Discovery of novel molecular and biochemical predictors of response and outcome in diffuse large B-cell lymphoma

Last, Kim William

Discovery of Novel Molecular and Biochemical Predictors of Response and Outcome in Diffuse Large B-cell Lymphoma

Kim William Last

A thesis submitted for the degree of
Doctor of Philosophy
At the University of London

Cancer Research UK Centre of Medical Oncology
St Bartholomew's Hospital
Queen Mary University of London,
London UK
2008

ABSTRACT

Discovery of Novel Molecular and Biochemical Predictors of Response and Outcome in Diffuse Large B-cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the commonest form of non-Hodgkin lymphoma and responds to treatment with a 5-year overall survival (OS) of 40-50%. Predicting outcome using the best available method, the International Prognostic Index (IPI), is inaccurate and unsatisfactory. This thesis describes research undertaken to discover, explore and validate new molecular and biochemical predictors of response and long-term outcome with the aims of improving on the inaccurate IPI and of suggesting novel therapeutic approaches. Two strategies were adopted: a rational and an empirical approach. The rational strategy used gene expression profiling to identify transcriptional signatures that correlated with outcome to treatment and from which a model of 13-genes accurately predict long-term OS. Two components of the 13-gene model, PKCβ and PDE4B, were studied using inhibitors in lymphoma cell-lines and primary cell cultures. PKCβ inhibition using SC-236 proved to be cytostatic and cytotoxic in the cell-lines examined and to a lesser extent in primary tumours. PDE4 inhibition using piclamilast and rolipram had no effect either alone or in combination with chemotherapy. The empirical approach investigated the trace element selenium in presentation serum and found that it was a biochemical predictor of response and outcome to treatment. In an attempt to provide evidence of a causal relationship as an explanation for the associations between presentation serum selenium, response and outcome, two selenium compounds, methylseleninic acid (MSA) and selenodiglutathione (SDG) were studied *in vitro* in the same lymphoma cell-lines and primary cell cultures. Both MSA and SDG exhibited cytostatic and cytotoxic activity and caspase-8 and caspase-9 driven apoptosis. For SDG reactive oxygen species generation was important for its activity in three of the four cell-lines. In conclusion, molecular and biochemical predictors of response and survival were discovered in DLBCL that led to viable targets for drug intervention being validated *in vitro*.

I Dr Kim William Last
hereby declare that this document
is the work of my own hand.

.....

I dedicate this thesis to my wife,
Marie-Christine and my son, Raphael
and to my late mum, without who's unfailing
love and support the completion of this work
would have been impossible.

I give heartfelt thanks to my brother and
my 'Aunty' Ann for steadfast encouragement
and belief in my abilities.

ACKNOWLEDGEMENTS

The work described in this thesis was carried out whilst I was a clinical research fellow at the Cancer Research UK Medical Oncology Unit at St Bartholomew's Hospital. I am indebted to numerous of my scientific, medical and nursing colleagues for their support and encouragement throughout my time within the Unit and to the patients that donated material for research.

In particular I wish to thank Dr Jude Fitzgibbon and Dr Simon Joel for scientific supervision, Dr's Margaret Shipp and Todd Golub and their teams at the Dana Farber Cancer Centre and the Whitehead Institute for the opportunity to participate in their micro-array project and Professor T Andrew Lister and Professor Ama Rohatiner for supervision of the translational and clinical aspects of the research undertaken.

I will always be grateful to CR-UK for their generous funding of the clinical research fellowship I held and the charity's enlightened and enabling ethos towards my time in research.

Of the many others who have helped me I would like to mention Dr Lindsey Goff, Ms Suzanne Jordan, Dr Andrew Norton, Dr Abigail Lee for assistance with immunohistochemistry; Dr Silvana Debarnardi, Dr Spouros Sgouros for assistance with gene expression profiling; Dr Wai Liu, Dr Sandra Strauss, Dr Theresa Davis, Ms Lenushka Maharaj, Ms Jackie Perrie and Mr Patrick Grimshaw for assistance with cell culture and FACS analysis; Dr Sandra Strauss with assistance with western blotting and Ms Jackie Perrie for assistance with annexin V and propidium iodide staining and FACS analysis and for performing the reactive oxygen species experiments; Mr Keith Adams, Ms Fauzia Khan and Mr Dave Wilkinson for assistance with patient material identification, reagent and equipment requirements; Mr Andrew Wilson and Dr Victoria Cornelius for clinical data collation and statistical analysis, Mr Linden and Mrs Ann Emery for proofreading and Mrs Margaret Cresswell and Ms Susan Chandler for tracking me down to ensure the thesis was submitted.

INDIVIDUAL CONTRIBUTIONS

Archived sample identification and retrieval: Kim Last

Cell cycle FACS analysis: Kim Last

Clinical data extraction and analysis: Kim Last and Andy Wilson

Ethics approval: Jude Fitzgibbon, Simon Joel and Andrew Lister

Histopathology review: Andrew Norton John Amess and Jon C. Aster

In vitro proliferation, cell count and viability experiments: Kim Last

Microarray project leadership Margaret A. Shipp & Todd R. Golub

Microarray sample preparation: Michelle Gaasenbeek

Microarray data analysis (US): Ken N. Ross, Pablo Tamayo, Michael Angelo, Michael Reich, Tane S. Ray, Jill Mesirov

Microarray data analysis (UK): Spouros Sgouros

N-acetyl cysteine *in vitro* experiments: Lenushka Maharaj

Novel drug identification for *in vitro* studies: Kim Last and Jude Fitzgibbon

Primary cell culture *in vitro* experiments: Kim Last and Lenushka Maharaj

Propidium iodide and annexin V FACS analysis: Jackie Perrie

Reactive Oxygen Species Measurement: Jackie Perrie

Serum selenium measurement: Trevor Delves, Christine Sieniawska

Statistics for serum selenium associations: Victoria Cornelius

Western blotting: Sandra Strauss

TABLE OF CONTENTS

LIST OF TABLES

Chapter 6

LIST OF FIGURES

Chapter 1

Chapter 2

LIST OF APPENDIX TABLES

Chapter 4

ABBREVIATIONS

A2780	human ovarian cancer cell-line
ABC	activated B-cell
ABC-	activated B-cell-like
ADP	adenosine diphosphate
ACVBP	dose intensive combination chemotherapy regimen
AGS	human gastric cancer cell-line
AID	activation-induced cytidine deaminase
AIDS	acquired immune deficiency syndrome
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
ANOVA	analysis of variance
APAF-1	apoptosis protease activating factor 1
ATM	ataxia telangiectasia mutated gene
AUC	area-under-the-curve
Ara-C	cytosine arabinoside
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated x protein
BCL-1/Bcl-1	gene and protein for B-cell lymphoma protein 1
BCL-2/Bcl-2	gene and protein for B-cell lymphoma protein 2
BCL-6/Bcl-6	gene and protein for B-cell lymphoma protein 6
BCL-x_L/Bcl-x_L	Bcl-2-related gene (long alternatively spliced variant of Bcl-x gene)
BCR	B-cell receptor
Bik	bcl-2 interacting killer – BH3 only pro-apoptotic Bcl-2 family memberprotein
Bim	Bcl-2-interacting protein
BL	Burkitt lymphoma
bp	base pairs
β pol	polymerase, DNA, beta
BSA	body surface area
BUB1B	budding uninhibited by benzimidazoles 1
c-JUN	v-JUN avian sarcoma virus 17 oncogene homologue
CASPASE-8	cysteine-aspartic acid protease-8
CASPASE-9	cysteine-aspartic acid protease-9
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CD19	cluster of differentiation 19
CD20	cluster of differentiation 20
CD22	cluster of differentiation 22
CD34	cluster of differentiation 34
CD79a	cluster of differentiation 79a
CDC6	cell cycle controller CDC6
CDC20	cell division cycle 20 homologue
CDC25A	cell division cycle 25 homologue A
CDK1	cyclin-dependent kinase 1
CDK2	cyclin-dependent kinase 2
CDK4	cyclin-dependent kinase 4
CDK6	cyclin-dependent kinase 6

CDKN2A	cyclin-dependent kinase inhibitor 2A gene
cDNA	complementary deoxyribonucleic acid
CDP	cytidine 5 -diphosphate
Cdw32Fc	Fc fragment of IgG, low affinity IIa, receptor (CD32)
CEM	human T-cell ALL cell-line
CGH	comparative genomic hybridisation
c-GMP	cyclic guanosine 3'-, 5'- monophosphate
CH_3SeH	methylselenol
$(CH_3)_2Se$	dimethylselenide
$(CH_3)_3Se^+$	trimethylselenide
CHK-1	checkpoint kinase 1
CHK-2	checkpoint kinase 2
CHO	Chinese Hamster Ovary cell-line
CHOEP-14	CHOP plus etoposide given every 14 days
CHOEP-21	CHOP plus etoposide given every 21 days
CHOP	cyclophosphamide, doxorubicin, vincristine prednisone
CHOP 14	CHOP chemotherapy given every 14 days
CHOP 21	CHOP chemotherapy given every 21 days
CHOP-R	CHOP chemotherapy plus rituximab
CI	confidence interval
CIBMTR	Center for International Blood and Marrow Transplant Research
CLL	chronic lymphocytic leukaemia
CNS	central nervous system
COPD	chronic obstructive airways disease
COX-1	cyclo-oxygenase-1
COX-2	cyclo-oxygenase-2
CpG	cytosine base followed immediately by a guanine base
CR	complete response
CRu	complete response undefined
GPR	good partial response
CRL-2261	DLBCL cell-line
CRL	shortened version of CRL-2261
cRNA	complementary ribonucleic acid
CRu	complete response unconfirmed
CR-UK	Cancer Research United Kingdom
CsCl	caesium chloride
CTP	cytidine triphosphate
c-MYC	cellular homologue of a transforming gene encoded by the avian myelocytomatosis virus, MC29
DAB	3'3'diaminobenzidine
DCDP	deoxycytidine 5-diphosphate
DCF-DA	2'7'-dichlorodihydrodifluoroscein diacetate
DFCI	Dana Farber Cancer Institute
DHAP	cisplatin-cytarabine-dexamethasone chemotherapy
DHL4	DLBCL cell-line
DHL6	DLBCL cell-line
DLBCL	diffuse large B-cell lymphoma
DMBA	7,12-dimethylbenz(a)anthracene

DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DoHH2	transformed FL cell-line
DRP2	dystrophin related protein 2
DU145	human prostate cancer cell-line
E	etoposide
E2F-1	E2F transcription factor 1
E2F-2	E2F transcription factor 2
EBV	Epstein-Barr virus
EGFR	epidermal growth factor receptor
ECOG	Eastern Co-operative Oncology Group
EC_{50}	effective concentration to achieve 50% response
E coli	Escherichia coli bacteria
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular signal-regulated kinase
cFLIP long	cellular FADD-like interleukin 1β converting enzyme inhibitory protein
FAS	a death receptor; aliases include CD95 and Apo-1
FL	follicular lymphoma
Fors	forskolin
G0	phase of the cell cycle where cells exist in a quiescent state
G1	first phase of interphase within the cell cycle
G1/S	transition between G1 and S phase in the cell cycle
G2/M	second checkpoint of the cell cycle
G2	third and final phase of interphase within the cell cycle
GADD45	growth arrest- and DNA damage-inducible gene 45
GADD153	growth arrest- and DNA damage-inducible gene 153
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA-1	GATA binding protein 1 (globin transcription factor1)
GC	germinal centre
GCB	germinal centre B-cell
GCB-	germinal centre B-cell-like
GELA	Groupe d'Etude des Lymphomes de l'Adulte
GPR	good partial response
GPx	glutathione peroxidase
G-CSF	granulocyte-colony stimulating factor
HAART	highly active antiretroviral therapy
HCL	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIF-1	hypoxia-inducible factor 1
HG-U133	human genome U133 Affymetrix gene chip
HG-U95A	human genome U95A Affymetrix gene chip
HIV	human immunodeficiency virus
HR	hazard ratio
HU6800	human genome 6800 Affymetrix microarray chip; alternative name HuGeneFl microarray chip
IC50	Inhibitor Concentration needed to achieve 50% enzyme inhibition
I_gG	immunoglobulin G

I_gG1	immunoglobulin G-1
Ig/BCL-6	chromosomal translocation involving an immunoglobulin chain locus and the *Bcl-6* gene locus
IgH	immunoglobulin heavy chain
IHC	immunohistochemistry
I lambda	immunoglobulin lambda light chain
I kappa	immunoglobulin kappa light chain
IκB	inhibitor of kappa light chain gene enhancer in B-cells
IκB υ	IκB
IL-4	interleukin-4
IMDM	Iscove's modified Dulbecco's medium
IPI	international prognostic index
JNK	Jun N-terminal Kinase
JNK-1	Jun N-terminal Kinase-1
JNK-2	Jun N-terminal Kinase-2
kb	kilo base pair
kDa	kilo Dalton
KH_2PO_4	potassium diphosphate
Ki	the dissociation constant for the enzyme-inhibitor complex
K-ras	Kirsten rat sarcoma viral oncogene homologue
LDH	lactate dehydrogenase
LNCaP	human prostate cancer cell-line
Ly379196	PKCβ inhibitor
M	mitotic phase of the cell cycle
MACOP	anthracycline-based combination chemotherapy regimen
MALT	extranodal marginal zone B-cell lymphoma of mucosa associated lymphoid tissue
MAPK1	mitogen-activated protein kinase 1 (ERK2)
MAPK3	mitogen-activated protein kinase 3 (ERK1)
MBP	methylated DNA binding proteins
MBR	major breakpoint region
MCF10AT1	human premalignant mammary epithelial cell-line
MCF10AT3B	human premalignant mammary epithelial cell-line
MCL	mantle cell lymphoma
MCL-1/Mcl-1	myeloid cell differentiation 1 gene/protein
MCM2	minichromosome maintenance 2
MCM3	minichromosome maintenance 3
MCM5	minichromosome maintenance 5
MCM6	minichromosome maintenance 6
MCM7	minichromosome maintenance 7
Mcr	minor cluster region
MDM2	mouse double minute 2 homologue
MEL	mouse erythroleukaemia cell-line
mer	oligomer
MES	2-[N-morpholino]ethansulphonic acid
MHC	major histocompatibility complex
MinT	MabThera International Trial
MINOR	mitogen induced nuclear orphan receptor
MOD	mouse mammary cancer cell-line
mRNA	messenger ribonucleic acid

MSA	methylseleninic acid
MSC	Se-methylselenocysteine
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2 (4-sulphophenyl)-2H-tetrazolium
NAC	N-acetyl cysteine
NaCl	sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NCI	national cancer institute of the United States of America
NFKB	nuclear factor-kappaB
NHL	non-Hodgkin lymphoma
nm23-H1	non-metastatic protein 23, homologue 1
NPM-ALK	nucleophosmin-anaplastic lymphoma kinase fusion protein
NSAID	non-steroidal anti-inflammatory drug
OCI-Ly3	human DLBCL cell-line
OCI-Ly10	human DLBCL cell-line
OD	optical density
oligo-Dt	short strings of thymidylate
OxPhos	oxidative phosphorylation
PI3-kinase	phosphatidylinositol 3-kinase
$p14^{ARF}$	14 kDa protein (alternative reading frame); beta transcript of *CDKN2A* gene
$p16^{INK4a}$	16 kDa protein (inhibitor of kinase CDK4); alpha transcript of *CDKN2A* gene
$p19^{INK4d}$	19 kDa protein (inhibitor of kinase CDK4); alternative name cyclin-dependent kinase inhibitor 2D (CDKN2D)
$p21^{WAF1}$	21 kDa protein (wild type p53-activated fragment 1) alternative name CDK-interacting protein 1 (CIP1)
$P27^{kip1}$	cyclin-dependent kinase inhibitor 1B
p38 MAP kinase	p38 mitogen-activated protein kinase; alternative name MAPK14
p53	tumour protein of 53 kDa
p63	tumour protein of 63 kDa
p73	tumour protein of 73 kDa
p90RSK	90 kDa ribosomal protein S6 kinase
P450	pigment at 450 nm
PARP	poly(ADP-ribose) polymerase
PAX5	paired domain gene 5
PBS	phosphate buffered saline
PC3	prostate cancer 3 cell-line
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDE	phosphodiesterase
Pic	piclamilast
PFS	progression free survival
PIM1	serine/threonine protein kinase PIM1
PKA	c-AMP-dependent protein kinase A protein
PKCβ	protein kinase C beta
PKCβ1	protein kinase C beta-1
PKCβ2	protein kinase C beta-2

PLK	polo-like kinase
PMA	phorbol-12-myristate-13-acetate
PMBCL	primary mediastinal B-cell lymphoma
PPR	poor partial response
PR	partial response
pRb	phosphorylated retinoblastoma protein
PTLPDs	post-transplant lymphoproliferative disorders
P values	probability values
PI	propidium iodide
PMSF	phenylmethanesulphonyl fluoride
PTEN	phosphatase and tensin homologue deleted on chromosome ten
Puma	p53-upregulated modulator of apoptosis
p48XPE	DNA damage-binding protein 2 p48 subunit
p-XSC	1,4-phenylenebis(methylene)selenocyanate
R	rituximab
RAD9	radiation gene 9
Rad-51	radiation gene 51
Rad-54	radiation gene 54
RAG	recombination-activating gene
RB1	retinoblastoma-1 gene
REAL	revised European and American lymphoma classification
REL	avian reticuloendotheliosis viral oncogene homologue
RhoH/TTF	RAS homolog gene family, member H/ translocation three four
RNA	ribonucleic acid
RQ-PCR	quantitative real-time polymerase chain reaction
Rol	rolipram
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RRM1	ribonucleotide reductase, M1 subunit
RRM2	ribonucleotide reductase, M2 subunit
S	S or synthesis phase of the cell cycle during which DNA synthesis or replication occurs
Se	Selenium
Sepp1	selenoprotein P
SAPE	streptavidin-phycoerythrin
SBH	St Bartholomew's Hospital
SDG	selenodiglutathione
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEER	surveillance, epidemiology and end results
SLL	small lymphocytic lymphoma
SNP	single nucleotide polymorphism
SNU-1	gastric adenocarcinoma cell-line
SOM	self-organising map
SPIB	spleen focus forming virus proviral integration oncogene B
STK15	serine/threonine protein kinase 15
Sub-G1	apoptotic cell fraction
SUD4	human DLBCL

SV40	simian virus 40
TGF-β	transforming growth factor β
TGF-βR1	transforming growth factor β receptor 1
TGF-βR2	transforming growth factor β receptor 2
Th2	T-helper cell type 2
TM12	mouse mammary epithelial cell-line
TM2H	mouse mammary epithelial cell-line
TNF	tumour necrosis factor
TP53	tumour protein 53 gene
TOP2A	topoisomerase II alpha
TR	thioredoxin reductase
TRAIL	tumour necrosis factor related apoptosis inducing ligand
Trx	thioredoxin
TYMS	thymidylate synthetase gene (TS)
tRNA	transfer ribonucleic acid
Tris-Cl	tris base and hydrochloric acid solution
UACC-903	melanoma cell-line
UK	United Kingdom
ULN	upper limit of normal
UTP	uridine triphosphate
US	United States
USA	United States of America
V(D)J	variable, diversity and joining genes
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor
VIM	etoposide-ifosfamide-methotrexate chemotherapy
w/	with
w/o	without
WHO	World Health Organisation

THESIS AIMS

- To discover, explore and validate new molecular and biochemical predictors of response and long-term outcome in diffuse large B-cell lymphoma (DLBCL).

- To improve on the International Prognostic Index in DLBCL.

- To discover novel therapeutic targets in DLBCL.

CHAPTER 1: INTRODUCTION

1.1 Pathogenesis of Lymphoma and Cancer

1.1.1 The Process of Carcinogenesis

The process of carcinogenesis involves the acquisition of at least six functional changes: resistance to growth inhibition, evasion of apoptosis, independence of mitogenic stimulation, angiogenesis, metastasis and invasion (Hahn and Weinberg, 2002b). It is hoped that the disruption of a limited number of regulatory pathways will underlie carcinogenesis and bring meaning to the ever-burgeoning number and complexity of described genetic and epigenetic alterations in tumours. The genetic and biochemical rules governing the transformation of human cells into malignant clones are the subject of intense worldwide research. The discovery and validation of such rules will redefine not only the diagnosis of tumours such as DLBCL but also prognostic accuracy and ultimately therapy development and deployment.

Strong, direct evidence supporting a genetic basis for carcinogenesis began to emerge in the 1980's with the demonstration of the malignant transformation of immortalised cells through the introduction of genetic material containing oncogenes and the identification of such dominantly acting, growth-controlling oncogenes in tumour derived cell-lines. In immortalised rodent cells the collaboration of just two oncogenes was sufficient to produce neoplastic cells, whilst in human cells more hurdles have to be overcome to achieve malignant transformation suggesting a more complex carcinogenic pathway. In lymphomas, the collaborative effect of oncogenes is illustrated by the presence in a minority of follicular lymphoma (FL) and DLBCL of both a *BCL-2* and a *c-Myc* translocation, an occurrence associated with aggressive, progressive disease (Yano *et al.*, 1992, Karsan *et al.*, 1993).

Each cancer develops through the clonal expansion of a single founder cell, which has acquired a survival advantage. In DLBCL, and other B-cell non-Hodgkin lymphomas (NHLs), the clonal nature of each tumour is readily proven by confirmation of a single idiotype common to each of the neoplastic cells.

Tumour development is considered to follow a multi-step pathway involving the disruption of critical genes involved in genome integrity, cell proliferation, differentiation and survival (Hahn and Weinberg, 2002a, Hahn and Weinberg, 2002b). The multi-stage progression from early to late, from pre- to metastatic cancer is conditional upon the acquisition of modifications in particular oncogenes and tumour

suppressor genes (Kinzler and Vogelstein, 1996b, Brat *et al.*, 1998). The acquisition of the critical genetic and epigenetic abnormalities for neoplastic transformation occurs as the clone undergoes many stochastic cycles of clonal selection. Many 'bystander' events are produced prior to a tumour's clinical presentation, with the consequence of wide-ranging alterations in gene and protein expression and also considerable diversity within each tumour, as different daughter cells of the original clone randomly acquire different lesions. Critical to carcinogenesis is the rate at which genetic aberrations are acquired. Under normal conditions, the rate of base-pair mutations is only 0.16 per genome per cell division and the occurrence of chromosomal abnormalities also develop infrequently and almost always result in the cell's elimination through programmed cell death (Drake *et al.*, 1998). Lymphomagenesis is expected to follow such a multi-step pathway, however, the absence of a pre-malignant forerunner of DLBCL and other NHLs in the immunocompetent has prevented detailed identification of early molecular events as has been possible in bowel, breast, lung and pancreatic cancer. Indeed, possibly most of the critical genetic, epigenetic and biochemical events in lymphomagenesis, beyond putative cell of origin and hallmark translocations, still await discovery. It is however already apparent that acquisition of critical carcinogenic properties occurs in DLBCL and other high-growth fraction lymphomas, including subversion of the cell cycle, resistance to apoptosis and independence of mitogenic stimulation. The normal cell cycle has three checkpoints built into it to prevent replication of a defective genome or division of misaligned daughter chromosomes as illustrated in Figure 1.1.

The acquisition of resistance to growth inhibition and the evasion of apoptosis are achieved by inactivation, through mutation, epigenetic silencing or deletion of recessive, tumour suppressor genes causing loss of their proteins and the failure of their regulatory pathways. In order to maintain homeostasis in normal tissues the balance between cell death and cell growth is tightly controlled. During carcinogenesis, this sensitive and dynamic process, controlled by the cell cycle regulators and checkpoints controls, is disrupted and lost (Hartwell and Kastan, 1994, Hanahan and Weinberg, 2000). Oncogenes, in contrast to tumour suppressor genes, positively promote cell proliferation by overcoming cell cycle checkpoints, by resisting apoptosis and preventing differentiation. Oncogenes become constitutively active through gain of function mutations or through excessive transcription, as produced through

chromosomal translocation. Chromosomal translocation of *c-Myc* and *CCND1* (the gene for cyclin-D1, originally named *BCL-1)* are examples of proto-oncogene deregulation in NHLs (Magrath, 1990, Kuroda *et al.*, 1995). The protein products of both these oncogenes and a third, Cyclin-D3, are overexpressed in a significant proportion of DLBCL.

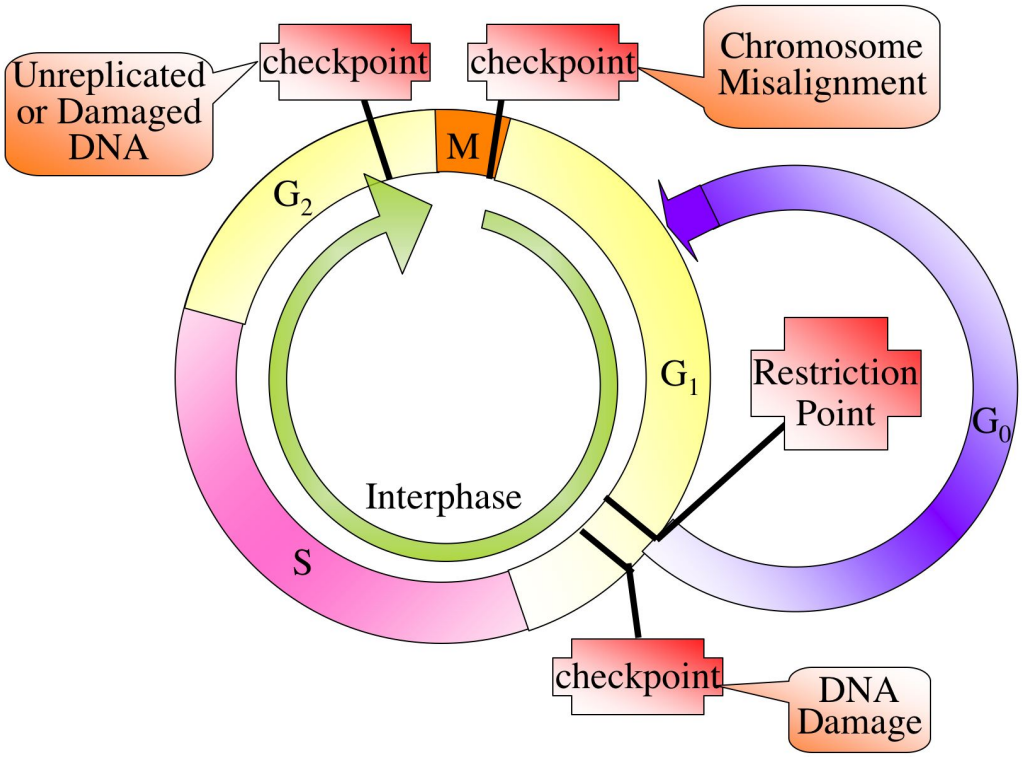


Figure 1.1 Phases and Checkpoints of the Cell Cycle The relative lengths of the phases of the cell cycle are typical of rapidly dividing human cells. If sufficient growth factors are available the cell can pass through the G_1 restriction point to move towards S phase. Otherwise the cell is forced to enter G_0. The cell grows during the three phases of interphase with DNA replication occurring during S phase. Chromosome separation and cell division occur during M phase. The three checkpoints prevent cell proliferation if DNA damage, faulty or incomplete replication has occurred or the chromosomes are not correctly aligned on the mitotic spindle. M = mitosis phase; G_1 = 1st growth phase of interphase; S = DNA synthesis phase; G_2 = 2nd growth phase of interphase; G_0 = quiescent/senescent phase. Adapted from (Cooper, 2002).

1.1.2 Impaired Apoptosis in NHL and Cancer

Two independent but convergent pathways, referred to as the 'intrinsic' and 'extrinsic' pathways, initiate programmed cell death, or apoptosis. The intrinsic pathway is centred on the cell's mitochondria where translocation of Bcl-2 family members into the mitochondria promotes changes in the mitochondrial membrane permeability, with the consequent release of cytochrome c. The association of cytochrome c with APAF-1

(apoptotic protease-activating factor-1) allows activation of the initiator caspase (cysteine-aspartic acid protease), caspase-9, which in turn triggers the effector caspase apoptotic cascade. The binding of ligands to cell surface death receptors, e.g. Fas ligand binding to Fas and tumour necrosis factor-α (TNFα) binding to TNF receptor-1, activates the extrinsic apoptotic pathway. Once a death receptor complex is formed, the initiator caspase, caspase-8 is recruited and activated, leading to ignition of the effector caspase apoptotic cascade. The apoptotic cell shows cell surface membrane blebbing, chromatin condensation and internucleosomal DNA fragmentation (Kerr *et al.*, 1972, Savill and Fadok, 2000). These changes are accompanied by the cell surface exposure of certain ligands, which indicate that the cell is marked for phagocytosis. The impairment of programmed cell death not only allows tumourigenesis to begin, but facilitates the accumulation of other lesions critical to the development of an overt tumour as proliferation and cell accumulation become uncontrolled. Apoptosis is subdued in DLBCL and other tumour cells by the combination of overexpression of anti-apoptotic, pro-survival genes such as *BCL-2*, the inhibitors of apoptosis proteins (IAPs) and NFκB; decreased expression and response to cell surface death receptors and their ligands, for example, Fas and Fas ligand; and inactivation of *TP53* and its pathways (Wickremasinghe and Hoffbrand, 1999, Schimmer *et al.*, 2001, Johnstone *et al.*, 2002, Evan and Vousden, 2001, Igney and Krammer, 2002, Salvesen and Duckett, 2002, Baldwin, 2001).

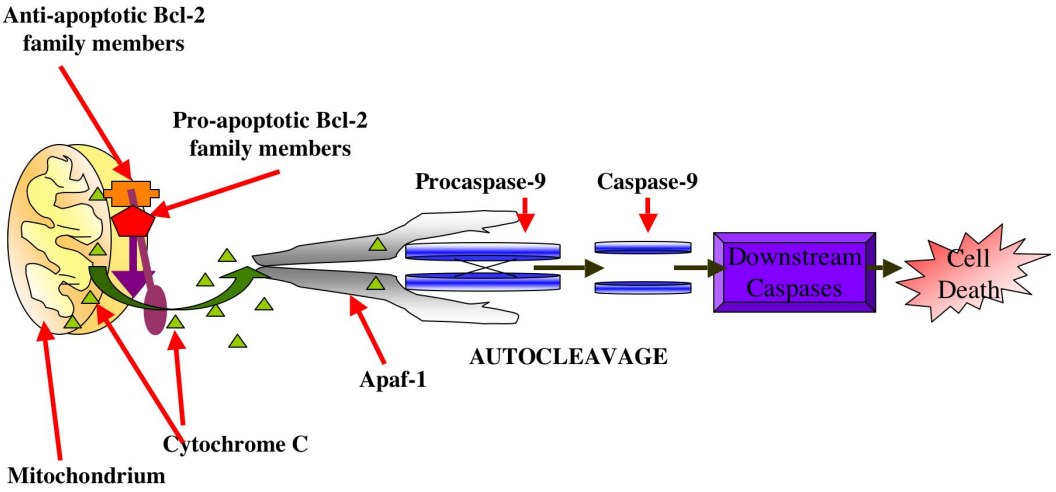


Figure 1.2 The Intrinsic Apoptotic Pathway. The pro- and anti-apoptotic Bcl-2 family members vie to cause and prevent release of cytochrome C from the mitochrondria. When cyctochrome C release occurs, it binds to the adaptor protein APAF-1 (apoptotic protease-activating factor-1) facilitating the activation of the initiator caspase-9 by autocleavage. Activated caspase-9 then cascades its signal via other caspases to produce programmed cell death – apoptosis. Adapted from (Cooper, 2002).
⊣ Inhibits cytochrome C release. → Promotes cytochrome C release.

1.1.3 Impairment of the Intrinsic Apoptotic Pathway: Alterations in The Bcl-2 Family of Proteins

The Bcl-2 family of nearly twenty proteins are divisible into three subgroups, the Bcl-2-like subgroup of anti-apoptotic proteins (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, Boo and A1), and two subgroups of pro-apoptotic proteins, the Bax/Bak-like proteins (Bax, Bak, Bok, Bcl-x_S and Bcl-G_L) and the BH-3-only subgroup (Bmf, Bad, Hrk, Noxa, Puma, Bik, Bim and Bid) (Coultas and Strasser, 2003, Strasser *et al.*, 2000). As the Bcl-2 family has grown and its members roles explored, it has become clear that considerable functional overlap exists amongst them; that interaction of the pro- and anti-apoptotic Bcl-2 family proteins is integral to their regulation of cell death and that their relative levels determine the pro- or anti- apoptotic inclination of cells (Yi *et al.*, 2003, Huang and Strasser, 2000, Cheng *et al.*, 2001) – see Figure 1.2.

The Bcl-2 family of proteins are critical for normal embryonic development and, once born, tissue homeostasis, including regulation of the death of lymphocytes and other blood cells as a counterbalance to haematopoiesis (Jacobson *et al.*, 1997, Newton and Strasser, 2000). This protein family is involved in the intrinsic apoptotic pathways initiated by cytokine withdrawal and cytotoxic stress conditions but are not required for

death receptor initiated cascades mediated by the TNF-receptor family (Strasser *et al.*, 2000, Strasser *et al.*, 1995, Ashkenazi and Dixit, 1998). The Bax/Bak-like subclass and the Bcl-2 subgroup are involved in controlling the integrity of the mitochondrial membrane, breach of which is essential if the intrinsic caspase pathway is to bring about apoptosis. The Bax/Bak-like subclass promotes cytochrome c release from the mitochondria, whilst the Bcl-2 subgroup prevents cytochrome c escape from the mitochondrial inter-membrane space, so by disabling cell death signal promotion (Gross *et al.*, 1999, Wang, 2001). The BH-3 only subgroup function upstream of the Bax/Bak-like proteins promoting cell death signal initiation (Huang and Strasser, 2000, Zong *et al.*, 2001, Cheng *et al.*, 2001, Bouillet *et al.*, 2002). In DLBCL, inactivating mutations in pro-apoptotic Bcl-2 family members have only been described in *Bik*, being seen in 18% of DLBCLs (Arena *et al.*, 2003). Whether epigenetic silencing of the Bax/Bak-like and BH-3 proteins occurs, as part of lymphomagenesis, is unknown at present.

BCL-2 was the first proto-oncogene to be described that exerted its transforming action by preventing cell death instead of promoting cell proliferation (Tsujimoto *et al.*, 1984). Transgenic overexpression of Bcl-2 subgroup members *BCL-2*, *MCL-1* and *Bcl-x_L* in mice results in B-cell lymphomas and plasma cell lesions (McDonnell and Korsmeyer, 1991, Strasser *et al.*, 1993, Zhou *et al.*, 2001, Linden *et al.*, 2003, Naik *et al.*, 1996). A synergistic interaction, in terms of tumourigenesis, has been observed in transgenic experiments between pro-survival *BCL-2* and the pro-cell growth and cell cycling genes, *Pim-1* and *v-abl* and *Bcl-x_L* and *c-Myc* (Strasser *et al.*, 1990, Acton *et al.*, 1992, Jaiswal *et al.*, 2003, Harris *et al.*, 1997, Naik *et al.*, 1996). Although transgenic mice overexpressing Bcl-2 display a preneoplastic, polyclonal lymphoproliferative condition that foretells the appearance of tumours (McDonnell *et al.*, 1989), frustratingly an analogous phenomenon has not been described in humans despite Bcl-2's evident function as a proto-oncogene. In DLBCL, overexpression of Bcl-2 is seen due to the presence of a t(14;18)(q32;q21) translocation or gene amplification in a minority of cases, with mRNA levels of *BCL-2* correlating with Bcl-2 protein levels (Shen *et al.*, 2004, Iqbal *et al.*, 2004).

1.1.4 Impairment of the Extrinsic Apoptotic Pathway: Fas and TRAIL Death Receptor Signalling in NHL

Fas (APO-1/CD95) is a cell surface death receptor belonging to the TNF-receptor family, which functions as a tumour suppressor gene in NHL (Straus *et al.*, 2001).

Stimulation of Fas by its natural ligand (FasL) results in receptor trimerisation and recruitment of procaspase-8 and FADD (Fas associated death domain) to form the death-inducing signalling complex (DISC) (Walczak and Krammer, 2000, Krammer, 2000). The resultant activation of caspase-8 triggers cell death through the extrinsic apoptotic pathway (illustrated in Figure 1.3). Fas signalling is the principle means of activated T-cell elimination and appears important for B-cell removal and malignant lymphocyte cell death (Rathmell and Goodnow, 1998, Krammer, 2000, Dhein *et al.*, 1994). Fas is encoded by nine exons found at 10q23 (Behrmann *et al.*, 1994). The inherited, usually autosomal dominant, 'autoimmune lymphoproliferative syndrome' is caused by mutations in the ligand binding and death domain exons of Fas, which result in benign lymphadenopathy, hepatosplenomegaly, an increased incidence of lymphomas and failure of T-cells to undergo apoptosis in response to FasL stimulation (van den Berg *et al.*, 2002, Martin *et al.*, 1999). Escape from apoptotic cell death through inactivation of Fas appears important in NHLs. Sporadic mutations of Fas occur in 20-30% of NHLs and Hodgkin lymphoma cases (Gronbaek *et al.*, 1998, Muschen *et al.*, 2000). Other NHLs inactivate the extrinsic Fas death pathway through silencing of Fas expression (Clodi *et al.*, 1998, Laytragoon-Lewin *et al.*, 1998). Hueber *et al* demonstrated that c-Myc-induced apoptosis requires interaction on the cell surface between Fas and its ligand. His findings linked two apoptotic pathways previously thought to be independent and established the dependence of c-Myc on Fas signalling for its killing activity (Hueber *et al.*, 1997). Recent Fas mutation analysis in a series of 117 DLBCL samples found eight cases (7%) with death domain mutations that resulted in loss of the death domain (Takahashi *et al.*, 2006). The frequency of Fas death domain mutations was highest, 15%, in DLBCL with an 'oxidative phosphorylation (*OxPhos*)' expression profile (see page Chapter 5). In the same series, overexpression of cFLIP long (cellular FADD-like interleukin 1β converting enzyme inhibitory protein) the competitive antagonist of FADD was seen in the 'host response' transcriptional signature subtype of DLBCL, suggesting that suppression of the extrinsic apoptotic pathway by Fas signalling silencing is important in these DLBCL.

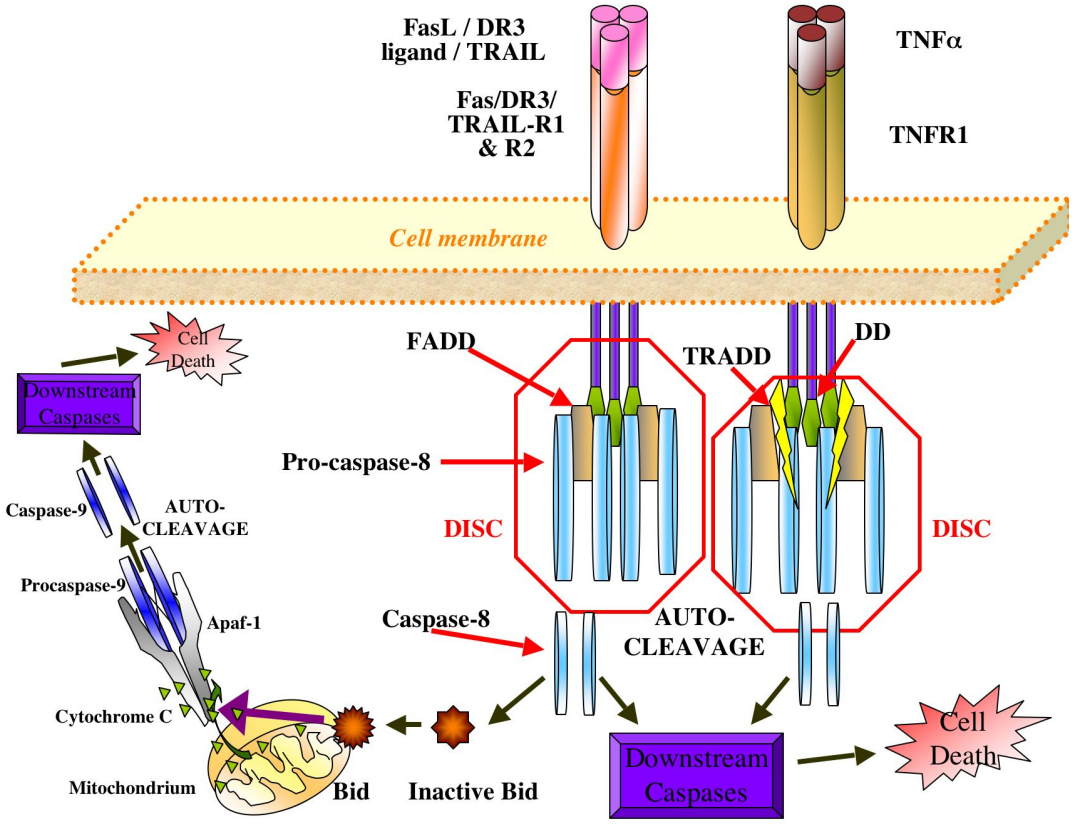


Figure 1.3 The Extrinsic Apoptotic Pathway. Activated caspase-8 then cascades its signal via caspases-3-7 to produce apoptosis. Caspase-8 can also activate the intrinsic apoptotic pathway via activation of Bid, which then binds to mitochondria, promoting cytochrome C release. FasL = Fas-ligand; DR3 ligand = death receptor 3 ligand; TRAIL = TNF-related apoptosis-inducing ligand; DR3 = death receptor 3; TRAIL-R1 & R2 = ; TNFα = tumour necrosis factor alpha; TNFR1 = tumour necrosis factor receptor-1; DD = death domain; TRADD = tumour necrosis factor receptor-1-associated death domain; FADD = Fas-associated death domain; DISC = death-inducing signalling complex; Bid = BH3-interacting domain death agonist; Apaf-1 = apoptotic protease activating factor-1; caspase = cyteine aspartate-specific proteinase.
Adapted from (Zimmermann *et al.*, 2001, Cooper, 2002). → Promotes cytochrome C release.

Study of the frequently deleted chromosome 8p21.3 in 45 B-cell NHL cell-lines defined a minimal deleted region of 600 kb that contains the TRAIL-R1 and –R2 genes. Further investigation, including FISH analysis of >100 primary NHL samples suggest that deletion-induced haploinsufficiency of TRAIL-R1/R2 can impair TRAIL-induced extrinsic pathway apoptosis in NHL and may therefore contribute to lymphomagenesis in B-cells through a gene dosage effect (Rubio-Moscardo *et al.*, 2005). Mutations in the death domain of TRAIL-R1/R2 have been found in 7% of NHL (Lee *et al.*, 2001).

1.1.5 Cell Cycle Deregulation in Cancer and Lymphoma

Tumour suppressor genes prevent unrestrained cell growth, cycling, and survival. Tumour suppressor genes were divided into two classes: 'caretaker' genes and 'gatekeeper' genes by Kinzler and Vogelstein, based on their observations of genetic abnormalities in colorectal cancer (Kinzler and Vogelstein, 1996a). 'Caretaker' genes are critical for maintaining genetic stability. Mutations in 'caretaker' genes make mutation in other genes much more likely so by creating a state of genetic instability. Examples of 'caretaker' genes include the DNA mismatch repair genes *MLH1* and *MSH2,* alteration of which predisposes to colorectal cancer; the breast and ovarian cancer susceptibility genes *BRCA-1* and *–2* and *ATM* (ataxia telangiectasia mutated) in MCL (mantle cell lymphoma) and other NHLs. 'Gatekeeper' genes and their proteins control aspects of cell proliferation, with the consequence of uncontrolled cell proliferation on their mutation. 'Gatekeeper' genes include *RB1* (retinoblastoma gene) in retinoblastoma, *APC* and *b-catenin* genes in colorectal cancer and *VHL* gene in renal cell cancer. Kinzler and Vogelstein proposed that only one additional somatic mutation would be necessary if a 'gatekeeper' gene was disrupted as opposed to three or more additional somatic mutations if a 'caretaker' gene was mutated (Kinzler and Vogelstein, 1997, Michor *et al.*, 2003, Frank, 2003).

Two pathways dominate the control of the cell cycle: the pRb protein pathway ($p16^{INK4a}$-Cyclin D-cdk4-pRb) and the p53 pathway ($p14^{ARF}$-mdm2-p53-$p21^{WAF1}$). Both pRb and p53 are 'gatekeeper' tumour suppressor proteins. p53 also functions as a 'caretaker' tumour suppressor. Members of these two pathways and a third, the $p27^{KIP1}$-cyclin E-cdk2, are found mutated or silenced in virtually every human cancer including DLBCL, with particular genetic and epigenetic alterations being seen in different tumour types (Giaccia and Kastan, 1998, Sherr and McCormick, 2002, Dyson, 1998). The pRb and p53 pathways interact at both the genetic and protein level (Stewart *et al.*, 2001, Yamasaki, 2003). The transforming potential of defective pRb and p53 is illustrated by the results of mouse embryo fibroblast knockout research, where the addition of just a Ras oncogene to either *TP53*-/-,*p19*-/- (the mouse homologue of $p14^{ARF}$) or *RB1*/*p107*/*p130*-/- cells was sufficient to induce transformation. Clinical consequences appear to stem from the concurrent disruption of *p16INK4a* and *p14ARF-TP53* with a significantly worse 5-year OS of only 7% compared to 38% in those

DLBCL patients with retention of one or both pathways (P = 0.005) (Gronbaek *et al.*, 2000).

1.1.6 Resistance to Cell Cycle Arrest

Disruption of the pRb Pathway in DLBCL

pRb regulates the G1/S checkpoint according to its phosphorylation status. Unphosphorylated Rb blocks G1/S progression by sequestration of the transcription factor E2F. Conversely, the phosphorylation of different sites on the pRb protein by the different cyclins/cdks removes pRb's inhibition of the G1/S checkpoint, releasing E2F and allowing irreversible progression into S phase and consequent cell division, even in the absence of growth signals. Normally pRb protein levels rise and fall in relation to the proliferative activity of the haematological cells concerned (Martinez *et al.*, 1993). This relationship is preserved in many NHLs where low growth fraction tumours express little pRb, whilst high growth fraction tumours display higher levels (Kiviniemi *et al.*, 2000). *Rb* is itself mutated in various solid tumours and inactivated in a third of lymphoid malignancies (Hangaishi *et al.*, 1996). Three members of the pRb pathway, $p16^{INK4a}$, cyclin-D1 and D3, are frequently disrupted in lymphomas and other cancers (Tsutsui *et al.*, 2002, Steeg and Zhou, 1998, Tetsu and McCormick, 1999, Ruas and Peters, 1998, Beasley *et al.*, 2003, Bai *et al.*, 2003). Overexpression of cyclin-D1 or D3 and suppression of $p16^{INK4a}$ cause sustained hyperphosphorylation and therefore inactivity of pRb. $p16^{INK4a}$ and $p15^{INK4b}$ suppress cell cycling by binding cdk-4 and -6, so by displacing bound Cyclin-D and preventing Cyclin-D rebinding (Sandhu *et al.*, 1997). The $p15^{INK4b}$ gene *CDKN2B* is located adjacent to the $p16^{INK4a}/p14^{ARF}$ gene locus *CDKN2A* at 9p21 (Hannon and Beach, 1994). 9p21 deletion, small homozygous deletions and hypermethylation all result in the silencing of $p16^{INK4a}$ (Nakahara *et al.*, 2001, Cairns *et al.*, 1995). The importance of the pRb pathway in DLBCL is illustrated by the high frequency of its disruption, with 53% of cases displaying $p16^{INK4a}$ inactivation, Cyclin-D3 overexpression or aberrant pRb expression and E2F-1 low expression being a poor prognostic factor for OS on multivariate analysis (relative risk = 6.9; P = 0.0037) (Moller *et al.*, 2000).

Resistance to Cell Cycle Arrest, Apoptosis and Induction of Genetic Instability: Disruption of the p53 Pathway in DLBCL

p53 functions as both a 'gatekeeper' and a 'caretaker' gene through its ability to induce cell cycle arrest and apoptosis and as a preserver of genomic integrity respectively

(Giaccia and Kastan, 1998, Morris, 2002). The key role p53 plays at the intersection of multiple pathways regulating cell fate, in response to internal and external stresses is illustrated in Figure 1.4 and is borne out by it being the most frequently mutated gene yet discovered in human cancer. Indeed *TP53* mutations, usually in the DNA binding domain, are seen in >50% of human solid tumours, 20% of DLBCLs (16/84) and 22% (22/102) of aggressive NHLs (DLBCL and Burkitt lymphoma (BL)) (Ichikawa *et al.*, 1997, Koduru *et al.*, 1997). Wild-type *TP53* serves as a 'guardian of the genome' preventing proliferation of genetically damaged cells. After DNA damage, p53 activates transcription of genes to mediate its biologic role as a tumour suppressor, including transcription of $p21^{WAF1}$ to induce a G2 cell cycle arrest (Bunz *et al.*, 1998). Surprisingly, the cdk inhibitor $p21^{WAF1}$ is rarely mutated in human malignancies (Kawamata *et al.*, 1995). In tumour cells with inactive p53 cell cycle progression cannot be blocked permitting upregulated growth. The half-life of p53 is short, due to binding of its antagonist the oncoprotein mdm2. Binding by mdm2 allows p53's efficient removal by ubiquitination, followed by proteosomal degradation (Haupt *et al.*, 1997). The phenotype of wild-type *TP53* cases revealed by IHC is therefore p53-/p21+. This changes to p53+/p21- in mutated *TP53* cases (Villuendas *et al.*, 1997). Where wild-type *TP53* persists, mdm2 overexpression frequently occurs in NHLs (22-28%) and serves as a means of p53 pathway deregulation (Moller *et al.*, 1999c, Watanabe *et al.*, 1994).

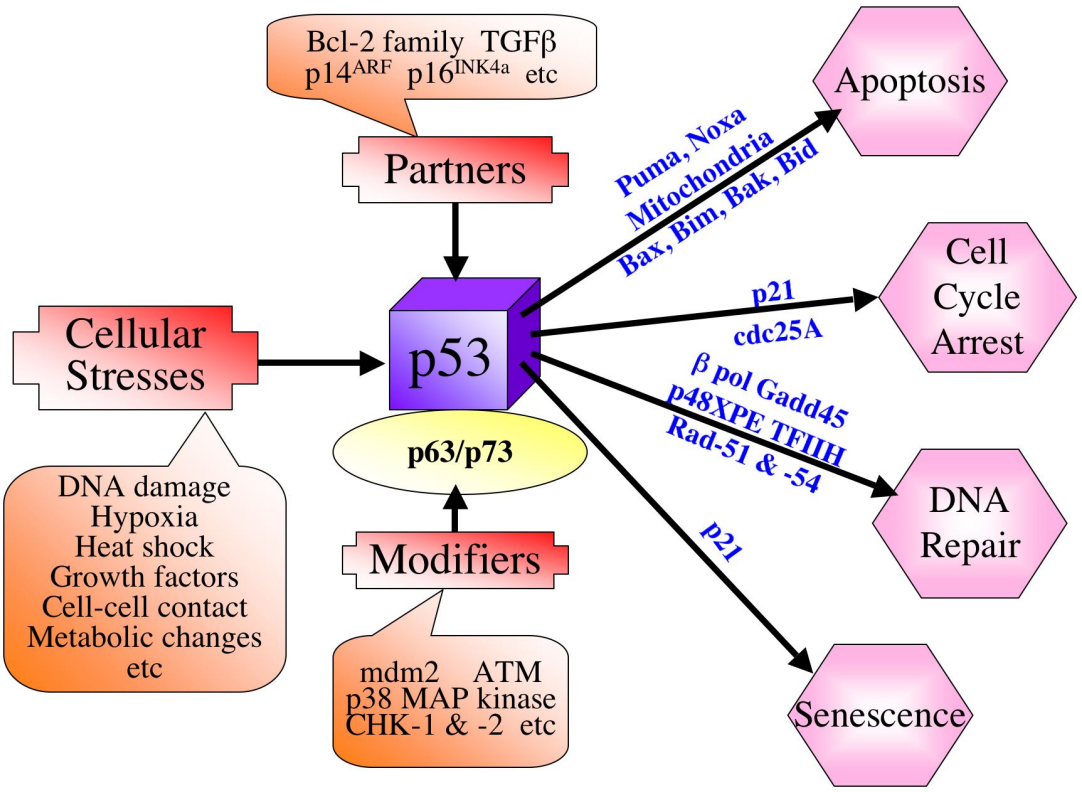


Figure 1.4 The Role of p53 at the Crossroads of Cellular Stress Response Pathways
The upregulation of p53 in response to different types of cellular stress can result in apoptosis, cell cycle arrest, DNA repair or senescence according to the cellular stress, the availability of partners and modifiers of p53 and its isoforms. TGF-β = transforming growth factor beta; $p14^{ARF}$ = 14 kDa protein (alternative reading frame); $p16^{INK4a}$ = 16 kDa protein (inhibitor of kinase CDK4); Puma = p53-upregulated modulator of apoptosis; Bax = Bcl-2-associated x protein; Bim = Bcl-2-interacting protein; Bak = Bcl-2 antagonist killer; Bid = BH3 Interacting domain Death agonist; p21 = 21 kDa protein (wild-type p53-activated fragment 1); cdc25A = cell division cycle 25 homologue A; β pol = polymerase, DNA, beta; Gadd45 = growth arrest- and DNA damage-inducible gene 45; p48XPE = DNA damage-binding protein 2 p48 subunit; TFIIH = transcription factor IIH, 62-kDa subunit; Rad-51 and -54 = radiation genes 51 and 54; mdm2 = mouse double minute 2 homologue; ATM = ataxia telangiectasia mutated; p38 MAP kinase = p38 mitogen-activated protein kinase; CHK-1 and -2 = checkpoint kinase 1 and 2; p63 = tumour protein of 63 kDa; p73 = tumour protein of 73 kDa. Adapted from (Braithwaite *et al.*, 2005, Smith and Seo, 2002).

p53 has been proposed to induce apoptosis through a three step process: transcriptional induction of redox-related genes; formation of reactive oxygen species; and oxidative degradation of mitochondrial components. Multiple interactions between p53 and members of the Bcl-2 family to effect apoptosis have been described. p53 translocates to mitochondria following DNA damage and directly induces permeability of the outer

mitochondrial membrane by forming complexes with the anti-apoptotic Bcl-x_L and Bcl-2 proteins, resulting in cytochrome c release (Mihara *et al.*, 2003). Cytosolic p53 can induce apoptosis by directly activating pro-apoptotic Bax, and releasing pro-apoptotic BH3-only proteins sequestered by Bcl-x_L (Chipuk *et al.*, 2004). Following cellular stress, p53 can interact with pro-apoptotic Bak, causing Bak oligomerisation and cytochrome c release from mitochondria (Leu *et al.*, 2004). Coincident with formation of p53-Bak complexes, loss of Bak sequestration by the anti-apoptotic Mcl-1 protein is seen. The Bcl-2 family member Bid is also induced by p53 and mediates p53-induced apoptosis (Sax *et al.*, 2002).

Interaction between p53 and another protein important in lymphomagenesis, c-Myc, has also been described. c-Myc can determine the choice between p53 induced cell cycle arrest or p53 induced apoptotic cell death, by blocking $p21^{WAF1}$ induction by p53, so by switching the response to DNA damage from cytostatic to apoptotic (Seoane *et al.*, 2002). Such an effect is seen in the majority of BL, which are exquisitely sensitive to chemotherapy-induced DNA damage and constitutionally overexpress c-Myc.

The importance of disruption of the p53 pathway in DLBCL is illustrated by the discovery of homozygous deletion of *CDKN2A, the* $p14^{ARF}$ locus, in 19%, *TP53* mutations in 22% and mdm2 overexpression in 43% of DLBCL examined, resulting in 62% of tumours having alterations of one or more p53 pathway components (*TP53*, $p14^{ARF}$ and mdm2) (Moller *et al.*, 1999b). Overall, 82% of DLBCLs displayed pRb and/or p53 pathway disruption (Moller *et al.*, 2000).

1.2 Diffuse Large B-cell Lymphoma

1.2.1 Pathology

Thomas Hodgkin made the first description of tumours arising within the lymphoid system in 1832 (Hodgkin, 1832). In 1864 and 1871 Virchow and Bilroth respectively referred to these illnesses as ‘lymphosarcomas’ and ‘malignant lymphomas’. Since that time it has become apparent the lymphomas are divisible into Hodgkin lymphoma and the NHLs, which together constitute the fifth commonest form of cancer in developed countries (Baris and Zahm, 2000). Several classifications of the lymphomas were developed and used in Europe and North America through the twentieth century. The World Health Organisation (WHO) classification of neoplastic diseases of the haemopoetic and lymphoid tissues is the most recent (Harris *et al.*, 1999). The lymphoid

neoplasms are divided into distinct disease entities in the WHO classification and its forerunner the REAL classification (Armitage and Weisenburger, 1998, Harris *et al.*, 1994) according to a combination of morphology, immunophenotype, genetic features and clinical syndromes. Numerous NHL subtypes are now recognised of both B-cell and T-cell origin. The B-cell NHLs constitute the major group, with diffuse large B-cell lymphoma (DLBCL) being the commonest, representing 30-40% of all NHLs, followed by FL, which constitutes 20-30% of NHLs, as illustrated in Figure 1.5. The B-cell NHLs can be segregated into low and high-growth fraction tumours. The low-growth fraction NHLs display a relatively low proliferation index due to deregulated apoptosis rather than subversion of cell cycle control genes. The consequence is cell accumulation due to the failure of cells to die. Thus the low-growth fraction NHLs tend to present with advanced stage disease, which follow an indolent treatable but incurable course. The high growth-fraction NHLs, which include DLBCL, exhibit a raised proliferative index due to alterations in cell cycle regulators in combination with apoptotic pathway defects. Clinically such tumours follow a more aggressive course, yet often present with more localised disease. A proportion is curable with radical treatment.

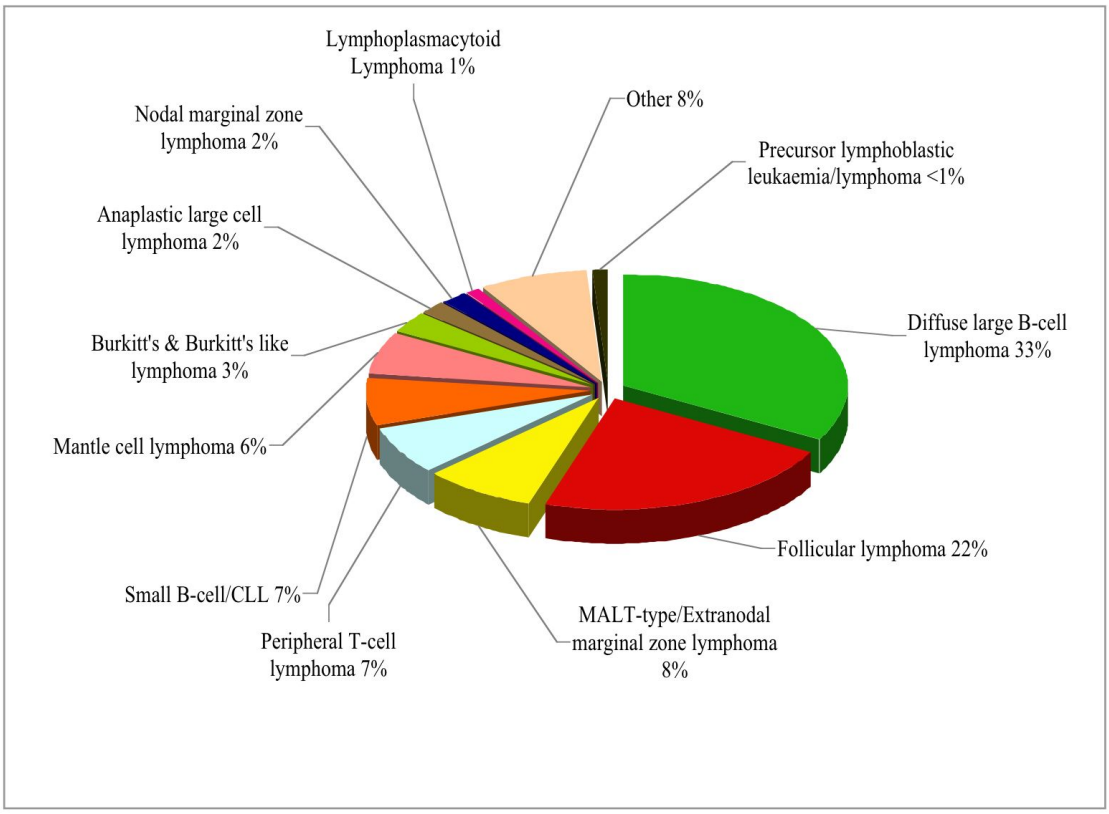


Figure 1.5 Frequency of Non-Hodgkin Lymphoma Subtypes Revised European American Lymphoma Classification Project Clinical Validation Data. More than 2000 cases of NHL from eight centres were reviewed and reclassified as the above subtypes resulting in the percentage frequencies in the Figure (Armitage and Weisenburger, 1998).

For the conclusive diagnosis of a lymphoma and assignment of a particular subtype, biopsy for histology with immunohistochemistry (IHC) is required. DLBCL supplants the normal architecture of invaded lymph nodes and extranodal sites with a diffuse pattern of large transformed lymphoid cells with various cytological appearances (Jaffe *et al.*, 2001). Despite clearly discernable variations in the microscopic appearance of DLBCL, highly reproducible histological subcategories of DLBCL have not been defined to date (Dick *et al.*, 1987). The sub-typing of DLBCL into immunoblastic and centroblastic in the Kiel classification (Lennert and Feller, 1992) was problematic in terms of reproducibility between pathologists (Harris *et al.*, 1994) and added little in the way of clinical relevance, as response to treatment and survival were usually the same for the two subtypes (Melnyk *et al.*, 1997, Salar *et al.*, 1998). The lack of clinical utility and inconsistent sub-classification in both the Kiel and Working Formulation systems

(the European and North American predecessors of the REAL classification) led to the optional, rather than compulsory, cytological subdivision of DLBCL in the REAL classification of 1994 (Harris *et al.*, 1994) and the later WHO classification, launched in 1999 (Harris *et al.*, 1999). Immunohistochemically, the malignant cells in DLBCL express some if not all of the pan-B-cell markers such as CD19, CD20, CD22 and CD79a and are negative for T-cell markers, such as CD4, CD8 and T-cell receptors. Surface and/or cytoplasmic immunoglobulin is displayed by half to three quarters of DLBCL cases. Staining for Ki-67 demonstrates the proliferative index of DLBCL to be >40% in the vast majority, and >90% in a minority (Miller *et al.*, 1994).

1.2.2 Clinical Presentation and Natural History

DLBCL can masquerade as numerous other illnesses and affect any part of the body. In the majority of patients DLBCL presents with the appearance of a swollen mass of lymph nodes in the neck, axilla or groin. Night sweats; fevers and/or weight loss - the constitutional (B) symptoms of the lymphomas - are described by a third of patients at the time of diagnosis (Armitage and Weisenburger, 1998). Three quarters of patients have at least one extranodal site involved at presentation. Left untreated, DLBCL proves fatal within months of diagnosis in the majority of patients. The more advanced the disease at diagnosis, the shorter the time until death.

The Importance of Staging

Accurate assessment of the sites and extent of disease in DLBCL is important, as the more widespread the illness, the lower the probability is of long-term survival following treatment. From the REAL classification clinical validation project, at presentation, half of patients with DLBCL have limited stage disease (stage I or II) just 13% have disease above and below the diaphragm without major organ involvement (stage III), leaving a third of patients with disseminated disease involving at least one extranodal site (stage IV disease) (Armitage and Weisenburger, 1998). Bone marrow involvement is seen in 20% at presentation in contrast to 42% of patients presenting with FL (Armitage and Weisenburger, 1998). In the 687 DLBCL cases treated within the Medical Oncology Unit, St Bartholomew's Hospital (SBH) between February 1970 and November 2003, 23% had stage I, 24% stage II, 12% stage III and 40% stage IV disease at presentation (see Table 1.1).

Response	CR/CRu	PR	OR	Fail	N/A	Total
Stage I	123 (76%)	13 (8%)	126 (84%)	19 (12%)	6 (4%)	161 (23%)
Stage II	109 (66%)	21 (13%)	130 (79%)	28 (17%)	6 (4%)	164 (24%)
Stage III	52 (62%)	13 (15%)	65 (77%)	15 (18%)	4 (5%)	84 (12%)
Stage IV	114 (41%)	42 (15%)	156 (56%)	104 (37%)	18 (6%)	278 (40%)
All	398 (58%)	89 (13%)	487 (71%)	166 (24%)	34 (5%)	687 (100%)

Table 1.1 Response According To Stage For The 687 DLBCL Patients Treated At St Bartholomew's Hospital CR/CRu= Complete Response/Complete Response unconfirmed PR= Partial Response; OR=CR/CRu + PR= Overall Response; Fail= Stable or Progressive Disease; N/A = Not available/ Not Applicable

1.2.3 Treatment

'CHOP': the Gold Standard of Care from the 1980's to 2002

A combination of four drugs, given the acronym 'CHOP' (cyclophosphamide, hydroxydaunomycin (now referred to as doxorubicin), Oncovin (more commonly referred to as vincristine), and prednisolone) was the best available treatment for DLBCL for 25 years. A standard course of CHOP therapy involves six to eight cycles administered every three weeks, side-effects permitting. The overall response (OR) rate to CHOP has been consistently in the order of 65%, with a complete response (CR) rate of $\sim$50% for advanced (stage III and IV) disease and up to 99% CR rate in limited stage (I or II) disease (Gottlieb *et al.*, 1990, Armitage *et al.*, 1984, Jones *et al.*, 1989). The response according to stage and for all 687 patients with DLBCL treated at SBH is illustrated in Table 1.1. In the 154 patients with DLBCL treated with CHOP at SBH the OR and CR/CRu (Complete Response unconfirmed) rates were 77 and 59% respectively (see Table 1.2). Through the late 1980's and 1990's numerous empirical variants of CHOP were investigated. Disappointingly, in randomised, controlled trials no novel combination or schedule produced clinically important, superior results compared to standard 3-weekly CHOP (Fisher *et al.*, 1994). At SBH a phase II study investigated a weekly multi-drug regimen called VAPEC-B in 154 newly diagnosed DLBCL patients (Radford *et al.*, 1994). The resulting CR/CRu and OR rates of 55 and

76% were identical to the 59 and 77% CR/CRu and OR rates in the 154 DLBCL SBH patients treated with CHOP (See Table 1.2).

Regimen	CR/CRu	PR	OR	Fail	N/A	Total
CHOP	91 (59%)	27 18%)	**118 (77%)**	**33 (21%)**	3 (2%)	154 (100%)
VAPEC-B	84 (55%)	33 (21%)	**117 (76%)**	**34 (22%)**	3 (2%)	154 (100%)

Table 1.2 Response According Regimen For The 154 DLBCL Patients Treated with CHOP and VAPEC-B At St Bartholomew's Hospital CR/CRu= Complete Response/Complete Response unconfirmed PR= Partial Response; OR=CR/CRu + PR= Overall Response; Fail= Stable or Progressive Disease; N/A = Not available/ Not Applicable

Despite being unbeaten in terms of percentage of long-term survivors produced, CHOP and variants resulted in only 40-50% of patients being cured of their lymphoma, as illustrated by the overall survival (OS) curves for the 687 SBH DLBCL patients and this group's CHOP and VAPEC-B subsets (Figures 1.6, 1.7 and 1.8).

Figure 1.6 Overall Survival of SBH DLBCL Patients 30 year follow-up illustrates the clinical problem of treatment failure and early relapse as the cause of the steep decline in survival in the first three years post treatment followed by a steady decline due to deaths from other causes.

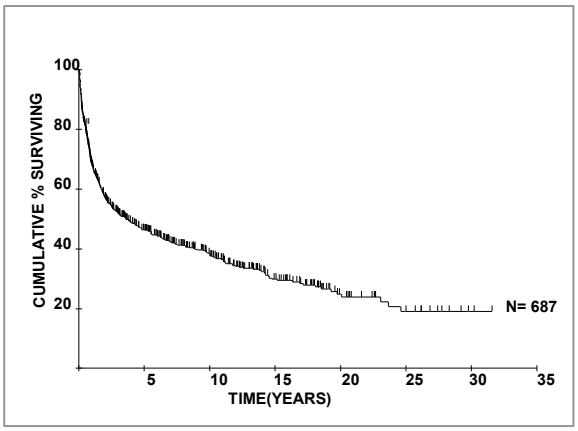


Figure 1.7 Overall Survival of SBH DLBCL Patients treated with CHOP 15-year follow-up illustrates the clinical problem of treatment failure and early relapse as the cause of the steep decline in survival in the first three years post treatment followed by a steady decline due to deaths from other causes.

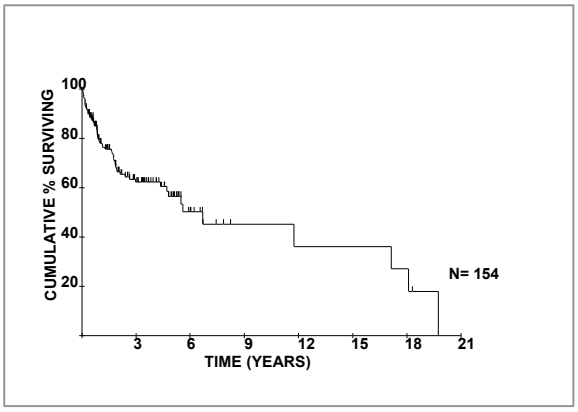
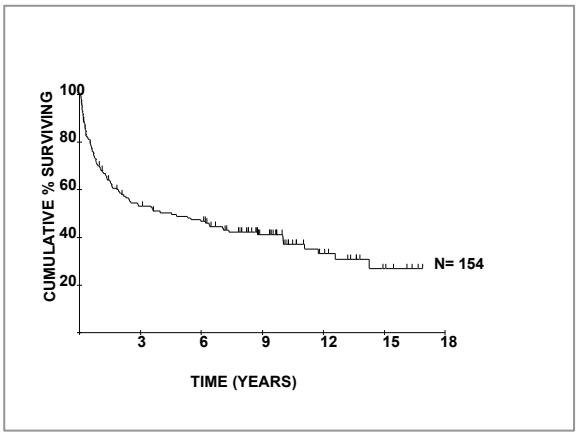


Figure 1.8 Overall Survival of SBH DLBCL Patients treated with VAPEC-B 15-year follow-up illustrates the clinical problem of treatment failure and early relapse as the cause of the steep decline in survival in the first three years post treatment followed by a steady decline due to deaths from other causes.



Improved Outcome with Newer Regimens

In the last six years six randomised controlled trials have advanced the treatment of DLBCL, four in the elderly (>60) (Coiffier *et al.*, 2002, Tilly *et al.*, 2003, Pfreundschuh *et al.*, 2004b, Pfreundschuh *et al.*, 2008) and two in the ≤60 years old (Pfreundschuh *et al.*, 2006, Pfreundschuh *et al.*, 2004a), have reported treatments superior to CHOP in terms of response rates, event-free survival (EFS) and OS. These trials investigated CHOP combined with another agent (rituximab (R) four of the trials or etoposide (E) two of the trials) and/or increased dose-intensity achieved by use of G-CSF (granulocyte colony stimulating factor) to decrease the frequency and duration of neutropenia (four of the trials).

In the first of these randomised controlled trials run by the GELA (Groupe d'Etude des Lymphomes de l'Adulte) group, CHOP plus the humanised anti-CD20 monoclonal antibody rituximab vs. CHOP alone was investigated in the elderly (Coiffier *et al.*, 2002). CD20 is a phosphoprotein present on the cell surface of nearly all B-cells. It plays a role in many cellular signalling events including activation, differentiation, proliferation and apoptosis upon cross-linking. As CD20 is not shed, internalised or present as a soluble factor in serum it became an attractive target for immunotherapy in the late 1980's and early 1990's (Stashenko *et al.*, 1980). Preclinical work confirmed that antibody blockade of CD20 produced b-cell death in malignant and benign cells (Reff *et al.*, 1994, Liu *et al.*, 1987). Phase I human studies confirmed the humanised anti-CD20 antibody rituximab as safe (Maloney *et al.*, 1997). The following Phase II trials in aggressive NHL demonstrated rituximab to have single agent activity of ≤30% (OR rate) (Coiffier *et al.*, 1998) and an OR rate when added to CHOP of 94% (CR rate of 61%) (Vose *et al.*, 2001). In the GELA phase III study of six cycles of CHOP ± R, 'CHOP-R', produced a higher CR rate of 76% vs. 63% for CHOP alone ($p = 0.005$) and significantly better 2-year EFS of 57% vs. 38% ($P < 0.001$) and 2-year OS of 70% vs. 57% ($p = 0.007$) for CHOP-R vs. CHOP alone (Coiffier *et al.*, 2002). Unlike many trials where data is released early, the significance of the OS results has increased with time. Furthermore, CHOP-R overcame the resistance to chemotherapy associated with Bcl-2 protein overexpression, improving OR rate from 60 to 78% ($P = 0.01$) and 2-year OS from 48 to 67% ($P = 0.004$) in those with Bcl-2 positive tumours (>50% tumour cells expressing Bcl-2) (Mounier *et al.*, 2003). Multivariate analysis confirmed the significant benefit for survival and EFS of CHOP-R in Bcl-2+ patients. Rituximab

appears to overcome Bcl-2 overexpression through inhibition of the NFKB and ERK 1/2 pathways and by causing increased expression of the pro-apoptotic proteins Bax and Bak and down regulation of the anti-apoptotic factors Bcl-x_L, Mcl-1 and Apaf-1 in addition to Bcl-2 itself (Olejniczak *et al.*, 2008, Jazirehi *et al.*, 2005, Jazirehi *et al.*, 2004, Jazirehi *et al.*, 2003). Rituximab has been confirmed to overcome the negative prognostic effect of Bcl-2 overexpression in several other patient series/trials (Wilson *et al.*, 2008, Wilson *et al.*, 2007, Shivakumar and Armitage, 2006).

The subsequent MinT (MabThera International Trial) study confirmed the better efficacy of six cycles of CHOP-R like treatment over CHOP-like chemotherapy in those $\leq$60 years old with good prognosis DLBCL (Pfreundschuh *et al.*, 2006).

In the first positive study of dose intensification, an aggressive chemotherapy induction regimen (ACVBP 3-weekly with G-CSF support (doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone including CNS prophylaxis)) followed by sequential consolidation (2 courses high-dose methotrexate plus leucovorin rescue, four courses of etoposide and ifosfamide and two courses of cytosine-arabinoside – all fortnightly with G-CSF support) was compared to eight cycles of standard CHOP. The trial was conducted in 708 poor-risk, elderly patients (age-adjusted IPI $\geq$1; see page 65) with aggressive NHL (80% DLBCL) (Tilly *et al.*, 2003). Despite treatment-related mortality being 13% vs. 7% (P = 0.1) and the CR rates being similar (58% vs. 56%) in the ACVBP and CHOP groups respectively, both 5-year EFS (39% vs. 29%, P = 0.005) and OS (46% vs. 38%, P = 0.036) were significantly longer for the ACVBP patients compared to the CHOP patients. CNS recurrence was significantly more frequent in the CHOP group (8% overall, 9% in DLBCL patients) compared to the ACVBP group (3% overall, 3% in DLBCL patients), P = 0.004. Recent results of a phase II GELA investigation of four cycles of fortnightly R-ACVBP with pegfilgrastim support followed by BEAM autograft have been published in abstract form (Belhadj *et al.*, 2007). The CR/CRu rate post R-ACVBP induction was 63% and the PR rate 25% with 3% (2 patients) dying during the induction phase. Seventy three% experienced febrile neutropenia. After a median follow-up of two years, 2-year PFS was 71% (95% confidence interval (CI)=61–84), and OS was 91% (95% CI=74–97). A longer follow-up is needed to see whether the addition of rituximab definitely decreases the relapse rate cf. ACVBP alone.

The other two studies of dose intensification of chemotherapy compared CHOP with CHOEP (CHOP plus etoposide, the long-established topoisomerase II inhibitor) ± the haemopoietic growth factor G-CSF in a '2 x 2' multifactorial design (Pfreundschuh *et al.*, 2004b). The use of G-CSF lessened the frequency and duration of neutropenia, allowing the treatment interval to be shortened to 14 days from the standard 21 days. Thus the 689 patients aged 61-75 years old in the NHL-B2 trial were randomised to receive six cycles of CHOP-21, CHOP-14, CHOEP-21 or CHOEP-14. The CR rates were CHOP-14 (76.1%), CHOP-21 (60.1%), CHOEP-14 (71.6%) and CHOEP-21 (76.1%). The toxicity of CHOP-14 was similar to that of CHOP-21, whilst CHOEP-21 and particularly CHOEP-14 were more toxic. CHOP-14 produced significant 5-year event-free survival (EFS) and OS advantages compared to standard CHOP-21: EFS 43.8% vs. 32.5% respectively (relative risk reduction = 0.66, $P = 0.003$) and OS 53.3% vs. 40.6% respectively (relative risk reduction = 0.58, $P < 0.001$). CHOEP-21 produced a non-significant improvement in OS compared to CHOP-21 whilst CHOEP-14 was poorly tolerated and had similar OS to CHOP-21. The German High-Grade Non-Hodgkin Lymphoma Study Group therefore recommended CHOP-14 as the new standard of care in the elderly (>60 years old).

As in their elderly patient study, the German High-Grade Non-Hodgkin Lymphoma Study Group's NHL-B1 trial in 710 patients aged 18-60 years old with good prognosis DLBCL (normal LDH) saw patients randomised to receive six cycles of CHOP or CHOEP given every 21 or 14 days with G-CSF support (Pfreundschuh *et al.*, 2004a). CHOEP achieved a better CR rate (87.6% vs. 79.4%; $P = 0.003$) and 5-year EFS rate (69.2% vs. 57.6%; $P = 0.004$, the primary endpoint) than CHOP. Interval reduction from 21 to 14 days improved OS ($P = 0.05$; $P = 0.044$ in the multivariate analysis). All regimens were well tolerated, although the CHOEP regimens induced more myelosuppression. The German High-Grade Non-Hodgkin Lymphoma Study Group therefore recommended CHOEP as the new standard of care in the good prognostic under-60's.

The outstanding question following the demonstration of superior survival through dose intensification and addition of rituximab was would the combination of these two strategies bring a further incremental improvement in survival? The answer to this question has started to emerge and from the single published study of this approach is positive.

The German High-Grade Non-Hodgkin Lymphoma Study Group RICOVER-60 trial randomly assigned 1,222 elderly patients (>60 years old) to six or eight cycles of CHOP-14 with or without rituximab (Pfreundschuh *et al.*, 2008). Using multivariate analysis (after adjusting for known prognostic factors) and six cycles of CHOP-14 without rituximab as the reference, all three intensified regimens improved 3-year EFS, both R-CHOP-14 regimens improved PFS but only six cycles of R-CHOP-14 improved OS. The 3-year EFS were 47.2% after six cycles of CHOP-14 (95% CI 41.2-53.3) and 66.5% (95% CI 60.9-72.0) after six cycles of R-CHOP-14, a 19.3% increase (relative risk reduction 0.51; 95% CI 0.40-0.65, P <0.0001). The 3-year OS was 67.7% (95% CI 62.0-73.5) for six cycles of CHOP-14 and 78.1% (95% CI 73.2-83.0) for six cycles of R-CHOP-14, a 10.4% advantage (relative risk reduction 0.63; 95% CI 0.46-0.85, P = 0.0031). In patients with a partial response after four cycles of chemotherapy, eight cycles were not better than six cycles. The German High-Grade Non-Hodgkin Lymphoma Study Group has therefore reasonably concluded that six cycles of R-CHOP-14 is the new standard of care for elderly patients with DLBCL. The UK R-CHOP 14 vs. 21 has addressed the same question and its results are awaited.

Not only have these studies resulted in improvements in responses and survival in DLBCL but they also suggest that the long recognised clinical and biological heterogeneity of DLBCL may respond to, and require different, scheduling of treatment to allow optimisation of response. Still to be clarified is which patients don't need/benefit from rituximab and which if any need a dose intensified schedule akin to ACVBP as first-line treatment?

Retreatment of DLBCL

The introduction of high-dose chemotherapy treatment with autologous stem cell rescue to retreatment strategies has improved outcome, producing durable long-term remissions in half of responders to retreatment (Philip *et al.*, 1995). However, only half of patients respond to standard-dose retreatment and many patients are not fit enough to safely undergo high-dose treatment. Consequently, less than half of all relapsing patients undergo high-dose consolidation, resulting in less than 25% of patients becoming long-term survivors after retreatment at first relapse. Therefore the majority of patients who relapse or fail to achieve remission from their DLBCL will die of their lymphoma (or its treatment). This sequence of events explains the 5-year overall and failure-free survival figures of 46% and 41% respectively derived from the ~400

DLBCL patients surveyed by the REAL classification clinical validation project (Non-Hodgkin's-Lymphoma-Classification-Project, 1997). What is clear is that CR/CRu patients are more likely than PR patients and especially stable/progressive disease patients to achieve long-term remission from their DLBCL when consolidated with high-dose treatment. Addition of rituximab to combination second-line chemotherapies appears to improve response rates and critically CR rates from single-arm phase II studies (Joyce *et al.*, 2003). In a subsequent randomised phase III trial those randomised to rituximab plus chemotherapy (2 cycles of DHAP (cisplatin-cytarabine-dexamethasone)-VIM (etoposide-ifosfamide-methotrexate)-DHAP) achieved a higher ORR of 75% vs. 54% in the chemotherapy alone arm (P = 0.01). After two chemotherapy courses responding patients were eligible for autologous stem-cell transplantation. At 24 months median follow-up there was a significant difference in progression-free survival (PFS) of 52% vs. 31% $P < 0.002$) for those in the rituximab chemotherapy vs. chemotherapy alone arm (Vellenga *et al.*, 2008).

Further confirmation of the superior outcome produced by adding rituximab to pre-autologous transplant chemotherapy in relapsed/refractory DLBCL comes from a review of the Center for International Blood and Marrow Transplant Research (CIBMTR) database (Fenske *et al.*, 2007). Between 1996 and 2003 1,006 such patients were reported to the CIBMTR, of which 188 received R-chemotherapy and 818 chemotherapy alone, prior to their autologous transplant. PFS at one and three years was superior in the R-chemotherapy group (62% vs. 49% at 1 year, P = 0.002; 49% vs. 38% at three years, P = 0.010). OS was also superior in the R-chemotherapy group (68% vs. 60% at 1 year, P = 0.032; 57% vs. 45% at three years, P = 0.003). In multivariate analysis, pre-transplant R, age <55, and fewer than three lines of chemotherapy were associated with improved PFS and OS.

Treatment Conclusions

DLBCL is highly responsive to chemotherapy, however just less than half of patients became long-term survivors pre-rituximab and dose intensification of CHOP/CHOEP. In those that experience relapse of their DLBCL, long-term EFS in response to retreatment is unlikely without high-dose consolidation of a CR/CRu. The heterogeneous clinical presentation of DLBCL is thus also apparent in terms of the response DLBCL to treatment and curability. There is therefore a pressing need for means of dividing DLBCL into robust and biologically meaningful homogeneous

subtypes for which considerably more accurate prognostic factors and predictors of response can be discovered and novel treatments pioneered.

1.2.4 Epidemiology of DLBCL and NHL

NHL, without Hodgkin lymphoma, is now the sixth commonest cancer (excluding non-melanomatous skin cancers, which account for half of all tumours) in the UK and the rest of the developed world, constituting 3% of the total non-skin cancer incidence and mortality in the UK in 1999 and 2001 respectively (CR-UK, 2003, Ferlay *et al.*, 2001). This amounts to 9,000 new cases and 4,600 deaths per annum (CR-UK, 2003). Diffuse large B-cell lymphoma (DLBCL) is the most frequently seen subtype of NHL in the developed world, accounting for up to 40% of NHL diagnoses (Anderson *et al.*, 1998, Armitage and Weisenburger, 1998). The incidence of DLBCL is currently 5/100,000 adults per annum and has been rising at 4% per annum since the 1950's (Weisenburger, 1994). More males than females develop NHL and DLBCL (Ferlay *et al.*, 2001, Ferlay *et al.*, 1998). The predominance of male patients with DLBCL at 55% is less than seen for NHL overall (62%) (Armitage and Weisenburger, 1998). of the 687 DLBCL patients treated at SBH 379 (55%) were male.

In the retrospective clinical validation of the REAL classification (conducted in nine lymphoma centres on four continents), the median age of those affected by DLBCL (422) was 64 years (range 14 – 83) (Armitage and Weisenburger, 1998). Globally, DLBCL makes up a similar proportion of NHL cases from one continent to the next, despite the fluctuation in FL incidence (Anderson *et al.*, 1998). In terms of racial distribution, DLBCL is seen more frequently in white Americans than black, or Hispanic Americans (SEER and Surveillance, 2002).

1.2.5 Clinical Aetiolology of DLBCL

Inherited Risk Factors for Lymphoma: Familial Lymphoma

Familial lymphoma can be seen as part of the Li-Fraumeni syndrome, caused by germ-line mutations in *TP53*, the gene encoding the p53 protein. However, in the majority of familial cases of lymphoma the relative risk for a first-degree relative of an affected case is raised only 2-3 fold compared to that of the general population (cf. 25-fold in Li-Fraumeni families (Evans and Lozano, 1997)) and mutations in multiple rather than single genes would appear to underlie the predisposition with no descriptions of *TP53* germline mutations (Last *et al.*, 2000, Weintraub *et al.*, 1996, Potzsch *et al.*, 1999).

DLBCL and Oncogenic Infections

The causes of DLBCL in the general population remain unknown. Despite repeated screens for oncogenic viruses - spurred on by the consistent observation of Epstein-Barr virus (EBV) in virtually all cases of endemic BL (Xue *et al.*, 2002) – no infectious agent has been found in the majority of DLBCLs. EBV infection of normal B-lymphocytes results in their polyclonal activation *in vivo* (Thorley-Lawson and Gross, 2004). This manifests itself clinically as infectious mononucleosis or glandular fever. *In vitro*, EBV infection causes B-cell immortalisation. Where T-lymphocytes are concurrently immunosuppressed, as is the case in many West African children due to coincident malarial infection, polyclonal B-cell proliferation proceeds unchecked. This increases the probability of t(8;14)(q24;q32) translocation development, which in turn heralds the evolution of BL. EBV is found in the malignant cells of 33% of Hodgkin lymphoma, where late first exposure to EBV is a significant risk factor for EBV+ Hodgkin lymphoma (Alexander *et al.*, 2003). In DLBCLs, EBV is aetiologically related but only in the context of immunosuppression as detailed below. Two other viruses have been linked with DLBCL outside of the context of HIV infection: SV40 and hepatitis C.

The polyomavirus simian virus 40 (SV40) is a potent oncovirus in laboratory animals, where tumours of the brain, mesothelium, bone and lymphoid system develop following inoculation with SV40 (Vilchez and Butel, 2003). Collation of data from the more 'stringent' studies investigating the presence of SV40 DNA in human cancers has revealed the presence of SV40 more frequently in human NHLs, mesotheliomas, brain and primary bone tumours than in corresponding tissue from controls (odds ratios and 95% CI of 5.4 [3.1 – 9.3], 17 [10 –28], 3.9 [2.6 – 5.8], 25 [6.8 – 88] respectively) (Vilchez *et al.*, 2003). In contrast to the 40% incidence of SV40 in US NHL, not one of the 152 UK NHL samples examined carried SV40 DNA nor was exposure to SV40-contaminated poliovirus vaccine in childhood associated with an increased cancer or NHL incidence in Denmark (Engels *et al.*, 2003, Vilchez *et al.*, 2002, MacKenzie *et al.*, 2003). A possible explanation for this major discrepancy comes from a recent study of mesotheliomas where the 60% incidence of SV40 in the samples was due entirely erroneous. False-positive results accounted for the findings due to contamination by common laboratory plasmids containing SV40 sequences (Lopez-Rios *et al.*, 2004).

A recent meta-analysis of 48 case-control studies, comprising 5,542 patients, found the prevalence of hepatitis C in patients with B-cell NHL to be ~15%. This is higher than that reported in both the general population (1.5%) and in patients with other

haematological malignancies (2.9%) (Gisbert *et al.*, 2003). Hepatitis C may therefore play a role in the aetiology of B-cell NHL whilst SV40 probably does not.

DLBCL and Chemical Exposures

Pesticides and hair dyes have been implicated in the aetiology of DLBCL but far from conclusively so, as the associations, when present, have been weak (McDuffie *et al.*, 2001, De Roos *et al.*, 2003, Cantor *et al.*, 2003, Altekruse *et al.*, 1999). The most recent population-based case-control study was conducted in Connecticut between 1996 and 2002 (Zhang *et al.*, 2004b). A small increased risk of NHL was observed among women who began use of hair dyes before 1980, but not afterwards (odds ratio (OR) = 1.3, 95% CI = 1.0 - 1.8).

DLBCL and Chronic Immunosuppression

Chronic immunosuppression and/or immune stimulation are risk factors for DLBCL development for reasons still to be fully elucidated. The lymphoproliferative disorders that develop in the immunosuppressed tend to be of B-cell origin and diffuse large cell histology, to present with extranodal site involvement and to be associated with EBV. The dysfunctional T-cell activity seen in immunodeficiency states appears crucial for EBV+ B-cells to proliferate and transform.

Congenital Immunodeficiency Syndromes and Autoimmune Disease

In those with congenital immunodeficiencies the incidence of NHL ranges from 0.7% in those with X-linked agammaglobulinaemia to 12-15% in patients with ataxia telangiectasia (Oertel and Riess, 2002). The relative risk of DLBCL is increased 2-fold in patients with the autoimmune disease rheumatoid arthritis rising to an OR of 26-fold in those with high levels of inflammatory activity compared to those with low inflammatory activity (OR = 1) (Beauparlant *et al.*, 1999, Baecklund *et al.*, 1998). 67% of the NHLs seen in rheumatoid arthritis are of the DLBCL subtype, with only 2% being EBV+ outside the setting of methotrexate immunosuppression where up to 30% are EBV+ and can be considered to be post-transplant lymphoproliferative disorders (Kamel *et al.*, 1999, Dawson *et al.*, 2001, Baecklund *et al.*, 2003).

DLBCL in the Context of HIV Infection

In HIV positive individuals the incidence of aggressive lymphoma is much higher than in the general population, with a prevalence of 5.4% in European AIDS sufferers in 2000 (Dal Maso and Franceschi, 2003). The relative risk of developing aggressive NHL (principally DLBCL of immunoblastic subtype, Burkitt and Burkitt-like NHL) in the

HIV+ has been up to 400-fold that seen in the general population (Dal Maso and Franceschi, 2003). The relative risk has dramatically reduced since the introduction of effective antiretroviral drug combinations in the mid 1990's, the so-called HAART (highly active antiretroviral therapy) era. In Denmark the incidence of NHL has fallen from 1.99 cases/100 person years of prospective follow-up before September 1995 to just 0.3 cases/100 person years of prospective follow-up after March 1999 (Kirk *et al.*, 2001). Not surprisingly, after starting HAART, poor response, in terms of failure of CD4+ count to rise and HIV viral load to fall, predicts for those most at risk of NHL development (Kirk *et al.*, 2001). Although the survival of AIDS-related NHL patients has improved from 6 to 20 months, AIDS-related NHL still remains incurable in all but a few (Besson *et al.*, 2001).

DLBCL in the Context of Allogeneic Transplantation

In those immunosuppressed to prevent allogeneic transplant rejection and graft versus host disease, DLBCL is seen at the aggressive end of a spectrum of EBV driven lymphoproliferative disorders collectively termed post-transplant lymphoproliferative disorders (PTLPDs) (Jaffe *et al.*, 2001, Nalesnik, 2002). The cumulative incidence of PTLPDs rises from 1.0% +/- 0.3% at 10 years following allogeneic bone marrow transplantation to 8% in recipients of allogeneic lung transplants (Curtis *et al.*, 1999, Armitage *et al.*, 1991). The WHO classification splits the PTLPD spectrum into four categories of increasing severity: plasmacytic hyperplasia; polymorphic PTLPDs; the uncommon Hodgkin lymphomas and Hodgkin lymphoma-like PTLPDs; and monomorphic PTLPDs, the majority of which are DLBCLs (Jaffe *et al.*, 2001). The progression from polyclonal, plasmacytic hyperplasia to monoclonal DLBCL graphically illustrates the multi-step nature of lymphomagenesis in the EBV+ immunosuppressed, and like the similar progressive spectrum of lymphoproliferations seen in mice overexpressing Bcl-2, illustrates that EBV alone is insufficient to render a normal B-cell lymphomatous (Strasser *et al.*, 1990). The oncogenic drive provided by EBV is confirmed by the finding that 30-50% of EBV-naïve patients who seroconvert after lung transplantation develop a PTLPD; a calamity preventable in most by continuous anti-viral prophylaxis (Malouf *et al.*, 2002).

Potential Protective Factors Against NHL Development

A recent study found plant or animal allergies, previous mononucleosis and receipt of $\geq$5 vaccinations to be protective against DLBCL development (Holly and Bracci, 2003).

Prescription medications associated with a protective effect against NHL include benzodiazepines (OR 0.34; 95% CI 0.18, 0.64), and H(2) blockers (OR 0.29; 95% CI 0.12, 0.69) and, in women, non-steroidal anti-inflammatory drugs (NSAIDs) (OR 0.40; 95% CI 0.22, 0.71) (Beiderbeck *et al.*, 2003b, Beiderbeck *et al.*, 2003a). Calcium channel blocker use was inversely associated with DLBCL incidence (OR = 0.35) and NSAIDs use was inversely associated with FL risk (OR = 0.67) (Holly and Bracci, 2003). Results from the recent European EPILYMPH Case-Control Study involved included 2,362 cases of incident B- and T-cell lymphoma and 2,206 hospital or population controls from six European countries suggest that regular statin use decreases the risk of lymphoma (Fortuny *et al.*, 2006). Information was collected via face-to-face interviews on drug use, diagnosis at admission (for hospital controls), and putative risk factors for lymphoma. The odds ratio for regular statin use was 0.61 (95% CI, 0.45-0.84), with all major lymphoma subtypes showed similarly decreased risks. Duration of statin use was not associated with a greater reduction in the risk of lymphoma and decreased risk of developing lymphoma was observed in all centres. Replication of these findings is desirable.

Transformation of an Indolent Lymphoma

Transformation of follicular and other indolent lymphomas to DLBCL was thought to account for up to a third of all cases of DLBCL. This was suggested by the presence of the hallmark translocations of follicular (17-30%), and lymphoplasmacytoid (LPCL) (1.6%) lymphomas in 20-30% of DLBCLs (Weiss *et al.*, 1987, Dalla-Favera *et al.*, 1994, Cigudosa *et al.*, 1999). In *BCL-2/IgH*+ FL, disruption of cell cycle regulators such as *TP53*, *c-Myc* and $p16^{INK4A}$ appear to be important transformation events. Recent data however suggest that transformation of FL to DLBCL occurs by two distinct processes: direct evolution in about half of cases and FL and DLBCL arising from a common precursor cell in the other half of cases (Carlotti *et al.*, 2007). The aetiology of indolent lymphomas (beyond *Helicobacter pylori*, *Chlamydia psittaci*, *Borrelia burgdorferi* and *Campylobacter jejuni* as the causative pathogen of gastric extranodal marginal zone lymphoma of Mucosa-Associated Lymphoid Tissue (MALT) type, ocular adnexal lymphomas, skin MALT-type NHLs, and small intestine MALT-type NHLs respectively (Cerroni *et al.*, 1997, Lecuit *et al.*, 2004, Ferreri *et al.*, 2004, Lehours *et al.*, 2004)) remains obscure, as do the processes that engender transformation itself.

In summary, rare germ-line mutations predispose to DLBCL directly or by causing congenital immunosuppression; acquired immunosuppression, often driven by EBV, predisposes to DLBCL but such cases account for <5% of the total DLBCL incidence; hepatitis C virus infection may predispose to DLBCL; no chemical exposure has been strongly linked to DLBCL development and transformation of an indolent NHL into DLBCL may account for a third of DLBCLs. The aetiology of DLBCL therefore remains poorly understood in all but a small minority of cases.

1.2.6 Prognostic Factors in DLBCL and other Lymphomas

Definition of Prognostic And Predictive Factors

Prognostic factors estimate the risk of relapse or death, allowing the stratification of patients according to the outcome measures chosen, be they response to therapy, duration of remission or OS. Prognostic factors are associated with the metastatic potential and/or the growth rate of the tumour. Predictive factors are able to foretell the response to a particular treatment due to the existence of a clearly-defined relationship between the factor and the mechanisms underlying response to the intervention. Predictive factors are associated with relative sensitivity and/or resistance to specific therapeutic agents. A factor can be both prognostic and predictive. A poor prognostic factor separates poor from favourable groups independent of treatment. The outcome of the groups is equally separated regardless of whether the patient receives no therapy or is treated with a systemic treatment, as is illustrated in Figure 1.9. For a purely predictive factor, in the absence of the specific treatment the factor is predictive for, patient outcome is the same for those patients positive and negative for the marker in question. When the specific treatment is used for which the factor predicts sensitivity or resistance, the group that score highly for the predictive factor fare better than those that score low, as depicted in Figure 1.10 for a visual depiction of a pure predictive factor. Truly predictive factors are rare. In the lymphomas, CD20 expression has no prognostic significance but is a predictive factor for rituximab efficacy (as CD20 can not bind to the CD20 negative B-cell lymphomas and so by can have no possibility of activity against them). Beyond NHLs, *K-Ras* and epidermal growth factor receptor (*EGFR*) gene mutation status have proven to be positive predictive factors for cetuximab in colorectal cancer (as single agent or in combination with chemotherapy (Van Cutsem *et al.*, 2008)) and gefitinib therapy as single agent treatment in non-small cell lung cancer (Paez *et al.*, 2004, Lynch *et al.*, 2004). A mixed prognostic and predictive factor

separates groups in the absence of the specific treatment it is predictive for (its prognostic ability) and goes on to separate the low and high expressing/scored groups much more in the presence of the relevant therapy. In Figure 1.11 the factor is both a favourable prognostic and predictive factor. A factor can be a poor prognostic factor and a favourable predictive factor. The best example of such a predictive and prognostic factor in routine use in oncology is of Her2/neu protein overexpression (as determined by IHC) or *Her2/neu* gene amplification (as detected by FISH), which is a poor prognostic factor and accurately predicts for response to trastuzumab, a humanised anti-Her2/neu monoclonal antibody (Ross *et al.*, 2003)

Description of Individual Prognostic Factors in DLBCL

The prognostic factors discovered in DLBCL can be grouped into the following categories: patient characteristics independent of their lymphoma diagnosis, macroscopic disease characteristics, parameters related to the patient-lymphoma interaction, parameters related to the patient's doctor, biological disease characteristics and molecular disease characteristics. Each type will be reviewed in turn. The number of potential prognostic factors in DLBCL has increased rapidly over the last two decades. However, most still await further investigation to enter standard clinical care.

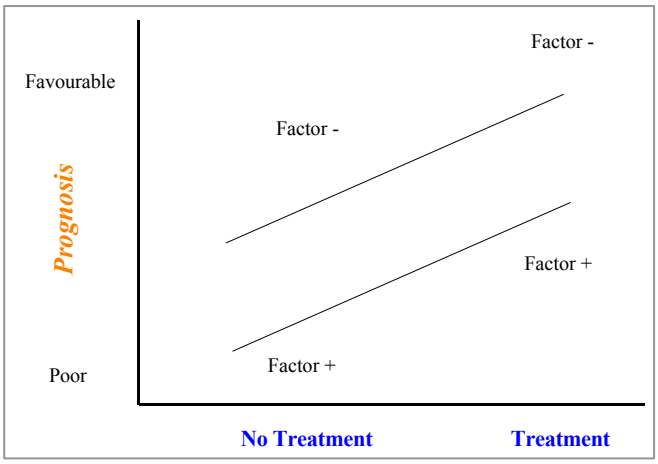


Figure 1.9 Graphical Illustration of a Pure Prognostic Factor Presence of the poor prognostic factor reduces prognosis equally in the untreated and treated

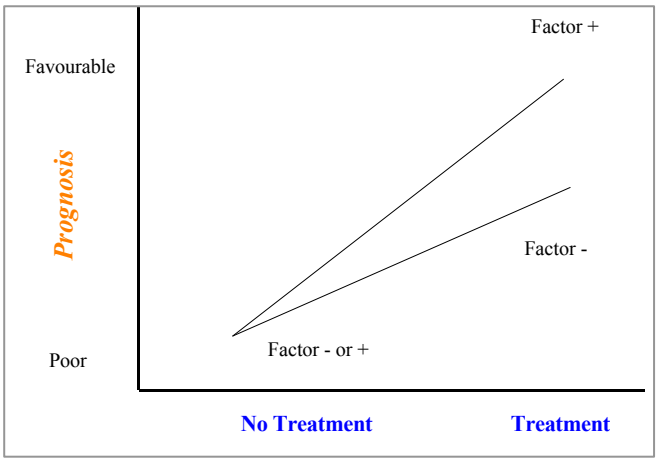


Figure 1.10 Graphical Illustration of a Pure Predictive Factor Presence of the positive predictive factor has no effect on the prognosis of the untreated but its presence significantly improves prognosis upon treatment

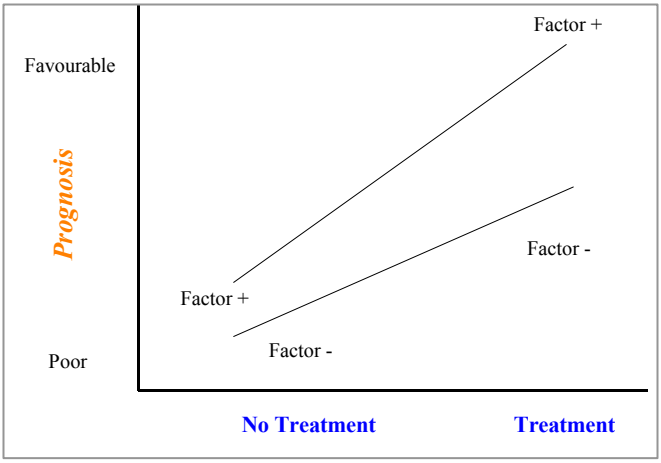


Figure 1.11 Graphical Illustration of a Mixed Prognostic and Predictive Factor Presence of the good prognostic and predictive factor improves prognosis in the untreated (its prognostic effect) and results in a further improvement in prognosis upon treatment for those that express the factor compared to those without it (its predictive effect).

Patient characteristics independent of their DLBCL

Age has been repeatedly demonstrated to be a poor prognostic risk factor in DLBCL with older patients (>60 or >65 years old) responding less well to treatment and relapsing more often, due to reduced dose-delivery and being more likely to die during the first cycles of treatment than younger patients (Vose *et al.*, 1988). Of the 687 DLBCL patients treated at SBH, 370 were < 60 years old at diagnosis and 317 were ≥ 60 years old. The OR and CR/CRu rates were 76 and 64% and 66 and 51% for the <60 and ≥60 years old respectively. Their OS according to age ≤ or > 60 years old is illustrated for all 687 patients in Figure 1.12 and Figures 1.13 and 1.14 for the subsets of CHOP and VAPEC-B treated patients respectively. HIV infection is the other profound negative prognostic factor in patients with DLBCL, changing the disease from potentially curable to a fatal diagnosis even with HAART and better-tolerated chemotherapy regimens (Besson *et al.*, 2001).

Patient-Lymphoma Related Prognostic Factors

Presence of B symptoms (night sweats, fevers >38^{0}C, weight loss >10% of body weight in preceding 6 months) has been linked to adverse outcome in DLBCL compared to their absence. A more reliable prognostic factor subsequently discovered is performance status, whether assessed by the Eastern Co-operative Oncology Group (ECOG) or Karnovsky indices (Shipp *et al.*, 1990). The two other well-recognised prognostic factors in this category are haemoglobin and serum albumin level (Conlan *et al.*, 1991, Dhaliwal *et al.*, 1993).

Treatment-Related Prognostic Factors

Although easily overlooked in such an introduction of prognostic factors, the effect treatment choice has on outcome can be profound e.g. the choice of CHOP-R or CHOP in those less than 60 years old and the elderly patients suffering from DLBCL (Pfreundschuh *et al.*, 2008, Coiffier *et al.*, 2002, Pfreundschuh *et al.*, 2006); or the decision of physician and patient to dose-reduce therapy, as dose-intensity of chemotherapy has been shown to have prognostic significance for adriamycin-based treatment, with delivery of >75% of the adriamycin dose being the single most important predictor of survival (Kwak *et al.*, 1990). The latter example has recently been borne out by the improved outcome following treatment with day-14 vs. day-21 CHOP and CHOEP in the NHL-B1 study and 14-day CHOP vs. 21-day CHOP in the NHL-B2 study (Pfreundschuh *et al.*, 2004a, Pfreundschuh *et al.*, 2004b).

Figure 1.12 Overall Survival of SBH DLBCL Patients According to Age Patients ≥60 years old have a significantly worse overall survival compared to patients <60 years old both before and following five years of follow-up.

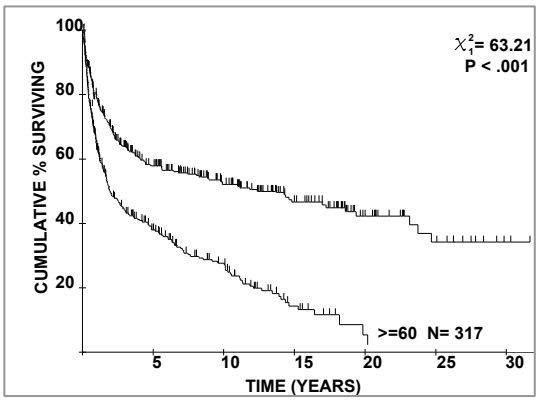


Figure 1.13 Overall Survival of SBH DLBCL Patients treated with CHOP According to Age. Patients ≥60 years old have a significantly worse overall survival compared to patients <60 years old following CHOP chemotherapy.

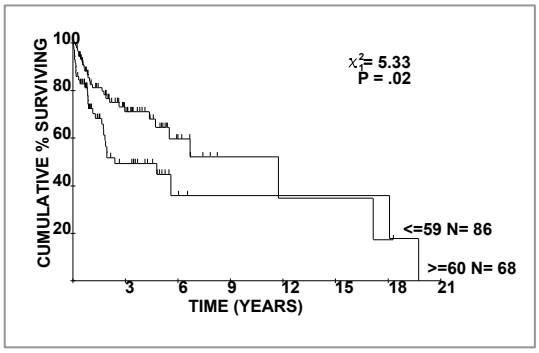
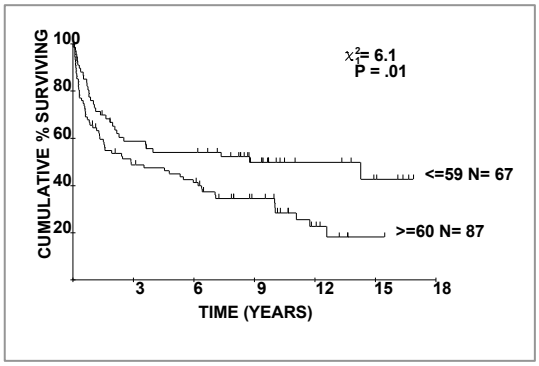


Figure 1.14 Overall Survival of SBH DLBCL Patients treated with VAPEC-B, According to Age Patients ≥60 years old have a significantly worse overall survival compared to patients <60 years old following VAPEC-B chemotherapy.



Disease-Related Serum Prognostic Factors

Lactate dehydrogenase (LDH) is measurable in serum by virtue of its release from the cytoplasm of lysed cells. In lymphomas and certain other tumours, LDH is a surrogate marker of cell turnover and tumour mass. Elevated serum LDH has been consistently demonstrated to be an adverse prognostic factor in DLBCL (Coiffier and Lepage, 1989). Similarly elevated serum β-2 microglobulin >3 mg/ml is associated with reduced OS in patients with large cell lymphomas (Swan *et al.*, 1989). Subsequently, elevated serum levels of nm23-H1 have been demonstrated to be a poor prognostic factor in both indolent and aggressive (majority DLBCL) lymphomas (Niitsu *et al.*, 2001a, Niitsu *et al.*, 2001b). nm23-H1 is an inhibitor of differentiation and is normally expressed in lymphoid tissue.

Prognostic Macroscopic Disease Characteristics

Increasing stage has proven to be an adverse prognostic factor in almost all large studies of DLBCL e.g. (Coiffier *et al.*, 1991). In the SBH DLBCL patients, response and OS according to stage are illustrated in Table 1.1 and Figure 1.15 respectively. It can be seen that increasing stage is associated with falling CR/CRu and OR rates: stage I-III vs. stage IV OR rates of 81% vs. 56%. The two other macroscopic disease characteristics with adverse prognostic significance in DLBCL are presence of extranodal disease and presence of tumour bulk. Increasing number of extranodal sites is a strong negative prognostic factor and is considered to reflect greater metastatic ability of certain DLBCL (Coiffier and Lepage, 1989). Bulky tumour mass ($\geq$10 cm) was an adverse prognostic factor in a series of 94 *de novo* DLBCL, in addition to the IPI (see page 65) and Bax protein expression (Sohn *et al.*, 2003).

Cytogenetic Prognostic Factors

In a multivariate analysis of 104 cases of presentation diffuse large cell lymphoma, breaks at 1q21-23 and presence of more than four marker chromosomes were found to be significant, independent estimators of reduced OS (Offit *et al.*, 1991). Breaks at 6q21-25 predicted for a decreased probability of achieving remission, whilst patients with breaks at 1q21-23 or 1p32-36 had a shorter duration of complete remission. The presence (or absence) of *BCL-2* or *c-Myc* translocations in DLBCL has not been found to have prognostic significance (Kramer *et al.*, 1998). *BCL -6* translocations have been variably associated with better, worse and no different OS: *BCL -6* translocation, found in 25% of cases was an adverse prognostic factor in the series examined by Barrans *et*

al (Barrans *et al.*, 2002b); presence of a 3q27 translocation was a positive prognostic factor in the group of patients investigated by Offit *et al* (Offit *et al.*, 1995); whilst Kramer *et al* found no prognostic power attached to the presence of a 3q27 translocation vs. germline DLBCL (Kramer *et al.*, 1998).

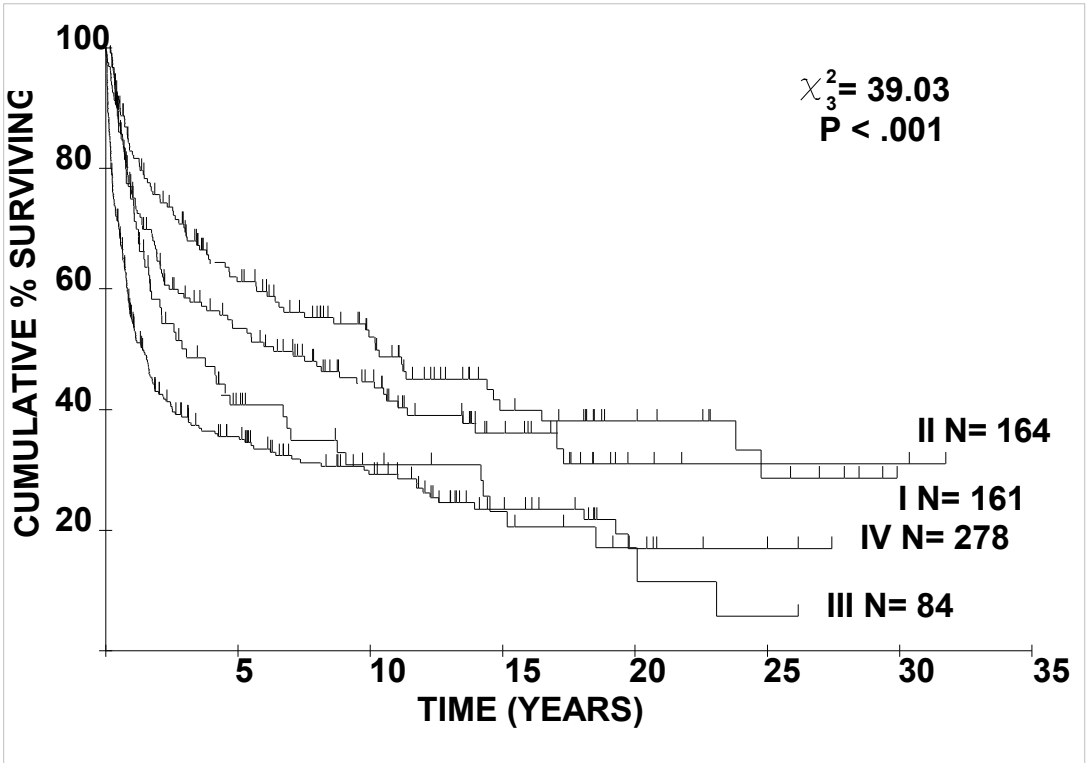


Figure 1.15 Overall Survival of SBH DLBCL Patients According to Stage

Molecular Prognostic Factors: Gene Mutations

Mutation of *TP53* appears to be a poor prognostic factor in DLBCL. In the 61 patients with a low/low-intermediate IPI (see page 65) reviewed by Leroy *et al*, *TP53* mutated DLBCL was associated with a significantly reduced OS on multivariate analysis, as it was in the NHL series examined by Moller *et al* (Moller *et al.*, 1999a, Leroy *et al.*, 2002). Similarly, mutated *TP53* was revealed as a poor predictive factor in terms of response (33% CR rate vs. 91% in *TP53* mutated and wild-type cases respectfully, $P < 0.001$) and OS (27% vs. 81% alive at five years in *TP53* mutated and wild-type cases respectfully, $P < 0.001$) in DLBCL patients with low/low-intermediate IPI (see page 65)

(Ichikawa *et al.*, 1997). However, a fourth series of 177 presentation DLBCL examined by Barrans *et al*, *TP53* mutation status was not a prognostic factor (Barrans *et al.*, 2002a). Recently 24 mutations in 113 DLBCL cases (21%) were found to be associated with poorer prognosis, confirmed on multivariate analysis and to be linked to low TRAIL receptor-2 expression (Young *et al.*, 2007). This year mutated *TP53* has been shown to have prognostic significance in a series of nearly 200 FL patients, where it was mutated in 6% of cases (O'Shea *et al.*, 2008).

Polymerase chain reaction (PCR) determined mutation status of *TP53, $p16^{INK4a}$* and *$p14^{ARF}$* was found to be abnormal in 40% of 87 aggressive NHL investigated by Gronbaek *et al*. Concurrent disruption of *$p16^{INK4a}$* and *$p14^{ARF}$-TP53* was associated with a significantly worse outcome with only 7% of patients alive at five years compared to 38% in those with retention of one or both pathways (P = 0.005) (Gronbaek *et al.*, 2000).

Molecular Prognostic Factors: Protein Expression as Detected by Immunohistochemistry

Immunohistochemical (IHC) assessment of proteins involved in the tumourigenesis has been undertaken in various retrospective NHL and DLBCL series. As the techniques involved in IHC and formalin-fixed material are both readily available as part of the diagnostic service for NHLs, production of IHC prognostic and predictive factors is both practical and affordable and can be expected to increase in importance and number.

Good Prognostic Protein Factors

High Bax protein expression was seen in 38% of 94 *de novo* DLBCL and was significantly associated with a better disease-free and OS (Sohn *et al.*, 2003), although Bax protein expression was inconsequential in the other series to date (Gascoyne *et al.*, 1997). No prognostic significance has been identified for other Bax family members to date. E2F-1 expression has been linked with a favourable outcome on multivariate analysis in one DLBCL series to date (Moller *et al.*, 2000) and high pRb and low mdm2 (mouse double minute-2 homologue) expression with OS in another DLBCL cohort (Sanchez *et al.*, 1998). High expression of VEGFR1 (vascular endothelial growth factor receptor 1) by DLBCL cells was associated with improved OS (P = 0.044) in the series examined by Gratzinger and colleagues only for the opposite result to be found by Ganjoo *et al* who (Gratzinger *et al.*, 2008, Ganjoo *et al.*, 2008). Co-expression of Bcl-6

and CD10 were found to predict for better outcome and is now considered indicative of a germinal centre-like phenotype (Hans *et al.*, 2004). Bcl-6 protein expression was also shown to be a positive predictor of disease-free survival in two other series of DLBCL patients (Barrans *et al.*, 2002a, Zhang *et al.*, 1999).

Poor Prognostic Protein Factors

Elevated Bcl-2 protein expression is a poor prognostic factor for disease-free survival and OS in three series where it was expressed in 26% of 177 *de novo* DLBCL cases, 24% of 116 diffuse aggressive NHL and 24% of 73 DLBCL patients with a low/low-intermediate IPI (see page 65) score (Gascoyne *et al.*, 1997, Barrans *et al.*, 2002a, Sohn *et al.*, 2003). In the cohorts of 141 DLBCL examined by Sanchez *et al* and 165 cases scrutinised by Kramer *et al*, Bcl-2 expression exhibited an inverse association with disease-free survival, substantiated on multivariate analysis (Sanchez *et al.*, 1998, Kramer *et al.*, 1996).

The consequence of mutated *TP53* is usually a p53 protein with an abnormal conformation, which poorly binds DNA and has a prolonged half-life due to disrupted binding of its antagonist mdm2. Immunohistochemically detected nuclear expression of p53 is therefore indicative of *TP53* mutation and is seen in ~20% of *de novo* DLBCL (Ichikawa, 2000). p53 protein expression appears to be an adverse prognostic factor in DLBCL as its expression was associated with curtailed OS on multivariate analysis by Zhang *et al* in a cohort of 158 DLBCL patients, in the 61 low/low-intermediate IPI (see page 65) risk DLBCL patients studied by Leroy *et al* in association with concurrent absence of $p21^{WAF1}$ expression (15/61) (47% vs. 74% 6-year OS, $P = 0.05$) and in 50 aggressive NHL patients examined by Navaratnam *et al* (Navaratnam *et al.*, 1998, Leroy *et al.*, 2002, Zhang *et al.*, 1999). Despite two other series failing to find prognostic significance for positive p53 staining (Sohn *et al.*, 2003, Barrans *et al.*, 2002a), simultaneous expression of p53 and Bcl-2 was noted to be associated with a worse prognosis than p53 expression alone by Piris *et al* in their examination of 119 patients with high-grade B-cell lymphomas (Piris *et al.*, 1994).

Cyclin-D2 and Cyclin-D3 overexpression were confirmed to be poor prognostic factors on multivariate analysis in 152 and 81 DLBCL respectively (Filipits *et al.*, 2002, Hans *et al.*, 2004). Cyclin-D3 overexpression was associated with a reduced CR rate (17% vs. 74%, $P < 0.001$) and 3-year OS (18% vs. 74%, $P < 0.001$) (Filipits *et al.*, 2002). Cyclin-D1 overexpression has not been shown to influence outcome in DLBCL. To date c-Myc

protein expression has only been found to be a poor prognostic factor in a single series of 61 aggressive NHLs (Pagnano *et al.*, 2001). A single study has identified $p27^{kip1}$ paradoxical overexpression on multivariate analysis in 133 DLBCL to be a negative predictive factor (Saez *et al.*, 1999). Additionally, high cell surface expression of nm23-H1 was found to be a poor prognostic factor in aggressive NHL, in agreement with the prognostic status of high serum nm23-H1 (Niitsu *et al.*, 2003). A high proliferation rate assessed by Ki-67 staining of >60% of malignant cells (19/105 diffuse large cell lymphomas) was associated with a median OS of 8 months compared to 39 months in those with <60% Ki-67 positive cells (P = 0.003) (Grogan *et al.*, 1988). Multivariate analysis confirmed Ki-67+ >60% as a predictor of poor outcome in the 63 cases with DLBCL (P = 0.002).

Combined Prognostic Factor Models – the International Prognostic Index

During the early 1990's an international collaboration of 16 co-operative groups and single centres investigated the available prognostic factors in a combined cohort of ~2000 patients to see if a combination of the best of them would result in a more useful prognostic model that could be used to risk-stratify patients. The result, the International Prognostic Index (IPI), remains the best available model to date (The-International-Non-Hodgkin's-Lymphoma-Prognostic-Factors-Project, 1993). The IPI comprises the patient characteristics of age and performance status with the disease characteristics of LDH, stage of disease and number of extranodal sites. Each factor is scored as 0 or 1. The higher the aggregate score, the less likely a patient will achieve a meaningful response to treatment and the less likely they will become a long-term survivor. Four risk groups: low, low-intermediate, high-intermediate and high were created based upon the total scores of the five prognostic factors (total scores: low = 0 or 1, low-intermediate = 2, high-intermediate = 3 and high risk = 4 or 5). The predicted 5-year OS rates for the four groups were significantly different: low = 73%, low-intermediate = 51%, high-intermediate = 43% and high = 26% (The-International-Non-Hodgkin's-Lymphoma-Prognostic-Factors-Project, 1993). For the 1274 patients 60 years old or younger, an age-adjusted model (age-adjusted IPI) based on tumour stage, lactate dehydrogenase level, and performance status identified four risk groups with predicted 5-year OS rates of 83%, 69%, 46%, and 32%.

Failure of The IPI To Resolve The Heterogeneity of DLBCL

Despite the ability to stratify patients according to the IPI, it is a crude tool upon which to base treatment decisions. Clearly, for those with low IPI risk (0-1) CHOP is an effective treatment and for those with high risk (IP 4-5) it is an ineffective treatment in most cases (Armitage and Weisenburger, 1998). Yet, half of patients do not fall into the low or high-risk categories but into the intermediate categories where 5-year OS is ~40% with CHOP chemotherapy i.e. equivalent to that of the DLBCL population considered as a whole (Armitage and Weisenburger, 1998). As such the IPI-determined prognosis for intermediate risk patients remains uncertain at the point of treatment. Furthermore, although the IPI can provide a gauge of a patient's likelihood to achieve a meaningful response, it is not a direct measure of chemosensitivity. In short the IPI is not a predictive factor model.

There is, therefore, an urgent need to improve on the IPI with more accurate dissection of the heterogeneity of response and outcome to treatment contained within the intermediate categories of the IPI in particular. Studies of large numbers of patients treated uniformly to evaluate the place of IHC prognostic factors will hopefully allow a tissue based prognostic index to be developed and validated that may supersede or at least be combined with the IPI. In the first such report using tissue microarrays, the expression of 52 selected proteins was investigated in a series of 152 DLBCLs (Saez *et al.*, 2004). A biological predictor, which used eight proteins, was built and validated and identified the probability of failure for a given patient with 78% accuracy. Combining this biological score and the International Prognostic Index (IPI) indeed improved the capacity for predicting failure and survival.

The discovery of predictive factors in DLBCL should help comprehension of treatment failure, allow patient stratification before clinical trial entry to aid response and other outcome measure interpretation and aid treatment decision making. An important caveat to consider when reviewing predictive and prognostic factors is that they are, by their nature, retrospective and, as such, may no longer be valid following introduction of a new therapy, schedule or strategy. This caveat has recently been illustrated in DLBCL where the poor prognostic status of Bcl-2 protein expression in DLBCL was lost/overcome on addition of rituximab to CHOP chemotherapy (Mounier *et al.*, 2003).

1.3 Genetic Events in Lymphoma and DLBCL

1.3.1 Genetic Instability: an Integral Part of Tumourigenesis

Integral to the process of neoplasia is genetic instability, whereby genetic lesions are acquired and sustained at an increased rate compared to normal cells. Genetic instability occurs early on in carcinogenesis at both the chromosomal and nucleotide levels (Lengauer *et al.*, 1998). The occurrence of genetic instability at the chromosomal level is seen in virtually all human tumours, including lymphomas and DLBCL, and in a minority of solid cancers at the nucleotide level. Genetic instability fuels the development and progression of tumours and their intra- and inter- heterogeneity. At the chromosomal level, genetic instability manifests as variations in chromosomal numbers (aneuploidy), chromosomal translocations, gene amplifications and gene deletions. At the cellular level genetic instability is clinically relevant because it causes tumour heterogeneity, which is one of the causes of mixed responses to treatment and of relapses despite excellent responses. It is hoped that specifically attacking the molecular causes of chromosomal and nucleotide instabilities will yield new, rational approaches to tumour treatment. DLBCL is chromosomally unstable and cytogenetically heterogeneous, with rife aneuploidy, several recurrent as well as many non-recurrent translocations, amplifications and chromosomal deletions (Mitelman *et al.*, 1994, Johansson *et al.*, 1995). This cytogenetic and genetic heterogeneity is presumed to contribute to the clinical heterogeneity of DLBCL. These chromosomal and genetic abnormalities are described in the sections below.

Nucleotide Instability

Nucleotide instability is produced in cancers by defects in two DNA repair pathways: the nucleotide excision and the mismatch repair systems. The genetic lesions produced by nucleotide instability take the form of deletions, insertions and base substitutions.

Nucleotide excision repair defects are seen principally in suffers of xeroderma pigmentosum, who are supersensitive to ultraviolet light DNA damage and consequently have a very high incidence of skin cancers (Cleaver, 1968). Surprisingly, despite nucleotide excision repair correcting DNA damage caused by many types of mutagens in addition to UV light, the incidence of non-skin cancers in xeroderma pigmentosum is raised (compared to the general population) to a much lower extent than the incidence of skin cancers in these patients. The most likely explanation for this discrepancy is that environmental agent exposure is not the rate-limiting step in the

development of most common cancers, with the exception of skin cancers (Ames and Gold, 1991).

Mismatch repair defects are responsible for microsatellite instability, which is usually caused by inactivating mutations of one of the six human homologues of the *mutS* and *mutL* genes (Peltomaki and de la Chapelle, 1997). The consequence of mismatch repair deficiency is the failure of DNA error correction during DNA replication. The DNA mutation rate is therefore dramatically increased in mismatch repair deficient neoplasms. Microsatellite instability, a consequence of mismatch repair defects, is seen in 13% of colorectal (mostly in hereditary non-polyposis coli suffers), and a minority of gastric and endometrial cancers, but is uncommon in NHL (Aaltonen *et al.*, 1993, Ionov *et al.*, 1993, Perucho, 1996).

1.3.2 Chromosomal Instability: Gains and Losses

Chromosomal gains and losses affect parts, or entire chromosomes and also the chromosome copy number present per cell (aneuploidy). These cytogenetic abnormalities are present in almost all malignancies, including the majority of NHLs (Mitelman *et al.*, 1994). Loss of heterozygosity affects about 25% of all alleles in the common solid cancers and the majority of DLBCL (Cigudosa *et al.*, 1999, Seymour *et al.*, 1994, Vogelstein *et al.*, 1989). Gains, losses and loss of heterozygosity have been found to occur in malignant cells at $\geq$10 times more often than in normal cell equivalents (Phear *et al.*, 1996, Lengauer *et al.*, 1997). The molecular basis of chromosomal instability includes the silencing of 'caretaker' tumour suppressor genes, which allows cytogenetic disruption to begin; and defective mitotic and spindle checkpoints, with the consequence of re-entry into S phase without cell death or repair of the gains, losses or translocations produced during previous defective cell cycles (Paulovich *et al.*, 1997, Jin *et al.*, 1998). The two 'caretaker' tumour suppressor genes shown to be frequently inactivated in DLBCL are *TP53* and *ATM*. p53 may cause genetic instability by affecting centrosome duplication. Absence of p53 results in multiple copies of functionally competent centrosomes being generated during a single cell cycle (Fukasawa *et al.*, 1996). The abnormally amplified centrosomes profoundly affected mitotic fidelity, resulting in unequal segregation of chromosomes. *ATM* plays a central role in the cellular response to DNA *double-strand* breaks, modulating numerous signalling pathways (Shiloh, 2003). ATM expression is down-regulated at those stages of lymphoid development where physiological DNA *double-strand* breaks

occur (Starczynski *et al.*, 2003). Patients with ataxia telangiectasia have bi-allelic inactivation of *ATM* and develop lymphomas and leukaemias at a higher rate than the general population (Gumy-Pause *et al.*, 2004). Mutations of *ATM* are seen in 10-20 % of DLBCLs and up to 45% of MCLs (Gronbaek *et al.*, 2002, Fang *et al.*, 2003), whilst deletion of the gene due to loss of 11q is seen frequently in MCL and CLL (Boultwood, 2001, Monni and Knuutila, 2001). At the protein level most follicular and DLBC lymphomas are negative for, or weakly express, ATM (Starczynski *et al.*, 2003).

Recurrent genetic changes, such as the hallmark translocations of FL, MCL and BL are considered to be primary events, essential for the development and phenotype of an NHL subtype. The many non-recurrent genetic changes seen in NHLs are considered to be secondary to, rather than a cause of, the inherent genetic instability that develops during the oncogenic process

Gains and Losses in DLBCL due to Chromosomal Instability

In the largest series of DLBCL subjected to cytogenetic examination only 13% of tumours displayed a normal karyotype (Cigudosa *et al.*, 1999). The majority of cases (63%) were hyperdiploid (>46 chromosomes/cell), 16% were pseudodiploid (approximately 46 chromosomes/cell but with gains and losses of individual chromosomes) and 8% were hypodiploid (<46 chromosomes/cell). The modal chromosomal number ranged from 38 to 107. The identification of 11 regions of common cytogenetic deletions on chromosomes 1, 3, 6 and 7 in DLBCL suggests that several tumour-suppressor genes involved in lymphomagenesis still await identification (Cigudosa *et al.*, 1999). Rearrangements at 1q25, 6p23, 7p36 and 19q13 occur more often in treated (52%) than untreated cases (7%, P <0.01) suggesting such rearrangements could contribute to treatment resistance frequently encountered at relapse (Cigudosa *et al.*, 1999). An example of a frequent homozygous deletion where the genetic consequences have been identified is that of *CDKN2A* and *CDKN2B* at 9p21, which code for the cdk-4 and –6 inhibitors $p15^{INK4b}$ and $p16^{INK4a}$ as well as $p14^{ARF}$. These cell cycle inhibitors are co-deleted in 15% of DLBCLs (Elenitoba-Johnson *et al.*, 1998, Koduru *et al.*, 1995). Array formatted, genome-wide comparative genomic hybridisation (CGH), single nucleotide polymorphism (SNP) analyses and systematic application of 24 colour fluorescent *in-situ* hybridisation to large series of DLBCL will undoubtedly reveal yet more changes. For example, array-based CGH and gene-expression microarray analysis of ~50 B-cell NHL cell-lines found 20

homozygous deletions at seven chromosome areas, all of which contained putative tumour suppressor genes including *BIM*, *NOXA* and *p18*^{INK4c} (Mestre-Escorihuela *et al.*, 2007). Very recently examination of 203 DLBCL samples by high-resolution, genome-wide copy number analysis coupled with gene-expression profiling found 30 recurrent chromosomal aberrations that were differentially associated with the *'cell-of-origin'* DLBCL subtypes (Lenz *et al.*, 2008). Of these, an amplicon on chromosome 19 containing the overexpressed SPIB gene was detected in 26% of ABC-DLBCLs but in only 3% of GCB-DLBCLs, with *in vitro* experiments suggesting SPIB is an oncogene in ABC-DLBCL. Deletion of the p16^{INK4a}/p14^{ARF} tumour suppressor locus and trisomy 3 also occurred almost exclusively in the ABC-DLBCL subgroup and was associated with inferior outcome within this subtype. In GCB-DLBCL, amplification of the oncogenic mir-17–92 microRNA cluster and deletion of the tumour suppressor PTEN were recurrent. In conclusion recurrent patterns of chromosomal aberrations and orders of changes and predispositions to DLBCL are being discerned that suggest different oncogenic pathways lead to different subtypes of DLBCL arising.

1.3.3 Chromosomal Instability: Translocations in the NHLs

The leukaemias, myeloma and lymphomas are distinct from most other adult cancers by virtue of the presence of recurrent, simple, reciprocal chromosomal translocations, where sections of DNA not normally adjacent to each other become juxtaposed. In solid cancers the translocations seen are complex, involving multiple chromosomes in non-recurrent rearrangements (Johansson *et al.*, 1996). In most simple, recurrent translocations the breakpoints occur within the same small sections of DNA. The occurrence of such translocations as the sole karyotypic aberration, in a proportion of cases, supports the hypothesis that simple, recurrent translocations play an early aetiological role in lymphomagenesis and are critical for the development of particular NHL subtypes.

The molecular causes of translocations so far discovered include failure of double-strand DNA break repair before cells undergo mitosis; dysfunctional somatic hypermutation; and abnormal recombination of the immunoglobulin genes in B-cells and T-cell receptor genes in T-cells (e.g. through deregulated recombinase-activating gene (RAG) enzyme activity) (Tsujimoto *et al.*, 1985, Jager *et al.*, 2000, Hiom *et al.*, 1998, Tycko *et al.*, 1989). The formation of specific translocations in human lymphomas is probably determined, in part, by the higher-order spatial organisation of

the genome. Evidence for this theory comes from the discovery that the *c-Myc*, *BCL* and immunoglobulin loci exist in close spatial proximity relative to each other, towards the nuclear interior of normal B-cells and that such locus proximity is caused by higher-order genome structure rather than the properties of the individual genes (Roix *et al.*, 2003). The consequence of simple translocations is either the activation of an oncogene by its juxtaposition to a strong promoter or creation of an oncogene by deregulated expression of a fusion protein. In B-cell NHL, the immunoglobulin genes (immunoglobulin heavy chain gene (*IgH)* at 14q32 and the λ (22q11) and κ (2p12) light-chain genes) are the predominant translocation partners and the source of such powerful promoters. Overexpression of Bcl-2 results from the juxtaposition of the *BCL-2* gene to the *IGH* gene in the t(14;18) translocation, with transcription of *BCL-2* being driven by *IGH's* powerful Eμ promoter. Indeed in the WHO classification of haematological malignancies the presence of a *c-Myc-IgH/Iλ/Iκ* t(8;14)(q24;q32) is now required for the diagnosis of BL and a *CCND1-IgH/Iλ/Iκ* t(11;14)(q13;q32) for the diagnosis of MCL (Jaffe *et al.*, 2001).

c-Myc, the first oncogene to be discovered, was cloned and sequenced by virtue of its involvement in the t(8;14)(q24;q32) translocation (Taub *et al.*, 1982, Dalla-Favera *et al.*, 1982, Zech *et al.*, 1976). The consequence of a t(8;14) or a t(8;22) translocation in lymphoma cell-lines and primary BL tissue is indeed transcriptional overexpression, as such cells expressed 2- to 5-fold more *c-Myc* RNA than normal B-cells or B-cell-lines without a t(8;14) translocation (Maguire *et al.*, 1983, Saez *et al.*, 2003).

Recurrent translocations that create a fusion protein have been described in anaplastic large cell lymphoma (ALCL) and MALT lymphoma but have not been discovered in DLBCL. In ALCL, its hallmark t(2;5)(p23;q35) creates an oncogenic fusion protein: the nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) (Drexler *et al.*, 2000). NPM-ALK binds to, and activates, signal transducer and activator of transcription 3 (STAT3), resulting in constitutional STAT3 activity, which in turn results in deregulated expression of multiple target proteins that are involved in the control of apoptosis and cell cycle progression (Amin *et al.*, 2004). The importance of the *NPM-ALK* gene fusion in ALCL is borne out by studies of *NPM-ALK* transgenic mice, which spontaneously develop B-cell lymphomas with marked overexpression of Jun and ERK kinases (Turner *et al.*, 2003). The frequency and NHL subtypes in which the commonest translocations in NHL occur are illustrated in Table 1.3.

Translocation	Disease	Frequency	Genes Involved	Function	References
t(14;18)(q32;q21)	DLBCL	20%	*BCL-2*	Anti-apoptosis	(Weiss *et al.*, 1987)
t(8;14)(q24;q32)	DLBCL	10%	*c-Myc*	Proliferative factor	(Kawasaki *et al.*, 2001)
t(3;x)(q27;x) (Several partners)	DLBCL	20-40%	*BCL-6*	Transcription repressor	(Barrans *et al.*, 2002b)
t(11;14)(q13;q32)	DLBCL	15%	*CCND1*	Cell cycle promoter	(Kawasaki *et al.*, 2001)
t(14;18)(q32;q21)	Follicular Lymphoma	80-85%	*BCL-2*	Anti-apoptosis	(Weiss *et al.*, 1987)
t(8;14)(q24;q32) and light chain variants	Burkitt Lymphoma	100%	*c-Myc*	Proliferative factor	(Magrath, 1990)
t(11;14)(q13;q32)	Mantle Cell Lymphoma	>85%	*CCND1*	Cell cycle promoter	(Li *et al.*, 1999)
t(9;14)(p13; q32)	Lymphoplasmacytoid Lymphoma	50%	*PAX5*	Transcription factor	(Offit *et al.*, 1992)
t(1;14)(p22;q32)	Extranodal Marginal Zone Lymphoma	<5%	*BCL-10*	NFkB activation	(Wotherspoon *et al.*, 1992)
t(14;18)(;q21)	Extranodal Marginal Zone Lymphoma	20%	*MALT1*	Unknown	(Streubel *et al.*, 2003)
t(11;18)(q21;q21)	Extranodal Marginal Zone Lymphoma	20-35%	*MALT1 and API2*	Inhibition of apoptosis and NFkB activation	(Ott *et al.*, 1997) (Remstein *et al.*, 2000)
t(2;5)(p23;q35)	Anaplastic Large Cell Lymphoma	30-50%	*ALK and NPM*	Anti-apoptotic tyrosine kinase Activation of STAT3	(Drexler *et al.*, 2000)

Table 1.3 Chromosomal translocations in non-Hodgkin lymphomas

Recurrent Translocations in DLBCL

DLBCL is distinguished from most other subtypes of NHL by the lack of a dominant, hallmark translocation. Indeed, only 46% of DLBCLs carry a translocation involving the *IgH* gene at 14q32, in stark contrast to nearly all FL, MCL and BL (Cigudosa *et al.*, 1999). The DLBCL carrying a 14q32 translocation are more likely to harbour additional rearrangements (P <0.001) (Cigudosa *et al.*, 1999). Translocations involving the cyclin-D1 gene *CCND1* of MCL and *c-Myc* gene of BL are seen in 15% and 10% of DLBCLs respectively (Kawasaki *et al.*, 2001). The hallmark translocations of FL, t(14;18)(q32;q21), and LPCL, t(9;14)(p13; q32), are seen in 17-30% and 1.6% of DLBCLs in keeping with the origin of up to a third of DLBCLs being a low-growth fraction NHL that has undergone transformation (Weiss *et al.*, 1987, Dalla-Favera *et al.*, 1994, Cigudosa *et al.*, 1999). The chromosomal breakpoints occur primarily in the 3'-non-coding regions of *BCL-2* and cluster in three sections, the major breakpoint region (MBR) (50-70% of translocations), the intermediate cluster region (icr), the minor cluster region (mcr) and ~10% in the 5' end of the gene (Cleary *et al.*, 1986, Albinger-Hegyi *et al.*, 2002).

3q27 translocations are seen in ~30% of DLBCLs and are the only recurrent rearrangement seen more often in DLBCL than in other NHL subtypes (Otsuki *et al.*, 1995). Unlike other common translocations in NHL, breakpoint partners of 3q27 are heterogeneous, involving non-immunoglobulin loci in half of cases (Cigudosa *et al.*, 1999). The *BCL-6* gene was cloned from the 3q27 breakpoint by three groups in the early 1990's (Ye *et al.*, 1993, Miki *et al.*, 1994, Kerckaert *et al.*, 1993). The 3q27 region is also subject to amplifications in 5% of cases and to *BCL-6* somatic mutation events as will be discussed below (Martinez-Climent *et al.*, 2003). The section of *BCL-6* involved in most translocations is the major breakpoint region, which contains the regulatory sequences for the gene's expression and is restricted to 4 kb that span the first non-coding exon and the 5' portion of the first intron. In 2002, an alternative breakpoint cluster region 5' to *BCL-6* was described that proved to be involved in many of the cytogenetically detected 3q27 translocations in which *BCL-6* remains intact (Butler *et al.*, 2002). The presence of a *BCL-6* translocation in a minority of DLBCLs suggests that although *BCL-6* is likely to be a proto-oncogene, a 3q27 translocation is not essential for DLBCL development. The effect of the translocation in terms of patient outcome has become clearer since the discovery that the partner in the translocation

affects the expression of Bcl-6. In *Ig/BCL-6* translocation tumours, the expression of Bcl-6 is higher than in *non-Ig/BCL-6* translocation-bearing tumours, with the former being associated with a better clinical outcome (Ueda *et al.*, 2002). In keeping with this finding, somatic mutations in the mutation 'hotspot' between 423 and 443 are associated with higher expression of Bcl-6 and a better clinical outcome (Artiga *et al.*, 2002).

Bcl-6 is a zinc-finger/POZ protein that acts as a sequence-specific transcriptional repressor, a function elicited in part through recruitment of class II histone deacetylases (Lemercier *et al.*, 2002). Normally, expression of Bcl-6 is seen in cells with a high proliferation index. In FL Bcl-6 expression is therefore restricted to the proliferating cells and is down-regulated in the interfollicular compartment. It is expressed in germinal centre B-cells and CD4+ T-cells, where it controls germinal centre formation, Th2-type (T-helper cell) immune response/T-cell-dependent antigen response and B-cell differentiation (Ye *et al.*, 1997, Reljic *et al.*, 2000). Bcl-6 can down-regulate the expression of the cell cycle kinase inhibitor $p27^{kip1}$ (Shaffer *et al.*, 2000). Overexpression of Bcl-6 in cell-lines has been found to inhibit cell growth and induced apoptotic cell death, suggesting that preceding abnormalities need to occur in B-cells for Bcl-6 to function as an oncogene (Yamochi *et al.*, 1999, Albagli *et al.*, 1999). In the last five years, Bcl-6 overexpression was found to inhibit apoptosis in response to etoposide by preventing the generation of reactive oxygen species (Kurosu *et al.*, 2003).

After 14q32 and 3q27, the third commonest site of recurrent breakpoints and translocations in DLBCL is 1q21 (Cigudosa *et al.*, 1999). This observation, taken together with the occurrence of a 1q21 translocation as the sole chromosomal abnormality in several DLBCLs, raises the possibility of a novel oncogene important in lymphomagenesis existing at 1q21. The presence of chromosomal instability in DLBCL is well illustrated by the high incidence of non-recurrent translocations (65% of the total) in DLBCL.

Gene Amplification in DLBCL

Gene amplifications or 'Amplicons' contain extra copies of particular sections of DNA between 0.5 and ten megabases in size. Amplicons usually contain a growth-promoting gene and exist as double minutes or inserts into different chromosomes. In DLBCL amplicons have been described involving *BCL-2* (18q21) (11% of cases), a small region of chromosome 2 containing the *REL* gene (2p12-16) (23%), *c-Myc* (8q24) (16%) and

the cdk -4 (*CDK-4)* gene (11%) (Rao *et al.*, 1998). In the series quoted, no overlap between DLBCLs exhibiting translocations and amplifications of *REL*, *c-Myc* and *BCL-2* was seen, indicative of translocation and amplification being potentially independent pathways to overexpression of these oncogenes. The importance of *REL* and *BCL-2* to lymphomagenesis is further suggested by the frequent development of amplification these oncogenes on transformation of FL to DLBCL (Martinez-Climent *et al.*, 2003).

Somatic Mutation in DLBCL

The occurrence of individual base substitutions or the insertion or deletion of several nucleotides in certain genes can have dramatic consequences and be critical determinants of the progression of carcinogenesis. Inactivating mutations of tumour suppressor genes alter the gene product structure rendering it ineffective or inefficient at its cancer-inhibiting process. Less commonly, mutations can change a gene into an oncogene through constitutive activation or change of function. *TP53* is the most frequently mutated tumour suppressor gene in solid cancers (50%) and in DLBCL, where mutations are seen in 20% of cases (Ichikawa, 2000). Eighty % of *TP53* mutations are dominant negative missense mutations. The most frequently mutated genes in DLBCL are listed in Table 1.4 below.

Gene Name	Mutation frequency in DLBCL	Mutation frequency in other Cancers	Gene Function	References
Bik	18%		Pro-apoptotic Bcl-2 family member	(Arena *et al.*, 2003)
Fas	20-30%	20-30% Hodgkin lymphoma	Cell surface death receptor	(Gronbaek *et al.*, 1998, Muschen *et al.*, 2000)
Rb	33%		Regulates G1/S checkpoint	(Hangaishi *et al.*, 1996)
TP53	20%	>50% solid tumours	Cell cycle arrest and apoptosis; guardian of genomic integrity	(Ichikawa *et al.*, 1997, Koduru *et al.*, 1997).
ATM	10-20 %	45% Mantle Cell Lymphoma	Cellular response to DNA double-strand breaks	(Gronbaek *et al.*, 2002, Fang *et al.*, 2003)

Table 1.4 Frequency of Somatic Gene Mutations in DLBCL

Somatic Hypermutation In DLBCL

The term 'somatic hypermutation' refers to the process first noted in the immunoglobulin heavy chain gene where, post-V(D)J rearrangement, point mutations are actively created, resulting in further refinement of the antibody to its target antigen. The process of somatic hypermutation is RAG and activation-induced cytidine deaminase (AID) enzyme dependent and occurs in B-cells within the germinal centres of lymph node follicles. Recent animal work has shown the critical need for AID for the development of Bcl-6 but not c-Myc driven NHLs (Pasqualucci *et al.*, 2008).

Due to the high fidelity with which the genome is maintained and replicated in normal cells, the mutation rate is only 0.16 nucleotide per genome each time a cell divides. The rate of somatic mutation in the immunoglobulin gene sections favoured by somatic hypermutation occurs at a frequency of more than a million times greater than the spontaneous mutation rate in other genes i.e. 1 in 10^3 base pairs (bp) per cell division. The discovery of somatic hypermutations in ~70% of DLBCLs within a confined area of the regulatory region of *BCL-6* was the first description of this process occurring outside of the immunoglobulin genes (Migliazza *et al.*, 1995). The somatic hypermutation region of *BCL-6* covers 700 bp and overlaps with the major breakpoint region, however, the presence and number of somatic hypermutations is unaffected by the presence or absence of a translocation involving *BCL-6* (Migliazza *et al.*, 1995). Several mutations caused by hypermutation result in deregulated *BCL-6* expression, in part through preventing Bcl-6 protein binding to its own gene promoter region, thereby disrupting its auto-regulatory negative feedback system (Capello *et al.*, 2000, Pasqualucci *et al.*, 2003). Subsequent to the discovery of *BCL-6* somatic hypermutation in DLBCL and other B-cell NHLs, examination of 18 genes revealed aberrant hypermutation in *PIM1*, *c-Myc*, *RhoH/TTF* and *PAX5* in >50% of DLBCLs examined (Pasqualucci *et al.*, 2001). It is not yet known if somatic hypermutation significantly affects the expression profile, phenotype or clinical course of DLBCL.

1.3.4 Epigenetic Changes in Lymphoma

Epigenetic alterations affect the heritable state of gene expression and chromatin organisation without changes to the DNA sequence itself. Methylation of the DNA base cytosine to form 5-methylcytosine was the first epigenetic silencing mechanism described. Approximately 45,000 small genomic regions (200 bp to 5 kb), termed CpG islands, that are rich in cytosine-guanosine dinucleotides (>60%) exist throughout the

genome ~100 kb apart (Bird, 1986, Cross and Bird, 1995). The DNA methyltransferase enzymes control the heritable phenomenon of cytosine methylation in CpG dinucleotides. CpG islands are found commonly in the promoter regions of genes and in repetitive DNA regions of the genome such as *Alu* repeats (Graff *et al.*, 1997, Bird, 1986). The consequence of DNA methylation is chromatin structure modification, which prevents gene transcription (Baylin *et al.*, 1998). Through the association of methylated DNA binding proteins (MBPs) at methylated CpG islands, a complex of co-repressors is assembled. A critical group of co-repressors are the histone deacetylases (Kim *et al.*, 2003a). The silencing produced by cytosine methylation and histone deacetylation is thus brought together in what is probably a synergistic manner (Jones *et al.*, 1998, Cameron *et al.*, 1999). A further mechanism of epigenetic silencing has been demonstrated in the last five years: methylation of histones, catalysed by the histone methyltransferases (Jaenisch and Bird, 2003, Kouzarides, 2002). Hypermethylation of the CpG islands in a gene's promoter region is now recognised as a means of creating a 'hit' critical to Knudson's hypothesis of recessive tumour suppressor gene inactivation (Chim *et al.*, 2002). The other 'hit' being met by deletion, mutation or epigenetic silencing of the other allele.

The methylation status of a number of genes has been investigated in haematological malignancies - see (Claus and Lubbert, 2003). $p15^{INK4B}$, the cdk-4 and -6 inhibitor, is unmethylated in normal CD34+ cells but is methylated in 10-88% of NHL cases, 30-90% of acute myeloid leukaemia (AML) cases and <10% of CLL cases. The related $p16^{INK4A}$ tumour suppressor gene is methylated in 20-40% of DLBCL, in 15-73% of NHL cases, in 17-50% % of AML cases and <10% CLL cases (Drexler, 1998, Chim *et al.*, 2002, Sanchez-Beato *et al.*, 2001, Guo *et al.*, 2000). Hypermethylation of $p16^{INK4A}$ has been shown to translate into absent expression of the $p16^{INK4A}$ protein (Klangby *et al.*, 1998). A detailed, genome-wide investigation of the frequency and genes affected by cytosine methylation in DLBCL and other NHLs is eagerly awaited.

1.4 Application of Empirical and Rational Research Strategies to Discover and Validate Novel Factors Predictive of Response and Outcome in DLBCL

There is a clear need for more accurate prognostic factors in DLBCL and for predictive factors that would allow a move away from generic, homogeneous treatment to more

individualised, heterogeneous therapeutic strategies. The discovery and validation of such novel predictors of response and outcome in DLBCL is the subject of this thesis.

Two strategies were used in the hope of discovering and validating novel prognostic and predictive factors in DLBCL: the traditional empirical approach of scientific research and a modern rational strategy. The empirical method of scientific enquiry uses evidence derived from experiment and observation (empiric evidence) to construct new scientific theories. To prove or discount a scientific theory, testable hypotheses with explicit, observable predictions are generated. This approach, called the hypothetico-deductive method is at the heart of the scientific method. 'Trial and error' is implicit to empiric drug discovery in oncology, as expounded by the testing of thousands of potential drugs in panels of cell-lines considered representative of a broad spectrum of cancers. The majority of existing drugs have been discovered empirically, either by their isolation as the active ingredients in traditional remedies (e.g. the vinca alkaloids derived from the Madagascar periwinkle *Catharanthus roseus*, which has been used in Chinese traditional medicine to treat malaria, skin diseases, diarrhoea, hypertension, and diabetes (Gidding *et al.*, 1999)), by serendipity (e.g. the anthracyclines were originally discovered as antibiotic fermentation products of *Streptomyces peucetius* var. *caesius* in the late 1960's (Arcamone *et al.*, 1969) and the serendipitous discovery of cisplatin was made following the observation of bacterial growth inhibition during experiments on the effects of electric current on bacterial growth using a platinum electrode. Several platinum compounds were isolated from the media, of which cisplatin was the most active (Rosenberg *et al.*, 1969).) or by active screening of natural sources for anticancer drugs (e.g. paclitaxel (Taxol) isolated in 1971 following the discovery in the 1963 that the bark extract from the Pacific yew *Taxus brevifolia* had cytotoxic properties (Wani *et al.*, 1971)).

Rational scientific research involves the reasoned generation of theory and hypothesis, where their creation depends more on the logical application of ideas than on experimentally acquired data. The application of the rational approach to scientific research has been well illustrated in the field of cancer drug discovery in the last decade, where understanding of the disease at the molecular and physiological levels has been used to target specific pathways and molecules. Examples of rational drug developments include imatinib, the tyrosine kinase inhibitor, which targets the molecular cause of chronic myeloid leukaemia, the oncogenic fusion protein bcr-abl,

rituximab the chimeric monoclonal antibody which targets the CD20 surface antigen of malignant and benign B-cells and bevacizumab a humanised anti-VEGF (vascular endothelial growth factor) antibody (Hoff *et al.*, 2004, Druker, 2004, Grillo-Lopez, 2003).

1.4.1 The Empirical Research Strategy: To Investigate Serum Selenium for Associations with Response and Outcome in DLBCL

The empirical hypothesis I chose to test was *"If selenium influences the short-term toxicity and the short- and long-term efficacy of treatment in patients with DLBCL and other aggressive lymphomas, then presentation serum selenium should be inversely associated with measures of treatment delivery, response and OS."* This hypothesis was based on *in vitro* data, generated using non-haematological tumour cell-lines, that supported the empirical theories 'selenium is a chemosensitiser; selenium can prevent chemoresistance; and selenium can reduce chemotherapy associated toxicity'. These laboratory data will be discussed in detail in section 1.4.2 through 1.4.8 below.

If my hypothesis proved to be true in part or whole, then the investigation of selenium compounds, as novel therapeutics in DLBCL, would be pursued. Such an attempt at drug discovery would be empirical, as it would be based on laboratory data generated using non-haematological tumour cells, where the targets and actions of cytotoxic selenium compounds remain far from clear. A summary of the clinical, epidemiological and laboratory data linking selenium deficiency with carcinogenesis is given in sections 1.4.1 through 1.4.8 below and the results of this empirical research in Chapter 3 and 4.

1.4.2 The Physiological Role of Selenium

Selenium (Se) is an essential trace element in humans, best known for its function as an antioxidant (Stadtman, 1996). Se is the defining component of selenocysteine, the 21st amino acid (Leinfelder *et al.*, 1988, Soll, 1988). For selenocysteine to be incorporated into the protein, selenocysteine insertion sequences (SECIS) within the 3' untranslated region require a UGA codon to be alternatively read thanks to binding of several proteins including Se binding protein 2, nuclease sensitive binding protein 1, SECp43 and soluble liver antigen (Xu *et al.*, 2005, Shen *et al.*, 2006). The function of proteins is also affected by the non-specific incorporation of selenomethionine during protein translation. Se is increasingly considered to be of importance to human physiology, and conversely its deficiency to the pathophysiology of conditions ranging from depression to atherosclerosis to cancer (Rayman, 2000).

The Selenoproteome

Selenocysteine is critical to the function of nearly 40 enzymes, collectively termed the selenoproteome (Moghadaszadeh and Beggs, 2006). The best-described selenoproteins are the four glutathione peroxidases (GPx) (Arthur, 2000), the three thioredoxin reductases (TR) (Gladyshev and Hatfield, 1999) and the three iodothyronine deiodinases, which are involved in the production of triiodothyronine from thyroxine (Kohrle, 2000). Others include selenoprotein P (Sepp1), selenoprotein K, selenoprotein W and selenophosphate synthetase. In the majority of selenoproteins there is a single selenocysteine at the active site of the enzyme, which plays a pivotal role in a redox reaction; in contrast, mouse and human Sepp1 has ten selenocysteines at the active site (Hill *et al.*, 2003). The first selenocysteine of Sepp1 functions as an antioxidant seleno-enzyme whilst the other nine function as Se transporters/suppliers (Saito *et al.*, 2004). Sepp1 carries 40-60% of Se in plasma, transporting Se to peripheral tissues. Sepp1 is capable of associating with endothelial membranes and restoring GPx and TR activity in endothelial cells, preventing oxidative damage (Steinbrenner *et al.*, 2006). The GPx and TR genes contain antioxidant response elements within their promoter regions. The four glutathione peroxidase enzymes (classical GPx1, gastrointestinal GPx2, plasma GPx3, phospholipid hydroperoxide GPx4) catalyse the reduction of peroxides preventing oxidative intra- and extra- cellular damage. Glutathione represents the major low molecular weight antioxidant redox recycling thiol in mammalian cells and plays a central role in the cellular defence against oxidative damage. Thioredoxin reductase (TR) provides a ubiquitous intracellular oxidoreductase system, with antioxidant and redox regulatory roles, catalysing the NADPH dependent reduction of thioredoxin (Trx). TR can reduce multiple substrates involved in the antioxidant network and cellular proliferation by providing reducing equivalents either directly or via Trx. The TR-Trx system also maintains the redox state of many transcription factors including p53, AP-1 and NF-κB (Smart *et al.*, 2004, Gebel and Muller, 2001). Selenoproteins K and W also protect against oxidative stress and are highly expressed in the heart and proliferating myoblasts respectively (Loflin *et al.*, 2006, Lu *et al.*, 2006a).

Selenomethionine and Protein Function

As $tRNA^{met}$ cannot discriminate between selenomethionine and methionine, local availability rather than specific $tRNA^{met}$ signalling determines the inclusion of selenomethionine into proteins. Thus, the less methionine present, the more

selenomethionine is incorporated into proteins and the less is metabolised to chemopreventive forms (Ip, 1988). Where selenomethionine is incorporated near the active site of enzymes, it can affect enzyme activity in comparison to the incorporation of methionine. The replacement of more than half the 150 methionine amino acids in β-galactosidase of *E. coli* by selenomethionine results in the inactivation of the enzyme (Huber and Criddle, 1967), whilst selenomethionine substitution in thymidylate synthase of *E. coli* causes a 40 % increase in its activity and an 8-fold lower thermal stability (Boles *et al.*, 1991).

1.4.3 Dietary Sources of Selenium

Se belongs to the group VI transitional metals and can exist in four oxidation states (-2, +1, +2 and +4). The main human dietary foods contain very little Se (only 0.01 – 0.1 mg/kg) (Morris and Levander, 1970). Most of this trace nutrient is obtained from the ingestion of cereal grains, meats and seafood (Wen *et al.*, 1997). Se intake varies depending upon food types consumed and the regional Se content of the staple cereal crops. This in turn is determined by the content and bioavailability of inorganic Se compounds to plants in the local soil. The poor content and/or bioavailability of Se in the soils of certain provinces of China, Finland, New Zealand and the UK are manifest in the local population's absolute or relative Se deficiency. Conversely, the high Se content and bioavailability in most of North America explains the high Se content of North American people's body tissues. The principle Se compound in the normal diet is selenomethionine with small quantities of selenite and other chemical forms such as Se-methylselenocysteine (MSC) and selenocysteine (Guo and Wu, 1998). One of the few foods naturally rich in Se is the Brazil nut (0.25 mg/kg). By comparison, offal contains 0.42 mg/kg, fish 0.32 mg/kg, eggs 0.16 mg/kg and cereals 0.02 mg/kg (Minerals, 2003). In seleniferous corn, wheat and soybeans, selenomethionine accounts for 80% of total Se (Yang *et al.*, 1997); whilst in selenium-enriched garlic and broccoli, MSC is the principle selenocompound (Cai *et al.*, 1995).

1.4.4 Normal Daily Intake of Selenium

Se is an essential dietary nutrient for all mammalian species. The normal dietary requirement of Se in mammalian animal diets is between 0.1 and 1 part per million (ppm) or mg/kg of Se (Jacobs and Forst, 1981b, Jacobs and Forst, 1981a). This can be met by ingestion of 55 μg of Se a day in women and men. The daily intake of Se in American adults varies between 80 and 165 μg/day (Schrauzer and White, 1978).

Although the US Institute of Medicine, Food and Nutrition Board reduced the recommended daily reference intake of Se from 70 to 55 μg/day for adults in 2000 (Institute of Medicine, 2000), intakes up to 700 μg/day appear to be safe, leading to the recommendation that total daily intake via food and supplements should not exceed 350-450 μg/day (Minerals, 2003, Patterson and Levander, 1997). The WHO has set a lower limit of safe daily intake at 40 μg/day. Selenomethionine is absorbed from the small intestine. Any excess, not immediately metabolised, is stored in organs with high rates of protein synthesis. These include skeletal muscle, erythrocytes, liver, pancreas, kidney and the mucosa of the gastrointestinal tract. The Se content of human skeletal muscle correlates with dietary selenomethionine intake, being highest in adult Japanese (1700 ng/g), followed by Canadians (370 ng/g), Americans (240 ng/g) and lowest in New Zealanders (61 ng/g) (Oster *et al.*, 1988). In plasma, selenomethionine is found mostly in the albumin fraction. The percentage contribution of albumin selenomethionine to total plasma Se varies from 20% in the Se deficient to 47% in those replete in the micronutrient (Whanger and Butler, 1994).

1.4.5 Selenium Deficiency

In humans, Se deficiency occurs when daily intake falls below 40 μg/day. Severe Se deficiency is associated with two illnesses: Keshan and Kashin-Beck diseases. In Keshan disease a cardiomyopathy is seen principally in children and pre-menopausal women endemically in areas of China and North-East Asia, whilst sufferers of Kashin-Beck disease experience a musculoskeletal disorder. Se deficiency is inducible in laboratory mammals by feeding a diet containing < 0.01 ppm selenium. The essential requirement for Se in trace quantities (50 nmol/l) in cell culture has been recognised for many years (McKeehan *et al.*, 1976). Supplementation of selenium-free media such as RPMI-1640 with 5% foetal calf serum, which contains 13 nM Se, is sufficient to meet physiological requirements of Se (Zhao *et al.*, 2004). In the last decade, Zeng *et al* demonstrated the up-regulation of several key cell cycle related gene transcripts in HL60 cells grown in serum-free RPMI media with 0.25 μM selenomethionine or selenite compared to HL60 cells grown in the selenium-free media (Zeng, 2002). The cells grown in the presence of Se proliferated, with removal of the G2/M block seen in the selenium-deprived cells. In addition, the cells grown in the presence of low-level Se had a >50% increase in total protein phosphorylation compared to the cells grown

without Se (Zeng, 2002). Se deficiency therefore has far-reaching effects on cell cycle regulation and protein phosphorylation.

1.4.6 Selenosis: Selenium Toxicity

In excess, Se compounds are toxic to mammals and their cells *in vitro* (Buell, 1983, Spallholz, 1994). The toxicity is cumulative. Se toxicity is inducible in laboratory mammals by feeding a diet containing 8-10 ppm Se (Jacobs and Forst, 1981b, Jacobs and Forst, 1981a). Signs of toxicity induced by selenite include skin lesions, degeneration of the muscles, bone marrow and liver necrosis (Herigstad *et al.*, 1973). The toxicity of Se can be ascribed, in part, to the oxidation of thiols and the generation of superoxide (Yan and Spallholz, 1993). In humans both acute and chronic toxicity syndromes have been described. Acute toxicity is characterised by vomiting, hypersalivation and a garlic odour on the breath due to excretion of the volatile Se metabolite dimethylselenide. Additional symptoms and signs that may be present are diarrhoea, severe vomiting, hair loss, restlessness, spasms, tachycardia and lethargy. Acute Se poisoning occurs at doses $\geq$500 μg/kg body weight. Selenosis, or chronic Se poisoning manifests as nail and hair changes and loss, skin lesions and neurological effects, which begin as altered sensation with hypoaesthesia, acroparasthaesia, pain and hyperreflexia, and progress on to convulsions, numbness and paralysis. For selenosis to develop in humans an intake in excess of 910 μg/day (equivalent to 14 μg/kg/day for a 60 kg person) is required and was found to range from 3.2-7.0 mg/day in areas with endemic selenosis in China (Yang and Zhou, 1994, Yang *et al.*, 1989a, Yang *et al.*, 1989b).

1.4.7 Selenium Metabolites And Chemoprevention

The biological activity of Se comes not from the element *per se* but from Se in a diverse assortment of chemical compounds, both organic and inorganic. Small changes in the structure of Se compounds can lead to dramatic changes in biological activity and toxicity *in vitro* and *in vivo* (Ip, 1998). Furthermore, the observation by Ip *et al* 1989 that selenomethionine was less effective than selenite in preventing the development of breast cancers in rats poisoned with DMBA (7,12-dimethylbenz(a)anthracene) despite achieving higher blood and tissue Se levels suggested that the metabolites of Se compounds determine the parent compound's efficacy as a chemopreventive agent (Ip and Hayes, 1989). Two inter-related pathways for Se compound metabolism are

recognised: the 'hydrogen selenide pool' and the 'methylselenol pool' (depicted in Figure 1.16).

The Hydrogen Selenide Pool

Sodium selenite, selenate, selenomethionine and other compounds, such as selenodioxide and selenocystine, are referred to as belonging to the *'hydrogen selenide pool'* (Figure 1.16). The metabolism of sodium selenite involves serial reduction by glutathione first to selenodiglutathione (SDG; GSSeGS) and then to glutathione selenopersulphide (GS-SeSH), before conversion to the highly toxic hydrogen selenide (H_2Se) (Ganther, 1968, Hsieh and Ganther, 1977). Selenomethionine and the benzyl selenocyanates are metabolised, principally in the liver, by a multi-step trans-sulphuration pathway and are ultimately reduced to hydrogen selenide. The fate of hydrogen selenide depends on the Se status of the treated cells and animal. Under normal physiological conditions, hydrogen selenide is converted to the amino acid selenocysteine prior to incorporation into selenoproteins under the control of the translational code UGA (Stadtman, 1996). Where hydrogen selenide is generated beyond physiological requirements of Se, it is detoxified by methylation to mono-, di- and tri-methylated derivates prior to excretion. The monomethylated derivative, methylselenol (CH_3SeH), is unstable and, like hydrogen selenide, highly toxic. The dimethyl metabolite, dimethylselenide ($(CH_3)_2Se$) is excreted in the breath (giving it a garlic odour) and trimethylselenide ($(CH_3)_3Se^+$), the trimethylated derivative of hydrogen selenide, is excreted in the urine. Trimethylselenide excretion predominates over that of dimethylselenide, except during Se toxicity when the further methylation from di- to tri-methylselenide becomes rate-limiting (Foster *et al.*, 1986).

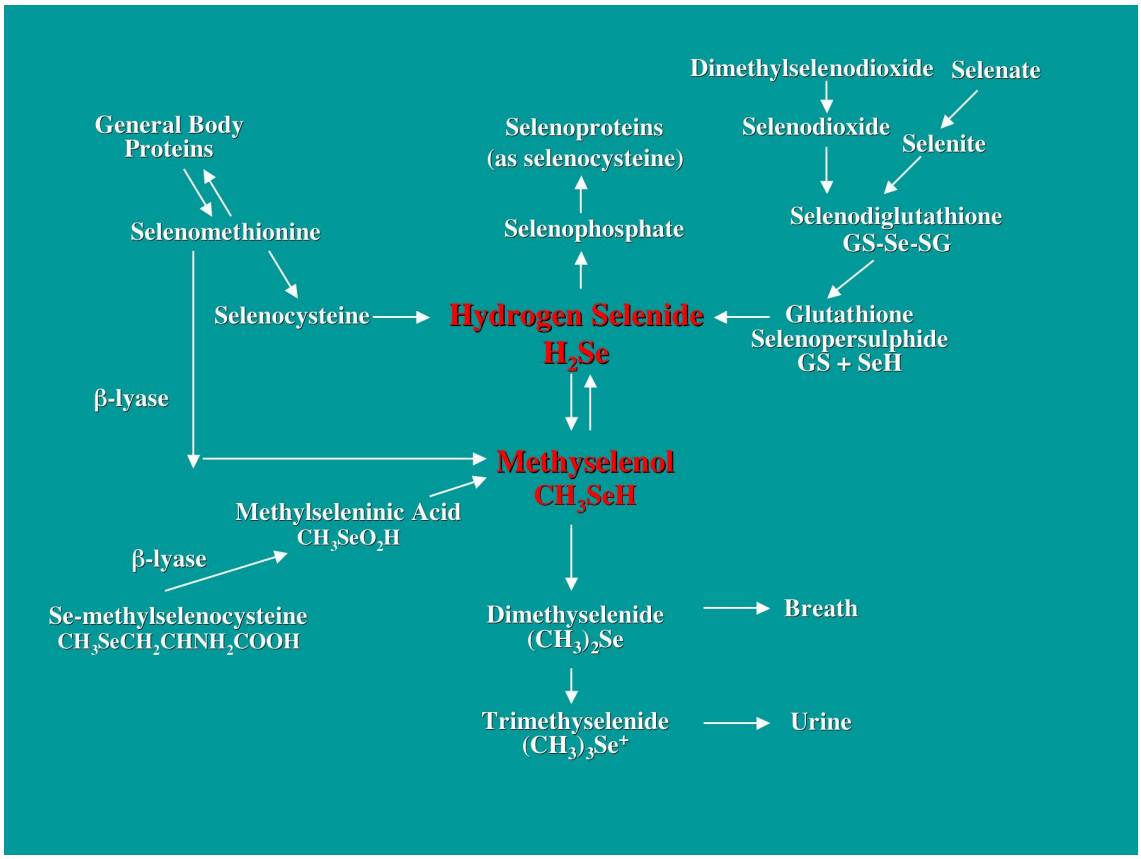


Figure 1.16 Selenium Metabolic Pathways: the Hydrogen Selenide and the Methylselenol Pools Adapted from (Schrauzer, 2000, Ip, 1998, Ganther and Lawrence, 1997)

Although selenite can inhibit cell growth (Ip *et al.*, 1981, Fico *et al.*, 1986), cell attachment (Yan and Frenkel, 1992)**,** microtubule formation (Leynadier *et al.*, 1991) and the syntheses of DNA, RNA and protein (Medina and Oborn, 1984, Cox, 1985, Jiang *et al.*, 1992, a lag period of 24 hours has been reported repeatedly before the *in vitro* activity of selenite is seen {Lanfear, 1994 #390). This is indicative of the need for selenite to be converted to more active metabolites before activity is seen. Evidence in support of this hypothesis includes:

i. the demonstration of increased efficacy of selenite *in vitro* upon the simultaneous addition of glutathione, critical for its efficient serial reduction to hydrogen selenide (Kuchan and Milner, 1991)
ii. selenite activity varies depending upon the intracellular glutathione level (Kuchan and Milner, 1992)

iii. SDG, the principle metabolite of selenite, is significantly more toxic than selenite *in vitro* (Lanfear *et al.*, 1994, Poirier and Milner, 1983)

iv. no lag period is observed as regards the *in vitro* activity of SDG in comparison to sodium selenite (Lanfear *et al.*, 1994).

The Methylselenol Pool

The formation of hydrogen selenide was shown to be unnecessary for Se compounds to exert chemopreventive activity, provided that the compounds produced monomethylated Se metabolites (Ip *et al.*, 1991) (Figure 1.16). In the presence of the intracellular enzyme β-lyase, selenomethionine and the plant organic Se compound MSC ($CH_3SeCH_2CH(NH_2)COOH$) are converted to the highly active methylselenol (Foster *et al.*, 1986). Because methylselenol is highly reactive, it cannot be studied directly, but rather needs to be locally generated from precursors such as MSC, and the β-lyase independent methylseleninic acid (MSA). In comparative, *in vivo* rat studies of selenite, selenomethionine and MSC, MSC was found to be the most effective chemopreventive agent (Ip *et al.*, 1991, Ip and Ganther, 1990, Ip and Ganther, 1992)**.** The dose of Se required to induce 50% tumour inhibition was 2 ppm Se for MSC, 3 ppm Se for selenite and 4-5 ppm Se for selenomethionine (Ip *et al.*, 1999, Ip and Ganther, 1992, Ip and Hayes, 1989, Ip *et al.*, 1991). Furthermore, MSC is not incorporated into proteins as occurs with selenomethionine. In contrast to methylselenol, the dimethyl and trimethyl metabolites of hydrogen selenide are ineffective as chemopreventive agents *in vivo*, not in small part due to their rapid elimination via the breath and urine respectively (Ip and Ganther, 1988, Ip *et al.*, 1991, Vadhanavikit *et al.*, 1993).

1.4.8 Selenium Intake and Cancer Risk

Observational Studies

Two types of epidemiological study, namely geographic correlation and case-control studies, have produced data indicating that Se can protect humans from cancer. A possible protective effect of Se against human malignancy was first suggested in 1969, following the observation of a decreased cancer incidence in populations of the US with a relatively higher Se intake (Shamberger and Frost, 1969). Subsequent epidemiological evidence in support of this observation has been mixed. Many studies have demonstrated a similar inverse association whilst others found no relationship between Se intake and cancer risk. Two important epidemiological papers, however, have led

many to conclude that an inverse association does exist. The first noted an inverse relationship between forage-crop Se and county cancer incidence in the US (Clark *et al.*, 1991) and the second revealed an inverse correlation between dietary Se intake and total age-adjusted cancer mortality in more than 25 countries (Schrauzer *et al.*, 1977).

Prospective case-control studies have demonstrated an association between low-blood Se and cancer in the US population and in Finland when the population was Se deficient (Salonen *et al.*, 1984, Salonen *et al.*, 1985, Willett *et al.*, 1983, Comstock *et al.*, 1992). For prostate cancer in particular, an inverse association between serum or toenail Se and the subsequent risk of developing prostate cancer was shown by several epidemiological studies (Brooks *et al.*, 2001, Helzlsouer *et al.*, 2000, Nomura *et al.*, 2000, Willett *et al.*, 1983, Yoshizawa *et al.*, 1998) but not all (Ghadirian *et al.*, 2000). Clarke *et al* found that serum Se at the time of diagnosis was lower in patients with skin cancer or adenomatous colonic polyp than in matched controls and a Canadian group observed a statistically significant inverse association between toenail Se level and the risk of colon cancer (OR, 0.42; 95% CI 0.19-0.93; $P = 0.009$) (Clark *et al.*, 1984, Clark *et al.*, 1993, Ghadirian *et al.*, 2000). However, other studies have failed to find an association between higher Se serum/tissue content and reduced colorectal-cancer incidence in Finland (Knekt *et al.*, 1988), lung-cancer incidence in the US (Menkes *et al.*, 1986) and overall cancer incidence in US citizens (Coates *et al.*, 1988, Ringstad *et al.*, 1988, Nomura *et al.*, 1987). Recent results from the US Third National Health and Nutrition Examination Survey Study found a nonlinear association between serum Se levels and all-cause and cancer mortality (Bleys *et al.*, 2008). This survey involved 13,887 healthy adults, who were recruited between 1988 and 1994, and followed up for mortality for up to 12 years. The mean serum Se level of the study participants was 125.6 ng/ml and the multivariate adjusted hazard ratios comparing the highest ($\geq$130.39 ng/ml) with the lowest (< 117.31 ng/ml) serum Se level tertile were 0.83 (95% CI= 0.72-0.96) for all-cause mortality, 0.69 (95% CI= 0.53-0.90) for cancer mortality, and 0.94 (95% CI= 0.77-1.16) for cardiovascular mortality. There was an inverse association at low Se levels (< 130 ng/ml) and a modest increase in mortality at high Se levels (> 150 ng/ml).

Taken together, these observational studies in humans suggest an inverse association between Se consumption, as assessed by serum or tissue levels, and subsequent risk of developing cancer and the possibility of a threshold level beyond which serum selenium

modestly increases mortality. This data is considerably strengthened by the interventional study results described in the next subsection.

Intervention Studies: Selenium Supplementation In Animal Models of Cancer

Many of the intervention studies performed in laboratory animal models of cancer have used the inorganic Se compound sodium selenite or the organic compound selenomethionine, due to their cheap and easy commercial availability. Most studies have increased dietary intake from the normal nutritional requirement of ~ 0.1 ppm to between 1 to 5 ppm Se (Medina, 1986, Thompson *et al.*, 1984, Ip, 1986, Medina and Morrison, 1988). Se chemoprevention, without apparent signs of toxicity, occurred in a dose-dependent manner (Clark *et al.*, 1996, Clark and Jacobs, 1998, Medina *et al.*, 2001, Duffield-Lillico *et al.*, 2002, el-Bayoumy, 1994, Rao *et al.*, 2001, Reddy *et al.*, 1994, Ip and White, 1987, Davis *et al.*, 2002). In the last 15 years, attempts to refine the chemopreventive effects of Se have led investigators to experiment with novel organic selenocompounds such as the synthetic benzyl selenocyanates (due to their superior therapeutic index compared to selenite and selenomethionine) and the naturally occurring plant Se compound MSC, which is readily converted to the putative highly active chemopreventive metabolite methylselenol *in vivo*. Selenocompounds have been proven to reduce the incidence of tumours of the colon, breast, pancreas, liver, lung, skin and several other sites in organ-specific carcinogen models (Thompson *et al.*, 1984, el-Bayoumy, 1985, Tanaka *et al.*, 1985, el-Bayoumy *et al.*, 1993, Reddy *et al.*, 1994). The chemopreventive effect of Se compounds occurs at the early stages of carcinogenesis, at both the initiation (the prevention of DNA damage by carcinogens) (Ejadi *et al.*, 1989, el-Bayoumy *et al.*, 1992, Fiala *et al.*, 1991) and post-initiation stages (Medina, 1986, Ip, 1998, Thompson *et al.*, 1984). In addition to a reduction in the incidence of carcinogen-induced cancers, experiments have demonstrated the prevention of spontaneous cancers and a reduction in the number and size of transplanted tumours in different animal models through Se supplementation (Watrach *et al.*, 1982, Poirier and Milner, 1983, Schrauzer, 1992, Yan *et al.*, 1997).

Human Selenium Supplementation Trials: The Clark Study

Clark *et al* conducted the first double-blind, randomised, placebo-controlled trial of Se supplementation (Clark *et al.*, 1996). It began in 1986 and closed early in 1992, due to the results detailed below. Patients with a history of resected non-melanomatous skin cancer (i.e. squamous and basal cell carcinomas) were randomised to receive placebo or

selenised brewer's yeast equivalent to 200 μg of Se a day. Selenised brewer's yeast contains a variety of Se compounds dominated by selenomethionine. Patients received placebo or supplementation for a mean of 4.5 years, giving 1271 years of follow-up for the 1312 patients enrolled to the study. No difference in the incidence of further squamous and basal-cell carcinomas was found between the treatment and control arms of the study. Unfortunately with further follow-up Se supplementation remained ineffective at preventing basal cell carcinoma and it increased the risk of squamous cell carcinoma (hazard ratio, HR = 1.25, 95% CI = 1.03 to 1.51) (Duffield-Lillico *et al.*, 2003). The adverse effect of Se supplementation appeared to increase with increasing baseline plasma Se concentration; skin cancer patients with baseline plasma Se in the upper tertile (≥122.4 ng/ml) experienced a 60% increase in probability of a new skin cancer as a result of Se supplementation, compared to no increased risk in the lower two tertiles. A disappointing answer at odds with findings that topical application of selenium protects humans against ultraviolet B radiation, the protective effect of selenomethionine against skin cancer development in mice subjected to ultraviolet irradiation and the result of Clarke's observational study (Uddin *et al.*, 2005, Oh *et al.*, 1995, Burke *et al.*, 1992, Clark *et al.*, 1984). Thus, the initiation of Se supplementation may need to begin many years before the overt development of skin cancer (due to the natural lifelong exposure to ultraviolet light), require co-administration of vitamin E (Uddin *et al.*, 2005) or be restricted to those with a serum Se of ≤105.2 ng/ml (Duffield-Lillico *et al.*, 2003); or Se deficiency may simply not be an aetiological factor in skin carcinogenesis. However, Se treatment decreased total cancer incidence by a statistically significant 25%, with the incidence and deaths from three of the most common solid organ cancers, namely prostate, lung and colon, were significantly reduced. The relative risks of developing cancer of the prostate, lung and colon were 0.42 ($P = 0.03$), 0.54 ($P = 0.04$) and 0.37 ($P = 0.002$) in the selenised brewer's yeast vs. placebo arms (Clark *et al.*, 1996). In 2002 an updated analysis of the data with an additional six years follow-up found the benefits of Se supplementation had diminished (Duffield-Lillico *et al.*, 2002). Total cancer incidence was still reduced (HR = 0.75, 95% CI = 0.58-0.97) as was prostate cancer incidence (HR = 0.48, 95% CI = 0.28-0.80) but the incidence of lung cancer (HR = 0.74, 95% CI = 0.44-1.24) and colorectal cancer (HR = 0.46, 95% CI = 0.21-1.02) was no longer significantly reduced. The protective effect of Se supplementation was confined to males (HR = 0.67, 95% CI = 0.50-0.89)

and was most pronounced in former smokers. Participants with baseline plasma Se concentrations in the lower two tertiles (<121.6 ng/ml) experienced reductions in total cancer incidence, whereas those in the highest tertile showed a nonsignificant elevated incidence (HR = 1.20, 95% CI = 0.77-1.86) (Duffield-Lillico *et al.*, 2002).

Although numerous studies have demonstrated a chemopreventive effect of Se compounds against carcinogen-induced breast and colon cancers in animal models, breast cancer incidence was unaffected and colon cancer was reduced only during the initial study period and not during the extended follow-up period in the selenised yeast arm of the Clark study. Three possible explanations for these observations are:-

1 That too few cases of each cancer occurred in this patient group to provide the requisite statistical power

2 That supplementation was stopped too soon whilst follow-up continued years beyond the end of the trial and long after any chemopreventive action had worn off (as in the Yu study below). Indeed for aspirin to reduce the risk of colorectal cancer it needs to be taken for a minimum of five years with a latency of 10 years before benefit is seen (Flossmann and Rothwell, 2007).

3 That Se deficiency and Se supplementation are respectively irrelevant to the aetiology and prevention of breast and colon cancers in humans.

An important observation in the Clark study was that the chemopreventive effect was strongest in those subjects with the lowest plasma Se level before supplementation. A physiological threshold for Se may therefore exist, below which supplementation is beneficial and above which it is inconsequential or potentially harmful.

Human Supplementation Trials: The Yu Study

The Yu study was a population-based study in 130,000 people in an area of China with low Se intake, a 15% prevalence rate of hepatitis B, and an incidence of primary liver cancer of ~ 50/10,000/annum (Yu *et al.*, 1997). After eight years of intervention with selenised table salt, the incidence of primary liver cancer had fallen by 35% in the intervention-township vs. the non-supplemented control townships. On withdrawal of Se from the treated group, primary liver cancer incidence rate began to increase. However, the inhibitory response to hepatitis B was sustained during the three years of follow-up beyond the cessation of treatment. An associated clinical sub-study among 226 hepatitis B surface antigen positive persons provided either daily 200 μg of Se in the form of a selenised yeast tablet or an identical placebo yeast tablet for four years.

This sub-study showed that seven of 113 subjects were diagnosed as having primary liver cancer in the placebo group, whereas no incidence of primary liver cancer was found in 113 subjects supplemented with selenium. Again on cessation of treatment, primary liver cancer developed at a rate comparable to that in the control group. Yu *et al* concluded that a continuous intake of Se is essential to sustain its chemopreventive effect.

Human Supplementation Trials: The SU.VI.MAX Study

This French randomised, double-blind, placebo-controlled primary prevention trial investigated a daily capsule of combined antioxidants (120 mg vitamin C, 30 mg vitamin E, 6 mg beta-carotene, 100 μg Se, and 20 mg zinc) vs. a placebo (Hercberg *et al.*, 2004). Just over 13,000 French adults (7876 women aged 35-60 years and 5141 men aged 45-60 years) were recruited and followed up for 7.5 years. Low-dose antioxidant supplementation lowered total cancer incidence in men (relative risk, 0.69; 95% CI 0.53-0.91) and all-cause mortality in men (relative risk, 0.63; 95% CI 0.42-0.93) but not in women. The risk of non-melanomatous skin cancer was unaffected by taking the antioxidant combination in either sex, but there was a increased incidence of melanoma in the women who received the supplement, although the number of events was small (adjusted HR = 4.31; $P = 0.02$) (Hercberg *et al.*, 2007).

Human Supplementation Trials: The Linxian Study

Between March 1986-May 1991 29,584 adults were recruited in Linxian County, China, which has one of the world's highest rates of gastro-oesophageal cancer and a population with chronically low intake of several micronutrients, to a primary chemoprevention trial (Blot *et al.*, 1993). The subjects were randomly assigned four combinations of nutrients: (A) retinol (5000 IU, as retinol palmitate) and zinc (22.5 mg, as zinc oxide); (B) riboflavin (3.2 mg) and niacin (40 mg); (C) vitamin C (120 mg) and molybdenum (30 μg, as molybdenum yeast complex); and (D) beta-carotene (15 mg), alpha-tocopherol (30 mg), and selenium (50 μg, as selenium yeast) i.e. all doses 1-2 times U.S. Recommended Daily Allowances. During the 5-year intervention period 2,127 deaths occurred amongst the trial subjects, with cancer being the leading cause of death. Indeed, 32% of all deaths were due to gastro-oesophageal cancer. Total mortality was significantly lowered only by factor D (beta carotene, vitamin E, and selenium; relative risk = 0.91, 95% CI = 0.84–0.99). This reduction was largely due to lower total cancer rates (RR = 0.87; 95% CI = 0.75–1.00), particularly stomach cancer (RR = 0.79;

95% CI = 0.64–0.99). The reduction in risk began between one to two years after the start of supplementation. These results are provocative, as the dose of Se was physiological rather than the equivalent chemopreventive doses using in animals. Unanswered questions include 'how relevant are the results to Western populations?', where micronutrient deficiency is unusual, and 'are all three supplements needed?', particularly as beta carotene supplementation is detrimental in smokers.

Human Supplementation Trials: The Limburg Study

Limburg *et al* conducted a randomised, placebo-controlled chemoprevention trial in Linxian province, China, where oesophageal cancer is the leading cause of cancer mortality (Limburg *et al.*, 2005). The aim was to prevent the development of squamous cell cancer of the oesophagus from its precursor squamous dysplasia using a Se compound and/or a COX-2 inhibitor. Two hundred sixty-seven asymptomatic adults, with histologically confirmed mild or moderate oesophageal squamous dysplasia, were randomised to receive placebo or 200 μg selenomethionine daily and 200 mg celecoxib twice daily or placebo ('2 x 2' factorial design). After 10 months of intervention selenomethionine demonstrated a chemopreventive effect, favourably changing dysplasia grade among the 115 subjects with mild oesophageal squamous dysplasia at baseline (P = 0.02), but not among the 123 subjects with moderate oesophageal squamous dysplasia at baseline (P = 1.00). Celecoxib status did not influence changes in dysplasia grade overall (P = 0.78) or by baseline histology subgroup. This is the first report of a possible chemopreventive agent in mild squamous dysplasia of the oesophagus.

Summary of Human Supplementation Trials

These five studies still require further investigation. Should only Western men and those with serum Se levels <130 μg/l receive Se supplementation? What dose and Se compound should be used? Are the doses and duration of intervention different in the primary and secondary cancer chemoprevention settings? In the field of prostate cancer large-scale phase III, double-blinded, randomised placebo-controlled studies are being undertaken with the PRECISE trial in Europe and the SELECT (Selenium and Vitamin E Cancer Prevention Trial) trial in the US, which is investigating Se supplementation in 32,000 men prospectively for 12 years (Klein *et al.*, 2000).

1.4.9 The Selenoproteome and Cancer

Understanding of the selenoproteome's influence on carcinogenesis is beginning to emerge. Transgenic mice displaying reduced selenoprotein synthesis due to a mutant selenocysteine transfer RNA gene, were found to exhibit increased colon cancer susceptibility (Irons *et al.*, 2006). Bigenic mice co-exhibiting reduced selenoprotein levels due to expression of an altered selenocysteine-tRNA (i6A-) and targeted expression of SV40 large T and small t oncogenes in prostate were found to develop precancerous prostate lesions faster than control WT/Tag mice (Diwadkar-Navsariwala *et al.*, 2006). Similarly, mice with a double knock-out for GPx-1 and -2 exhibit an increased incidence of gastrointestinal tumours (Chu *et al.*, 2004). GPx-1 loss of heterozygosity (LOH) is frequently seen in human breast and colon cancers (Hu *et al.*, 2005, Hu and Diamond, 2003). Whilst, enforced expression of GPx-1 resulted in resistance to doxorubicin and inhibition of Fas-induced effector caspase activation, DNA fragmentation, and apoptotic cell death human breast cancer T47D cells (Gouaze *et al.*, 2001, Gouaze *et al.*, 2002). Sepp1 has been shown to be downregulated in murine and human prostate cancer cell-lines and primary human prostate carcinomas compared to normal prostate tissue (Calvo *et al.*, 2002) and Sepp1 mRNA expression is significantly reduced or lost in human colon cancers (Al-Taie *et al.*, 2004). Increased levels of Trx-1 in human tumours can lead to functional inhibition of PTEN (Meuillet *et al.*, 2004) and Trx-1 protein levels are significantly elevated in several human cancers and are an independent poor prognostic factor in colon cancer (Raffel *et al.*, 2003). A positive correlation between Trx-1 expression and cisplatin resistance was observed in a panel of 11 ovarian cancer cell-lines (Yamada *et al.*, 1996). Overexpression of selenophosphate synthetase (sps1) results in increased sensitivity *in vitro* to radiation through upregulation of p53 (Chung *et al.*, 2006). In humans, TR-1 expression is increased in several cancers and is associated with aggressive tumour growth and decreased patient survival (Engman *et al.*, 2006). *In vitro*, TR-1 overexpression results in increased tumour growth, HIF-1 and VEGF levels (Lincoln *et al.*, 2003) and TR-1 inhibition/siRNA knockdown results in cell death and malignant cell phenotype recapitulation (Lu *et al.*, 2006b, Yoo *et al.*, 2006). p53 activity has a specific requirement for an intact Trx system (Merwin *et al.*, 2002). Chemical (electrophilic disruption of the C-terminal selenocysteines) and mutational (lacking the two C-terminal selenocysteines) modification of TR-1 results in two gain-of-function roles for

modified TR-1: conformational misfolding of p53, rendering p53 incapable of transactivation of its effector genes; and, despite inactivation of p53, induction of apoptosis via caspase-3 activation (Anestal and Arner, 2003, Cassidy *et al.*, 2006). Reduction of TR-1 levels protected p53 conformation in colon and breast cancer cells treated with endogenous electrophiles and also antagonised electrophile-induced apoptosis (Cassidy *et al.*, 2006).

1.4.10 Molecular Mechanisms of Selenium Chemoprevention

The hydrogen selenide pool members such as selenite exert their activity in part through the generation of ROS, which can induce general or specific DNA damage (Zhou *et al.*, 2003, Stewart *et al.*, 1999). Selenite is also reported to cause topoisomerase II/DNA cleavable complex formation resulting in double-strand DNA breaks and apoptosis (Zhou *et al.*, 2003). This activity resulted from the modification of important thiol groups in topoisomerase II by selenite generated ROS, an action reversed by glutathione.

Intracellular Se compounds (not incorporated into proteins) can alter the activity of transcription factors and kinases, such as c-Jun N-terminal kinase (JNK) and caspase-3, by oxidation of reactive cysteine residues within the proteins (Park *et al.*, 2000, Ganther, 1999, Adler *et al.*, 1996). Both *in vitro* and *in vivo*, Se can mimic insulin (Stapleton, 2000), and can activate the signal transduction factors mitogen-activated protein kinase -1 (MAPK1/ERK2) and -3 (MAPK3/ERK1) (Stapleton *et al.*, 1997, Hei *et al.*, 1998).

MSA and MSC both induce phase II liver and kidney detoxification enzymes, so by detoxifying carcinogens by glucuronidation, as part of their mode of cancer chemoprevention against carcinogens (Zhao *et al.*, 2004, Ip and Lisk, 1997). Recently human tissue expression profiling results suggest that Se supplementation enhances immune surveillance against premalignant cells (Joshi *et al.*, 2006).

The original hypothesis to explain the inverse association between Se status and cancer risk was that optimal activity of the antioxidant seleno-enzymes glutathione peroxidase and TR eliminated tumour promoting reactive oxygen species (ROS). However, from animal supplementation experiments (as mentioned above) the quantity of Se that must be ingested to demonstrate a chemopreventive effect is $\geq$10 times (1-30 ppm Se/day) that required to restore enzyme function to 100% (~0.1 ppm Se/day). Furthermore null mice lacking the *Gpx1* gene, which codes for the cytosolic and most abundant

glutathione peroxidase, are no more susceptible to cancer than wild-type mice up to an age of 15 months (Cheng *et al.*, 1997, Ho *et al.*, 1997). Indeed *Gpx1-/-* appear phenotypically normal until subjected to oxidative stress (Cheng *et al.*, 1998). In contrast, mice overexpressing *Gpx1* developed more skin cancers following carcinogen exposure than their normal counterparts (Lu *et al.*, 1997). As regards TR, the enzyme can be inhibited by high levels of Se supplementation due to thiol cross-linking of cysteine residues within its catalytic domain (Ganther, 1999). The molecular mechanisms by which Se supplementation prevents cancer are thus still being determined as will be described in Chapter 4. One intriguing observation is that the effectiveness of Se compounds *in vitro*, in terms of growth inhibition and cell death induction, occurs irrespective of functional p53 status (Kaeck *et al.*, 1997, Lanfear *et al.*, 1994).

1.4.11 The Rational Research Strategy: Identification of Gene Expression Signatures Predictive of Outcome in DLBCL

The rational process was used to construct a research programme to discover prognostic factors and potential therapeutic targets in DLBCL as follows: *"DLBCL is a heterogeneous disease where the genetic aberrations of each tumour determine the genes and proteins expressed. Each tumour's molecular genotype determines its phenotype, which in turn determines its chemosensitivity or resistance and therefore the response and long-term outcome to treatment for the patient. Global assessment of gene expression in DLBCL material from patients that succumbed or were cured of their DLBCL should therefore yield distinct expression profiles reducible to a small subset of common genes".* The modern rational approach adopted, utilised the recently developed technique of gene expression profiling using Affymetrix oligonucleotide microarrays. This technique allows the simultaneous and parallel measurement of the transcriptional expression of thousands of genes, escaping the need for *a priori* empiric knowledge of association and activity of individual genes in carcinogenesis and chemosensitivity. RNA from archived, presentation DLBCL material would therefore be prepared and arrayed and the resultant dataset explored for transcriptional profiles associated with long-term outcome to treatment. If this rational strategy indeed yielded a group of genes whose profiles were strongly linked to treatment outcome in DLBCL, the genes with feasible molecular roles in lymphocyte biology would be investigated as rational drug targets. Demonstration of novel molecular drug targets would strengthen the argument

of a causative rather than correlative association between expression of the genes concerned and outcome to treatment.

Evidence in support of genetic aberrations underlying the transcriptional differences seen in tumours compared to their normal tissue counterparts comes from many studies of single gene or chromosomal changes as well as more recently from genome-wide surveys linked to the corresponding transcriptome. In regard to tumour suppressor gene mutation and consequent expression alteration, mutation of *TP53* has been repeatedly demonstrated to result in excessive, abnormal protein expression in DLBCL, other NHLs and cancers (Koduru *et al.*, 1997). The *c-Myc* oncogene is translocated to an immunoglobulin gene locus in all cases of BL and 10% of DLBCL, and amplified in 16% of DLBCL, with the consequence of several fold increased *c-Myc* RNA expression (Kawasaki *et al.*, 2001, Maguire *et al.*, 1983, Rao *et al.*, 1998). The far-reaching expressional consequences of *c-Myc* gene amplification and overexpression have been confirmed by the discovery that no less than 15% of ~500 genes examined in a genome-wide survey were transcriptionally activated by c-Myc (Li *et al.*, 2003). Demonstration of the impact of genetic copy number changes on gene expression due to partial chromosomal deletions and amplifications comes from investigations of trisomy 8 in AML, where numerous chromosome 8 genes were proportionally over expressed (Virtaneva *et al.*, 2001). Similarly, scrutiny of the expression patterns of genes from chromosome 17 in breast cancer, where 30% of cases have partial gains and/or losses in chromosome 17, revealed an appropriate increase or decrease in gene expression according to whether the respective segment of chromosome 17 was deleted or amplified. Genes affected included *c-erbB2,* which was overexpressed due to its region of chromosome 17 being amplified. *c-erbB2* not only has prognostic significance in breast cancer but also predictive significance for response to trastuzumab monoclonal antibody therapy (Orsetti *et al.*, 2004). In DLBCL, amplification of the oncogene *BCL-2* has been associated with overexpression of Bcl-2 protein and altered mRNA expression, the level of which predicts Bcl-2 protein expression (Shen *et al.*, 2004, Monni *et al.*, 1997).

In the light of these examples, and many others, examination of the transcriptome for molecular signatures of prognosis was not only a valid and rational strategy, but transcriptional profiles can be anticipated to reflect and reveal the genomic changes unique and necessary for DLBCL.

1.4.12 An Introduction to Microarray Technology

The means of discovery of molecular signatures of response, long-term outcome and subtype definition took a great leap forward with the advent of microarray technology (Pease *et al.*, 1994, Schena *et al.*, 1995). By allowing the simultaneous measurement of the mRNA expression of hundreds to thousands of genes from across the genome, array technology offered the potential to screen thousands of genes without the need for preconceived hypotheses.

Microarrays can investigate mRNA or genomic DNA. mRNA targeted arrays allow gene expression to be studied, whilst genomic DNA arrays can take two forms: CGH arrays where bacterial artificial chromosome clones are arrayed and used to reveal partial chromosomal gains and losses with much greater definition than the 20Mb of conventional CGH and SNP arrays, which allow the detection of genetic mutations and polymorphisms. An array is constructed on a solid substrate, often termed the *'platform'*, made of either glass (microarray) or nitrocellulose or nylon (macroarrays). On the substrate, hundreds (macroarrays) to thousands (microarrays) of cDNA amplicons or oligonucleotides are immobilised in an ordered, grid pattern. Confusingly, the arrayed cDNA amplicons or oligonucleotides are called *'targets'* in most systems but *'probes'* in Affymetrix chips. As this thesis refers to the use of the Affymetrix system, the cDNA amplicons or oligonucleotides will be referred to as *'probes'* henceforth. To the *'probes'*, labelled complementary RNA (cRNA), cDNA or genomic DNA is hybridised in a manner similar to that of Southern and Northern blotting. In the Affymetrix system, and the remainder of this thesis, the labelled cRNA, cDNA or genomic DNA is referred to as the *'target'*, whilst in most other systems it is referred to as the *'probe'*. Each of the immobilised *'probes'* acts as an assay for its specific partner strand in the complex nucleic acid mixture of the *'targets'*. The *'target'* labels can be fluorophores, biotin or radioactive phosphorus. By quantifying the intensity of label signal from each hybridised *'probe-target'* set, the relative expression of each gene, presence or absence of a chromosomal region or SNP is detected. Arrays designed for radioactive labelled *'target'* (macroarrays) require as little as 50 ng RNA (5,000 cells), compared to the minimum of 5 μg (500,000 cells) required for fluorescent systems. ^{32}P - based arrays, however, produce reliable read-outs only for highly expressed genes, which are the minority. Fluorecence-dependent arrays allow detection of low and medium abundance genes, which are the majority, as well as those genes highly

expressed at the mRNA level. It is the fluorescence-dependent arrays that have been used to great effect in the study of clinical material, including DLBCL, and that will now be described in more detail.

Two types of array dominate the expression profiling field: *'chips'* where oligonucleotide *'probes'* are grown out from the substrate surface and *'dot-blot'* slides or membranes, where tiny dots of probe solution are blotted on to the substrate. A third type of array has been developed called *'Oligonucleotide Ink-Jet/Piezo'* and will be briefly described.

1.4.13 The Different Types of Microarray Platform

'Dot-blot' Arrays

The *'probes'* for *'dot-blot'* slides are created from cDNA clone libraries using the PCR to amplify part of the cDNA clone insert of each bacteriophage in the library. The arrayer, a robot, arrays 0.25-1 nl of probe cDNA solution in the same place on the 20+ chemically-treated slides under production. Up to 40,000 different *'probes'* can be arrayed per standard size slide. After the probe solutions have dried, the *'probes'* are covalently fixed to the slide using UV light. Maintaining the clone libraries required for *'dot-blot'* slides makes slide manufacture too expensive for all but the largest non-commercial organisations, unless the number of *'probes'* arrayed is compromised, and has sensibly led to the establishment of core facilities capable of providing arrays to a network of labs.

Because the volume of each probe spot deposited can vary, apparent expression changes can be produced by spot differences alone. To compensate for this artefact, two *'target'* samples are used, the test sample and a reference RNA. The reference or control RNA is usually created from pooled cell-lines in sufficient quantity to be used with all the test samples in a particular experiment. In time exposure experiments, the time zero sample is used as the reference. The test sample RNA is converted into labelled cDNA *'target'* using one fluorophore whilst the reference RNA is converted into labelled cDNA *'target'* using a different fluorophore. Equal amounts of test and control *'target'* are added to each array, allowing the test and reference cDNA fragments to competitively hybridise with the *'probes'*. If a test cDNA is in excess the resultant signal will come from its fluorophore, if the control cDNA dominates the fluorescence detected will come from the control cDNA. By employing competitive hybridisation the problem of dot variability is overcome.

'Oligonucleotide Ink-Jet/Piezo' Arrays

A development from *'dot-Blot'* arrays, *'Oligonucleotide Ink-Jet/Piezo'* arrays use no-contact, less wasteful piezo electric pulse or ink-jet technology to propel a tiny volume of synthesised oligonucleotide solution on to the platforms. Oligomers of 60 bp length were found to offer optimal sensitivity and specificity by Hughes *et al* (Hughes *et al.*, 2001). Sensitivity down to one transcript per cell can be achieved. Standard and custom-made 25- or 60-mer oligonucleotide arrays are available from several commercial sources (e.g. Agilent Technologies, Santa Clara, USA). The release of the POSaM (piezoelectric oligonucleotide synthesiser and microarrayer) platform for academic research allows the production of multiple microarrays of up to 9,800 unique reporter sequences on modified glass microscope slides (Lausted *et al.*, 2004). The microarray synthesis is relatively cheap and certainly rapid with new arrays ready for hybridisation the same day that they are designed (if all goes according to plan).

'Chip' Arrays

'Chip' arrays consist of oligonucleotide *'probes'* synthesised *in situ* on silica wafers under the control of photolithographic chemistry (Lockhart *et al.*, 1996). The lengths of both the *'probes'* and *'targets'* are critical to the sensitivity and specificity of the hybridisation reactions. Probe oligomers between 20 and 60 bases in length provide the best balance between discriminate and sensitive hybridisation. In the Affymetrix system (see www.affymetrix.com/), the sense *'probes'* are 25-mer (high specificity but reduced sensitivity cf. longer oligomers) and are referred to as *'Perfect Matches'*. As well as the correct oligonucleotides, missense oligonucleotides are created in parallel for each probe. The missense *'probes'* are termed *'Mismatches'*, differing from their sense probe by just their middle i.e. 13^{th} base. This allows the specificity of the hybridisation reaction to be determined by comparison of the extent of hybridisation to the missense and sense *'probes'*, as well as background noise reduction by subtraction of missense from sense signal. The lower the value of the missense:sense signal ratio, the better. For each gene, a set of 11 - 20 perfect match and mismatch probe pairs is laid down, which increases the sensitivity for detecting a gene transcript to, at best, 1 in 300,000 transcripts. The area each oligomer is arrayed into is called the *'feature'*. Through the reduction in *'feature'* size from 20 to 11 μm and probe sets per gene, up to 54,000 gene *'probes'* can be included on one chip. As with *'dot-blot'* slides, the *'target'* for oligonucleotide chips is created from test sample RNA. The RNA is reverse transcribed

into cDNA followed by transcription back to RNA, as cRNA. Because this two-step procedure results in amplification of the mRNA, less RNA is required for chip arrays compared with *'dot-blot'* arrays (5-10 μg vs. 30 μg). To produce the labelled *'target'*, biotin-conjugated bases are used in the synthesis of the cRNA. The labelled cRNA requires fragmentation into lengths of 20 to 200 bp, followed by denaturing before application to the array. Unlike *'dot-blot'* arrays, competitive hybridisation and the need for a reference RNA are not required for inter-chip array expression profile comparison. This is because the oligonucleotide synthesis of each *'feature'* is so precisely controlled that inter-chip variation is minimal. The cost of commercially available generic chips has reduced substantially since their introduction, making the technology affordable to an increasing number of research facilities. Furthermore customised chips can be ordered containing only the gene feature of interest from previous experiments or considered critical to a particular illness, tissue or set of experimental conditions.

1.4.14 Expression Profiling in Cancer and DLBCL

From Schena *et al*'s proof-of-principle paper of 1995 that a high-capacity system could monitor the expression of genes in parallel using a *'dot-blot'* cDNA array for just 45 *Arabidopsis* genes, the first report of the use of a microarray platform to investigate the gene expression of cancer was the following year in 1996 using a high-density *'dot-blot'* array containing 1,161 cDNAs (Schena *et al.*, 1995, DeRisi *et al.*, 1996). DeRisi *et al* looked for expression differences between the parent melanoma cell-line UACC-903 and its less tumourigenic derivative with an inserted normal chromosome 6. Without using sophisticated analysis tools, gene expression differences were clearly apparent in a range of mostly unexpected genes. In 1998 the first report of microarrays being used to search for transcriptional changes unique to a particular type of neoplasm were reported (Khan *et al.*, 1998). Using a *'dot-blot'* array of 1,238 cDNAs and the data handling technique of multidimensional scaling seven alveolar rhabdomyosarcoma cell-lines were clustered together and distinguished from control cell-lines. In 1999 the potential of the oligonucleotide array for molecular classification of human malignant tissue was first described (Golub *et al.*, 1999). Using the Affymetrix Hu6800 chip containing oligonucleotides for 6,817 genes, 38 AML and acute lymphoblastic leukaemia (ALL) specimens were arrayed and the resultant dataset subjected to a newly-devised type of mathematical cluster analysis called self-organising maps (SOMs), that was capable of recognising and classifying features within the complex,

multidimensional data (Tamayo *et al.*, 1999). Distinguishing the AML from the ALL samples based upon transcriptional differences proved possible. Furthermore, the derived class predictor was able to correctly classify additional AML and ALL samples, confirming the general strategy of using microarrays for discovering and predicting cancer classes independent of previous biological knowledge. When a four-cluster SOM was created, the first subgroup was exclusively AML, the second subgroup contained all eight T-ALLs, and the third and fourth subgroups contain the majority of the B-ALL samples. This further subdivision of the tumour samples indicated for the first time that transcriptional signatures would allow increasingly sophisticated molecular subclassification of cancer. Expression profiling experiments have subsequently been reported for all the common cancers and many rare tumours.

The variability in clinical response and course of DLBCL and the recognition of morphological subtypes and nuances has led to the hypothesis that DLBCL is in fact composed of several distinct subtypes with differing outcomes to treatment. As such subtypes cannot be successfully reproduced on the basis of morphological appearance alone, the search for molecular methods of subdividing DLBCL into meaningful clinical subtypes has long been the goal of lymphoma clinicians, pathologists and scientists.

The two major platforms for expression profiling have been used in DLBCL and have produced remarkable and reproducible expression signatures that reveal molecularly defined subtypes of DLBCL and predict for response to treatment and long-term outcome (Shipp *et al.*, 2002, Rosenwald *et al.*, 2002, Alizadeh *et al.*, 2000). Indeed these papers were the first to specifically search for and find gene expression profiles predictive of long-term outcome. The Shipp *et al* paper is the subject and basis of Chapter 5.

In the Alizadeh *et al* and Rosenwald *et al* papers, the clustering analyses were performed using a different approach termed hierarchical clustering (Eisen *et al.*, 1998). Alizadeh *et al* demonstrated that molecular subclassification of DLBCL on the basis of gene expression was possible. The two subtypes produced had expression profiles similar to those of different putative cells of origin (Alizadeh *et al.*, 2000). This finding was confirmed and extended in the much larger series of Rosenwald *et al*, in which DLBCL was divided into three subtypes termed germinal centre-like, activated B-cell-like and Type 3 DLBCL (Rosenwald *et al.*, 2002). In both papers, the patients with lymphomas subclassified as germinal centre-like fared better than the patients with

lymphoma subclassified as activated B-cell-like, suggesting clinical relevance to DLBCL subclassification according to the putative cell of origin.

1.4.15 Exploration and Validation of The Empirical and Rational Research Strategy Results

Both research strategies proved fruitful: the empirically driven investigation discovered that presentation serum Se was predictive of chemotherapy dose-delivery, response to chemotherapy, first remission Duration and OS in DLBCL and associated aggressive NHLs (subject of Chapter 3); the rational approach of gene expression profiling archived, diagnostic lymph node material from DLBCL patients also proved to be worthwhile, as the gene expression signatures created were independently capable of outcome prediction for the patients whose material was arrayed (subject of Chapter 5). In particular, a model composed of just 13 genes proved capable of accurately delineating the patients from whom the samples came into *'alive and cured'* and *'dead from disease/refractory'* groups. These results opened new avenues for exploration and validation, which have grown to become the subjects of Chapters 4 and 6.

Investigation of Selenium Compounds For Causality And As Novel Treatment Targets

Following the empirically led discovery of serum Se as a prognostic factor in DLBCL, I chose two Se compounds to study *in vitro*, one from the methylselenol pool (MSA) and one from the hydrogen selenide pool (SDG) of Se compound metabolic pathways. SDG had not been studied in human lymphoma cells *in vitro* before, yet had demonstrated activity in other cell culture systems and was an active metabolite of the naturally occurring inorganic sodium selenite. MSA is known to be readily converted to the highly active methylselenol *in vitro* without the need for β-lyase. As β-lyase is not present in lymphocytes, study of other methylselenol pool members (e.g. selenomethionine and MSC) would have proved ineffective *in vitro*. MSA, like SDG, was also appealing, as it had not previously been studied in human NHL cell-lines and primary cultures (see Chapter 4).

Investigation of Components of The 13-Gene Model For Causality And as Novel Treatment Targets

Of the different gene expression signatures created that could predict for outcome in the DLBCL patients from whom the diagnostic material came, the model containing just 13-genes proved to be the most robust. To validate members of this 13-gene model and

to search for rational novel treatments for DLBCL, inhibitors of two of the 13-gene model genes, PKCβ and PDE4B (phosphodiesterase-4B), were studied in established DLBCL cell-lines and primary lymphoma cell cultures searching for cytostatic and cytotoxic activity. I chose PKCβ and PDE4B as the two targets for further investigation because: -

i. The expression PKCβ and PDE4B was predictive of poor OS in the series described in Chapter 5: both genes were significantly overexpressed in DLBCL that proved fatal/refractory, as opposed to curable.

ii. Furthermore, PKCβ and PDE4B were predictive of OS in the series described by Alizadeh *et al* (Alizadeh *et al.*, 2000).

iii. The alternatively-spliced PKCβ1 and PKCβ2 isoforms are the major PKC isoforms expressed by B-lymphocytes (Mischak *et al.*, 1991). PKCβ-deficient mice have profoundly impaired humoral and B-cell proliferative responses, indicating a critical role for PKCβin B-cell signalling and survival (Leitges *et al.*, 1996). *In vitro* the consequences of B-cell receptor (BCR) signalling have been shown to be dependent upon PKCβ activation (King *et al.*, 1999). In the presence of an intact PKCβ pathway, B-cell–receptor engagement resulted in B-cell proliferation; however, B-cell–receptor signalling induced apoptosis when mature B-cells are either PKC depleted or stimulated in the presence of PKC inhibitors. These data suggest that PKCβ activity enhances B-cell proliferation and survival and therefore that PKCβ overexpression could contribute to a fatal/refractory DLBCL phenotype.

iv. PDE4B is a class-4 cyclic AMP (c-AMP)-specific phosphodiesterase. PDE4 is the predominant class of PDEs in lymphocytes, where they catalyse the hydrolysis of c-AMP, so by terminating its activity (Manning *et al.*, 1999, Lerner *et al.*, 2000). c-AMP-dependent protein kinase A (PKA) signalling inhibits lymphocyte chemotaxis, cytokine release and cellular proliferation (Manning *et al.*, 1999). Because PDE4B reduces c-AMP availability, the PDE also limits the negative effects of PKA signalling in lymphocytes. For this reason, PDE4A and -4B inhibitors are being evaluated in the treatment of CLL/SLL (small lymphocytic lymphoma) where they are reported to induce B-cell apoptosis (Moon *et al.*, 2002, Moon and Lerner, 2003). These facts suggested that PDE4B overexpression in

presentation DLBCL material may be causally related to the chemoresistance and poor outcome of the affected patients and their DLBCLs.

These facts suggested that the applicability of PKCβ and PDE4B as poor prognostic markers in DLBCL is likely to be genuine and that a causal relationship between their expression and the presence of a chemoresistant phenotype is more likely.

CHAPTER 2. MATERIALS AND METHODS

2.1 Microarray and Expression Validation Materials and Methods

2.1.1 Patients and their Samples

Samples were chosen on the basis of the following criteria: snap-frozen and archived at –80^{o}C, presentation lymph node material with a diagnosis of DLBCL or FL; treatment with an anthracycline-based chemotherapy regimen for the DLBCL patients; availability of comprehensive clinical data and follow-up; histological confirmation of original diagnosis with exclusion of DLBCL variants such as T-cell-rich and mediastinal large B-cell DLBCL and tumours with excessive necrosis. After application of these criteria and exclusion of samples where production of an array of sufficient quality was not possible, 58 DLBCL cases and 19 FL were investigated. For each of the DLBCL patients the presentation IPI was calculated and response to treatment, date and outcome of relapse and OS updated. The 58 patient's IPI, OS and living status are given in Table 2.1.

Nineteen of the DLBCL cases were treated at SBH (Table 2.2). Prior to their addition to the Dana Farber Cancer Institute (DFCI) cohort, no significant results were produced, not least due to significantly shorter follow-up. Eleven of the SBH patients were male and eight were female. The median age of the 19 was 55 years old, with 58% of them being less than 60 years old. Four had stage I disease, six stage II, five stage III and four stage IV disease at presentation. Eleven of the 19 had a low-risk total IPI score, five had an intermediate-risk score and two had a high-risk score. The IPI was not known for one case. Twelve of the 19 patients (63%) were alive and disease-free as of April 2000 (the month and year of data censorship) and seven (37%) were *'dead from disease'*.

Sample	Full IPI	OS	Status	Outcome	Sample	Full IPI	OS	Status	Outcome
DLBCL1	Low	72.9	Alive w/o disease	0	DLBCL40	High	3.2	Dead w/disease	1
DLBCL2	Low	143.1	Alive w/o disease	0	DLBCL41	L/I	4.9	Dead w/disease	1
DLBCL3	L/I	144.2	Alive w/o disease	0	DLBCL42	H/I	12	Dead w/disease	1
DLBCL4	H/I	61	Alive w/o disease	0	DLBCL43	H/I	60.4	Dead w/disease	1
DLBCL5	Low	86.5	Alive w/o disease	0	DLBCL44	L/I	16.3	Dead w/disease	1
DLBCL6	Low	84.2	Alive w/o disease	0	DLBCL45	H/I	16.4	Dead w/disease	1
DLBCL7	H/I	112.5	Alive w/o disease	0	DLBCL46	H/I	9.5	Dead w/disease	1
DLBCL8	Low	133.2	Alive w/o disease	0	DLBCL47	H/I	15.6	Dead w/disease	1
DLBCL9	Low	22.1	Alive w/o disease	0	DLBCL48	H/I	17.8	Dead w/disease	1
DLBCL10	L/I	182.4	Alive w/o disease	0	DLBCL49	L/I	56.9	Dead w/disease	1
DLBCL11	Low	66.4	Alive w/o disease	0	DLBCL50	Low	13.3	Dead w/disease	1
DLBCL12	.	146.8	Alive w/o disease	0	DLBCL51	L/I	12.3	Dead w/disease	1
DLBCL13	L/I	62.9	Alive w/o disease	0	DLBCL52	Low	44.6	Alive w/disease	1
DLBCL14	L/I	50.9	Alive w/o disease	0	DLBCL53	H/I	4.6	Dead w/disease	1
DLBCL15	Low	26.3	Alive w/o disease	0	DLBCL54	High	7.5	Dead w/disease	1
DLBCL16	.	48.6	Alive w/o disease	0	DLBCL55	H/I	19.3	Dead w/disease	1
DLBCL17	H/I	55.9	Alive w/o disease	0	DLBCL56	Low	30.1	Dead w/disease	1
DLBCL18	Low	12.6	Dead w/o disease	0	DLBCL57	Low	33.6	Alive w/disease	1
DLBCL19	L/I	50.2	Dead w/o disease	0	DLBCL58	H/I	13.9	Dead w/disease	1
DLBCL20	H/I	58	Alive w/o disease	0	FL1				
DLBCL21	L/I	66.4	Alive w/o disease	0	FL2				
DLBCL22	Low	65.7	Alive w/o disease	0	FL3				
DLBCL23	Low	50.2	Alive w/o disease	0	FL4				
DLBCL24	Low	26.9	Dead w/o disease	0	FL5				
DLBCL25	Low	34.4	Alive w/o disease	0	FL6				
DLBCL26	Low	26	Alive w/o disease	0	FL7				
DLBCL27	Low	30	Alive w/o disease	0	FL8				
DLBCL28	L/I	31.7	Alive w/o disease	0	FL9				
DLBCL29	Low	32.2	Alive w/o disease	0	FL10				
DLBCL30	Low	19.2	Alive w/o disease	0	FL11				
DLBCL31	Low	33	Alive w/o disease	0	FL12				
DLBCL32	Low	21.4	Alive w/o disease	0	FL13				
DLBCL33	Low	15.7	Dead w/disease	1	FL14				
DLBCL34	H/I	11.6	Dead w/disease	1	FL15				
DLBCL35	H/I	3.4	Dead w/disease	1	FL16				
DLBCL36	Low	36.6	Dead w/disease	1	FL17				
DLBCL37	H/I	5.0	Dead w/disease	1	FL18				
DLBCL38	Low	9.5	Dead w/disease	1	FL19				
DLBCL39	High	3.2	Dead w/disease	1					

Table 2.1 Sample Identification and associated Patient characteristics
OS = overall survival in months; w/o without; w/ with. Cases in red are from SBH

Variable	Number	Percentage
Gender Male : Female	11: 8	58% : 42%
Age Median (Range)	55	23-83
Age <60: ≥60 years old	11 : 8	58% : 42%
Stage I	4	21%
Stage II	6	32%
Stage III	5	26%
Stage IV	4	21%
LDH ≤ Upper Limit of Normal: > ULN - Presentation LDH unavailable for one case	12 : 6	63% : 32%
Extranodal Sites <2: ≥2	18 : 1	95% : 5%
IPI Low Risk	11	58%
IPI Intermediate Risk	5	26%
IPI High Risk	2	11%
IPI Unknown	1	5%
Response to Treatment CR/CRu: PR: Fail	12 : 5 : 2	63% :26% :11%
Long-term Outcome *'Alive and cured'*: *'Dead from disease'*	12 : 7	63% : 37%

Table 2.2 Characteristics of the 19 SBH Patients Arrayed

2.1.2 *'Target'* preparation for Microarray

Total RNA was extracted from each frozen tumour specimen and converted to double-stranded cDNA. Tissue samples were homogenised (Polytron, Kinematica, Lucerne) in guanidinium isothiocyanate and RNA was isolated by centrifugation over a CsCl gradient. RNA integrity was assessed either by northern blotting or by gel electrophoresis. The amount of starting total RNA for each reaction varied between 10 and 12 μg. First strand cDNA synthesis was generated using a T7-linked oligo-dT primer, followed by second strand synthesis. An *in vitro* transcription reaction was done to generate the cRNA containing biotinylated UTP and CTP, which was subsequently chemically fragmented at 95 °C for 35 minutes. Ten μg of the fragmented, biotinylated cRNA was hybridised in MES buffer containing 0.5 mg/ml acetylated bovine serum albumin (Sigma, St. Louis) to Affymetrix (Santa Clara, CA) HU6800 oligonucleotide

arrays at 45 °C for 16 hours. The HU6800 arrays contained 5920 known genes and 897 expressed sequence tags (total = 6,800). Arrays were washed and stained with streptavidin-phycoerythrin (SAPE, Molecular Probes). Signal amplification was performed using a biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) at 3 μg/ml. A second staining with SAPE followed this. Normal goat IgG (2 mg/ml) was used as a blocking agent. Scans were performed on Affymetrix scanners and the expression value for each gene was calculated using Affymetrix GeneChip software. Minor differences in microarray intensity were corrected using a linear scaling method as detailed in the next section.

2.1.3 Data processing and analysis

The raw expression data from the HU6800 GeneChips was rescaled to account for differences in intensities between chips. Each column (sample) in the dataset was multiplied by *1/slope* of a least squares linear fit of the sample vs. the reference (the first sample in the dataset). This linear fit was done using only genes that have *'present'* calls in both the sample being re-scaled and the reference. The reference sample was chosen from the series on the basis of it having the number of *'present'* calls closest to the average over all samples in the dataset. A ceiling of 16,000 'average difference' units was chosen due to fluorescence saturation of the scanner at and above this level. A lower threshold for expression levels was set at 20 'average difference' units to minimise noise effects while avoiding missing any potentially informative marker genes. After this pre-processing, gene expression values were subjected to a variation filter that excluded genes showing minimal variation across the samples being analysed. The variation filter tested for both a fold-change and absolute variation over samples (comparing maximum/minimum and maximum-minimum with predefined values and excluding genes not obeying both conditions). For maximum/minimum fold variation, genes were excluded with <3-fold variation and, for maximum-minimum absolute variation, genes were excluded with <100 units absolute variation.

A supervised classifier was built as follows: a target class was defined based on morphology, tumour class or treatment outcome information. The *'marker'* genes with the highest correlation with the target class using a class separation statistic (signal-to-noise ratio) were selected. The class separation statistic used was 'signal-to-noise ratio', where class-0 and class-1 were identified by sorting all of the genes on the array according to the signal-to-noise statistic (μ class-0 - μ class-1)/(σ class-0 + σ class-1),

with μ and σ representing the mean and standard deviation of expression for each class respectively (Golub *et al.*, 1999). A permutation test was also applied to the top ranked genes to assess their class-correlation statistical significance. The permutation test was applied to answer the following question: what was the likelihood that the set of marker genes selected by the signal-to-noise of phenotype (FL vs. DLBCL or *'alive and cured'* vs. *'fatal/refractory disease'*) was due to chance correlations and not the hoped-for biological determinants. The algorithms used to classify according to phenotype distinction were weighted voting, κ-nearest neighbours and support vector machines. Hierarchical clustering was used to reassess the Alizadeh *et al* dataset reduced to just the 90 genes common to the *'Lymphochip'* and the Affymetrix HU6800. A classifier was built through cross-validation using the leave-one-out technique. One sample was removed and the remaining samples used as a training set. This reduced the risk of over-fitting whilst acknowledging the limitation of a relatively small cohort size. Ideally, the classifier would have been built using a training set of samples and validated in a second cohort. Several models were built using different numbers of marker genes, allowing the final model to be chosen with the lowest error rate in cross-validation. The Kaplan-Meier method was used to calculate predicted OS curves and the log rank test was used to calculate the respective *P* values (Kaplan and Meier, 1958). These marker genes were used to build the κ-nearest neighbour and weighted voting classifiers. Support vector machines used different methods to select marker genes.

2.1.4 Immunohistochemical Staining

For a subset of the DFCI patients only (n = 21), five representative 0.6 mm cores were obtained from diagnostic areas of each paraffin-embedded formalin-fixed DLBCL and inserted in a grid pattern in a single recipient paraffin block using a tissue arrayer (Beecher Instruments, Silver Spring, MD). Five μm sections cut from this "tissue array" were stained for PKCβ using an immunoperoxidase method. Slides were deparaffinised and pre-treated in 1 mM EDTA, pH 8.0, for 20 minutes at 95°C. All further steps were performed at room temperature in a hydrated chamber. Slides were pre-treated with peroxidase block (DAKO, USA) for 5 minutes to quench endogenous peroxidase activity, and a 1:5 dilution of goat serum in 50 mM Tris-Cl, pH 7.4, for 20 minutes to block non-specific binding sites. Primary antibody (murine monoclonal antibody specific for PKCβ (Serotec, UK)) was applied at a 1:1000 dilution in 50 mM Tris-Cl, pH 7.4 with 3% goat serum for 1 hour. After washing, secondary goat anti-mouse

horseradish peroxidase-conjugated antibody (Envision detection kit, DAKO, USA) was applied for 30 minutes. After further washing, immunoperoxidase staining was developed using a DAB chromogen kit (DAKO, USA) as per the manufacturer's instructions. Following counterstaining with haematoxylin, immunoperoxidase staining within the malignant cell population of each core was scored in a blinded fashion with respect to clinical outcome and expression profile results by three pathologists (Jon Aster, Andrew Weng, Jeffery Kutok). The intensity of staining on each core was graded from 0 (no staining) to 3 (maximal staining), and an average staining intensity (the mean of all five cores) was generated for each tumour. The *P* value for the association between staining intensities and the array-based transcript levels was evaluated by using the median to divide measured intensities into two levels and then using Fisher's exact test to evaluate the degree of association between the quantified measurements.

2.2 Presentation serum selenium materials and methods

2.2.1 Patient Samples, Histology and Clinical Variables Investigated

To ensure adequate patient follow-up, presentation serum samples were selected that had been archived between July 1986 and March 1999. The NHL classification system in operation at SBH during this time was the Kiel classification in which the different NHL subtypes were grouped into three broad categories termed low-, intermediate- and high-grade NHLs of B- or T-cell type. Therefore serum was searched for where a diagnosis of high-grade B-cell NHL had been made, as this Kiel subgroup would contain all the REAL and WHO defined DLBCL cases. Of the 383 patients with such a diagnosis, 143 had serum samples from presentation still archived. Only those samples that were haem-free were selected, as intracellular Se is released from lysed red cells, resulting in elevation of serum Se content. One hundred of the 143 archived sera were macroscopically haem-free. The histological subtype of the 100 high-grade B-cell NHLs with suitable archived sera was updated to the WHO classification by Dr Andrew Norton, Department of Pathology, SBH (Table 2.3).

The clinical variables investigated for an association with dose-delivery, response to first treatment and/or OS were serum Se, gender, and the recognised prognostic factors of age, B symptoms, stage, retrospective performance status, LDH (or hydroxybutyrate dehydrogenase if the patient presented prior to mid-1989), and number of extranodal sites (Table 2.4). The IPI score was calculated for the 95 patients with complete IPI data (Table 2.4).

WHO Histological Subtype	No of Cases
Diffuse large B-cell Lymphoma	77
Mediastinal Large B-cell Lymphoma	14
DLBCL, T-cell-rich	3
Lymphoplasmablastic Lymphoma with a High Content of Blasts	2
Angioimmunoblastic T-cell Lymphoma	2
Burkitt Lymphoma	1
Mantle Cell Lymphoma with a High Contents of Blasts	1
Total	**100**
Composite or Discordant Histology	15

Table 2.3 Revised Histological Classification According to WHO of the 100 Patients

Variable		N = 100
Serum Selenium (μmol/l)	Mean	0.92
	Range	0.33-1.51
Gender	Male : female	56 : 44
B symptoms	Present : Absent	15 : 85
Age, years old	Median	57
	Range	19-87
Stage	I, IE	25
	II, IIE	23
	III	13
	IV	39
ECOG Performance	0	34
Status* (96/100)	1	43
	2	19
LDH* (98/100)	< ULN	49
	> ULN	49
Number of extranodal	0	33
Sites* (98/100)	1	53
	2	12
IPI* (95/100)	0	17
	1	24
	2	23
	3	23
	4	7
	5	1

Table 2.4 Clinical Characteristics of the 100 Patients
Abbreviations: ULN, upper limit of normal; ECOG, Eastern Co-operative Oncology Group.
*Available for the number of patients in brackets only.

2.2.2 Treatment

All patients bar one (who died before treatment could begin) were treated with curative intent with anthracycline-based combination chemotherapy, radiotherapy or both (Table 2.5). Comprehensive dose-delivery data was available for 87 of the 93 patients given chemotherapy. By July 3 2000, the date up to which survival data was analysed, 47 of the 100 patients had died (43 from DLBCL), two had been lost to follow-up and 49 were alive. Thirty-six patients had relapsed by the date up to which survival data was analysed, only five of which received high-dose consolidation with autologous stem cell support as part of retreatment.

First Treatment Regimen	Regimen Details	Number of Cases
VAPEC-B	An alternating anthracycline-based weekly schedule given for 12 weeks of Vincristine, doxorubicin, prednisolone, etoposide, cyclophosphamide and bleomycin	64
VAPEC-B Variant		11
CHOP	Cyclophosphamide doxorubicin, vincristine, and prednisolone	12
MACOP	Methotrexate, doxorubicin, cyclophosphamide vincristine, and prednisolone	6
Radiotherapy		6
No treatment	Died before treatment could commence.	1
Total		100

Table 2.5 First Treatment Details for the 100 Patients used for the Presentation Serum Selenium Research

2.2.3 Selenium Concentration Measurement

Inductively coupled plasma mass spectrometry was used to measure total serum Se content. Christine Sieniawska undertook the measurement under the supervision of Trevor Dells in the Trace Elements Unit, Southampton General Hospital, Southampton, UK. Selenium-78, as the principle Se isotope, was assayed after a 1:15 dilution with 0.5% butan-1-ol and the addition of tellurium as an internal standard. In the five

samples with a high iodine content, caused by use of iodine as an antiseptic during their recent surgical procedure, performed to obtain tissue to reach their diagnosis of NHL, indium-115 was used as the internal standard. This was because of the overlap of the principal tellurium mass spectrometry peak with that of one of the principal iodine isotopes. Each sample was tested in duplicate, with standards of 0.2, 0.6, 1.2 and 1.7 μmol/l being run per 10 test duplicates. The between run precision ranged from 3.7% to 5.9% relative standard deviation and a within-run precision of 2.3% to 9.3% over the concentration range 0.25 to ± 1.75 μmol/l selenium.

2.2.4 Statistical Methods

Dose-Delivery Analysis

Dose-delivery associations were sought between the clinical variables investigated and doxorubicin, cyclophosphamide and vincristine. An area-under-the-curve summary measure, AUC_{ratio} was created by Victoria Cornelius, CR-UK Department of Statistics, Oxford, UK due to the inadequacy of *'relative dose'* and *'cumulative dose'* as dose-delivery summary measures. Neither *'relative dose'* nor *'cumulative dose'* dose-delivery measures could accurately account for variation in dosing schedules in both quantity and time duration. Relative dose intensity was not used, as this method merely calculates the rate of dose-delivery and takes no account of total dose delivered e.g. if a patient receives just two of six cycles of treatment, their relative dose intensity will be the same as that of a person receiving all six cycles assuming all courses were given on time. Cumulative dose was equally poor as a summary measure, as it only reveals dose differences between patients, taking no account of differences in time needed to deliver treatment. Taking the mean of the relative dose intensity for each course of chemotherapy will produce an indictor of the dose intensity but takes no account of when time delays occurred in treatment schedule. Our AUC methodology was created in order to more accurately reflect the temporal as well as quantity dimensions of dose intensity. The standard AUC measure used to distil serial measurements was adapted to account for variations in dosing schedules in terms of quantity and time duration (Matthews *et al.*, 1990). Comparing actual to planned AUCs of the proportion of cumulative dose vs. time created a standardised summary measure evaluable between patients regardless of regimen variant used. The AUC_{ratio} of cumulative dose vs. time reveals not only variation in time taken and dose administered, but also distinguishes between dose reductions and delays incurred at the beginning from those incurred at the

end of treatment. AUC_{ratio} therefore factors for the premise that timely dose-delivery early is more critical than toward the end of therapy.

For a schedule of n doses, where the proportion of cumulative dose at time t(i) was y(i) (where i = 1 to n) the AUC was defined as:

$$\mathrm{AUC} = \sum_{i=1}^{n-1} \frac{1}{2}\big(y(i) + y(i+1)\big)\big(t(i+1) - t(i)\big)$$

The proportion of cumulative dose was plotted against time for both the planned and actual dosing schedules, followed by calculation of the AUC of the planned and actual schedules ($AUC_{planned}$ and AUC_{actual}). The summary measure used, AUC_{ratio}, was then created by dividing AUC_{actual} by $AUC_{planned}$. AUC_{ratio} therefore represented the proportion of actual dose to planned dose over the duration of the treatment time. If the full planned dose was not given or a dose was delayed then AUC_{ratio} decreased from its maximum of 1.

Where actual doses were given after the final planned time, the time axis for the planned regimen was extended and the contribution was weighted using the formula:

$$w = \frac{t_1}{n(t_2 - t_1)} \qquad \text{when } (t_2 - t_1) > \frac{t_1}{n}$$

Where t_1 is the time from the first planned dose to the final planned dose, t_2 is the time from the first administered dose to the final administered dose and n is the number of planned doses. For those patients where treatment stopped early because of death or disease progression, the planned dose intensity was censored at the time of death or disease progression. An example of dose intensity calculation is illustrated in Table 2.6 and Figure 2.1.

Variables were included in the logistic regression and Cox multivariate models for dose-delivery if they had obtained significance at the univariate level (P <0.1). Age and Se were treated as continuous variables and the remaining variables as categorical.

t_1			y_1	t_2			y_2
Planned dose days	Planned Dose	Planned cumulative dose	Proportion of planned cumulative dose	Actual dose days	Actual dose	Actual cumulative dose	Actual dose as prop'n of planned cumulative dose
0	49	49	0.16	0	49	49	0.16
14	49	98	0.32	14	49	98	0.32
28	49	147	0.48	29	49	147	0.48
42	49	196	0.64	50	49	196	0.64
56	54	250	0.82	63	49	245	0.80
70	55	305	1.00	74	55	300	0.98

Table 2.6 Cumulative Dose of Doxorubicin (mg) for a Patient Receiving VAPEC-B (Doxorubicin Fortnightly At 35mg/m^2). Initial BSA (body surface area) = 1.39 m^2 increasing to 1.57 m^2. $AUC_{planned}$ = 43.76, AUC_{actual} = 40.27, AUC = 0.92

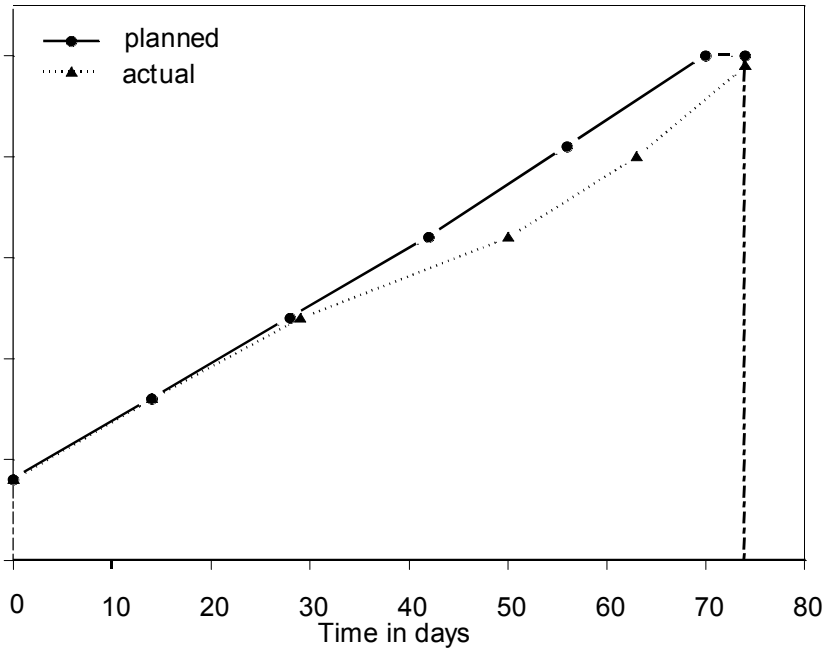


Figure 2.1 Cumulative Dose of Doxorubicin (mg) for a Patient Receiving VAPEC-B (Doxorubicin Fortnightly At 35mg/m^2). The x-axis intersection indicates the actual end of treatment administration 4 days later than the planned schedule.

Response Analysis

Response criteria in use at SBH during the years of patient presentation were different from the later 1997 consensus statement (Cheson *et al.*, 1999, Dhaliwal *et al.*, 1993). The definitions for complete response (CR) are the same, whereas good partial response (GPR) is comparable to CR uncertain and poor partial response (PPR) to partial response. Response was categorised into two groups according to similar OS observed amongst the 100 patients studied: complete response/good partial response and poor partial response/treatment failure/treatment-related death (Figure 2.2).

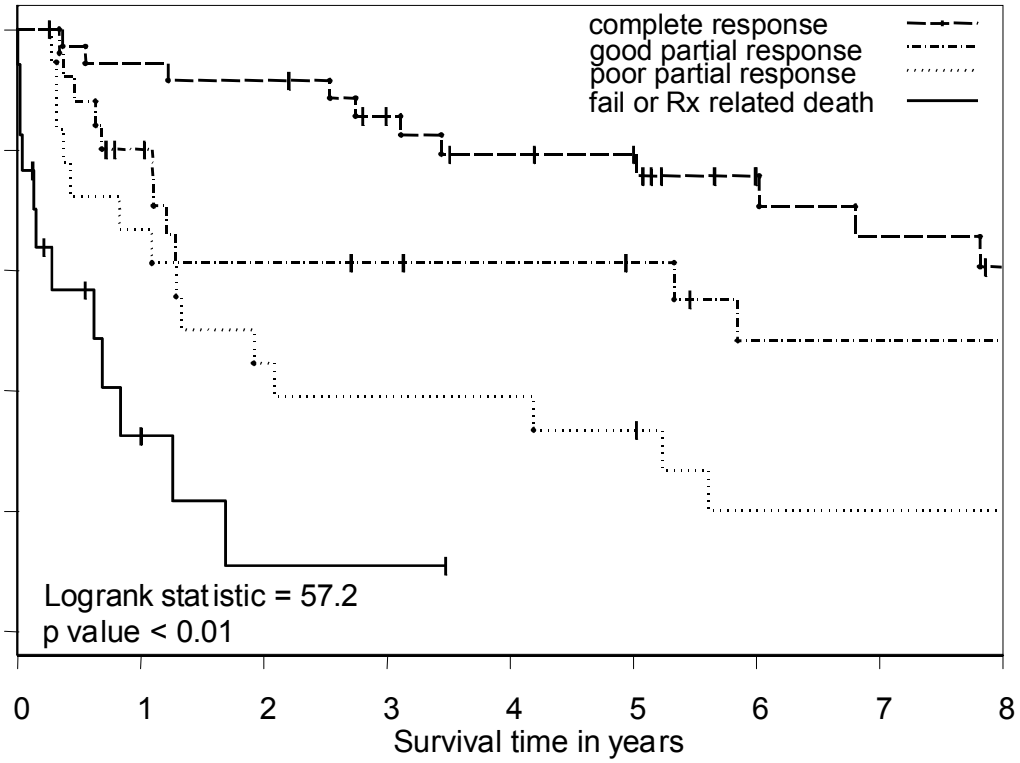


Figure 2.2 Overall Survival of The 100 Patients Grouped According to Response to First Treatment, from Response Documentation.

Logistic regression was fitted to estimate the odds ratio (OR), following employment of the χ^2 test and T-test to compare groups at the univariate level. The

patients were divided into quartiles, as defined by presentation serum Se concentration, and tested univariately with regard to response and response duration.

Survival Analysis

Survival curves were estimated using Kaplan-Meier methods and the log-rank test. The patients were divided into quartiles, as defined by presentation serum Se concentration, and tested univariately with regard to OS. Survival time was analysed using a Cox proportional hazards model (Cox, 1972).

2.3 *In Vitro* Target Validation Materials and Methods

2.3.1 Established Tumour Cell-lines

Four DLBCL/transformed FL cell-lines were used in these studies. DHL4 (t(8:14)) was obtained from the DFCI (gift from Dr Margaret Shipp), and DoHH2 (t(14:18)) (Kluin-Nelemans *et al.*, 1991), SUD4 (t(14:18)) (Epstein *et al.*, 1978) and CRL-2261 (t(14:18)) (Beckwith *et al.*, 1995) were provided by CR-UK cell services. The characteristics of the cell-lines are detailed in Table 2.7.

Cell-lines	Origin	Translocation	*TP53* Status	GC vs. ABC like phenotype	Chemosensitive or resistant?
DHL4	DLBCL (38 years old male 1975)	Complex karyotype including t(8;14)(q24;q32) [1]	Homozygous mutated & non-functional p53 [1]	GC like [2]	Resistant [1]
DoHH2	Tx FL (60 years old male 1991) [3]	Complex karyotype including t(14;18)(q32;q21) [1]	Heterozygous mutated & functional p53 [1]	GC like [1]	Highly sensitive [1]
CRL-2261	DLBCL (52 years old male 1989) [4]	Complex karyotype including t(14;18)(q32;q21) [1 & 4]	Homozygous mutated & non-functional p53 [1]	GC like [1]	Sensitive [1]
SUD4	DLBCL [5]	Complex karyotype including t(14;18)(q32;q21) [1]	Homozygous mutated & non-functional p53 [1]	GC like [1]	Sensitive [1]

Table 2.7 Characteristics of the Cell-lines used for *in vitro* experiments

GC vs. ABC like phenotype = Germinal centre vs. activated B-cell like phenotype
Tx FL = Transformed follicular lymphoma.

1 (Strauss *et al.*, 2007) and personal communication from PhD thesis
2 (Smith *et al.*, 2005)
3 (Kluin-Nelemans *et al.*, 1991)
4 (Beckwith *et al.*, 1995)
5 (Epstein *et al.*, 1978)

The cell-lines were maintained in suspension in RPMI-1640 growth media (Sigma, Poole, UK) supplemented with 10% foetal calf serum, 1% L-glutamine and 1% penicillin/streptavidin (all Sigma, Poole, UK) and kept at 37^{o}C in an humidified atmosphere containing 5% CO_2.

2.3.2 3-day Cell-line Exposure Experiments

Only exponentially growing cells with viability >90% were used. Cells were plated at 1-2x10^5 cells/ml in 5ml of RPMI-1640 supplemented with 10% foetal calf serum, 1 % L-glutamine and 1% penicillin/streptavidin in 6-well plates or in 2ml in 24-well plates. The cytostatic, cytotoxic and/or anti-proliferative activity of the different drugs against the cell-lines was investigated after continuous exposure for three days to a minimum of five non-zero drug concentrations. Cell number and viability were determined by light microscopy following trypan blue staining. Proliferation was estimated using the MTS assay described below. The data presented is the mean of a minimum of three experiments, unless otherwise stated. EC_{50}s (Effective Concentration to achieve 50% response) were calculated as detailed below using GraphPad Prism (San Diego, CA, USA).

Drug Stock Solutions

MSA (PharmaSe, Lubbock, USA) was prepared in DMSO at a concentration of 80 mM and SDG (PharmaSe, Lubbock, USA) was dissolved in 0.05M HCl at a concentration of 10 mM. SC-236 was a generous gift of Pharmacia UK. A stock solution of SC-236 of 10 mM was made in 60% ethanol, 40% sterile water. Piclamilast was a gift of Altana Pharma, Bad Homburg, Germany. A 10 mM stock solution of piclamilast using DMSO was made. Rolipram (Sigma chemicals, Poole, Dorset UK) was made up as 100 mM stock solution in 100% DMSO; forskolin (Sigma chemicals, Poole, Dorset UK) was made up as a 10 mM stock solution using anhydrous DMSO. Doxorubicin (Sigma chemicals, Poole, Dorset UK) was prepared using sterile water at a concentration of 1 mM. Cytosine arabinoside (ara-c; Sigma chemicals, Poole, Dorset UK) was dissolved in sterile water to create a stock solution of 0.1 mM. The final DMSO concentration was <1% in all cultures. The stock solutions were stored at –40 or -80^{o}C for up to three months. The stock solutions were diluted with RPMI-1640 media to make working drug concentrations on the day required.

Sigmoidal Dose/Concentration–Response Model

'Dose-response' curves were used to plot the results of the drug experiments using the sigmoidal dose–response model (GraphPad Prism, San Diego, CA, USA). The X-axis was used to plot the concentration of the drug under investigation and the Y-axis to plot the response, which was cell count, viability or proliferation relative to no treatment controls. As the term 'dose' refers to 'concentration' in cell-line *in vitro* experiments, the term 'concentration-response curve' is a more precise label for the results of these *in vitro* experiments.

The steepness of concentration-response curves is quantified by the slope factor, referred to as the sigmoid factor in sigmoidal concentration-response curves. A concentration-response curve with a standard slope has a slope factor of 1.0. For the experiments undertaken, the anticipated activity of each drug was expected to be antagonistic i.e. reducing cell count, viability or proliferation with increasing concentrations of drug relative to no treatment controls. The curves created were therefore expected to go downhill and the slope factor to be negative. A standard curve in such circumstances has a slope factor of –1, a steeper curve a higher slope factor e.g. -8 and a shallower curve a lower slope factor e.g. -0.5. The mid-point of such curves is the EC_{50}.

Curve fitting finds the curve that minimises the sum-of-squares of the vertical distance from the data points. By using an equation to fitting the concentration-response curve to the $logEC_{50}$ rather than the EC_{50}, the curve itself isn't fitted better but it dramatically improves the accuracy of the standard error and CI calculated. The reason being that the uncertainty of the EC_{50} is not symmetrical, causing the SE and CI to be highly asymmetric. Without the log transformation of the x-axis data this cannot be accounted for, resulting in the lower 95% CI frequently having a negative lower value – an impossibility in reality. Switching from a linear to log scale turns a symmetrical CI into a very asymmetrical interval, which can be reported. As the SE represents a distance along the x-axis rather than a point on the axis, to convert the SE of the log EC_{50} to the SE for the EC_{50} it needs to be considered as a multiplier. To calculate the 95 % CI for the EC_{50} the antilog of the SE was taken, the answer multiplied by a constant from the t distribution for 95% confidence and the appropriate number of degrees of freedom, lastly the best-fit EC_{50} was divided and multiplied by the result to produce the lower and upper 95% CI. Under normal circumstances, concentration-response graphs for cell count, viability and proliferation will follow a sigmoidal shaped curve when plotted

with log concentration values on the x-axis – hence the term sigmoidal concentration-response curve.

Therefore to fit a sigmoidal concentration-response curve with Prism, it was necessary to transform the concentration (x-axis) values to the logarithm of concentration. To this end the Prism software transforms the entered concentration values on your behalf. As the log of zero is undefined, all no treatment controls needed to be entered as low concentrations one or two log units below the lowest concentration for that experiment series. Because for virtually all the cell-line experiments performed there were plenty of data points due to using at least five different concentrations of each drug, the data was analysed using the 'sigmoidal dose-response curve with a variable slope' equation. Where the data points were scanty (as in some of the primary cell culture drug exposure experiments) the sigmoidal dose-response equation with a standard slope of –1 was used. After creation of a data table for all the replicates from a particular series of experiments, the Prism software calculated the mean value for the replicates at each log concentration value. To create a sigmoidal concentration-response curve from the now transformed mean values, the 'non-linear regression' tab was selected from the analysis list and then 'sigmoidal dose-response variable or standard slope' from the list of classic equations. Because all data points were relative to the no treatment controls, the data was in effect normalised. Therefore the maximal response possible was zero and the minimal response possible was one for all cell count, viability and proliferation assessments. The consequence of having normalised data for any Prism analysis was that there was no need for the software to attempt to fit the top and bottom values as well as the $\log EC_{50}$ and the slope factor. The top value was therefore constrained to constant equal to zero and the bottom value to a constant equal to one. The Prism software therefore only had to fit two of the four possible parameters. The equation used by the Prism software is:

$$Y=\text{Bottom} + (\text{Top}-\text{Bottom})/(1+10^{((\text{LogEC50}-X)*\text{Hill Slope})})$$

Cell Count and Viability Assessment by Trypan Blue Exclusion Staining

Trypan blue is a vital dye, which requires a damaged cell membrane for its negatively charged chromopore to be able to interact with the cell. Therefore cells stained blue are dying/dead and unstained cells are alive and viable. Cell number and viability were

determined by light microscopy following trypan blue staining using a 1:1 ratio of cell solution:trypan blue. This mixed solution was added to a haemocytometer. Only unstained cells were counted as viable and only cells stained blue were counted as dead. All data were expressed relative to control (untreated) cells and EC_{50} concentrations derived using a sigmoidal dose-response model in GraphPad Prism (San Diego, CA, USA). Final DMSO concentration was <1% in all cultures. The data presented is the mean of a minimum of three experiments.

MTS Cell Viability Assay

The CellTiter 96 Aqueous One Solution cell proliferation assay (Promega Wizard, Southampton, UK) was used to determine the number of viable cells. This solution contains a novel MTS tetrazolium compound, which is stabilised by the presence of an electron coupling reagent , PES (phenazine ethosulphate). The MTS tetrazolium compound is reduced to a soluble formazan product, changing the culture medium colour from yellow to brown. This reaction is due to the presence of dehydrogenase enzyme in metabolically active cells, which produce nicotinamide adenine dinucleotide phosphate (NADPH) or NADH. The rate of colour change is proportional to the number of viable cells present in the culture medium. The assay was carried out according to manufacturer's guidelines. In brief, 1-2x10^5 cells in 100µl of RPMI-1640 supplemented with 10% foetal calf serum, 1% L-glutamine and 1% penicillin/streptavidin were added to the inner 60 wells of 96 well plates. Drug or vehicle were added in a volume of 100µl to give a final volume per well of 200µl. 200µl of Hank's balanced salt solution was added to the outer 36 wells. 3 days later, 40µl CellTiter 96 Aqueous One reagent was added to each well and plates were incubated for 2 to 4 hours. Absorbance was then read at 490nm using a 96-well plate reader (Fluorostar optima).

2.3.3 Flow Cytometry and Staining Procedure

Cell Cycle Distribution

After washing in ice-cold nucleus buffer (0.15 M NaCl, 5 mM KH_2PO4, 1 mM EGTA, 0.1 mM dithiothreitol, 10 % glycerol in distilled water pH 6.5) cells (1x10^6) were resuspended in 4 ml of permeabilising solution (0.35% Triton X-100, 0.1 mM PMSF in nucleus buffer), and mixed by rotation at 4^{o}C for 20 min. Cells were then fixed in ice-cold methanol for 30 minutes and washed with ice-cold PBS (phosphate buffered saline) before staining with 500µl of PI stain (50µg/ml PI and 50µg/ml RNAse A in PBS). Acquisition of data was performed within 1 hour. Five to ten thousand cells were

collected and analysed for each data point using a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK) with the CellQuest software, and the percentage of cells in the sub-G1 (apoptotic fraction), G1, S and G2/M phases were analysed using a cell cycle analysis program (WinMDI 2.4).

Detection of Apoptosis

Apoptosis was confirmed by dual labelling using PI/annexin V staining of unfixed cells (Sigma, Poole Dorset, UK). After resuspending $1x10^6$ cells in 500µl of binding buffer (HEPES with NaOH, NaCl and $CaCl_2$), 5 µl annexin conjugate was added and the samples incubated for 10 minutes at room temperature in the dark. Propidium iodide (10 µl of 100 µg/ml) was then added, and the sample immediately analysed on a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK) using CellQuest software.

2.3.4 Primary Cell Culture

For primary culture experiments B-cell suspensions were prepared from ascitic fluid, peripheral blood or lymph nodes collected as part of normal clinical management from patients with histologically confirmed B-cell lymphoma or chronic lymphocytic leukaemia. A FL sample (01), a CLL/SLL sample (02) and two MCL samples (03) and (04) were used. All four patients from whom the samples came had been multiply pretreated for their malignancy and had chemoresistant/refractory disease. In addition, a pooled normal B-cell sample obtained from buffy coat from multiple blood donors was used. Local ethics committee approval was obtained and all patients gave informed consent. After disaggregation and/or density gradient separation, cells were plated at a density of $5x10^5$ cells/well into 96-well plates containing CHO cells transfected with the Cdw32Fc receptor , which, when irradiated to prevent further proliferation, provided a stromal layer supporting B-cell proliferation. Malignant or normal B-cells were cultured in IMDM medium supplemented with 10% human serum and 2ng/ml interleukin-4 (IL-4), cells could be maintained for up to eight days whilst retaining close phenotypic resemblance to the original sample, confirmed by flow cytometric quantitation of cell surface markers (personal communication Lenushka Maharaj). After 24 hours, drug was added at varying concentrations in at least triplicate, and cell number and viability were assessed after a further 48 hours using the trypan blue exclusion assay.

2.3.5 Western Blotting

Immunoblot Analyses

To confirm the mechanism of cell death induced by Se compounds, cell extracts were analysed for caspase and poly(ADP-ribose) polymerase (PARP) cleavage. Cells were exposed to an EC_{50} concentration of either MSA or SDG for 3 days, after which cell lysates were prepared using lysis buffer (75%) containing protease inhibitors and NuPAGE sample buffer (25%) 100µl per million cells (all Invitrogen Ltd, Paisley, UK). Prior to electrophoresis samples were diluted with NuPAGE reducing agent (Invitrogen Ltd, Paisley, UK) and heated at 70^{o}C for 10 minutes before gel loading. Proteins were resolved by SDS-PAGE electrophoresis using a 5% stacking gel and 12% running gel, electroblotted onto nitrocellulose membranes and then probed using primary antibodies against caspase-8 (1:100), caspase-9 (1:100) or PARP (1:100), (all Oncogene research Product, Boston, MA). Gel loading was controlled by probing for β-actin (1:2000, Dako Ltd, Cambridge UK). A horseradish peroxidase-conjugated anti-mouse IgG1 (1:1000, Source) was used as the secondary antibody for all immunoblots except caspase-9, where an anti-rabbit IgG antibody (1:2000) was used (both Dako Ltd). Bands were visualised using the ECL-plus detection system (Amersham Life Sciences, Little Chalfont, UK).

2.3.6 Measurement of Reactive Oxygen Species

To determine ROS generation, experiments were conducted at the cytotoxic EC_{50} concentration of either MSA or SDG for each cell-line. Cells were resuspended in PBS ($1x10^{6}$/ml) and incubated in 20 µM 2'7'-dichlorodihydrodifluoroscein diacetate (DCF-DA, Molecular Probes, Oregon, USA) for 15 minutes at 37^{o}C in 5% CO_2. After the addition of MSA, SDG, 25 µM H_2O_2 (positive control) or no treatment control (0.75% DMSO, equivalent to the highest concentration used in drug incubations for each cell-line) cells were incubated for a further 30 minutes. ROS generation was then immediately determined by measuring the mean fluorescent intensity of 10,000 cells at 530 nM on a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK) using CellQuest software.

To investigate the possible influence of ROS on the activity of SDG and MSA, cells were treated at the EC_{50} concentration of either drug for 3 days in the presence of 2.5

mM N-acetyl cysteine (NAC) (Sigma chemicals, Poole, Dorset UK) after which cell number and percentage viability, relative to controls, were determined.

2.3.7 Measurement of c-AMP

To measure intracellular c-AMP concentration, the c-AMP Biotrak Enzymeimmunoassay (EIA) System (96 wells) kit was used (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). One hundred μl of each cell-line suspension was seeded at 5 x 10^5 cells/ml in a 96-well plate and incubated overnight at 37^{o}C in 5% CO_2. The following morning cell counts were performed for each cell-line and 100 μl of drug at 2 x desired concentration was added to the wells and the plate returned to the incubator for four hours. The plate was centrifuged to pellet the cells and the media aspirated. Cells were resuspended in 200 μl of lysis reagent 1B (dodecyltrimethylammonium bromide 0.25%) and agitated until lysis was complete. Working on ice, the kit 96-well microplate was loaded with prepared standards (12.5 to 3200 fmol c-AMP/well) and controls and 100 μl of each cell-line lysate into respective wells. One hundred μl of antiserum was then added to all wells except the blank and non-specific binding wells. After two hours incubation at 3-5^{o}C, 50 μl of c-AMP peroxidase conjugate was added to all wells except the blank wells. After a further hour's incubation at 3-5^{o}C, all wells were washed four times with 400 μl of wash buffer. To the blotted wells 150 μl of enzyme substrate was added to each well and the plate agitated at room temperature for 60 minutes. One hundred μl of 1 M sulphuric acid was then added to each well to halt the reaction. The plate was then inserted into the fluorstar plate reader and the optical density of each well determined at 450 nm within 30 minutes. The average of the replicates was calculated and a standard curve constructed to allow sample well c-AMP content to be generated.

2.4 Ethical Considerations

The studies using clinical material had the approval of the East London and The City Health Authority Ethic Committee, and their stipulations regarding to matters of patient consent, confidentiality and data protection were followed. To ensure patient anonymity, archival DNA and tissue samples are labelled with a reference number and location position. The patient name and hospital number are held separately in a password protected computer file. The medical oncology department data managers hold patient identifiable clinical details separately. Correlation between patient

identifiable clinical details and laboratory results was made solely through these individuals.

2.5 Statistics

To assess the significance of differences observed between drugs, concentrations, combinations and cell-lines in the *in vitro* experiments one-way analysis of variance (ANOVA) was performed, controlling for cell-line and/or treatments. To ensure that the overall type I error rate remained 0.05, Bonferoni's method was used to calculate the type I error rate for individual paired *t* test comparisons. A *P* value of <0.05 was taken as the cut-off for statistical significance. These tests were performed using the statistical package incorporated into the GraphPad Prism software (San Diego, CA, USA).

CHAPTER 3: INVESTIGATION OF PRESENTATION SERUM SELENIUM AS A PROGNOSTIC FACTOR IN DLBCL

3.1 Chapter Summary

Serum Se was measured using inductively coupled plasma mass spectrometry in archived presentation sera from 100 patients with high-grade NHL (78% DLBCL). Ninety-three of the 100 patients were treated with CHOP-like chemotherapy, six with radiotherapy and one received no treatment. Four outcome measures were studied for an association with presentation serum selenium: chemotherapy dose-delivery, response to treatment, first remission duration and OS. Serum Se closely correlated with performance status but no other clinical variable. Significant inverse associations were found for serum Se and all four of the outcome measures investigated (dose-delivery, response to treatment, first remission duration and OS).

3.2 Introduction

The empirical scientific reasons for investigating serum Se as a potential prognostic factor were multiple and included the following. The epidemiological observation of an inverse association between Se intake and cancer risk, the chemopreventive effect of Se compounds against tumour formation in more than a hundred animal studies, the demonstration of a similar chemopreventive effect in several human Se supplementation trials and limited data indicating reduced serum Se levels in cancer patients. The clinical reasons for undertaking the research were threefold: if serum Se should prove prognostic, that sample collection during routine blood testing would be straight forward; and that the affordability of testing for serum Se would make it feasible to introduce as an additional clinical investigation for research or routine practice. More ambitiously, it was hoped that if serum Se was predictive of outcome following intervention in DLBCL, then Se supplementation might offer a novel therapeutic adjunct to conventional poly-chemotherapy. The empirical hypothesis tested was that serum Se at presentation would predict for response to first treatment, dose-delivery, and OS in patients with DLBCL.

3.3 Methods

I identified the patient samples and Andy Wilson and I extracted relevant clinical data from the departmental database and the patients case notes. Reclassification of the corresponding presentation NHL histological material to the serum samples was undertaken by Dr Andrew Norton, Department of Histopathology and where bone

marrow material Dr John Amess Department of Haematology, St Bartholomew's Hospital, London, UK. The mesurement of the Se content of the frozen sera was conducted by Dr Trevor Delves and Dr Christine Sieniawska Trace Elements Unit, Southampton General Hospital, Southampton, UK. Victoria Cornelius, CR-UK Department of Statistics, Oxford, UK, performed all statistical analyses for the research described in this Chapter.

Between July 1986 and March 1999 of the 383 patients with a diagnosis likely to be DLBCL on updated classification, frozen, archived, presentation serum from 100 patients was suitable for measurement of serum Se. The updated histological subtype of NHL according to the WHO classification is illustrated in Table 2.3. The clinical variables investigated for an association with dose-delivery, response to first treatment and/or OS were serum Se, gender, and the recognised prognostic factors of age, B symptoms, stage, retrospective performance status, LDH (or hydroxybutyrate dehydrogenase if the patient presented prior to mid-1989), and number of extranodal sites (Table 2.4). The IPI score was calculated for the 95 patients with complete IPI data (Table 2.4). All patients bar one (who died before treatment could begin) were treated with curative intent with anthracycline-based combination chemotherapy, radiotherapy or both (Table 2.5). Comprehensive dose-delivery data was available for 87 of the 93 patients given chemotherapy. Survival data was analysed up until July 3 2000. Total serum Se content was measured by inductively coupled plasma mass spectrometry. Dose-delivery associations were sought between the clinical variables investigated and doxorubicin, cyclophosphamide and vincristine. An area-under-the-curve summary measure, AUC_{ratio} was created especially for the project by Victoria Cornelius (see also Chapter 2.2.4). The AUC_{ratio} of cumulative dose vs. time revealed not only variation in time taken and dose administered, but also distinguishes between dose reductions and delays incurred at the beginning from those incurred at the end of treatment. AUC_{ratio} therefore factors for the premise that timely dose-delivery early is more critical than toward the end of therapy.

3.4 Results

3.4.1 Serum Selenium

The presentation serum Se concentration was normally distributed for the 100 patients with a mean of 0.92 μmol/l (standard deviation = 0.25) and range of 0.33 to 1.51 μmol/l. A trend of decreasing mean serum Se was observed with increasing year of

presentation. Seventy-three patients had a Se level below the UK reference range from the early 1980's (UK adult reference range = 1.07-1.88 μmol/l (Matthews *et al.*, 1990)). Serum selenium concentration was highly associated with performance status but no other clinical variable (P <0.001). As a consequence of this association, only one of these variables was included in the multivariate analyses at the same time, as they explained similar variation. As the primary interest was in assessing the relationship between Se levels, response, dose-delivery and survival rather than developing a new prognostic model, the multivariate analyses containing the Se variable are presented here. Exchanging Se with performance status gave similar results. Logistic regression and Cox multivariate models for dose-delivery, response, OS were performed on variables if had obtained significance at the univariate level (P <0.1). Survival curves were estimated using Kaplan-Meier methods and the log-rank test. The patients were divided into quartiles, as defined by presentation serum Se concentration, and tested univariately with regard to OS.

3.4.2 Dose-delivery

In the 93 patients with complete treatment data, there were 129 treatment delays and/or dose reductions, associated with 42 in-patient episodes for neutropenic fever/sepsis, 29 admissions for other treatment-related complications, 45 episodes of neutropenia without fever and 13 delays for other reasons.

At the univariate level (P <0.1), two variables correlated positively with the dose-delivery summary measure AUC_{ratio}: age and serum selenium; and one variable correlated negatively: stage (Table 4.1). Stepwise inclusion of these three variables in the multivariate model revealed that a significantly better dose-delivery (summarised by AUC_{ratio}) was associated with younger age, advanced stage, and higher serum Se (see Table 3.2).

Clinical variable	Dose-delivery $^{\alpha}$	Response $^{\beta}$	Survival time $^{\gamma}$
Age	$<0.001^{\delta}$	0.532	0.042^{δ}
Selenium	0.063^{δ}	0.009^{δ}	0.026^{δ}
Gender	0.175	0.156	0.092^{δ}
Stage	0.053^{δ}	0.517	0.089^{δ}
B symptoms	0.786	0.791	0.570
Performance status	0.234	0.007^{δ}	$<0.001^{\delta}$
LDH	0.403	0.892	0.280
No. of extranodal sites	0.282	0.265	0.383

Table 3.1 Univariate Analysis With Probability Values
The *P* values are derived from: α – significance of regression coefficient (H_0: β=0); β - χ^2 for categorical and ANOVA for continuous variables; γ – log rank test for categorical and significance of hazard ratio for continuous variables.
δ - variables indicating significance at the univariate level (*P* < 0.1).

Clinical Variable	Dose-delivery		Response		Survival time	
	Regression Coefficient (Standard Error)	*P* value	Odds Ratio (95% CI)	*P* value	Hazard Ratio (95% CI)	*P* value
Age	-0.006 (0.001)	$<0.001^{\gamma}$	-	-	1.09 (0.99, 1.18)$^{\alpha}$	0.066
Selenium	0.215 (0.099)	0.032^{γ}	0.62 (0.43, 0.90)$^{\beta}$	0.011^{γ}	0.76 (0.60, 0.95)$^{\beta}$	0.018^{γ}
Gender	-	-	-	-	1.66 (0.94, 2.93)	0.083
Stage	0.051 (0.015)	0.001^{γ}	-	-	-	-

Table 3.2 Multivariate Analyses With Probability Values
The Regression Coefficient is the change in AUC for a one-unit increase in that variable.
α - for 5-year change in age. β for 0.2 μmol/l change in serum selenium.
γ indicates significance at the multivariate level (*P* <0.01)

3.4.3 Response To Therapy

Only two patient variables, Se concentration and performance status, were associated with response to first treatment at the univariate level (using a statistical significance of

10%) (Table 3.1). Fitting a logistic regression model produced an odds ratio of 0.62 for every 0.2 μmol/l increase in serum Se (95% CI 0.43, 0.90) and $P = 0.011$ (Table 3.2). A higher Se level was therefore associated with a lower probability of having a poor outcome to treatment (i.e. PPR, treatment failure or treatment-related death). For example, a patient with a serum Se of 0.8 μmol/l has estimated odds of CR or GPR 38% lower than a patient with a serum Se level 1.0 μmol/l. Similar results were obtained by exchanging the variable performance status with Se in the logistic regression model. A cross-tabulation of response and IPI score revealed no obvious association between IPI and response to first treatment (χ^2 $P = 0.45$). Table 3.3 displays response according to Se quartiles (χ^2=9.52, $P = 0.023$).

Response		Lowest quartile	3rd quartile	2nd quartile	Highest quartile
CR/GPR	Number	13	14	14	22
	%	54.2%	56.0%	56.0%	88.0%*
PPR/ fail/ Treatment - related death	Number	11	11	11	3
	%	45.8%	44.0%	44.0%	12.0%

Table 3.3 Response to First Treatment According to Selenium Quartiles.
There were 99 assessable patients as one died before treatment could commence.
* significantly different compared to the other quartiles (χ^2=9.52, $P = 0.023$).

3.4.4 Remission Duration

When treated as a continuous variable, serum Se was not significant at the univariate level (p = 0.298). However, when serum Se was treated as a categorical variable (4 quartiles) it was significant with regard to remission time (log rank test = 4.38, P = 0.036). This difference was due to response to treatment only being significantly better in the top quartile of patients as defined by serum Se level. This result suggests that above a threshold of 1.096 μMol/l, response is significantly improved. Performance status was also significant with regard to remission time (log rank = 15.7, $P < 0.001$) whilst LDH indicated significance at the univariate level ($P = 0.07$).

3.4.5 Overall Survival

The OS of the 100 studied patients did not differ significantly from the 283 remaining aggressive B-cell NHL patients who did not have archived presentation sera (Figure 3.1).

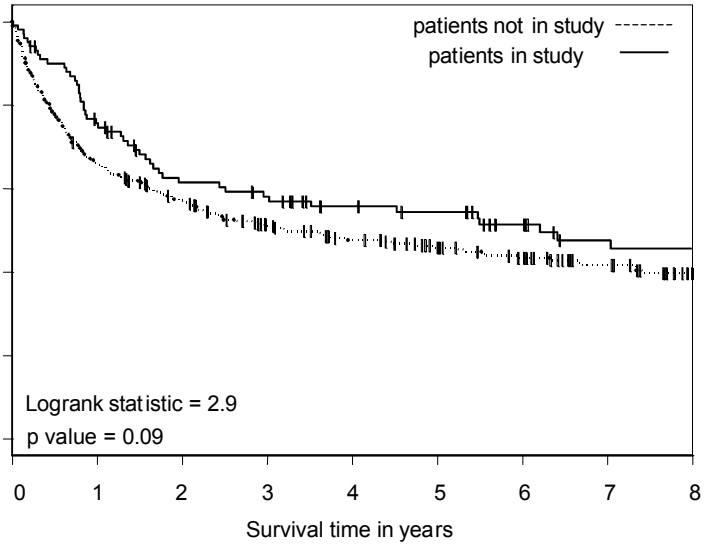


Figure 3.1 Overall Survival for the patients included in and excluded from the study

…… OS from diagnosis of the 283 patients treated between July 1986 and March 1999 without available presentation frozen sera.

——— OS from diagnosis of the 100 patients investigated in the study.

The median OS of the 100 patients was 6.2 years (range one week to 13 years, 95% CI 1.9, 10.7) and 6.1 years for the 51 surviving patients (range 4 months [1 patient lost to follow-up at 128 days] -13.5 years). When considering IPI as a continuous variable, it was found to be prognostic with regard to OS with a hazard ratio of 1.58 (95% CI 1.18, 2.13). The results of univariate analysis indicated that age, Se concentration, gender, stage and performance status correlated with survival time (Table 3.1). On multivariate analysis, using a Cox model and excluding PS, Se was the most significant factor, with a hazard ratio of 0.76 for every increase of 0.2 μmol/l in serum Se level (Table 3.2). Thus, a lower serum Se concentration was associated with shorter survival. For

example, a patient with a serum Se concentration of 1.0 μmol/l had a 24% lower risk of death than a patient with a concentration of 0.8 μmol/l, over the duration of the study. Replacing Se with performance status gave similar results i.e. a decrease in performance status was significantly associated with shorter survival . OS for each Se quartile is displayed in Figure 3.2.

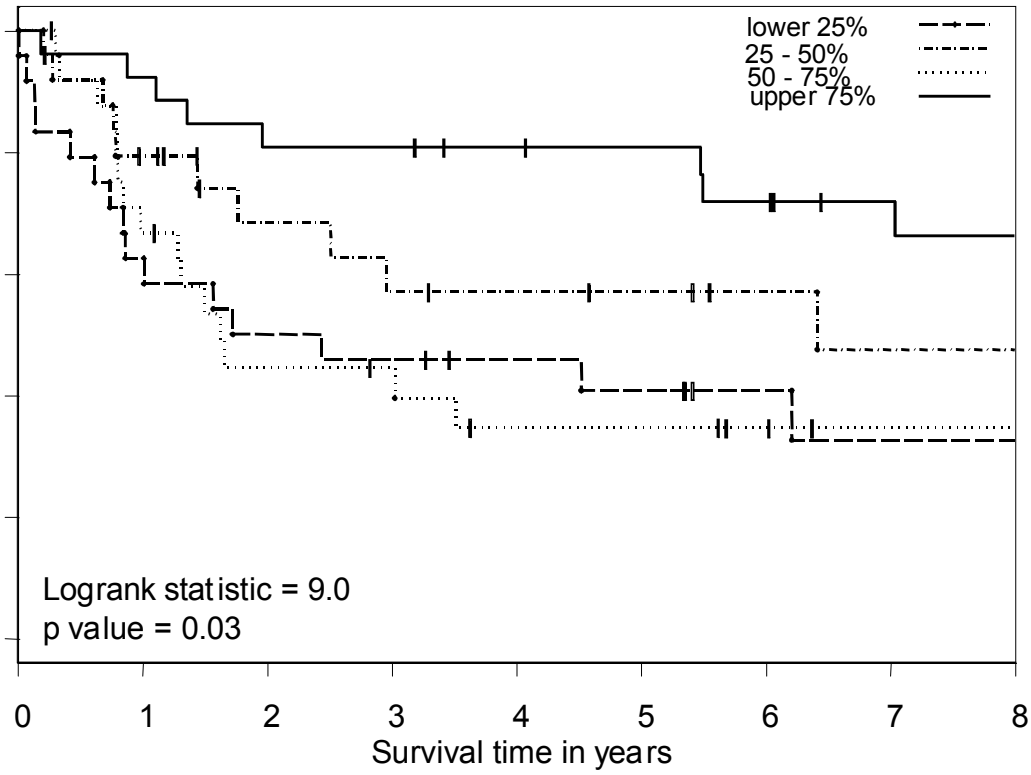


Figure 3.2 Overall survival, from diagnosis, of the 100 patients According to serum Selenium quartiles

Lowest quartile = serum Se from 0.33 to 0.74 μMol/l
Second quartile = serum Se from 0.75 to 0.91 μMol/l
Third quartile = serum Se from 0.92 to 1.095 μMol/l
Highest quartile = serum Se from 1.096 to 1.51 μMol/l

3.5 Discussion

This study was undertaken to test the hypothesis that serum Se at presentation would predict for either response to first treatment, dose-delivery or OS in patients with DLBCL, the commonest NHL (Armitage and Weisenburger, 1998). Correlations between presentation serum Se, dose-delivery, response to first therapy, first remission duration and OS were discovered in patients with aggressive NHL, the majority of which were DLBCL or the related mediastinal large B-cell lymphoma (94/100) on histology re-evaluation. Dose-delivery, assessed using actual to planned AUCs, was also associated with stage and inversely associated with age. Serum Se was therefore found to be a prognostic factor in DLBCL. As all but one of the patients received treatment i.e. no best supportive care cohort of patients, it was not possible to identify if serum Se was a pure prognostic factor or also a predictive factor for outcome measures to CHOP-like treatment.

Performance status was the only clinical variable that was highly associated with serum selenium. This correlation may be the result of general nutritional deficiency in the months and weeks pre-diagnosis and/or an acute phase response of serum Se to illness (Nichol *et al.*, 1998). The associations found in this study could therefore be due to a bystander phenomenon with no causality related to Se level. Alternatively, the correlations described may indeed reflect a causal relationship and account, in part, for the prognostic power of performance status in aggressive NHL. As regards the possibility of the low Se values seen in this study being partly due to an acute phase response, this need not imply lack of causality nor scope for therapeutic intervention. For example, the low serum Se seen in patients with acute pancreatitis (Wereszczynska-Siemiatkowska *et al.*, 2004) has been reported to respond to supranutritional intravenous infusions of Se, resulting in a marked decline in the mortality rate associated with this frequently fatal condition (Kuklinski *et al.*, 1991, Kuklinski *et al.*, 1995) and aggressive selenite supplementation of Se deficient patients with severe sepsis on a medical intensive care unit (with performance status IV) resulted in reduced morbidity (Angstwurm *et al.*, 2004).

The importance of these findings is unknown at present and will require validation in other patient series in the first instance. Critical to their relevance is the issue of whether causality exists in the relationships discovered. Does Se deficiency, as indicated by reduced serum Se, cause side-effects of treatment to be worse, allow chemoresistance to

develop more readily and impair an immune response to tumour? A second question arises upon assumption of causality in associations described, namely in what direction does the causality exist? Does Se deficiency cause/predispose to the tumour, as is evident from the epidemiological data described earlier, or does the tumour cause the observed Se deficiency? The animal data reviewed indicates that Se supplementation can prevent the initiation of cancer and kill preneoplastic cells in the early post-initiation stage and reduce the growth of xenografts. The recovery of serum Se levels to normal on successful treatment of acute myelogenous leukaemia implies that the illness causes the deficiency in Se (Beguin *et al.*, 1989). Some take such a scenario to mean that intervention will be fruitless. Such a nihilistic viewpoint overlooks the possibility that advanced, clinically apparent tumours, may obtain a survival advantage from inducing a Se deficiency state, an advantage that could be corrected by Se supplementation as an adjunct to conventional therapy. Others have shown the opposite to occur: that Se status remains suboptimal despite successful cancer treatment (Ujiie and Kikuchi, 2002), a result supporting the former relationship of low Se predisposing to neoplasia.

In the laboratory, *in vitro* and *in vivo* experiments have revealed that Se compounds can exert cytotoxic activity, synergise with cytotoxic agents and favourably influence cancer cell phenotype (Thompson *et al.*, 1994, Vadgama *et al.*, 2000, Caffrey and Frenkel, 2000). No clinical evidence confirming Se as a disease-modifier in patients with established cancer so far exists. If proven, the argument for exploration of Se compounds as a therapy adjunct to aid improvement of the long-term remission rate of only 40-50% in this illness would be strengthened. Indirect evidence supporting an association between Se and the clinical course of lymphoma comes from a study, which found that pre-treatment serum Se concentration correlated with response to treatment in a group of 51 epidermotrophic T-cell lymphomas, and the observation that serum Se levels were significantly lower in patients with NHL than in normal controls (Deffuant *et al.*, 1994, Avanzini *et al.*, 1995).

Several compatible mechanisms may underlie the prediction of dose-delivery, response and outcome by presentation serum selenium. These include prevention of chemoresistance, enhancement of immune function, direct cytotoxic activity of Se compounds and reduction of treatment-related side-effects. Evidence of Se preventing chemoresistance comes from *in vitro* and mouse studies. No confirmatory *in vivo*

human data yet exists. Selenite prevented melphalan resistance and cisplatin cross-resistance when co-administrated with low-dose melphalan to the human ovarian cancer cell-line A2780 *in vitro* (Caffrey *et al.*, 1998). A2780 xenografts in nude mice readily develop melphalan and cisplatin resistance upon low-dose melphalan or cisplatin administration (Caffrey and Frenkel, 2000, Caffrey *et al.*, 1998). Concurrent administration of selenite or selenomethionine prevented chemoresistance development (Caffrey and Frenkel, 2000, Caffrey *et al.*, 1998). Also in a murine model, using Dalton's lymphoma, pre-inoculation with selenomethionine increased survival by 31 and 112% compared to those mice that received selenomethionine at inoculation and those that received no selenomethionine supplementation (Rana *et al.*, 1996). Multiple processes are likely to account for this survival advantage. Although limited, evidence suggesting a role for Se in immune response enhancement includes a study where selenite, given to volunteers with normal Se levels, resulted in clonal expansion of activated T-cells in response to *in vitro* incubation with a cancer cell-line. T-cell cytotoxicity increased by 118% and natural killer cell-mediated lytic activity by 82% compared with baseline (P values < 0.05) (Kiremidjian-Schumacher *et al.*, 1994, Roy *et al.*, 1994). The same researchers conducted a randomised, double-blind, placebo-controlled trail of sodium selenite 200 μg/day vs. placebo in patients beginning treatment for their head and neck squamous cell carcinomas (Kiremidjian-Schumacher *et al.*, 2000). They found that the selenium-supplemented group experienced a significantly enhanced cell-mediated immune responsiveness both during therapy and after its completion compared to a decline in immune responsiveness in the control group during treatment (Kiremidjian-Schumacher *et al.*, 2000). A recent report from the UK assessed gene expression in lymphocytes isolated from 39 healthy subjects pre- and post- 6-weeks of 100 μg sodium selenite a day (Pagmantidis *et al.*, 2008). Upregulation of ribosomal protein and translation factor genes were seen in response to Se supplementation. More convincing evidence in support of the immunomodulatory potential of Se supplementation comes from the microarray results from pre and post 10-month Se supplementation in people with asymptomatic oesophageal squamous dysplasia (Joshi *et al.*, 2006). Only genes related to the immune response pathway were significantly overrepresented in the 149 genes differentially expressed between samples from patients whose dysplasia regressed and those whose dysplasia worsened. The cytostatic and cytotoxic activity of different Se compounds has been confirmed in

numerous *in vitro* and animal experiments (Ip, 1998, Lanfear *et al.*, 1994, Medina *et al.*, 2001). Vadgama *et al* noted synergy between Se and the cytotoxic agents paclitaxel and doxorubicin (in terms of increased cell death and growth arrest) in the majority of cell-lines investigated (Vadgama *et al.*, 2000). A possible means of Se enhancing dose-delivery may be through reducing the side-effects of therapy. Se supplementation has been shown to reduce cisplatin-induced nephrotoxicity and bone marrow suppression in a crossover patient study and prevent doxorubicin-induced cardiomyocyte cytotoxicity *in vitro* (Hu *et al.*, 1997, Yoshida *et al.*, 2000, Kotamraju *et al.*, 2000). One potential explanation for this effect may be a differential alteration in the apoptotic threshold between Se replete normal cells (threshold raised, so that cells are able to repair damage rather than die) and malignant cells (threshold lowered so that cells more prone to undergo programmed cell death rather than DNA repair). This hypothesis is supported by two reports. One in elderly dogs where Se supplementation caused the amount of DNA damage in peripheral blood mononuclear cells to be lower in supplemented animals vs. controls, while the number of apoptotic cells in prostate epithelium was increased vs. controls (Waters *et al.*, 2003). The second study found that Se supplementation encouraged DNA repair via p53 in normal cells but promoted cell apoptosis in malignant cells with mutated *TP53* and null for p53 protein (Fischer *et al.*, 2007).

A recent report also described reduced side-effects of chemotherapy as a result of Se supplementation, suggesting that Se status may affect chemotherapy dose-delivery in cancer and lymphoma patients through protection of normal tissues from cytotoxic damage (Sieja and Talerczyk, 2004). The less well a patient is at the start of treatment, the more likely they will experience side-effects from chemotherapy and the less likely they are to respond to treatment.

The majority of the patients investigated (73%) had a serum Se concentration below the UK reference range from the early 1980's. This finding is consistent with the reporting of lower Se levels in cancer and lymphoma patients compared to the general population (Alaejos *et al.*, 2000, Deffuant *et al.*, 1994), to the decline in Se intake in the UK population (attributed to the cessation of Canadian wheat importation) since this reference range was generated (Group, 1997, Group, 1999), and to the afore mentioned fall in serum Se as part of the acute phase response to illness (Nichol *et al.*, 1998).

The extent of Se deficiency in aggressive NHL patients, as assessed by serum Se levels, coming from general populations replete in Se remains to be seen. Se levels vary around the world from extremely low in areas of China, to low in many parts of Europe compared to the high-normal levels in most areas of North America and Japan. If Se is important in aggressive NHL phenotype evolution and treatment success, countries with a high-normal Se level in the population might be expected to have a better OS for aggressive NHL than countries with a low Se intake. This is not apparent, as UK survival rates are comparable to those in North America. Whether this reflects the need for supernormal Se intakes to alter disease outcome at the population level, or to aggressive NHL resulting in low Se levels regardless of premorbid Se status will require further investigation. The Se chemoprevention study performed in former non-melanomatous skin cancer patients, took place in the US (Clark *et al.*, 1996). Se supplementation was beneficial even in those replete in the micronutrient, although the benefit was greatest in the lower tertile of patients (serum Se <1.35 μmol/l) (Rayman and Clark, 2000). All but three patients in this study had a serum Se concentration within this tertile.

Prospective, multicentre/country substantiation of the findings described will be required. The discovery of presentation serum Se as a potential prognostic factor in aggressive NHL raises the possibility of investigating Se compounds as a novel strategy for both disease and outcome modification in this frequently curable malignancy. In search of empirical scientific evidence in support of a causal relationship in the associations found, I proceeded to study two Se compounds, MSA and SDG, for cytostatic and cytotoxic activity alone and in combination with established cytotoxics in a panel of B-cell NHL cell-lines and primary cultures.

CHAPTER 4: IN VITRO INVESTIGATION OF SELENODIGLUTATHIONE (SDG) AND METHYLSELENINIC ACID (MSA)

4.1 Chapter Summary

In the light of the positive results described in Chapter 3, the research in this Chapter was undertaken to establish if Se compounds could inhibit cell growth and viability in a panel of human DLBCL cell-lines and in a selection of primary lymphoma samples from multiply treated patients using a primary cell culture system. This research has not been previously reported. The two Se compounds selected, SDG and MSA, proved to exert concentration dependent cytostatic and cytotoxic actions against the DLBCL cell-lines and primary lymphoma samples but were much less effective against the pooled normal B-cell sample, raising the possibility of a therapeutic index between malignant and normal cells for both SDG and MSA. Next, the effect of the Se compounds on the cell cycle and the mode of cell death induced were investigated. Neither SDG nor MSA caused a block at a specific phase of the cell cycle and both caused apoptotic cell death through caspase-8 and -9 activation and PARP cleavage. Finally, the generation of ROS by SDG and MSA and the importance of ROS generation for effect cell death by the two Se compounds were investigated in the DLBCL cell-line panel. MSA was shown to exert its cytotoxic activity independent of ROS, as ROS generation did not occur to a significant degree with any of the cell-lines. In contrast, exposure to SDG led to ROS generation within 30 minutes in three of the four cell-lines and was critical to the cytotoxicity of SDG in two of the four cell-lines tested. In summary, SDG and MSA were active against DLBCL cell-lines and primary lymphoma samples, induced apoptotic cell death and acted via different mechanisms.

4.2 Introduction

In vitro investigations in DLBCL cell-lines and primary cell cultures using Se compounds were conducted for two reasons: to provide evidence in favour of a causal relationship between the associations described in Chapter 3 between presentation serum Se the clinical outcome measures of dose-delivery, response and OS and to investigate Se compounds as novel treatments for DLBCL. A number of Se compounds have been shown to be cytostatic and cytotoxic to cancer cell-lines and to exhibit chemopreventive activity in animal models of cancer. Se compounds are also effective in cell-lines lacking functional p53. For example, in the *TP53* mutated colon cancer cell-line HT29, selenomethionine inhibited cell growth in a concentration-dependent

manner (Redman *et al.*, 1997), whilst the synthetic, organic methylselenocyanate induced apoptosis in the *TP53* null mouse MOD mammary cancer cell-line (Kaeck *et al.*, 1997), and SDG has equivalent cytostatic and cytotoxic activity in mutated p53 cells as in wild-type p53 cells (Lanfear *et al.*, 1994). For the experiments conducted in this Chapter the choice of compound included three Se compounds from the hydrogen selenide pool, namely selenite, SDG and 1,4-phenylenebis(methylene)selenocyanate (p-XSC) and three compounds from the methylselenol pool: selenomethionine, MSC and MSA. A summary of the data on each of these six will now be presented and an explanation for the choice of SDG and MSA.

Selenite

Selenite inhibits cell growth and DNA synthesis, induces cell cycle arrest and cell death in a range of cell-lines (Lu *et al.*, 1995, Lu *et al.*, 1994, Kaeck *et al.*, 1997). The activity of selenite is concentration-dependent and occurs in the low micromolar range. Cell death is dominated by necrosis although apoptosis is also induced (Lu *et al.*, 1995, Lu *et al.*, 1994, Kaeck *et al.*, 1997, Stewart *et al.*, 1999). Genotoxicity is induced by selenite, as an early event, in a concentration-dependent manner, as evidenced by single- and then double-strand DNA breaks, an effect independent of apoptosis (Lu *et al.*, 1994, Lu *et al.*, 1995, Jiang *et al.*, 2001). The cell death and DNA laddering caused by selenite occurs at an 8-10 fold lower concentration than is required for selenomethionine and MSC (Stewart *et al.*, 1999). Selenite induces the generation of the ROS superoxide in the presence of glutathione and mitochondria; an effect abolished by the addition of a superoxide scavenger (Kim *et al.*, 2003b). Although sodium selenite has a proven track record as a cytotoxic agent *in vitro* and a chemopreventive agent *in vivo*, I did not choose it for my *in vitro* studies for two reasons. Firstly, the lag time of 24 hours or more until cytotoxic activity commences *in vitro*; and secondly because selenite has one of the narrowest therapeutic indices of the Se compounds *in vivo*.

1,4-phenylenebis(methylene)selenocyanate (p-XSC)

p-XSC was the most promising of several aromatic Se compounds developed in an attempt to widen the therapeutic index seen with selenite and selenomethionine in animal experiments (el-Bayoumy, 1985). The maximum tolerated dose of dietary p-XSC in rats was 32.5 ppm Se compared to $\leq$5 ppm Se for sodium selenite (Fan and Kizer, 1990, Conaway *et al.*, 1992). *In vitro*, the intracellular accumulation of Se was 3-6 fold higher following treatment with p-XSC than the equivalent dose of selenite and

the percentage of apoptotic cells was greater (Thompson *et al.*, 1994). Both the initiation and post-initiation/tumour promotion stages of carcinogenesis were inhibited by p-XSC in a rat DMBA breast cancer model and a rat azoxymethane colon cancer model (Reddy *et al.*, 1994, Ip and Lisk, 1994, el-Bayoumy *et al.*, 1992). The chemopreventive index of p-XSC appeared to be four times that of selenite (Ip *et al.*, 1994). p-XSC appears to have intrinsic activity as well as activity derived from its metabolism to hydrogen selenide (Thompson *et al.*, 1994, Ip and Lisk, 1994, el-Bayoumy *et al.*, 1993).

SDG

The variable efficacy of selenite *in vitro* is not seen with SDG. Fleming *et al* found 50 μM selenite to produce only 20% reduction in viability after 3-day exposure experiments compared to 100% for just 5 μM of SDG (Fleming *et al.*, 2001). They went on to confirm the metabolism of selenite to SDG in the presence of glutathione (Fleming *et al.*, 2001). Indeed SDG can be produced within the cell by the reaction of selenite or selenodioxide with glutathione. SDG is reduced to the selenopersulphide anion and then hydrogen selenide with the generation of superoxide by redox cycling (Spallholz, 1994).

Lanfear *et al* demonstrated the *in vitro* potency of SDG when they compared the effect of different Se compounds upon cell number after 3-day treatment of the mouse erythroleukaemia cell-line MEL and the ovarian carcinoma cell-line A2780. From these results it is evident that SDG was ten times more potent than selenite, dimethylselenoxide and MSC. The latter two Se compounds had widely different efficacies in the two cell-lines. MEL cells were resistant to MSC but sensitive to dimethylselenoxide, whilst A2780 cells were sensitive to MSC but resistant to dimethylselenoxide (Lanfear *et al.*, 1994). Morphologically the changes induced by SDG in MEL resemble those of anti-cancer agents such as cisplatin and etoposide (Lanfear *et al.*, 1994). The percentage of apoptotic cells increased from 1% in no treatment controls to ~10% following 6 hours treatment of MEL cells with 3 μM of SDG (Lanfear *et al.*, 1994). SDG was chosen for investigation because of its superior *in vitro* efficacy compared to selenite.

Selenomethionine

As the majority of Se in selenised yeast is in the form of selenomethionine (85%) and is more bioavailable than inorganic forms such as sodium selenite, animal and *in vitro*

investigation of selenomethionine has been pursued. In nude mice topical selenomethionine alone and combined with vitamin E provided protection from ultraviolet light-induced skin damage (Burke *et al.*, 2003). When given to elderly dogs for 7 months, selenomethionine reduced the DNA-damage detectable in peripheral blood lymphocytes and increased the percentage of apoptotic prostate epithelial cells (Waters *et al.*, 2003). Furthermore, selenomethionine at 3 ppm proved chemopreventive in the rat DMBA mammary carcinoma model (Ip, 1988). *In vitro* the 3-day cell proliferation IC50's (assessed by crystal violet) for selenomethionine were 1, 70 and 90 μM respectively for LNCaP, PC-3 and DU145 human prostate cancer cell-lines (Menter *et al.*, 2000) and ranged from 30-130 μM in a panel of lymphoma, leukaemia and hepatoma cell-lines (Kajander *et al.*, 1990). Selenomethionine is principally metabolised in the liver by β-lyase to a monomethylated intermediate for the expression of its anticancer activity and epithelial tissues typically retain a low capacity to generate a mono-methylated Se-metabolite from selenomethionine (Ip, 1998). Consequently concentrations of selenomethionine that are 20 - 100 times above those needed to meet physiological requirements have been used in many cultured cells, including lymphocytes. For this reason selenomethionine was not selected as the methylselenol pool member for the *in vitro* experiments.

Se-Methylselenocysteine (MSC)

MSC is a water-soluble organic form of Se that is naturally found in selenium-enriched plants, such as broccoli and garlic. It is converted to methylselenol *in vivo*, an event considered critical for its chemopreventive properties (Foster *et al.*, 1986). In a rat mammary carcinogenesis model, 2 ppm Se supplementation as MSC produced a 50% reduction in tumour formation (EC_{50} tumour yield), a dose 10-fold higher than required for restoration of seleno-enzyme function (Ip *et al.*, 1991). As with selenomethionine, the metabolism of MSC requires the action of β-lyase, an enzyme present in the liver, kidney, intestine and mammary glands but frequently absent in malignant cells (Ip *et al.*, 1999). As a consequence, MSC is less potent than selenite in cell culture experiments, causing less growth inhibition and cell death (Ip, 1998). The cell death seen is largely apoptotic in contrast to the necrotic cell death of selenite, without the DNA breaks seen with selenite. The dependence upon β-lyase activity for MSC to have efficacy *in vitro* was demonstrated by Dong *et al*, where a concentration of 200 μM MSC and 72 hours exposure was required before even a modest effect upon cell number

was seen in MCF10AT1, a human premalignant mammary epithelial cell-line, whilst just 2.5 μM of MSA induced a 70% reduction in cell number by 48 hours of treatment (Dong *et al.*, 2002). Because malignant cells contain little β-lyase, MSC was not chosen for *in vitro* study.

MSA

Methylselenol is considered to be the dominant *in vivo* chemopreventive metabolite of Se (Sinha *et al.*, 2001). The production of methylselenol from MSC and selenomethionine is believed to underlie much of the chemopreventive effects of Se supplementation in animal models (Ip *et al.*, 2000). MSA is a simplified form of MSC, lacking the amino acid moiety of MSC which β-lyase catalyses the cleavage of to produce methylselenol. Methylselenol is therefore formed intracellularly from MSA by facile reduction without the need for β-lyase. This facile reduction is anticipated to be both non-enzymatic by reaction with thiols to produce methylselenenylsulphide and then methylselenol, and enzymatic where the glutathione derived thiol intermediary CH3Se-SG would be reduced by glutathione reductase in the presence of NADPH to CH_3SeH (Ganther and Lawrence, 1997). Thus MSA is a β-lyase-independent synthetic precursor of methylselenol, merely requiring the presence of NADPH and glutathione for methylselenol to be generated *in vitro* in cells lacking β-lyase (Ip *et al.*, 2000). *In vitro*, MSA acts more rapidly and more potently upon cell number (assessed using crystal violet staining), at one-tenth the concentration of MSC (Ip *et al.*, 2000). After 24 hours exposure to 5 μM MSA the cell number reduced to 44% and 46% relative to no treatment controls in the mouse mammary epithelial cell-lines TM2H and TM12 respectively. In comparison, 24-hour exposure to 50 μM of MSC marginally decreased the cell number of TM2H and TM12 to 97% and 84% respectively, relative to no treatment controls. Unlike selenite, MSA is not genotoxic and can induce apoptosis in *TP53* null cells as well, as *TP53* wild type cells (Ip *et al.*, 2000).

Therefore, on the basis of the ability of MSA to overcome the dependence on β-lyase to produce monomethylated Se metabolites, MSA was chosen as the methylselenol pool member for the *in vitro* research described below.

4.3 Methods

The collection of human DLBCL cell-lines (SUD4, CRL, DoHH2 and DHL4) and primary lymphoma samples were cultured and used for 3-day and 2-day drug exposure experiments as described in Chapter 2. I performed this work with help and support,

particularly with the primary cell culture experiments, from Lenushka Maharaj. Each experiment was performed on at least three separate occasions unless stated. Cell count and viability were assessed by trypan blue exclusion assay as described in Chapter 2. For each cell-line the EC_{50} was calculated using the non-linear regression sigmoidal concentration–response model according to the GraphPad prism software (as described in Chapter 2.3.2). I assessed cell cycle phase distribution by FACS scanning of PI stained cells. Sandra Strauss looked for evidence of caspase-8, caspase-9 and PARP cleavage by western blotting following cell-line exposure to SDG and MSA. Jackie Perrie carried out FACS analysis of PI and annexin V stained cells to confirm the induction of apoptosis following exposure of cells to SDG and MSA. The production of ROS by the DLBCL cell-lines was assessed using DCF-DA, which generates a fluorescent product in the presence of ROS by Jackie Perrie. The established positive control, 25 μM H_2O_2, was used in each experiment. To assess the importance of ROS generation to the cytotoxicity of SDG and MSA, the free-radical scavenger and glutathione precursor N-acetyl cysteine (NAC) was employed. Lenushka Maharaj undertook the NAC exposure experiments.

4.4 Results

4.4.1 Cell Count and Viability in DLBCL Cell-lines after 3-day exposure to SDG

Cell count and viability of the DLBCL cell-lines SUD4, CRL, DoHH2 and DHL4 following 3-day exposure to SDG are illustrated in Figures 4.1 and 4.2. The EC_{50}s are displayed in Table 4.1 and the raw data for all four cell-lines in Appendix Tables A4.1 through A4.8. All four cell-lines were sensitive to SDG with very steep concentration-response curves for both cell count and viability. The concentration-response curves were steeper for SDG than MSA, particularly for SUD4 and DoHH2 where the sigmoid factor was –8.3 and –16.2 for SUD4 and DoHH2 SDG cell count compared to –2.5 and –1.9 for SUD4 and DoHH2 MSA cell count respectively; and –8.4 and –10.1 for SUD4 and DoHH2 SDG viability compared to –3.5 and –1.9 for SUD4 and DoHH2 MSA viability respectively. Consequently, although a number of drug concentrations were investigated across a relatively small range, it was difficult to obtain data points lying between the 20 % and the 80% cell number and viability effects. This is indicative of a trigger concentration above which, the effect was near maximal.

In summary SDG proved cytostatic and cytotoxic in the three *TP53* mutated/null cell-lines (SUD4, CRL, and DHL4) and the *TP53* heterozygous wild type/null DoHH2. This is a novel finding, as SDG has not previously been studied in human DLBCL cell-lines.

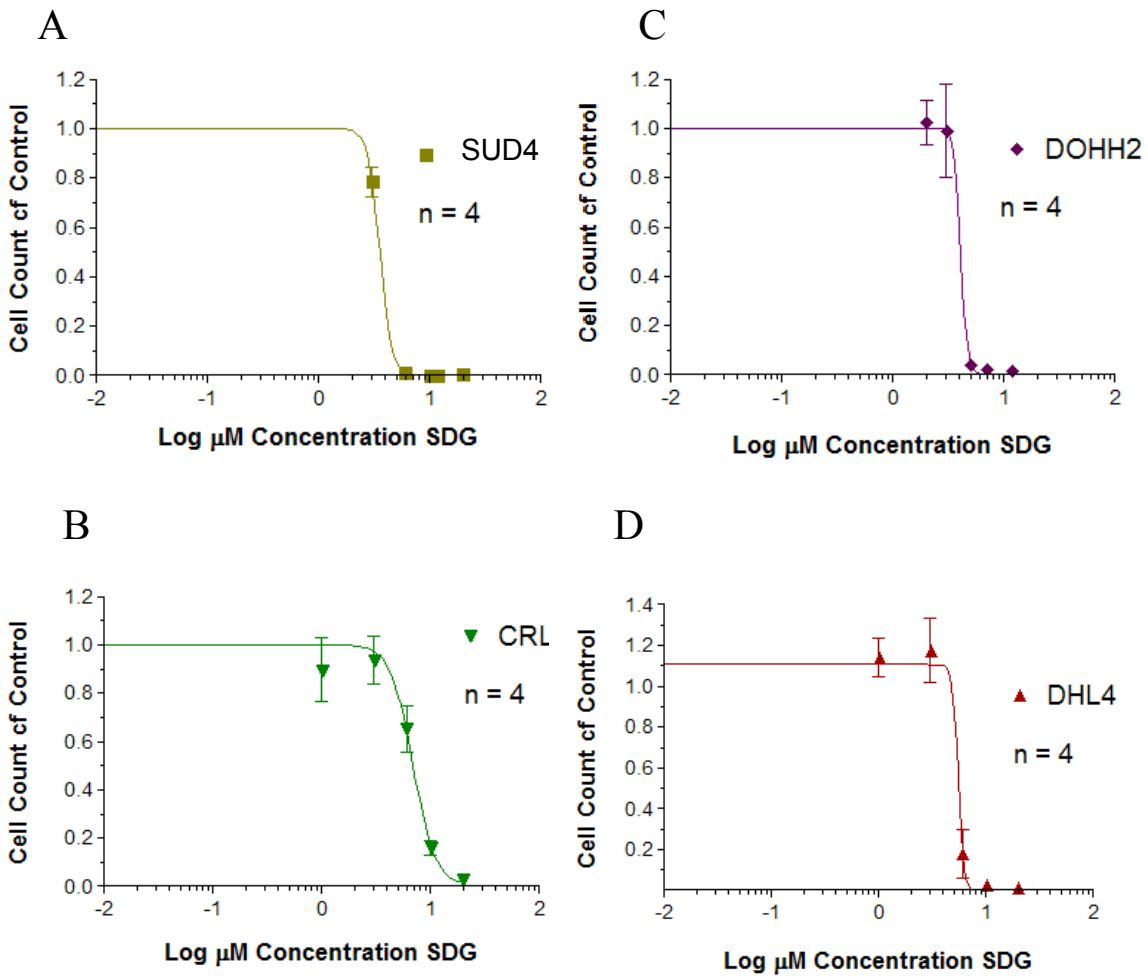


Figure 4.1 Cell-line Cell Count after 3-day exposure to SDG

After 3-day exposure to SDG the cell count of the cell-lines was assessed by trypan blue exclusion assay. The mean cell count (n = 4) is shown as a percentage relative to the no treatment control cells for each experiment. A concentration-response curve has been fitted using non-linear regression analysis. Error bars represent the standard deviation.

A = SUD4 B = CRL C = DoHH2 D = DHL4

SDG = selenodiglutathione

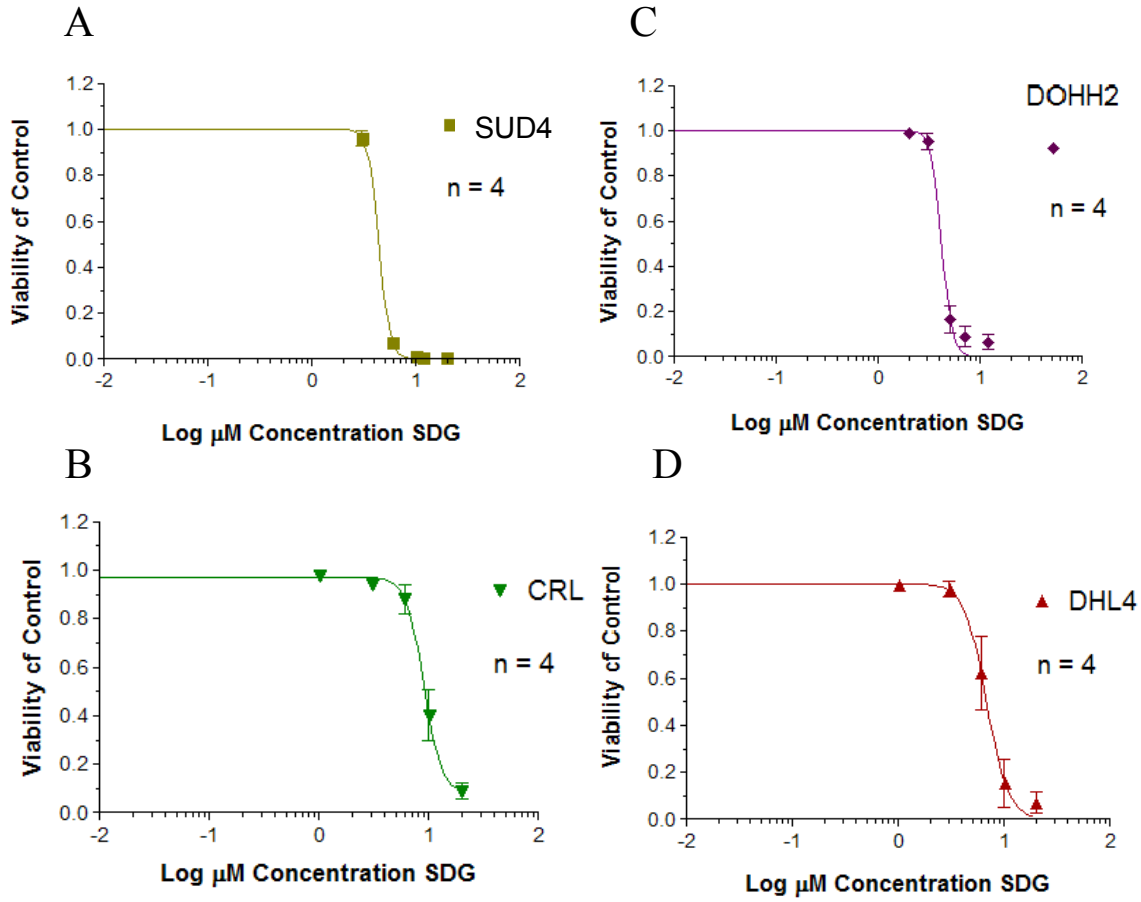


Figure 4.2 Cell-line Viability after 3-day exposure to SDG

After 3-day exposure to SDG the viability of the cell-lines was assessed by trypan blue exclusion assay. The mean viability (n = 4) is shown as a percentage relative to the no treatment control cells for each experiment. A concentration-response curve has been fitted using non-linear regression analysis. Error bars represent the standard deviation.

A = SUD4 B = CRL C = DoHH2 D = DHL4

SDG = selenodiglutathione.

SDG Cell Count	EC_{50} μM	95% CI μM
SUD4	3.5	3.3 to 3.7
CRL	6.9	6.3 to 7.5
DoHH2	4.1	3.3 to 5.1
DHL4	5.5	4.2 to 7.4

SDG Viability	EC_{50} μM	95% CI μM
SUD4	4.4	4.3 to 4.5
CRL	9.0	8.5 to 9.6
DoHH2	4.2	4.0 to 4.4
DHL4	6.8	6.3 to 7.3

MSA Cell Count	EC_{50} μM	95% CI μM
SUD4	5.9	5.1 to 6.8
CRL	4.9	4.6 to 5.1
DoHH2	1.0	0.9 to 1.2
DHL4	2.4	2.0 to 2.9

MSA Viability	EC_{50} μM	95% CI μM
SUD4	10.2	9.7 to 10.7
CRL	9.2	8.7 to 9.7
DoHH2	1.9	1.7 to 2.2
DHL4	166	143 to 193

Table 4.1 EC50s for SDG and MSA in DLBCL Cell-lines The 3-day EC_{50} for cell count and viability for SDG and MSA in the cell-lines was calculated from the trypan blue exclusion assay data from a minimum of three experiments using GraphPad prism software. The 95% confidence interval is given.
SDG = selenodiglutathione, MSA = methylseleninic acid and CI = Confidence Interval

4.4.2 Cell Count and Viability in DLBCL Cell-lines after 3-day exposure to MSA

MSA caused cytostasis in the low micromolar range in all four cell-lines. In terms of cytotoxicity DoHH2 was more sensitive than SUD4 and CRL with 3-day EC_{50}s for viability also in the low micromolar range. DHL4, however, was much less sensitive to the cytotoxic effects of MSA, with a 3-day viability EC_{50} of 166 μM. Low concentration MSA did not cause a potentiation of cell number in any of the four cell-lines. Cell count and viability of the DLBCL cell-lines SUD4, CRL, DoHH2 and DHL4 following 3-day exposure to SDG are illustrated in Figures 4.3 and 4.4, the EC_{50}s are displayed in Table 4.1 and the raw data for all four cell-lines in Appendix Tables A4.9 through A4.16.

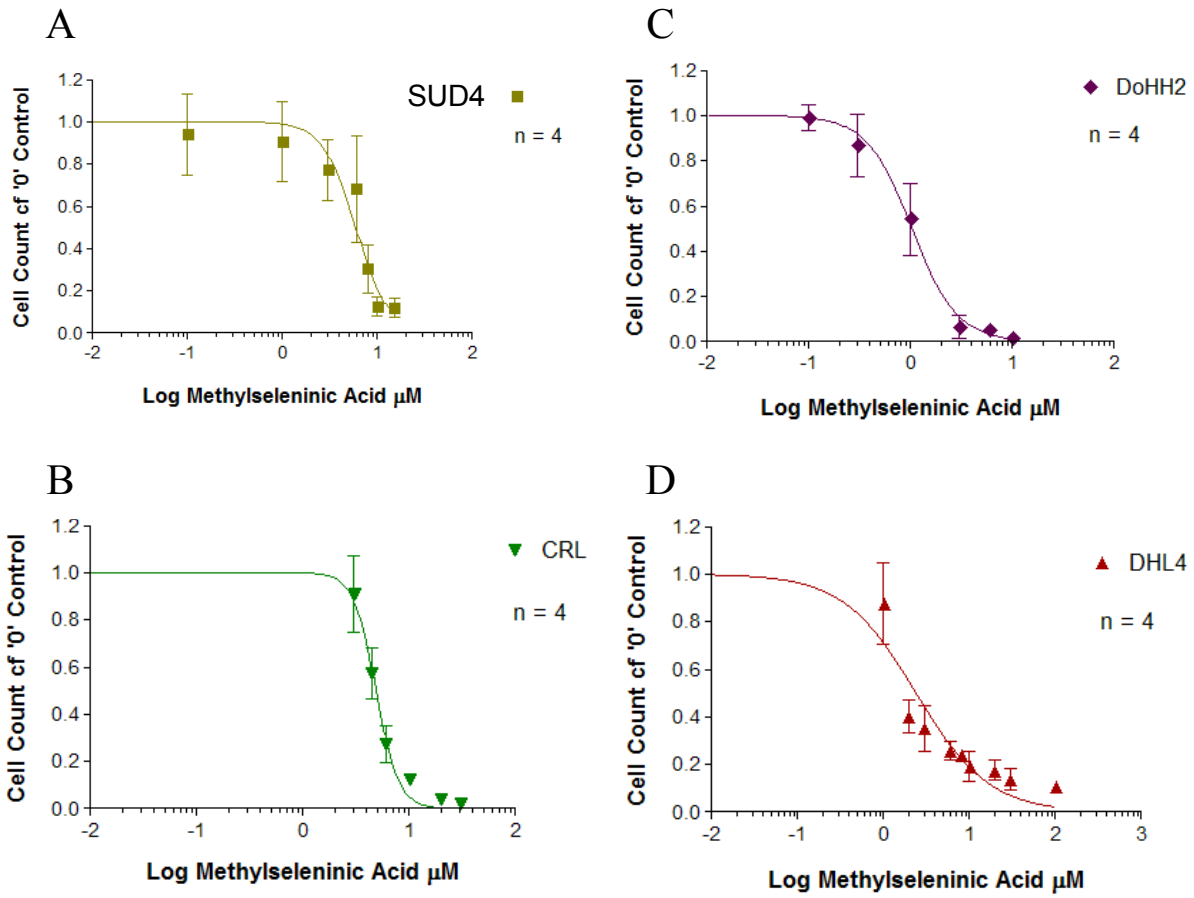


Figure 4.3 Cell-line Cell Count after 3-day exposure to MSA

After 3-day exposure to MSA the cell count of the cell-lines was assessed by trypan blue exclusion assay. The mean cell count (n = 4) is shown as a percentage relative to the no treatment control cells for each experiment. A concentration-response curve has been fitted using non-linear regression analysis. Error bars represent the standard deviation.

A = SUD4 B = CRL C = DoHH2 D = DHL4

MSA = methylseleninic acid

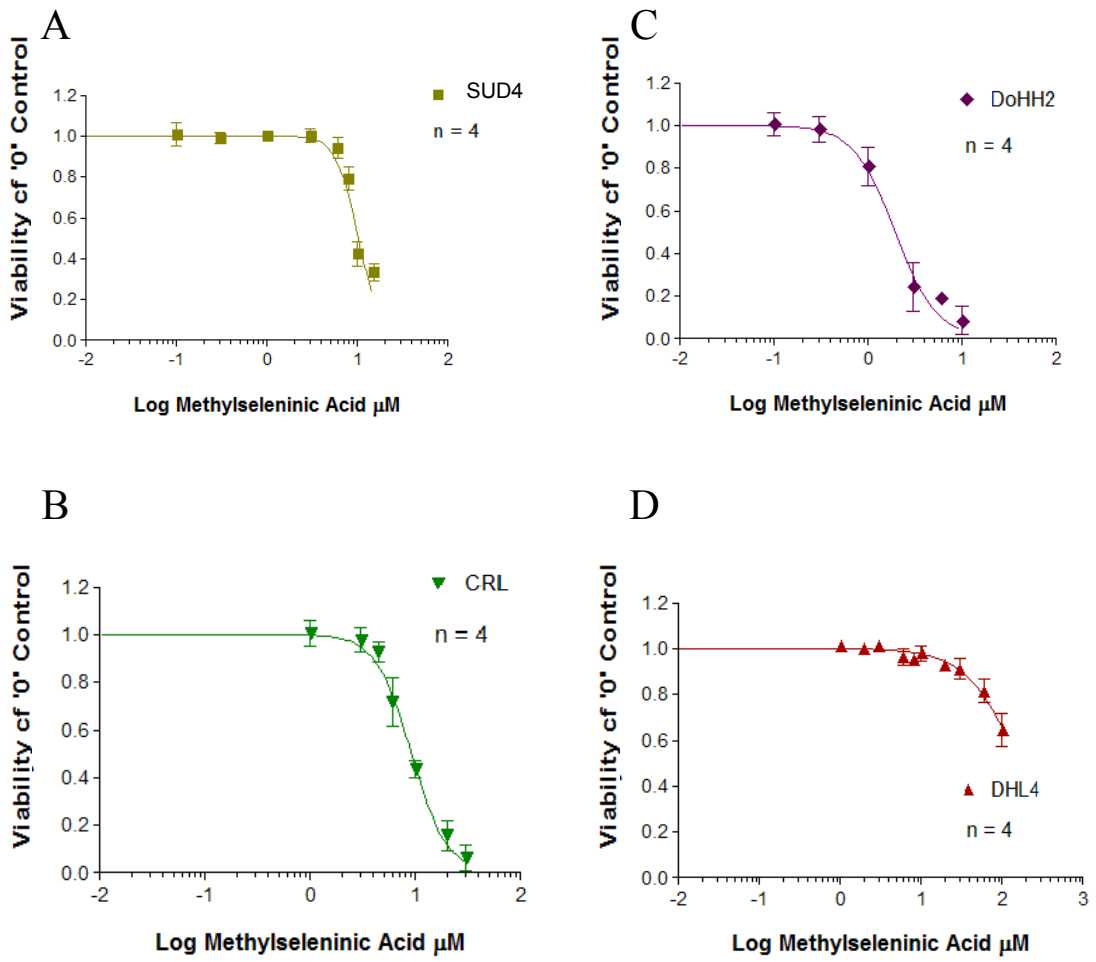


Figure 4.4 Cell-line Viability after 3-day exposure to MSA

After 3-day exposure to MSA the viability of the cell-lines was assessed by trypan blue exclusion assay. The mean viability (n = 4) is shown as a percentage relative to the no treatment control cells for each experiment. A concentration-response curve has been fitted using non-linear regression analysis. Error bars represent the standard deviation.

A = SUD4 B = CRL C = DoHH2 D = DHL4

MSA = methylseleninic.

4.4.3 Cell Count and Viability after 2-day Exposure to SDG in Primary Cell Cultures

SDG caused a concentration-dependent decrease in percentage cell count and viability following 2-day exposure to the primary NHL tumours and the pooled normal B-cell sample, as illustrated in Figures 4.5 and 4.6 and the EC_{50}s in Table 4.2. The raw data are displayed in Appendix Tables A4.17 through A4.26. The effect of SDG on cell count was variable with one of the two MCLs exhibiting sensitivity (03), the CLL/SLL (02) and the other MCL (04) showing relative sensitivity whilst the FL (01) and particularly the pooled normal B-cell sample were much less sensitive. The cytotoxic effect of SDG was more uniform. All four primary tumours had a similar 2-day EC_{50} that ranged between 15 and 28 μM. The viability 2-day EC_{50} for the pooled normal B-cell sample was considerably higher than that of the primary tumours at 109 μM. This result suggests that a therapeutic index may exist between malignant and normal lymphocytes for SDG *in vivo*. One surprising finding was that although SDG was not obviously stimulatory in terms of cell number at low concentration, the EC_{50}s for cell count were higher than the EC_{50}s for viability in 01, 02, 04 and the pooled normal B-cell sample.

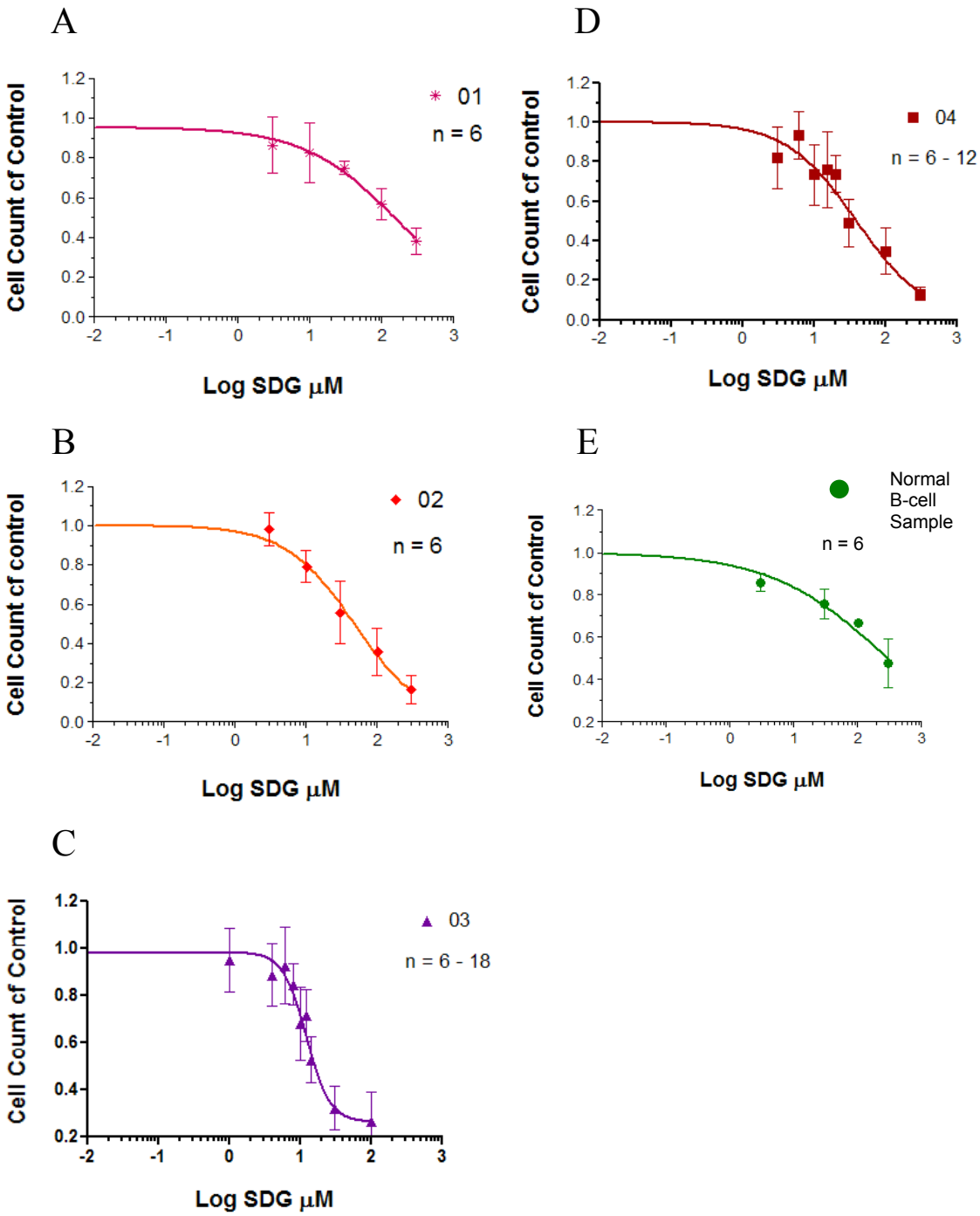


Figure 4.5 Cell Count of Primary Cell Cultures after 2-day exposure to SDG

After 2-day exposure to SDG the cell count of the primary cultures was assessed by trypan blue exclusion assay. The mean cell counts (n = 6-18*) are shown relative to that of the no treatment control cells. A concentration-response curve has been fitted using non-linear regression analysis. Error bars represent the standard deviation. SDG = selenodiglutathione

* additional concentrations were tested for 03 to define better the EC_{50}s due to the very steep concentration-response curve seen with SDG

A = 01 B = 02 C = 03 D = 04 = Pooled normal B-cell sample

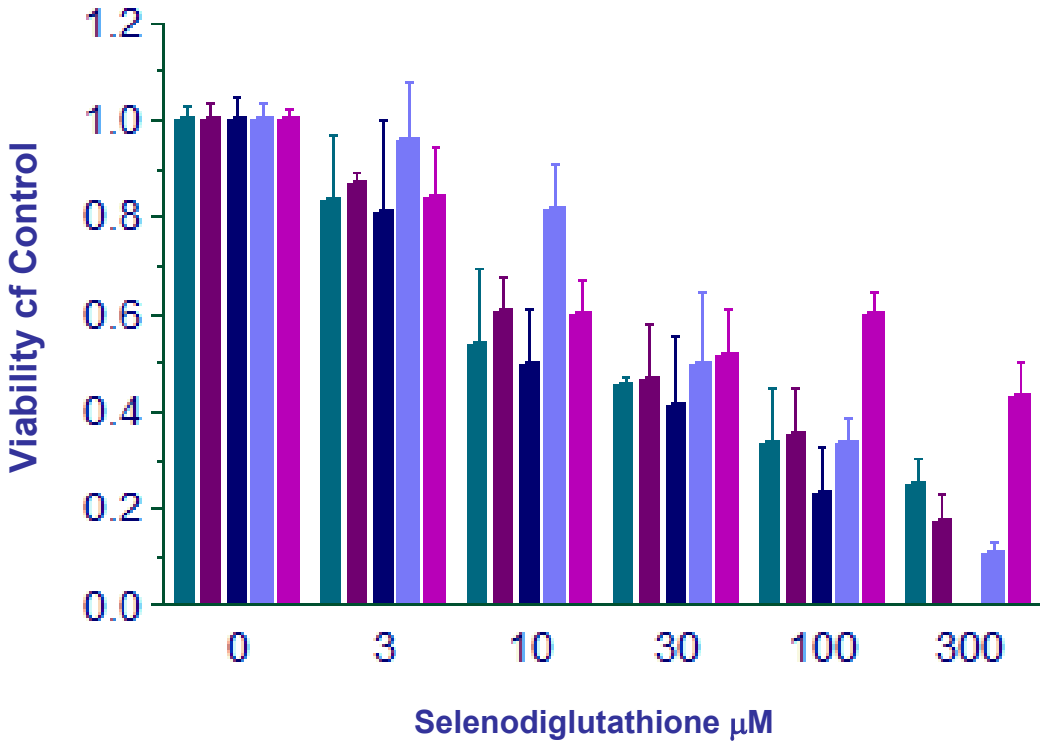


Figure 4.6 Viability of Primary Cell Cultures after 2-day exposure to SDG

After 2-day exposure to SDG the viability of the primary cultures was assessed by trypan blue exclusion assay. The mean viabilities (n = 6-18*) are shown relative to that of the no treatment control cells. Error bars represent the standard deviation. SDG = selenodiglutathione

01: Follicular lymphoma

02: small lymphocytic lymphoma

03: mantle cell lymphoma

04: mantle cell lymphoma

Pooled normal B-cell sample

* Additional concentrations were tested for 03 to define better the EC_{50}s due to the very steep concentration-response curve seen with SDG

SDG Cell Count	EC_{50} μM	95% CI μM
01	170	100 to 300
02	47	36 to 61
03	12	10 to 14
04	39	31 to 49
Normal B-cell Sample	290	150 to 550

SDG Viability	EC_{50} μM	95% CI μM
01	18	11 to 30
02	28	22 to 35
03	15	12 to 20
04	21	11 to 43
Normal B-cell Sample	110	61 to 200

MSA Cell Count	EC_{50} μM	95% CI μM
01	85	41 to 180
02	23	7.1 to 78
03	130	73 to 240
04	46	23 to 94
Normal B-cell Sample	880	240 to 3200

MSA Viability	EC_{50} μM	95% CI μM
01	100	88 to 120
02	68	57 to 82
03	300	150 to 580
04	39	26 to 60
Normal B-cell Sample	1070	480 to 2400

Table 4.2 EC_{50}s for SDG and MSA in NHL Primary Cell Cultures
The 2-day EC_{50} for cell count and viability for SDG and MSA in the primary culture samples was calculated from the trypan blue exclusion assay data using GraphPad prism software. The 95% confidence interval is given. SDG = selenodiglutathione, MSA = methylseleninic acid and CI = confidence interval

4.4.4 Cell Count and Viability after 2-day Exposure to MSA in Primary Cell Cultures

MSA proved to be less efficacious in the primary tumours than in the NHL cell-lines (Figures 4.7 and 4.8, EC_{50}s in Table 4.2 and raw data in Appendix Tables A4.27 through A4.36.). Two primary tumours, 01 (FL) and 03 (MCL) were poorly sensitive to both the cytostatic and cytotoxic effects of MSA, 02 (CLL/SLL) displayed relative cytostatic sensitivity with a 2-day cell count EC_{50} of 23 μM but was less sensitive to the cytotoxic actions of MSA with a 2-day viability EC_{50} of 68 μM. The final primary tumour 04 (MCL) exhibited relative cytostatic and cytotoxic sensitivity but at a level of MSA not achievable *in vivo* (2-day cell count and viability EC_{50}s of 46 and 39 μM respectively). Of concern, MSA stimulated proliferation at low concentration in the two resistant primary tumours (01 and 03), which raises the possibility of a similar effect *in vivo*. In contrast, one encouraging observation was that the pooled normal B-cell sample

was resistant to MSA in terms of both 2-day cell count and viability EC_{50}s, which were 878 and 1071 µM respectively.

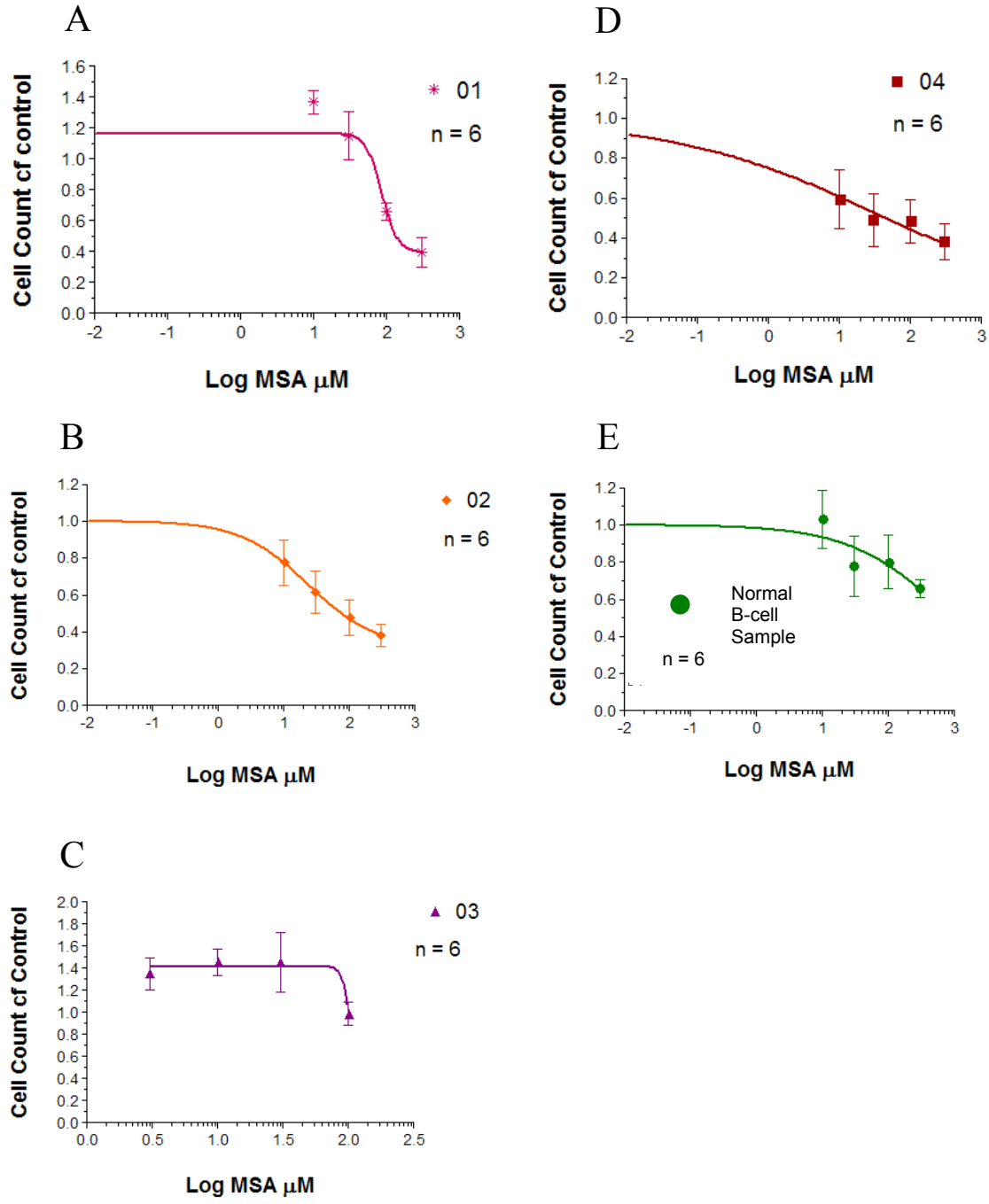


Figure 4.7 Cell Count of Primary Cell Cultures after 2-day exposure to MSA
After 2-day exposure to MSA the cell count of the primary cultures was assessed by trypan blue exclusion assay. The mean cell counts (n = 6) are shown relative to that of the no treatment control cells. A concentration-response curve has been fitted using non-linear regression analysis. Error bars represent the standard deviation. MSA = methylseleninic acid

A = 01 B = 02 C = 03 D = 04 = Pooled normal B-cell sample

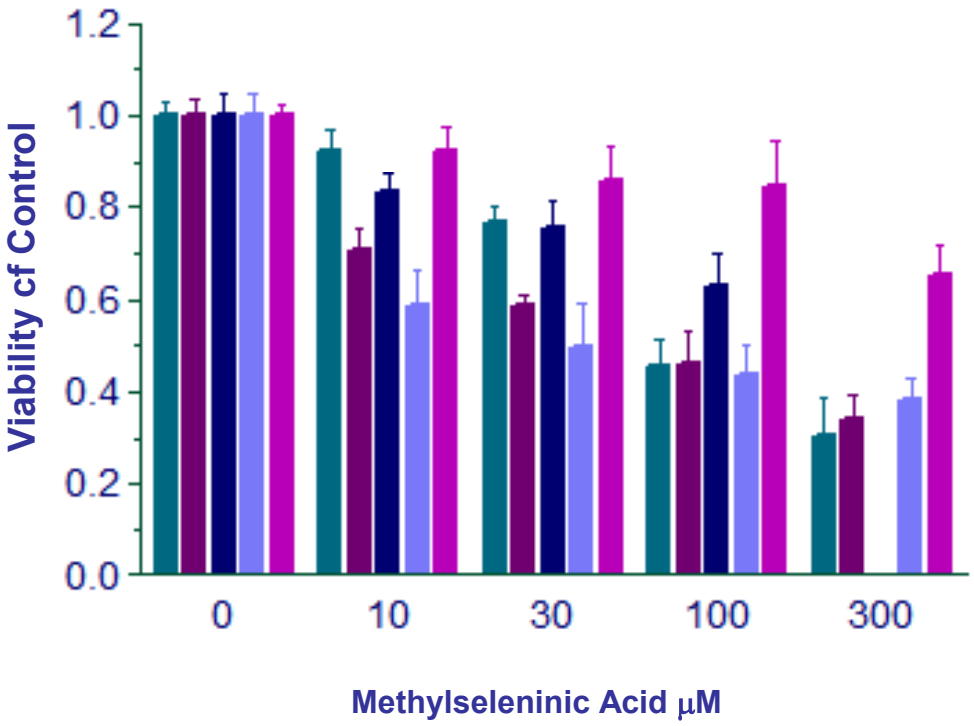


Figure 4.8 Viability of Primary Cell Cultures after 2-day exposure to MSA
After 2-day exposure to MSA the viability of the primary cultures was assessed by trypan blue exclusion assay. The mean viabilities (n = 6) are shown relative to that of the no treatment control cells. Error bars represent the standard deviation. MSA = methylseleninic acid

01: Follicular lymphoma
02: small lymphocytic lymphoma
03: mantle cell lymphoma
04: mantle cell lymphoma
Pooled normal B-cell sample

4.4.5 Effect of SDG and MSA on Cell Cycle Distribution

The effects of SDG and MSA on cell cycle distribution and any induction of a sub-G1 (apoptotic) fraction were assessed by flow cytometry of PI stained cells in all four cell-lines. The data revealed that increasing concentrations of SDG and MSA caused an emptying of the G1 fraction without an obvious phase-specific cell cycle block (Figures 4.9 and 4.10 for MSA; Figures 4.11 and 4.12 for SDG; and raw data in Appendix Tables A4.37 and A4.38).

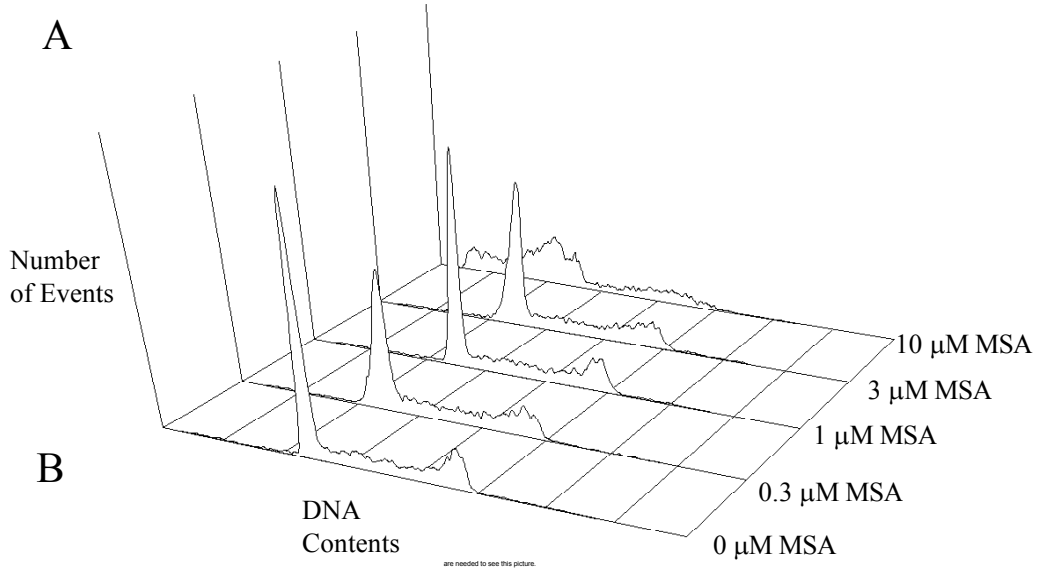


Figure 4.9 Cell Cycle Distribution Changes after 3-day Exposure of SUD4 and CRL to MSA Changes in cell cycle distribution were assessed by FACS analysis following propidium iodide staining. Cells were continuously exposed to increasing concentrations of MSA for three days. No clear arrest in a specific phase of the cell cycle was seen in either cell-line. Apoptotic cells (sub-G1, where low molecular weight, fragmented DNA is extracted during cell fixation) were apparent in SUD4 (A) AND CRL (B) cells.

SDG and MSA produced a concentration-dependent increase in the sub-G1 population in all four cell-lines, with the exception of MSA and DHL4 where the apoptotic fraction barely changed compared to untreated cells.

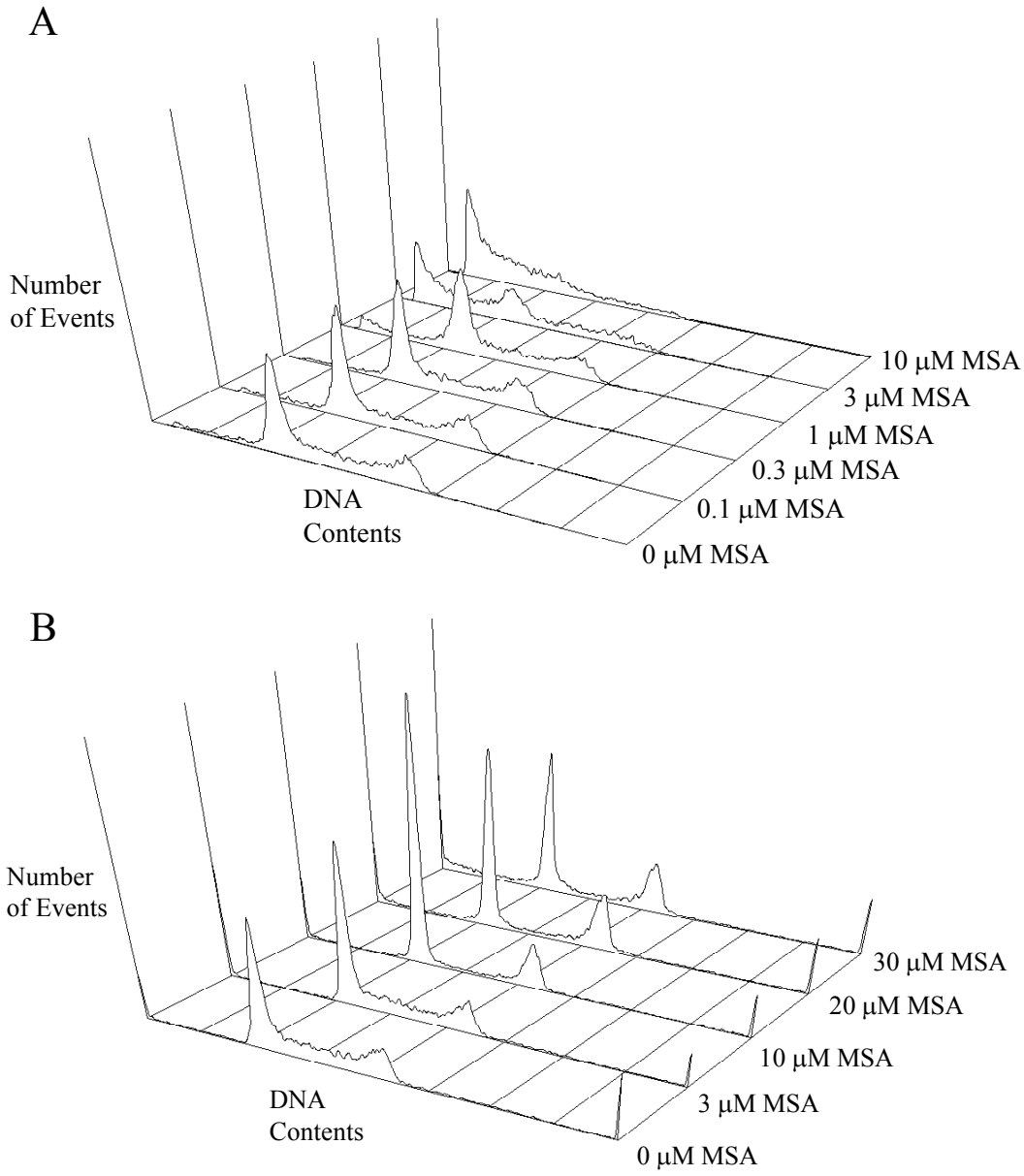


Figure 4.10 Cell Cycle Distribution Changes after 3-day Exposure of DoHH2 and DHL4 to MSA Changes in cell cycle distribution were assessed by FACS analysis following propidium iodide staining. Cells were continuously exposed to increasing concentrations of MSA for three days. No clear arrest in a specific phase of the cell cycle was seen in either cell-line. Apoptotic cells (sub-G1) were apparent in the sensitive DoHH2 (A) cells but not so in the markedly less sensitive DHL4 (B) cells.

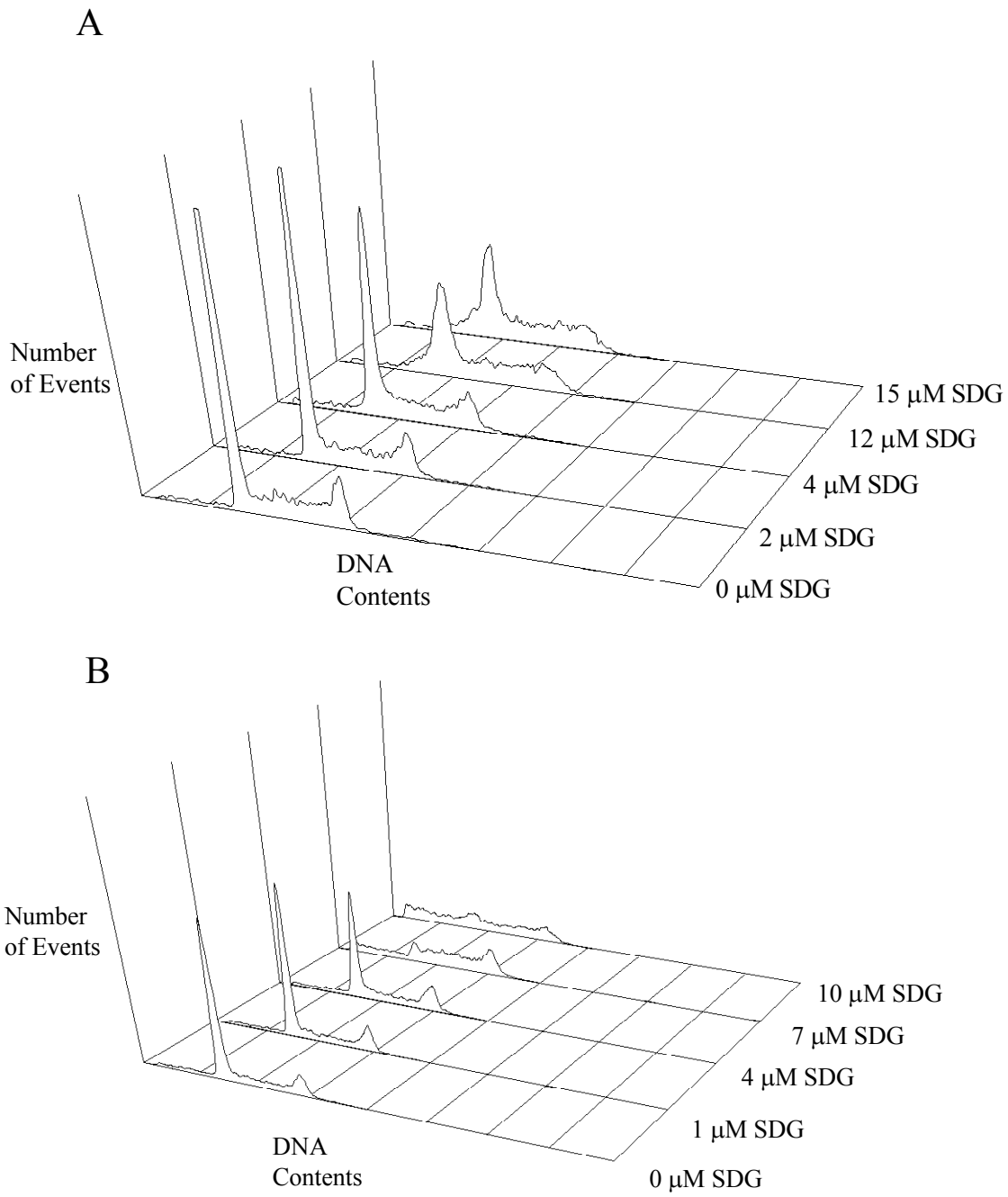


Figure 4.11 Cell Cycle Distribution Changes after 3-day Exposure of SUD4 and CRL to SDG Changes in cell cycle distribution were assessed by FACS analysis following propidium iodide staining. Cells were continuously exposed to increasing concentrations of SDG for three days. No clear arrest in a specific phase of the cell cycle was seen in either cell-line. Apoptotic cells (sub-G1) were apparent in both SUD4 (A) and CRL (B) cells.

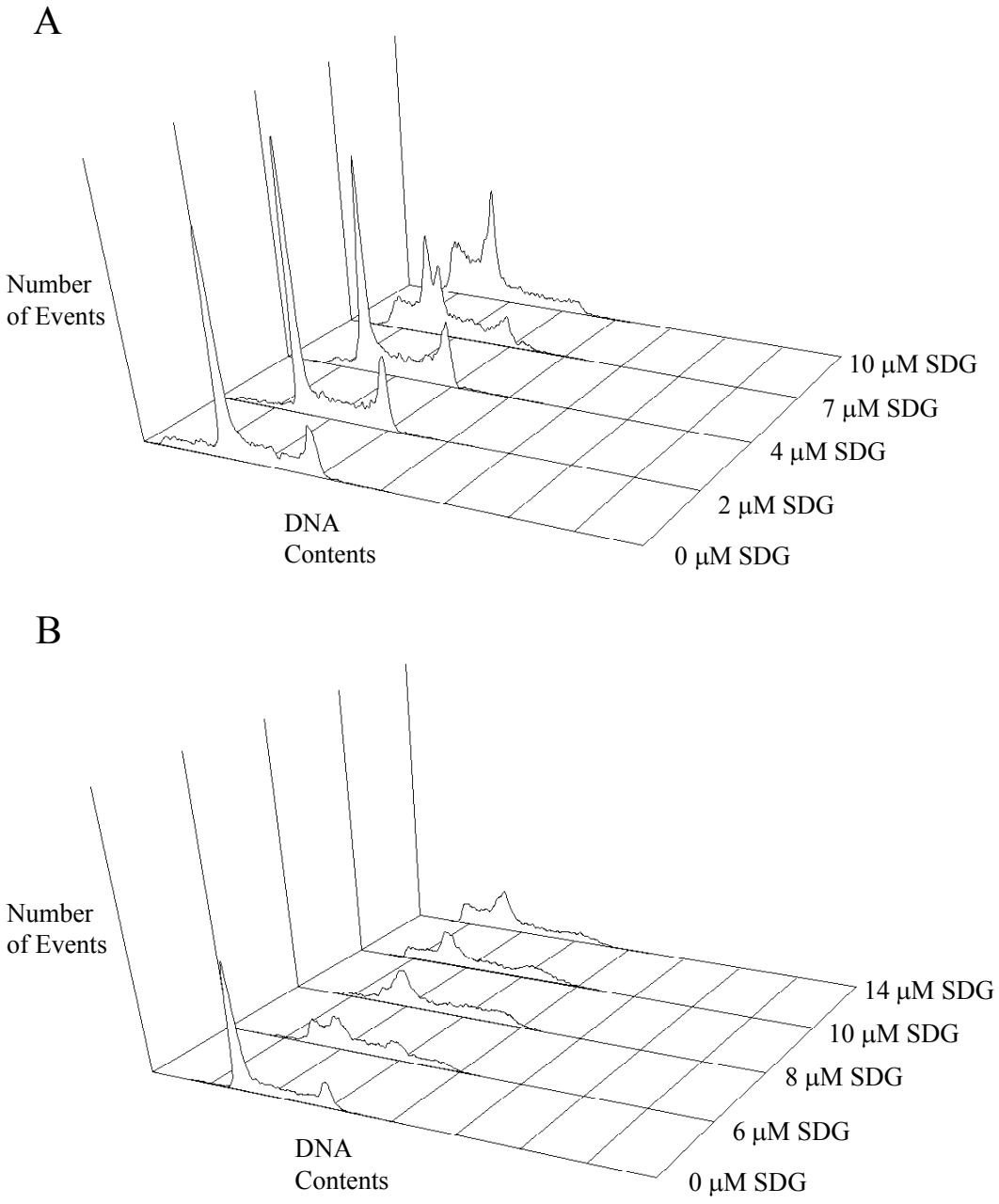


Figure 4.12 Cell Cycle Distribution Changes after 3-day Exposure of DoHH2 and DLH4 to SDG Changes in cell cycle distribution were assessed by FACS analysis following propidium iodide staining. Cells were continuously exposed to increasing concentrations of SDG for three days. No clear arrest in a specific phase of the cell cycle was seen in either cell-line. Apoptotic cells (sub-G1) were apparent in DoHH2 (A) and DHL4 (B) cells.

The effect of SDG on cell cycle phase distribution and induction of a sub-G1 population of cells was studied in two of the primary lymphoma samples, (03) and (04). In both, apoptosis was induced (as shown by the increase in sub-G1 cell fraction) without arrest during a specific phase of the cell cycle (Figure 4.13 and Appendix Table A4.39).

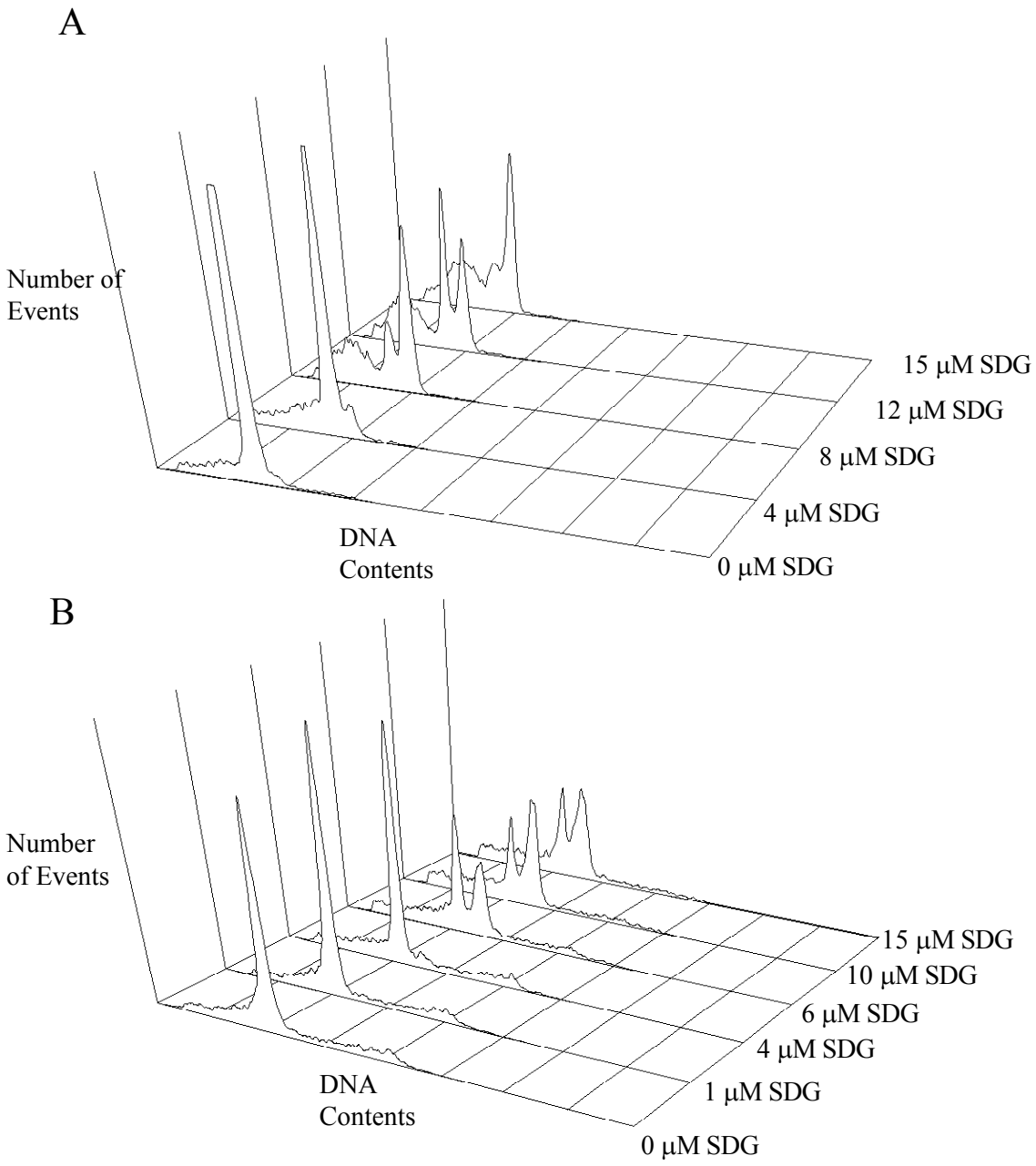


Figure 4.13 Cell Cycle Distribution Changes for Primary MCLs (03) and (04) after 2-day Exposure to SDG Changes in cell cycle distribution were assessed by FACS analysis following propidium iodide staining. Cells were continuously exposed to increasing concentrations of SDG for two days. No clear arrest in a specific phase of the cell cycle was seen in either primary MCL sample. Apoptotic cells (sub-G1) were apparent in both (03) and (04).

Investigation of the effect of 2-day MSA and SDG exposure on cell cycle phase distribution and induction of a sub-G1 fraction was undertaken in the pooled normal B-cell sample. Neither MSA nor SDG produced a cell cycle arrest or significant apoptosis induction (as shown by the lack of increase in sub-G1 cell fraction) (Figure 4.14 and Appendix Table A4.39).

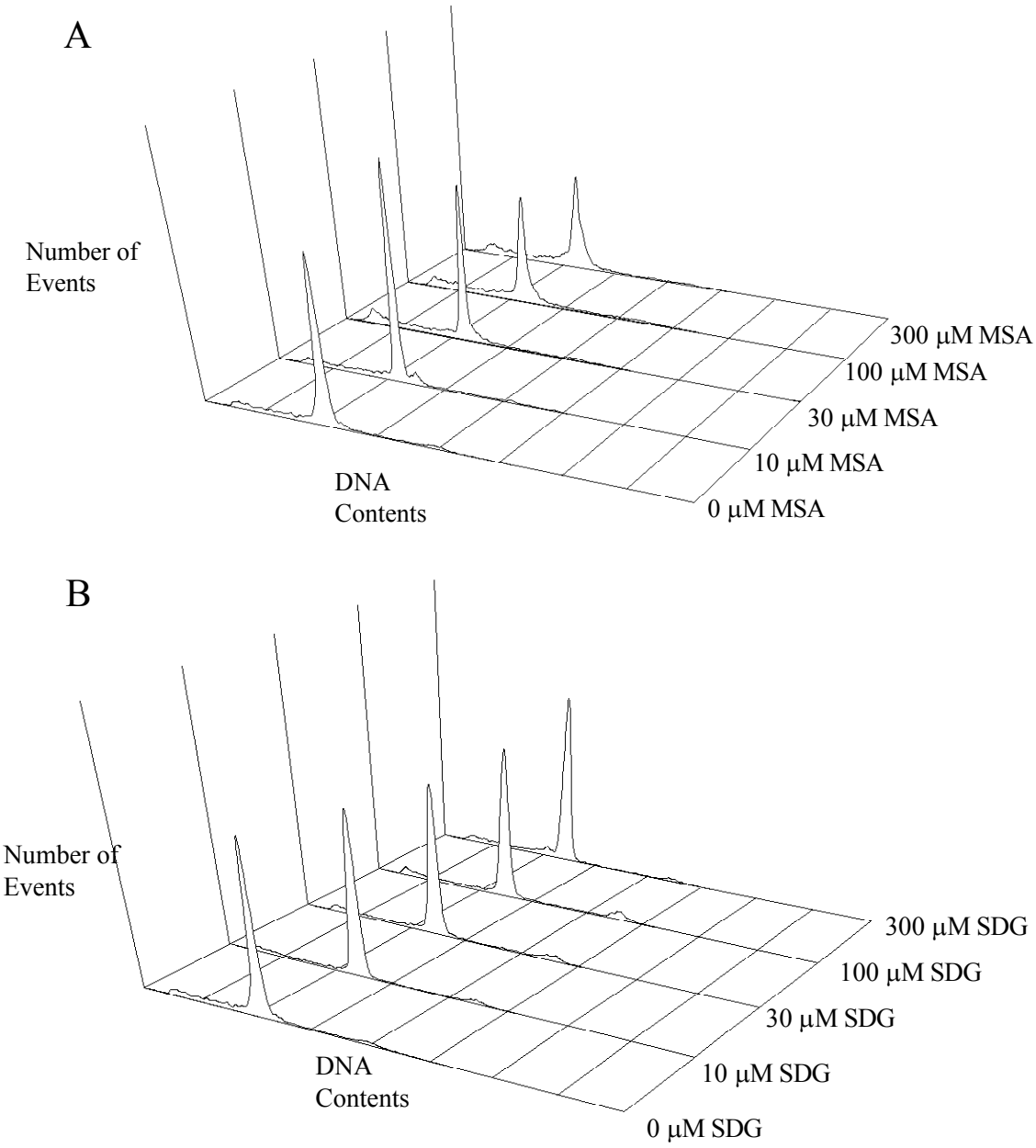


Figure 4.14 Cell Cycle Distribution Changes after 2-day exposure of the Pooled Normal B-cell Sample to SDG Changes in cell cycle distribution were assessed by FACS analysis following propidium iodide staining. Cells were continuously exposed to increasing concentrations of MSA (A) or SDG (B) for two days. No clear arrest in a specific phase of the cell cycle was seen. Apoptotic cells (sub-G1) were barely apparent for MSA (A) or SDG (B).

4.4.6 Apoptotic Cell Death Induction by SDG and MSA

FACS analysis of PI- and annexin V-stained cells, treated for three days at the viability EC_{50} for SDG or MSA, was performed to explore the nature of the cell death induced by both agents. A shift in cells from the lower left to the lower right quadrant was followed by a shift to the upper right quadrant. This transition from staining by annexin V to staining by both annexin V and PI indicates that cell death was by apoptosis, as illustrated in Figure 4.15.

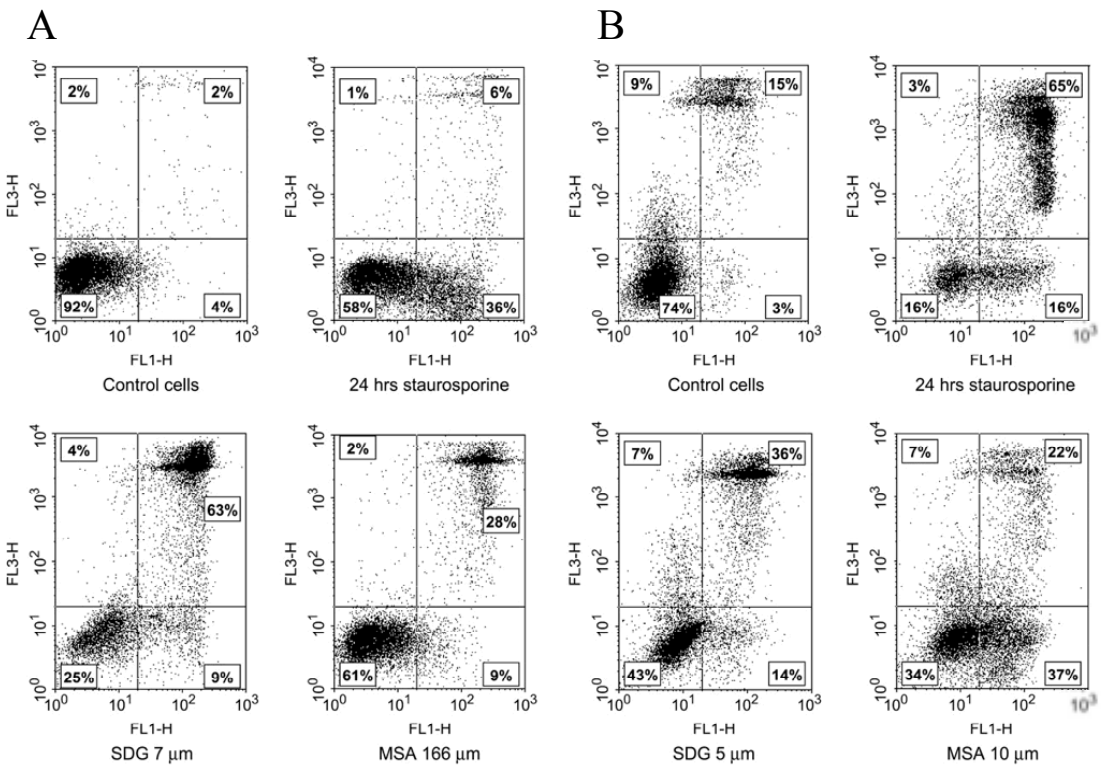


Figure 4.15 Annexin V and Propidium Iodide Staining after 3-day Exposures to the cytotoxic EC_{50} concentrations of SDG or MSA

Dual labelling with propidium iodide (FL3-H) and annexin V (FL1-H) in cells treated with the positive control staurosporine (24 hours), or the cytotoxic EC_{50} concentrations of SDG or MSA for 3 days (percentage of cells in each quadrant). Cell death with SDG and MSA was associated with increased signal from both labels, confirming cell death by apoptosis.

A = DHL4 B = SUD4 SDG = selenodiglutathione MSA = methylseleninic acid

Confirmation and elaboration of this finding for MSA and SDG was produced by the western blot results for caspase-8, caspase-9 and PARP, illustrated in Figures 4.16 and 4.17. Both SDG and MSA led to cleavage of full length PARP (116 kDa) to its 85 kDa product and to smaller products as the concentrations of MSA and SDG were increased further.

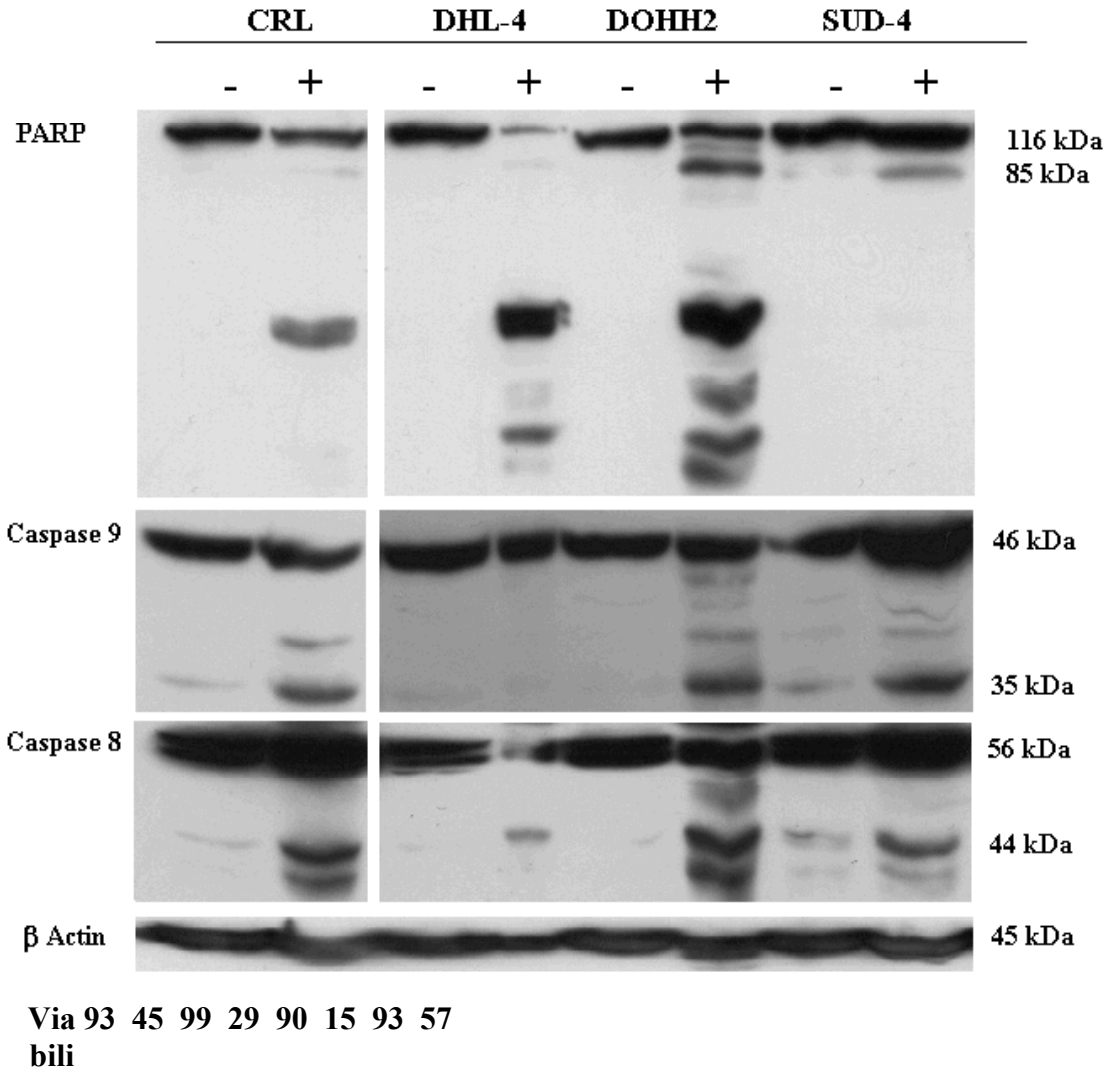


Figure 4.16 Effect of 3-day Exposure to SDG EC_{50} Viability on PARP, Caspase-8 and Caspase-9 Cleavage in the DLBCL Cell-lines The actual percentage viability relative to untreated cells for each of the cell-lines in the experiment is given below the respective lane.
- = untreated controls; + = cell-line exposed to EC_{50} viability SDG; β actin = positive control

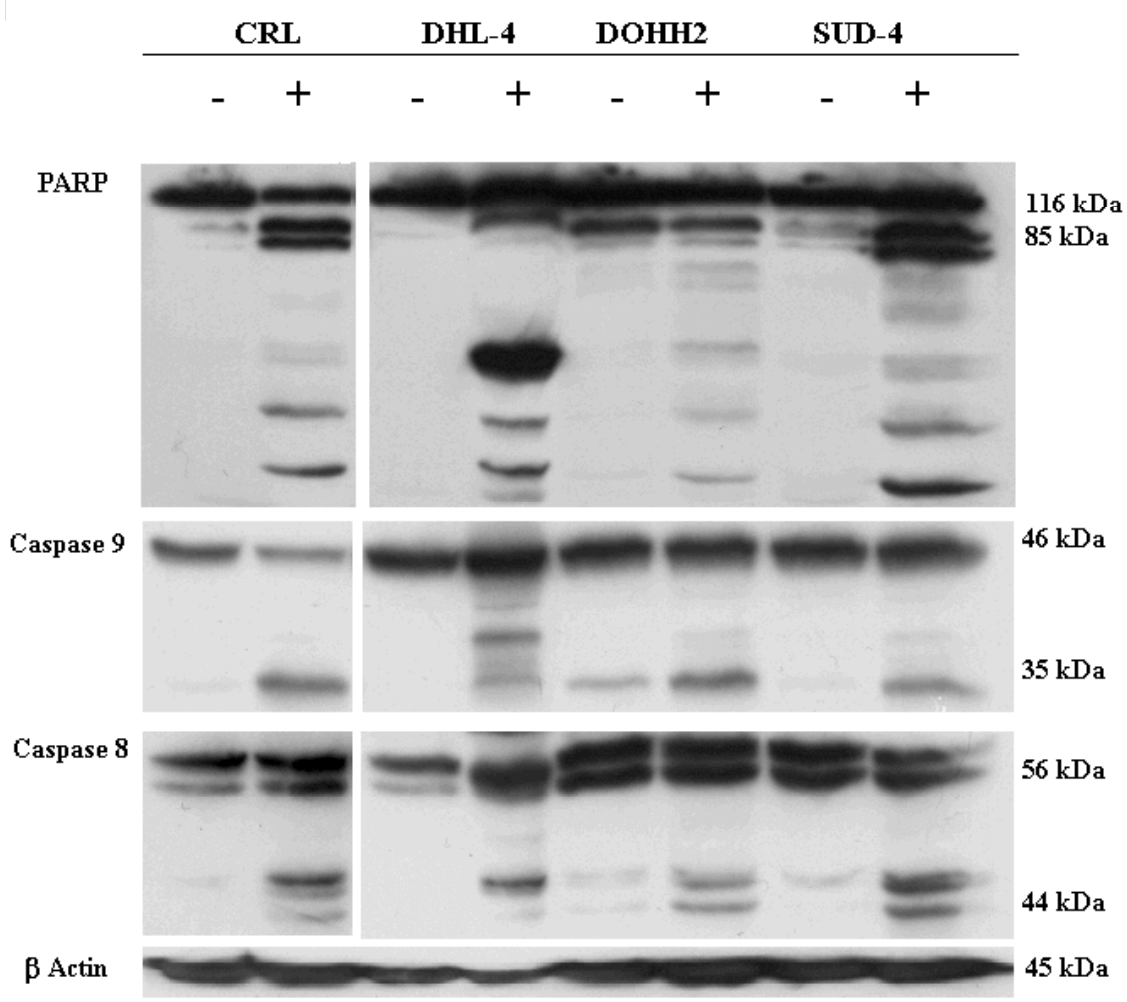


Figure 4.17 Effect of 3-day Exposure to MSA EC_{50} Viability on PARP, Caspase-8 and Caspase-9 Cleavage in the DLBCL Cell-lines The actual percentage viability relative to untreated cells for each of the cell-lines in the experiment is given below the respective lane.
- = untreated controls; + = cell-line exposed to EC_{50} viability SDG; β actin = positive control

PARP cleavage was associated with cleavage of the full length caspase-8 (56 kDa) and caspase-9 (46.5 kDa) for cells incubated with SDG or MSA. These results suggest that SDG and MSA induced apoptosis through activation of both the intrinsic and the extrinsic apoptotic pathways leading to caspase-3 activation and PARP cleavage. The only exception was DHL4 following exposure to SDG, where only cleavage of caspase-8, and not caspase-9, was demonstrated.

4.4.7 Evidence of ROS generation by SDG and MSA

The production of ROS by the DLBCL cell-lines was assessed using DCF-DA, which generates a fluorescent product in the presence of ROS. The generation of ROS varied between the cell-lines following 30-minute exposure to 25 μM H_2O_2. Compared to no treatment controls, H_20_2 did not result in a significant change in DCF-DA fluorescence in DoHH2 and DHL4, whilst ROS increased significantly in CRL and SUD4 (Figure 4.18 and Appendix Table A4.40). In DHL4, CRL and SUD4 30-minute exposure to the 3-day viability EC_{50} concentration of SDG generated significant increases in ROS compared with no treatment controls and greater than the H_2O_2 positive control. For MSA, 30-minute exposure to the respective 3-day viability EC_{50} concentration did not produce ROS in CRL, DoHH2 or DHL4 and showed only a minor but significant rise in SUD4 to a level less than that achieved using 25 μM H_2O_2.

Next the effect of ROS on the cytotoxic activity of SDG or MSA was investigated by 3-day incubation of SUD4, CRL, DoHH2 and DHL4 with the glutathione precursor NAC alone or with the 3-day viability EC_{50} concentration of SDG or MSA in the presence or absence of NAC, as depicted in Figure 4.19 and Appendix Table A4.41. As expected, NAC alone had no effect on cell viability and NAC added to MSA had no effect on the activity of MSA in any of the cell-lines studied. Similarly, in DoHH2, in which SDG did not generate ROS, the addition of NAC had no effect on the activity of SDG. In contrast, the cytotoxic activity of SDG was dramatically reduced by NAC in DHL4 and SUD4 cells, in which SDG resulted in significant increases in ROS. Indeed in SUD4, where the EC_{50} viability concentration of SDG resulted in a 450% increase in ROS compared to no treatment controls (Figure 4.18), 30-minute co-exposure with NAC almost abolished the cell kill activity of SDG (Figure 4.19). In CRL, where exposure to H_20_2 or SDG produced ROS, the cell-kill activity of SDG was unaffected by co-incubation of CRL with NAC and SDG. This surprising result confirms that SDG can produce its *in vitro* cytotoxicity via ROS dependent and independent mechanisms.

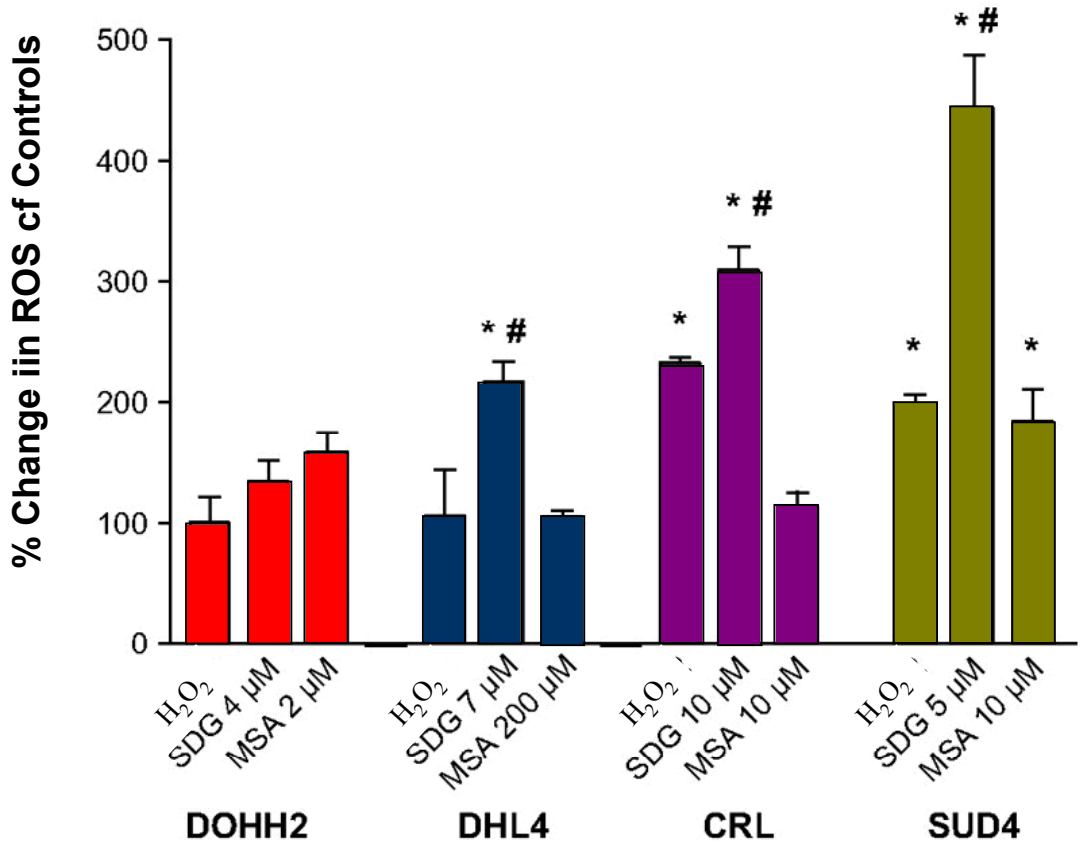


Figure 4.18 ROS Generation after 30-minute Exposures to the Cytotoxic EC_{50} Concentrations of SDG or MSA The generation of ROS in lymphoma cells relative to control (0.75% DMSO) after exposure to selenium compounds for 30 minutes at the 3-day cytotoxic EC_{50} concentration of SDG or MSA. 25 µM H_2O_2 was used as the positive control.
SDG = selenodiglutathione MSA = methylseleninic acid
* significantly different compare to no treatment controls # SDG significantly different to MSA

= DoHH2 = DHL4 = CRL = SUD4

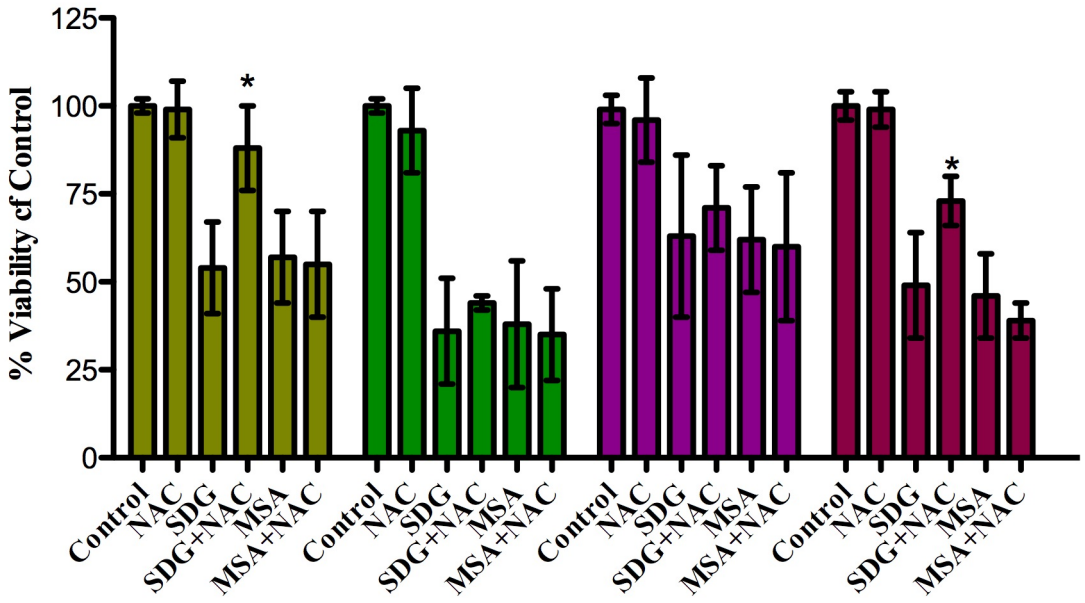


Figure 4.19 Effect of N-acetyl cysteine on the Cytotoxicity of SDG and MSA after 3-day Exposures to the cytotoxic EC_{50} Concentrations of SDG or MSA Cells were continuously co-exposed to EC_{50} viability concentrations of SDG or MSA ± N-acetyl cysteine (NAC) or N-acetyl cysteine alone. SDG concentration: SUD4, CRL, DoHH2, DHL4 and cells = 4.0, 9.0, 4.2 and 7.0 µM, respectively; MSA concentration SUD4, CRL DoHH2 and DHL4 cells 10.0, 9.0, 1.9 and 166 µM, respectively, N-acetyl cysteine 2.5 µM throughout. * P = 0.0008 and 0.0053 respectively for SUD4 and DHL4 SDG vs. SDG + NAC.

A = SUD4 B = CRL C = DoHH2 D = DHL4

SDG = selenodiglutathione MSA = methylseleninic acid NAC = N-acetyl cysteine

4.5 Discussion

4.5.1 Review of Experiment findings in Comparison with Published Data

Cytostatic and Cytotoxic Activity of SDG and MSA

Following the observation that presentation serum selenium was an independent predictor of response to treatment and OS in patients with aggressive NHL, the activity of specific selenium compounds has now been studied for the first time in a panel of DLBCL cell-lines and in a primary lymphoma culture system. Dietary forms of Se, such as selenomethionine, require activation by ß-lyase, typically in the liver, so have little *in vitro* activity. Activated selenium species were therefore used in these studies, namely MSA, which is rapidly converted to the highly reactive methylselenol, and SDG (an

active metabolite of the naturally occurring sodium selenite), which is activated further to hydrogen selenide with the generation of ROS.

Both SDG and MSA demonstrated cytostatic and cytotoxic activity in DLBCL cell-lines and NHL primary tumours. Relative cytotoxicity insensitivity was only encountered for MSA in DHL4. Both SDG and MSA induced cytostasis and cytotoxicity in a concentration and time-dependent manner in the lymphoma cell-lines studied. The MSA cell count EC_{50} (1–7 μM) or percentage viability EC_{50} (2–10 μM; DHL4 166 μM) is in the range reported by others using human cancer cell-lines of epithelial origin [15–17], and the activity of SDG is in keeping with that found in primary cultures of oral carcinomas by Ghose *et al* (Ghose *et al.*, 2001). It is noteworthy that the concentration effect curves were much steeper for SDG than for MSA, most notably so in DoHH2 and SUD4 cells (sigmoid factor for DoHH2 and SUD4 cells –16.2 and –8.3, respectively, for cell number and –10.1 and –8.4, respectively, for percentage viability). This suggests a threshold concentration, above which the effect increases dramatically.

SDG and MSA Activity is Independent of p53 Status

The activity of SDG and MSA proved independent of *TP53* status in the cell-lines studied, as three of the four (SUD4, DHL4 and CRL) are *TP53* homozygous mutated and produce non-functional p53 and the fourth cell-line (DoHH2) is *TP53* heterozygous ((Strauss *et al.*, 2007); Richard Camplejohn, personal communication). The results set out in this Chapter confirm previous reports that although SDG induces functional p53, this is in response to DNA damage and is not required for the induction of apoptosis (Lanfear *et al.*, 1994).

Cell cycle effects of Se Compounds

Both drugs inhibited all phases of the cell cycle with an associated increase in the sub-G1 (apoptotic) fraction and induced caspase dependent apoptosis. In terms of the effect of MSA upon cell cycle, Zhao *et al* found a similar effect to that described in this Chapter, where all phases of the cell cycle were retarded. Zhao *et al* went on to show that cell cycle promoters were inhibited and cell cycle inhibitors were enhanced in all phase of the cell cycle by MSA (Zhao *et al.*, 2004). Similarly, in the human prostate cancer cell-line PC3, MSA retarded cell cycle progression at multiple transition points without changing the proportion of cells in different phases of the cell cycle (Dong *et al.*, 2003). In contrast, in the premalignant human breast epithelial cell-lines

MCF10AT1 and MCF10AT3B MSA blocked cell cycle progression at the G0/G1 phase of the cell cycle (Dong *et al.*, 2002).

There are no other reports beyond that presented here of the effect of SDG on the cell cycle progression/arrest. A single paper describes sodium selenite inducing an S-phase block in the human prostate cancer cell-line DU145 (Jiang *et al.*, 2001).

ROS generation by SDG and MSA

ROS generation was critical to the cytotoxic activity of SDG in two out of the four cell-lines examined but not for the activity of MSA. The ROS and NAC data detailed above also demonstrated that the cytotoxicity of SDG can be independent of ROS as the compound was active in DoHH2 cells in the absence ROS generation, and in the presence of NAC and in CRL, which generated ROS on exposure to SDG yet was still killed by SDG in the presence of NAC. The report in this Chapter of the antioxidant NAC, a precursor of glutathione, preventing the cytotoxic activity of SDG in DHL4 and SUD4 supports the results of Fleming *et al*, who found NAC blocked the activity of SDG and p-XSC in human oral cancer cells (Fleming *et al.*, 2001). Similarly, the actions of p-XSC were blocked by NAC and the putative hydroxyl scavengers mannitol and pyrrolidine, confirming that the activity of p-XSC is mediated, in part at least, through the generation of superoxide (Fleming *et al.*, 2001). In keeping with these results, others have shown ROS are produced by selenite and selenocystine treatment of breast cancer or hepatoma cells *in vitro* upon glutathione reduction (Yan and Spallholz, 1993, Shen *et al.*, 2002).

The production of ROS by SDG but not the positive control (H_2O_2) in DHL4 cells suggests the primary ROS generated in DHL4 by SDG is superoxide rather than hydrogen peroxide. Fleming *et al* found p-XSC generated superoxide; whilst SDG generated hydrogen peroxide in the oral cancers they studied (Fleming *et al.*, 2001). Similarly, Jiang *et al* found that superoxide rather than hydrogen peroxide generation was critical for the cytostatic and cytotoxic actions of selenite in the DU145 prostate cancer cell-line (Jiang *et al.*, 2001).

The findings detailed in this Chapter indicate that SDG exerts its antiproliferative and cytotoxic activity by ROS dependent and independent pathways. SDG can therefore act through both ROS dependent and independent mechanisms.

From the results given in this Chapter it is clear that the actions of MSA do not require the build up of ROS to induce cell death and that addition of NAC to MSA had no

effect on the cytotoxicity of MSA. In contrast, in the human hepatoma cell-line HepG(2) augmentation of intracellular glutathione by addition of NAC resulted in a significant increase in MSA induced apoptosis (Shen *et al.*, 2002). Further investigation showed that intracellular glutathione was rapidly depleted by exposure of HepG(2) to MSA. In contrast, depletion of intracellular glutathione by addition of buthionine sufoximine caused a marked reduction in MSA-related cell death. It is clear that glutathione is involved in the metabolism of many methylated forms of Se to methylselenol as well as being of importance to the efficacy of hydrogen selenide pool members (Shen *et al.*, 2002). These data, generated from a methylselenol pool member, also support the hypothesis that higher intracellular glutathione level present in many tumours compared to their equivalent normal tissues could account for part of the evident therapeutic index for Se compounds in animal studies (Shen *et al.*, 2002).

Primary cell culture activity of SDG and MSA

The cytotoxic activity of SDG and MSA was also studied in primary lymphoma samples from patients who had received multiple previous lines of chemotherapy and in a pooled normal B-cell sample. All primary samples showed a concentration dependent loss of viability after a 2-day exposure to SDG or MSA, with the potentially genotoxic compound SDG showing slightly greater potency than MSA (EC_{50} values 15–28 μM compared to 39–300 μM respectively). Although only one pooled normal B-cell sample was studied, by virtue of it having come from multiple donors it provided a population estimate of the effect of the Se compounds on normal B-cells. It is noteworthy that the EC_{50} concentrations for both SDG and MSA were higher in this pooled normal B-cell sample than for the lymphoma samples (EC_{50} viability SDG 110 μM and MSA 1070 μM). The primary cell culture results presented in this Chapter therefore raise the possibility of a therapeutic index existing for SDG and MSA *in vivo* by virtue of the relative resistance of pooled normal B-cell sample to both agents. This finding is supported by the primary cell cultures results of Fleming *et al*, who studied human normal oral mucosa and oral carcinomas (Ghose *et al.*, 2001). This group found that SDG-induced apoptosis more readily in the malignant cells than their normal counterparts (Ghose *et al.*, 2001). Wan *et al* also found a therapeutic index for p-XSC between normal and cancerous prostate cells *in vitro* (Wan *et al.*, 2003). Taken together these results demonstrate the differential sensitivity of tumour cells and normal cells to SDG and p-XSC from the hydrogen selenide pool and MSA from the methylselenol

precursor pool, suggesting that these agents may be useful as cytotoxics in their own right or as chemotherapy adjuncts, potentiating the activity of established cytotoxics against malignant cells whilst protecting normal cells against cytotoxic damage.

Apoptotic Cell Death Induction by SDG and MSA

Contrary to the statements by Ip *et al* that SDG causes necrotic rather than apoptotic cell death(Ip, 1998), this Chapter details the occurrence of caspase-mediated apoptosis, confirmed by FACS analysis of PI/annexin V stained cells. Lanfear *et al* were also able to demonstrate apoptosis induction by SDG (Lanfear *et al.*, 1994). In keeping with the findings stated in this Chapter, Jiang *et al* found that 5 μM MSA caused caspase-3, -7, -8, and -9 activation and PARP cleavage (Jiang *et al.*, 2001). This group also failed to demonstrate selenite-induced caspase-dependent apoptosis but rather found DNA fragmentation associated with phosphorylation of JNK1/2 and p38/MAPK (Jiang *et al.*, 2001).

Cell Proliferation Promotion by SDG and MSA

One concern raised by some of the data detailed in this Chapter is that low concentration Se supplementation could in fact stimulate rather than inhibit malignant cell proliferation particularly in resistant cells. In DHL4, low concentration SDG increased the cell count compared to no treatment controls and in the primary tumour experiments, the 2-day cell count EC_{50}s for SDG were paradoxically higher than the 2-day viability EC_{50}s in three of the four NHL primary tumours tested and the pooled normal B-cell sample and MSA increased the cell count of two of the four NHL primary tumours, although the 2-day cell count EC_{50}s for MSA were all lower than the 2-day viability EC_{50}s. These results are not unique. In the gastric adenocarcinoma cell-line SNU-1, cells responded to selenomethionine with a biphasic proliferative curve: enhanced incorporation of 3H-thymidine into DNA within a very narrow range of selenomethionine concentrations followed by decreased 3H-thymidine uptake at higher levels (Verma *et al.*, 2004). The findings of Verma *et al* suggest that, at certain concentrations, selenomethionine induces mild oxidative stress that, in turn, stimulates DNA synthesis. From the 24-hour investigation of selenite, campthotecin and their combination upon the cervical carcinoma cell-line Hep-2 HeLa by Rudolf *et al*, it is clear that selenite at the lowest concentrations actually stimulated cell growth and slightly reduced cytotoxicity and proapoptotic effects of campthotecin (Rudolf *et al.*, 2004). Selenomethionine in the culture medium of three hepatoma cell-lines promoted

cell growth at subcytotoxic levels (1-20 μM), but the growth of malignant lymphoid and myeloid cells was not stimulated (Kajander *et al.*, 1990). MSA was found to mildly stimulate proliferation at 2.5 μM before inhibiting proliferation at 5 μM (Zu and Ip, 2003). Therefore subtoxic concentrations of hydrogen selenide and methylselenol pool members can stimulate cell growth.

The repercussions of these observations as regards animal and human *in vivo* studies could be as follows:-

1) They may not hold true *in vivo.*
2) They could be true and beneficial, as any increased tumour cell cycling and proliferation could increase the tumour's sensitivity to other cytotoxics and prevent chemoresistance – one of the hypotheses put forward in Chapter 3 to account for the discovered associations between serum Se and response and overall survival.
3) A serum threshold concentration of Se supplementation may need to be passed to guarantee cytotoxic single-agent activity *in vitro*.

4.5.2 Literature Review of the Gene Expression Changes Induced by Selenium Compounds

The Pre-Microarray Data

Beyond ROS generation, protein thiol oxidation appears to be an important means of hydrogen selenide pool members' activity *in vitro*. Bjørnstedt *et al* showed that SDG is a highly efficient oxidant of NADPH and a substrate for mammalian TR, with the consequence of dramatic inhibition of Trx-dependent reduction of CDP (cytidine 5 - diphosphate) to dCDP (deoxycytidine 5-diphosphate) by ribonucleotide reductase (Bjornstedt *et al.*, 1992), and Kim *et al* demonstrated protein oxidised thiol cross-linking capable of inducing apoptosis (Kim *et al.*, 2003b). Therefore the ability of Se compounds to induce apoptosis is likely to be due, in part, to their oxidation of thiols, such as cysteine residues of proteins and enzymes in addition to the generation of superoxide in the presence of glutathione.

Selenite induced apoptosis in DU145 prostate cancer cells was associated with decreased expression of $p27^{kip1}$ and $p21^{WAF1}$ and increased phosphorylation of AKT, JNK-1, JNK-2 and p38 MAP kinase (Jiang *et al.*, 2001). In keeping with these findings, Ghose *et al* found marked induction of Fas-ligand with both SDG and p-XSC, which in turn, correlated closely with the level of apoptosis seen and was associated with

activation of the JNK and MAPK stress kinase pathways (Ghose *et al.*, 2001). Ghose *et al* speculate that the upstream regulator of JNK/p38 MAP kinases, apoptosis signal-regulating kinase-1 is released from inhibition by reduced levels of Trx and glutathione due to their oxidation by SDG (Ghose *et al.*, 2001). Phosphorylation of MAPK-1 and -3 is also seen in a concentration-dependent manner with selenomethionine-induced growth arrest, and is associated with the consequent phosphorylation of histone H3 and p90RSK (Goulet *et al.*, 2005). These results suggest that activation of the MAPK pathway is an important means of expression of activity for both hydrogen selenide and methylselenol pool members. An interesting discovery in the light of the SC-236 data described in Chapter 6 is that selenomethionine regulates COX-2 (cyclo-oxygenase-2) at the transcriptional level by modulating the activity of NFκB (Cherukuri *et al.*, 2005).

Recently redox modification of thiol/disulphide interchange in proteins by MSA was shown to produce protein unfolding in the endoplasmic reticulum (Zu *et al.*, 2006). This process, known as the unfolded protein response, blocks general protein synthesis in order to reduce the burden of more unfolded proteins forming within the cell. Persistence of the unfolded protein response forces the endoplasmic reticulum to switch from attempting to keep the cell alive to triggering apoptotic cell death. In PC3 cells the unfolded protein response was rapidly initiated on exposure to MSA, resulting in increased expression of $p21^{WAF1}$ in a p53-independent manner (PC3 cells are p53 null). Zu *et al* concluded that their findings support the hypothesis that the endoplasmic reticulum's unfolded protein response is an important mechanism in mediating the anticancer activity of selenium (Zu *et al.*, 2006).

Microarray Derived Effects of Se Compounds

Evidence of widespread effects of selenocompounds upon RNA synthesis first came to light nearly 30 years ago when selenite was found to cause a widespread reduction in RNA synthesis (Gruenwedel and Cruikshank, 1979). Since the advent of micro-array technology and genome-wide gene expression profiling six papers have investigated the effects of Se compounds using microarray technology: three papers describing *in vitro* experiments using MSA, one paper detailing *in vivo* studies with p-XSC, one paper which compared *in silico* gene expression data generated from normal and malignant human prostate tissue, *in vitro* prostate cancer cell-line experiments with Se compounds and androgen stimulation, and one paper from a human intervention study where

normal oesophageal tissue pre- and post- 10-month supplementation with selenomethionine was gene expression profiled. Each paper will be reviewed in turn.

Investigation of the mRNA changes over the 48-hours of exposure induced by the 2-day cell count EC_{50} of MSA in the human prostate cancer cell-line PC3 revealed transcript variance of >2 and <0.5 in 2650 genes (20% of those present on the U95A Affymetrix chip) (Dong *et al.*, 2003). Clustering analysis using the SOM algorithm produced 12 clusters, defined by their different kinetic patterns of modulation by MSA. There were three early response clusters (at 12 hours), five intermediate response clusters (between 24 and 32 hours) and four late clusters (after 36 hours). Only the analysis of the early response clusters was given. Multiple targets were affected by MSA in PC3 cells including induction of inhibitors of cell cycle progression ($p19^{INK4d}$, $p21^{WAF1}$, RB binding protein 1, GADD45, RAD9 and CHK2) and repression of promoters of cell cycling (cyclins A & E2, cdk-1, -2 & -4, MCM (minichromosome maintenance) -3, -5, -6 & -7, thymidylate synthase, dihydrofolate reductase and PCNA). Few apoptosis-related genes were affected at the 12-hour time point (caspase-9 and toll-like receptor-2 were induced, and survivin was repressed). The gene expression of a host of transcription factors and signal transduction genes was affected (e.g. PKCα (induced) and PI3-kinase (repressed) and several members of the MAPK cascade). There was a 70% correlation between the array findings and western blotting results for the corresponding protein expression.

In the human breast cell-line MCF10AT1 exposure to 5 μM MSA caused significant transcript changes at 6 and/or 12 hours in 30 genes out of around 200 on the membrane based array of cell cycle and apoptosis related genes (Dong *et al.*, 2002). Expression of *CDK-1, -2 & -4*, *cyclin A* was decreased, whilst the expression of *GADD153*, *MAPK-3* and *c-JUN* genes was increased.

The exposure of LNCaP, an androgen-sensitive human prostate cancer cell-line, to 3-30 μM MSA over the time course of 48 hours, induced significant concentration- and time-dependent changes in the expression of 951 genes (Zhao *et al.*, 2004). Transcript levels of many cell cycle-regulated genes changed in response to MSA, suggesting that MSA inhibited proliferation via multiple proteins (e.g. DNA replication initiation (CDC6, MCM2, and MCM6), DNA repair (PCNA), and cell cycle control (CDC25A and E2F1 - expressed in G1/S phase), DNA replication (RRM1, RRM2, and TYMS - expressed in S phase), chromosome condensation and organisation (TOP2A and CENPA), mitotic

spindle checkpoint (CDC20 and BUB1B), and centrosome duplication (PLK and STK15 - expressed in G2 and M phase)). Consistent with these gene expression changes, cells accumulated at the G0/G1 phase of the cell cycle (as detected by flow cytometry). MSA also modulated expression of many androgen-regulated genes, suppressed androgen receptor expression at both mRNA and protein level, and decreased levels of prostate specific antigen secreted into the medium. Low concentrations of MSA also induced significant increases in transcript levels of phase II detoxification enzymes (Zhao *et al.*, 2004). These results suggest that MSA may protect against prostate cancer by inhibiting cell proliferation, by modulating the expression of the androgen receptor and androgen receptor-regulated genes and by inducing carcinogen defences (Zhao *et al.*, 2004).

Microarray analysis of the mammary adenocarcinomas formed in rats fed DMBA receiving a standard diet or one supplemented with 10 ppm pXSC revealed induction of specific phase II detoxifying enzymes including selenoprotein P and several glutathione S transferase forms (El-Bayoumy *et al.*, 2003). Phase I P450 enzymes were significantly inhibited and the cell cycle genes cyclin-D1 and -D2 were downregulated (El-Bayoumy *et al.*, 2003). One concern with these results is that the genes overexpressed in the adenocarcinomas that developed on a diet of 10 ppm p-XSC were not those that were critical to the significantly fewer tumours that formed in the supplemented rats compared to controls but rather were the genes whose expression led to resistance and development on p-XSC.

Using a custom-built *'dot-blot'* array, the Roswell Park group studied the effect of MSA on genes transcripts in the LNCaP human prostate cancer cell-line (Zhang *et al.*, 2005a). Zhang *et al* then compared the gene expression profiles obtained *in silico* with their own from experiments with MSA and the PC3 prostate cancer cell-line detailed above ((Dong *et al.*, 2003)) and those of others of gene signatures considered important to human prostate carcinogenesis and gene transcripts affected by androgen stimulation of prostate cancer cell-lines. They found that Se reverses the expression of genes implicated in prostate carcinogenesis and counteracted the effect of androgen on the expression of androgen-regulated genes (Zhang *et al.*, 2005a).

In the Limburg study the chemopreventive activity of selenomethionine was studied in asymptomatic adults with squamous dysplasia of the oesophagus in Linxian province, China (Limburg *et al.*, 2005). As well as taking biopsies of the areas of dysplasia,

biopsies of adjacent normal tissue were taken pre- and post- the 10-month intervention with selenomethionine and/or placebo and celecoxib and/or placebo. From the 117 subjects in the selenomethionine and placebo arms suitable paired samples of histologically confirmed normal mucosa taken from 29 individuals (11 individuals whose dysplasia regressed, 13 who had stable dysplasia, and five subjects whose dysplasia progressed) (Joshi *et al.*, 2006). Microarray analysis was performed using Affymetrix HU 133A chips to search for genes associated with regression and progression of squamous dysplasia. Joshi *et al* discovered that the higher expression of 20 genes in the normal oesophageal mucosa was associated with regression and the higher expression of 129 genes in the normal oesophageal mucosa was associated with progression in neighbouring squamous dysplasia samples. These 149 differentially expressed genes were categorised into known or probable functional categories using the Gene Ontology database. The top five categories included immune response (15 genes), cell cycle (15 genes), metabolism (15 genes), calcium transport or calcium ion activity (10 genes) and regulation of transcription (9 genes). Using the EASE software package, which aids discovery of biological themes within gene lists, only the 15 genes associated with the immune response pathway were overrepresented compared to the total number of immune response genes on the microarray chip ($P < 0.01$). Of these 15 genes, those associated with dysplasia regression had functions associated with upregulation of the immune response, including antigen presentation (HLA-DPA1, HLA-DRA, and HLA-DQB1), protection of T cells from cell death (CD58), and T-cell activation (FCERA1). In contrast, the genes associated with progression of oesophageal squamous dysplasia were involved in immunosuppression (CNR2) and NFATC4), acute phase response and inflammation (CRP, ORMS, SERPINA7 and SERPINA1), or a decrease in the level of B- and T-cells (NFRKB) (Joshi *et al.*, 2006).

4.5.3 Should Se Compounds be used as Adjuvant or Maintenance Treatment?

***In vitro* Data**

A recurrent and critical question and concern in research is whether *in vitro* effects will be reproduced *in vivo*. Where *in vitro* and *in vivo* effects appear comparable the issue is whether the results will be the same in humans. The potential for additive or synergistic interactions between Se compounds and cytotoxics, through preferential increase in oxidative cell stress in tumours, is supported by work of Frenkel and Caffrey. They discovered the potential for Se compounds to prevent chemoresistance to low-level

continuous cytotoxic agent exposure, the principal reason for treatment failure in DLBCL (Caffrey and Frenkel, 2000). Additionally, moderate and higher concentrations of selenite enhanced the cytotoxic and proapoptotic effects of campthotecin, changing cell death from apoptosis into more necrosis-like cell death (Rudolf *et al.*, 2004).

***In vivo* Studies**

In athymic nude mice MSC and selenomethionine reduced the toxicity and improved the anti-tumour activity of several anticancer drugs against human carcinoma xenografts (Cao *et al.*, 2004). By starting the Se compounds for a month seven days before chemotherapy, the maximum tolerated dose of irinotecan, a topoisomerase I poison, could be quadrupled and resistant cell-line xenografts rendered chemosensitive with a high cure rate. The Se compounds therefore selectively protected normal tissues from irinotecan toxicity, whilst sensitising the malignant cells to cytotoxics.

Human Trials

The same group has gone on to conduct several human phase I studies of high-dose oral selenomethionine supplementation prior to irinotecan treatment in a patients with chemoresistant/refractory cancer (Fakih *et al.*, 2006, Fakih *et al.*, 2008). The results are contrary to those of the nude mouse model experiments summarised above. No selective protective effect of Se supplementation for normal tissues against the side-effects of irinotecan were observed, even at 7.2 mg of selenomethionine twice daily for a week followed by once daily dosing, which lead to Se plasma concentrations >150 μmol/l. However, three PRs and nine stable disease cases were seen out of 41 patients investigated (disease control rate of 30%). The authors concluded that selenomethionine, at the dose and schedule used, may induce irinotecan sensitivity in a minority of patients and on this basis plan further clinical studies to verify this proposition (Fakih *et al.*, 2006, Fakih *et al.*, 2008).

These results are disappointing and encouraging. The failure of high-dose selenomethionine to protect normal tissues from irinotecan poisoning may be due to any of the following:

1 Selenomethionine is ineffective and its mechanisms of protection in athymic nude mice do not hold true in humans with end-stage cancer.

2 The dose of selenomethionine was too high, so as to be toxic to normal tissues i.e. not only was the putative chemosensitising serum concentration passed but also the possible cytoprotective concentration for normal cells exceeded.

3 The wrong Se compound was used.

4 The wrong cytotoxic agent was used.

In the light of the above and the finding detailed in this Chapter that low concentration Se exposure can stimulate proliferation, the concerns are that Se supplement interactions may be no more than neutral and possibly antagonistic. Alternatively, stimulation of proliferation via oxidative stress may be the very mechanism by which Se compounds can prevent chemoresistance and potentiate the activity of established cytotoxics, as exemplified in the work of Cao (Cao *et al.*, 2004). From the data described in Chapter 3 it is clear that the majority of DLBCL patients are Se deficient at presentation. Therefore, it is likely that Se supplementation will need to go beyond restoration of physiological function to achieve chemosensitising levels. The potential effect of 'supraphysiological but low-dose chemosensitising' Se supplementation on cell cycling and proliferation will need investigation as to whether it occurs preferentially in malignant cells vs. normal and whether this effect augments (e.g. by depleting intracellular glutathione leading to increased sensitivity to cytotoxic drugs that are inactivated by conjugation with glutathione, such as cisplatin or doxorubicin.) or antagonises the efficacy of conventional cytotoxics. Other potential roles for Se supplementation are as a maintenance treatment to reduce the risk of lymphoma recurrence and to prevent the development of treatment-related malignancy.

4.5.4 Choice of Se compound for Human Investigation

The metabolism and bioavailability of Se compounds are influenced by absorption in the gastrointestinal tract, transport in the blood, metabolism and storage in tissues, and rate of excretion in the urine and faeces (Daniels, 1996). The organic forms of Se such as selenomethionine are more bioavailable than are inorganic forms such as sodium selenite (Daniels, 1996). In terms of which methylselenol-pool precursor would be suitable for clinical trials, selenomethionine has already been used and proved effective (Clark *et al.*, 1996). However excess selenomethionine accumulates in tissues through competition with methionine during protein transcription and the results from the Roswell Park group's phase I clinical studies failed to show protection of normal tissues from chemotherapy toxicity. Beyond selenomethionine, MSC looks to be a promising *in vivo* methylselenol precursor with equivalent efficacy to MSA as a chemopreventive agent in animal studies, with the tumour reducing effect of 2 ppm/day Se being the same for MSA and MSC i.e. 10-fold more than the 0.2 ppm needed to restore the

function of seleno-enzymes in deplete animals (Ip *et al.*, 2000). In terms of production, MSC generation is simpler than MSA, as it is the principal Se compound of selenium-enriched vegetables such as garlic and broccoli. Both MSC and MSA are stable for at least 7-10 days in the prepared feed of rats (Ip *et al.*, 2000). From the hydrogen selenide pool selenite and p-XSC are the two candidate Se compounds. The latter was specifically developed as a potential Se supplement in humans, due to its superior therapeutic index *in vivo* compared to selenite (Ip *et al.*, 1994).

In summary, the results presented in this Chapter demonstrated that both a hydrogen selenide Se compound (SDG) and a methylselenol pool member (MSA) are cytostatic and cytotoxic towards human DLBCL cell-lines and *ex vivo* cultured human primary lymphoma samples and induced caspase-8 and -9 dependent apoptosis. The next stage of investigation suggested by these results would be the investigation of SDG and MSA in combination with established cytotoxics to search for any chemosensitising ability in human DLBCL cell-lines and primary lymphomas.

CHAPTER 5: DISCOVERY OF OUTCOME PREDICTIVE GENE EXPRESSION SIGNATURES IN DLBCL USING MICROARRAY PROFILING

5.1 Chapter Summary

This Chapter describes the *rational* approach of gene expression profiling used to search for predictors of outcome to anthracycline-based treatment in previously untreated DLBCL. RNA was extracted and converted into labelled cDNA from cryopreserved, presentation lymph node material from 58 DLBCL patients and 19 FL patients. The labelled cDNA was hybridised onto Affymetrix Hu6800 gene expression chips and the resultant intensities of *'probe-target'* hybridisations measured using a laser scanner. The raw array data was pre-processed as detailed in Chapter 2 to allow comparison of sample data. The supervised learning classification algorithm *'weighted voting'* was used in an attempt to delineate DLBCL from the closely related GC (germinal centre) B-cell-lineage malignancy, FL. Following the successful delineation of DLBCL from FL, the weighted voting algorithm and two other prediction methods were used to try and predict the post-treatment alive and cured DLBCL patients from the dead/with refractory disease DLBCL patients using their respective microarray expression data. Within the 58 DLBCL group, application of each of the three different prediction methods, supported by *'leave-one-out'* cross-validation, could significantly stratify the patients into *'alive and cured'* and *'fatal/refractory disease'* groups. Furthermore, a model of just 13 out of the 6,800 genes arrayed could define *'alive and cured'* and *'fatal/refractory disease'* groups alone and within the different IPI risk groups. The molecular gene expression model was therefore extracting additional prognostic information to that of the clinical IPI. *In silico* and immunohistochemical validations were performed and supported the legitimacy of the findings. When the SBH cohort of 19 patient samples was investigated alone, a separate 19-gene model proved highly effective at outcome prediction within this cohort.

5.2 Introduction

The paucity of precision in the description of the molecular events and players in DLBCL suggests that many if not most of the critical participant genes and proteins underlying and explaining the clinical heterogeneity of DLBCL are unknown and unlinked to the illness. To date, investigation of potential molecular prognostic factors has been labour intensive, due to each target requiring individual scrutiny. The discovery and validation of individual prognostic factors in DLBCL has thus been slow.

Furthermore, the factors investigated were already established as having a role in the pathogenesis of tumours in general (e.g. *TP53*) or DLBCL in particular (*BCL-6*). The advent of microarray technology broke the restraints of individual gene scrutiny by allowing thousands of genes to be interrogated in parallel. In the research described below 6,800 different gene transcripts from across the human genome were investigated simultaneously using the HU6800 Affymetrix chip. This obviated the need for *empirical* hypotheses justifying individual or multiple target investigation. The aims of the project were guided by the reproduction and extension of traditional acute leukaemia classification using Affymetrix array technology and supervised learning analyses. In addition to the acute leukaemia specimens arrayed being correctly classified as ALL and AML based upon their expression signatures further, novel gene expression signature generated subclasses were suggested (Golub *et al.*, 1999). The lymphoma project set out to answer the following *rational* as opposed to *empirical* questions:

- Could gene expression profiling allow DLBCL to be distinguished from the related B-cell NHL FL using supervised learning analyses? As the histological distinction between DLBCL and FL is considerable this question was anticipated to be easily addressed by analyses of gene expression profiling.
- Could supervised learning methods distinguish *'alive and cured'* patients from *'fatal/refractory disease'* patients based solely on the gene expression profiles of their respectively diagnostic lymphoma material? To date such a distinction has not been possible by any method. This was therefore expected to be a considerably more difficult question to address.
- Could a treatment outcome predictor for DLBCL be developed from expression signature differences? If this was possible, then the potential application of such a predictor to other DLBCL series could have far reaching and positive benefits for patients, clinicians and trialists.

5.3 Methods

The research relating to the use of gene expression profiling was conjoint work. My role in this research was limited. I identified and collected over 30 frozen diagnostic samples of diffuse large B-cell lymphoma (DLBCL) from the tissue archives at St Bartholomew's Hospital. For each sample I extracted key clinical data from the lymphoma database and the original case notes, retrospectively assigning a performance

status to the patient at presentation based on recorded clinical details. Where no presentation serum LDH was available I identified archived frozen serum samples from presentation and obtained an LDH level. I identified the corresponding formalin fixed paraffin embedded diagnostic tissue blocks and slides to the frozen specimens in the St Bartholomew's Hospital histopathology department archive. I then transported the samples to the Whitehead Institute microarray facility and Dana Farber Cancer Institute. Jon Aster, the project's principal histopathologist, examined the corresponding histopathology blocks and slides to confirm the diagnosis of DLBCL. At the Whitehead Institute microarray facility I helped prepare the samples I had transported for expression profiling. Only nineteen of the more than 30 specimens processed were of sufficient quality and of standard DLBCL rather than several variants excluded which were excluded from the study. The initial expression profiling of the samples was performed by Michelle Gaasenbeek in my presence. Michelle conducted the repeat profiling after my return to the UK. The subsequent data analyses were performed by the team at the Whitehead Institute and repeated on only the St Bartholomew's Hospital samples by Spouros Sgouros in the UK.

In brief, total RNA was extracted from each frozen tumour specimen and converted to double-stranded cDNA before *in vitro* transcription of biotinylated cRNA. Ten μg of fragmented, biotinylated cRNA was hybridised to HU6800 oligonucleotide Affymetrix arrays at 45 °C for 16 hours (see Chapter 2 for more details). The arrays were then washed and stained twice with SAPE prior to scanning on an Affymetrix scanner. The expression value for each gene was then calculated using Affymetrix GeneChip software. The raw data was cleaned and processed as detailed in Chapter 2 to allow application of supervised learning methodologies as detailed in Chapter 2. The algorithms used to classify according to phenotype distinction were weighted voting, κ-nearest neighbours and support vector machines. Hierarchical clustering was used to reassess the Alizadeh *et al* dataset reduced to just the 90 genes common to the *'Lymphochip'* and the Affymetrix HU6800. A classifier was built through cross-validation using the *'leave-one-out'* technique. Several models were built using different numbers of marker genes, allowing the final model to be chosen with the lowest error rate in cross-validation. The Kaplan-Meier method was used to calculate predicted OS curves and the log rank test was used to calculate the respective *P* values (Kaplan and Meier, 1958).

5.4 Results

The 5-year OS of the 58 DLBCL patients was 54%, a result better than expected from larger series treated with the same therapy (Figure 5.1). The most likely explanation for this difference is the higher number of good-risk and low-intermediate risk IPI patients in this series compared to others. At time of data censuring (April 2000) 32 of 58 patients were alive and disease-free and 26 had died of DLBCL (23) or had refractory disease (3).

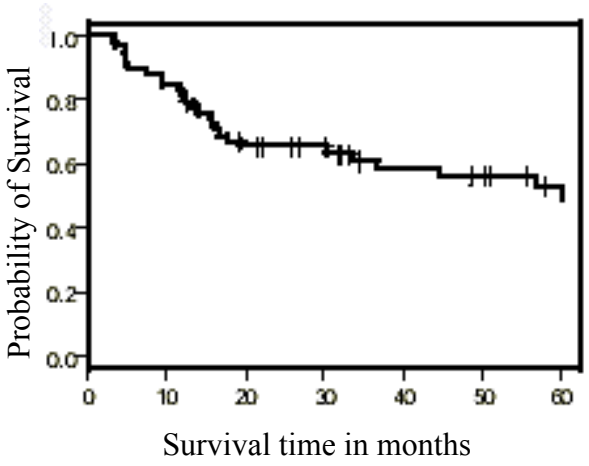


Figure 5.1 Kaplan-Meier Plot of 5-year overall survival for the 58 DLBCL Patients The 5-year OS was 54%, which is slightly better than expected for DLBCL but in keeping with the favourable IPI score of the majority of patients (see Chapter 2).

5.4.1 Distinguishing DLBCL from FL

The 6,817 genes were sorted by the degree of their expression correlation with the DLBCL vs. FL distinction using their mean signal-to-noise ratios. The 100 best gene markers for the DLBCL versus FL distinction are illustrated in Figure 5.2. Using the weighted voting algorithm and the leave-one-out cross-validation method a model containing 30 of the most discriminating genes predicted the correct class of 71 of the 77 (91%) samples ($p < 1.4 \times 10^{-9}$). The 30-gene predictor incorrectly classified six DFCI DLBCL as FL (Table 5.1).

		Weighted Voting Assigned Class		
		DLBCL	FL	Total
True Class	DLBCL	52	**6**	58
	Follicular	**0**	19	19
	Total	52	25	77

Table 5.1: confusion matrix for the prediction of the DLBCL versus FL distinction using the 30-gene weighted voting algorithm generated model. None of the FL cases were incorrectly assigned to the DLBCL subclass but six DLBCL were incorrectly assigned to the FL subclass of NHL by the weighted voting algorithm.

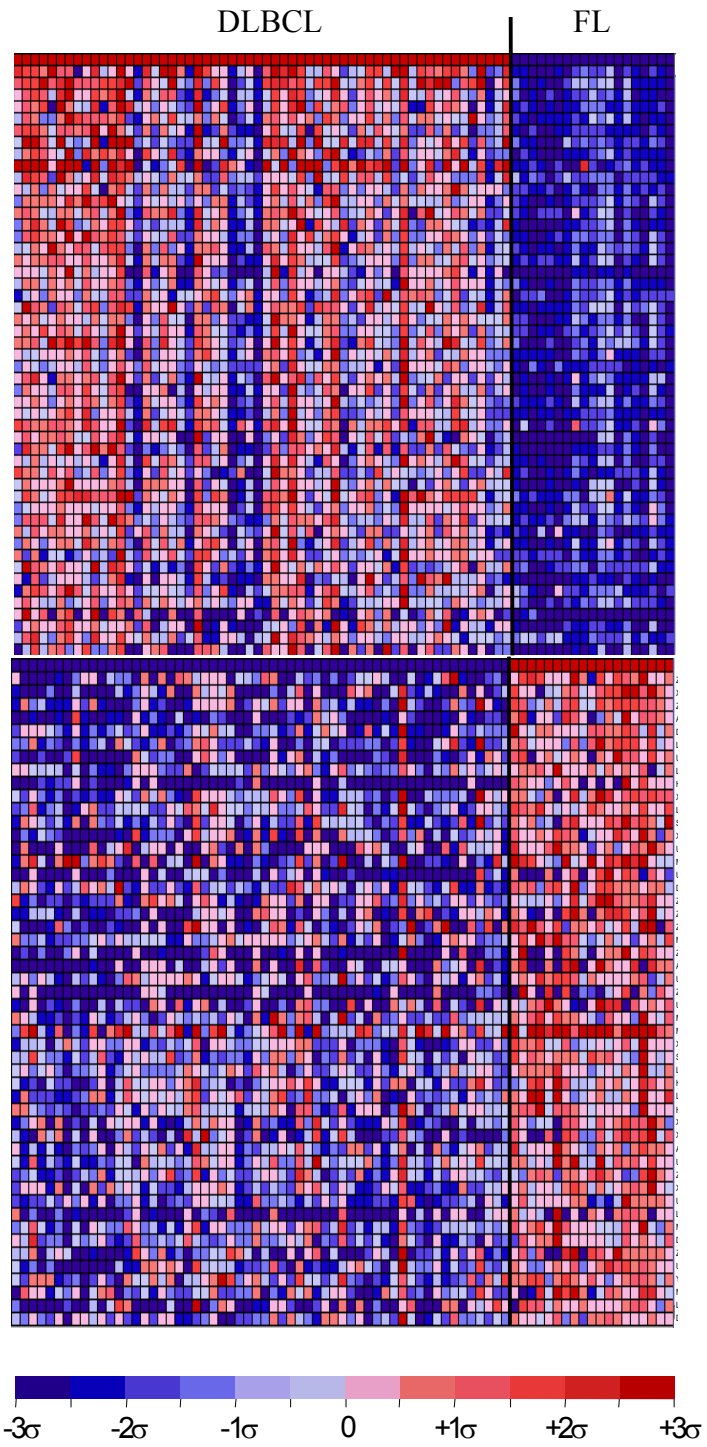


Figure 5.2 Pinkogram of the 100 most discriminating genes between DLBCL and FL
The 50 genes most differentially expressed at higher levels in DLBCL compared to FL are shown at the top of the '*pinkogram'* while the 50 genes that were most highly expressed in FL compared to DLBCL are shown at the bottom. Red indicates a high relative expression while blue represents a low relative expression. Each column represents a sample and each row a gene (with the first rows of the DLBCL and FL sections showing an idealised expression profile). Expression profiles for the 58 DLBCL samples are on the left while the profiles for the 19 FL samples are on the right.

σ = standard deviation from mean

5.4.2 Distinguishing DLBCL 'Alive and cured' from DLBCL 'Fatal/refractory disease'

The 6,817 genes were sorted according to the degree of expression correlation with the *'alive and cured'* vs. *'fatal/refractory disease'* distinction using the mean signal-to-noise ratios for each gene. The 100 most discriminating genes, 50 for each class (*'alive and cured'* and *'fatal/refractory disease'*), are illustrated in Figures 5.3.

It is clear from Figure 5.3 that this class distinction was less clear-cut than that achieved for DLBCL vs. FL. Indeed, taken individually, the top marker genes for treatment outcome prediction did not show statistical significance - unlike the top marker genes for morphological distinction of FL from DLBCL. Nevertheless, from the approximately 700 genes that demonstrated differential expression in relation to the supervised learning class divider *'alive and cured'* vs. *'fatal/refractory disease'*, a pool of genes could be extracted that, when combined, effectively predicted for the *'alive and cured'* vs. *'fatal/refractory disease'*. Application of the weighted voting algorithm and leave-one-out cross validation testing revealed that predictors containing a minimum of eight and maximum of 16 genes produced statistically significant class distinctions. Of these models, the 13-gene model was the most accurate. For each patient's sample, the leave-one-out cross-validation method created a slightly different set of 13 genes as the treatment outcome predictor. Seven genes were common to all 58 cross-validation 13-gene models; four genes were included in 54 or more models, three genes were included in 20-34 models, and five additional genes were used in 3-8 of the 58 cross-validation models. The top 13 of these 19 genes were used to create the final 13-gene outcome prediction model. Details of these 19 genes are given in Table 5.2 and the expression values of the 13-gene model are illustrated in Figure 5.4.

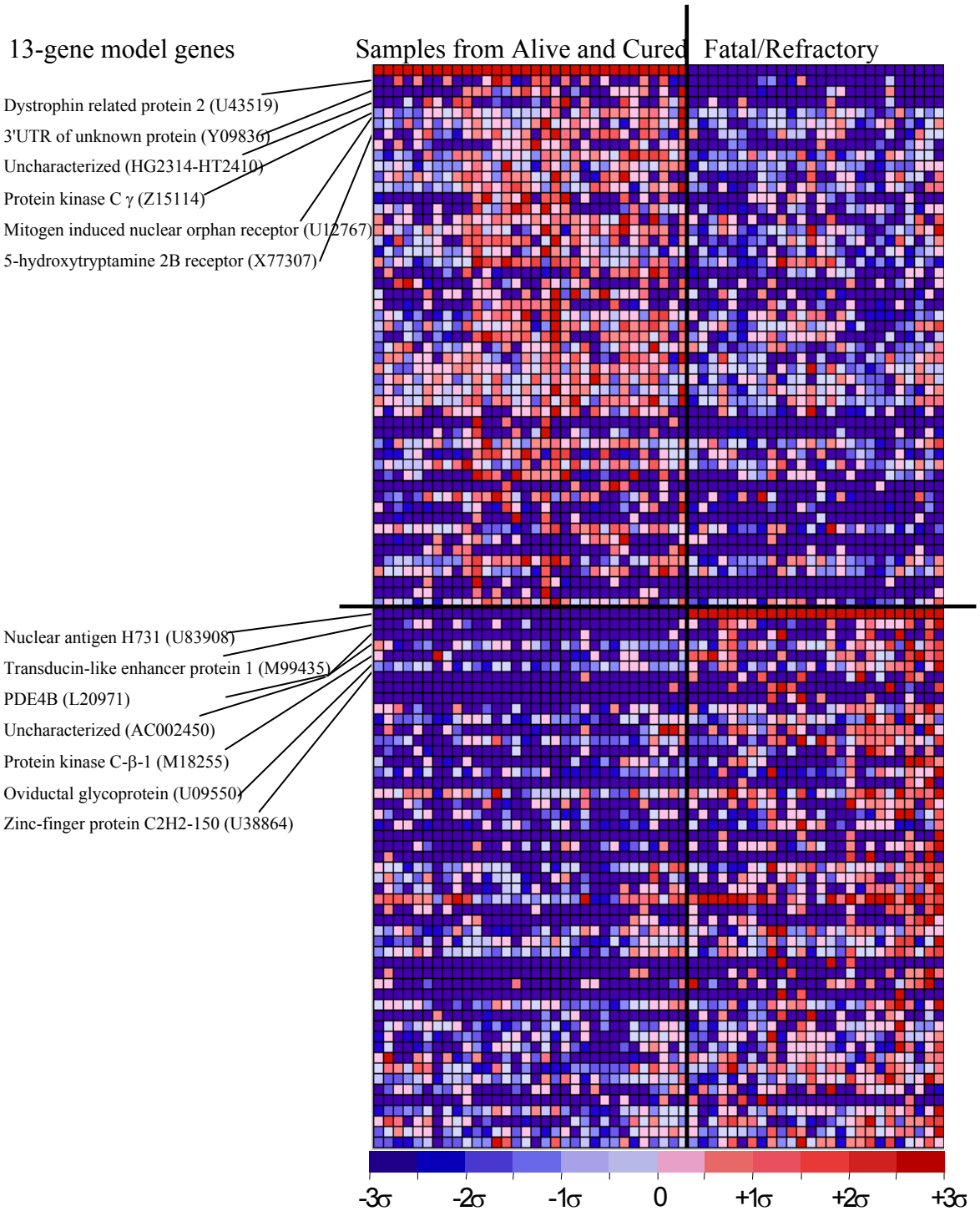
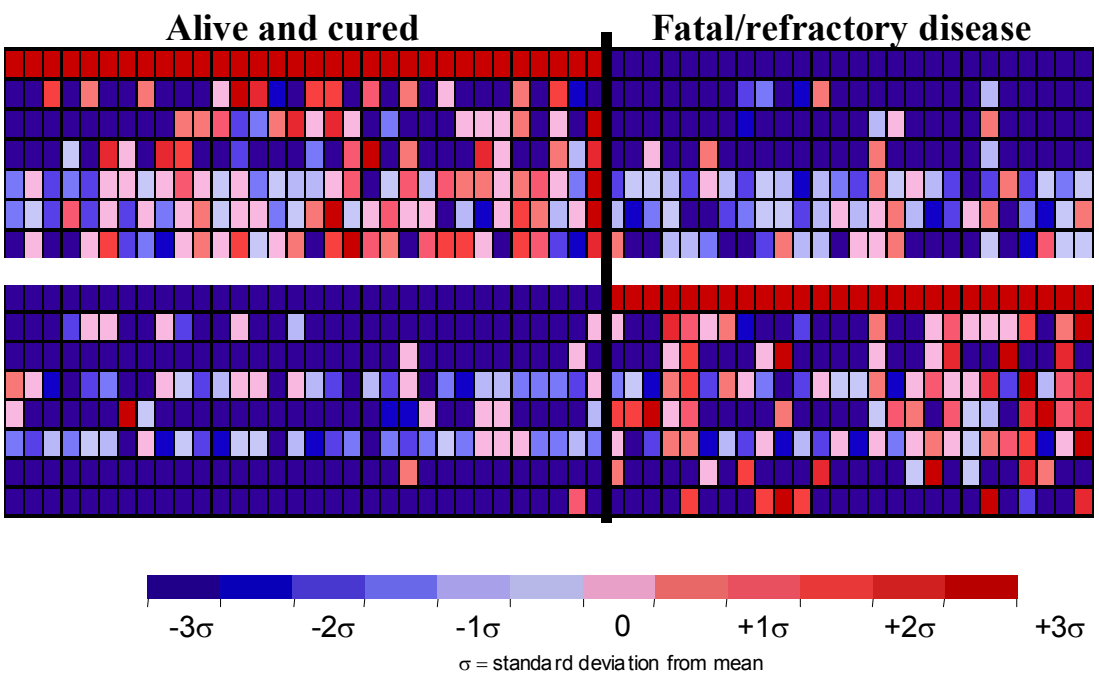


Figure 5.3 100 most discriminating genes for DLBCL samples from patients with *'Alive and cured'* vs. from patients *'dead/with refractory disease'*. The genes that were expressed at higher levels in samples from *'alive and cured'* patients are shown at the top while the genes that were more highly expressed in the tumours of those with *'fatal/refractory disease'* are shown at the bottom. Red indicates a high relative expression whilst blue represents a low relative expression and each column represents a sample and each row a gene (with the first rows of the two sections showing an idealised expression profile). Expression profiles for the 32 samples from the *'alive and cured'* patients are on the left while the profiles for the samples from the 26 patients with *'fatal/refractory disease'* are on the right. σ = standard deviation from mean

Application of the 13-gene model created using the top 13 genes detailed in Table 5.2 created an *'alive and cured'* group with a 5-year OS of 70% and a *'fatal/refractory disease'* group with a 5-year OS of 12% (p = 0.00004) (Figure 5.5). Use of two further analysis tools, κ-nearest neighbours and support vector machines, produced *'alive and cured'* and *'fatal/refractory disease'* groups from the 58 patients with similar 5-year OS to that produced by the weighted voting algorithm 13-gene model. For κ-near neighbour the 5-year OS were 72% and 12% respectively (log rank *P* = 0.00002) and for support vector machine the 5-year OS were 68% and 23% (log rank *P* = 0.002) respectively for the *'alive and cured'* and *'fatal/refractory disease'* groups.

	Unigene Identifier	Number of Cross Validation Models Using Gene	Description
U43519	**Hs.159291**	**58**	**Dystrophin related protein 2 (DRP2)**
M18255_cds2	**Hs.77202**	**58**	**Protein kinase C β1 (PKCβ)**
U83908	**Hs.100407**	**58**	**Nuclear antigen H731**
Y09836	**Hs.82503**	**58**	**3'UTR of unknown protein**
M99435	**Hs.28935**	**58**	**Transducin-like enhancer protein 1**
AC002450		**58**	**Uncharacterised**
L20971	**Hs.188**	**58**	**Phosphodiesterase 4B (PDE4B)**
HG2314-HT2410		**57**	**Uncharacterised**
Z15114	**Hs.2890**	**57**	**Protein kinase C γ (PKCγ)**
U09550	**Hs.1154**	**55**	**Oviductal glycoprotein**
U12767	**Hs.80561**	**54**	**MINOR (Mitogen induced nuclear orphan receptor)**
U38864	**Hs.108139**	**34**	**Zinc-finger protein C2H2-150**
X77307	**Hs.2507**	**23**	**5-Hydroxytryptamine 2B Receptor**
L40377	Hs.41726	20	Cytoplasmic antiproteinase 2 (CAP2)
U65093	Hs.82071	8	Msg1-related gene 1 (mrg1)
Z30644	Hs.123059	7	Chloride channel (putative) 2163bp
U02609	Hs.114416	5	Transducin-like protein
Z83802		4	Axonemal dynein heavy chain (partial, ID hdhc3)
M55531	Hs.33084	3	SLC2A5 Solute carrier family 2

Table 5.2 Details of the 19 genes included in the 58 individually generated 13-gene models using the weighted voting algorithm and leave-one-out cross-validation testing for predicting outcome in the 58 DLBCL patients. The upper 13 genes highlighted in bold type constitute the final 13-gene model applied to all 58 samples to generate the OS curves illustrated in Figure 5.5.



Affymetrix Identifier	Description	No. of Models Using Gene
U43519	Dystrophin related protein 2 (DRP2)	58
Y09836	3'UTR of unknown protein	58
HG2314-	Uncharacterised	57
Z15114	Protein kinase C γ (PKCγ)	57
U12767	MINOR (Mitogen induced nuclear orphan receptor)	54
X77307	5-Hydroxytryptamine 2B Receptor	23
U83908	Nuclear antigen H731	58
M99435	Transducin-like enhancer protein 1	58
L20971	Phosphodiesterase 4B (PDE4B)	58
AC002450	Uncharacterised	58
M18255	Protein kinase C β1 (PKCβ)	58
U09550	Oviductal glycoprotein	55
U38864	Zinc-finger protein C2H2-150	34

Figure 5.4 13-gene outcome prediction model for DLBCL *'alive and cured'* vs. *'fatal/refractory disease'*.
The six genes that were expressed at higher levels in samples from *'alive and cured'* patients are shown at the top while the seven genes that were more highly expressed in the tumours of those with *'fatal/refractory disease'* are shown at the bottom. Red indicates a high relative expression whilst blue represents a low relative expression. Each column represents a sample and each row a gene (with the first rows of the two sections showing an idealised expression profile). The vertical black line separates the expression profiles for the 32 samples from the *'alive and cured'* patients to the left from the profiles for the samples from the 26 patients with *'fatal/refractory disease'* to the right

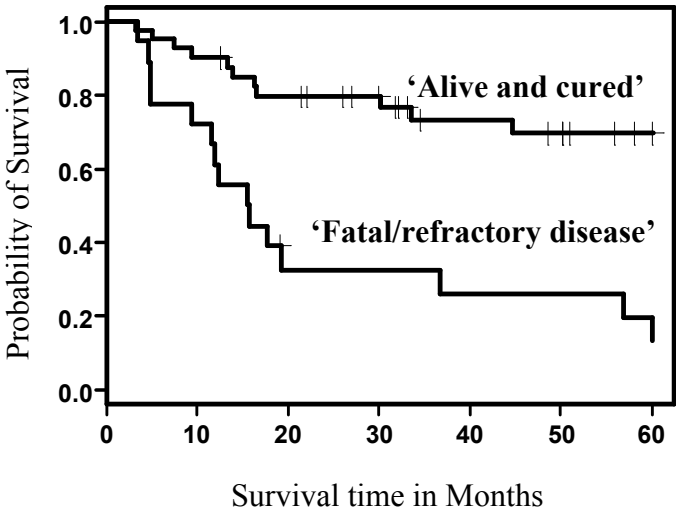


Figure 5.5 Overall Survival for the Predicted *'Alive and cured'* and *'Fatal/refractory disease'* Groups
Kaplan-Meier plot for the 5-year OS for the predicted *'alive and cured'* and *'fatal/refractory disease'* risk groups where the predicted groups were defined by the 13-gene model described above. The patients in the array model-predicted *'alive and cured'* group had 70% survival at five years compared to only 12% 5-year OS for the patients in the model-predicted *'fatal/refractory disease'* group (log-rank P = 0.00004).

The confusion matrix summarising the leave-one-out cross-validation of the 13-gene predictor is shown in Table 5.3. It can be seen that the principle failing of the 13-gene predictor was the incorrect classification of 11 *'fatal/refractory disease'* cases as cured. This gives a false-positive rate of 42% and constitutes 27.5% of the cured cases predicted by the model. This figure in turn explains why the 5-year OS of the predicted cured cases was 70% and not 100%. Only three cured patients were predicted to have *'fatal/refractory disease'* by the 13-gene model, a false negative rate of 9%. In turn, this error explains why the 5-year OS for the 13-gene model generated *'fatal/refractory disease'* group was 12% and not 0%.

		Predicted Class		
		Alive and cured	Fatal/Refractory disease	Total
True Class	Alive and cured	29	**3**	32
	Fatal/Refractory disease	**11**	15	26
	Total	40	18	58

Table 5.3 Confusion Matrix Summarising the Leave-One-Out Cross-Validation Prediction Results Generated by Application of the 13-Gene Model for *'Alive and cured'* vs. *'Fatal/refractory disease'*.

5.4.3 Application of the 13-gene predictor within the IPI categories

Application of the 13-gene model to the patients within the low/low-intermediate and high-intermediate IPI categories resulted in further stratification. This suggests that the clinical and molecular prediction models captured distinct information. Within the combined low/low intermediate IPI patients, the 13-gene model produced *'alive and cured'* and *'fatal/refractory disease'* groups with 5-year OS of 75% and 32% respectively (log rank $P = 0.02$) and within the high intermediate patients the 13-gene model produced *'alive and cured'* and *'fatal/refractory disease'* groups with 5-year OS of 57% and 0% respectively (log rank $P = 0.02$). Figures 5.6 through 5.8 illustrate the Kaplan-Meier plots of 5-year OS for the 58 patients stratified according to the IPI alone, and the low/low-intermediate and high-intermediate IPI patients stratified by the 13-gene model respectively.

5.4.4 *In silico* and Immunohistochemical Validation of the 13-gene Model

Two approaches were used in an attempt to validate and provide biological plausibility to the 13-gene outcome predictor model. Firstly, *in silico* validation was explored using the NCI (national cancer institute, USA) group's microarray results generated using the 'lymphochip' from 40 pretreatment DLBCL samples (Alizadeh *et al.*, 2000). Hierarchical clustering was performed using just the 90 Unigene clusters from the 'lymphochip' class-defining *'cell-of-origin'* set that were also present on the Affymetrix HU6800. These 90 Unigene clusters were represented by 139 clones on the Lymphochip and by 100 *'probes'* on the oligonucleotide arrays. This hierarchical clustering analysis confirmed the delineation of two groups, a GCB-like class and an ABC-like class within both the original Alizadeh *et al* series of 40 DLBCL and also within the 58 DFCI/SBH cases. However, only in the NCI series did the GCB-like group have a more favourable 5-year OS than the ABC-like class ($X^2 = 0.2$ for one degree of freedom, $P = 0.631$). In the DFCI/SBH series the *'cell-of-origin'* class divider created using the common 90 genes did not predict for outcome (Figure 5.9). However for the 19 SBH cases the '*cell-of-origin'* classifier proved moderately effective: for the GCB-like subgroup the 5-year OS was 77% compared to 50% for the ABC-like subgroup.

Figure 5.6 OS Curves for the Patients within each IPI group

Kaplan-Meier OS curves for the 56 patients with an IPI score, subdivided into the four risk groups of the IPI. OS data has been censured at five years follow-up.

IPI Groups

	Low risk	n = 26
	Low-intermediate	n = 11
	High-intermediate	n = 17
	High-risk	n = 2
Total		n = 56

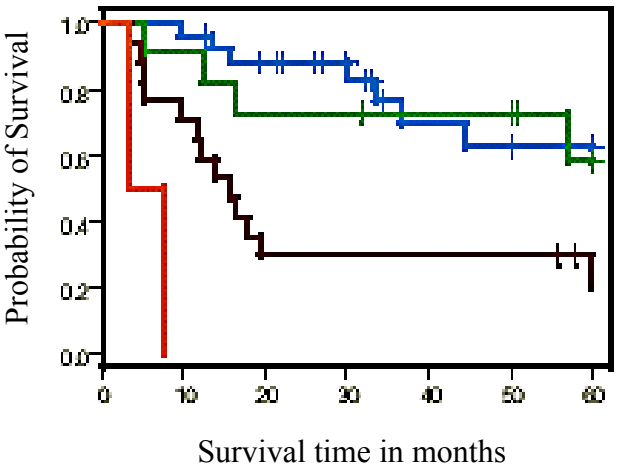


Figure 5.7 OS curves for the low/low-intermediate IPI group stratified according to the 13-gene model.

Kaplan-Meier 5-year OS plots following application of the 13-gene model to the 37 of the 58 DLBCL patients that were classified as low/low-intermediate risk according to the IPI. The upper curve represents the 13-gene model generated *'alive and cured'* group with 5-year OS of 75% and the lower curve represents the significantly different 13-gene model generated *'fatal/refractory disease'* group with 5-year OS of 32%; nominal log-rank $P = 0.02$. OS data has been censured at five years follow-up.

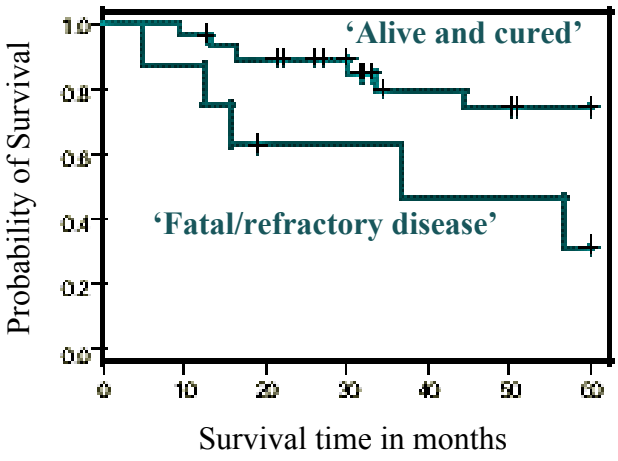
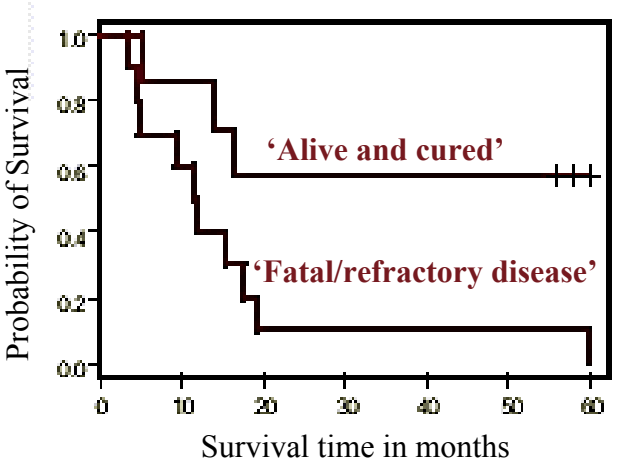


Figure 5.8 OS curves for the high-intermediate IPI group stratified according to the 13-gene model.

Kaplan-Meier 5-year OS plots following application of the 13-gene model to the 17 of the 58 DLBCL patients that were classified as high-intermediate risk according to the IPI. The upper curve represents the 13-gene model generated *'alive and cured'* group with 5-year OS of 57% and the lower curve represents the significantly different 13-gene model generated *'fatal/refractory disease'* group with 5-year OS of 0%; nominal log-rank $P = 0.02$. OS data has been censured at 5 years follow-up.



Only three of the thirteen genes from the 13-gene model, namely PKCβ, PDE4B and MINOR, were also represented on the 'Lymphochip'. Taken individually, each of these three genes was found to predict for OS in the NCI dataset, with *P* values of 0.04, 0.07 and 0.05 respectively for PKCβ, PDE4B and MINOR.

Immunohistochemical staining for PKCβ protein was performed using a tissue array for 19 of the DFCI patients. A significant correlation between outcome and OS for these 19 cases was found using a *t* test (p = 0.03) and the PKCβ protein level showed a trend towards a correlation with the Affymetrix PKCβ probe M18255_cds2 expression using Fisher's exact test (p = 0.08).

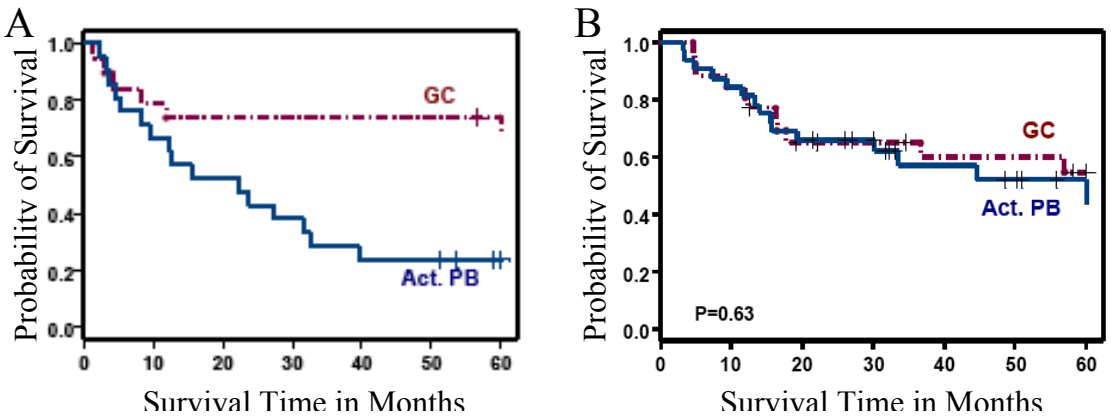


Figure 5.9 OS curves for the 90 Unigene cluster defined germinal centre-like and activated B-cell-like subgroups. Kaplan-Meier 5-year OS plots following application of the subset of 90 common Unigene clusters from the cell-of-origin signature used to predict OS in the LLMPP/NCI series. OS data has been censored at five years follow-up.
A = OS curves for the 40 DLBCL from the LLMPP/NCI series
B = OS curves for the 58 DLBCL from the DFCI/SBH series

GC = germinal centre-like **Act. PB = Activated peripheral blood B-cell-like**

5.4.5 Independent Analysis of the St Bartholomew's Hospital Cohort

Evidence suggesting that a different set of genes may also serve as predictors of OS in only the SBH subset of patients was first suggested by the earliest representations of the 50 most discriminating and highly expressed genes in the *'alive and cured'* vs. *'fatal/refractory disease'* where the SBH cases were placed together as illustrated in Figure 5.10. It can be seen that these 50 genes could be clustered into six groups with the second group being highly expressed in the SBH *'alive and cured'*. The six genes in this cluster were zinc finger protein 91, transporter protein G17, retinoic acid receptor related orphan receptor A, a (1,2) fucosyltransferase 5' untranslated region (UTR) partial sequence, GATA-1 transcription factor and 3' UTR of an unknown protein (Figure 5.10).

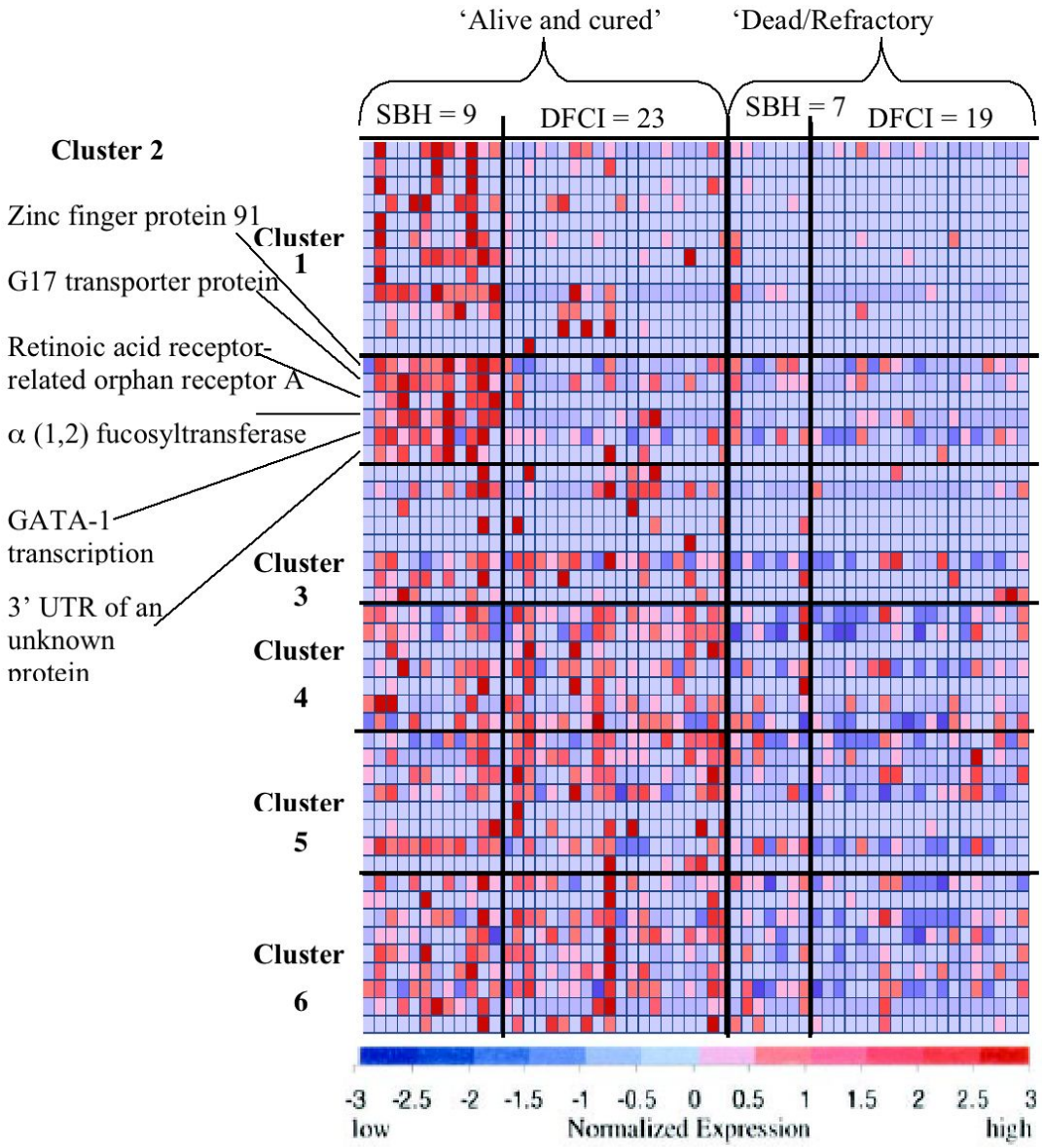


Figure 5.10 Pinkogram of the 50 most discriminating and highly expressed genes in *'Alive and cured'* compared to *'Fatal/refractory disease'* patients. Expression profiles for the samples from the 32 alive and cured patients are to the left of the vertical black line, while the profiles for the samples from the 26 patients with fatal/refractory disease are on the right. Each column represents a sample and each row a gene. Red indicates a high relative expression while blue represents a low relative expression. The genes have been grouped into six clusters according to similarity of co-expression. The six genes belonging to cluster 2 were more highly expressed in the SBH rather than the DFCI alive and cured patient samples and have been named.

In order to test for an SBH-specific outcome predictor model based on expression data, a supervised analysis using hierarchical clustering was performed using the microarray data from just the 19 SBH patients. The supervised class distinction of *'alive and cured'* and *'dead from disease'* was applied. A model consisting of the 19 most discriminating genes correctly predicted the class of 92% of the *'alive and cured'* cases and 100% of the dead-from-disease (Table 5.4, Figures 5.11 and 5.13). The genes in the model are named in Figure 5.16. Eight of the genes were more highly expressed in the *'alive and cured'* patients compared to the *'dead from disease'* cases and 11 were expressed more highly in the *'dead from disease'* patients compared to the *'alive and cured'*. Only one of the 19 genes was shared in common with the 100 most discriminating genes for *'alive and cured'* and *'fatal/refractory disease'*: retinoic acid receptor related orphan receptor A. This gene is expressed in mesenchymal stem cells derived from bone marrow, where cells undergoing osteogenic differentiation show increased expression. Frustratingly, application of the SBH generated 19-gene model to the whole cohort of 58 patients failed to accurately predict the outcome class, as illustrated in Figure 5.12.

		Predicted Class		
		Alive and cured	Dead from disease	Total
Observed Class	Alive and cured	11	**1**	12
	Dead from disease	**0**	7	7
	Total	11	8	19

Table 5.4: Confusion matrix summarising the leave-one-out cross-validation prediction results.

Figure 5.11 Hierarchical Cluster Dendrogram Produced by the 19 most discriminating Genes for *'Alive and cured'* vs. *'Dead From Disease'* in the SBH Patients.
ND = Not Dead = Sample from an Alive and cured Patient

D = Dead = Sample from a patient dead-from-disease/ with refractory DLBCL.

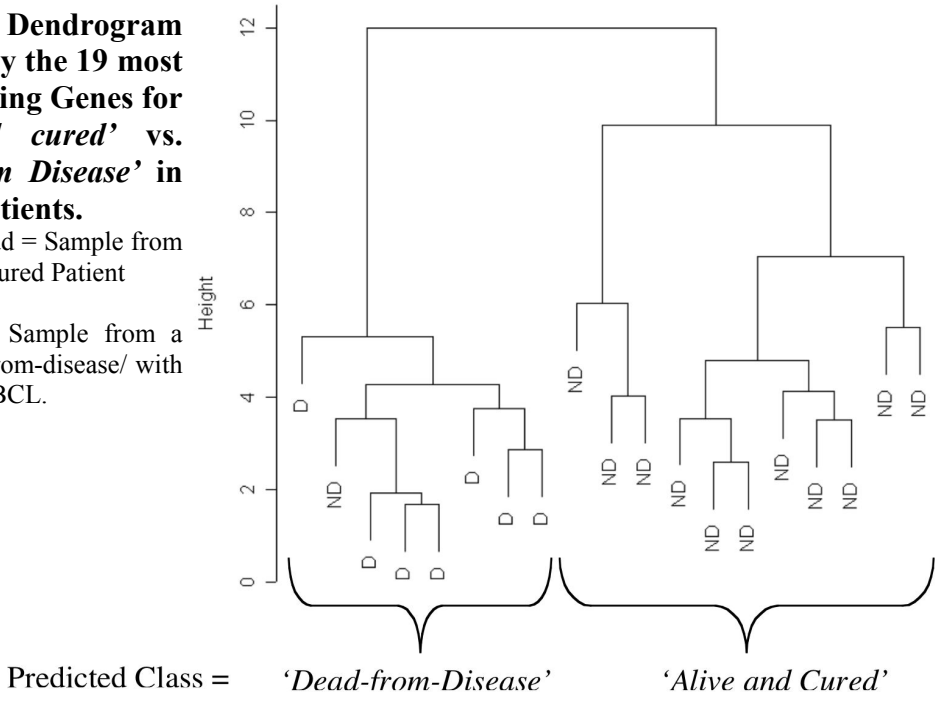
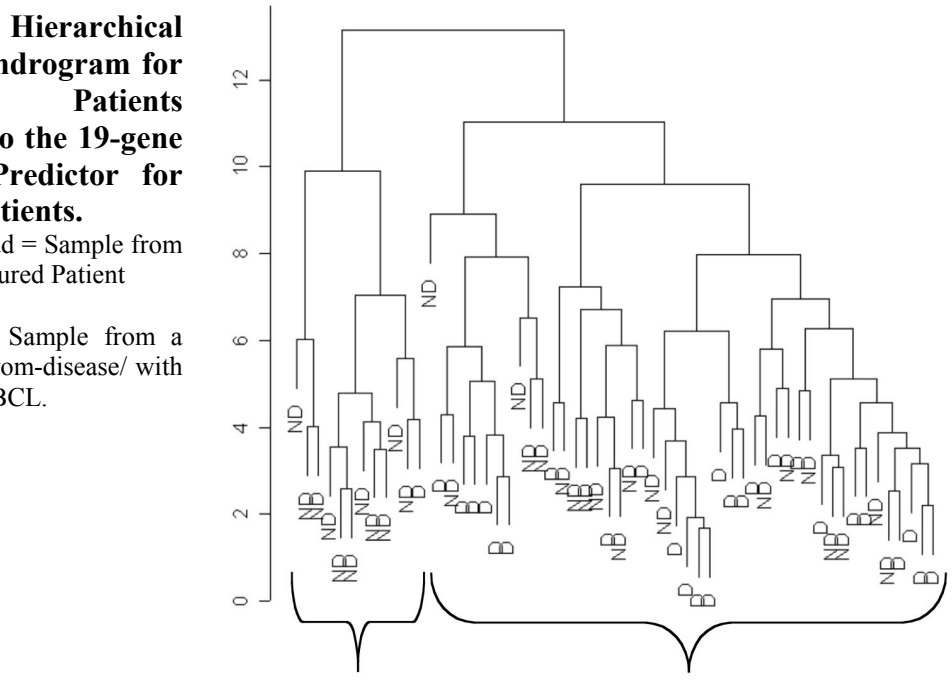


Figure 5.12 Hierarchical Cluster Dendrogram for the 58 Patients according to the 19-gene Outcome Predictor for the SBH Patients.
ND = Not Dead = Sample from an Alive and cured Patient

D = Dead = Sample from a patient dead-from-disease/ with refractory DLBCL.



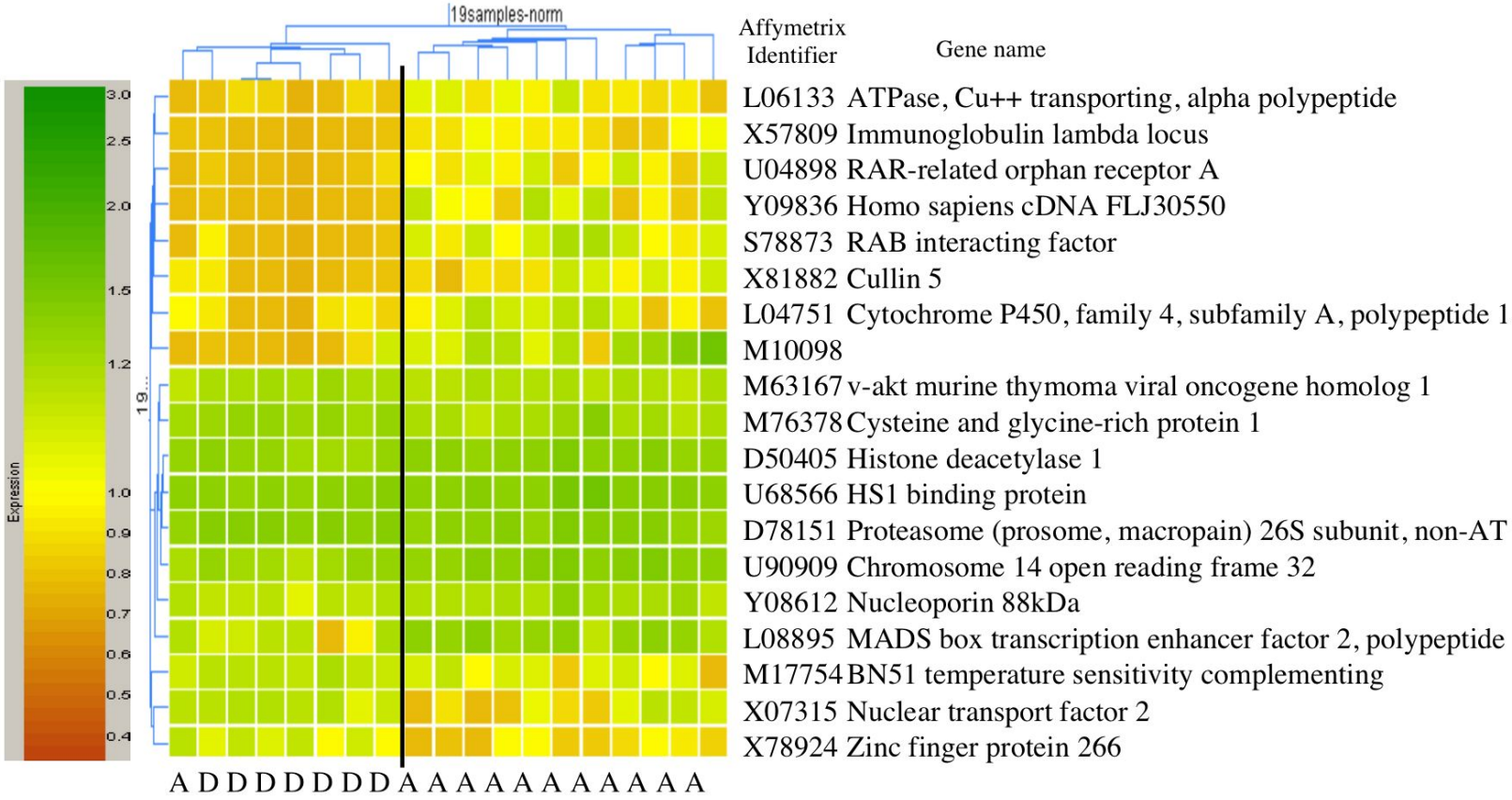


Figure 5.13 Dendrogram Produced by the 19 most discriminating Genes for *'Alive and Cured*' vs. *'Dead From Disease'* in the SBH Patients Illustrating the 19 Genes Identifiers. Each column represents a patient sample and each row a gene. The eight samples classified as *'dead-from-disease'* are to the left of the black line and the 11 samples classified as *'alive and cured'* are to the right of the line. To the right of the Figure, the Affymetrix identifiers and names of the 19 genes are displayed. Green represents high relative expression and orange represents low relative expression. To the left of the Figure the threefold variance in expression is colour quantified.
A = *'alive and cured'*. D = *'dead-from-disease'*

5.5 Discussion

5.5.1 Review of Experimental Findings in Comparison with Published Data

Chapter Results Summary

The research detailed in this Chapter achieved the ambitious aims of creating a molecular classifier capable of distinguishing DLBCL from the closely related FL; of identifying genes that were differentially expressed in pre-treatment DLBCL samples according to the post-treatment survival outcome of the corresponding patients; and producing a highly effective 13-gene outcome predictor model that was at least as effective as the best clinical tool, the IPI. The 13-gene model captured aspects of DLBCL biology independent of the IPI and, in conjunction with the IPI, produced the most accurate predictor of outcome to treatment in DLBCL yet described. *In silico* and IHC validation of the 13-gene model confirmed components of it to have prognostic significance. Finally, a separate analysis of the SBH cohort resulted in the creation of a tailored SBH specific 19-gene outcome prediction model. Because all the patients from whom the DLBCL material came received chemotherapy, it was not possible to clarify if the 13- and 19-gene models were also predictive of outcomes to treatment or simply prognostic models.

Summary of the Preceding NCI Microarray Paper

The preceding 2000 paper from the NCI-led consortium, which became the LLMPP, took a different approach to array profiling in DLBCL to that of the DFCI-led group's results described in this Chapter. Alizadeh *et al* also sought to realise a very different goal, namely discovery of molecular subtypes of DLBCL defined by *'cell-of-origin'*. As well as using the alternative type of array, a '*dot-blot*' cDNA array, Alizadeh *et al* analysed different types of malignant and non-malignant lymphoid samples and employed hierarchical clustering to segregate the samples. Specifically, a custom-made cDNA array, the Lymphochip, was created, which comprised 17,856 cDNA clones from a GC B-cell library, B-cell lymphoid malignancies and from genes of importance to tumour biology (Alizadeh *et al.*, 2000). Forty-two presentation DLBCL cases were arrayed alongside 56 other samples derived from benign T- and B-cells, tonsil and lymph node, malignant B-cell-lines and primary FL and CLL samples. A reference cDNA probe was prepared from a pool of mRNAs isolated from nine different lymphoma cell-lines. After preprocessing, a dataset containing 4,026 array elements remained. Hierarchical clustering segregated the samples from the three different

lymphoid malignancies correctly in all but two cases (Figure 5.14). The following gene expression 'signatures' were then created: *'T-cell'*, *'Activated B-cells (ABC)'*, *'lymph node'*, *'resting B-cell'*, *'germinal centre B-cell (GCB)'*, *'proliferation'* and *'major histocompatibility complex'* ('MHC' signature). The DLBCL samples were found to express the *'lymph node'* signature, the *'proliferation'* signature (silent in the FL and CLL samples) and to variably express the *'T-cell'* and *'GCB'* signatures. In the belief that *'GCB'* defined clustering would reveal subtypes of DLBCL arising from different stages of normal B-cell differentiation, Alizadeh *et al* reclustered the DLBCL samples using only the expression pattern of the genes that defined their *'GCB'* signature. A two branch Dendrogram resulted, with one subgroup being named GCB-DLBCL and the other being named ABC-DLBCL.

Alizadeh *et al* went on to create Kaplan-Meier OS curves for the 40 patients whose clinical data was available, and found the 5-year OS of the patients with DLBCL subclassified as GCB was 76% compared to only 26% for the patients with array-defined ABC DLBCL. This difference in OS led the group to make this statement: *"the molecular differences between these two kinds of lymphoma were accompanied by a remarkable divergence in clinical behaviour, suggesting that GC B-like DLBCL and activated B-cell DLBCL should be regarded as distinct diseases"*. The GCB vs. ABC distinction added to the outcome predictive ability of the IPI in the low-risk but not the high-risk IPI subgroupings.

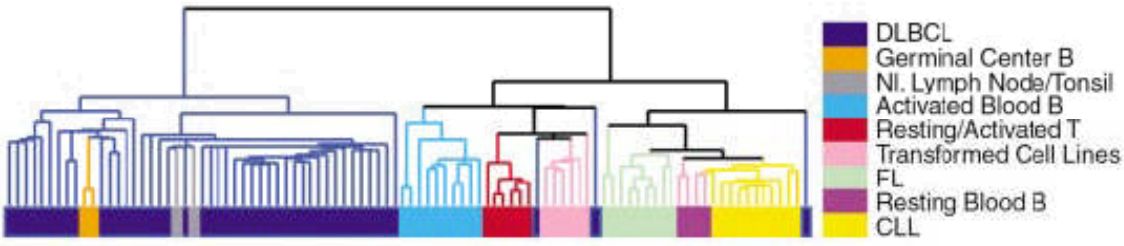


Figure 5.14 Dendrogram of hierarchical clustering of the Lymphochip derived gene expression data. The dendrogram illustrates the hierarchical clustering of gene expression data, depicting the relationships between the 96 samples of normal and malignant lymphocytes analysed. The closer a sample is to another in the dendrogram the greater the relatedness of gene expression between the samples. The dendrogram is colour-coded according to the category of mRNA sample studied as per the right-hand key. It can be seen that all the FL and all the CLL samples are clustered together and that the germinal centreB-cells and lymph node and tonsil samples sit within the DLBCL samples, which are bordered by the activated B-cell samples (adapted from (Alizadeh *et al.*, 2000)).

Critique of the Preceding NCI Microarray Paper

I consider that the Alizadeh *et al* paper was a milestone in DLBCL translational research. The limitations of the paper have come to light as this research field has matured. Firstly, too few samples were used to define the pivotal signatures of *'T-cell'*, *'ABC'*, *'GCB'*, *'proliferation'* and *'lymph node'*. Additionally, the process of signature assignment and selection was informal and subjective, using no criteria or algorithm. The definition of the non-GCB subgroup as ABC was imposed from amongst the hundreds of genes differentially expressed between the GCB and non-GCB DLBCL. It is likely that a mixture of other genes, particularly those from the *'proliferation'* signature, could have better defined the non-GCB group.

A potential weakness of both the Lymphochip and the Affymetrix Hu6800 is their lack of comprehensive representation of the entire genome, an issue addressed by the latest Affymetrix chips. The limited gene repertoire of the two arrays has hindered comparative studies of the rival group's strategies and assertions due to only a minority of genes that were differentially displayed on each array being present on the other. The unanswered question for both arrays is just how many genes critical to DLBCL biology were excluded or absent.

In terms of providing an efficient predictor of outcome to empirical chemotherapy, the differential post-treatment outcome of GCB vs. ABC DLBCL was a beguiling finding. Nevertheless, I suspect that more effective outcome prediction clusterings were missed due to the application of the *'cell-of-origin'* supervised classifier rather than performance of a separate, supervised search specifically addressing this critical clinical issue and I concur with others that this secondary assessment of the *'cell-of-origin'* classifier is inferior to the use of direct supervised methods (Smyth *et al.*, 2003).

When compared to the 13-gene model's results, the GCB vs. ABC classifier proved less efficient within its own series and proved ineffective at predicting OS post-treatment within the series described in this Chapter. Part of this discrepancy will come from the fact that the 13-gene model was the optimal for the 58 samples/patients studied by ourselves, whilst the GCB vs. ABC classifier was created for another purpose and found to have a secondary ability to predict OS. For both series the issue of overfitting and transferability is raised, due to the absence of validation series.

In conclusion, the Alizadeh *et al* paper, established a benchmark in terms of gene expression-defined subclassification of lymphoma and that the majority of the

reservations relating to it have been addressed by subsequent research, as will be described below.

Summary of the Subsequent LLMPP DLBCL Microarray Paper

The follow-on 2002 LLMPP paper confirmed and extended the findings of Alizadeh *et al* and validated the strategy of outcome prediction pursued in this Chapter (Rosenwald *et al.*, 2002). On this occasion, 240 presentation DLBCL samples were arrayed using the Lymphochip, so by overcoming the common weakness of the DFCI/SBH paper described in this Chapter and the 2000 NCI paper, namely small series size. For each sample, corresponding patient's clinical data was available and all patients had received anthracycline-based chemotherapy, with an average follow-up of 4.4 years and 138 deaths in the cohort. As in their preceding paper (Alizadeh *et al.*, 2000), the group sought to impose a *'cell-of-origin'* expression based classifier on the series using hierarchical clustering, and to search for an expression-based predictor of outcome (OS) independent of the *'cell-of-origin'* classifier using supervised learning. The dendrogram produced by application of hierarchical clustering according to the top 100 from the nearly 400 genes used in the Alizadeh *et al* series for the GCB vs. ABC division yielded three not two subgroups. Two of the three corresponded to the GCB (48%) and ABC (22%) subtypes of the first paper, along with a third robustly distinct subgroup named *'Type 3'* DLBCL (30%), which expressed neither set of genes at a high level. OS after anthracycline-based chemotherapy differed significantly among the subgroups, as in the Alizadeh *et al* series (P <0.001). Patients with GCB-DLBCL had a significantly better 5-year OS rate of 60%, compared to 39% for patients with Type 3 DLBCL and 35% for those with ABC-DLBCL, in keeping with the previous study (P < 0.01). However, it is clear from these percentages that the *'cell-of-origin'* molecular classifier was less effective at outcome prediction than in the first series of 40 patients and failed to capture a significant proportion of the determinants of OS.

The remarkable ability of gene expression profiling to extract critical innate molecular properties from presentation DLBCL that heavily influence treatment outcome, as first demonstrated in the research detailed in this Chapter, was then confirmed by the LLMPP. Application of a Cox proportional-hazards model by Rosenwald *et al* identified 670 significantly associated with a good or bad outcome in the training group of 180 samples (P <0.01). Just over half of the genes associated with OS could be classified into four of their signatures: *'GCB'*=15 (all genes associated with a

favourable outcome), *'MHC'*=35 (all genes associated with a favourable outcome), *'lymph node'*=32 (30 genes associated with a favourable outcome), *'proliferation'*=293 (287 associated with a poor outcome), leaving 44% (295) OS-associated genes as unclassified. Rosenwald *et al* chose 16 out of 17 genes from amongst their signatures to create an outcome predictor (3 GCB genes, four MHC class II genes, six lymph node genes, and three proliferation genes). After ranking the patients according to their 17-gene model score and dividing them into quartiles, a significant difference in 5-year OS was produced in both the training and validation sets of patients/samples. For the whole group the 5-year OS rates were 73 %, 71 %, 34 %, and 15 % in quartiles -1 to -4 respectively. Although the 17-gene model captured different prognostic information from the IPI in this cohort of patients and samples, the upper two and the lower two quartiles needed to be combined to produce differences in OS within the low/intermediate risk IPI and within the few high-risk IPI patients. The 17-gene outcome predictor score could also be used to subdivide the patients within each of the *'cell-of-origin'* subgroups into distinct survival risk groups, confirming the 17-gene model's greater prognostic power compared to the *'cell-of-origin'* classifier.

Critique of the Subsequent LLMPP DLBCL Microarray Paper

The creation of a third, unclassified, subgroup by the GCB/ABC classifier, which constitutes a third of cases and is in fact larger than the ABC subgroup, confirms that the *'cell-of-origin'* classifier is far from comprehensive. *'Type 3'* DLBCL may truly represent a hidden stage (or stages) of B-cell development or it could indicate that this classifier is inappropriately named, reflecting instead dominant molecular pathways disrupted in DLBCL. The imposition of the '*cell-of-origin'* order on the gene expression data may in fact have merely imposed constraints and as such risk having missed important associations and findings. Louis Staudt, head of the LLMPP, has speculated that the cell of origin for the ABC subclass is the plasmablastic B-cell on the brink of exiting the germinal centre and that GCB-DLBCL are derived from normal germinal centre B-cells and retain much of their biology (Staudt and Dave, 2005). The extrapolation from the LLMPP array data by Staudt *et al* to the unproven is illustrated well by this statement *"These three DLBCL subgroups [ABC, GCB and PMBCL [*primary mediastinal B-cell lymphoma*]] should be considered separate disease entities since they arise from B-cells at different stages of differentiation, utilise different oncogenic pathways, and have distinct clinical behaviours."*(Staudt and Dave, 2005).

Gone is the *'Type 3'* DLBCL subclass conveniently swept under the array bench and in comes the long recognised clinical subtype of DLBCL, PMBCL, demonstrated, not surprisingly by both the DFCI and LLMPP consortia to display a reproducibly distinct expression signature from DLBCL (Feuerhake *et al.*, 2005, Rosenwald *et al.*, 2003).

Nevertheless, the different approach taken by the LLMPP, compared to the DFCI consortium, has opened up a tantalising area of research: the division of the heterogeneous DLBCL subtype of NHL into distinct, molecularly defined subtypes according to the stage in normal B-cell development that malignant transformation arose.

As with the 100-gene '*cell-of-origin'* classifier, imposition of 16 genes in the 17-gene model for post treatment outcome prediction from amongst the LLMPP's signatures is likely to have imposed constraints and forfeited inclusion of important genes of unknown function. Frustratingly, no gene present in the 13-gene model is present in the 17-gene model for outcome prediction and vice versa. Certainly, the lack of genes in common between the two different arrays used will partly explain this. The concern remains that for both series, the outcome prediction models were curated for their datasets to the extent that they demonstrated maximal intrinsic predictive power but lost the critical ability to be universally applicable to patients and their samples treated with anthracycline-based chemotherapy.

The Rosenwald *et al* paper successfully confirmed and extended the results of the 2000 NCI paper (Alizadeh *et al.*, 2000). Consequent IHC, CGH and molecular studies have supported GCB-DLBCL and ABC-DLBCL being distinct subtypes of DLBCL with different aetiologies. The *'cell-of-origin'* classifier retained prognostic significance, whilst a 17-gene model predicted outcome to CHOP-like chemotherapy and captured distinct prognostic information compared to the *'cell-of-origin'* classifier and the IPI.

Non-array validation of distinct pathways in DLBCL Lymphomagenesis

In terms of the ABC vs. GCB as an aetiological classifier, supporting evidence has been provided by cytogenetic and CGH studies. *BCL-2* translocations are seen in 45% of GCB-DLBCL and are absent in ABC-DLBCL cases (Rosenwald *et al.*, 2002, Huang *et al.*, 2002). Similarly, amplification of the *REL* locus on chromosome 2p appears restricted to a minority of GCB-DLBCL and is absent in ABC-DLBCL (Bea *et al.*, 2005, Rosenwald *et al.*, 2002). Another distinction between ABC- and GCB- DLBCL is the presence of ongoing somatic hypermutation only in the GCB subgroup (Lossos *et*

al., 2004). Lastly, certain CGH detected gains and losses were seen more frequently in the different subtypes: ABC-DLBCL (trisomy 3, gains of 3q and 18q21-q22, and losses of 6q21-q22), GCB-DLBCL (gains of 12q12) and PMBCL (gains of 9p21-pter and 2p14-p16) (Bea *et al.*, 2005). However only gain of 3p11-p12 was an independent adverse prognostic factor, an abnormality not unique to the ABC subset.

Summary of the Subsequent DFCI DLBCL Microarray Paper

In 2005 the DFCI consortium published their second paper reporting the results of expression profiling of presentation DLBCL material – the follow-on paper to that described in this Chapter (Monti *et al.*, 2005). In this research two weaknesses of the experiments described in this Chapter and the first paper from the LLMPP were overcome, namely small cohort size and a restricted gene set on the microarray. A large cohort of 176 presentation DLBCL samples were arrayed and the most comprehensive array developed, the Affymetrix HG-U133 A and B series (39,000 transcripts, representing 33,000 genes) was used (Monti *et al.*, 2005). For 130 samples, corresponding patient clinical data was available and all had received CHOP-based treatment. Monti *et al* set out to molecularly subdivided DLBCL into robust subtypes. Instead of using supervised hierarchical clustering and the 100-gene '*cell-of-origin'* classifier of the LLMPP, the DFCI consortium followed the example of Todd Golub (the DFCI microarray project leader) in his seminal report on the molecular classification of acute leukaemia using self-organising maps in an unsupervised analysis, as described in Chapter 1.4.14 (Golub *et al.*, 1999). To ensure the reproducibility of any classifier created by the self-organising maps method, two other analysis tools were used to verify the findings: model-based probabilistic clustering and hierarchical clustering. Monti *et al* discovered that the DLBCL cohort could be divided into subgroups of between two and nine in number. After testing for stability by consensus clustering, a consensus matrix was constructed based upon sample assignment agreement. The result was the same using the three analysis tools: three distinct clusters, with 80% of the samples being assigned to the same cluster by the three algorithms. Again a different strategy to that of the LLMPP was used in order to bring meaning to the three clusters/subgroups produced. Rather than impose *'cell-of-origin'* signatures upon the three clusters, gene set enrichment analysis was performed to identify groups of genes differentially expressed in the three clusters from within 281 recognised gene sets. The first cluster (termed *'OxPhos'*) was found to be significantly

enriched for oxidative phosphorylation, mitochondrial function and electron transport chain genes. The second cluster (termed *'BCR/Proliferation'*) had greater expression of cell cycle regulatory genes, DNA repair genes, and components of the BCR-signalling cascade. The third cluster (termed *'Host Response'*) appeared to be largely defined by the host response rather than the tumour itself, due to increased expression of T-cell receptor components, molecules associated with T/NK (natural killer) -cell activation and the complement cascade and markers of ongoing inflammation/immune response. Using all three analysis techniques, 28% of samples were assigned to the *'OxPhos'* cluster, 28% to the *'BCR/Proliferation'* cluster, 24% to the *'Host Response'* cluster and 16% were inconsistently classified. The 5-year OS for the patients assigned to each of the three clusters were similar. Importantly, thanks to the comprehensive array used, sufficient genes were found in common with the Lymphochip for the three consensus clusters to be successfully reproduced from within the LLMPP series of 240 DLBCL. The DFCI molecular classifier of DLBCL was therefore reproducible in the other large DLBCL gene expression dataset created using a different array. Importantly, no correlation between *'cell-of-origin'* subgrouping and the *'OxPhos, BCR/Proliferation, Host Response'* consensus clustering was discovered, indicating that the two classification systems were capturing largely different aspects of DLBCL biology.

Critique of the Subsequent DFCI DLBCL Microarray Paper

The major disappointment with the paper of Monti *et al* is in what it didn't say rather than what it did. There is no mention of any attempt to investigate the ability of the 13-gene or the 17-gene models, or the *'cell-of-origin'* classifier at outcome prediction in the 130 samples with allied clinical data. This constitutes a surprising omission, given that this was the major achievement of the group's first paper - the subject of this Chapter. No independent analysis of this dataset has been published addressing this issue.

In conclusion, Monti *et al* successfully used a different approach to gene expression profiling to that of the LLMPP, so by creating a novel molecular classifier of DLBCL and revealed new insights into the molecular aetiology of DLBCL. In particular, this paper gives credence as much to the non-malignant milieu as to the malignant characteristics of DLBCL in terms of influencing tumour biology and response to treatment.

DLBCL Microarray Data from Tumour Samples of Patients Treated with CHOP-R-like Treatment

IN 2007 an ASH meeting oral presentation by the LLMPP described the results of gene expression profiling of 156 DLBCL samples from previously untreated patients using the Affymetrix U133 plus chips (Lenz *et al.*, 2007). All patients received rituximab and CHOP-like chemotherapy. Seventy one DLBCL samples were classified as GCB-DLBCL, 63 as ABC-DLBCL, and 22 as Type 3. After a median follow-up of 2.3 years, addition of rituximab to CHOP-like chemotherapy GCB-DLBCL had a superior OS compared to ABC-DLBCL, with 3-year OS rates of 86% for GCB-DLBCL vs. 68% for ABC-DLBCL ($P = 0.014$). The 3-year OS rate of unclassified DLBCLs was 69%. The *'lymph node'* signature was associated with favourable OS ($p = 0.023$) and the *'proliferation'* signature with inferior OS ($p = 0.009$). Therefore the previously described findings of the LLMPP (detailed above) have held true on addition of rituximab to chemotherapy.

Quantitative Real-Time Polymerase-Chain-Reaction Analyses: an Alternative to Microarray Gene Expression Profiling?

Lossos *et al* sought to make the findings described in this Chapter and those of the LLMPP simpler to replicate and of prognostic significance in both of these, and hopefully future, series by restricting the genes investigated to 36 and using RQ-PCR to quantify gene expression (Lossos *et al.*, 2004). The 36 genes were investigated for correlation with OS in 66 newly diagnosed DLBCL patients using univariate Cox proportional models. The six most significant genes were included in a multivariate proportional hazards model, coefficients derived and the cases stratified into three prognostic groups – low-, intermediate- and high-risk. Lossos *et al* then validated their 6-gene model in both the DFCI/SBH 58 DLBCL series and the LLMPP 240 DLBCL series, demonstrating that the model could stratify into three groups with different OS in the Rosenwald *et al* series and two groups in the Shipp *et al* series with the intermediate and high risk having similar OS in the latter series.

Last year the same 6-gene model was proven to still be predictive of outcome following R-CHOP therapy for DLBCL (Malumbres *et al.*, 2007). Using RNA extracted from 100 patient's paraffin-embedded specimens (up to six years old) the expression of the six genes comprising the model was measured in these samples and the mortality-prediction score was calculated for each patient. The 3-year OS was 80% in the low risk

and 50% in the high risk groups, respectively. The predictive power of the 6-gene model was independent of the IPI prognostic factors for prediction of OS ($P = 0.06$) and PFS ($P = 0.01$) in these patients. If validated in an independent patient series, the 6-gene model could be easily added to routine clinical investigation in major treatment centres.

***In Silico* Reanalyses of DLBCL Microarray Datasets**

At least five papers have reanalysed the DFCI/SBH dataset described in this Chapter and/or the LLMPP dataset of 240 DLBCL to produce models predictive of OS. Matsui used his group's novel prediction models to create an 85-gene model to predict OS for the LLMPP patients (Matsui, 2006). Bair and Tibshirani arrived at a 23-gene model predictive of OS using semi-supervised methods and est statistics (Bair and Tibshirani, 2004).

Because the *'cell-of-origin'* classifier failed to predict for survival in the DFCI/SBH series described in this Chapter, Wright *et al* reanalysed the later LLMPP dataset of 240 DLBCL in an attempt to reconcile the disparate findings of the two groups in regard to the outcome predictive ability of the *'cell-of-origin'* classifier (Wright *et al.*, 2003). Using Bayes rule and 'compound covariates/linear predictor scores' to distinguish between the subtypes of GCB- and ABC-DLBCL, a 27-gene model was derived from those genes differentially expressed between the *'cell-of-origin'* subgroups in the Rosenwald *et al* series. Using this model, Wright *et al* were able to reproduce GCB and ABC subgroups with significantly different 5-year OS in the LLMPP series and also in the DFCI/SBH series of samples and patients (using the 14 out of the 27 genes form the model present on the Hu6800). For the DFCI/SBH dataset, the 5-year OS rates for the GCB and ABC DLBCL patients were 62% and 26%, respectively ($P = 0.005$) (Wright *et al.*, 2003). Significant results but still considerably less predictive than the 5-year OS of 70% and 12% for the *'alive and cured'* and *'fatal/refractory disease'* groups defined by the 13-gene model ($p = 0.00004$) (Shipp *et al.*, 2002).

In a further paper, two of the principal concerns with the data analysis methods of Rosenberg *et al*, namely the subjective and informal means of signature assignment and selection, led Segal *et al* to reanalyse the LLMPP 240 series data using a range of techniques (Senftleben *et al.*, 2001). Segal *et al* concentrated on searching for gene expressions associated with outcome. They found that an L_1 penalised proportional hazards method (Gui and Li, 2005) produced the best predictor of outcome followed by the original 17-gene model of Rosenberg *et al*. Using area under time dependent ROC

curves, Segal *et al* found that the predictive powers were 67% and 65% for the L_1 penalised proportional hazards method and 17-gene model respectively. Segal *et al* also sought evidence that the outcome predictive models from the LLMPP and the DFCI/SBH series were transferable to the competitor's dataset. After restriction to the genes from the Lymphochip common to the Hu6800 Affymetrix array, use of the L_1 penalised proportional hazards method resulted in a 3-gene model, which had a 65% predictive power in the LLMPP series it was derived from, but a lower 60% predictive power in the DFCI/SBH series. Conversely, the 6-gene model, produced from the DFCI/SBH dataset, when subjected to the L_1 penalised proportional hazards method resulted in a 78% predictive power for post-treatment outcome in the DFCI/SBH series it was derived from but only a 53% predictive power for OS estimation in the rival LLMPP series i.e. little better than by chance alone. The 6-gene model of Lossos *et al*, described above (Lossos *et al.*, 2004), fared badly in both series, with OS predictive power of 59% and 61% in the LLMPP series and the DFCI/SBH series respectively.

Are the Results of this Chapter still of Importance?

Distinguishing FL from DLBCL by Gene Expression Profiling

This was a major achievement. The utility of the 30-gene discriminator in other datasets and the development of a successor created from the more recent and comprehensive Affymetrix arrays are awaited. It is expected that investigation of differential expressed genes between FL and DLBCL will help unravel their respective pathogeneses and suggest potential treatment targets for investigation.

Distinguishing *'alive and cured'* from *'dead-from-disease/refractory'* Patients with DLBCL

This was an attention grabbing achievement and remains so as an exercise in proof of principle: namely supervised analysis of gene expression data can produce a post-treatment outcome predictor from pre-treatment malignant tissue. That such a predictor can be distilled into a small number of genes from the many differentially expressed between the two outcome groups is remarkable. The independence of the molecular predictor from the clinical IPI gives the promise of long-term utility of such models and strengthened the predictive power of a combined molecular and clinical score.

SBH Cohort Specific Results

The finding that a completely different 19-gene set to the 13-gene model proved more effective at outcome prediction within the 19 SBH but not the 37 DFCI patients and

samples is initially alarming but on reflection not surprising. There is a massive imbalance between data points and samples in microarray experiments, with a multitude of varying genes exhibiting differential expression. The top genes for such discriminations vary depending firstly on the genes present on the arrays, next according to the thresholding used and lastly according to the analysis techniques employed. Thus for the DFCI/SBH dataset three outcome predictors are effective in the SBH cohort (the original 13-gene model, the Wright *et al 'cell-of-origin'* 14 of 27-gene model and the SBH specific 19-gene model), whilst in the LLMPP 240 DLBCL series five different models have been published that are capable of outcome prediction (as detailed above), none of which contain anything but a few overlapping genes with the original 17-gene model. The disappointment of this revelation is that the holy grail of a universally applicable molecular outcome predictor is no longer readily in sight. I anticipate that many hundreds to several thousands of similarly treated patients will need presentation material to be arrayed using the same array for the core of universally applicable outcome predictive genes to be truly identified. Furthermore, the change of empirical treatment from chemotherapy to immunochemotherapy necessitates repeat microarray investigation to ascertain the relevance of the described outcome predictive expression models and to search for new prognostic expression signatures.

Shipp vs. Staudt: which strategy will triumph?

Microarray Platform

Realistically the lymphochip, as used in the LLMPP papers will not become standard clinical kit nor will a version with a greatly reduced number of features. It simply cannot be manufactured with adequate provision of identical control cDNA for worldwide research or clinical utility. The Affymetrix system is more quantitative and allows results to be interpreted and combined between series and institutions, as no control cDNA is required. Equally the Affymetrix chips used to date will not be widely used at the clinical level: the Hu6800 has already been withdrawn due to larger, more comprehensive chips succeeding it (e.g. the HG-U133 PLUS chip featuring 47,000 transcripts, which represent 38,000 genes). Indeed the most recent LLMPP DLBCL microarray study used the HG-U133 PLUS chip (Lenz *et al.*, 2007). Even the most recent chips are not useful to molecular pathology laboratories long-term due to the gross redundancy of features. Quite simply there are too many genes of no relevance present, making the complexity of analysis and the cost prohibitive. The development of

custom-made chips with a much reduced number of DLBCL relevant features capable of translating outcome predictive molecular insights from DLBCL into sufficiently robust, affordable and practical tests to allow them to be used clinically. The fashion for Bcl-6 and CD-10 IHC may be a complementary response by pathologists to the ABC vs. GCB distinction but it remains of little clinical relevance, as there are no ABC vs. GCB defined treatment decisions to be made as yet. The treatment of DLBCL remains empirical and still largely generic despite the introduction of rituximab and dose-dense therapy as described in Chapter 1. Until more treatment choices are available, the molecular prediction of which treatment to use remains impossible and all outcome predictive gene expression models remain solely prognostic and not predictive.

Aetiological Molecular Classification

In terms of aetiological molecular classifications I think Staudt's *'cell-of-origin'* classifier will be (and needs) refinement due to the '*Type 3*' subgroup comprising more than the 'ABC' subgroup and the latter subgroup being arbitrarily if apparently successfully defined. The DFCI consortium approach of consensus clustering followed by gene set enrichment analysis looks more promising in terms of identifying the multiple pathways and processes critical to lymphomagenesis and potentially allowing complex mapping of serially acquired abnormalities along the lines of Horsman *et al* with CGH abnormalities in FL (Hoglund *et al.*, 2004). One potential unifier of the LLMPP and DFCI hypotheses is that the malignant cell signatures are divisible by a *'cell-of-origin'* classifier and that consensus clustering dissects the non-malignant cellular component of the malignant phenotype. It will be important to perform analyses on the non-malignant and malignant cell populations individually and assess their contributions to the *'GCB*', *'ABC'* and *'Type 3'* division and *'OxPhos, BCR/Proliferation* and *Host Response'* subgrouping.

Gene Expression Outcome Prediction

In terms of gene expression models for outcome prediction, I consider Staudt *et al* to be ahead. Their 17-gene model, despite its subjective and informal construction, produced impressive results within the three risk groups defined by the IPI. If this model continues to stratify within the IPI subgroups following CHOP-R treatment it could well become established as an aid to risk estimation for patients and therefore treatment stratification within and outside of clinical trials. The hurdle of rapid and affordable

results production for such a labour intensive and costly test may limit such a molecular predictive tool's accessibility.

Although the 13-gene model was created in a more rational way to the rival 17-gene model (and proved highly effective in the 58 DLBCL cases and samples investigated) I feel it is unlikely to be used, due to it remaining of unproven utility beyond its original dataset. I anticipate that Shipp *et al* will create a successor to the 13-gene model for outcome prediction following rituximab-polychemotherapy using the vastly more comprehensive modern Affymetrix chips.

My concerns with gene expression models are that the choice of genes predictive of OS can clearly be influenced by the methods and dataset used to create it; that the variation in genes between models suggests that the number of genes with predictive power is likely to be considerably larger than at first thought and can be expected to grow, rather than shrink, as more genes and DLBCL samples are surveyed; and that application of a particular model to a new sample or series may not produce the individualised risk stratifying predictions so hoped for from gene expression profiling. The potential clinical application of upfront gene expression profiling to stratify patients and determine treatment is therefore likely to be further off than hoped. Ultimately I believe that expression profiling of pretreatment DLBCL will only ever provide part of the answer to treatment outcome, even in combination with the IPI or equivalent clinical variables. Potential closure of this area of uncertainty in prediction may come through pharmacogenomic analyses to create a tripartite predictor, which quantifies risk due to the patient's clinical condition, their lymphoma's biology and their drug handling characteristics.

Subsequent Research for this Thesis - Validation of the 13-gene Model

Through rational investigation using gene expression profiling a molecular predictor of OS was produced. To further investigate the 13-gene model constituents as molecular targets in DLBCL I went on to study inhibitors of PKCβ and PDE4B, two of the 13 gene-model's poor prognostic genes common to all of the 58 DLBCL individual predictor models on 'leave-one-out' cross-validation. Furthermore, both PKCβ and PDE4B were predictive of OS in the Alizadeh *et al* dataset and have recognised functions in haematological cells. If the overexpression of these two genes indeed has a direct influence upon DLBCL response and resistance to chemotherapy, inhibitors of

PKCβ and PDE4B used alone or in conjunction with established cytotoxics *in vitro* should induce cytostasis or cytotoxicity.

CHAPTER 6: IN VITRO INVESTIGATION OF THE RATIONAL TARGETS PDE4B AND PKCβ

6.1 Chapter Summary

Two of the seven poor-risk genes (PDE4B and PKCβ from the 13-gene outcome to treatment prediction model described in Chapter 5 were selected for *in vitro* inhibition to ascertain if either could be a novel rational target for therapy. *In vitro* studies were performed using the panel of DLBCL cell-lines and primary lymphoma cultures employed in Chapter 4.

PDE4B inhibition using the PDE4 inhibitors rolipram and the more potent and PDE4B selective piclamilast proved incapable of diminishing cell count, proliferation or viability, either alone or with the established chemotherapy drugs doxorubicin and cytosine arabinoside. This inactivity was despite evidence of PDE4 inhibition producing c-AMP elevation. When the c-AMP augmenter forskolin was used, cell count inhibition was seen in all four DLBCL cell-lines and the primary CLL/SLL sample 02. Forskolin produced a meaningful decrease in viability only in the most chemo-sensitive cell-line DoHH2. Combined exposure to a PDE4 inhibitor and forskolin proved no more effective than forskolin use alone. Isolated PDE4B inhibition therefore does not offer a rational, novel treatment strategy in DLBCL.

In contrast, the PKCβ inhibitor SC-236 was found to inhibit cell count and viability in all four DLBCL cell-lines and the four primary lymphoma samples cultured. PKCβ inhibition may therefore be a potential novel and rational means of increasing treatment efficacy in DLBCL.

6.2 Introduction

6.2.1 PDE4B as a Rational Target in DLBCL

The PDEs play a decisive role in cyclic nucleotide-mediated intracellular signalling through destruction, by hydrolysis of the 3'-5'-phosphodiester bond, of the ubiquitous second messengers cyclic guanosine 3'-, 5'- monophosphate (c-GMP) and cyclic adenosine 3'-, 5-' monophosphate (c-AMP). Intracellular c-AMP levels are also controlled by the rate of synthesis of c-AMP by adenylate cyclase, an enzyme activated by extracellular signals, so by allowing diverse extracellular signals to mediate intracellular responses via c-AMP and c-GMP. PKA, a serine-threonine type of protein, and EPAC (Exchange Protein directly Activated by c-AMP) are the best-described effectors of c-AMP mediated signals (Figure 6.1).

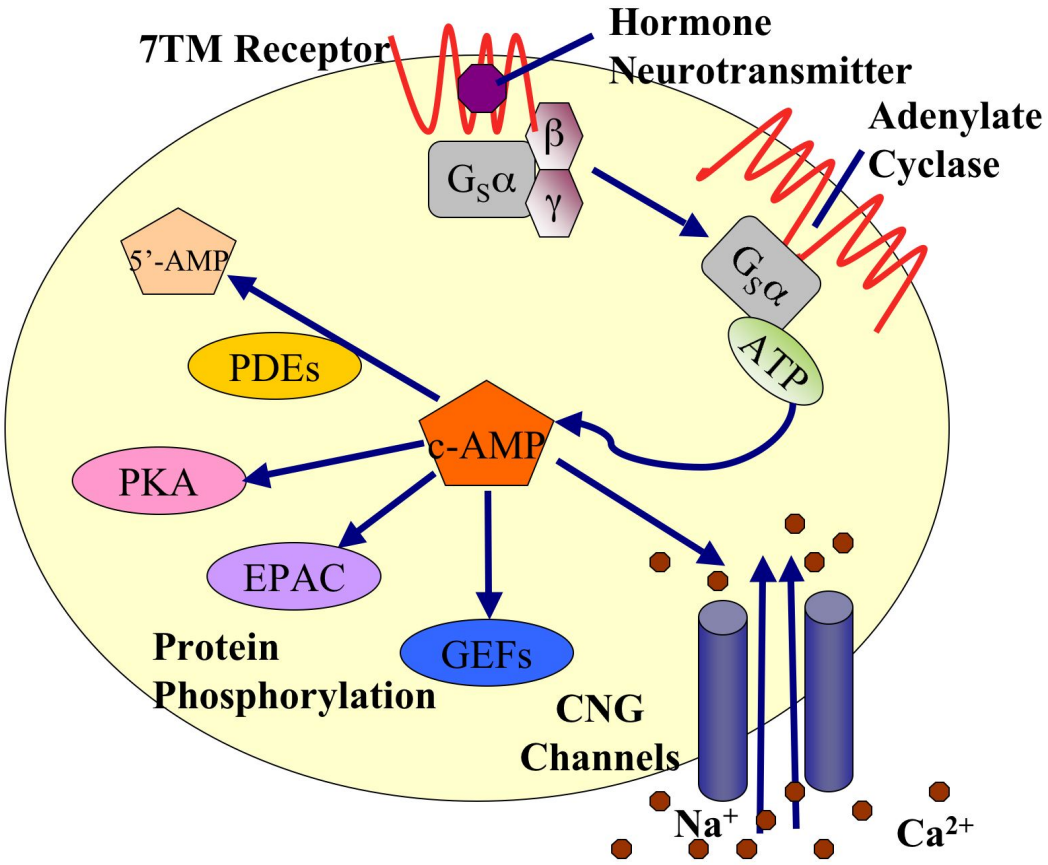


Figure 6.1 Basic c-AMP Regulation and Function. The diagram depicts the basic intracellular synthetic and regulatory pathways for c-AMP. CNG channels = Cyclic Nucleotide Gated channels; GEFs = Guanine-nucleotide Exchange Factors; EPAC = Exchange Protein directly Activated by c-AMP; PKA = Protein Kinase A; PDEs = Phosphodiesterases; 5'-AMP = 5' adenosine monophosphate; Gsα, β, γ = guanine nucleotide-binding stimulatory protein/adenylate cyclase stimulatory protein, alpha beta and gamma subunits; ATP = adenosine triphosphate; c-AMP = cyclic adenosine monophosphate. Adapted from (Beavo and Brunton, 2002)

Eleven PDE families have been described containing 50 different proteins and splice variants encoded by more than 20 genes, as summarised in Table 6.1 (Beavo, 1995, Soderling and Beavo, 2000). PDE iso-enzymes differ from each other in molecular structure, catalytic properties, intracellular regulation and location, and sensitivity to selective inhibitors. The common catalytic core of each PDE has 25-40% homology between PDE families and >80% homology within each PDE class.

PDE Isoenzyme	No of Genes	No. of Isoforms	Substrate	Km (μM) c-AMP	Km (μM) c-GMP	Tissue Expression	Specific/Selective inhibitors *	References
PDE 1	3	8	Ca^{2+}/calmodulin-stimulated	1–30	3	Heart, brain, lung, smooth muscle	KS-505a (0.17-13) Vinopocetine (5-25)	(Han *et al.*, 1999, Ichimura *et al.*, 1996)
PDE 2	1		c-GMP-stimulated	50	50	Adrenal gland, heart, lung, liver, platelets	EHNA (1) 8-MM-IBMX (0 4)	(Mery *et al.*, 1995)
PDE 3	2	4	c-GMP-inhibited, c-AMP-selective	0.2	0.3	Heart, lung, liver, platelets, adipose tissue, inflammatory cells	Cilostamide (0.005) Milrinone (0.3) Siguazodan (3)	(Nicholson and Shahid, 1994, Manganiello *et al.*, 1995, Sudo *et al.*, 2000)
PDE 4	4	20	c-AMP-specific	4		Sertoli cells, kidney, brain, liver, lung, inflammatory cells	Rolipram (1) Roflumilast Piclamilast (0.001)	(Hatzelmann and Schudt, 2001, Ashton *et al.*, 1994, Reeves *et al.*, 1987)
PDE 5	1	3	c-GMP-specific	150	1	Lung, platelets, vascular smooth muscle	Sildenafil (0.003) Zaprinast (0.3) Dipyridamole (0.9)	(Stacey *et al.*, 1998, Turko *et al.*, 1999)
PDE 6	4	4	c-GMP-specific		60	Photoreceptor	Zaprinast (0.15) Dipyridamole (0.4)	(Zhang *et al.*, 2005b, Gillespie and Beavo, 1989)
PDE 7	2	3	c-AMP-specific, high-affinity	0.2		Skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes	BRL-50481 (0.2)	(Smith *et al.*, 2004)
PDE 8	2		c-AMP-selective,	0.06		Testes, eye, liver, skeletal muscle, heart, kidney, ovary, brain, T lymphocytes	Dipyridamole (4-9)	(Soderling *et al.*, 1998)
PDE 9	1	4	c-GMP-specific,		0.17	Kidney, liver, lung, brain	BAY 73-6691 (0.06)	(Wunder *et al.*, 2005)
PDE 10	1	2	c-GMP-sensitive, c-AMP-selective	0.05	3.0	Testes, brain	SCH 51866 (1)	(Soderling *et al.*, 1999)
PDE 11	1	4	c-GMP-sensitive, dual specificity	0.7	0.6	Skeletal muscle, prostate, kidney, liver, pituitary and salivary glands, testes	Tadalafil (0.07) Zaprinast (11–33) Dipyridamole (1.8)	(Bischoff, 2004)

Table 6.1 The PDE Family. The PDE enzyme's ability to metabolise c-AMP and c-GMP, their tissue expression and best pharmacological inhibitors are displayed. * The numbers in brackets are the Ki or IC50 values, in mM, for the inhibitor against the PDE family in question. Table adapted from (Boswell-Smith *et al.*, 2006, Feil *et al.*, 2003).

The PDEs are differentially expressed in various cell types by virtue of different transcription initiation sites or by alternative mRNA splicing (Beavo, 1995, Soderling and Beavo, 2000). Three PDE families are c-AMP specific: PDE4, PDE7 and PDE8 (Conti *et al.*, 2003, Bloom and Beavo, 1996, Kobayashi *et al.*, 2003). PDE1 and PDE3 hydrolyse both c-AMP and c-GMP, whilst PDE2, PDE5 and PDE9 hydrolyse c-GMP preferentially (Degerman *et al.*, 1997, Meyer *et al.*, 2000, Rybalkin *et al.*, 2003, Huai *et al.*, 2004). PDE6 (rod PDE) is the key effector enzyme of vertebrate visual transduction and is specific to the retina (He *et al.*, 2000). PDE11A is the most recently cloned PDE belonging to a new family of unknown physiological significance (Fawcett *et al.*, 2000). PDE4 was selected as a rational target for inhibition in DLBCL because the gene appeared to be actively contributing to the chemoresistant phenotype rather than merely being a bystander gene for the following reasons: -

i. PDE4B was overexpressed in poor prognosis DLBCL patients in three independent microarray series.
ii. PDE4 is the principle phosphodiesterase in B- and T- lymphocytes.
iii. PDE inhibition has been proven to be cytotoxic in two other lymphoproliferative malignancies *in vitro*.
iv. PDE inhibitors are being actively developed for clinical use and a whole class of orally administered PDE4 selective inhibitors are approaching licensing.

i. PDE4B was overexpressed in poor prognosis DLBCL patients in three independent microarray series.

In the DLBCL microarray series described in Chapter 5, PDE4B was a component of the 13-gene model of all 58 cases, being overexpressed in the *'fatal/refractory disease'* patients (Table 5.2). Furthermore PDE4B was overexpressed in the poorer prognosis ABC-like patients in the LLMPP consortium series (Rosenwald *et al.*, 2002, Alizadeh *et al.*, 2000) and was overexpressed in the transformed FL biopsy series microarrayed by Davies *et al* (personal communication).

ii. PDE4 is the principle phosphodiesterase in B- and T- lymphocytes.

In B- and T- lymphocytes PDE4 is the major PDE family expressed followed by PDE7, with marginal PDE3 activity (Gantner *et al.*, 1998). The PDE4 family consists of PDE4A, B, C and D, with isoforms of each existing. PDE4B isoforms are the principal subtype seen in lymphocytes, where they exert differing effects depending on the type and physiological state of the lymphocytes. PDE4 isoforms, by virtue of their unique

intracellular targeting, play a pivotal role in controlling functionally and spatially distinct pools of c-AMP. Targeting occurs through association with proteins, such as arrestins, SRC family tyrosyl kinases, A-Kinase Anchoring Proteins (AKAPs) and Receptor for Activated C kinase-1 (RACK1) – reviewed in (Houslay and Adams, 2003). In T-cells, the c-AMP pathway promotes apoptosis and has an inhibitory action on T-cell proliferation (Kizaki *et al.*, 1990, Averill *et al.*, 1988). Specific inhibition of PDE4 preferentially blocks the production of Th1 versus Th2 effector cytokines *in vitro* (Claveau *et al.*, 2004). Similarly in GC B-cells and resting human peripheral blood B-cells, elevation of c-AMP levels promotes apoptosis (Knox *et al.*, 1993). For example the c-AMP-inducing agent forskolin caused a significant, concentration-dependent increase in cell death (relative to spontaneous death in medium alone) in resting human peripheral blood B-cells, an effect reversed by B-cell activation using IL-4 and TPA (O-tetradecanoylphorbol-13-acetate) (Lomo *et al.*, 1995). On the contrary, peripheral B-cell proliferation (in response to a mitogenic stimulus) can be further enhanced by pharmacological elevation of c-AMP (Gantner *et al.*, 1998). On stimulation of CD19+ peripheral blood lymphocytes with lipopolysaccharide and IL-4, c-AMP levels rose and increased further on addition of the PDE4 inhibitors rolipram and piclamilast, with further augmentation of their proliferative response. The effect of the PDE4 inhibitors could be partly replicated by using c-AMP analogues, whilst addition of an inhibitor of PKA, the major c-AMP effector, led to a decreased proliferative response in control cells and reversed the proliferative response induced by rolipram (Gantner *et al.*, 1998). These data suggest that the c-AMP-dependent second messenger system plays dramatically different roles in resting and activated peripheral B-cells and germinal centre B-cells, promoting apoptosis in resting B-cells and survival and proliferation in activated B-cells. These data indicate the potential importance of PDE4 inhibition in DLBCL but also raise the possibility of stimulation not inhibition of ABC-like DLBCL, if the findings of Gantner *et al* are valid and applicable to malignant B-cells.

iii. PDE inhibition has been proven to be cytotoxic in two other lymphoproliferative malignancies *in vitro*.

Data indicating cytostatic and cytotoxic activity of PDE4 inhibition in lymphoproliferative malignancies comes from *in vitro* work in ALL and CLL. In a glucocorticoid-resistant subclone of the human T-cell ALL cell-line CEM (CEM-GH), non-specific PDE inhibition by methylxanthines, PDE4 inhibition by rolipram and c-

AMP augmentation by forskolin-induced adenylate cyclase stimulation, all led to decreased CEM-GH cell proliferation in a concentration-dependent manner (Ogawa *et al.*, 2002). Forskolin proved the most potent of these drugs at inducing dexamethasone sensitivity in this glucocorticoid-resistant cell-line, increasing the sensitivity to dexamethasone 1,000-fold compared to just 5-fold for the PDE inhibitors (Ogawa *et al.*, 2002). In primary CLL cells *ex vivo* cell death was induced by PDE4 inhibition in conjunction with c-AMP level elevation by the adenylate cyclase potentiator forskolin, an effect further augmented by simultaneous PDE3 inhibition (Moon *et al.*, 2002, Moon and Lerner, 2003). Beyond Lerner and Moon's laboratory research a randomised clinical trial of chlorambucil ± theophylline in CLL found that the CR rate and the EFS were significantly better in the combination therapy arm (Mabed *et al.*, 2004). These results suggest the potential for efficacy of PDE4B inhibition in DLBCL.

iv. PDE inhibitors are being actively developed for clinical use and a whole class of orally administered PDE4 selective inhibitors are approaching licensing.

The clinical use of PDE inhibitors goes back more than 30 years to the introduction of the non-selective PDE inhibitors theophylline and aminophylline for the treatment of reactive airways diseases, due to their anti-inflammatory properties. The interaction of theophylline and aminophylline with other drugs and their narrow therapeutic indices has limited their use. Rolipram was the first selective PDE4 inhibitor to enter clinical practice, as an antidepressant, in the 1980's, leading to the second-messenger dysbalance hypothesis of affective disorders (Zeller *et al.*, 1984, Wachtel, 1990). However, the efficacy of rolipram proved to be no better than that of the tricyclic antidepressants with production of the troublesome side-effect of nausea (Scott *et al.*, 1991). In neurons, elevation of c-AMP promotes cell survival not cell death, in contrast to resting thymocytes and lymphocytes (Li *et al.*, 2000). Resurgent medical interest in rolipram and PDE4 inhibition has followed reports of its ability to promote functional recovery, axon growth, and attenuation of glial scarring after spinal cord injury, when combined with embryonic spinal or Schwann cell grafts in rodents (Nikulina *et al.*, 2004, Pearse *et al.*, 2004).

The development of more selective PDE4 inhibitors than rolipram has been actively pursued for the treatment of asthma and chronic obstructive pulmonary disease (COPD). Such inhibitors have demonstrated immunosuppressive and anti-inflammatory effects (Souness *et al.*, 2000). PDE4B inhibition appears to result in anti-inflammatory

effects whilst PDE4D inhibition leads to the undesirable side-effect of nausea through stimulation of neurons in the vomiting centre of the area postrema (Manning *et al.*, 1999). The two most advanced PDE4 inhibitors in clinical development are roflumilast and cilomilast, the latter being ten times more selective for PDE4D than PDE4B with more nausea associated with its use (Lipworth, 2005). Roflumilast is in late phase III studies for use in both asthma and COPD, whilst cilomilast is in late phase III studies for COPD only, due to lack of efficacy in asthma. In mild to moderate persistent asthma, roflumilast has proven as effective as the gold standard treatment, low-dose inhaled corticosteroids (Izquierdo *et al.*, 2003). The commonest side-effects of roflumilast are higher in the first weeks of therapy and then settle to the following incidences: headache (6%), diarrhoea (3%), nausea (1%) and abdominal pains (1%) (Izquierdo *et al.*, 2003). Such a side-effect profile would be readily accepted in the field of oncology and indicates the feasibility of combining PDE4 inhibition with conventional polychemotherapy.

Choice of PDE4 Inhibitors for in vitro Experiments

Taken together these data support the investigation of PDE4B inhibition in DLBCL and suggest that PDE4 inhibition may contribute to the reversal of the chemoresistant phenotype present in half of DLBCL. In addition to rolipram, a selective PDE inhibitor, piclamilast, was obtained for *in vitro* cell-line and primary culture experiments in DLBCL. The Ki (the dissociation constant for the enzyme-inhibitor complex) for rolipram against human PDE4 and PDE3 are 2.4 μM and >25 μM respectively (Reeves *et al.*, 1987). In contrast, the Ki for piclamilast against PDE4 has consistently been shown to be only 1nM (Ashton *et al.*, 1994).

6.2.2 PKCβ as a Rational Target in DLBCL

The protein kinase C (PKC) family of serine-threonine kinases consists of at least ten members classified as conventional (PKC α, β1, β2, and γ), novel (δ, ε, θ and η) or atypical (PKCζ and PKCι) according to their activation by the lipid second messenger diacylglycerol, calcium or phosphatidylserine – reviewed in (Mellor and Parker, 1998). A related enzyme, PKCμ (alternative name protein kinase D) displays multiple unique features that make it a distant relative of the PKC enzymes (Johannes *et al.*, 1994). With the exception of PKCβ1 and β2, which are alternative spliced variants, each PKC enzyme is the product of a separate gene. Evidence indicates that the α, β, ε and the

atypical isoforms are anti-apoptotic in their action, whereas the δ and θ isoforms are usually involved in the promotion of apoptosis – reviewed in (Gutcher *et al.*, 2003).

In vitro exploration of PKCβ inhibition as a novel and rational means of DLBCL control and eradication was undertaken for the following reasons: -

i. The microarray data described in Chapter 5 and in the LLMPP series.
ii. The importance of PKCβ for effective BCR signalling.
iii. *In vitro* and animal data demonstrating a causal link between PKCβ and the malignant phenotype of cell-lines and the development of gastrointestinal tumours.

i. The microarray data described in Chapter 5 and in the LLMPP series.

In the DLBCL microarray series described in Chapter 5, PKCβ2, like PDE4B, was a component of the 13-gene model of all 58 cases, being overexpressed in the *'fatal/refractory disease'* patients. PKCβ was also overexpressed in the poorer prognosis ABC-DLBCL patients in the LLMPP consortium series, indicative of a potentially causal role of PKCβ in poor prognosis/chemoresistant DLBCL (Rosenwald *et al.*, 2002, Alizadeh *et al.*, 2000).

ii. The importance of PKCβ for effective BCR signalling.

PKCβ1 and –2 have been shown to play a critical role in BCR dependent NFκB survival signalling. PKCβ1 and PKCβ2 (and PKCθ) deficient mice exhibit severe immunodeficiencies because B-cell and T-cell receptor engagement cause cell death rather than proliferation (Sun *et al.*, 2000, Leitges *et al.*, 1996). In contrast, PKCδ deficiency in mice causes B-cell hyperactivity with the development of autoimmune disease (Miyamoto *et al.*, 2002). Subsequent investigation has shown that PKCβ is required for the correct formation of the lipid rafts of the BCR signalosome, which allow the recruitment of IκB kinase (Su *et al.*, 2002).

iii. *In vitro* and animal data demonstrating a causal link between PKCβ and the malignant phenotype of cell-lines and the development of gastrointestinal tumours.

PKCβ2 has been linked to carcinogenesis in animal models of gastrointestinal tumours. PKCβ2 overexpression in the colon of transgenic mice produces hyperproliferation and an increased incidence of colon carcinoma (Murray *et al.*, 1999). This activity is achieved through induction of COX-2, suppression of TGF-β signalling, and establishment of a TGF-β-resistant, hyperproliferative state in the colonic epithelium (Yu *et al.*, 2003). When PKCβ2 is overexpressed in rat intestinal epithelial cells, an

invasive phenotype develops, which is blocked by use of the PKCβ1 and -2 inhibitor Ly379196. Invasion is produced through PKCβ activating K-Ras, which in turn activates Rac1 leading to Mek activation (Zhang *et al.*, 2004a).

Choice of PKCβ Inhibitor for in vitro Experiments

The choice of SC-236 as the PKCβ inhibitor to investigate *in vitro* was made on the basis of its ability to induce apoptosis in the human gastric cancer cell-line AGS (Jiang *et al.*, 2002). In this cell-line PKCβ proved to be a survival mediator, with overexpression of PKCβ protecting the cells against SC-236. Importantly, the ability of SC-236 to decrease PKCβ protein expression and kinase activity was found to be independent of the COX-2 inhibitory properties of SC-236 (Jiang *et al.*, 2002).

6.3 Methods

All work described in this Chapter was performed by myself. Three-day cell culture experiments were conducted using DLBCL cell-lines in exponential growth. Two-day cell culture experiments were undertaken with primary lymphoid malignant material (see Chapter 2). Different concentrations of PDE4 inhibitor or SC-236 alone or in combination with the established cytotoxics cytosine arabinoside and doxorubicin or the adenylate cyclase potentiator forskolin were added on day-0. Day-3 cell proliferation was measured by the MTS assay; day-3 cell count and viability were assessed by trypan blue exclusion assay. Each experiment was performed on at least three separate occasions, unless stated otherwise. The data analysis package, GraphPad prism software, was used to analyse results. One-way ANOVAs were performed to identify statistically significant (P <0.05) differences in outcome variable (proliferation, cell count, viability) of the different concentrations and combinations of drugs used. To minimise the risk of false-positive differences being identified due to the multiple comparisons undertaken, Bonferoni's multiple comparison test was applied to each set of one-way ANOVAs.

6.4 Results

6.4.1 Effect of Piclamilast on Cell Proliferation of DLBCL Cell-lines

Cell proliferation (assessed by MTS assay) of the DLBCL cell-lines SUD4, DoHH2 and CRL following 3-day exposure to piclamilast and DMSO is illustrated in Figure 6.2 and Appendix Table A6.1. For each cell-line no difference was observed between piclamilast 300 nM (DMSO = 0.03%), piclamilast 1000 nM (DMSO = 0.1%) and vehicle-only controls (P >0.05). DMSO, the initial solvent for piclamilast, had no effect

on cell proliferation until a 1% solution was used (present in 10,000 nM piclamilast solution) (P <0.001). Thus, although piclamilast 10,000 nM appeared to inhibit cell proliferation (64.9%, 49.7% and 63.3% cf. no treatment controls for SUD4, CRL and DoHH2 respectively), this effect was due to the 1% DMSO content of the 10,000 nM piclamilast solution (cell proliferation for 1% DMSO controls cf. no treatment controls being 58.6%, 40.1% and 65.1% for SUD4, CRL and DoHH2 respectively). In summary, the proliferation of SUD4, DoHH2 and CRL was not altered after 3-day exposure to piclamilast, despite use of concentrations up to 10,000-fold higher than the Ki for PDE4 inhibition.

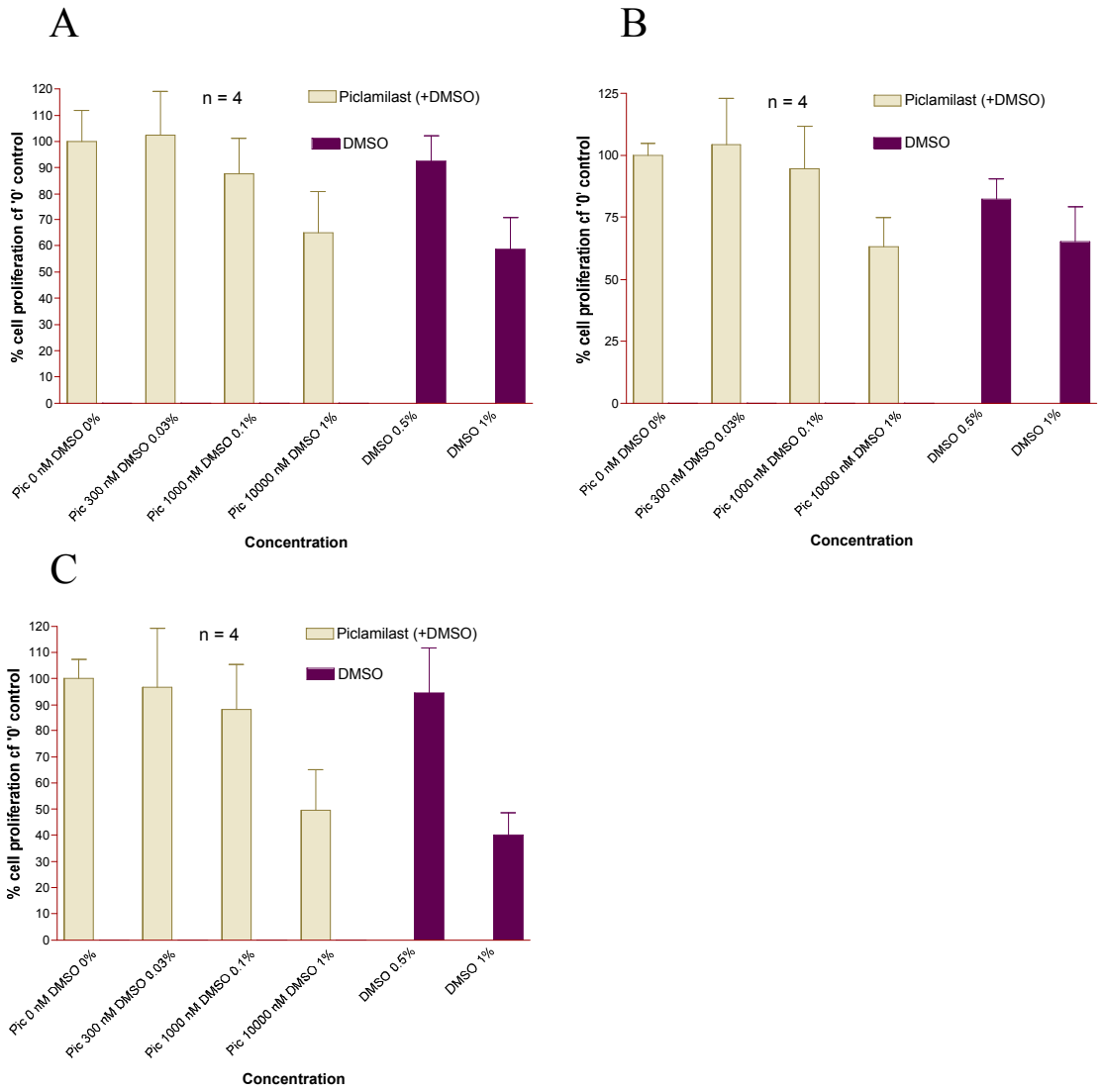


Figure 6.2 Cell Proliferation after 3-day Exposure to Piclamilast assessed by MTS Assay After 3 days of exposure to piclamilast, media alone or DMSO controls, cell proliferation was assessed by MTS assay. Cell proliferation is displayed relative to no treatment controls. The results displayed are the mean of at least four independent experiments. The error bars represent the standard deviation.

A = SUD4 B = CRL
C = DoHH2 Pic = Piclamilast

6.4.2 DLBCL Cell-line Proliferation after 3-day Exposure to Piclamilast, Cytosine Arabinoside and Doxorubicin

The effects of 3-day exposure to piclamilast, cytosine arabinoside, doxorubicin and DMSO (as a control) upon cell proliferation in SUD4, DoHH2 and CRL are illustrated in Figures 6.3 and 6.4. For all three cell-lines a concentration-response effect to cytosine arabinoside alone was confirmed (P <0.001). For SUD4, CRL and DoHH2 no differences between the 0.25 and 0.5 x EC_{50} concentrations of cytosine arabinoside with or without piclamilast 300 nM (DMSO = 0.03%) and 1000 nM (DMSO = 0.1%) respectively were seen (P >0.05) (Appendix Table A6.2). In each cell-line piclamilast 10,000 nM (DMSO 1%) added to the 1- and 2- x EC_{50} concentrations of cytosine arabinoside appeared to inhibit cell proliferation compared to the 1- and 2- x EC_{50} concentrations of cytosine arabinoside alone (P <0.001). However, in all three cell-lines this effect was due to the DMSO content of the piclamilast solution, as the one-way ANOVA of piclamilast 10,000 nM and the 1- x EC_{50} concentration of cytosine arabinoside compared to DMSO 1% and the 1- x EC_{50} concentration of cytosine arabinoside was not significant (P >0.05), whilst the combination of DMSO 1% and the 1- x EC_{50} concentration of cytosine arabinoside compared to the 1- x EC_{50} concentration of cytosine arabinoside alone was significant, confirming that the antiproliferative effect was due to the solvent DMSO and not the PDE4 inhibitor (P <0.001) (Appendix Table A6.2).

For SUD4, CRL and DoHH2 3-day exposure experiments revealed the expected concentration-response effect to doxorubicin alone (P <0.001). For each of the cell-lines no differences between the 0.25- and 0.5- x EC_{50} concentrations of doxorubicin with or without piclamilast 300 nM (DMSO = 0.03%) and 1000 nM (DMSO = 0.1%) respectively were seen (P >0.05), with the exception of CRL and doxorubicin 20 nM with and without piclamilast 300 nM (DMSO = 0.03%), where the addition of piclamilast increased cell proliferation (P >0.05) (Appendix Table A6.3). Piclamilast 10,000 nM (DMSO 1%) added to the 1- and 2- x EC_{50} concentrations of doxorubicin appeared to inhibit cell proliferation compared to the 1- and 2- x EC_{50} concentration of doxorubicin alone (P <0.001). However, as seen in the above-described experiments, the effect was due to the 1% DMSO content of the piclamilast solution and not piclamilast itself.

In summary, both cytosine arabinoside and doxorubicin inhibited proliferation of the three cell-lines, whilst piclamilast in combination with the chemotherapy drugs cytosine arabinoside or doxorubicin had no effect on cell proliferation independent of the DMSO content remaining from the initial piclamilast stock solution. Piclamilast was therefore ineffective against the DLBCL cell-lines examined as a single agent and in combination with standard chemotherapy agents.

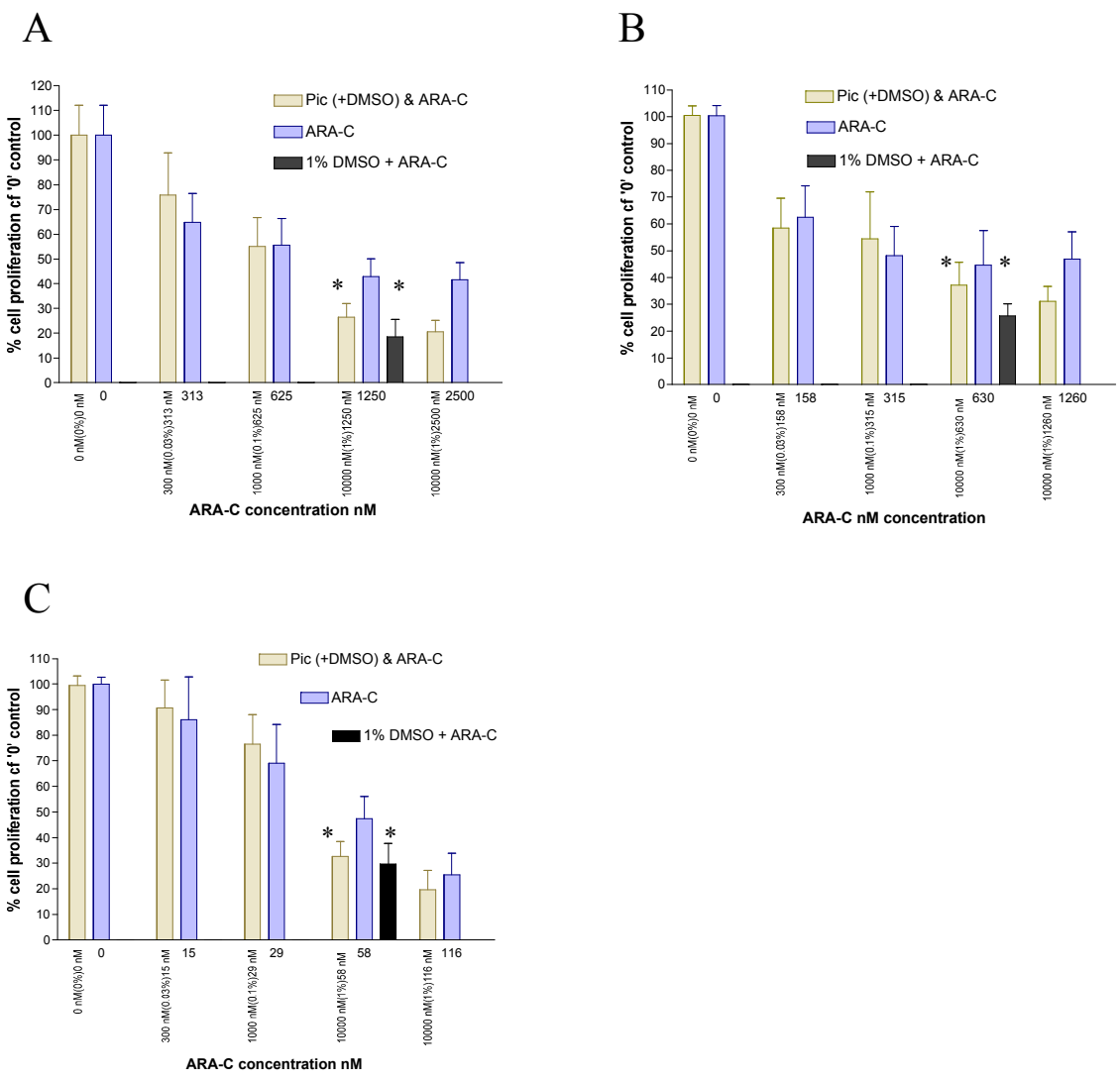


Figure 6.3 Cell Proliferation after 3-day Exposure to Piclamilast &/or Cytosine Arabinoside assessed by MTS Assay After a 3-day exposure to piclamilast, ara-c, DMSO control or neither the proliferation of the cell-lines was assessed by MTS assay. The mean proliferation is shown as a percentage relative to that of the no treatment control cells. A minimum of three independent experiments was conducted. Error bars represent the standard deviation.
Pic = piclamilast Ara-c = cytosine arabinoside A = SUD4; B = CRL; C = DoHH2
* Piclamilast 1000 nM +Ara-c vs. Ara-c with equivalent DMSO concentration = non-significant

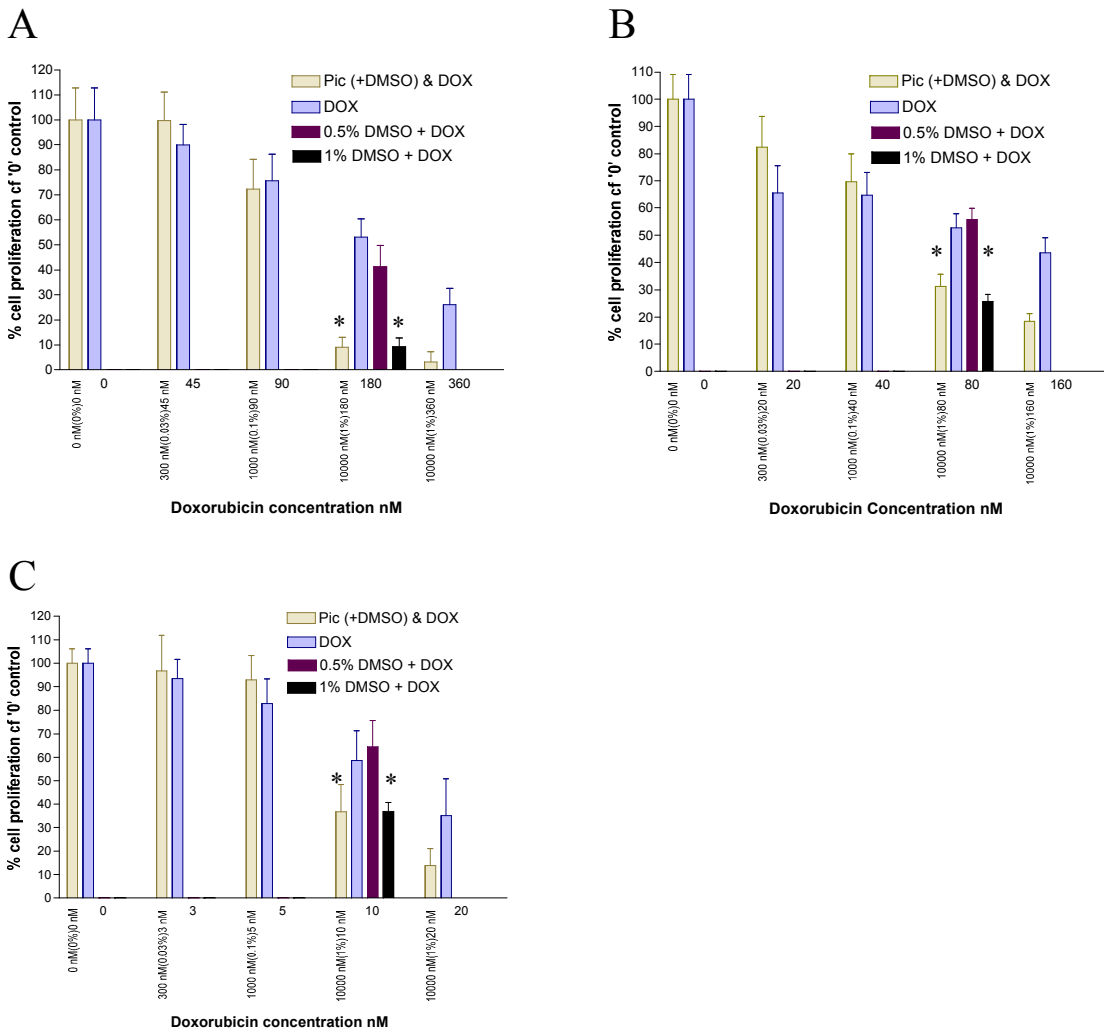


Figure 6.4 Cell Proliferation after 3-day Exposure to Piclamilast &/or Doxorubicin assessed by MTS Assay After a 3-day exposure to piclamilast, doxorubicin, DMSO control or neither the proliferation of the cell-lines was assessed by MTS assay. The mean proliferation is shown as a percentage relative to that of the no treatment control cells. A minimum of three independent experiments was conducted. Error bars represent the standard deviation.
Pic = piclamilast Dox = doxorubicin A = SUD4; B = CRL; C = DoHH2
* Piclamilast 1000 nM +Dox EC_{50} vs. Dox EC_{50} with equivalent DMSO concentration = non-significant

6.4.3 Cell Count and Viability Results after 3-day Exposure to Piclamilast, Rolipram and Forskolin in DLBCL Cell-lines

The cell count results following 3-day exposure of SUD4, CRL, DoHH2 and DHL4 to piclamilast, rolipram and forskolin alone and in combination are displayed in Figure 6.5 and Appendix Table A6.4. In CRL piclamilast 1 μM and rolipram 100 μM alone

reduced cell count to a mean of 74% (P <0.05) and 72% (P <0.01) respectively. DHL4 cells were also affected by piclamilast 1 μM and rolipram 100 μM, as cell count reduced to a mean of 81% (P <0.05) and 76% (P <0.05) respectively. For DoHH2 cells, no cytostatic activity was seen for piclamilast 1 μM (81% P >0.05), whilst rolipram 100 μM reduced cell count to a mean of 68% (P <0.01). In contrast, SUD4 was oblivious to 3-day exposure to piclamilast 1 μM and rolipram 100 μM alone, retaining cell counts of 97 and 89% respectively (P >0.05).

In contrast, single agent forskolin demonstrated cytostatic activity in all four cell-lines. This effect was most marked in DoHH2 where forskolin 40 μM reduced cell count to a mean of 10% (P <0.001). SUD4 exhibited a concentration-response effect to forskolin, as cell count fell to 60% with 20 μM and a further significant reduction when incubated with forskolin 40 μM to 40% (P <0.05). For CRL, Forskolin 20 and 40 μM also significantly decreased 3-day cell count compared to no treatment controls to 60% (P <0.001), with no concentration-response effect being evident. Lastly DHL4 proved the least sensitive cell-line to forskolin 20 and 40 μM, as cell count decreased only moderately to 75 and 57% respectively (P <0.001). The activity of forskolin was not accountable for by the DMSO content of the different concentrations. Only in SUD4 did the maximum concentration of DMSO used in the forskolin and PDE4 inhibitor combinations – 0.4% - have a minor effect on cell count. In none of the four cell-lines tested did the addition of rolipram or piclamilast to forskolin add to the cytostatic activity of forskolin alone (P >0.05).

The viability results following 3-day exposure of SUD4, CRL, DoHH2 and DHL4 to piclamilast, rolipram and forskolin alone and in combination are displayed in Figure 6.6 and Appendix Table A6.4. Viability was unaffected in the four cell-lines by either the 0.2 and 0.4% DMSO controls, piclamilast 1 μM or rolipram 100 μM – in keeping with the 3-day cell proliferation results described above (P >0.05). The viability of CRL and DHL4 cells was unaffected by forskolin alone or in combination (P >0.05). In SUD4 forskolin caused a minor but significant fall in viability to 87% compared to no treatment controls with 40 μM (P <0.05). Addition of rolipram and piclamilast to forskolin did not enhance the cytotoxic activity of forskolin alone (P >0.05). Only in DoHH2 did single agent forskolin 40 μM produce a marked decrease in viability compared to no treatment controls (50%; P <0.001). The addition of piclamilast 1 μM or rolipram 100 μM to forskolin 20 μM further and significantly increasing the

cytotoxic activity compared to single agent forskolin 20 μM (37% P <0.05; 26% P <0.001 respectively vs. 58% for forskolin 20 μM compared to no treatment controls).

In summary, the PDE4 inhibitors failed to impact meaningfully on DLBCL cell-line viability after 3-day exposure when used alone or in combination with the c-AMP potentiator forskolin in all but the most sensitive cell-line, DoHH2. In contrast, single-agent forskolin demonstrated cytostatic activity in all four cell-lines (cell count reduced to 10-57% relative to no treatment controls), minor cytotoxic activity against SUD4 and appreciable cytotoxicity against DoHH2 (viability decreased to 87% and 50% respectively compared to no treatment controls) further enhanced by addition of a high concentration of PDE4 inhibitor.

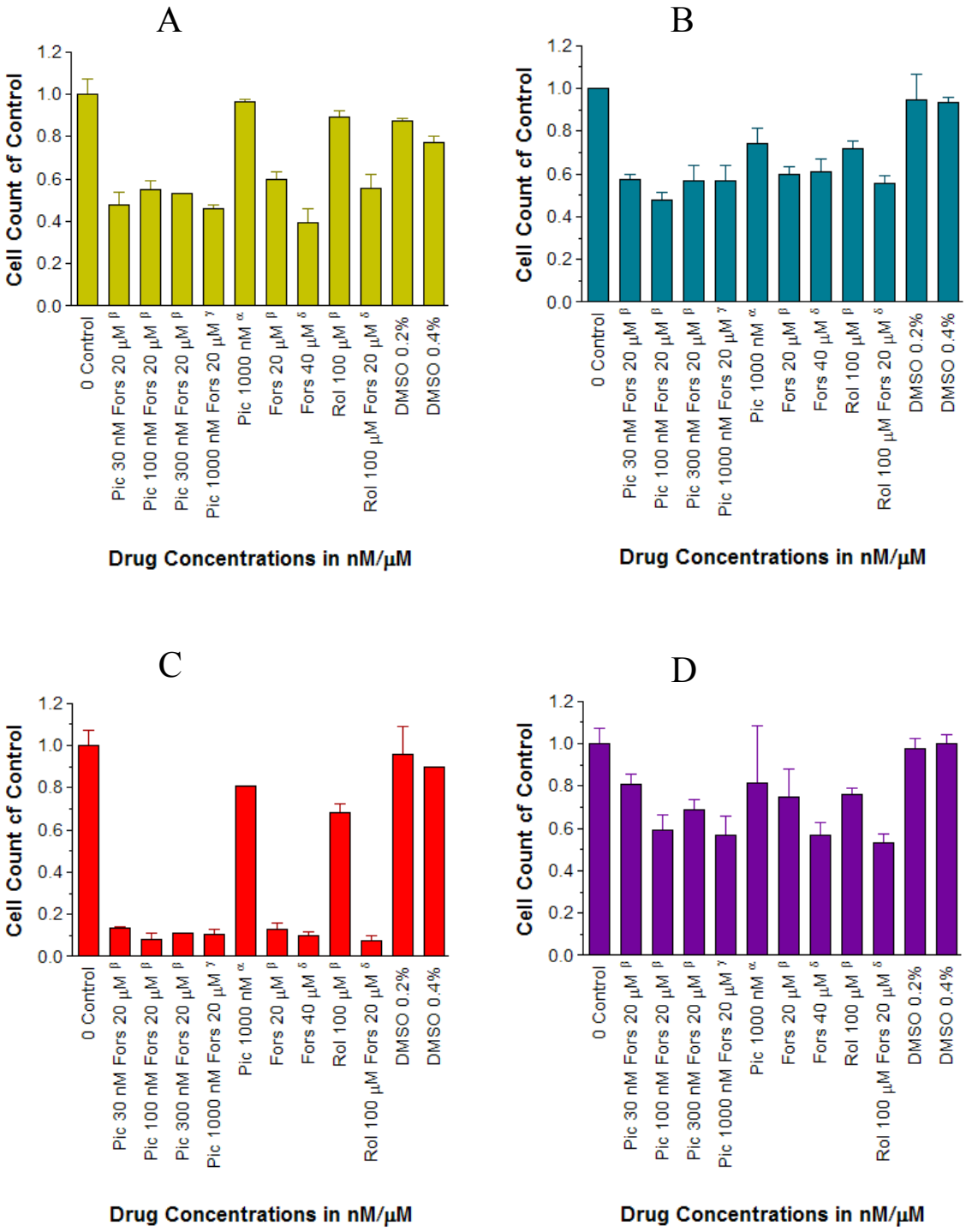


Figure 6.5 DLBCL Cell-line Cell Count after 3-day Exposure to a PDE4 Inhibitor and/or Adenylate Cyclase Potentiator Forskolin. After a 3-day exposure to piclamilast, forskolin, rolipram, DMSO control or neither the cell count of the cell-lines was assessed by trypan blue exclusion assay. The mean cell count (n = 2) is shown as a percentage relative to the no treatment control cells for each experiment. Error bars represent the standard deviation. Pic = piclamilast; Fors = forskolin; Rol = rolipram The final DMSO content was α = 0.1% DMSO; β = 0.2% DMSO; γ = 0.3% DMSO; δ = 0.4% DMSO. A = SUD4; B = CRL; C DoHH2; D = DHL4

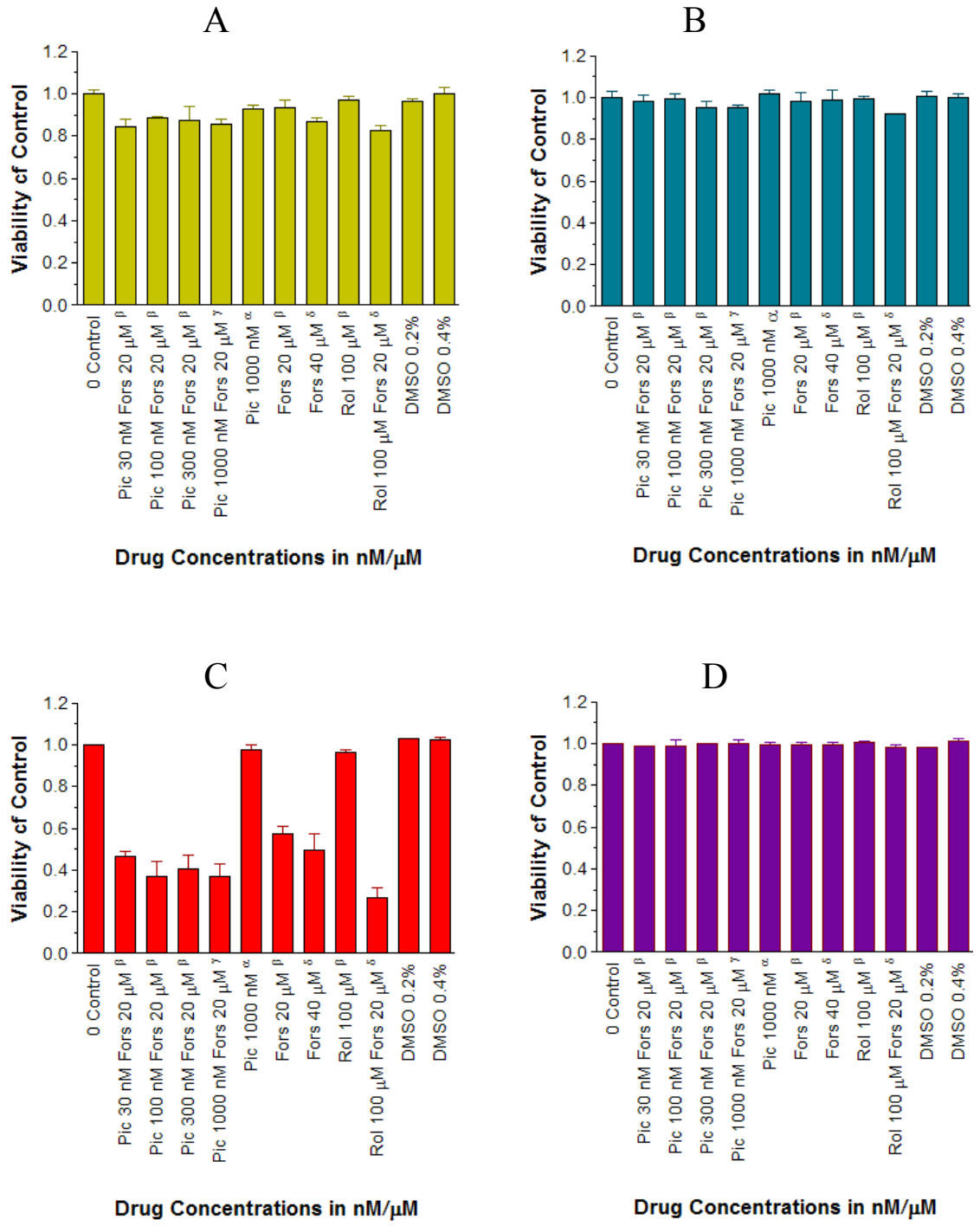


Figure 6.6 DLBCL Cell-line Viability after 3-day Exposure to a PDE4 Inhibitor and/or Adenylate Cyclase Potentiator Forskolin. After a 3-day exposure to piclamilast, forskolin, rolipram, DMSO control or neither the viability of the cell-lines was assessed by trypan blue exclusion assay. The mean viability (n = 2) is shown as a percentage relative to the no treatment control cells for each experiment. Error bars represent the standard deviation. Pic = piclamilast; Fors = forskolin; Rol = rolipram The final DMSO content was α = 0.1% DMSO; β = 0.2% DMSO; γ = 0.3% DMSO; δ = 0.4% DMSO. A = SUD4; B = CRL; C DOHH2; D = DHL4

6.4.4 Primary CLL 48-hour *Ex Vivo* Culture With PDE4 Inhibitors And Forskolin

The results for the 48-hour primary culture exposure experiments with the CLL/SLL sample 02 are illustrated in Figures 6.7 and 6.8. Single agent piclamilast produced a minor, non-significant reduction in cell count (89.5% for 1 μM piclamilast), no reduction in viability and no evidence of a concentration-response effect (P >0.05). Rolipram 60 μM induced a concentration-dependent reduction in cell count (73% P <0.001) but only a minor reduction in viability (93% P <0.01). Similarly, forskolin 20 μM alone produced a non-significant decrease in cell count to 76.3% and viability to 95.8% (P >0.05). When piclamilast was combined with forskolin, a significant reduction in cell count and viability was seen (e.g. 51.7% and 72.6% respectively for 1 μM piclamilast and 20 μM forskolin (p <0.001)). The combination of forskolin and rolipram also produced a significant reduction in cell count and viability (e.g. 62% and 66.5% respectively for 100 μM rolipram and 20 μM forskolin (P <0.001)). Rolipram combined with forskolin produced a greater effect than either agent used alone (P <0.01). The combination results for a PDE4 inhibitor and the adenylate cyclase potentiator forskolin were not due to the DMSO content of the solutions, as the maximum DMSO concentration (0.4%) had no effect on cell count or viability compared to no treatment controls (P >0.05).

In summary, single agent PDE4 inhibitor or forskolin had no or little activity compared to significant activity for a combination of high concentration PDE4 inhibitor and forskolin in the *ex vivo* culture primary CLL/SLL sample 02.

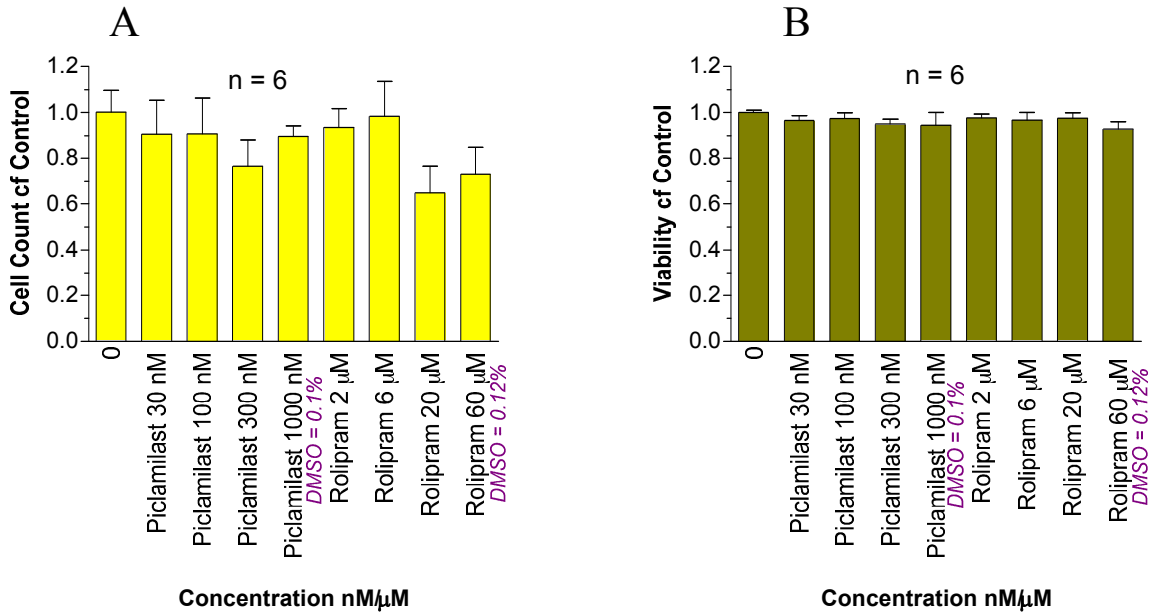


Figure 6.7 CLL Sample 02 Primary Culture Cell Count (A) and Viability (B) after 2-day exposure to Piclamilast and Rolipram. After 48-hour exposure to piclamilast, rolipram or vehicle alone, the cell count and viability of sample 02 was assessed by trypan blue exclusion assay. The mean cell counts (n = 6) and viabilities (n = 6) are shown as a percentage relative to that of the no treatment control cells. Error bars represent the standard deviation. The maximum DMSO content was 0.12%. In the subsequent experiment with sample 02, 0.4% DMSO controls had no effect on cell count and viability (see Figure 6.8) **Pic = piclamilast; Rol = rolipram**

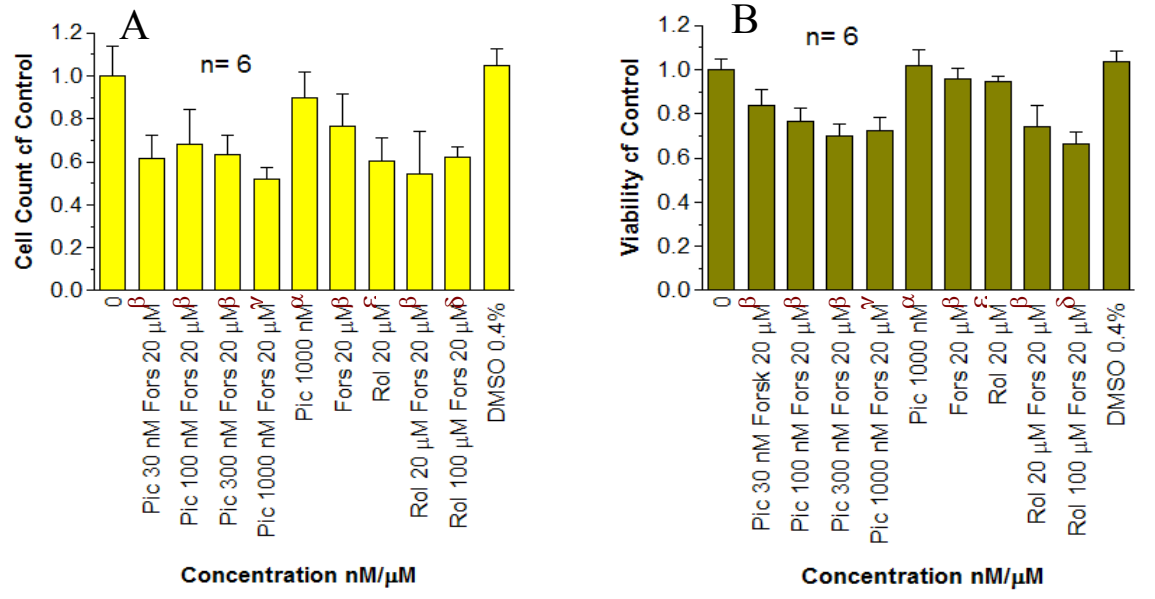


Figure 6.8 CLL Sample 02 Primary Culture Cell Count (A) and Viability (B) after 2-day Exposure to PDE4 Inhibitors and Forskolin. After a 2-day exposure to piclamilast, forskolin, rolipram, DMSO control or neither the cell count and viability of sample 02 was assessed by trypan blue exclusion assay. The mean cell counts (n = 6) and viabilities (n = 6) are shown as a percentage relative to that of the no treatment control cells. Error bars represent the standard deviation. The final DMSO content was α = 0.1% DMSO; β = 0.2% DMSO; γ = 0.3% DMSO; δ = 0.4% DMSO, ε = 0.04% DMSO

Pic = piclamilast; Fors = forskolin; Rol = rolipram

6.4.5 c-AMP Assay with cell-lines after Exposure to Piclamilast and Rolipram

The c-AMP assay was undertaken qualitatively on four separate occasions and quantitatively once. Further repeats were not possible due to time constraints. The results from the quantitative assay are illustrated in Figure 6.9 and followed the trend of the preceding qualitative experiments. Piclamilast and rolipram alone and in combination produced a significant rise in c-AMP levels compared to no treatment controls in CRL and SUD4 but not in DoHH2 or DHL4. No concentration-response effect was seen for either agent. The elevation of c-AMP did not correlate with either the effect of piclamilast or rolipram upon cell count, as rolipram reduced cell count to approximately 70% of no treatment controls in CRL, DHL4 and DoHH2 yet had no effect on cell count in SUD4 (Figure 6.5).

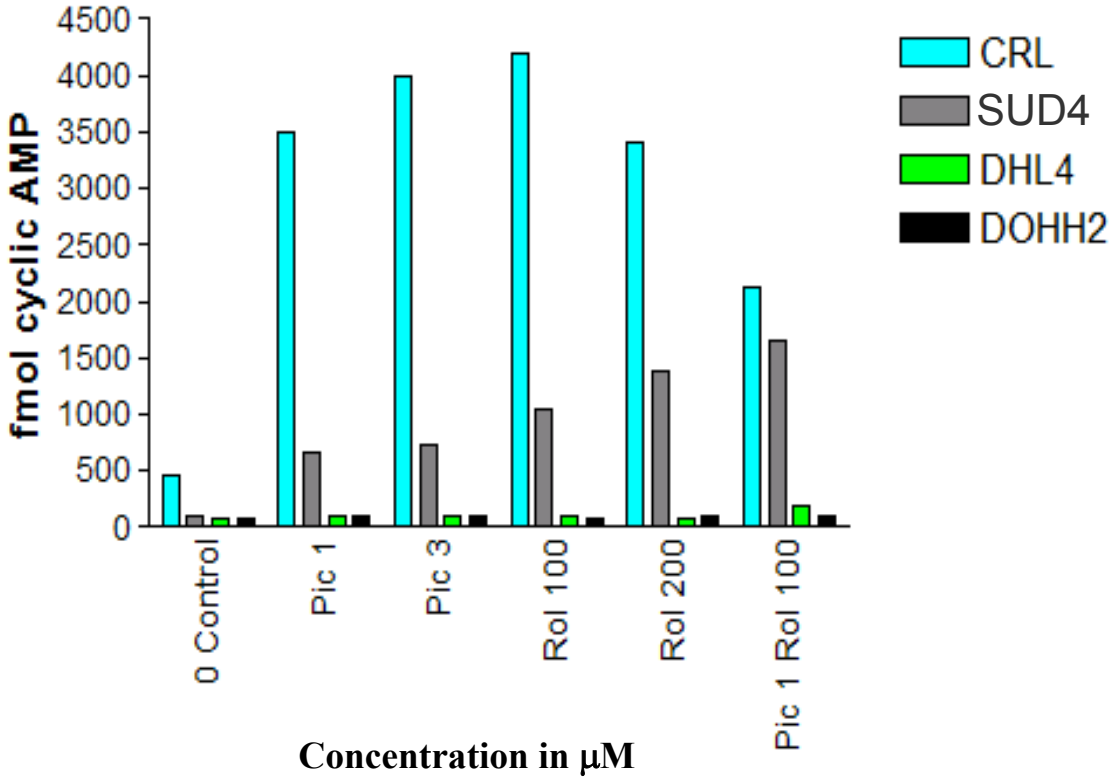


Figure 6.9 DLBCL Cell-line c-AMP levels after 4 hours exposure to PDE4 Inhibitors. c-AMP was measured in fmol. The magnitude of c-AMP augmentation was different in each cell-line. Thus, for DoHH2 and DHL4 there was less than a fold rise in c-AMP with piclamilast &/or rolipram compared to a 7 to 17-fold rise for SUD4 and 5 to 9-fold rise from a much higher baseline for CRL. Pic = piclamilast; Rol = rolipram

6.4.6 Effect of the PKCβ Inhibitor SC-236 on Cell Count and Viability of DLBCL cell-lines

In 3-day exposure experiments, the cell count and viability of SUD4, DHL4, CRL and DoHH2 were significantly inhibited in a concentration-dependent manner by SC-236 (Figures 6.10 and 6.11). The EC_{50} and 95% CI for cell count and viability are given in Table 6.2 and the raw data in Appendix Tables A6.5 through A6.12. DoHH2 and SUD4 were more sensitive than CRL or DHL4. Unfortunately it was not possible to pursue the mechanism of action of SC-236 due to time constraints.

SC-236	Cell Count		Viability	
Cell-lines	EC_{50} μM	95% CI μM	EC_{50} μM	95% CI μM
SUD4	15	13 to 17	28	24 to 31
CRL	26	21 to 32	41	38 to 44
DoHH2	14	12 to 16	22	20 to 25
DHL4	25	22 to 27	68	64 to 71

SC-236	Cell Count		Viability	
Primary Cultures	EC_{50} μM	95% CI μM	EC_{50} μM	95% CI μM
O3	131	115 to 149	141	134 to 149
O4	103	64 to 166	78	67 to 92

Table 6.2 DLBCL Cell-line and Primary Culture Cell Count and Viability EC50s following 3-day and 2-day exposure to SC-236 respectively
CI = Confidence Interval

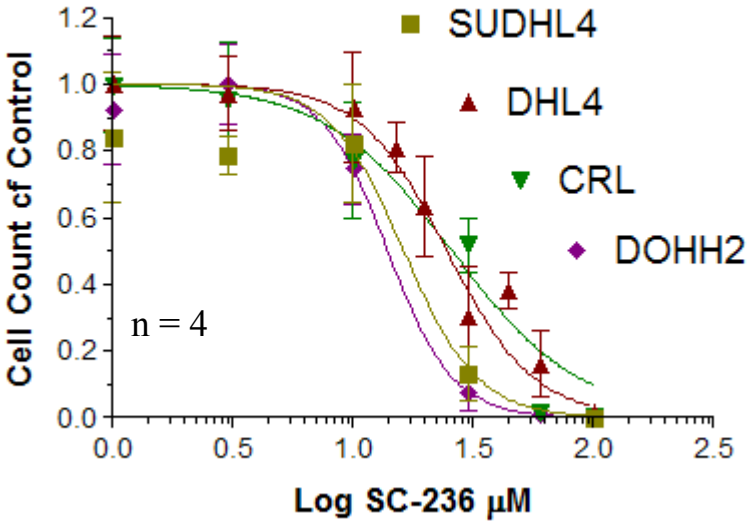


Figure 6.10 DLBCL Cell-line Cell Count after 3-day exposure to SC-236
After a 3-day exposure to SC-236 or media alone the cell count of the cell-lines was assessed by trypan blue exclusion assay. The mean cell count (n = 4) is shown as a percentage relative to that of the no treatment control cells. Error bars represent the standard deviation.

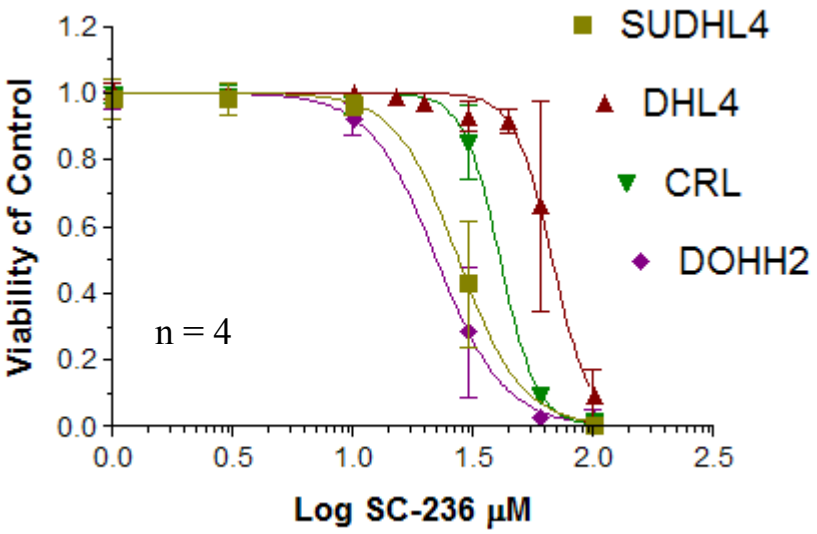


Figure 6.11 DLBCL Cell-line Viability after 3-day exposure to SC-236
After a 3-day exposure to SC-236 or media alone the viability of the cell-lines was assessed by trypan blue exclusion assay. The mean viability (n = 4) is shown as a percentage relative to that of the no treatment control cells. Error bars represent the standard deviation.

6.4.7 Effect of SC-236 on Cell Count and Viability of Primary NHL Cell Culture

In 2-day exposure experiments, the cell count and viability of the MCL samples 03 and 04 was significantly inhibited in a concentration-dependent manner by SC-236 (Figure 6.12). The EC_{50}s for cell count and viability were significantly higher than in the cell-lines examined (Table 6.2; raw data in Appendix Tables A6.13 through A6.16).

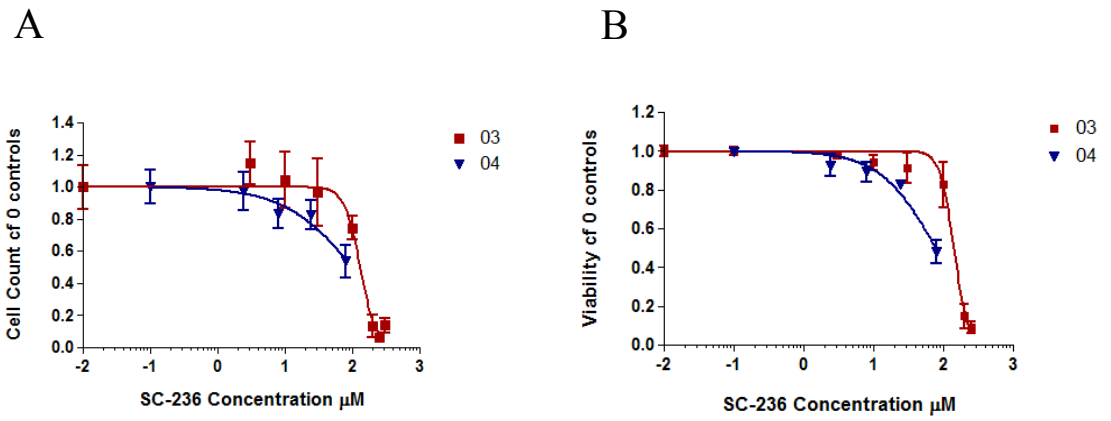


Figure 6.12 Primary Culture Cell Count (A) and Viability (B) after 2 days exposure to SC-236 for Samples 03 & 04 After a 2-day exposure to SC-236 or media alone the cell count and viability of samples 03 and 04 was assessed by trypan blue exclusion assay. The mean cell counts (n = 6) and viabilities (n = 6) are shown as a percentage relative to that of the no treatment control cells. Error bars represent the standard deviation.

6.5 Discussion

6.5.1 Review of Experimental Findings in Comparison with Published Data

PDE4 Inhibition and c-AMP Potentiation in DLBCL Cell-lines

From the 3-day cell count experiments it is clear that the PDE4 inhibitors rolipram and piclamilast have only modest cytostatic activity (68 – 89% for rolipram 100 µM and 74 – 96.5% for piclamilast 1 µM relative to no treatment controls) and no cytotoxic or anti-proliferative effects on the four DLBCL cell-lines (Tables A6.2, A6.3 and A6.4). This is despite using concentrations of both PDE4 inhibitors 50 to 1,000-fold higher than their Ki values for PDE4 inhibition of 2.4 µM only 1nM for rolipram and piclamilast. Even

when the PDE4 inhibitors were added to the chemotherapy drugs cytosine arabinoside and doxorubicin they produced no additional impact on cell proliferation.

At the outset of the PDE4 inhibitor experiments, the finding of Ogawa *et al* of reduced cell proliferation following exposure to rolipram and forskolin for four days in the dexamethasone resistant T-cell ALL cell-line CEM-GH was encouraging (Ogawa *et al.*, 2002). However, after discovering the PD4 inhibitors to be inactive, the CEM cell-line was tested and rolipram found to have no cytotoxic activity against it (data not shown). A subsequent personal communication from group head for Ogawa *et al* acknowledged his group's subsequent failure to reproduce Ogawa's results.

One possible explanation for the lack of efficacy of the PDE4 inhibitors could be the subtype of cell-line used. All four were GCB-DLBCL rather than ABC-DLBCL, whilst PDE4 was overexpressed in ABC-DLBCL rather than GCB-DLBCL in the LLMPP series. Although logical, this argument doesn't stand up, as rolipram has been found to be ineffective against ABC-like DLBCL cell-lines in subsequent experiments and produced a small but significant reduction in cell proliferation in the chemosensitive GCB-like DHL6 (to 80% compared to no treatment controls) (Smith *et al.*, 2005). Furthermore, it is clear that the *'cell-of-origin'* distinction captures only a part of the chemoresponsive/resistant phenotype of DLBCL (see Chapter 5 discussion).

In contrast to the PDE4 inhibitors, the c-AMP potentiator forskolin exhibited cytostatic activity, producing a significant fall in 3-day cell count in all four cell-lines, especially DoHH2, compared to no treatment controls (forskolin 40 μM: SUD4 39.5%, CRL 61%, DoHH2 10% and DHL4 56.5% *P* values <0.001). Addition of piclamilast or rolipram to forskolin did not enhance the cell count reduction produced by single agent forskolin. Forskolin 40 μM produced a noticeable fall in viability in DoHH2 to 50% ($P < 0.001$) and a minor reduction in SUD4 to 87% compared to no treatment controls ($P < 0.05$). In DoHH2 alone, addition of a PDE4 inhibitor (piclamilast 30-1000 nM or rolipram 100 μM) to forskolin 20 μM reduced viability further from a mean of 58% to 35% ($P < 0.01$). Similarly, experiments by Margaret Shipp's group found forskolin reduced proliferation to 25% compared to no treatment controls in the very chemosensitive DHL6 and modestly to 75% in the other three DLBCL cell-lines tested (Smith *et al.*, 2005). A possible explanation for the sensitivity of DoHH2 to forskolin and the relative insensitivity of the other three cell-lines tested in this Chapter could be their differential *TP53* status - DoHH2 possessing functional p53 (heterozygous) whilst the other three

cell-lines are null for the p53 protein. In support of this hypothesis, Ogawa *et al* found CEM cell death induced by rolipram and forskolin was via the intrinsic apoptotic pathway associated with rapid induction of p53 and then $p21^{WAF1}$ (Ogawa *et al.*, 2002).

In all four cell-lines studied in this Chapter the basal levels of c-AMP were found to be very low (70-100 fmol in SUD4, DoHH2 and DHL4 and was 350 fmol in CRL), as in normal peripheral blood B- and T-cells (Diamond *et al.*, 1987). Margaret Shipp's group also found c-AMP levels to be very low or undetectable in the seven DLBCL cell-lines they tested (Smith *et al.*, 2005). c-AMP levels were unaffected by PDE4 inhibition in DoHH2 and DHL4, whilst they rose markedly on exposure to rolipram and piclamilast in SUD4 and CRL. Therefore, despite the PDE4 inhibitors elevating c-AMP in SUD4 and CRL they were inactive agents in the DLBCL cell-lines studied. Three possible explanations of this lack of efficacy are:- (1) That PDE4 is not important for DLBCL survival. (2) That the inhibition of PDE4 is ineffective due to the presence or rapid induction of the other c-AMP PDEs PDE3 and PDE7. Against this hypothesis is the fact that the high concentrations of PDE4 inhibitor used by others and myself meant that PDE3 and PDE7 would have been effectively inhibited and yet the agents tested remained inactive. (3) That sustained high levels of c-AMP are needed to induce cytostasis, for which use of a c-AMP augmenter or analogue is required. Unfortunately, due to time constraints, it was not possible to measure the effect of forskolin ± PDE4 inhibitor on c-AMP levels in the four DLBCL cell-lines.

More recently, inhibition of PDE4B in conjunction with c-AMP promotion by forskolin improved the activity of dexamethasone and rapamycin in the DLBCL cell-lines DHL6 and OCI-Ly3 (Batra *et al.*, 2007). This brings the PDE4 inhibition story full circle to the results of Ogawa *et al* – suggesting that glucocorticoid sensitivity could be promoted by the use of a PDE4 inhibitor and a c-AMP potentiator (Ogawa *et al.*, 2002).

PDE4 Inhibition and c-AMP Potentiation ex vivo in Primary CLL/SLL Sample 02

The data generated from the 2-day primary culture experiments with the CLL/SLL sample 02 was interesting and different from those in the DLBCL cell-lines. Used alone, piclamilast, rolipram and forskolin were largely ineffective, whilst the use of either piclamilast or rolipram with forskolin produced significant falls in cell count and viability (e.g. cell count reduced to 51.7% and viability to 72.6% following exposure to 1 μM piclamilast and 20 μM forskolin ($p < 0.001$)).

Moon and Lerner have previously demonstrated that CLL primary cells cultured simply in RPMI and 10% calf serum undergo apoptosis via the intrinsic apoptotic pathway when exposed to rolipram 10 μM ± forskolin 40 μM for 24 hours (Moon and Lerner, 2003). Caspase-9 is activated following mitochondrial membrane depolarisation, resulting in caspase-3 activation and PARP cleavage (Moon and Lerner, 2003). However, by 48 hours 27% of the no treatment control cells were apoptotic. This suggests that their primitive culture conditions were acting as a significant primer to cell death. Moon and Lerner found that c-AMP analogues were capable of down-regulating Bcl-2 and Bcl-x_L, up-regulating Bax and Bad and inducing apoptosis in primary CLL cells (Moon and Lerner, 2003).

In conclusion, the results presented in this Chapter indicate that targeting PDE4B alone is ineffective in DLBCL and certainly not a means of sensitising chemoresistant cell-lines to established cytotoxics and by extrapolation PDE4 inhibition is unlikely to improve response and outcome to chemotherapy in poor prognostic DLBCL.. Although forskolin proved to be cytostatic it was cytotoxic in only the most chemosensitive cell-line DoHH2. Combination therapy with a PDE4 inhibitor and a c-AMP potentiator was similarly disappointing in the cell-lines investigated and only modestly effective against the primary CLL sample, 02. In keeping with these results, Smith *et al* found some efficacy for the selective PDE4B inhibitor PLX563 combined with forskolin in the very chemosensitive DHL6, an effect mediated via PI3-kinase down-regulation. Therefore PDE4 inhibition in conjunction with c-AMP elevation is not worthy of further investigation as a rational target in DLBCL application as it is not likely to have efficacy in any but the most sensitive lymphomas, the very ones that don't require a novel treatment strategy. At best such a strategy may still be a viable novel treatment strategy in CLL/SLL.

SC-236 Activity and PKCβ Inhibition

SC-236 demonstrated cytostatic and cytotoxic activity in all four cell-lines examined, with the chemotherapy insensitive cell-line DHL4 being relatively less sensitive to the cytotoxic actions of SC-236 (EC_{50} cell count 14-26 μM; EC_{50} viability 22-68 μM). In primary culture studies, the chemoresistant MCL samples 03 and 04 were less susceptible to SC-236, with considerably higher EC_{50}s for cell count and viability (EC_{50} cell count 103-131 μM; EC_{50} viability 78-141 μM). The results presented in this Chapter were produced only in GCB-DLBCL cell-lines, as the two described ABC-

DLBCL cell-lines OCI-Ly3 and OCI-Ly10 were not available for study. These results suggest that SC-236 and other PKCβ inhibitors merit further investigation as novel agents in DLBCL regardless of *'cell of origin'*, and support the hypothesis that PKCβ overexpression may indeed by causally associated with chemoresistance and poor outcome in DLBCL as per PKCβ's inclusion in the 13-gene model described in Chapter 5. The potential for PKCβ inhibition in poor prognosis ABC-like DLBCL is suggested by the overexpression of PKCβ in such cases.

Use of the specific PKCβ inhibitor, Ly37919, in human DLBCL cell-lines inhibited BCR-dependent IκB kinase activation, so by blocking the PKCβ-dependent NFκB pathway and causing a concentration-dependent reduction in cell viability in seven of eight cell-lines examined (Su *et al.*, 2002). As chemotherapy can induce NFκB, this may be a mechanism to secondary chemoresistance and a means of novel therapeutic attack via NFκB or PKCβ inhibition, with the potential that PKCβ could prove a more specific target than NFκB.(Das and White, 1997, Cheng *et al.*, 2000).

Two other sets of data indicate that the down-regulation of PKCβ can have an anti-tumour effect. Firstly, the omega-3 fatty acid eicosapentaenoic acid (present in fish oils), inhibits colon carcinogenesis by suppression of PKCβ2 activity, which in turn causes inhibition of COX-2 protein expression, re-expression of TGF-βR2, and restoration of TGF-β1-mediated transcription in rat intestinal epithelial cells (Yu *et al.*, 2003). Secondly, the total PKC activity was reduced in TM6 cells by the Se compound MSC (50 μM) within 30 minutes of treatment for calcium-dependent and independent PKCs, both in cytosolic (55.4 and 77.6% respectively) and membrane (35.2 and 34.1% respectively) fractions. Whilst PMA significantly elevated the PKC activity in the membrane fraction ($P < 0.01$), addition of MSC inhibited this activation by more than 57%. The effect of MSC was Se compound specific, as selenomethionine and sulphurmethyl-L-cysteine did not alter PKC activity either in the cytosolic or membrane fractions (Sinha *et al.*, 1999). Therefore certain Se compounds may be able to inhibit the activity of PKCβ as one of their routes to cytotoxicity.

Could COX-2 Inhibition be an Alternative Mechanism of SC-236 Activity?

`the efficacy of SC-236 demonstrated here could have been due in part, or in its entirety, to the COX-2 inhibitory properties of SC-236, as the anti-cancer activity of NSAIDs has been observed *in vitro* and *in vivo*. In human studies a decreased incidence of

gastrointestinal cancers has been observed in those taking NSAIDs compared to controls and in people with familial polyposis coli the incidence and rate of colonic polyp formation was reduced by their use (Steinbach *et al.*, 2000, Gupta and DuBois, 1998). COX-2, but not COX-1, up-regulation occurs in adenomas and adenocarcinomas compared with adjacent normal colonic mucosa (Sano *et al.*, 1995, Eberhart *et al.*, 1994, Kargman *et al.*, 1995). Furthermore the level of COX-2 overexpression correlates with angiogenesis (Cianchi *et al.*, 2001). The anti-neoplastic activity of NSAIDs may not be solely derived from COX-2 inhibition as COX-deficient cell-lines are still inhibited by NSAIDs (Richter *et al.*, 2001).

The worldwide withdrawal of COX-2 inhibitors from clinical use in 2004 due to the shock discovery of a clinically relevant increased risk of cardiac and cardiovascular events due to the prothrombotic side-effect of unopposed COX-2 inhibition has led to the abandonment of secondary prevention trials of COX-2 inhibitors in colorectal cancer patients. This makes the pursuit of agents such as SC-236 with COX-2 inhibitory properties less attractive in DLBCL.

Human Trials of PKCβ Inhibitors

The practicality of PKCβ inhibition is suggested by the entry of ruboxistaurin (Ly333531), a PKCβ inhibitor, into phase III trials for diabetic nephropathy and retinopathy and enzastaurin into phase II studies in combination with chemotherapy in patients with malignancies. Indeed the oral PKCβ inhibitor enzastaurin has rapidly passed from single-agent phase I to phase II studies in cancer and lymphoma patients. From phase I studies, fatigue and gastrointestinal toxicities were the most common seen and prolonged QTc interval on electrocardiogram monitoring was the dose-limiting toxicity. This led to the recommended dose for phase II trials of 525 mg once daily (Carducci *et al.*, 2006). Margaret Shipp has led a phase II study of enzastaurin in patients with relapsed or refractory DLBCL (Robertson *et al.*, 2007). No deaths or discontinuations due to toxicity were reported; only one grade 4 toxicity (hypomagnesaemia) occurred and six grade 3 toxicities included (fatigue, oedema, headache, motor neuropathy, and thrombocytopenia). Of the 55 patients studied, 22% (12 of 55) experienced freedom from progression (FFP) for two cycles (56 days) and four patients (three with CR and one with stable disease) remained free from progression for more than 20-50 months. In a similar phase II study of enzastaurin in patients with relapsed or refractory MCL, no responses were seen but 27% (6 of 22)

were free from progression for more than 6 months. Fatigue was the most common toxicity and there were only six grade 3 toxicities (Morschhauser *et al.*, 2008). Phase I studies of enzastaurin in combination with gemcitabine and cisplatin or capecitabine have shown the PKCβ inhibitor to be safe and to produce prolonged FFP in a minority of patients (Rademaker-Lakhai *et al.*, 2007, Camidge *et al.*, 2008). As SC-236 proved efficacious in GCB-DLBCL lines, the question of efficacy of PKCβ inhibition in PKCβ protein expressing NHLs, rather than solely ABC-like DLBCL, will be of interest.

Summary of PKCβ Inhibitor Results

In conclusion, SC-236 demonstrated cystostatic and cytotoxic activity as a single agent in DLBCL cell-lines and MCL primary cultures. More recent clinical research has shown PKCβ inhibition can stabilise and induce responses in a minority of chemoresistant/refractory lymphoma patients. This suggests that PKCβ inhibition is indeed a valid rational target for intervention, as suggested by the 13-gene model results of Chapter 5. Further investigation of PKCβ inhibition as a single agent and in combination with chemotherapeutics is therefore warranted.

CHAPTER 7: DISCUSSION

Summary of Introductory Chapter and Thesis Aims

DLBCL is the commonest subtype of NHL and is increasing in incidence. At the time of the initiation of the research described in this thesis the treatment of DLBCL was almost universally with CHOP-21 chemotherapy, which produced long-term remission in a minority of patients (Fisher *et al.*, 1994). Subsequently the boundaries of successful treatment have pushed further through the addition of rituximab, the humanised monoclonal antibody against CD20, to chemotherapy and dose intensification of therapy (Tilly *et al.*, 2003, Pfreundschuh *et al.*, 2004b, Pfreundschuh *et al.*, 2004a, Coiffier *et al.*, 2002). As a consequence the 5-year OS for DLBCL now exceeds 50%. Nevertheless the treatment of DLBCL remains empirical and virtually the same for all new patients and combination therapies still fail to cure a significant minority, due to primary or secondary chemoresistance or complications of treatment. The stratification of patients into meaningful risk groups following their diagnosis of DLBCL has long been sought for several decades. As outlined in Chapter 1, numerous individual prognostic factors have been discovered in DLBCL through retrospective analyses. Despite these findings, the majority of putative prognostic factors have not been validated in independent series. In the early 1990's a collaborative group investigated the existing prognostic factors and developed the IPI (The-International-Non-Hodgkin's-Lymphoma-Prognostic-Factors-Project, 1993). The IPI has proven capable of stratifying patients into its four risk groups for estimated chances of response and/or outcome to first treatment and at relapse in numerous patient series. Although the IPI provides a general estimate of risk of treatment success it is unable to provide a sufficiently accurate risk to allow individualised risk estimation. This is particularly true for a patient whose IPI score places them in one of the intermediate risk categories. No successor to the IPI had been described at the time the research for this thesis was started.

This thesis was undertaken to discover and validate novel prognostic and predictive factors in DLBCL through the rational strategy of gene expression profiling and the empirical investigation of presentation serum Se for correlations with dose-delivery, response to treatment and OS. Critical to the research was the hypothesis that the clinical and biochemical factors comprising the IPI represent only a part of the patient and tumour biology that go on to determine the success or failure of CHOP-based

treatment. As described in detail in Chapter 1 the genetic and molecular basis for cancer and DLBCL in particular is complex, heterogeneous and still poorly understood. If new prognostic and possibly predictive factors could be discovered in DLBCL then this heterogeneous illness may indeed become divisible into more tangible subtypes. This in turn would bring the promise of novel, rational, treatment adjuncts and regimens to at last afford more individualised treatment strategies based on the biology of a patient and their DLBCL.

Investigation of Presentation Serum Selenium as a Prognostic Factor in DLBCL

The empirical research into Se concentration in presentation serum as a prognostic factor in DLBCL produced positive results. Serum Se was measured using inductively coupled plasma mass spectrometry in archived presentation sera from 100 patients with high-grade NHL (78% DLBCL). Ninety-three of the 100 patients were treated with CHOP-like chemotherapy, six with radiotherapy and one received no treatment. The presentation serum Se concentration was normally distributed for the 100 patients with a mean of 0.92 μmol/l (standard deviation = 0.25) and range of 0.33 to 1.51 μmol/l. Seventy-three patients had a Se level below the UK adult reference range of 1.07-1.88 μmol/l (Matthews *et al.*, 1990). Four outcome measures were studied for an association with presentation serum selenium: chemotherapy dose-delivery, response to treatment, first remission duration and OS. Serum Se closely correlated with performance status but no other clinical variable.

An area-under-the-curve summary measure, AUC_{ratio} was created especially for the project to allow more accurate assessment of chemotherapy dose-delivery. The AUC_{ratio} of cumulative dose vs. time revealed not only variation in time taken and dose administered, but also distinguished between dose reductions and delays incurred at the beginning from those incurred at the end of treatment. Comprehensive dose-delivery data was available for 87 of the 93 patients given chemotherapy. Multivariate analysis revealed that a significantly better dose-delivery (as summarised by AUC_{ratio}) was associated with younger age, advanced stage, and higher serum Se.

In terms of response to treatment, fitting a logistic regression model produced an odds ratio of 0.62 for every 0.2 μmol/l increase in serum Se (P = 0.01; 95% CI 0.43, 0.90). A higher Se level was therefore associated with a lower probability of having a poor outcome to treatment. When serum Se was considered in quartiles response was

significantly better in the highest quartile compared to the lower three (88.0% vs. 56% of patients achieved a CR or GPR (χ^2=9.52; P = 0.023)).

When associations between OS and the variables collected were analysed using a Cox model, Se was the most significant factor, with a hazard ratio of 0.76 for every increase of 0.2 μmol/l in serum Se level (Table 3.2). Thus, a lower serum Se concentration was associated with shorter survival. For example, a patient with a serum Se concentration of 1.0 μmol/l had a 24% lower risk of death than a patient with a concentration of 0.8 μmol/l, over the duration of the study.

In conclusion, presentation serum Se predicted for dose-delivery of chemotherapy, response to first treatment and OS. Significant inverse associations were found for serum Se and all four of the outcome measures investigated (dose-delivery, response to treatment, first remission duration and OS). Reproduction of these results in a patient series treated with CHOP-R like chemotherapy is awaited, as is the exploration of a possible threshold effect for serum Se to promote dose-delivery, response to treatment and OS.

In Vitro Investigation of SDG and MSA

In vitro exploration of this discovery was undertaking using two Se compounds, SDG and MSA. Both agents were highly active in the DLBCL cell-lines, inducing caspase-dependent apoptosis. The 3-day viability EC_{50}s ranged from 4 to 9 μM for SDG and 2 to 10 μM for MSA with the exception of DHL4 which was relatively resistant to MSA (3-day viability EC_{50} of 166 μM). In the primary tumour cultures, SDG was also cytotoxic (2-day viability EC_{50} of 18 to 28 μM), whilst the pooled normal B-cell sample was less sensitive (2-day viability EC_{50} of 109 μM). For MSA, the results were less impressive as two of the four primary tumours were resistant (2-day viability EC_{50}s of 102 and 300 μM for 01 and 03 respectively) and two were relatively sensitive (2-day viability EC_{50}s of 38 and 68 μM for 04 and 02 respectively). More encouraging was the observation of the marked resistance of the pooled normal B-cell sample to MSA (2-day viability EC_{50} of 1071 μM) indicating the presence of a potentially clinically relevant therapeutic index between normal and malignant cells. In terms of the type of cell death induced by SDG and MSA, both agents were found to induce apoptotic cell death via caspase-8 and/or –9 activation and PARP cleavage. The generation of ROS by SDG and MSA and the importance of ROS generation for effect cell death by the two Se compounds were investigated in the DLBCL cell-line panel. In terms of mechanisms of

action, exposure to SDG led to ROS generation within 30 minutes in three of the four cell-lines and was critical to the cytotoxicity of SDG in two of the three cell-lines tested. In contrast, cell-line incubation with MSA failed to generate ROS, confirming the cytotoxic activity of MSA to be independent of ROS production.

This *in vitro* data generated using SDG and MSA was the first generated in human DLBCL cell-lines and primary NHL tumours and adds to the growing literature demonstrating the cytotoxic properties of Se compounds. Subsequent *in vitro* research combining MSA with established cytotoxic drugs used to treated NHLs found a surprising finding; that minimally toxic concentrations of MSA increased the efficacy of the chemotherapy drugs by up to 2.5-fold via the down-regulation of NFκB (Juliger *et al.*, 2007). Elsewhere, clinical research has taken place into the role of very high dose selenomethionine as an adjunct to irinotecan chemotherapy, with the goal of side-effect reduction and increased cytotoxicity. Unlike their animal model data, the Roswell Park group found no clinically meaningful reduction in side-effects was apparent, which prevented the anticipated dose-escalation of irinotecan (Fakih *et al.*, 2008, Fakih *et al.*, 2006, Cao *et al.*, 2004). Nevertheless, unexpected responses to treatment and cases of disease stabilisation were seen in the end-stage patients studied.

In conclusion, the empirical research undertaken proved fruitful, by not only finding a new serum prognostic factor in DLBCL but also by demonstrating the cytotoxic effects of two Se compounds against DLBCL cell-lines and primary lymphoid malignancies. The clinical potential for Se supplementation in DLBCL is now being actively pursued. Outstanding questions are the form of Se to use, the dose, which, if any, chemotherapeutics to use it in combination with. And whether Se compounds could be used alone as priming or maintenance therapy.

Discovery of Outcome Predictive Gene Expression Signatures in DLBCL using Microarray Profiling

The gene expression profiling research described in Chapter 5 used labelled cDNA created from the RNA from cryopreserved, presentation lymph node material from 58 patients with DLBCL and 19 patients with FL. This was subject to gene expression profiling using the Hu6800 Affymetrix microarray. The results proved remarkable; firstly, the supervised learning classification algorithm *'weighted voting'* correctly delineated DLBCL from FL, the closely related GC B-cell lineage malignancy. More importantly, class distinction proved possible within the group of 58 DLBCL samples.

Substantial gene expression differences were revealed between those that came from the 32 patients that were *'alive and cured'* and those DLBCL samples that came from the patients who died from DLBCL or had refractory disease. From the 100 genes that exhibited the clearest distinction between the *'alive and cured'* and *'fatal/refractory disease'* groups, application of the weighted voting algorithm analysis tool and *'leave-one-out'* cross-validation revealed that predictors of outcome to treatment containing a minimum of eight and a maximum of 16 genes out of the 6,800 genes arrayed could accurately separate the 58 sample profiles into *'alive and cured'* and *'fatal/refractory disease'* groups (P <0.05) (Shipp *et al.*, 2002). The 13-gene model proved the most accurate, creating an *'alive and cured'* group with 70% 5-year OS and a *'fatal/refractory disease'* group with only a 12% 5-year OS (P = 0.00004). Importantly, the 13-gene model predicted outcome both independently of the IPI and within the subgroups of the IPI. This discovery strongly suggests that the gene expression model was capturing different, additional prognostic information to that of the clinically based IPI outcome prediction model. *In silico* and immunohistochemical validations of components of the 13-gene model were performed and supported the legitimacy of the findings. Separate investigation of the SBH cohort of 19 DLBCL patient samples discovered that a distinct 19-gene model proved highly effective at outcome prediction within this cohort.

'Shipp vs. Staudt': Which Strategy is Best?

The subsequent expression profiling research in DLBCL from the DFCI collaboration and the LLMPP have followed different paths. The DFCI group has delved into what I term the *'social geography'* of DLBCL whilst the LLMPP has continued to explore the *'history'* of DLBCL. The DFCI group asked the simple question "what's different between DLBCL samples?" which to me constitutes an investigation into the 'social milieu' present in DLBCL bound by the anatomy of the host material – it's 'geography'. Using this approach resulted in the subclassification by gene expression profiling of 170 presentation DLBCL samples into three groups termed *'OxPhos, BCR/Proliferation* and *Host Response'* (Monti *et al.*, 2005). Thanks to the gene expression data having been generated on the very comprehensive Affymetrix HG-U133 A and B series (39,000 transcripts, representing 33,000 genes), interrogation of the LLMPP's 2002 DLBCL dataset created using the Lymphochip *'dot-blot'* slide was possible. This found that the *'OxPhos, BCR/Proliferation* and *Host Response'* classifier was successfully reproduced

in the LLMPP series of 240 DLBCL samples. Importantly, no correlation between *'cell-of-origin'* subgrouping and the *'OxPhos, BCR/Proliferation, Host Response'* consensus clustering was discovered, indicating that the two classification systems were capturing largely different aspects of DLBCL biology. The 5-year OS for the patients assigned to each of the three clusters were similar and no attempt was made to develop a prognostic model as in Chapter 5. This paper gives credence as much to the non-malignant milieu as to the malignant characteristics of DLBCL i.e. the tumour's *'social geography'* in terms of influencing tumour biology and response to treatment.

The original findings of the LLMPP research for the putative '*cells of origin'* of DLBCL were extended in their microarray examination of a second, large series of 240 biopsies from cases of DLBCL. Staudt's group revealed that DLBCL could be subclassified based upon molecular profiling into GCB-like, ABC-like, and Type 3 DLBCL with different OS following CHOP-like treatment (Rosenwald *et al.*, 2002). Staudt *et al* also developed a 17-gene model, which predicted outcome to CHOP-like chemotherapy more accurately than the '*cell of origin'* classifier and the IPI. Staudt's research into the origins, the *'history'* of DLBCL has yielded satisfy and tangible results borne out by the findings of others.

The *in silico* analyses by others of the DFCI results detailed in Chapter 5 and the 2002 LLMPP series make it clear that multiple models with prognostic power can be created capable of outcome prediction, but that they remain most effective within the dataset they were created and validated in. In view of the thousands of gene transcripts examined there is the risk that apparently robust gene-expression models may have arisen by chance. Hence the need for dedicated bioinformatics and statisticians to interpret and mine gene expression data will not diminish.

In summary both the LLMPP and the DFCI groups have successfully used different approaches to gene expression profiling to create novel molecular classifiers of DLBCL and revealed new insights into the molecular aetiology of DLBCL. Neither has created an outcome prediction model that has reached the clinic nor has either yet reproduced their experiments in a cohort of samples from patients treated with CHOP-R like therapy. As the two groups have used different microarray platforms, this has hampered comparison but may have helped their diverging lines of research to develop. Fortunately, the public accessibility of their datasets allows ongoing reinterpretation. My expectation is that their respective strategies will continue to reveal new insights

into the biology and heterogeneity of DLBCL and the interaction of the patients' tumours with their immune systems and other normal tissues. Ultimately I cannot see individualised treatment decisions coming from microarray data in isolation of intelligent integration of clinical factors. A further hurdle to be passed is the need to develop alternative treatments to CHOP-R. If nothing betters CHOP-R than profiling to know it won't work in a particular individual isn't going to be helpful without an idea of what to try in it stead. This of course brings out the other deficit of the DFCI and LLMPP models to date – they are at best prognostic for an historical treatment and have no predictive power, as they were developed from patient series treated identically. Much work therefore remains to be done.

In Vitro Investigation of the Rational Targets PDE4B and PKCβ

In an attempt to build upon and explore the 13-gene model described in Chapter 5, *in vitro* studies were undertaken of PDE4 and PKCβ inhibitors in a panel of DLBCL cell-lines and primary NHL with the ultimate goal of discovering a novel treatment for poor prognosis, chemoresistant DLBCL. PDE4 inhibition was undertaken with piclamilast and rolipram and proved unsuccessful in the four DLBCL cell-lines studied. With the exception of the highly chemosensitive DoHH2, the PDE4 inhibitors remained ineffective when combined with the c-AMP potentiator forskolin or the established cytotoxics doxorubicin and cytosine arabinoside. This lack of activity of the PDE4 inhibitors was despite their ability to raise c-AMP levels dramatically in two of the four cell-lines. For DoHH2 cell viability was unaffected by DMSO, piclamilast 1 μM or rolipram 100 μM alone (P >0.05), whilst forskolin 40 μM alone significantly decreased viability to 50% compared to no treatment controls (P <0.001). Addition of piclamilast 1 μM or rolipram 100 μM to forskolin 20 μM significantly increasing cytotoxic activity compared to forskolin alone 20 μM (37% P <0.05, 26% P <0.001 and 58% respectively).

In contrast to the findings in DLBCL cell-lines, the primary CLL/SLL tumour 02 was sensitive to PDE4 inhibition at high concentrations. Rolipram induced a dose-dependent reduction in cell count (73% P <0.001) but only a minor reduction in viability (93% P <0.01), whilst piclamilast or forskolin alone had no cytostatic or cytotoxic effect. When the PDE4 inhibitors were combined with forskolin they produced a greater effect than either agent used alone in terms of cytostasis (cell counts relative to no treatment controls for 20 μM forskolin with 100 μM rolipram = 62% and with 1 μM piclamilast =

51.7%; P <0.01) and cell kill (viability relative to no treatment controls for 20 μM forskolin with 100 μM rolipram = 66.5% and with 1 μM piclamilast = 72.6%; P <0.01). In summary, PDE4 inhibition was effective only in combination with the c-AMP potentiator forskolin in the most sensitive DLBCL cell-line, DoHH2, and the CLL/SLL primary tumour 02. The pursuit of PDE4 inhibition as a novel treatment strategy in high-risk DLBCL does not appear promising based upon these results but may still warrant further attention in CLL.

All four DLBCL cell-lines proved sensitive to SC-236, the PKCβ inhibitor investigated. The 3-day EC_{50} cell count ranged from 15 to 26 μM and the 3-day EC_{50} viability ranged from 22 to 68 μM. In the two chemoresistant primary MCL tumours examined, SC-236 also inhibited cell growth and induced cell death but at higher concentrations. Since this research was conducted the PKCβ inhibitor enzastaurin has entered clinical trials in patients DLBCL and other cancers. Encouragingly three of the 55 relapsed/refractory DLBCL patients achieved a CR and ongoing remissions of >20 months at the time of publication (Robertson *et al.*, 2007). From the results in relapsed/refractory MCL enzastaurin holds the promise of PKCβ inhibition proving to be an effective maintenance treatment or a useful adjunct to standard cytotoxic therapy in a minority of patients. Molecular clarification of which patients will benefit from this novel treatment would be a welcome proof of principle for this rational therapy.

Thesis Summary and Predictions

In summary, the aims and objectives of this thesis were achieved. Novel empirical and rational predictors of response and outcome were discovered and validated *in vitro*. Further work has been suggested by the results generated. The long-term goal of accurate risk-stratification and treatment selection for individual DLBCL patients requires greater understanding of the molecular pathogenesis of DLBCL and the mechanisms governing response and resistance to treatment. It is my expectation that a combined clinical, molecular and pharmacogenomic prognostic model will be capable of more accurately risk stratifying DLBCL patients than each model in isolation or in dual combinations and will ultimately allow individualised treatment strategies to be studied. The research described in this thesis suggests that such knowledge is now within reach of investigators and will lead to additional rational therapies for DLBCL.

APPENDIX

Data Tables for Chapter 4

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	1.00	1.00	1.00	1.00
3.00	0.48	0.74	0.82	0.85	0.72
6.00	0.78	0.01	0.01	0.02	0.01
10.00	1.00	0.00	0.00	0.00	0.00
12.00	1.08	0.00	0.00	0.00	0.00
20.00	1.30	0.00	0.00	0.01	0.00

Table A4.1 SDG 3-day cell count relative to no treatment controls for SUD4

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	1.00	1.00	1.00	1.00
3.00	0.48	0.92	0.98	0.94	0.99
6.00	0.78	0.08	0.08	0.06	0.05
10.00	1.00	0.01	0.01	0.01	0.01
12.00	1.08	0.00	0.00	0.01	0.01
20.00	1.30	0.00	0.00	0.02	0.00

Table A4.2 SDG 3-day viability relative to no treatment controls for SUD4

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	0.96	1.07	0.91	1.11
1.00	0.00	0.81	1.09	0.83	0.85
3.00	0.48	0.98	1.00	0.97	0.79
6.00	0.78	0.75	0.55	0.71	0.59
10.00	1.00	0.12	0.15	0.19	0.18
20.00	1.30	0.04	0.04	0.01	0.02

Table A4.3 SDG 3-day cell count relative to no treatment controls for CRL

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	1.00	0.98	0.99	1.00
1.00	0.00	0.99	0.99	0.95	0.98
3.00	0.48	0.96	0.91	0.95	0.93
6.00	0.78	0.97	0.84	0.87	0.84
10.00	1.00	0.26	0.38	0.46	0.50
20.00	1.30	0.10	0.13	0.05	0.08

Table A4.4 SDG 3-day viability relative to no treatment controls for CRL

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	1.00	1.00	1.00	1.00
2.00	0.30	0.99	1.08	0.91	1.11
3.00	0.48	1.08	1.21	0.84	0.83
5.00	0.70	0.05	0.02	0.05	0.05
7.00	0.85	0.01	0.02	0.03	0.02
12.00	1.08	0.01	0.01	0.02	0.02

Table A4.5 SDG 3-day cell count relative to no treatment controls for DoHH2

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	1.00	1.00	1.00	1.00
2.00	0.30	1.01	0.96	0.98	1.00
3.00	0.48	0.96	0.92	1.00	0.93
5.00	0.70	0.18	0.08	0.22	0.18
7.00	0.85	0.05	0.09	0.15	0.07
12.00	1.08	0.05	0.03	0.10	0.08

Table A4.6 SDG 3-day viability relative to no treatment controls for DoHH2

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	0.95	1.06	1.03	1.07
1.00	0.00	1.20	1.19	1.03	*1.40*
3.00	0.48	1.17	1.02	1.34	*1.78*
6.00	0.78	0.07	0.08	0.25	0.31
10.00	1.00	0.00	0.02	0.04	0.03
20.00	1.30	0.00	0.00	0.02	0.02

Table A4.7 SDG 3-day cell count relative to no treatment controls for DHL4
Values in italics were censored as outliers

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	1.00	1.01	0.99	1.00
1.00	0.00	0.99	1.00	0.98	1.00
3.00	0.48	0.93	1.00	1.00	0.98
6.00	0.78	0.53	0.46	0.80	0.70
10.00	1.00	0.02	0.13	0.25	0.21
20.00	1.30	0.03	0.04	0.12	0.10

Table A4.8 SDG 3-day viability relative to no treatment controls for DHL4

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1	1	1	1	1	1	1	1
0.10	-1.00	0.72	1.05	0.84	1.14				
0.30	-0.52	*0.72*	*0.74*	*0.84*	*1.19*				
1.00	0.00	0.68	0.85	0.96	1.13				
3.00	0.48	0.82	0.64	1	0.8	0.56	0.86	0.72	*1.41*
6.00	0.78	0.85	0.42	0.94	0.51				
8.00	0.90	0.43	0.2	0.37	0.21				
10.00	1.00	0.18	0.08	0.2	0.12	0.09	0.13	0.1	0.11
15.00	1.18	0.18	0.09	0.12	0.09				

Table A4.9 MSA 3-day cell count relative to no treatment controls for SUD4
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1	1	1	1	1	1	1	1
0.10	-1.00	1.02	0.98	1.08	0.95				
0.30	-0.52	1.02	0.97	1.01	0.96				
1.00	0.00	1.01	0.99	1.02	0.98				
3.00	0.48	1	0.97	0.98	0.98	1.04	1	0.99	1.06
6.00	0.78	0.94	0.95	0.88	1				
8.00	0.90	0.86	0.76	0.73	0.81				
10.00	1.00	0.41	0.48	0.37	0.34	0.49	0.49	0.43	0.38
15.00	1.18	0.37	0.3	0.29	0.37				

Table A4.10 MSA 3-day viability relative to no treatment controls for SUD4
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.00	0.00	*1.43*	*0.85*	*1.30*	*0.65*				
3.00	0.48	0.98	1.02	1.20	0.90	0.86	0.66	0.85	0.79
4.50	0.65	0.63	0.43	0.56	0.68				
6.00	0.78	0.28	0.17	0.24	0.22	0.26	0.23	0.39	0.38
10.00	1.00	0.15	0.12	0.11	0.12	0.12	0.12	0.12	0.11
20.00	1.30	0.02	0.04	0.04	0.06				
30.00	1.48	0.01	0.01	0.03	0.03				

Table A4.11 MSA 3-day cell count relative to no treatment controls for CRL
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.00	0.00	1.00	1.07	1.01	0.94				
3.00	0.48	1.00	1.04	1.04	1.01	0.94	0.90	0.93	0.96
4.50	0.65					0.88	0.90	0.97	0.96
6.00	0.78	0.61	0.64	0.66	0.64	0.72	0.77	0.91	0.79
10.00	1.00	0.37	0.44	0.45	0.48	0.42	0.48	0.41	0.43
20.00	1.30					0.08	0.17	0.15	0.23
30.00	1.48	0.01	0.03	0.12	0.10				

Table A4.12 MSA 3-day viability relative to no treatment controls for CRL
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.10	-1.00	1.03	*1.20*	*1.31*	0.95				
0.30	-0.52	0.85	*1.22*	1.06	0.84	1.05	0.69	0.76	0.82
1.00	0.00	0.51	0.46	0.88	0.48	0.45	0.43	0.44	0.68
3.00	0.48	0.02	0.00	0.07	0.02	0.11	0.07	0.12	0.12
6.00	0.78					0.06	0.05	0.04	0.07
10.00	1.00	0.00	0.00	0.01	0.00	0.03	0.03	0.03	0.03

Table A4.13 MSA 3-day cell count relative to no treatment controls for DoHH2
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1	1	1	1	1	1	1	1
0.10	-1.00	1	0.94	1.07	1.02				
0.30	-0.52	0.97	0.97	1.04	0.92	1	0.88	1.03	1.04
1.00	0.00	0.83	0.71	0.84	0.82	0.7	0.72	0.88	0.95
3.00	0.48	0.1	*0.01*	0.18	0.11	0.33	0.26	0.39	0.33
6.00	0.78					0.17	0.2	0.18	0.21
10.00	1.00	0.03	0.01	0.05	0.01	0.14	0.15	0.16	0.13

Table A4.14 MSA 3-day viability relative to no treatment controls for DoHH2
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.00	0.00	1.16	0.60	0.79	0.87		0.87	1.00	0.84
2.00	0.30	0.48	0.32	0.37	0.43				
3.00	0.48	0.29	0.28	0.28	0.35	0.52	0.33	0.28	0.48
6.00	0.78	0.31	0.26	0.24	0.22				
8.00	0.90	0.24	0.23	0.24	0.24				
10.00	1.00	0.23	0.24	0.27	0.20	0.21	0.08	0.12	0.18
20.00	1.30	0.18	0.19	0.22	0.23	0.16	0.15	0.10	0.17
30.00	1.48	0.18	0.18	0.17	0.17	0.11	0.06	0.10	0.13
60.00	1.78	*0.19*	*0.16*	*0.18*	*0.15*				
100.00	2.00	0.09	0.09	0.12	0.12				

Table A4.15 MSA 3-day cell count relative to no treatment controls for DHL4
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.00	0.00	1.01	0.99	0.99	1.01	1.02	1.03	1.02	1.02
2.00	0.30	1.01	1.01	0.99	1.00				
3.00	0.48	1.02	1.00	1.00	0.99	1.05	1.02	1.00	1.02
6.00	0.78	0.98	0.98	0.91	0.98				
8.00	0.90	0.96	0.95	0.95	0.94				
10.00	1.00	1.02	1.00	0.96	0.97	0.96	0.93	1.03	0.97
20.00	1.30	0.92	0.95	0.90	0.93	0.93	0.95	0.91	0.94
30.00	1.48	0.93	0.90	0.92	0.94	0.81	0.91	0.97	0.92
60.00	1.78	0.88	0.78	0.83	0.77				
100.00	2.00	0.68	0.54	0.69	0.68				

Table A4.16 MSA 3-day viability relative to no treatment controls for DHL4
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.09	0.95	0.90	*0.75*	*1.59*	*0.72*
3.00	0.48	1.06	*0.44*	0.92	0.78	0.69	0.87
10.00	1.00	*1.25*	0.96	0.91	0.81	0.62	*0.57*
30.00	1.48	0.75	0.73	0.72	0.80	*0.00*	*0.00*
100.00	2.00	*0.87*	0.68	0.55	0.53	0.51	*0.27*
300.00	2.48	0.44	*0.51*	0.34	0.44	0.29	0.40

Table A4.17 SDG 2-day cell count relative to no treatment controls for 01
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.04	0.98	1.03	0.97	1.00	0.98
3.00	0.48	0.79	0.97	0.92	0.92	0.79	0.60
10.00	1.00	0.74	0.69	0.55	0.50	0.42	0.32
30.00	1.48	0.47	0.43	0.44	0.47	*0.00*	*0.00*
100.00	2.00	0.52	0.39	0.31	0.28	0.31	0.19
300.00	2.48	0.28	0.33	0.24	0.26	0.18	0.22

Table A4.18 SDG 2-day viability relative to no treatment controls for 01
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.05	1.00	1.09	1.12	0.84	0.91
3.00	0.48	0.93	0.90	0.95	1.01	1.11	*1.23*
10.00	1.00	0.89	0.68	0.84	0.79	0.83	0.72
30.00	1.48	*0.79*	0.65	0.32	0.66	0.60	*0.24*
100.00	2.00	*0.57*	0.54	0.33	0.31	0.39	0.22
300.00	2.48	0.26	0.06	0.23	0.15	0.17	0.13

Table A4.19 SDG 2-day cell count relative to no treatment controls for 02
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.00	0.98	0.97	0.98	1.04	1.04
3.00	0.48	0.89	0.88	0.84	0.84	0.88	0.89
10.00	1.00	0.74	0.60	0.62	0.58	0.55	0.53
30.00	1.48	0.65	0.52	0.34	0.48	0.43	0.36
100.00	2.00	0.46	0.46	0.35	0.31	0.31	0.22
300.00	2.48	0.21	0.08	0.24	0.18	0.20	0.12

Table A4.20 SDG 2-day viability relative to no treatment controls for 02
[ ] = concentration μM; values in italics were censored as outliers

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9	Replicate 10	Replicate 11	Replicate 12
0.00	-3.00	0.87	1.03	0.77	1.26	1.03	1.05	1.17	0.98	1.03	0.89	0.97	0.97
1.00	0.00	0.91	0.87	0.82	0.95	1.21	0.94						
3.00	0.48	*1.31*	*1.19*	*1.04*	*1.25*	*1.17*	*0.79*	*1.23*	*1.08*	*0.95*	*0.89*	*0.87*	*1.05*
4.00	0.60	1.08	0.79	0.69	0.93	0.91	0.93						
6.00	0.78	0.73	0.82	0.87	1.15	0.90	1.10						
8.00	0.90	0.90	0.84	0.70	0.87	0.93							
10.00	1.00	0.73	0.66	0.58	0.91	0.87	0.89	0.62	0.78	0.89	0.70	0.55	0.75
12.00	1.08	0.74	0.79	0.81	0.77	0.66	0.52						
14.00	1.15	0.47	0.46	0.58	0.62	0.63	0.40						
30.00	1.48	*1.00*	*1.30*	*1.06*	*1.03*	*0.74*	0.30	0.27	0.25	0.42	0.47	0.33	0.21
100.00	2.00	*0.78*	*0.78*	0.47	0.47	0.16	0.22	0.21	0.21	0.37	0.18	0.21	0.17

Table A4.21 SDG 2-day cell count relative to no treatment controls for 03

[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9	Replicate 10	Replicate 11	Replicate 12
0.00	-1.00	0.95	0.99	1.01	1.02	1.03	1.01	1.00	1.05	0.99	1.09	0.93	0.93
3.00	0.48	1.00	0.98	1.01	0.92	1.02	0.94	0.67	0.71	0.69	0.64	0.67	0.42
10.00	1.00	0.65	0.56	0.72	0.50	0.50	0.49	0.49	0.43	0.42	0.52	*0.36*	*0.29*
30.00	1.48	0.30	0.37	0.50	0.50	0.33	0.23	0.56	0.60	0.59	0.43	0.36	0.18
100.00	2.00	0.23	0.21	0.30	0.17	0.19	0.15	0.37	0.42	0.28	0.22	0.10	0.13

Table A4.22 SDG 2-day viability relative to no treatment controls for 03

[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9	Replicate 10	Replicate 11
0.00	-3.00	1.07	0.89	1.02	1.06	0.96	0.99	1.08	0.87	1.05	0.98	1.02
1.00	0.00	*1.25*	*0.90*	*1.18*	*1.26*	*1.44*						
3.00	0.48	0.81	1.03	0.66	0.78							
4.00	0.60	*1.47*	*1.48*	*1.00*	*0.86*	*1.48*	*1.30*					
6.00	0.78	*1.30*	1.05	1.05	0.95	0.83	0.79					
10.00	1.00	*1.19*	0.77	1.20	0.47	1.02	0.68	0.87	0.77	0.71	0.66	0.66
12.00	*1.08*	*1.15*	*0.60*	*0.53*	*0.64*	*1.09*						
15.00	1.18	0.92	1.00	0.86	0.65	0.63	0.51					
20.00	1.30	0.79	0.79	0.63								
30.00	1.48	0.52	0.47	0.37	0.77	0.50	0.52	0.50	0.46	0.45	0.33	
100.00	2.00	0.50	0.49	0.30	0.25	0.29	0.25					
300.00	2.48	0.18	0.13	0.16	0.10	0.12	0.10					

Table A4.23 SDG 2-day cell count relative to no treatment controls for 04

[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9	Replicate 10	Replicate 11	Replicate 12
0.00	-3.00	1.06	1.04	0.93	1.00	0.98	1.00	0.98	0.98	1.01	1.02	1.04	0.98
1.00	0.00	1.05	1.01	1.08	1.08	1.00	0.95						
3.00	0.48	0.99	0.81	0.76	*0.24*	*0.17*							
4.00	0.60	*1.09*	*1.09*	*1.05*	*0.97*	*1.00*	*0.84*						
6.00	0.78	0.90	0.88	0.80	0.72	0.76	0.76						
10.00	1.00	0.78	0.83	0.78	0.69	*0.91*	*0.95*	*0.90*	*0.81*	0.69	*0.18*		
12.00	1.08	0.84	0.86	0.68	0.60	0.70	0.69						
15.00	1.18	0.68	0.83	0.70	0.65	0.68	0.57						
20.00	1.30	0.63	0.75	0.57	0.75	0.65							
30.00	1.48	*0.74*	0.67	0.39	0.39	0.37	0.41	0.51	0.68	0.55	0.56	0.42	0.24
100.00	2.00	0.40	0.40	0.33	0.31	0.29	0.29						
300.00	2.48	0.14	0.11	0.13	0.08	0.09	0.07						

Table A4.24 SDG 2-day viability relative to no treatment controls for 04

[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.25	1.03	0.90	0.96	0.99	0.87
3.00	0.48	0.83	*0.69*	0.91	0.84	0.64	0.63
10.00	1.00	*0.60*	*0.60*	*0.65*	*0.53*	*0.54*	*0.70*
30.00	1.48	0.83	0.76	*0.43*	*0.38*	*0.38*	0.69
100.00	2.00	*0.93*	0.68	0.68	0.65	*0.81*	*0.88*
300.00	2.48	0.38	0.49	0.49	0.69	0.45	0.37

Table A4.25 SDG 2-day cell count relative to no treatment controls for pooled normal B-cells

[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	0.99	0.99	1.03	1.02	1.00	0.97
3.00	0.48	0.80	0.82	0.85	1.01	0.86	0.69
10.00	1.00	0.60	0.71	0.63	0.59	0.51	0.55
30.00	1.48	0.57	0.62	0.46	0.38	0.45	0.60
100.00	2.00	0.62	0.62	0.61	0.49	0.61	0.62
300.00	2.48	0.49	0.45	0.47	0.48	0.38	0.32

Table A4.26 SDG 2-day viability relative to no treatment controls for pooled normal B-cells

[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.09	0.95	0.90	0.75	1.59	0.72
10.00	1.00	1.34	*1.72*	1.31	1.48	1.30	1.42
30.00	1.48	1.23	1.09	1.28	1.25	*1.47*	0.90
100.00	2.00	0.62	0.69	0.62	0.60	0.69	0.74
300.00	2.48	0.44	*0.70*	0.51	0.44	0.32	0.28

Table A4.27 MSA 2-day cell count relative to no treatment controls for 01
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.04	0.98	1.03	0.97	1.00	0.98
10.00	1.00	0.89	1.01	0.90	0.91	0.91	0.93
30.00	1.48	0.78	0.77	0.76	0.75	0.82	0.71
100.00	2.00	0.45	0.51	0.35	0.46	0.48	0.49
300.00	2.48	0.34	0.41	0.37	0.27	0.23	0.20

Table A4.28 MSA 2-day viability relative to no treatment controls for 01
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.05	1.00	1.09	1.12	0.84	0.91
10.00	1.00	*1.06*	0.88	0.86	0.85	0.61	0.68
30.00	1.48	0.66	0.73	0.49	0.68	0.45	0.67
100.00	2.00	*0.77*	0.56	0.54	0.36	0.38	0.54
300.00	2.48	0.32	0.37	0.42	0.33	*0.24*	0.46

Table A4.29 MSA 2-day cell count relative to no treatment controls for 02
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.00	0.98	0.97	0.98	1.04	1.04
10.00	1.00	0.79	0.69	0.70	0.71	0.65	0.68
30.00	1.48	0.61	0.57	0.60	0.59	0.59	0.54
100.00	2.00	0.57	0.48	0.46	0.36	0.43	0.47
300.00	2.48	0.29	0.39	0.41	0.29	0.32	0.35

Table A4.30 MSA 2-day viability relative to no treatment controls for 02
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.17	0.98	1.03	0.89	0.97	0.97
3.00	0.48	1.39	1.19	*2.36*	1.44	1.22	1.52
10.00	1.00	*2.06*	1.41	1.41	*2.11*	1.63	1.36
30.00	1.48	1.58	1.68	1.65	1.14	1.61	1.08
100.00	2.00	1.08	0.85	1.01	0.87	1.03	1.09

Table A4.31 MSA 2-day cell count relative to no treatment controls for 03
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	1.00	1.05	0.99	1.09	0.93	0.93
3.00	0.48	0.92	0.96	0.89	0.93	0.91	0.90
10.00	1.00	0.81	0.88	0.79	0.86	0.87	0.78
30.00	1.48	0.77	0.76	0.78	0.83	0.72	0.65
100.00	2.00	0.63	0.64	0.60	0.51	0.65	0.73

Table A4.32 MSA 2-day viability relative to no treatment controls for 03
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	0.89	0.86	1.09	1.06	1.12	0.99
10.00	1.00	0.43	0.69	0.74	0.49	0.46	0.75
30.00	1.48	0.68	0.54	0.47	0.53	0.28	0.43
100.00	2.00	0.64	0.49	0.33	0.48	0.55	0.4
300.00	2.48	0.42	0.39	0.52	0.28	0.28	0.39

Table A4.33 MSA 2-day cell count relative to no treatment controls for 04
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-1.00	0.98	1.00	0.99	1.00	1.02	1.00
10.00	1.00	0.63	0.59	0.68	0.50	0.49	0.62
30.00	1.48	0.63	0.54	0.50	0.45	0.35	0.51
100.00	2.00	0.53	0.49	0.36	0.44	0.40	0.39
300.00	2.48	0.42	0.38	0.44	0.31	0.36	0.39

Table A4.34 MSA 2-day viability relative to no treatment controls for 04
[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	0.99	0.99	1.03	1.02	1.00	0.97
10.00	1.00	1.29	1.05	0.82	0.94	1.08	0.99
30.00	1.48	0.74	0.55	0.90	0.73	1.02	0.73
100.00	2.00	0.96	0.74	0.96	0.82	0.60	0.71
300.00	2.48	0.67	0.63	0.67	0.74	0.63	0.60

Table A4.35 MSA 2-day cell count relative to no treatment controls for pooled normal B-cells

[ ] = concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	0.99	0.99	1.03	1.02	1.00	0.97
10.00	1.00	0.94	0.92	0.99	0.84	0.90	0.96
30.00	1.48	0.79	0.80	0.91	0.77	0.94	0.93
100.00	2.00	0.92	0.88	0.90	0.93	0.69	0.74
300.00	2.48	0.65	0.65	0.65	0.77	0.62	0.58

Table A4.36 MSA 2-day viability relative to no treatment controls for pooled normal B-cells

[ ] = concentration μM; values in italics were censored as outliers.

Cell-line and SDG Concentration μM	CRL 0	CRL 0	CRL 0	CRL 1	CRL 2	CRL 4	CRL 7	CRL 10	CRL 10
A %	9	9	7	9	8	11	22	40	40
G1 %	57	57	62	55	56	46	13	16	16
S %	20	18	17	19	18	21	41	28	26
G2 %	14	16	14	17	18	22	24	16	18
Total	99	100	100	100	100	100	100	100	100
Cell-line and SDG Concentration μM	DHL4 0	DHL4 0	DHL4 0	DHL4 6	DHL4 8	DHL4 10	DHL4 12	DHL4 14	
A %	2	1	1	24	11	18	25	28	
G1 %	54	51	52	36	40	34	35	39	
S %	29	29	28	20	26	23	22	17	
G2 %	16	18	18	19	23	23	18	16	
Total	100	100	100	100	100	98	100	100	
Cell-line and SDG Concentration μM	SUD4 0	SUD4 0	SUD4 0	SUD4 2	SUD4 4	SUD4 7.5	SUD4 12	SUD4 15	
A %	6	5	5	6	9	19	10	13	
G1 %	48	51	56	53	48	34	45	41	
S %	27	28	25	23	27	24	31	29	
G2 %	17	14	13	16	14	23	14	17	
Total	98	98	99	99	98	100	99	100	

Table A4.37 Cell Cycle Distribution for the four cell-lines following 3-day exposure to increasing concentrations of SDG. A % = percentage cells in Apoptosis i.e. sub-G1; G1 = percentage cells in Growth phase 1, S = percentage cells in Synthesis phase and G2 = percentage cells in Growth phase 2.

Cell-line and MSA Concentration μM	CRL 0	CRL 1	CRL 3	CRL 3	CRL 3	CRL 6	CRL 6	CRL 10	CRL 10	CRL 30	CRL 30	
A %	11	11	11	11	14	23	24	41	43	57	51	
G1 %	46	46	40	44	42	45	41	37	38	16	22	
S %	24	23	25	23	23	15	19	15	12	23	19	
G2 %	18	19	23	21	21	15	16	7	7	4	8	
Cell-line and MSA Concentration μM	DHL4 0	DHL4 1	DHL4 1	DHL4 3	DHL4 3	DHL4 10	DHL4 10	DHL4 20	DHL4 20	DHL4 30	DHL4 30	DHL4 30
A %	2	2	1	2	3	3	4	11	7	16	15	14
G1 %	43	49	49	44	50	67	67	67	53	44	48	45
S %	34	27	31	33	27	17	8	7	10	14	15	28
G2 %	20	22	19	22	20	12	21	15	29	26	22	13
Cell-line and MSA Concentration μM	DoHH2 0	DoHH2 0	DoHH2 0.1	DoHH2 0.1	DoHH2 0.3	DoHH2 0.3	DoHH2 1	DoHH2 3	DoHH2 10			
A %	8	7	8	10	7	16	51	78	72			
G1 %	44	42	47	48	46	44	22	5	5			
S %	32	32	28	24	27	23	20	16	21			
G2 %	16	19	16	18	20	17	6	1	2			
Cell-line and MSA Concentration μM	SUD4 0	SUD4 0	SUD4 0.1	SUD4 0.1	SUD4 0.3	SUD4 0.3	SUD4 1	SUD4 1	SUD4 3	SUD4 3	SUD4 10	SUD4 10
A %	3	3	2	3	5	3	3	4	3	5	52	65
G1 %	49	52	47	49	44	51	47	49	45	49	19	10
S %	30	25	31	27	29	26	31	31	32	30	25	22
G2 %	17	18	19	20	22	20	18	16	19	16	4	3

Table A4.38 Cell Cycle Distribution for all four cell-lines following 3-day exposure to increasing concentrations of MSA. A % = percentage cells in Apoptosis i.e. sub-G1; G1 = percentage cells in Growth phase 1, S = percentage cells in Synthesis phase and G2 = percentage cells in Growth phase 2.

Cell-line and SDG Concentration μM	B-cells 0	B-cells 0	B-cells 3	B-cells 3	B-cells 10	B-cells 10	B-cells 30	B-cells 30	B-cells 100	B-cells 100	B-cells 300	B-cells 300
A %	22	22	16	21	15	13	16	15	17	16	50	46
G1 %	69	66	78	74	78	81	75	78	73	73	45	49
S %	6	9	3	3	4	3	4	3	4	4	6	3
G2 %	3	2	3	3	4	3	5	4	6	6	1	1
Cell-line and MSA Concentration μM	B-cells 0	B-cells 0	B-cells 10	B-cells 10	B-cells 30	B-cells 30	B-cells 100	B-cells 100	B-cells 300	B-cells 300		
A %	22	22	22	23	34	35	31	34	31	32		
G1 %	69	66	66	62	53	50	54	47	58	45		
S %	6	9	11	12	10	12	12	17	9	19		
G2 %	3	2	1	2	2	2	2	2	2	2		
Cell-line and SDG Concentration μM	(04) 0	(04) 1	(04) 1	(04) 4	(04) 4	(04) 6	(04) 6	(04) 10	(04) 10			
A %	26	21	17	24	23	60	61	51	52			
G1 %	53	60	65	53	54	27	27	38	38			
S %	16	11	11	16	17	8	8	8	6			
G2 %	4	7	6	7	5	4	3	2	2			
Cell-line and SDG Concentration μM	(04) 12	(04) 12	(04) 15	(04) 15	(04) 20	(04) 20	(04) 30	(04) 30				
A %	54	53	53	53	49	49	49	50				
G1 %	35	37	37	38	42	42	43	42				
S %	7	7	6	6	6	6	5	5				
G2 %	3	3	2	2	2	2	1	1				

Table A4.39 Cell Cycle Distribution for the Pooled Normal B-cells and MCL sample (04) following 3-day exposure to increasing concentrations of MSA and SDG and SDG Respectively. A % = percentage cells in Apoptosis i.e. sub-G1; G1 = percentage cells in Growth phase 1, S = percentage cells in Synthesis phase and G2 = percentage cells in Growth phase 2.

Cell-line	SUD4	SUD4	SUD4	Cell-line	CRL	CRL	CRL
Replicate	1	2	3	Replicate	1	2	3
Luminescence	% cf Control	% cf Control	% cf Control	Luminescence	% cf Control	% cf Control	% cf Control
Control	100	100	100	Control	100	100	100
Control (DMSO)	93	104	97	Control (DMSO)	93	103	105
Control (H_2O_2)	191	208	195	Control (H_2O_2)	228	231	237
SDG 2 μM	375	341	402	5 μM SDG	264	305	323
SDG 5 μM	424	413	494	10 μM SDG	290	309	329
SDG 10 μM	415	418	521	25 μM SDG	283	302	326
MSA 5 μM	146	170	192	5 μM MSA	114	134	145
MSA 10 μM	157	186	210	10 μM MSA	102	113	125
MSA 25 μM	155	186	211	25 μM MSA	100	107	111

Cell-line	DoHH2	DoHH2	DoHH2	Cell-line	DHL4	DHL4	DHL4
Replicate	1	2	3	Replicate	1	2	3
Luminescence	% cf Control	% cf Control	% cf Control	Luminescence	% cf Control	% cf Control	% cf Control
Control	100	100	100	Control	100	100	100
Control (DMSO)	94	102	103	Control (DMSO)	98	105	108
Control (H_2O_2)	118	107	153	Control (H_2O_2)	78	90	149
SDG 2 μM	149	121	238	SDG 4 μM	220	218	243
SDG 4 μM	153	131	239	SDG 7 μM	206	208	236
SDG 10 μM	153	134	228	SDG 20 μM	172	186	217
MSA 1 μM	136	153	137	MSA 100 μM	119	108	121
MSA 2 μM	155	177	150	MSA 200 μM	106	99	110
MSA 5 μM	175	200	146	MSA 500 μM	97	91	107

Table A4.40 ROS Generation after 30-minute Exposures to the cytotoxic EC_{50} Concentrations of SDG or MSA The generation of ROS in lymphoma cells relative to control (0.75% DMSO) after exposure for 30 minutes to SDG and MSA at concentrations around the 3-day cytotoxic EC_{50} concentrations. 25 μM H_2O_2 was used as the positive control.

SUD4	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Control	100	100	101	101	97	100
NAC	83	100	103	104	102	101
SDG	61	55	69	62	46	33
SDG+NAC	77	71	98	103	88	94 *
MSA	47	61	70	76	46	46
MSA+NAC	53	51	74	73	38	41
CRL	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Control	99	99	103	99	0	0
NAC	88	78	101	103	0	0
SDG	15	50	40	37	0	0
SDG+NAC	43	43	46	47	0	0
MSA	16	33	43	60	0	0
MSA+NAC	23	28	36	53	0	0
DoHH2	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Control	105	102	97	94	100	99
NAC	108	104	80	81	101	101
SDG	66	62	39	35	88	89
SDG+NAC	67	78	54	62	82	85
MSA	63	68	43	46	82	72
MSA+NAC	65	72	33	33	80	77
DHL4	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
Control	104	101	96	96	102	103
NAC	104	104	93	94	101	100
SDG	52	34	32	46	57	71
SDG+NAC	63	70	70	78	80	80 *
MSA	47	40	30	40	59	58
MSA+NAC	34	34	46	42	34	41

Table A4.41 Effect of N-acetyl cysteine on the Cytotoxicity of SDG and MSA after 3-day Exposures to the cytotoxic EC50 Concentrations of SDG or MSA Cells were continuously co-exposed to EC50 viability concentrations of SDG or MSA ± N-acetyl cysteine (NAC) or N-acetyl cysteine alone. SDG concentration: SUD4, CRL, DoHH2, DHL4 and cells = 4.0, 9.0, 4.2 and 7.0 µM, respectively; MSA concentration SUD4, CRL DoHH2 and DHL4 cells 10.0, 9.0, 1.9 and 166 µM, respectively, N-acetyl cysteine 2.5 µM throughout. * P = 0.0008 and 0.0053 respectively for SUD4 and DHL4 SDG vs. SDG + NAC. SDG = selenodiglutathione, MSA = methylseleninic acid and NAC = N-acetyl cysteine

Data Tables for Chapter 6

SUD4	Mean ± Standard Deviation	Number of Replicates
Pic 0 nM DMSO 0%	100 ± 12	18
Pic 300 nM DMSO 0.03%	103 ± 17	24
Pic 1000 nM DMSO 0.1%	89 ± 14	24
Pic 10000 nM DMSO 1%	65 ± 16	24 *
DMSO 0.5%	92 ± 10	6
DMSO 1%	59 ± 12	15 *

CRL	Mean ± Standard Deviation	Number of Replicates
Pic 0 nM DMSO 0%	100 ± 8	13
Pic 300 nM DMSO 0.03%	97 ± 22	21
Pic 1000 nM DMSO 0.1%	88 ± 17	21
Pic 10000 nM DMSO 1%	50 ± 15	21 *
DMSO 0.5%	95 ± 17	3
DMSO 1%	40 ± 9	18 *

DoHH2	Mean ± Standard Deviation	Number of Replicates
Pic 0 nM DMSO 0%	100 ± 5	13
Pic 300 nM DMSO 0.03%	104 ± 18	24
Pic 1000 nM DMSO 0.1%	95 ± 17	24
Pic 10000 nM DMSO 1%	63 ± 12	24 *
DMSO 0.5%	82 ± 8	6
DMSO 1%	65 ± 14	18 *

Table A6.1 Effect of 3-day Exposure to Piclamilast on DLBCL Cell-line Proliferation

After a 3-day exposure to piclamilast, DMSO control or neither the proliferation of the cell-lines was assessed by MTS assay. The mean proliferation is shown as a percentage relative to that of the no treatment control cells. A minimum of three independent experiments was conducted with the total number of wells assayed being indicated in the Number column.

Pic = piclamilast

* Piclamilast 10000 nM vs. DMSO 1% = non-significant

SUD4	Mean Standard Deviation	Number of Replicates
Pic 0 nM (DMSO 0%) Ara-c 0 nM	100 ± 12	9
Pic 300 nM (DMSO 0.03%) Ara-c 313 nM	76 ± 17	12
Pic 1000 nM (DMSO 0.1%) Ara-c 625 nM	**55 ± 12**	**12 ***
Pic 10000 nM (DMSO 1%) Ara-c 1250 nM	26 ± 6	12
Pic 10000 nM (DMSO 1%) Ara-c 2500 nM	20 ± 5	9
Ara-c 0 nM	100 ± 12	9
Ara-c 313 nM	65 ± 12	12
Ara-c 625 nM	**56 ± 11**	**12 ***
Ara-c 1250 nM	43 ± 7	12
Ara-c 2500 nM	42 ± 7	12
DMSO 1% Ara-c 1250 nM	19 ± 7	9

CRL	Mean Standard Deviation	Number of Replicates
Pic 0 nM (DMSO 0%) Ara-c 0 nM	100 ± 3	12
Pic 300 nM (DMSO 0.03%) Ara-c 158 nM	59 ± 11	12
Pic 1000 nM (DMSO 0.1%) Ara-c 315 nM	**54 ± 17**	**12 ***
Pic 10000 nM (DMSO 1%) Ara-c 630 nM	37 ± 8	12
Pic 10000 nM (DMSO 1%) Ara-c 1260 nM	31 ± 6	12
Ara-c 0 nM	100 ± 4	12
Ara-c 158 nM	63 ± 12	12
Ara-c 315 nM	**48 ± 11**	**12 ***
Ara-c 630 nM	45 ± 13	12
Ara-c 1260 nM	47 ± 10	12
DMSO 1% Ara-c 630nM	26 ± 4	12

DoHH2	Mean Standard Deviation	Number of Replicates
Pic 0 nM (DMSO 0%) Ara-c 0 nM	99 ± 4	12
Pic 300 nM (DMSO 0.03%) Ara-c 15 nM	91 ± 11	9
Pic 1000 nM (DMSO 0.1%) Ara-c 29 nM	**76 ± 12**	**12 ***
Pic 10000 nM (DMSO 1%) Ara-c 58 nM	32 ± 6	12
Pic 10000 nM (DMSO 1%) Ara-c 116 nM	20 ± 7	12
Ara-c 0 nM	100 ± 3	12
Ara-c 15 nM	86 ± 17	6
Ara-c 29 nM	**69 ± 15**	**6 ***
Ara-c 58 nM	47 ± 9	12
Ara-c 116 nM	25 ± 8	12
DMSO 1% Ara-c 58 nM	30 ± 8	12

Table A6.2 Cell Proliferation After a 3-day Exposure to Piclamilast and Cytosine Arabinoside

After a 3-day exposure to piclamilast, ara-c, DMSO control or neither the proliferation of the cell-lines was assessed by MTS assay. The mean proliferation is shown as a percentage relative to that of the no treatment control cells. A minimum of three independent experiments was conducted with the total number of wells assayed being indicated in the Number column.

Pic = piclamilast; Ara-c = cytosine arabinoside

* Piclamilast 1000 nM +Ara-c vs. Ara-c with equivalent DMSO concentration = non-significant

SUD4	Mean ±Standard Deviation	Number of Replicates
Pic 0 nM (DMSO 0%) Dox 0 nM	100 ±13	9
Pic 300 nM (DMSO 0.03%) Dox 45 nM	100 ±11	12
Pic 1000 nM (DMSO 0.1%) Dox 90 nM	**72 ±12**	**12 ***
Pic 10000 nM (DMSO 1%) Dox 180 nM	9 ±4	12
Pic 10000 nM (DMSO 1%) Dox 360 nM	3 ±4	12
Dox 0 nM	100 ±13	9
Dox 45 nM	90 ±8	12
Dox 90 nM	**76 ±11**	**12 ***
Dox 180 nM	53 ±7	12
Dox 360 nM	26 ±7	12
DMSO 0.5% Dox 180 nM	41 ±8	6
DMSO 1% Dox 180 nM	9 ±3	6

CRL	Mean ± Standard Deviation	Number of Replicates
Pic 0 nM (DMSO 0%) Dox 0 nM	100 ±9	9
Pic 300 nM (DMSO 0.03%) Dox 20 nM	82 ±11	12
Pic 1000 nM (DMSO 0.1%) Dox 40 nM	**70 ±10**	**12 ***
Pic 10000 nM (DMSO 1%) Dox 80 nM	31 ±4	11
Pic 10000 nM (DMSO 1%) Dox 160 nM	18 ±3	11
Dox 0 nM	100 ±9	9
Dox 20 nM	66 ±10	9
Dox 40 nM	**64 ±8**	**9 ***
Dox 80 nM	52 ±5	9
Dox160 nM	43 ±5	9
DMSO 0.5% Dox 80 nM	56 ±4	3
DMSO 1% Dox 80 nM	26 ±3	6

DoHH2	Mean ±Standard Deviation	Number of Replicates
Pic 0 nM (DMSO 0%) Dox 0 nM	100 ±6	9
Pic 300 nM (DMSO 0.03%) Dox 3 nM	97 ±15	12
Pic 1000 nM (DMSO 0.1%) Dox 5 nM	**93 ±10**	**12 ***
Pic 10000 nM (DMSO 1%) Dox 10 nM	37 ±12	12
Pic 10000 nM (DMSO 1%) Dox 20 nM	14 ±7	12
Dox 0 nM	100 ±6	9
Dox 3 nM	93 ±8	12
Dox 5 nM	**83 ±10**	**12 ***
Dox10 nM	59 ±13	12
Dox 20 nM	35 ±16	12
DMSO 0.5% Dox 10 nM	64 ±11	6
DMSO 1% Dox 10 nM	37 ±4	6

Table A6.3 Cell Proliferation After a 3-day Exposure to Piclamilast and Doxorubicin

After a 3-day exposure to piclamilast, doxorubicin, DMSO control or neither the proliferation of the cell-lines was assessed by MTS assay. The mean proliferation is shown as a percentage relative to that of the no treatment control cells incubated for the same time period with vehicle alone. A minimum of three independent experiments was conducted with the total number of wells assayed being indicated in the Number column. Pic = piclamilast; Dox = doxorubicin

* Piclamilast 1000 nM +Dox EC_{50} vs. Dox EC_{50} with equivalent DMSO concentration = non-significant

Cell Count	SUD4	SUD4	SUD4	CRL	CRL	CRL	DoHH2	DoHH2	DoHH2	DHL4	DHL4	DHL4
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
0 Control	100	7	2	100	0	2	100	7	2	100	7	2
Pic 30 nM Fors 20 □Mβ	48	6	2	57	2	2	13	1	2	81	4	2
Pic 100 nM Fors 20 □M β	55	4	2	47	3	2	8	3	2	59	7	2
Pic 300 nM Fors 20 □M β	53	0	2	57	7	2	11	0	2	68	5	2
Pic 1000 nM Fors 20 □M γ	46	1	2	57	7	2	10	2	2	57	8	2
Piclamilast 1000 nM α	96	1	2	74	7	2	81	0	2	81	27	3
Forskolin 20 □M β	59	3	2	59	3	2	13	3	2	75	13	2
Forskolin 40 □M δ	39	6	2	61	6	2	10	1	2	56	6	2
Rolipram 100 □Mβ	89	3	2	71	3	2	68	4	2	76	3	2
Rol 100 mM Fors 20 □M δ	55	6	2	55	3	2	7	2	2	53	4	2
DMSO 0.2%	87	1	2	94	12	2	95	13	2	97	5	2
DMSO 0.4%	77	3	2	93	2	2	90	0	2	100	4	2

SD = Standard deviation

n = number

α = 0.1% DMSO
β = 0.2% DMSO
γ = 0.3% DMSO
δ = 0.4% DMSO

Pic = piclamilast
Rol = rolipram
Fors = forskolin

Viability	SUD4	SUD4	SUD4	CRL	CRL	CRL	DoHH2	DoHH2	DoHH2	DHL4	DHL4	DHL4
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
0 Control	100	1	2	100	3	2	100	0	2	100	0	2
Pic 30 nM Fors 20 □M β	84	3	2	98	3	2	46	2	2	99	0	2
Pic 100 nM Fors 20 □Mβ	88	1	2	99	2	2	37	7	2	99	3	2
Pic 300 nM Fors 20 □M β	87	6	2	95	3	2	40	6	2	100	0	2
Pic 1000 nM Fors 20 □M γ	85	2	2	95	1	2	37	6	2	100	1	2
Piclamilast 1000 nM α	92	2	2	101	2	2	97	2	2	99	1	2
Forskolin 20 □M β	93	3	2	98	4	2	57	3	2	99	1	2
Forskolin 40 □M δ	86	2	2	99	4	2	49	8	2	99	1	2
Rolipram 100 □M β	97	1	2	99	1	2	96	1	2	100	1	2
Rol 100 mM Fors 20 □M δ	82	2	2	92	0	2	26	5	2	98	1	2
DMSO 0.2%	96	1	2	100	2	2	103	0	2	98	0	2
DMSO 0.4%	100	3	2	100	1	2	102	1	2	101	1	2

SD = Standard deviation

n = number

α = 0.1% DMSO
β = 0.2% DMSO
γ = 0.3% DMSO
δ = 0.4% DMSO

Pic = piclamilast
Rol = rolipram
Fors = forskolin

Table A6.4 Viability and Cell Count After a 3-day Exposure to PDE4 Inhibitor &/or Forskolin After a 3-day exposure to piclamilast, forskolin, rolipram, DMSO control or neither the cell count and viability of the cell-lines was assessed by trypan blue exclusion assay. The mean cell counts and viabilities (n = 2) are shown relative to the control cells for each experiment. The standard deviation is given. Pic = piclamilast; Fors = forskolin; Rol = rolipram
The final DMSO content was: α = 0.1% DMSO; β = 0.2% DMSO; γ = 0.3% DMSO; δ = 0.4% DMSO

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	1.00	1.00	1.00	1.00
1.00	0.00	1.11	0.71	0.69	0.85
3.00	*0.48*	0.83	0.70	0.80	0.81
10.00	1.00	*1.08*	0.70	0.79	0.72
30.00	1.48	0.22	0.18	0.06	0.07
100.00	2.00	0.00	0.00	0.00	0.00

Table A6.5 SC-236 3-day cell count relative to no treatment controls for SUD4
Concentration μM; values in italics were censored as outliers.

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4
0.00	-3.00	1.00	1.00	1.00	1.00
1.00	0.00	0.94	0.96	1.07	0.95
3.00	0.48	0.93	1.00	1.04	0.96
10.00	1.00	0.92	0.99	0.97	0.97
30.00	1.48	0.61	0.57	0.24	0.29
100.00	2.00	0.00	0.00	0.00	0.01

Table A6.6 SC-236 3-day viability relative to no treatment controls for SUD4
Concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	*0.88*	*0.73*
1.00	0.00	1.00	1.11	0.79	1.22		0.90	1.02	0.93
3.00	0.48	1.07	0.98	0.69	0.81	1.17	0.80	1.16	0.93
10.00	1.00	0.75	0.85	0.54	0.77	1.12	0.70	0.81	0.62
30.00	1.48	0.42	0.54	0.49	0.57	0.68	0.52	0.48	0.44
60.00	1.78	0.02	0.01						
100.00	2.00	0.01	0.00	0.00	0.00				

Table A6.7 SC-236 3-day cell count relative to no treatment controls for CRL
[ ] = Concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.98
1.00	0.00	0.97	0.97	1.00	0.99	1.02	1.04	0.99	0.98
3.00	0.48	0.94	1.02	0.94	1.00	1.01	1.01	0.98	1.02
10.00	1.00	0.95	0.94	0.99	0.97	1.01	0.95	0.94	0.95
30.00	1.48	0.66	0.75	0.82	0.86	0.85	0.93	0.98	0.97
60.00	1.78	0.09	0.10						
100.00	2.00	0.02	0.00	0.02	0.01	0.04	0.01		

Table A6.8 SC-236 3-day viability relative to no treatment controls for CRL
[ ] = Concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.00	0.00	0.85	0.91	0.68	0.84	0.84	1.11	0.95	1.21
3.00	0.48	0.90	0.97	0.88	1.04	1.04	1.16	0.85	1.16
10.00	1.00	0.61	0.76	0.65	0.80	0.65	0.74	0.85	0.91
30.00	1.48	0.03	0.02	0.03	0.03	0.12	0.17	0.08	0.14
60.00	1.78	0.01	0.01						
100.00	2.00	0.01	0.00	0.00	0.00	0.00	0.02		

Table A6.9 SC-236 3-day cell count relative to no treatment controls for DoHH2
[ ] = Concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.00	0.00	0.98	0.96	1.02	0.96	0.94	1.02	0.98	0.96
3.00	0.48	0.95	0.94	1.00	1.00	0.97	1.00	0.99	1.00
10.00	1.00	0.88	0.86	0.89	0.92	1.00	0.93	0.98	0.93
30.00	1.48	0.12	0.06	0.17	0.10	0.45	0.53	0.32	0.52
60.00	1.78	0.04	0.02						
100.00	2.00	0.04	0.01	0.00	0.00	0.00	0.07		

Table A6.10 SC-236 3-day viability relative to no treatment controls for DoHH2
[ ] = Concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9	Replicate 10	Replicate 11	Replicate 12
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.09	0.94
1.00	0.00	0.92	0.92	0.95	0.81	1.06	1.15	0.96	1.31	0.98	1.08	0.82	1.07
3.00	0.48	0.81	0.97	0.91	0.96	0.82	1.17	0.88	1.15	0.97	1.01	1.01	1.01
10.00	1.00	0.84	0.69	1.11	0.85	0.72	1.08	0.82	1.05	1.19	0.98		0.89
15.00	1.18	0.83	0.89	0.71	0.81								
20.00	1.30	0.53	0.50	0.67	0.83								
30.00	1.48	0.11	0.12	0.16	0.14	0.48	0.42	0.48	0.51	0.31	0.36	0.20	0.34
45.00	1.65	0.45	0.33	0.34	0.40								
60.00	1.78	0.25	0.24	0.21	0.19			0.03	0.04				
100.00	2.00	0.00	0.00	0.00	0.00	0.03	0.02	0.02	0.03	0.02	0.02		

Table A6.11 SC-236 3-day cell count relative to no treatment controls for DHL4
[ ] = Concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9	Replicate 10	Replicate 11	Replicate 12
0.00	-3.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.03	1.00
1.00	0.00	1.00	0.99	1.00	0.98	0.98	1.01	0.99	1.04	1.03	1.02	1.03	0.96
3.00	0.48	0.98	1.00	0.98	1.01	0.99	1.00	0.99	1.02	1.03	1.01	1.00	1.01
10.00	1.00	1.00	0.97	0.98	0.98	0.99	1.01	1.00	1.03	1.02	1.01	1.01	1.02
15.00	1.18	1.00	1.00	0.97	0.99								
20.00	1.30	0.96	1.00	0.95	0.98								
30.00	1.48	0.94	0.97	0.92	1.01	0.87	0.90	0.93	0.89				
45.00	1.65	0.58	0.62	0.64	0.56								
60.00	1.78	0.28	0.23										
100.00	2.00	0.00	0.03	0.00	0.00	0.17	0.17	0.15	0.20	0.12	0.10		

Table A6.12 SC-236 3-day viability relative to no treatment controls for DHL4
[ ] = Concentration μM; values in italics were censored as outliers.

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9	Replicate 10	Replicate 11	Replicate 12
0.00	-3.00	1.00	1.00	1.35	0.98	0.91	0.76	1.05	0.95	1.03	1.03	0.94	1.00
3.00	0.48	1.25	1.26	1.09	1.21		0.94						
10.00	1.00	0.93	0.84	1.30	0.92	1.13	1.14						
30.00	1.48	1.22	1.12	0.81	1.21	1.21	0.84	0.98	*0.44*	*0.62*	0.66	0.75	0.87
100.00	2.00	0.81	0.74	0.63	*0.96*	0.74	0.81						
200.00	2.30	0.19	0.21	0.07	0.18	0.12	0.04						
250.00	2.40	0.08	0.06	0.07	0.08	0.04	0.06						

Table A6.13 SC-236 2-day cell count relative to no treatment controls for primary MCL (03)
[ ] = Concentration μM; values in italics were censored as outliers.

Concentration	Log Concentration	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9	Replicate 10	Replicate 11	Replicate 12
0.00	-3.00	1.00	0.98	1.03	1.02	0.98	0.98	1.03	0.96	1.00	0.99	1.03	1.00
3.00	0.48	0.96	1.00	0.99	0.96	0.99							
10.00	1.00	0.99	0.91	0.93	0.89	0.98	0.94						
30.00	1.48	0.97	0.95	0.98	0.92	1.00	0.96	0.97	0.71	0.80	0.84	0.83	0.80
100.00	2.00	0.98	0.93	0.89	0.96	0.93	0.88	0.70	0.78	0.65	0.78	0.74	0.70
200.00	2.30	0.16	0.22	0.11	0.20	0.15	0.05						
250.00	2.40	0.10	0.09	0.10	0.09	0.04	0.05						

Table A6.14 SC-236 2-day viability relative to no treatment controls for primary MCL (03)

[ ] = Concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	0.89	0.86	1.09	1.06	1.12	0.99
2.40	0.38	0.85	0.99	0.80	1.08	1.07	1.06
8.00	0.90	0.86	0.68	0.81	0.95	0.88	0.81
24.00	1.38	0.88	0.74	0.89	0.68	0.88	0.89
80.00	1.90	0.61	0.51	0.43	0.57	0.68	0.42

Table A6.15 SC-236 2-day cell count relative to no treatment controls for primary MCL (04)
[ ] = Concentration μM; values in italics were censored as outliers.

[ ]	Log [ ]	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
0.00	-3.00	0.98	1.00	0.99	1.00	1.02	1.00
2.40	0.38	0.85	0.89	0.89	0.97	0.98	0.98
8.00	0.90	0.82	0.88	0.90	0.96	0.94	0.86
24.00	1.38	0.81	0.82	0.82	0.82	0.83	0.86
80.00	1.90	0.47	0.55	0.43	0.48	0.55	0.40

Table A6.16 SC-236 2-day viability relative to no treatment controls for primary MCL (04)
[ ] = Concentration μM; values in italics were censored as outliers.

LIST OF PUBLICATIONS

The Induction of Apoptosis in Lymphoma Cell Lines and Primary Tumours by Methylselenininic Acid and Selenodiglutathione

Kim W. Last, L Maharaj, J Perry, S Strauss, Jude Fitzgibbon, T. Andrew Lister and S Joel

Clinical Cancer Research under review 2005

Molecular Diagnosis of Lymphoma: Outcome Prediction by Gene Expression Profiling in Diffuse Large B cell Lymphoma **Kim Last**, Silvana Debarnardi, and T. Andrew Lister Book chapter in 'Lymphoma: Methods and Protocols' ISBN: 1-58829-159-6 Humana Press 2005

Correspondence, In Reply to 'Serum Selenium in Lymphoma'

Kim W. Last, Ama Z.S. Rohatiner, Jude Fitzgibbon, T. Andrew Lister

Journal of Clinical Oncology 2004, 22(16):3430

The Management of Follicular Lymphoma. **Dr KW Last**, Dr AZS Rohatiner

Extensively revised book chapter in 'The Effective Management of Non-Hodgkin's Lymphoma' Second Edition; published by Aesculapius Medical Press 2004

Presentation Serum Selenium Predicts for Overall Survival, Dose Delivery, and First Treatment Response in Aggressive Non-Hodgkin's Lymphoma

Kim W. Last, Victoria Cornelius, Trevor Delves, Christine Sieniawska, Jude Fitzgibbon, Andrew Norton, John Amess, Andy Wilson, Ama Z.S. Rohatiner, and T. Andrew Lister

Journal of Clinical Oncology 2003, 21:2335-2341.

Diffuse Large B-cell Lymphoma Outcome Prediction by gene Expression Profiling and Supervised Machine Learning

Margaret A Shipp, Pablo Tamayo, Ken Ross, Andrew P Weng, Jeffery Kutok, Ricardo CT Aguiar, Michelle Gaasenbeek, Michael Angelo, Michael Reich, Geraldine S Pinkus, Tane S Ray, Margaret A Koval, **Kim W Last**, Andrew Norton, T Andrew Lister, Jill Mesirov, Donna S Neuberg, Eric S Lander, Jon C Aster, Todd R Golub

Nature Medicine 2002 January; (8) 1: 68-74.

The Management of Follicular Lymphoma. **Dr KW Last**, Dr AZS Rohatiner
Book chapter in 'The Effective Management of Non-Hodgkin's Lymphoma' published by Aesculapius Medical Press 2001

Familial Follicular Lymphoma: a Case report with Molecular Analysis
Kim W Last, Lindsey K Goff, Karin E Summers, Michael Neat, Michael Jenner, Charles Crawley, Ama Z Rohatiner, Jude Fitzgibbon, T Andrew Lister
British Journal of Haematology 2000 September; 110 (3): 744-5.

Presentation Serum Selenium Predicts for Overall Survival, Dose Delivery, and First Treatment Response in Aggressive Non-Hodgkin's Lymphoma

By Kim W. Last, Victoria Cornelius, Trevor Delves, Christine Stenlawska, Jude Fitzgibbon, Andrew Norton, John Ames, Andy Wilson, Ama Z.S. Rohatiner, and T. Andrew Lister

Purpose: This study was undertaken to test the hypothesis that serum selenium concentration at presentation correlates with dose delivery, first treatment response, and overall survival in patients with aggressive B-cell non-Hodgkin's lymphoma.

Patients and Methods: The patients presented between July 1986 and March 1999 and received anthracycline-based chemotherapy, radiotherapy, or both. The total selenium content was retrospectively analyzed in 100 sera, frozen at presentation, using inductively coupled plasma mass spectrometry.

Results: The serum selenium concentration ranged from 0.33 to 1.51 μmol/L (mean, 0.92 μmol/L; United Kingdom adult reference range, 1.07 to 1.88 μmol/L). Serum selenium concentration correlated closely with performance status but with no other clinical variable. Multivariate analysis revealed that increased dose delivery, summarized by an area under the curve, correlated positively with younger age ($P < .001$), advanced stage ($P = .001$), and higher serum selenium concentration ($P = .032$). Selenium level also correlated positively with response (odds ratio, 0.62; 95% confidence interval [CI], 0.43 to 0.90; $P = .011$) and achievement of long-term remission after first treatment (log-rank test, 4.38; $P = .036$). On multivariate analysis, selenium concentration was positively predictive of overall survival (hazard ratio [HR], 0.76 for 0.2 μmol/L increase; 95% CI, 0.60 to 0.95; $P = .018$), whereas age indicated negative borderline significance (HR, 1.09; 95% CI, 0.99 to 1.18; $P = .066$).

Conclusion: Serum selenium concentration at presentation is a prognostic factor, predicting positively for dose delivery, treatment response, and long-term survival in aggressive non-Hodgkin's lymphoma. Unlike most existing prognostic factors in aggressive non-Hodgkin's lymphoma, selenium supplementation may offer a novel therapeutic strategy in this frequently curable malignancy.

J Clin Oncol 21:2335-2341. © *2003 by American Society of Clinical Oncology.*

SELENIUM IS an essential trace element in humans, best known for its function as an antioxidant. With the classification of selenocysteine as the 21st amino acid in 1988,[1,2] and the increase in the number of discovered selenoproteins to ~ 40, selenium is increasingly considered to be important to human physiology; conversely, its deficiency is considered to be important to the pathophysiology of conditions ranging from depression to atherosclerosis to cancer.[3]

A possible protective effect of selenium against human malignancy was first suggested in 1969, after the observation of a decreased cancer incidence in populations within the United States with a higher selenium intake.[4] Subsequent epidemiologic evidence in support of this hypothesis has been equivocal. Two important epidemiologic studies, however, have led many to believe that an association exists. In the United States, an inverse relationship between forage-crop selenium and county cancer incidence was observed[5] and examination of the dietary intake of selenium in more than 25 countries found an inverse correlation with total age-adjusted cancer mortality.[6]

More recently, data from intervention studies have lent support to the role of selenium in human cancer prevention. In two trials published to date, selenium was given as the sole chemopreventive agent. The first was a population-based study in an area of China with low selenium intake, a 15% prevalence rate of hepatitis B, and an incidence of primary liver cancer of ~ 50/10,000/annum.[7] After 8 years of intervention with selenized table salt, the incidence of primary liver cancer had decreased by 35% in the intervention township versus the nonsupplemented control townships. In the second study, patients with a history of nonmelanomatous skin cancer were treated for a mean of 4 years with placebo or selenium (as selenized brewer's yeast).[8] Selenium supplementation was found to be significantly associated with reductions in the secondary end points of total cancer incidence and total cancer mortality. The primary end point, a reduction in basal and squamous cell carcinoma incidence, was not reached. In the laboratory, in vitro and in vivo experiments have revealed that selenium compounds can exert cytotoxic activity, synergize with cytotoxic agents, and favorably influence cancer cell phenotype.[9-11] No clinical evidence confirming selenium as a disease modifier in cancer so far exists. However, the reduction in cisplatin-induced bone marrow and renal toxicity by

From the Cancer Research UK Medical Oncology Unit, Department of Medical Oncology, St Bartholomew's Hospital, London; Cancer Research UK Department of Statistics, Oxford; Trace Elements Unit, Southampton General Hospital, Southampton, United Kingdom.

Submitted June 25, 2002; accepted March 26, 2003.

Supported by Cancer Research UK, Lincoln's Inn Fields, London, UK.

Address reprint requests to Kim W. Last, MD, Cancer Research UK Medical Oncology Unit, Department of Medical Oncology, 45 Little Britain, St Bartholomew's Hospital, London EC1M 6BQ, United Kingdom; email: kim.last@cancer.org.uk.

0732-183X/03/2112-2335/$20.00

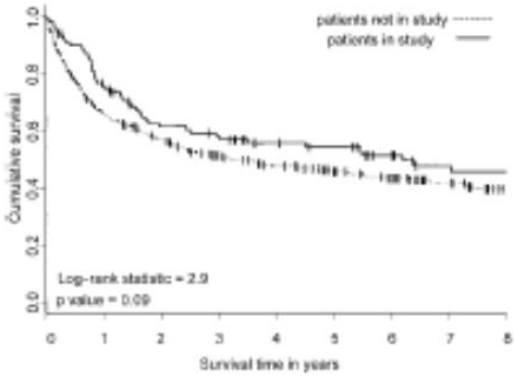


Fig 1. Overall survival, from diagnosis, of the 283 patients treated between July 1986 and March 1999 without available presentation frozen sera and the 100 patients investigated.

selenium supplementation, and the amelioration of doxorubicin cardiomyocyte toxicity by an organic selenium compound, indicate that selenium status may affect chemotherapy dose delivery in cancer and lymphoma patients.[12-14]

This study was undertaken to test the hypothesis that serum selenium at presentation would predict for response to first treatment, dose delivery, and overall survival in patients with high-grade, B-cell, non-Hodgkin's lymphoma, the most common non-Hodgkin's lymphoma.[15] If proven, the argument for exploration of selenium compounds as a therapy adjunct to aid improvement of the long-term remission rate of only 40% to 50% in this illness would be strengthened. Indirect evidence supporting an association between selenium and the clinical course of lymphoma comes from a study indicating that pretreatment serum selenium concentration correlated with response to treatment in a group of 51 epidermotropic T-cell lymphomas[16] and the observation that serum selenium levels were significantly lower in patients with non-Hodgkin's lymphoma than in normal controls.[17]

PATIENTS AND METHODS

Patients

Patient samples were selected on the basis of an original diagnosis of high-grade, B-cell, non-Hodgkin's lymphoma and the availability of heme-free frozen sera. Of the 383 patients treated at St Bartholomew's Hospital between July 1986 and March 1999 for high-grade, B-cell, non-Hodgkin's lymphoma, 143 had sera frozen at diagnosis; 100 heme-free sera were still available. The overall survival of the 283 patients without suitable sera was the same as that of the 100 patients studied (Fig 1). Histology review of the 100 samples investigated by one of the authors (A.N.) according to the new World Health Organization classification was as follows: diffuse large B-cell lymphoma (DLBCL), 77 samples; mediastinal large B-cell lymphoma, 14 samples; DLBCL, T-cell–rich, three samples; lymphoplasmacytic lymphoma with a high content of blasts, two samples; angioimmunoblastic T-cell lymphoma, two samples; Burkitt's lymphoma, one sample; and mantle-cell lymphoma with a high content of blasts, one sample. Fifteen patients had composite or discordant histology at presentation (eight with evidence of follicular lymphoma, one with evidence of lymphoplasmacytic lymphoma, two with evidence of unclassifiable low-grade non-Hodgkin's lymphoma, and four with evidence of marginal zone lymphoma of mucosa-associated lymphoid tissue type). Clinical information was prospectively collected as part of the department's clinical database. Where necessary, additional treatment administration details were retrieved from patient records. Presentation clinical variables investigated for an association with either overall survival, response to first treatment, or dose delivery were serum selenium, age, sex, B symptoms, stage, retrospective performance status, lactate dehydrogenase (or hydroxybutyrate dehydrogenase if the patient presented before mid-1989), and number of extranodal sites of disease (Table 1). As the best available current predictor of overall survival, the International Prognostic Index (IPI) score was calculated for the 95 patients with complete IPI data (Table 1).[18]

Table 1. Clinical Characteristics of the 100 Patients

Clinical Variable	N = 100
Serum selenium, μmol/L	
Mean	0.92
Range	0.33-1.51
Sex	
Male	56
Female	44
B symptoms	
Present	15
Absent	85
Age, years	
Median	57
Range	19-87
Stage	
I, IE	25
II, IIE	23
III	13
IV	39
ECOG performance status* (96 of 100)	
0	34
1	43
2	19
LDH* (98 of 100)	
≤ ULN	49
> ULN	49
Number of extranodal sites* (98 of 100)	
0	33
1	53
2	12
IPI* (95 of 100)	
0	17
1	24
2	23
3	23
4	7
5	1

Abbreviations: ULN, upper limit of normal; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; IPI, International Prognostic Index.
*Available only for the number of patients in parentheses.

Treatment

Patients received anthracycline-based chemotherapy, radiotherapy, or both with curative intent in all but one instance, in which the patient died before treatment could be initiated. Seventy-five patients received vincristine, doxorubicin, prednisolone, etoposide, cyclophosphamide and bleomycin (VAPEC-B; on an alternating, anthracycline-based weekly schedule given for a total of 12 weeks)[19] or a variant of it (11 patients); 12 patients received cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP);[20] six patients received methotrexate, doxorubicin, cyclophosphamide, vincristine and prednisolone (MACOP);[21] and six patients received radiotherapy as their first treatment. No patient received granulocyte colony-stimulating factor. Comprehensive first-treatment dose-delivery data were available for 87 of

the 93 patients receiving chemotherapy. Thirty-six patients had relapsed by July 3, 2000 (the date up to which survival was analyzed); five of these patients received high-dose treatment with autologous stem-cell rescue as part of their re-treatment therapy. Only one of the 11 patients who failed to respond to first treatment entered long-term remission with additional therapy. Forty-nine of the 100 patients were alive, two had been lost to follow-up, and 47 had died by July 3, 2000. Of the 47 patients who died, 43 died of disease, one died from a cerebrovascular accident with disease, and three died of other causes in long-term remission (bronchoalveolar cell carcinoma, ovarian carcinoma, and congestive cardiac failure, respectively). One other patient has developed a second malignancy (bladder cancer).

Selenium Concentration Measurement

Total selenium content of the sera was measured using inductively coupled plasma mass spectrometry. Selenium-78 was determined after a 1 in 15 dilution with a diluent containing 0.5% butan-1-ol, which minimized interference from argon adducts, and tellurium as an internal standard to compensate for instrumental fluctuations.[22] However, high concentrations of iodine (> 3,000 μmol/L) in five samples analyzed produced spectral overlap from hydrogen-1–iodine-127 with the tellurium-128 isotope monitored. For these samples, indium-115 was used as the internal standard. The calibration was carried out using matrix-matched standards containing bovine serum. Internal quality control sera were prepared by adding selenium to bovine sera at 0.2, 0.6, 1.2, and 1.7 μmol/L. These were analyzed with every 10 duplicate test samples, providing a between-run precision that ranged from 3.7% to 5.9% relative SD, and a within-run precision of 9.3% to 2.3%, over the concentration range 0.25 to ± 1.75 μmol/L selenium. Analysis of a certified quality control serum (Seronorm 704121, SERO AS, Asker, Norway), with an assigned value of 0.92 mmol/L as measured by electrothermal atomic absorption spectrometry, gave a daily mean variation of 0.93 ± 0.03 mmol/L throughout the analysis of the samples. External quality control was provided by participation in two quality assessment schemes from Centre du Toxicologie de Quebec (Canada) and Trace Element Quality Assurance Scheme (UK-TEQAS, University of Surrey, Guildford, United Kingdom).

Statistical Methods

Area under the curve summary measure, AUC_{ratio}. A summary measure for dose delivery was calculated and the relationship with patient variables was explored using linear regression. The usual dose summary measures (eg, relative dose-intensity, cumulative dose) were not satisfactory summary statistics for this application because of the variation in dosing schedules (both in quantity and time duration). Instead, an adaptation of the standard AUC measure, often used to summarize serial measurements, was applied.[23] The AUC was calculated using the trapezium rule. For a schedule of n doses, where the proportion of cumulative dose at time $t(i)$ was $y(i)$ (where $i = 1$ to n), the AUC was defined as

$$\text{AUC} = \sum_{i=1}^{n-1} \frac{1}{2}[y(i) + y(i+1)][t(i+1) - t(i)] \qquad (1)$$

Dose-delivery associations were sought for doxorubicin, cyclophosphamide, and vincristine. Data are shown for doxorubicin only because the findings for cyclophosphamide and vincristine were comparable. Each patient had a planned chemotherapy schedule determined by the regimen prescribed. After treatment was completed, the patient's actual schedule was available, which differed from the planned schedule when drug doses and dates were modified. The proportion of the cumulative dose was plotted against time for both the planned and the actual dosing schedules. The area under the planned and actual curves ($AUC_{planned}$, AUC_{actual}) was then calculated. The summary measure used, AUC_{ratio}, represents AUC_{actual} divided by $AUC_{planned}$ and is thus a measure of the proportion of actual dose to planned dose over the duration of the treatment time. If the planned schedule was administered, the AUC_{ratio} was 1; if a full planned dose was not given or a dose was delayed, then AUC_{ratio} decreased. If doses were given after the final planned time, then the time axis for the planned regimen was extended and the contribution was weighted using

$$w = \frac{t_1}{n(t_2 - t_1)} \qquad \text{when } (t_2 - t_1) > \frac{t_1}{n} \qquad (2)$$

where t_1 is the time from the first planned dose to final planned dose, t_2 is the time from the first administered dose to the final administered dose, and n is the number of planned doses. Where treatment stopped early because of death or disease progression, the planned dose intensity was censored at the time of death or disease progression.

Variables included in the multivariate model for dose delivery were selected using a stepwise procedure. Age and selenium were treated as continuous variables and the remaining variables were treated as categorical. Variables were included in the Cox and logistic regression model if they indicated significance at the univariate level ($P < .1$). Analysis of residuals was performed to check model assumptions.

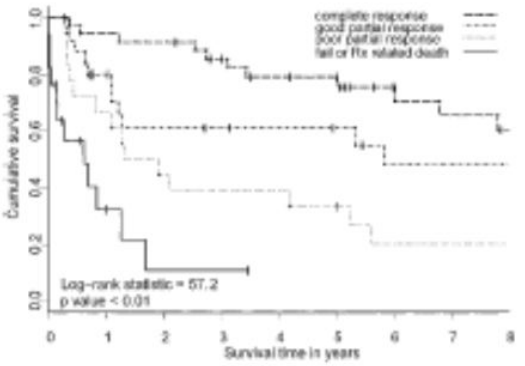


Fig 2. Overall survival of the 100 patients grouped according to response to first treatment, from response documentation.

Response Analysis

Response was categorized according to the local criteria in use at the time.[24] The response criteria used predate the 1997 consensus statement.[25] The definitions for complete response (CR) are the same, whereas good partial response (GPR) is comparable to $CR_{unconfirmed}$ and poor partial response (PPR) is comparable to partial response. Response was grouped according to similar overall survivals: CR/GPR and PPR, treatment failure, or treatment-related death (Fig 2). The χ^2 and t tests were used to compare groups at the univariate level and logistic regression was fitted to estimate the odds ratio. The patients were divided into quartiles as defined by presentation serum selenium concentration, and tested univariately with regard to response and response duration. Remission duration was defined as time to relapse or death from documentation of response to first treatment.

Survival Analysis

Survival time was analyzed using a Cox proportional hazards model.[26] Survival time was defined as time from diagnosis to death from any cause. Univariate analysis included estimated survival curves using Kaplan-Meier methods and the log-rank test. For presentation purposes, the patients were divided into quartiles, as defined by presentation serum selenium concentration, and tested univariately with regard to overall survival.

RESULTS

The presentation serum selenium concentration was normally distributed for the 100 patients, with a mean of 0.92 μmol/L (SD, 0.25 μmol/L) and range of 0.33 to 1.51 μmol/L. A trend of

Table 2. Univariate Analysis for Dose-Delivery, Response, and Overall Survival With Probability Values

Clinical Variable	Dose-Delivery*	Response†	Survival Time‡
Age	< .001§	.532	.042§
Selenium	.063§	.009§	.026§
Sex	.175	.156	.092§
Stage	.053§	.517	.089§
B symptoms	.786	.791	.570
Performance status	.234	.007§	< .001§
LDH	.403	.892	.280
No. of extranodal sites	.282	.265	.383

Abbreviation: LDH, lactate dehydrogenase.

NOTE. The *P* values are derived from: *significance of regression coefficient (H_0: $\beta = 0$); †χ^2 for categorical and analysis of variance for continuous variables; ‡log-rank test for categorical and significance of hazard ratio for continuous variables.

§Variables indicating significance at the univariate level ($P < .1$).

decreasing mean serum selenium was observed with increasing year of presentation. Seventy-three patients had a selenium level below the United Kingdom reference range from the early 1980s (United Kingdom adult reference range, 1.07 to 1.88 μmol/L[27]). Serum selenium concentration correlated closely with performance status but no other clinical variable ($P < .001$, data not shown). As a consequence of this association, only one of these variables was included in the multivariate analyses at the same time because the variables explain similar variation. Our primary interest was in assessing the relationship between selenium levels, response, dose delivery, and survival rather than developing a new prognostic model. As a result, the multivariate analyses containing the selenium variable are presented here. Exchanging selenium with performance status gave similar results.

Dose Delivery

In the 87 chemotherapy patients with complete treatment data, there were 128 treatment delays or dose reductions (or both) associated with 42 in-patient episodes for neutropenic fever or sepsis, 28 admissions for other treatment-related complications, 45 episodes of neutropenia without fever, and 13 delays for other reasons. Stage and serum selenium positively and age-negatively correlated with the dose-delivery summary measure AUC_{ratio} at the univariate level (Table 2). These three variables were then selected by a stepwise procedure for the multivariate model. The results indicate that a significantly better dose delivery (summarized by AUC_{ratio}) correlated positively with younger age, advanced stage, and higher serum selenium (Table 3).

Table 4. Response to First Treatment According to Selenium Quartiles (99 assessable patients; one died before treatment could commence)

Response	Lowest Quartile	Third Quartile	Second Quartile	Highest Quartile
CR/GPR				
Number	13	14	14	22
%	54.2	56.0	56.0	88.0
PPR/Fail/treatment-related death				
Number	11	11	11	3
%	45.8	44.0	44.0	12.0

Abbreviations: CR, complete response; GPR, good partial response; PPR, poor partial response.

Response

Univariate analysis (using a statistical significance of 10%) indicated that selenium concentration and performance status were the only two patient variables associated with response to first treatment (Table 2). Fitting a logistic regression model produced an odds ratio of 0.62 for every 0.2 μmol/L increase in serum selenium (95% CI, 0.43 to 0.90) and *P* value of .011 (Table 3). A higher selenium level was therefore associated with a lower probability of having a poor outcome to treatment (ie, PPR, treatment failure, or treatment-related death). For example, a patient with a serum selenium level of 0.8 μmol/L has estimated odds of CR or GPR 38% lower than a patient with a serum selenium level of 1.0 μmol/L. Similar results were obtained by exchanging the variable performance status with selenium in the logistic regression model (data not shown). A cross-tabulation of response and IPI score revealed no obvious association between IPI and response to first treatment (data not shown; χ^2 $P = .45$). No confounding factors were identified. Table 4 displays response according to selenium quartiles ($\chi^2 = 9.52$; $P = .023$).

Remission Duration

When treated as a continuous variable, serum selenium was not significant at the univariate level ($P = .298$). However, when serum selenium was treated as a categorical variable (four quartiles), it was significant with regard to remission time

Table 3. Multivariate Analyses for Dose-Delivery, Response, and Overall Survival With Probability Values

	Dose-Delivery		Response			Survival Time		
Clinical Variable	Regression Coefficient (standard error)	*P*	Odds Ratio	95% CI	*P*	Hazard Ratio	95% CI	*P*
Age	−0.006 (0.001)*	< .001§	—		—	1.09	0.99 to 1.18*	.066
Selenium	0.215 (0.099)†	.032§	0.62	0.43 to 0.90†	.011§	0.76	0.60 to 0.95†	.018§
Sex	—	—	—		—	1.66	0.94 to 2.93	.083
Stage	0.051 (0.015)‡	.001§	—		—	—		—

NOTE. The regression coefficient is the change in area under concentration-time curve for a one-unit increase in that variable: *for 5-year increase in age; †for 0.2 μmol/L increase in serum selenium; ‡for increasing stage.

§Significance at the multivariate level ($P < .01$).

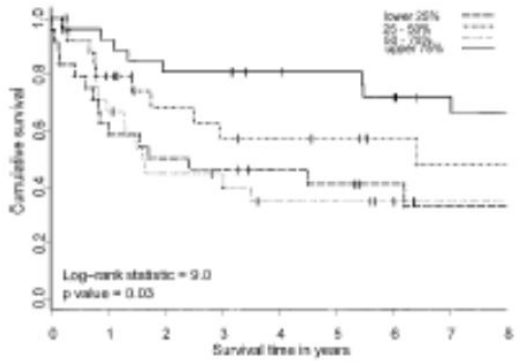


Fig 3. Overall survival, from diagnosis, of the 100 patients split into serum selenium quartiles.

(log-rank test, 4.38; $P = 0.036$). Performance status was also significant with regard to remission time (log-rank test, 15.7; $P < .001$), whereas lactate dehydrogenase level indicated significance at the univariate level ($P = .07$).

Overall Survival

The calculated median overall survival of the 100 patients was 6.2 years (range, 1 week to 13 years; 95% CI, 1.9 to 10.7). Completeness of follow-up data was confirmed because the actual median overall survival of the 51 surviving patients was almost identical at 6.1 years (range, 4 months [one patient lost to follow-up at 4 months] to 13.5 years). When IPI was considered as a continuous variable, it was found to be prognostic with regard to overall survival, with a hazard ratio of 1.58 (95% CI, 1.18 to 2.13). The results of univariate analysis indicated that age, selenium concentration, sex, stage, and performance status correlated with survival time (Table 2). On multivariate analysis, using a Cox model, selenium was the most significant factor, with a hazard ratio of 0.76 for every increase of 0.2 μmol/L in serum selenium level; that is, a higher serum selenium concentration correlated with longer survival (Table 3). For example, a patient with a serum selenium concentration of 1.0 μmol/L had a 24% lower risk of death than a patient with a concentration of 0.8 μmol/L, over the duration of the study. Replacing selenium with performance status gave similar results; that is, a decrease in performance status was significantly associated with shorter survival (data not shown). Overall survival for each selenium quartile is displayed in Fig 3.

DISCUSSION

This study has discovered positive correlations between presentation serum selenium, dose delivery, response to first therapy, first remission duration, and overall survival in patients with aggressive non-Hodgkin's lymphoma. Dose delivery was assessed using actual compared with planned AUCs because relative dose intensity, cumulative dose, and other standard methods failed to account adequately for delays incurred and total dose delivered. Comparison of the actual with planned AUCs of the proportion of cumulative dose versus time created a standardized summary measure assessable among patients regardless of regimen variant used. The AUC_{ratio} of cumulative dose versus time revealed not only variation in time taken and dose administered, but also distinguished between dose reductions and delays incurred at the beginning from those incurred at the end of treatment. AUC_{ratio} therefore factored for the premise that timely dose delivery early in therapy is more critical than timely dose delivery toward the end of therapy. Dose delivery also correlated positively with stage and was inversely associated with age.

Several compatible mechanisms may underlie the prediction of dose delivery, response, and outcome by presentation serum selenium. These include prevention of chemoresistance, enhancement of immune function, direct cytotoxic activity of selenium compounds, and reduction of treatment-related side effects. Selenomethionine prevented cisplatin resistance when administered in vitro and to nude mice with tumors derived from the same ovarian cancer cell line.[11] In addition, in a murine model, using Dalton's lymphoma, preinoculation with selenomethionine increased survival by 31% and 112% compared with those mice that received selenomethionine at inoculation and those that received no selenomethionine supplementation, respectively.[28] Multiple processes are likely to account for this survival advantage.

Evidence indicating a role for selenium in immune response enhancement includes a study in which selenite, given to volunteers with normal selenium levels, resulted in clonal expansion of activated T cells in response to in vitro incubation with a cancer cell line. T-cell cytotoxicity increased by 118% and natural-killer cell–mediated lytic activity increased by 82% compared with baseline ($P < .05$).[29,30] In vitro, selenium compounds produce direct antitumor effects, as demonstrated by tumor cell growth inhibition and apoptosis promotion,[10] and exhibit synergy with the cytotoxic agents paclitaxel and doxorubicin (in terms of increased cell death and growth arrest) in the majority of cell lines investigated by Vadgama et al.[9] It is possible that selenium enhances dose delivery by reducing the side effects of therapy. Selenium supplementation has been shown to reduce cisplatin-induced nephrotoxicity and bone marrow suppression in a crossover patient study, and to prevent doxorubicin-induced cardiomyocyte cytotoxicity in vitro.[12,14]

Performance status was the only clinical variable that correlated closely with serum selenium. This correlation may be the result of general nutritional deficiency in the months and weeks before diagnosis or an acute phase response of serum selenium to illness.[31] The associations found in this study could therefore be due to a bystander phenomenon with no causality related to selenium level. Alternatively, the correlations described may indeed reflect a causal relationship and account, in part, for the predictive power of performance status in aggressive non-Hodgkin's lymphoma. Regarding the possibility that the low selenium values seen in this study are partly due to an acute phase response, this need not imply lack of causality or scope for therapeutic intervention. For example, the low serum selenium levels seen in patients with acute pancreatitis have been reported

to respond to supranutritional intravenous infusions of selenium, resulting in a marked decline in the mortality rate associated with this frequently fatal condition.[32,33]

The majority of the patients investigated (73%) had a serum selenium concentration below the United Kingdom reference range from the early 1980s. This finding is consistent with the reporting of lower selenium levels in cancer and lymphoma patients compared with the general population,[17,34] with the decline in selenium intake in the United Kingdom population since this reference range was generated (attributed to the cessation of Canadian wheat importation),[35,36] and with the aforementioned decrease in serum selenium as part of the acute phase response to illness.[31]

The extent of selenium deficiency in aggressive non-Hodgkin's lymphoma patients (as assessed by serum selenium levels) who come from general populations replete in selenium remains to be seen. Selenium levels vary throughout the world from extremely low in areas of China, to low in many parts of Europe, to the high-normal levels in most areas of North America. If selenium is important in aggressive non-Hodgkin's lymphoma phenotype evolution and treatment success, countries with a high-normal selenium level in the population might be expected to have a better overall survival for aggressive non-Hodgkin's lymphoma than countries with a low selenium intake. This is not apparent, because United Kingdom survival rates are comparable to those in North America. Whether this reflects the need for supernormal selenium intakes to alter disease outcome at the population level or reflects aggressive non-Hodgkin's lymphoma resulting in low selenium levels regardless of premorbid selenium status will require additional investigation.

The selenium cancer chemoprevention study, performed in former nonmelanomatous skin cancer patients, took place in the United States.[8] Selenium supplementation was beneficial even in those replete in the micronutrient, although the benefit was greatest in the lower tertile of patients (serum selenium < 1.35 μmol/L).[37] All but three patients in our study had a serum selenium concentration within Clark's lower tertile.

Prospective, multicenter or multicountry substantiation of the findings described will be required. The discovery of presentation serum selenium as a potential prognostic factor in aggressive non-Hodgkin's lymphoma is intriguing. Our results, in conjunction with the chemopreventive and laboratory data reviewed, indicate that incorporation of selenium into an overall therapeutic strategy is worthy of study, with the aim to improve prognosis in this frequently curable malignancy.

ACKNOWLEDGMENT

We thank Margaret Rayman, University of Surrey, United Kingdom, for comments on the manuscript, and the radiology department and many staff members at St Bartholomew's hospital involved in caring for the patients studied.

REFERENCES

1. Leinfelder W, Zehelein E, Mandrand-Berthelot MA, et al: Gene for a novel tRNA species that accepts L-serine and cotranslationally inserts selenocysteine. Nature 331:723-725, 1988

2. Soll D: Genetic code: Enter a new amino acid. Nature 331:662-663, 1988

3. Rayman MP: The importance of selenium to human health. Lancet 356:233-241, 2000

4. Shamberger RJ, Frost DV: Possible protective effect of selenium against human cancer. Can Med Assoc J 100:682, 1969

5. Clark LC, Cantor KP, Allaway WH: Selenium in forage crops and cancer mortality in U.S. counties. Arch Environ Health 46:37-42, 1991

6. Schrauzer GN, White DA, Schneider CJ: Cancer mortality correlation studies: III. Statistical associations with dietary selenium intakes. Bioinorg Chem 7:23-31, 1977

7. Yu SY, Zhu YJ, Li WG: Protective role of selenium against hepatitis B virus and primary liver cancer in Qidong. Biol Trace Elem Res 56:117-124, 1997

8. Clark LC, Combs GF Jr, Turnbull BW, et al: Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin: A randomized controlled trial—Nutritional Prevention of Cancer Study Group. JAMA 276:1957-1963, 1996

9. Vadgama JV, Wu Y, Shen D, et al: Effect of selenium in combination with Adriamycin or Taxol on several different cancer cells. Anticancer Res 20:1391-1414, 2000

10. Thompson HJ, Wilson A, Lu J, et al: Comparison of the effects of an organic and an inorganic form of selenium on a mammary carcinoma cell line. Carcinogenesis 15:183-186, 1994

11. Caffrey PB, Frenkel GD: Selenium compounds prevent the induction of drug resistance by cisplatin in human ovarian tumor xenografts in vivo. Cancer Chemother Pharmacol 46:74-78, 2000

12. Hu YJ, Chen Y, Zhang YQ, et al: The protective role of selenium on the toxicity of cisplatin-contained chemotherapy regimen in cancer patients. Biol Trace Elem Res 56:331-341, 1997

13. Yoshida M, Iizuka K, Terada A, et al: Prevention of nephrotoxicity of cisplatin by repeated oral administration of ebselen in rats. Tohoku J Exp Med 191:209-220, 2000

14. Kotamraju S, Konorev EA, Joseph J, et al: Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen: Role of reactive oxygen and nitrogen species. J Biol Chem 275:33585-33592, 2000

15. Armitage JO, Weisenberger DD: New approach to classifying non-Hodgkin's lymphomas: Clinical features of the major histologic subtypes—Non-Hodgkin's Lymphoma Classification Project. J Clin Oncol 16:2780-2795, 1998

16. Deffuant C, Calerier P, Boiteau HL, et al: Serum selenium in melanoma and epidermotropic cutaneous T-cell lymphoma. Acta Derm Venereol 74:90-92, 1994

17. Avanzini P, Vinceti M, Ilariucci F, et al: Serum selenium concentrations in patients with newly diagnosed lymphoid malignancies. Haematologica 80:505-511, 1995

18. A predictive model for aggressive non-Hodgkin's lymphoma: The International Non-Hodgkin's Lymphoma Prognostic Factors Project. N Engl J Med 329:987-994, 1993

19. Radford JA, Whelan JS, Rohatiner AZ, et al: Weekly VAPEC-B chemotherapy for high grade non-Hodgkin's lymphoma: Results of treatment in 184 patients. Ann Oncol 5:147-151, 1994

20. McKelvey EM, Gottlieb JA, Wilson HE, et al: Hydroxydaunomycin (Adriamycin) combination chemotherapy in malignant lymphoma. Cancer 38:1484-1493, 1976

21. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. Ann Intern Med 102:596-602, 1985

22. Delves HT, Sieniawska C: Simple method for the accurate determination of selenium in serum by using ICP-MS. J Anal At Spectrom 12:387-389, 1997

23. Matthews JN, Altman DG, Campbell MJ, et al: Analysis of serial measurements in medical research. BMJ 300:230-235, 1990

24. Dhaliwal HS, Rohatiner AZ, Gregory W, et al: Combination chemotherapy for intermediate and high grade non-Hodgkin's lymphoma. Br J Cancer 68:767-774, 1993

25. Cheson BD, Horning SJ, Coiffier B, et al: Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas: NCI Sponsored International Working Group. J Clin Oncol 17:1244-1253, 1999

26. Cox DR: Regression models and life tables. J R Stat Soc (B) 34:187-220, 1972

27. Lloyd B, Lloyd RS, Clayton BE: Effect of smoking, alcohol, and other factors on the selenium status of a healthy population. J Epidemiol Community Health 37:213-217, 1983

28. Rana MP, Sardar S, Chatterjee M: Selenomethionine in the inhibition of a transplantable murine lymphoma: Reflection on hepatic drug metabolizing enzymes. Tumour Biol 17:102-109, 1996

29. Kiremidjian-Schumacher L, Roy M, Wishe HI, et al: Supplementation with selenium and human immune cell functions: II. Effect on cytotoxic lymphocytes and natural killer cells. Biol Trace Elem Res 41:115-127, 1994

30. Roy M, Kiremidjian-Schumacher L, Wishe HI, et al: Supplementation with selenium and human immune cell functions: I. Effect on lymphocyte proliferation and interleukin 2 receptor expression. Biol Trace Elem Res 41:103-114, 1994

31. Nichol C, Herdman J, Sattar N, et al: Changes in the concentrations of plasma selenium and selenoproteins after minor elective surgery: Further evidence for a negative acute phase response? Clin Chem 44:1764-1766, 1998

32. Kuklinski B, Buchner M, Schweder R, et al: [Acute pancreatitis: A free radical disease—Decrease in fatality with sodium selenite (Na_2SeO_3) therapy]. Z Gesamte Inn Med 46:145-149, 1991

33. Kuklinski B, Zimmermann T, Schweder R: [Decreasing mortality in acute pancreatitis with sodium selenite: Clinical results of 4 years antioxidant therapy]. Med Klin 90:36-41, 1995 (suppl 1)

34. Alaejos MS, Diez Romero FJ, Diez Romero C: Selenium and cancer: Some nutritional aspects. Nutrition 16:376-383, 2000

35. Joint Food Safety and Standards Group: Food Surveillance Information Sheet 126. London, United Kingdom. Dietary intake of selenium. Ministry of Agriculture, Fisheries and Food, 1997

36. Joint Food Safety and Standards Group: Food Surveillance Information Sheet 191. London, United Kingdom. 1997 Total diet study-Aluminium, arsenic, cadmium, chromium, copper lead, mercury, nickel, selenium, tin and zinc. Ministry of Agriculture, Fisheries and Food, 1999

37. Rayman MP, Clark LC: Selenium in cancer prevention, in Rousel AM, Favier A, Anderson RA (eds): Trace Elements in Man and Animals 10: Proceedings of the 10th International Symposium on Trace Elements in Man and Animals. New York, Plenum Press, 2000, pp 575-580

original article

Annals of Oncology 17: 773–779, 2006
doi:10.1093/annonc/mdl004
Published online 9 February 2006

The activity of methylated and non-methylated selenium species in lymphoma cell lines and primary tumours

K. Last, L. Maharaj, J. Perry, S. Strauss, J. Fitzgibbon, T. A. Lister & S. Joel*

Cancer Research UK Medical Oncology Unit, St Bartholomew's Hospital, London, UK

Received 31 August 2005; accepted 23 December 2005

Background: Diffuse large B-cell lymphoma patients with low serum selenium concentration at presentation have a lower response rate and overall survival than patients with higher serum selenium. The co-administration of selenium with conventional chemotherapy may be useful in these patients.
Patients and methods: We investigated the activity of two selenium species, methylseleninic acid (MSA) and selenodiglutathione (SDG) in a panel of human lymphoma cell lines and in a primary lymphoma culture system.
Results: Both compounds demonstrated cytostatic and cytotoxic activity with EC_{50} values in the range 1.0–10.2 μM. Cell death was associated with an increase in the sub-G1 (apoptotic) fraction by flow cytometry and was not preceded by any obvious cell cycle arrest. SDG, but not MSA, resulted in marked increases in intracellular ROS, particularly in CRL2261 and SUD4 cells in which the cytotoxic activity of SDG was partly, or completely, inhibited by n-acetyl cysteine, suggesting a dependence on ROS for activity in some cells. Both MSA and SDG showed a concentration dependent reduction in percentage viability after a 2-day exposure in primary lymphoma cultures, with EC_{50} values in the range 39–300 μM and 9–28 μM, respectively.
Conclusion: The selenium compounds MSA and SDG induce cell death in lymphoma cell lines and primary lymphoma cultures, which with SDG may be partly attributable to the generation of ROS.
Key words: lymphoma, methylseleninic acid, primary culture, selenium, selenodiglutathione

introduction

Selenoproteins play a critical role in a number of biochemical pathways, notably in cellular anti-oxidant systems such as glutathione peroxidase and thioredoxin reductase [1, 2]. Many studies have reported an association between selenium intake or serum selenium concentration and cancer incidence [3] or overall age adjusted cancer mortality [4]. Studies in animals have described the chemopreventive activity of selenium compounds (reviewed in [5]), while clinical trials have also reported the protective effect of selenium supplementation in the development of prostate, lung and colorectal cancer [6], with the benefit being clearest in prostate cancer [7].

Serum selenium concentration at diagnosis was recently reported to be independently predictive of both treatment response and long-term survival in patients with aggressive non-Hodgkin's lymphoma [8]. In a group of 99 assessable patients, response to first treatment was 54% in the lowest serum quartile compared with 88% in the highest quartile, with a lower overall survival in patients with lower serum selenium. Serum selenium remained predictive of outcome in a multivariate analysis that included clinical variables as cofactors, as also reported by Deffuant et al. [9] in cutaneous T-cell lymphoma patients.

One explanation for this effect may involve the role of selenium in apoptosis induction and may reflect decreased apoptosis in the presence of similar amounts of cytotoxic drug-induced DNA damage in selenium deplete, compared with selenium replete, cells. In a recently reported supplementation study in dogs, the amount of DNA damage in peripheral blood mononuclear cells was lower in supplemented animals, while the number of apoptotic cells in prostate epithelium was increased, again suggesting an alteration in the apoptotic threshold in selenium replete cells [10].

These data suggest a possible role for selenium in the treatment of established malignancy, most likely with DNA damaging agents. There are currently no data describing the activity of selenium compounds in human lymphomas. Against the background of the clinical data above, a study of the effects of methylseleninic acid (MSA) and selenodiglutathione (SDG), representing methylated and non-methylated selenium species, respectively, in a lymphoma cell line panel and in a primary lymphoma culture system has been undertaken.

*Correspondence to: Dr S. Joel, Cancer Research UK Medical Oncology Unit, St Bartholomew's Hospital, West Smithfield, London EC1A 7BE, UK.
Tel: +44-207-601-8906; Fax: +44-207-600-4265; E-mail: s.p.joel@qmul.ac.uk

patients and methods

cell lines and culture conditions

Four non-Hodgkin's lymphoma cell lines were used in these studies. DHL-4 was obtained from the Dana Farber Cancer Institute (gift from Dr Margaret Shipp) and DoHH2 [11], SUD4 [12] and CRL2261 from Cancer Research UK cell services. All lines were maintained in RPMI-1640 growth media with 10% fetal calf serum, 1% glutamine and 1% penicillin/streptamycin at 37°C in a humidified atmosphere with 5% CO_2.

MSA (PharmaSe, Lubbock, TX, USA) was prepared in DMSO at a concentration of 80 mM and SDG (PharmaSe) in 0.05 M HCl at a concentration of 10 mM. These solutions were stored at −40°C for up to 3 months. The cytotoxic activity of these compounds was investigated after 3 day's continuous exposure, with cell number and viability determined by light microscopy following trypan blue staining. All data were expressed relative to control (untreated) cells and IC_{50} concentrations derived using a sigmoidal dose–response model (Graphpad Prism, San Diego, CA, USA). The final DMSO concentration was <1% in all cultures. The data presented is the mean of a minimum of three experiments.

To investigate the possible influence of reactive oxygen species (ROS) on the activity of SDG and MSA, cell lines were treated at the EC_{50} concentration of either drug for 3 days in the presence of 2.5 mM N-acetyl cysteine (NAC, Sigma Chemicals, Poole, Dorset, UK).

primary culture system

B-cell suspensions were prepared from ascitic fluid, peripheral blood or lymph nodes collected as part of normal clinical management from patients with histologically confirmed B-cell lymphoma or chronic lymphocytic leukaemia. All subjects gave informed consent and the study received approval from the institutional ethics review board. After disaggregation and/or density gradient separation, cells were plated at a density of 5×10^5 cells/well into 96-well plates containing CHO cells transfected to express CD40 ligand. When irradiated to prevent further proliferation these cells provide a stromal layer supporting B-cell proliferation. Malignant or normal B-cells were cultured in IMDM medium supplemented with 10% human serum and 2 ng/ml IL-4, and retained close phenotypic resemblance to the original sample (confirmed by flow cytometric analysis of cell surface markers) for up to 8 days. MSA or SDG were added after 24 h (in triplicate) and cell number and viability were assessed after a further 48 h using the trypan blue exclusion assay.

flow cytometry

To determine cell cycle distribution cells were washed in ice-cold nucleus buffer (0.15 M NaCl, 5 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM EGTA, 0.1 mM dithiothreitol, 10% glycerol in distilled water pH 6.5), cells (1×10^6) re-suspended in 4 ml of permeabilising solution (0.35% Triton X-100, 0.1 mM PMSF in nucleus buffer) and mixed by rotation at 4°C for 20 min. Cells were then fixed in ice-cold methanol for 30 min and washed with ice-cold PBS before staining with 500 µl of PI stain (50 µg/ml propidium iodide and 50 µg/ml RNAse A in PBS). Acquisition of data was performed within 1 h. Ten thousand cells were analysed for each data point using a Becton Dickinson FACSCalibur flow cytometer with CellQuest software, and the percentage of cells in the sub-G1 (apoptotic fraction), G1, S and G2/M phases were analysed using a cell cycle analysis program (WinMDI 2.8).

Apoptosis was confirmed by dual labelling using propidium iodide/annexin staining (Sigma, Poole Dorset, UK) of unfixed cells treated at the cytotoxic IC_{50} concentration for that cell line. Briefly, 1×10^6 cells were suspended in 500 µl of binding buffer (HEPES with NaOH, NaCl and $CaCl_2$) to which was added 5 µl annexin conjugate, after which the sample was incubated in the dark for 10 min. Propidium iodide (10 µl of 100 µg/ml) was then added, and the sample immediately analysed on a Becton Dickinson FACSCalibur using CellQuest software.

To determine ROS generation, experiments were conducted at the cytotoxic EC_{50} concentration of either MSA or SDG for each cell line. Cells were suspended in PBS (1×10^6/ml) and incubated in 20 µM 2′7′-dichlorodihydrodifluorescein diacetate (DCF-DA, Molecular Probes, OR, USA) for 15 min at 37°C in 5% CO_2. After the addition of MSA, SDG, 25 µM H_2O_2 (positive control) or 0.75% DMSO (negative control), cells were incubated for a further 30 min. ROS generation was then immediately determined by measuring the mean fluorescent intensity of 10 000 cells at 530 nM using a Becton Dickinson FACSCalibur flow cytometer and CellQuest software.

statistical analyses

Differences between treatments, or cell lines, were compared using an analysis of variance controlling for cell line and/or treatments. If this indicated significant differences within the data, pairs of treatments or cell lines were compared using a paired *t*-test. A *P* value of <0.05 was taken as the cut-off for statistical significance.

results

MSA and SDG are cytotoxic in lymphoma cell lines

Exposure to either MSA or SDG resulted in cytostasis and cytotoxicity in all lymphoma cell lines studied (Figure 1, Table 1). The EC_{50} values for these effects were typically between 1 and 10 µM, with the exception of MSA in the DHL-4 cell line where the response was mainly cytostatic (EC_{50} concentrations for cell number and 9% viability 1.7 µM and 166 µM, respectively).

Both MSA and SDG resulted in an increase in the apoptotic fraction with increasing concentration (as shown in Figure 2A for DoHH2 cells with MSA). Neither SDG nor MSA caused an apparent block in the cell cycle, but resulted in a reduction of cells from all phases as apoptosis increased with increasing selenium concentration. Similarly, the decreased sensitivity of DHL-4 cells to MSA was not associated with a cell cycle block (as shown in Figure 2B).

Three-day incubations at the cytotoxic EC_{50} concentration of SDG or MSA increased both annexin and PI staining, resulting in cells moving from the lower left to lower right quadrant and then to the upper right quadrant, indicating increased annexin staining initially, followed by an increase in PI staining (as shown in Figure 3A for DHL4 cells and Figure 3B for SUD4 cells), indicative of cell death by apoptosis.

Cell lines varied in their ability to generate or inactivate ROS, as evidenced by the change induced by a 30-min exposure to 25 µM H_2O_2. This treatment did not result in a significant change in DCF-DA fluorescence in DoHH2 and DHL-4 cells compared with control cells (100%), but did increase ROS in CRL2261 and SUD4 cells (Figure 4A). In three of the four cell lines studied the cytotoxic EC_{50} concentration of SDG generated significant increases in ROS compared with control cells, and greater than the H_2O_2 positive control. MSA at the EC_{50} concentration resulted in little or no change in ROS in any cell line. The increase in ROS with SDG was most apparent at 30 min, had declined by 2 h and showed no difference at 24 h (data not shown).

To investigate whether ROS contributed to the cytotoxic activity of SDG or MSA, cells were incubated at the cytotoxic

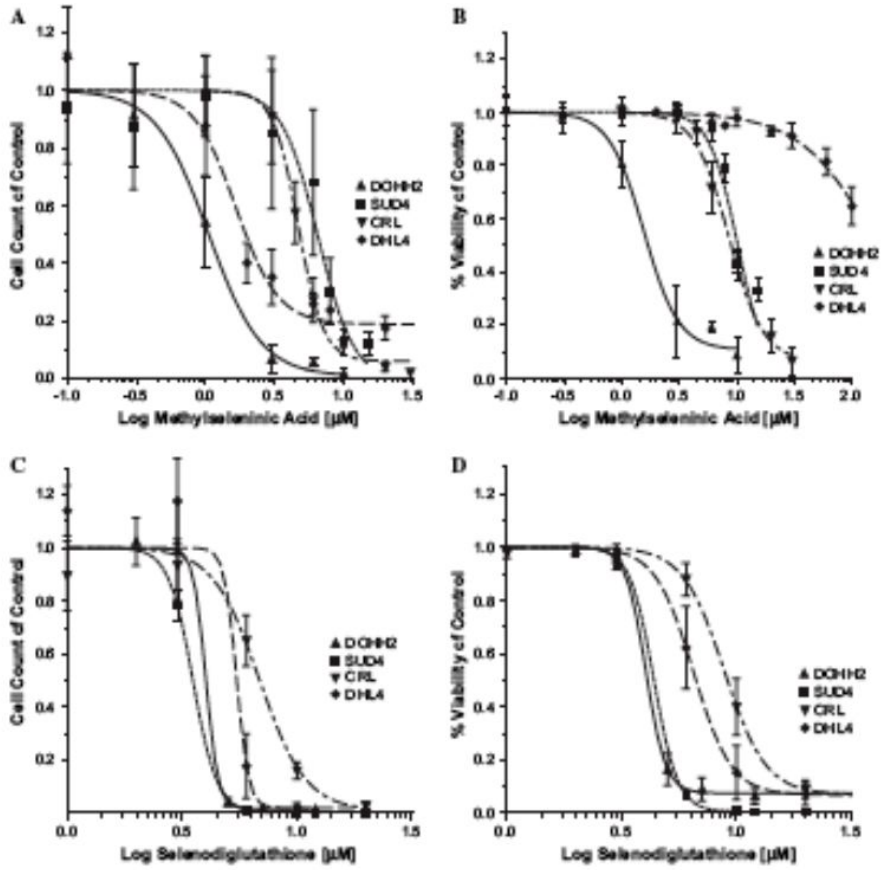


Figure 1. The effect (mean ± SD of at least three separate experiments) of MSA (A and B) and SDG (C and D) on proliferation (A, C) and viability (B, D) in four lymphoma cell lines.

Table 1. EC_{50} values for cell number and percentage viability (95% confidence intervals, at least three separate experiments for each) for MSA and SDG in four lymphoma cell lines after a 3-day continuous exposure

	Mean EC_{50} concentration, μM, (95% CI)	
Cell line	Cell number	% Viability
Methylseleninic acid		
SUD4	6.5 (6.9–8.7)	10.2 (9.7–10.8)
DoHH2	1.0 (0.9–1.5)	1.6 (1.3–1.8)
CRL2261	4.7 (4.5–5.2)	8.5 (7.8–9.5)
DHL4	1.7 (1.5–1.9)	166 (141–196)
Selenodiglutathione		
SUD4	3.5 (3.5–3.8)	4.4 (4.2–4.5)
DoHH2	4.0 (3.1–5.2)	4.0 (3.8–4.2)
CRL2261	6.8 (6.1–7.7)	8.8 (8.3–9.4)
DHL4	4.9 (3.5–6.8)	6.5 (6.0–7.0)

BC_{50} concentration of either compound for 3 days in the presence or absence of 2.5 mM NAC (Figure 4B). NAC alone had no effect on cell viability. In the DoHH2 cell line, in which SDG did not generate ROS, the addition of NAC had no effect on the activity of SDG. However, in DHL4 and SUD4 cells, in which SDG resulted in significant increases in ROS, NAC reduced the cytotoxic activity of SDG, and almost abolished its activity in SUD4 cells in which the cytotoxic BC_{50} concentration of SDG resulted in a 450% increase in ROS over 30 min. NAC had no effect on the activity of MSA in any of the cell lines studied.

SDG and MSA are active in primary lymphoma cultures

The activity of a 48-h exposure to SDG or MSA was then investigated in primary B-cell malignancy samples from

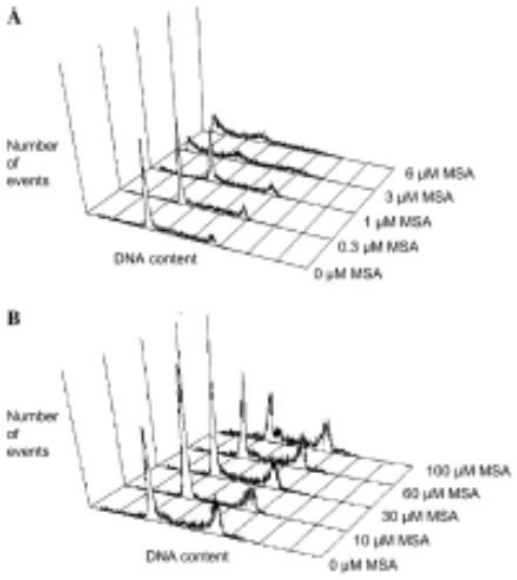


Figure 2. Changes in cell cycle distribution (DNA content) on day 5 with increasing exposure concentration, including sub-G1 (apoptotic) cells with MSA in DoHH2 (A) and DHL4 (B) cells. There is no clear arrest in a specific cell cycle phase in either cell line. Apoptotic cells (sub G1) are apparent in the sensitive DoHH2 cells but not in the markedly less sensitive DHL4 cells.

patients with mantle cell lymphoma (No. 03 and No. 04), follicular lymphoma (No. 01), chronic lymphocytic leukemia (No. 02) and a normal B-cell sample from a healthy blood donor. All samples showed a concentration-dependent decrease in percentage viability with MSA and SDG (Figure 5A, B). Although these data are limited, cells appeared more sensitive to SDG (EC_{50} concentrations 9–28 μM) than MSA (EC_{50} values 39–300 μM). Cytotoxic EC_{50} concentrations in a normal B-cell sample were 109 μM and 1071 μM for SDG and MSA, respectively.

discussion

Following the observation that presentation serum selenium was an independent predictor of response to treatment and overall survival in patients with aggressive non-Hodgkin's lymphoma, the activity of specific selenium compounds has now been studied in a panel of lymphoma cell lines and in a primary lymphoma culture system. Dietary forms of selenium, such as L-selenomethionine, require activation by β-lyase, typically in the liver, so have little *in vitro* activity [13]. Activated selenium species were therefore used in these studies, namely MSA, which is rapidly converted to the highly reactive methylselenol, and SDG (an active metabolite of the naturally occurring sodium selenite), which is activated further to hydrogen selenide with the generation of ROS in the process [14].

While the activity of these compounds has been reported in other cancer types, there are no data describing the activity of selenium compounds in human lymphomas. Both MSA and SDG induced cytostasis and cytotoxicity in the lymphoma cell line panel studied, in a concentration and time-dependent manner. The MSA cell number EC_{50} (1–7 μM) or percentage viability EC_{50} (2–10 μM; DHL-4 166 μM) is in the range reported by others using human cancer cell lines of epithelial origin [15–17], and the activity of SDG is in keeping with that found in primary cultures of oral carcinomas by Ghose et al. [18]. It is noteworthy that the concentration effect curves were much steeper for SDG than for MSA, most notably so in DoHH2 and SUD4 cells (sigmoid factor for DoHH2 and SUD4 cells –16.2 and –8.3, respectively, for cell number and –10.1 and –8.4, respectively, for percentage viability). This suggests a possible threshold concentration above which the effect increases dramatically.

Other reports have described the induction of G1 cell cycle arrest in response to MSA in cancer cell lines, and S phase arrest in response to selenite, an SDG precursor [15]. This was not apparent in our studies, with a decrease in all phases of the cell cycle concomitant with an increase in the sub-G1 (apoptotic) fraction.

As part of other studies we have determined that all of these lines contain p53 mutations, confirmed using a functional assay in yeast [19], which found no transcriptional p53 activity in SUD4, DHL-4 and CRL2261 cells, and reduced activity in DoHH2 cells that contain only one mutated p53 gene copy (Strauss et al., manuscript in preparation; Richard Camplejohn, personal communication). The activity of these selenium compounds is therefore not dependent on functional p53 activity, confirming previous reports that although SDG induces functional p53, this is in response to DNA damage and is not required for the induction of apoptosis [20].

The cell lines studied differed markedly in their ability to generate, or inactivate, ROS, with DoHH2 and DHL-4 cells showing no change in ROS in response to H_2O_2, but marked increases in CRL2261 and SUD4 cells. A similar result was seen with SDG, with no change in ROS in DoHH2 cells, a significant increase in DHL-4 cells and a more marked increase still in CRL2261 and SUD4 cells. MSA generated little or no increase in ROS in any cell line. The potential importance of ROS in the cytotoxic activity of MSA and SDG was investigated using NAC, which increases intracellular cysteine, resulting in increased glutathione (GSH) concentration [21]. NAC had no effect on the activity of MSA or SDG in DoHH2 cells, in which these compounds alone induced little or no change in ROS. In DHL-4 cells, in which SDG increased ROS, NAC significantly reduced SDG cytotoxicity, while in SUD4 cells that had shown the biggest increase in ROS in response to SDG, the addition of NAC almost abolished cytotoxic activity. NAC had no effect on the activity of MSA in any cell line studied. As MSA is activated to methylselenol in a reaction requiring GSH, increased intracellular GSH has been reported to increase the activity of MSA [21], an effect not seen in our studies. In contrast, GSH has a dual role in the activation of SDG in that it acts as a cofactor in the generation of ROS by SDG, but also acts as an antioxidant by mopping up the ROS produced [22]. GSH, or NAC, may

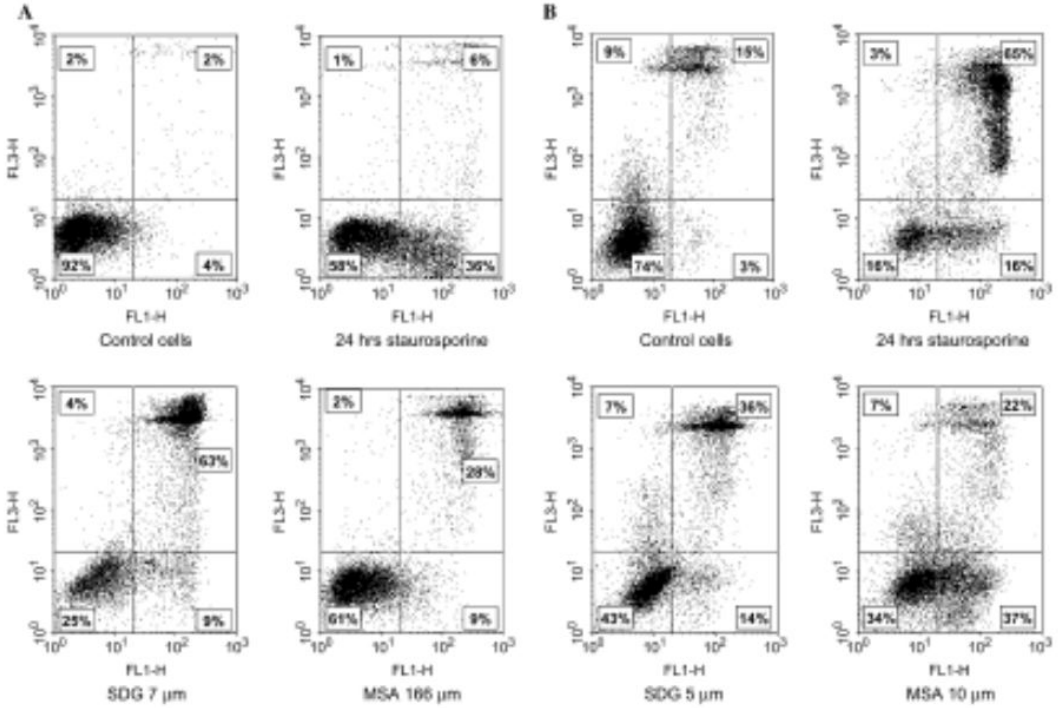


Figure 3. Dual labeling with PI (FL3-H) and annexin (FL1-H) in DHL4 (A) and SUD4 (B) cells treated with staurosporine (24 h), or the cytotoxic EC_{50} concentrations of SDG or MSA for 3 days (percentage of cells in each quadrant). Cell death with SDG and MSA is associated with increased signal from both labels, confirming cell death by apoptosis. Staurosporine is included as a positive control.

therefore reduce the activity of selenite, or SDG [22, 23]. These data clearly highlight the importance of ROS in the induction of apoptosis by SDG in some cell lines, but also suggest that this can be independent of ROS as the compound was active in DoHH2 cells in the absence of ROS generation, and in the presence of NAC.

These data also suggest mechanisms whereby SDG may be exerting its activity. ROS are potentially DNA damaging and other reports have described the induction of general [23] or specific [24] DNA damage with sodium selenite. Using a similar assay MSA did not generate DNA damage [25]. Selenite is also reported to cause topoisomerase II/DNA cleavable complex formation resulting in double-strand DNA breaks and apoptosis [23], albeit at concentrations higher than those used in our own studies. This activity resulted from the modification of important thiol groups in topoisomerase II by selenite generated ROS, an action reversed by GSH.

In addition to effects mediated by selenoproteins, both of these selenium species deplete intracellular GSH during their activation, so may make cells more sensitive to cytotoxic drugs that are inactivated by conjugation with GSH, such as cisplatin or doxorubicin.

The cytotoxic activity of MSA and SDG was also studied in primary lymphoma samples from patients who had received multiple previous lines of chemotherapy and in a normal B-cell sample. All primary samples showed a concentration dependent loss of viability after a 2-day exposure to MSA or SDG, with the potentially genotoxic compound SDG showing slightly greater potency than MSA (EC_{50} values 9–28 µM compared to 39–300 µM respectively). Although only one normal B-cell sample was studied it is noteworthy that the EC_{50} concentrations for both SDG and MSA were higher than for the lymphoma samples.

These studies were conducted to determine the activity of different selenium species as single agents, prior to examining the role of selenium in mediating chemosensitivity *in vitro* and *in vivo* in the lymphoma setting. The finding that SDG cytotoxicity is, at least in part, dependent on ROS generation further suggests that this compound may be directly genotoxic. SDG is not currently available in a form that can be administered to patients. In contrast other selenium species, most notably Se-Methylselenocysteine (MSC), are available for clinical use and in the case of MSC are rapidly converted by β-lyase to methylselenol [25], the same activated species as with MSA used in our studies. MSC has also shown impressive synergy with cytotoxic agents in a colorectal cancer mouse xenograft study, by both protecting normal tissue and by increasing the sensitivity of tumour tissue [26].

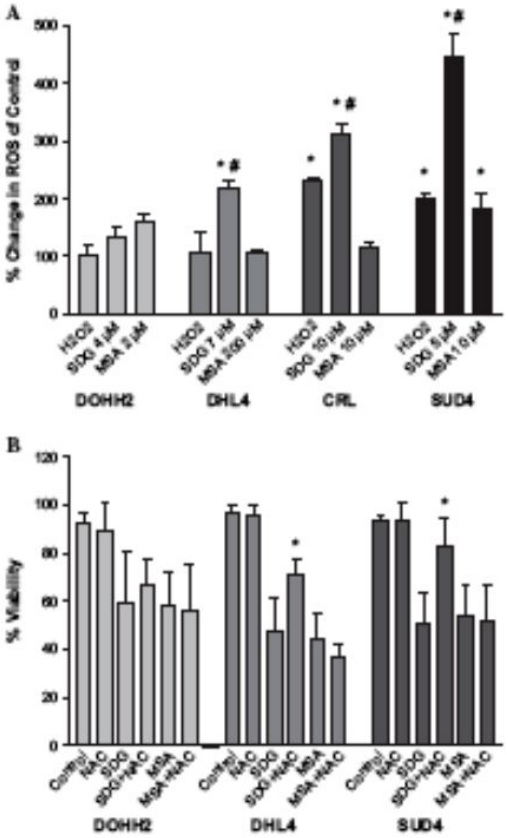


Figure 4. (A) The generation of ROS in lymphoma cells relative to control (0.75% DMSO) after exposure to selenium compounds for 30 min at the 5-day cytotoxic EC_{50} concentration (*denotes significantly different to control cells, # denotes SDG significantly different to MSA). (B) N-acetyl cysteine (NAC) significantly reduced the cytotoxic activity of SDG in DHL4 and SUD4 cells, but had no effect on the activity of MSA (SDG concentration DoHH2, DHL4 and SUD4 cells 4.2, 7.0 and 4.0 µM, respectively, MSA concentration DoHH2, DHL4 and SUD4 cells 1.9, 166 and 10.0 µM, respectively, N-acetyl cysteine 2.5 µM throughout, *$P < 0.01$ compared to SDG alone).

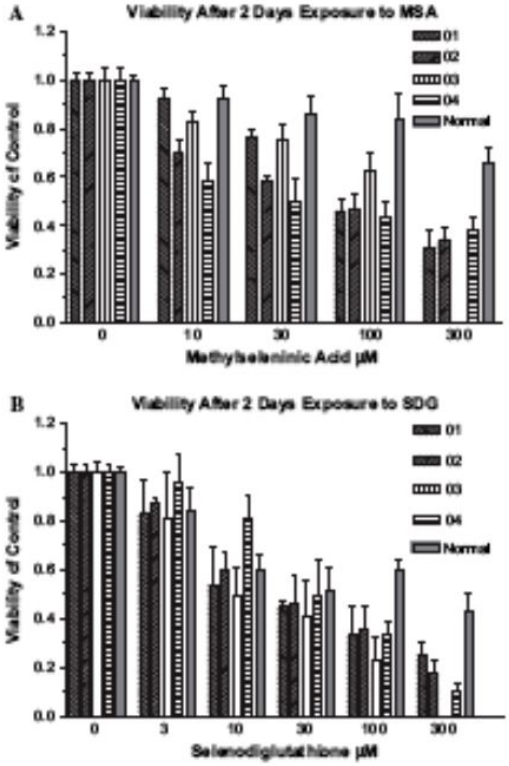


Figure 5. The effect of MSA (A) and SDG (B) on the viability of primary lymphoma cultures (samples 01–04) and normal B-cells after a 2-day continuous exposure. The data is from triplicate observations in two independent experiments.

Further studies investigating the potential role of selenium compounds, particularly those activated rapidly to methylselenol, as an adjunct to established chemotherapy in the treatment of lymphomas, are clearly warranted.

acknowledgements

The authors acknowledge the support of Cancer Research UK in conducting these studies and thank Professor Youcef Rustum for helpful comments in the preparation of the manuscript.

references

1. Allan CB, Lacourciere GM, Stadtman, TC. Responsiveness of selenoproteins to dietary selenium. Annu Rev Nutr 1999; 19: 1–16.
2. Thomson CD. Assessment of requirements for selenium and adequacy of selenium status: a review. Eur J Clin Nutr 2004; 58: 391–402.
3. Clark LC, Cantor KP, Allaway WH. Selenium in forage crops and cancer mortality in U.S. counties. Arch Environ Health 1991; 46: 37–42.
4. Schrauzer GN, White DA, Schneider CJ. Cancer mortality correlation studies-III: statistical associations with dietary selenium intakes. Bioinorg Chem 1977; 7: 23–31.
5. El-Bayoumy K. The protective role of selenium on genetic damage and on cancer. Mutat Res 2001; 475: 123–139.
6. Clark LC, Combs GF, Jr., Turnbull BW et al. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. JAMA 1996; 276: 1957–1963.
7. Duffield-Lillico AJ, Dalkin BL, Reid ME et al. Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. BJU Int 2003; 91: 608–612.
8. Last K, Mehanaj L, Perry J et al. Presentation serum selenium predicts for overall survival, dose delivery, and first treatment response in aggressive non-Hodgkin's lymphoma. J Clin Oncol 2003; 21: 2335–2341.

9. Delhaut C, Colerier P, Boileau HL et al. Serum selenium in melanoma and epidermotropic cutaneous T-cell lymphoma. Acta Derm Venereol 1994; 74: 90–92.
10. Waters DJ, Shen S, Cooley DM et al. Effects of dietary selenium supplementation on DNA damage and apoptosis in canine prostate. J Natl Cancer Inst 2003; 95: 237–241.
11. Kluin-Nelemans HC, Limpens J, Meerabux J et al. A new non-Hodgkin's B-cell line (DoHH2) with a chromosomal translocation t(14;18) (q32;q21). Leukemia 1991; 5: 221–224.
12. Epstein AL, Levy R, Kim H et al. Biology of the human malignant lymphomas. IV. Functional characterization of ten diffuse histiocytic lymphoma cell lines. Cancer 1978; 42: 2379–2391.
13. Klein EA. Selenium: epidemiology and basic science. J Urol 2004; 171 (2 Pt 2): S50–53.
14. Davis RL, Spallholz JE. Inhibition of selenite-catalyzed superoxide generation and formation of elemental selenium (Se(0)) by copper, zinc, and ethylenediaminetetraacetic acid (EDTA). Biochem Pharmacol 1996; 51: 1015–1020.
15. Jiang C, Wang Z, Ganther H et al. Distinct effects of methylseleninic acid versus selenite on apoptosis, cell cycle, and protein kinase pathways in DU145 human prostate cancer cells. Mol Cancer Ther 2002; 1: 1059–1066.
16. Zhong W, Oberley TD. Redox-mediated effects of selenium on apoptosis and cell cycle in the LNCaP human prostate cancer cell line. Cancer Res 2001; 61: 7071–7078.
17. Kim TS, Yun BY, Kim IY. Induction of the mitochondrial permeability transition by selenium compounds mediated by oxidation of the protein thiol groups and generation of the superoxide. Biochem Pharmacol 2003; 66: 2301–2311.
18. Ghose A, Fleming J, El-Bayoumy K et al. Enhanced sensitivity of human oral carcinomas to induction of apoptosis by selenium compounds: involvement of nitrogen-activated protein kinase and Ras pathways. Cancer Res 2001; 61: 7479–7487.
19. Lomax ME, Barnes DM, Gilchrist R et al. Two functional assays employed to detect an unusual mutation in the oligomerisation domain of p53 in a Li-Fraumeni like family. Oncogene 1997; 14: 1869–1874.
20. Lanfear J, Fleming J, Wu L et al. The selenium metabolite selenodiglutathione induces p53 and apoptosis: relevance to the chemopreventive effects of selenium? Carcinogenesis 1994; 15: 1387–1392.
21. Shen HM, Ding WX, Ong CN. Intracellular glutathione is a cofactor in methylseleninic acid-induced apoptotic cell death of human hepatoma HEP(2) cells. Free Radic Biol Med 2002; 33: 552–561.
22. Shen H, Yang C, Liu J et al. Dual role of glutathione in selenite-induced oxidative stress and apoptosis in human hepatoma cells. Free Radic Biol Med 2000; 28: 1115–1124.
23. Zhou N, Xiao H, Li TK et al. DNA damage-mediated apoptosis induced by selenium compounds. J Biol Chem 2003; 278: 29532–29537.
24. Stewart MS, Stewart MS, Spallholz JE, Neldner KH et al. Selenium compounds have disparate abilities to impose oxidative stress and induce apoptosis. Free Radic Biol Med 1999; 26: 42–48.
25. Ip C, Thompson HJ, Zhu Z et al. *In vitro* and *in vivo* studies of methylseleninic acid: evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention. Cancer Res 2000; 60: 2882–2886.
26. Cao S, Durrani FA, Rustum YM. Selective modulation of the therapeutic efficacy of anticancer drugs by selenium containing compounds against human tumor xenografts. Clin Cancer Res 2004; 10: 2561–2569.

Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning

Margaret A. Shipp[1], Ken N. Ross[2], Pablo Tamayo[2], Andrew P. Weng[3], Jeffery L. Kutok[3], Ricardo C.T. Aguiar[3], Michelle Gaasenbeek[2], Michael Angelo[2], Michael Reich[2], Geraldine S. Pinkus[3], Tane S. Ray[4], Margaret A. Koval[1], Kim W. Last[4], Andrew Norton[5], T. Andrew Lister[6], Jill Mesirov[2], Donna S. Neuberg[1], Eric S. Lander[2,7], Jon C. Aster[3] & Todd R. Golub[1,2]

[1]*Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, USA*
[2]*Whitehead Institute for Biomedical Research/Massachusetts Institute of Technology Center for Genome Research, Cambridge, Massachusetts, USA*
[3]*Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA*
[4]*ICRF Medical Oncology Unit and [5]Pathology Unit, St. Bartholomew's Hospital, London, UK*
[6]*Department of Computer Science, Maths and Physics, University of West Indies, Bridgetown, Barbados*
[7]*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA*
K.N.R. and P.T. contributed equally to this study.
Correspondence should be addressed to M.A.S.: email: margaret_shipp@dfci.harvard.edu; or T.R.G.: email: golub@genome.wi.mit.edu

Diffuse large B-cell lymphoma (DLBCL), the most common lymphoid malignancy in adults, is curable in less than 50% of patients. Prognostic models based on pre-treatment characteristics, such as the International Prognostic Index (IPI), are currently used to predict outcome in DLBCL. However, clinical outcome models identify neither the molecular basis of clinical heterogeneity, nor specific therapeutic targets. We analyzed the expression of 6,817 genes in diagnostic tumor specimens from DLBCL patients who received cyclophosphamide, adriamycin, vincristine and prednisone (CHOP)-based chemotherapy, and applied a supervised learning prediction method to identify cured versus fatal or refractory disease. The algorithm classified two categories of patients with very different five-year overall survival rates (70% versus 12%). The model also effectively delineated patients within specific IPI risk categories who were likely to be cured or to die of their disease. Genes implicated in DLBCL outcome included some that regulate responses to B-cell–receptor signaling, critical serine/threonine phosphorylation pathways and apoptosis. Our data indicate that supervised learning classification techniques can predict outcome in DLBCL and identify rational targets for intervention.

Diffuse large B-cell lymphomas (DLBCLs) are the most common lymphoid neoplasms, composing 30–40% of adult non-Hodgkin lymphomas[1]. Although a subset of DLBCL patients are cured with current chemotherapeutic regimens, most succumb to the disease[2]. Clinical prognostic models such as the International Prognostic Index (IPI) have been developed to identify DLBCL patients who are unlikely to be cured with standard therapy[3]. However, the clinical factors of the IPI (age, performance status, stage, number of extranodal sites and serum lactate dehydrogenase (LDH))[3] are likely to be surrogate markers for the intrinsic molecular heterogeneity in this disease. Therefore, it is not surprising that IPI is imperfect in its identification of high-risk patients. In addition, in the absence of molecular insights into the clinical heterogeneity of DLBCL, therapeutic approaches to high-risk patients have primarily included increased doses of conventional chemotherapeutic agents and additional stem-cell support[4]. However, the value of high-dose therapy has not been confirmed in this setting[4], underscoring the need to identify more rational, molecularly defined approaches to treatment.

Molecular analyses of clinical heterogeneity in DLBCL have largely focused on individual candidate genes, with particular emphasis on genes with known functions in other malignancies or in normal lymphocyte development. Examples include adhesion molecules that influence the trafficking of normal activated B cells and tumor cells[5,6], proteins that regulate apoptosis in other B-cell lymphomas and normal B-cell subpopulations[7–9], and angiogenic peptides that promote the development of an effective tumor vasculature[10]. Additional individual genes, such as *BAL* (B-aggressive lymphoma), have been identified on the basis of their differential expression in fatal high-risk DLBCL and cured low-risk tumors[11]. Although some of these candidate genes correlate with DLBCL treatment outcome, a comprehensive molecular approach to outcome prediction is still lacking.

The recent development of DNA microarrays provides an opportunity to take a genome-wide approach to predicting DLBCL treatment outcome. One strategy is to use gene-expression profiling to extend current biological insights into the disease. Such an approach was recently described by Alizadeh *et al.*, who built on the hypothesis that DLBCL derives from normal B cells located within the germinal centers (GCs) of lymphoid organs[12,13]. Customized cDNA ('lymphochip') microarrays enriched in genes related to the GCs were used to obtain the gene-expression pat-

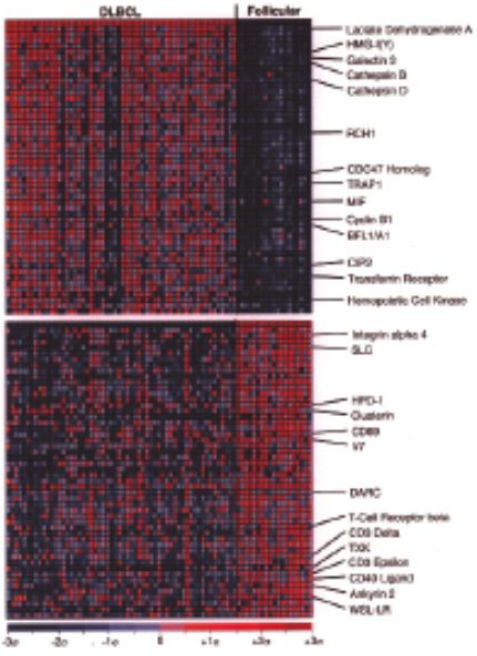


Fig. 1 Expression profiles of DLBCLs and FLs. The genes that were expressed at higher levels in DLBCL are shown on top, the ones which were more abundant in FL, on bottom. Red indicates high relative expression; blue, low expression. Color scale at bottom indicates relative expression in standard deviations from the mean. Each column is a sample, each row is a gene. Expression profiles of the 58 DLBCLs are on the left (58 columns); profiles of the 19 FLs are on the right (19 columns).

terns of DLBCL and normal lymphocytes, including B cells from GC B cells and *in vitro*-activated peripheral blood (PB) B cells. Using the unsupervised learning technique of hierarchical clustering, Alizadeh *et al.*[11] demonstrated that the DLBCLs fell into two groups: those with expression patterns similar to normal GC B cells, and those with expression patterns similar to *in vitro*-activated PB B cells. Alizadeh *et al.*[12] found the GC-like DLBCLs to have a more favorable outcome compared with the PB-like DLBCLs, suggesting that putative cell of origin might be predictive of response to treatment in this disease.

An alternative strategy for the prediction of DLBCL outcome is to use supervised learning methods to directly develop a gene-expression–based outcome model that is independent of *a priori* hypotheses. Here we report the successful prediction of outcome in a series of 58 DLBCL patients using gene-expression data from oligonucleotide microarrays together with supervised learning methods. Notably, this supervised approach identifies molecular correlates of outcome that are independent of the previously described putative cell of origin[12].

Delineating DLBCL from follicular lymphoma

We have described a supervised learning classification algorithm ('weighted voting') which delineated acute leukemias that arise from different lineages (lymphoid versus myeloid)[14]. Before attempting to apply this method to distinguish cured versus fatal/refractory DLBCLs, we investigated whether the algorithm could identify tumors within a single (B-cell) lineage. Specifically, we asked whether we could distinguish DLBCL from a related GC B-cell lymphoma, follicular lymphoma (FL). Although these two malignancies have very different clinical presentations, natural histories and responses to therapy[1,2], FLs frequently evolve over time and acquire the morphologic and clinical features of DLBCLs. In addition, a subset of *de novo* DLBCLs have the t(14;18) chromosomal translocation characteristic of most FLs (ref. 7). The t(14;18) results in overexpression of the anti-apoptotic protein BCL2 (ref. 15); however, the mechanism by which most DLBCLs circumvent normal apoptotic signals is not known.

Pre-treatment biopsies obtained from 77 patients with DLBCL ($n = 58$) or FL ($n = 19$) were subjected to transcriptional profiling using oligonucleotide microarrays containing probes for 6,817 genes. The gene-expression data are available in their entirety in Supplementary Information (www.genome.wi.mit.edu/MPR/lymphoma). The 6,817 genes were sorted by their degree of correlation with the DLBCL versus FL distinction, and the most highly correlated genes are shown in Fig. 1. Genes expressed at higher levels in DLBCL patients than in FL patients included known DLBCL markers such as lactate dehydrogenase[3] and transferrin receptor (Fig. 1). Genes associated with cellular proliferation (cyclin B1 and a CDC47 homolog) and invasion and metastasis (cathepsins B and D) were also expressed at higher levels in DLBCLs versus FLs. DLBCLs also overexpressed: 1) the high-mobility group protein isoforms I and Y (HMGIY), known to be a MYC target and encoded by a potential oncogene[16]; 2) the hematopoietic cell kinase (HCK) which has been linked with CD44 signaling[17]; and 3) inhibitors of apoptosis such as the carbohydrate-binding protein, galectin 3 (ref. 18) and the B-cell lymphoma-2 (BCL2)-related protein, BFL1A1 (ref. 19; also known as BCL2A1).

BFL1A1 overexpression in DLBCL is of particular interest because this anti-apoptotic molecule is induced by CD40 signaling and is required for CD40-mediated B-cell survival[19]. BFL1A1 is also a direct transcriptional target of nuclear factor-κB (NF-κB), which suppresses both chemotherapy- and tumor necrosis factor–associated apoptosis[21,22]. These observations raise the possibility that BFL1A1 overexpression may represent an important anti-apoptotic mechanism for reducing the chemosensitivity of DLBCLs.

Genes overexpressed in FLs compared with DLBCLs included additional regulators of apoptosis such as human programmed death-1 (*HPD1*)[23] and *WSL-LR* (also known as *TNFRSF12*)[24]. FLs also had more abundant expression of genes encoding cytoskeletal components (ankyrin 2) and adhesion molecules (α_4 integrin) and genes expressed by follicular dendritic cells (clusterin[25]) and infiltrating T cells (T-cell receptor-β, CD3-ε, CD3-δ, CD40 ligand, TXK tyrosine kinase[26], T-cell activation antigens, CD69 (ref. 27) and V7 (ref. 28)) and the T-cell chemoattractant, SLC (ref. 29; also known as SCYA21). The presence of a prominent T-cell and follicular dendritic-cell signature in the FLs also indicates that microarray profiling can be used to capture additional non-malignant components of the tumor microenvironment. This non-malignant component of the FL versus DLBCL signature would have been missed had purified tumor cells, rather than primary tumor specimens, been analyzed.

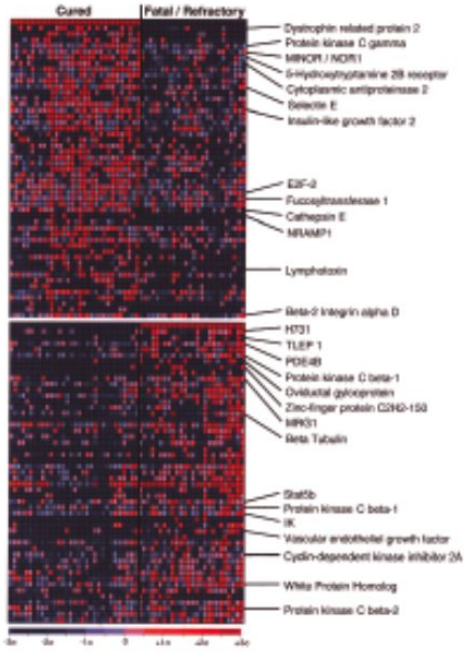


Fig. 2 Expression profiles of cured and fatal/refractory DLBCLs. The genes that were expressed at higher levels in cured disease are shown on top, those that were more abundant in fatal disease are shown on bottom. Red indicates high level expression; blue, low level expression. Color scale at bottom indicates relative expression in standard deviations from the mean. Each column is a sample, each row a gene. Expression profiles of the 32 cured DLBCLs are on the left; profiles of the 26 fatal/refractory tumors are on the right.

To determine whether the gene-expression patterns associated with DLBCL and FL (Fig. 1) were sufficiently robust to predict the lymphoma type of an unknown sample, we used the weighted-voting algorithm, which calculates the weighted combination of informative marker genes to make a class distinction (that is, DLBCL versus FL)[24]. To avoid the statistical problem of over-estimating prediction accuracy that occurs when a model is trained and evaluated with the same samples, we used a 'leave-one-out' cross-validation testing method. In this procedure, 1 of the 77 samples is withheld, and the remaining 76 samples are used to train a gene-expression-based model and predict the class of the withheld sample. The process is repeated until all 77 samples are predicted in turn. A 30-gene predictor correctly classified 71 of 77 tumors (91%) with respect to the DLBCL versus FL distinction ($P < 1 \times 10^{-4}$ compared with random prediction).

Predicting outcome in DLBCL

The success in distinguishing DLBCL from FL with supervised learning suggested that a similar approach might be used to delineate clinically relevant subsets of DLBCL. Long-term clinical follow-up was available for all 58 DLBCL patients in the study. These patients were divided into two groups: those with cured disease ($n = 32$) and those with fatal or refractory disease ($n = 26$).

The genes most highly correlated with the cured versus fatal/refractory distinction included genes that have been previously associated with DLBCL outcome, such as VEGF, linked with adverse outcome[18] and overexpressed in fatal/refractory DLBCLs, and E2F, associated with favorable outcome[25] and overexpressed in cured DLBCLs (Fig. 2). The presence of known prognostic markers among our outcome-correlated genes indicates that the gene-expression signatures are likely to be *bona fide*.

We next used a supervised learning classification approach (weighted-voting algorithm and cross-validation testing) to develop a DLBCL outcome predictor and assess its accuracy. Predictors containing between 8 and 16 genes all yielded statistically significant outcome predictions, with the highest accuracy obtained using 13 genes (Fig. 3). Although each of the cross-validation loops generated a new 13-gene model, each of these models contained mostly the same genes (see Methods and www.genome.wi.mit.edu/MPR/lymphoma).

The predictor separated the 58 patients, who had a 5-year overall survival (OS) of 54% (Fig. 4*a*), into 2 groups: those predicted to be cured and those predicted to have fatal/refractory disease. Kaplan–Meier survival analyses indicated that the patients predicted to be cured had significantly improved long-term survival compared with those predicted to have fatal/refractory disease (5-year OS, 70% versus 12%; nominal log rank P-value = 0.00004; Fig. 4*b*). Other classification algorithms, including support vector machines (SVM) and *k*-nearest neighbors (*k*-NN) performed similarly (5-year OS for SVM, 72% versus 12%, $P = 0.00002$; for *k*-NN, 68% versus 23%, $P = 0.001$; www.genome.wi.mit.edu/MPR/lymphoma). These results indi-

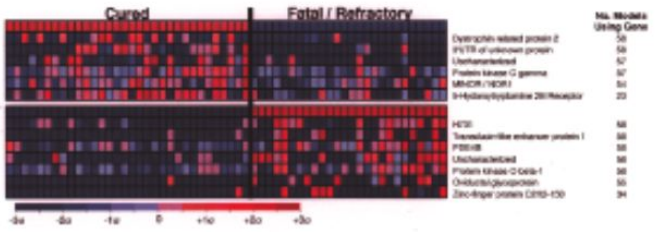


Fig. 3 Genes included in the DLBCL outcome model. Genes expressed at higher levels in cured disease are listed on top and those that were more abundant in fatal disease are shown on bottom. Red indicates high expression; blue, low expression. Color scale at bottom indicates relative expression in standard deviations from the mean. Each column is a sample, each row a gene. Expression profiles of the 32 cured DLBCLs are on the left; profiles of the 26 fatal/refractory tumors are on the right. Models with the highest accuracy were obtained using 13 genes. Although each of the 58 cross-validation loops generates a new 13-gene model, 7 of the genes were common to all 58 models; 4 additional genes were included in 54 or more models and 2 genes were included in 23–34 models.

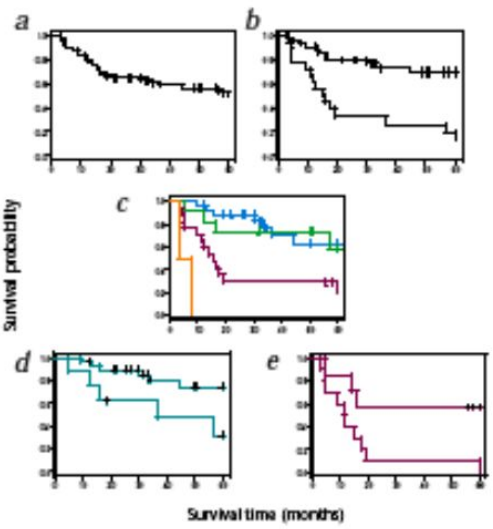


Fig. 4 Overall survival predictions for DLBCL study patients. *a*, 5-year OS for the entire study group. 33 of 58 DLBCL study patients remained alive at a median of a 58-month follow-up. The predicted 5-year OS for the group as a whole was 54%. *b*, 5-year OS for favorable and unfavorable risk groups defined by the 13-gene model (70% versus 12%, $P = 0.00004$). Top line, cured; bottom, fatal/refractory. *c*, 5-year OS for patients in L-risk (green line), LI-risk (blue line), HI-risk (red line) and H-risk (orange line) categories as defined by the IPI: L, 26 pts; LI, 11 pts; HI, 17 pts; H, 2 pts. *d*, 5-year OS for combined L/LI-risk patients with favorable or unfavorable disease as defined by the molecular model (75% versus 32%, nominal $P = 0.02$) Top line, cured; bottom, fatal/refractory. *e*, 5-year OS for HI-risk patients with favorable or unfavorable disease as defined by the molecular model (57% versus 0%; nominal $P = 0.02$). Top line, cured; bottom, fatal/refractory.

cate the existence, at diagnosis, of a gene-expression signature of outcome in DLBCL.

The clinically based IPI outcome predictor is effective in predicting the outcome of subsets of DLBCL patients[2]. In the current series, all of the IPI-defined H-risk patients died of their disease (Fig. 4*c*). However, the IPI incorrectly predicted the outcome of many of the patients in the other IPI risk groups (high intermediate (HI), low intermediate (LI) and low (L)) (Fig. 4*c*). For this reason, we investigated whether the gene-expression-based outcome predictor contained additional information not captured by the IPI. L/LI-risk patients with the 'cured' gene-expression signature had significantly higher OS rates than L/LI-risk patients with the 'fatal/refractory' signature (5-year OS, 75 versus 32%; $P = 0.02$) (Fig. 4*d*). Similarly, the outcome of HI-risk patients could be further predicted by the application of the gene-expression model (5-year OS, 57 versus 0%; $P = 0.02$) (Fig. 4*e*). These results indicate that the microarray-based outcome predictor provides additional information not reflected in the clinical prognostic model and suggests a possible strategy for further individualization of patient treatment. However, the gene-expression-based predictor did not eliminate outcome differences between L/LI-risk and HI-risk patients (Fig. 4*d* and *e*), suggesting that the clinical and molecular models contain at least partially independent information. Additional studies will be required to determine how to optimally combine such models.

Validating the model

Having defined an outcome predictor for DLBCL, we investigated the connection, if any, between this model and the cell-of-origin classification described by Alizadeh *et al.* Such comparisons are admittedly difficult, given that, 1) different genes were measured on the arrays, 2) the microarray technology was different (oligonucleotide versus cDNA arrays), 3) different computational approaches were employed, and 4) different patient samples were studied. Nevertheless, we determined that 90 of the previously described cell-of-origin signature genes[12] were also represented on our oligonucleotide arrays (see Methods and www.genome.wi.mit.edu/MPR/lymphoma).

We first used a hierarchical clustering algorithm to sort the DLBCL samples of Alizadeh *et al.* based on expression of the 90 cell-of-origin signature genes represented on both the cDNA and oligonucleotide microarrays. Two major branches of the hierarchical tree were observed; these branches were closely associ-

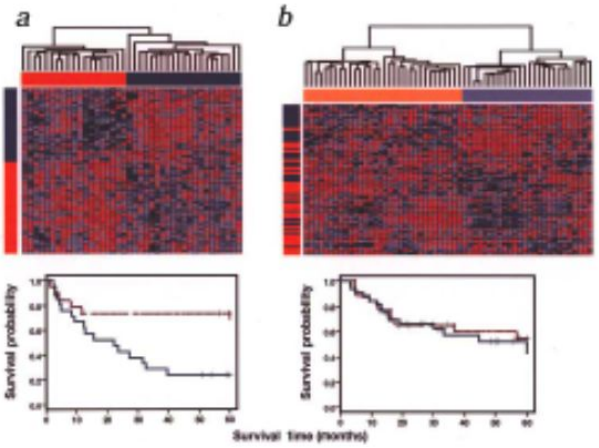


Fig. 5 Predictive value of GC-B-cell and activated B-cell signatures. *a* and *b*, The GC and activated B-cell markers common to our dataset (*b*) and that of Alizadeh *et al.*[12] (*a*). (90 common UniGene clusters) were hierarchically clustered[8] with respect to patient samples. In each dataset, 2 major branches of the hierarchical tree were observed. In the Alizadeh *et al.* dataset, the 2 major branches corresponded exactly to the previously described cell-of-origin distinction (GC-like DLBCLs, orange and activated B-like DLBCLs, blue). Genes (rows) correlated with these 2 categories are similarly indicated with the same color scheme. In our dataset, the 2 major branches of the hierarchical tree were also associated with putative cell of origin. Genes correlated with the left branch were predominantly GC-like (orange) genes, whereas genes selectively expressed in the right branch were predominantly activated B-like (blue) genes ($P = .00001$, χ^2 test). In the bottom panels, the 5-year OS for patients whose tumors exhibited the GC (top lines) and activated B-cell (bottom lines) signature are shown.

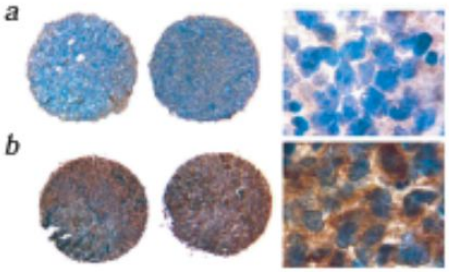


Fig. 6 Immunohistochemical staining for PKC-β. *a* and *b*, Representative PKC-β immunostaining of duplicate core samples from a cured DLBCL (*a*) and a fatal DLBCL (*b*) are shown at low (×4, left) and high (×1000, right) power.

ated with the cell-of-origin distinction[12], confirming that the 90 overlapping signature genes were sufficient to make this determination (Fig. 5*a*). As expected, the 90-gene cell-of-origin distinction was associated with outcome in the DLBCL samples of Alizadeh *et al.* (Fig. 5*a*).

We then used these same 90 genes to cluster our own 58 DLBCL samples (Fig. 5*b*). Again, two major branches of the hierarchical tree were observed, and these branches were highly correlated with the cell-of-origin distinction ($P = 0.00001$, χ^2 test) (Fig 5*b*). However, this distinction was not significantly correlated with patient outcome in our DLBCL series (Fig. 5*b*). This observation suggests that although the signature genes may reflect cell of origin, they do not explain a significant portion of the clinical variability seen in this DLBCL dataset.

We next investigated whether we could find support for our outcome predictor in the expression data of Alizadeh *et al.* Of the 13 genes in our supervised DLBCL outcome predictor, 3 were represented on the lymphochip: *NOR1* (also known as *NR4A3*), *PDE4B* and *PKC-β* (also known as *PRKCBI*). When evaluated as single markers in the dataset of Alizadeh *et al.*, *NOR1* ($P = 0.05$) and *PDE4B* ($P = 0.07$) were clearly correlated with outcome. Multiple *PKC-β* cDNAs are present on the lymphochip; these clones gave discordant expression results in the DLBCL patients, perhaps reflecting varying degrees of specificity for the β isoforms of PKC. However, two clones (clone 1308435 and 685194), specific for the PKC-β2 isoform, were indeed correlated with outcome in the DLBCL patient series of Alizadeh *et al.* ($P = 0.04$). These results from an independent dataset confirm our initial observations and highlight the value of publicly accessible gene-expression databases for rapid, computational validation of hypotheses.

The potential extension of microarray-based outcome prediction to the clinical setting was further explored using immunohistochemical detection methods. For this purpose, we generated a tissue array containing the study DLBCLs for which formalin-fixed, paraffin-embedded tumor tissue was available ($n = 21$). PKC-β protein expression was analyzed because of the critical role of PKC pathways in B-cell signaling and the commercial availability of a monoclonal antibody against PKC-β known to function in immunohistochemistry assays. PKC-β protein expression was highly associated with microarray-determined transcript abundance in the DLBCL specimens ($P = 0.08$, Fisher exact test; Fig. 6). In addition, PKC-β protein expression was closely associated with clinical outcome in the DLBCL patients ($P = 0.03$). This result both validates the microarray measurements, and demonstrates how microarray-based studies can be extended using methods that are more widely available in routine clinical practice.

Discussion

Herein, we report the successful prediction of outcome in a series of DLBCL patients using oligonucleotide microarray gene-expression data and supervised learning methods. Genes implicated in DLBCL outcome included ones that regulate responses to B-cell–receptor signaling, critical serine/threonine phosphorylation pathways and apoptosis. For example, all three of the computationally validated microarray-based outcome genes, *NOR1*, *PDE4B* and *PKC-β*, regulate apoptotic responses to antigen-receptor engagement and, potentially, cytotoxic chemotherapy.

The mitogen-inducible nuclear orphan receptor (MINOR) or NOR1 is overexpressed in cured, as opposed to fatal/refractory, DLBCL (Figs. 2 and 4). NOR1 is a member of the nerve growth factor-1B (NGF1B, also known as NR4A1) subfamily (NGF1B/TR3/Nur77, Nurr-1) of nuclear orphan receptors[31]. NGF1B family members are induced by antigen-receptor engagement and external stressors such as seizures or ischemia[31,32]; in addition, these factors directly promote the apoptosis of affected cells[31,33,34]. Recent studies indicate that at least one NGF1B family member (NGF1B/TR3/Nur77) translocates from the nucleus to the mitochondria where it directly exerts its proapoptotic effects[31,33]. Given the functions of related NGF1B family members, it is possible that NOR1 increases the apoptotic response to chemotherapy in curable DLBCL.

The cyclic AMP (cAMP)-specific phosphodiesterase PDE4B is overexpressed in fatal/refractory, as opposed to cured, DLBCL (Figs. 2 and 4). PDE4s are the predominant class of phosphodiesterases in lymphocytes[35,37], catalyzing the hydrolysis of cAMP and terminating its activity[37]. cAMP-dependent protein kinase A (PKA) signaling inhibits lymphocyte chemotaxis, cytokine release and cellular proliferation[38]. Because PDE4B reduces cAMP-availability, the phosphodiesterase also limits the negative effects of PKA signaling in lymphocytes. For this reason, PDE4A and -4B inhibitors are being evaluated in the treatment of certain B-cell malignancies where they are reported to induce B-cell apoptosis[37,38]. Together, these data suggest that PDE4B may also be an attractive therapeutic target in fatal/refractory DLBCLs.

Like PDE4B, PKC-β is overexpressed in fatal/refractory, rather than cured, DLBCL (Figs. 2,3 and 6). The alternatively-spliced PKC-β1 and -β2 isoforms are the major PKC isoforms expressed by B-lymphocytes[39]. The pivotal role of PKC-β in B-cell signaling and survival was recently demonstrated in PKC-β-deficient mice which have profoundly impaired humoral and B-cell proliferative responses[40]. In additional *in vitro* analyses, the consequences of B-cell–receptor signaling were dependent upon associated activation of PKC-β (ref. 41). In the presence of an intact PKC-β pathway, B-cell–receptor engagement resulted in B-cell proliferation; however, B-cell–receptor signaling induced apoptosis when mature B cells are either PKC depleted or stimulated in the presence of PKC inhibitors[41]. Taken together, these studies suggest that PKC-β activity enhances B-cell proliferation and survival, consistent with our observation that the enzyme is overexpressed in fatal/refractory DLBCLs. Recently, synergy between PKC-β inhibitors and chemotherapeutic agents in murine tumor

models[42] has been reported, further suggesting that pharmacologic inhibition of PKC-β may have a therapeutic role in the future treatment of fatal/refractory DLBCL.

These studies demonstrate the potential of DNA microarray-based recognition of gene-expression patterns for the prediction of outcome in DLBCL patients. This work also illustrates the important difference between unsupervised (clustering) and supervised machine learning analytical approaches. The previously reported cell-of-origin distinction[23] was originally identified using an unsupervised clustering algorithm and this distinction was subsequently associated with disease outcome. In our series, the cell-of-origin distinction was not associated with significant outcome differences (Fig. 5), suggesting that additional factors may be important in determining DLBCL response to therapy.

One limitation of the supervised classification method employed here is that it reduces the classification problem to a dichotomous distinction (cured versus fatal/refractory disease). However, it is likely that these distinct clinical behaviors are explained by different molecular mechanisms in different patients. More refined outcome prediction may thus require the use of alternative feature selection algorithms capable of capturing more complex DLBCL substructure, or the application of non-linear classification strategies. Moreover, optimal outcome prediction may require not only gene-expression data but also the inclusion of tumor genotype information.

Nevertheless, the DLBCL outcome-correlated genes described here were highly informative, including key intermediaries in signaling pathways that regulate apoptotic responses to receptor engagement, and potentially, to cytotoxic therapy. These studies suggest strategies for both optimizing the use of existing therapy for DLBCL and developing more rationally designed therapies in this disease. The computational validation of our DLBCL outcome predictor using publicly available gene-expression databases further illustrates the important role of computational genomics in biomedical research.

Methods

Samples. Frozen diagnostic nodal tumor specimens from 58 DLBCL patients and 19 FL patients were analyzed according to an Institutional Review Board approved protocol. The histopathology and immunophenotype of each tumor were centrally reviewed to confirm diagnosis and uniform involvement with tumor. The DLBCL study patients were those for whom frozen tumor tissue and complete clinical information (presenting clinical characteristics, treatment records and long-term follow-up) were available. Treatment records of all 58 DLBCL patients were reviewed to confirm that patients had received adequate doses of cyclophosphamide, adriamycin, vincristine and prednisone (CHOP)-like combination chemotherapy[2] for 6 or more cycles or until documented disease progression and to document outcome and clinical IPI risk group[3]. The IPI was not determined in 2 patients because of missing LDH levels in these patients. DLBCL study patients (predicted 5-year OS 54%, median follow-up 58 months) were divided into 2 discrete categories: 1) 29 patients who achieved CR and remained free of disease plus 3 additional patients who died of other causes (total of 32 'cured' patients); and 2) 23 patients who died of lymphoma plus 3 additional patients who remained alive with recurrent refractory or progressive disease (total of 'fatal/refractory' 26 patients).

Target cRNAs and oligonucleotide microarrays. Total RNA was extracted from each frozen tumor specimen and biotinylated cRNAs were generated as described[43] and as detailed (see Supplementary Information). Samples were hybridized overnight to Affymetrix HU6800 oligonucleotide arrays (Affymetrix, Santa Clara, California)[44]. Arrays were subsequently developed with phycoerythrin-conjugated streptavidin (SAPE) and biotinylated antibody against streptavidin, and scanned to obtain quantitative gene-expression levels[34,41]. The raw gene-expression values were then scaled in order to account for any minor differences in global chip intensity. Expression levels below 20 units were assigned a value of 20, and those exceeding 16,000 units were assigned a value of 16,000. Genes whose expression did not vary across the dataset were removed (see Supplementary Information).

Supervised prediction. Classes (classes 0 and 1) were defined based on morphology (DLBCL versus FL) or treatment outcome (cured versus fatal/refractory disease). Marker genes were then identified using a signal-to-noise calculation: $S_x = (\mu_{true} - \mu_{false}) \div (\sigma_{true} + \sigma_{false})$ where, for each gene, μ_{true} represents the mean value of arrays with true class equal to class 0, and σ_{true} represents the standard deviation of class 0 samples[34]. Thereafter, a weighted-voting classification algorithm was applied as previously described, and was tested by 'leave-one-out' cross-validation[34]. The total number of prediction errors in cross-validation was calculated using a variable number of genes, and a final model chosen which minimized cross-validation errors. Analyses of error rates, confusion matrices (false negatives versus false positives) and Kaplan–Meier survival curves were performed using S-Plus (http://www.splus.mathsoft.com/products/splus/splusintro.html). The log-rank test was used to assess the differences between the survival curves and nominal *P* values were calculated.

The *P* value for the prediction of lymphoma type (DLBCL versus FL) was predicted using the proportional chance calculation[45] as described (http://marketing.byu.edu/htmlpages/tutorials/discriminant.htm).

Analysis of lymphochip microarray data. The raw lymphochip data from the 40 DLBCL specimens and the associated outcome information was obtained from the Lymphoma/Leukemia Molecular Profiling Project (http://llmpp.nih.gov/lymphoma). RAT2 values were pre-processed by setting minimum values to 0 and normalizing arrays to a mean value of 0 and variance of 1. Computational model validation was performed by identifying genes from our outcome predictor (Fig. 3) that were represented on the lymphochip. For the lymphochip data, we mapped the clone IMAGE (Integrated molecular analysis of genomes and their expression) numbers to GenBank accession numbers (using the list at http://llmpp.nih.gov/lymphoma/data/clones.txt) and then mapped the accession numbers to UniGene clusters (National Center for Biotechnology Information, Bethesda, Maryland). Similarly, we mapped accession numbers for our oligonucleotide array data to UniGene clusters. Predictors using single genes (*PKC-β*, *PDE4B*, MINOR/MOR1) were constructed by finding the boundary halfway between the classes ($b_x = (\mu_{true} + \mu_{false}) \div 2$) in the dataset and predicting the unknown sample according to its gene-expression value with respect to that boundary. This method is equivalent to performing weighted voting with only 1 gene.

The 90 UniGene clusters common to both arrays are represented by 139 clones in the data of Fig. 3c of Alizadeh *et al.* and by 100 probe sets on the oligonucleotide arrays. The DLBCL series of Alizadeh *et al.* and our DLBCL series were separately clustered using these common cell-of-origin signature genes by average linkage hierarchical clustering, and the results visualized using TreeView (from M. Eisen)[41].

Immunohistochemical staining. Five representative 0.6-mm cores were obtained from diagnostic areas of each paraffin-embedded formalin-fixed DLBCL tumor and inserted in a grid pattern in a single-recipient paraffin block using a tissue arrayer (Beecher Instruments, Silver Spring, Maryland). Five-micron sections cut from this 'tissue array' were stained for PKC-β using an immunoperoxidase method. Briefly, slides were deparaffinized and pre-treated in 1 mM EDTA (pH 8.0) for 20 min at 95 °C. All further steps were performed at room temperature in a hydrated chamber. Slides were pre-treated with Peroxidase Block (DAKO, Carpinteria, California) for 5 min to quench endogenous peroxidase activity, and a 1:5 dilution of goat serum in 50 mM Tris-Cl (pH 7.4) for 20 min to block non-specific binding sites. Primary antibody (murine monoclonal antibody specific for PKC-β (Serotec, Oxford, UK) was applied at a 1:1000 dilution in 50 mM Tris-Cl (pH 7.4) with 3% goat serum for 1 h. After washing, secondary goat anti-mouse horseradish-peroxidase-conjugated antibody (Envision Detection Kit, DAKO) was applied for 30 min. After further washing, immunoperoxidase staining was developed using a DAB chromogen kit (DAKO) according to manufacturer's instructions. Following counterstaining with hema-

toxylin, immunoperoxidase staining within the malignant cell population of each core was scored in a blinded fashion with respect to clinical outcome and expression profile results by 3 experienced hematopathologists (J.C.A., A.P.W. and J.L.K.). The intensity of staining on each core was graded from 0 (no staining) to 3 (maximal staining), and an average staining intensity (mean of all 5 cores) was generated for each tumor. Median values were used to divide both the PKC immunostaining intensities and the array-based transcript levels into two categories. The Fisher exact test was then used to evaluate the association between these measurements.

Acknowledgments

We thank members of the Center for Genome Research and members of the Shipp and Aster laboratories for technical assistance and helpful comments. This work was supported in part by grants from Bristol-Myers Squibb, Millennium Pharmaceuticals and Affymetrix (E.S.L.).

RECEIVED 18 MAY; ACCEPTED 26 NOVEMBER 2001

1. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. *Blood* **89**, 3909–3918 (1997).
2. Shipp, M., Harris, N. & Mauch, P. The non-Hodgkin's lymphomas. In *Cancer Principles & Practices of Oncology* (eds. DeVita, V.T., Hellman, S. & Rosenberg, S.A) 2165–2220 (Lippincott, Philadelphia, 1997).
3. A predictive model for aggressive non-Hodgkin's lymphoma. The International NHL Prognostic Factors Project. *N. Engl. J. Med.* **329**, 987–994 (1993).
4. Shipp, M.A. *et al.* International consensus conference on high-dose therapy with hematopoietic stem cell transplantation in aggressive non-Hodgkin's lymphomas: Report of the jury. *J. Clin. Oncol.* **17**, 423–429 (1999).
5. Yakushijin, Y. *et al.* A directly spliced exon 10-containing CD44 variant promotes the metastasis and homotypic aggregation of aggressive non-Hodgkin's lymphoma. *Blood* **91**, 4292–4297 (1998).
6. Terol, M.-J. *et al.* Expression of the adhesion molecule ICAM-1 in non-Hodgkin's lymphoma: Relationship with tumor dissemination and prognostic importance. *J. Clin. Oncol.* **16**, 35–40 (1998).
7. Gascoyne, R. *et al.* Prognostic significance of bcl-2 protein expression and bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. *Blood* **90**, 244–251 (1997).
8. Kramer, M. *et al.* Clinical relevance of BCL2, BCL6, and MYC rearrangements in diffuse large B-cell lymphoma. *Blood* **92**, 3152–3162 (1998).
9. Ambrosini, G., Adida, C. & Altieri, D. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nature Med.* **3**, 917–921 (1997).
10. Salven, P., Teerenhovi, L. & Joensuu, H. A high pretreatment serum vascular endothelial growth factor concentration is associated with poor outcome in non-Hodgkin's lymphoma. *Blood* **90**, 3167–3172 (1997).
11. Aguilar, R. *et al.* BAL is a novel risk-related gene in diffuse large B-cell lymphomas which enhances cellular migration. *Blood* **96**, 4328–4334 (2000).
12. Alizadeh, A. *et al.* Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* **403**, 503–511 (2000).
13. Kuppers, R., Klein, U., Hansmann, M.-L. & Rajewsky, K. Cellular origin of human B-cell lymphomas. *N. Engl. J. Med.* **341**, 1520–1529 (1999).
14. Golub, T. *et al.* Molecular classification of cancer: Class discovery and class prediction by gene expression monitoring. *Science* **286**, 531–537 (1999).
15. Cleary, M. *et al.* Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B cell lymphoma. *Cell* **44**, 97–106 (1985).
16. Wood, L. *et al.* HMG-I/Y, a new c-Myc target gene and potential oncogene. *Mol. Cell Biol.* **20**, 5490–5502 (2000).
17. Rangaswamy, S., Borisch, B. & Hoessli, D. Signal transduction via CD44: role of plasma membrane microdomains. *Leuk. Lymphoma* **35**, 455–468 (1999).
18. Matarrese, P. *et al.* Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties. *Internat. J. Cancer* **85**, 545–554 (2000).
19. Zhang, H. *et al.* Structural basis of BFL-1 for its interaction with BAX and its anti-apoptotic action in mammalian and yeast cells. *J. Biol. Chem.* **275**, 11062–11068 (2000).
20. Lee, H., Dadgostar, H., Cheng, Q., Shu, J. & Cheng, G. NF-κB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. *Proc. Natl. Acad. Sci. USA* **96**, 9136–9141 (1999).
21. Wang, J., Saunthararajah, Y., Redner, R.L. & Liu, J.M. Inhibitors of histone deacetylase relieve ETO-mediated repression and induce differentiation of AML1-ETO leukemia cells. *Cancer Res.* **59**, 2766–2769 (1999).
22. Zong, W., Edelstein, L., Chen, C., Bash, J. & Gelinas, C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κB that blocks TNFα-induced apoptosis. *Genes Dev.* **13**, 382–387 (1999).
23. Ishida, Y. *et al.* The human PD-1 gene: complete cDNA, genomic organization, and developmentally regulated expression in B cell progenitors. *Gene* **197**, 177–187 (1997).
24. Ellison, J. *et al.* A death-domain-containing receptor that mediates apoptosis. *Nature* **384**, 372–375 (1996).
25. Wellmann, A. *et al.* Detection of differentially expressed genes in lymphomas using cDNA arrays: identification of clusterin as a new diagnostic marker for anaplastic large-cell lymphomas. *Blood* **96**, 398–404 (2000).
26. Schimmen, C. *et al.* A role for the Tec family tyrosine kinase Tsk in T cell activation and thymocyte selection. *J. Exp. Med.* **190**, 1427–1438 (1999).
27. Testi, R., D'Ambrosio, D., De Maria, R. & Santoni, A. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunol. Today* **15**, 479–483 (1994).
28. Ruegg, C. *et al.* V7, a novel leukocyte surface protein that participates in T cell activation. II. Molecular cloning and characterization of the V7 gene. *J. Immunol.* **154**, 4434–4443 (1995).
29. Saeki, H., Moore, A., Brown, M. & Hwang, S. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J. Immunol.* **162**, 2472–2475 (1999).
30. Moller, M. *et al.* Frequent disruption of the 8&1 pathway in diffuse large B cell lymphoma: prognostic significance of E2F-1 and p16INK4A. *Leukemia* **14**, 888–894 (2000).
31. Brenner, C. & Kroemer, G. Mitochondria—the death signal integrators. *Science* **289**, 1150–1151 (2000).
32. Youn, H., Sun, L., Prywes, R. & Liu, J. Apoptosis of T cells mediated by Ca²⁺-induced release of the transcription factor MEF2. *Science* **286**, 790–793 (1999).
33. Kuang, A., Cado, D. & Winoto, A. Nur77 transcriptice activity correlates with its apoptotic function *in vivo*. *Eur. J. Immunol.* **29**, 3722–3728 (1999).
34. Xue, Y. *et al.* Positive and negative thymic selection in T cell receptor-transgenic mice correlate with Nur77 mRNA expression. *Eur. J. Immunol.* **27**, 2048–2056 (1997).
35. Li, H. *et al.* Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. *Science* **289**, 1159–1164 (2000).
36. Manning, C. *et al.* Suppression of human inflammatory cell function by subtype-selective PDE4 inhibition correlates with inhibition of PDE4A and PDE4B. *Br. J. Pharmacol.* **128**, 1393–1399 (1999).
37. Lerner, A., Kim, D. & Lee, S. The cAMP signaling pathway as a therapeutic target in lymphoid malignancies. *Leuk. Lymphoma* **37**, 39–51 (2000).
38. Kim, D. & Lerner, A. Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. *Blood* **92**, 2484–2494 (1998).
39. Mischak, H. *et al.* Expression of protein kinase C genes in hemopoietic cells is cell-type- and B cell-differentiation stage specific. *J. Immunol.* **147**, 3981–3987 (1991).
40. Leitges, M. *et al.* Immunodeficiency in protein kinase Cb-deficient mice. *Science* **273**, 788–791 (1996).
41. King, L., Norvell, A. & Monroe, J. Antigen receptor-induced signal transduction imbalances associated with the negative selection of immature B cells. *J. Immunol.* **162**, 2655–2662 (1999).
42. Teicher, B. *et al.* Enzymatic rationale and preclinical support for a potent protein kinase C beta inhibitor in cancer therapy. *Adv. Enzymolog.* **38**, 313–327 (1998).
43. Tamayo, P. *et al.* Interpreting patterns of gene expression with self-organizing maps: Methods and application to hematopoietic differentiation. *Proc. Natl. Acad. Sci. USA* **96**, 2907–2912 (1999).
44. Huberty, C.J. *Applied Discriminant Analysis*. (John Wiley and Sons, Inc., 1994).
45. Eisen, M.B., Spellman, P.T., Brown, P.O. & Botstein, D. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* **95**, 14863–14868 (1998).

REFERENCES

Aaltonen, L. A., Peltomaki, P., Leach, F. S., Sistonen, P., Pylkkanen, L., Mecklin, J. P., Jarvinen, H., Powell, S. M., Jen, J. and Hamilton, S. R. (1993). Clues to the pathogenesis of familial colorectal cancer, *Science,* **260,** 812-6.

Acton, D., Domen, J., Jacobs, H., Vlaar, M., Korsmeyer, S. and Berns, A. (1992). Collaboration of pim-1 and bcl-2 in lymphomagenesis, *Curr Top Microbiol Immunol,* **182,** 293-8.

Adler, V., Pincus, M. R., Posner, S., Upadhyaya, P., El-Bayoumy, K. and Ronai, Z. (1996). Effects of chemopreventive selenium compounds on Jun N-kinase activities, *Carcinogenesis,* **17,** 1849-54.

Al-Taie, O. H., Uceyler, N., Eubner, U., Jakob, F., Mork, H., Scheurlen, M., Brigelius-Flohe, R., Schottker, K., Abel, J., Thalheimer, A., Katzenberger, T., Illert, B., Melcher, R. and Kohrle, J. (2004). Expression profiling and genetic alterations of the selenoproteins GI-GPx and SePP in colorectal carcinogenesis, *Nutr Cancer,* **48,** 6-14.

Alaejos, M. S., Diaz Romero, F. J. and Diaz Romero, C. (2000). Selenium and cancer: some nutritional aspects, *Nutrition,* **16,** 376-83.

Albagli, O., Lantoine, D., Quief, S., Quignon, F., Englert, C., Kerckaert, J. P., Montarras, D., Pinset, C. and Lindon, C. (1999). Overexpressed BCL6 (LAZ3) oncoprotein triggers apoptosis, delays S phase progression and associates with replication foci, *Oncogene,* **18,** 5063-75.

Albinger-Hegyi, A., Hochreutener, B., Abdou, M. T., Hegyi, I., Dours-Zimmermann, M. T., Kurrer, M. O., Heitz, P. U. and Zimmermann, D. R. (2002). High frequency of t(14;18)-translocation breakpoints outside of major breakpoint and minor cluster regions in follicular lymphomas: improved polymerase chain reaction protocols for their detection, *Am J Pathol,* **160,** 823-32.

Alexander, F. E., Lawrence, D. J., Freeland, J., Krajewski, A. S., Angus, B., Taylor, G. M. and Jarrett, R. F. (2003). An epidemiologic study of index and family infectious mononucleosis and adult Hodgkin's disease (HD): evidence for a specific association with EBV+ve HD in young adults, *Int J Cancer,* **107,** 298-302.

Alizadeh, A. A., Eisen, M. B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., Boldrick, J. C., Sabet, H., Tran, T., Yu, X., Powell, J. I., Yang, L., Marti, G. E.,

Moore, T., Hudson, J., Jr., Lu, L., Lewis, D. B., Tibshirani, R., Sherlock, G., Chan, W. C., Greiner, T. C., Weisenburger, D. D., Armitage, J. O., Warnke, R., Levy, R., Wilson, W., Grever, M. R., Byrd, J. C., Botstein, D., Brown, P. O. and Staudt, L. M. (2000). Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling, *Nature,* **403,** 503-11.

Altekruse, S. F., Henley, S. J. and Thun, M. J. (1999). Deaths from hematopoietic and other cancers in relation to permanent hair dye use in a large prospective study (United States), *Cancer Causes Control,* **10,** 617-25.

Ames, B. N. and Gold, L. S. (1991). Endogenous mutagens and the causes of aging and cancer, *Mutat Res,* **250,** 3-16.

Amin, H. M., McDonnell, T. J., Ma, Y., Lin, Q., Fujio, Y., Kunisada, K., Leventaki, V., Das, P., Rassidakis, G. Z., Cutler, C., Medeiros, L. J. and Lai, R. (2004). Selective inhibition of STAT3 induces apoptosis and G(1) cell cycle arrest in ALK-positive anaplastic large cell lymphoma, *Oncogene,* **23,** 5426-34.

Anderson, J. R., Armitage, J. O. and Weisenburger, D. D. (1998). Epidemiology of the non-Hodgkin's lymphomas: distributions of the major subtypes differ by geographic locations. Non-Hodgkin's Lymphoma Classification Project, *Ann Oncol,* **9,** 717-20.

Anestal, K. and Arner, E. S. (2003). Rapid induction of cell death by selenium-compromised thioredoxin reductase 1 but not by the fully active enzyme containing selenocysteine, *J Biol Chem,* **278,** 15966-72.

Angstwurm, M. W., Schopohl, J. and Gaertner, R. (2004). Selenium substitution has no direct effect on thyroid hormone metabolism in critically ill patients, *Eur J Endocrinol,* **151,** 47-54.

Arcamone, F., Cassinelli, G., Fantini, G., Grein, A., Orezzi, P., Pol, C. and Spalla, C. (1969). Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from S. peucetius var. caesius, *Biotechnol Bioeng,* **11,** 1101-10.

Arena, V., Martini, M., Luongo, M., Capelli, A. and Larocca, L. M. (2003). Mutations of the BIK gene in human peripheral B-cell lymphomas, *Genes Chromosomes Cancer,* **38,** 91-6.

Armitage, J. M., Kormos, R. L., Stuart, R. S., Fricker, F. J., Griffith, B. P., Nalesnik, M., Hardesty, R. L. and Dummer, J. S. (1991). Posttransplant lymphoproliferative disease in thoracic organ transplant patients: ten years of

cyclosporine-based immunosuppression, *J Heart Lung Transplant,* **10,** 877-86; discussion 886-7.

Armitage, J. O., Fyfe, M. A. and Lewis, J. (1984). Long-term remission durability and functional status of patients treated for diffuse histiocytic lymphoma with the CHOP regimen, *J Clin Oncol,* **2,** 898-902.

Armitage, J. O. and Weisenburger, D. D. (1998). New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project, *J Clin Oncol,* **16,** 2780-95.

Arthur, J. R. (2000). The glutathione peroxidases, *Cell Mol Life Sci,* **57,** 1825-35.

Artiga, M. J., Saez, A. I., Romero, C., Sanchez-Beato, M., Mateo, M. S., Navas, C., Mollejo, M. and Piris, M. A. (2002). A short mutational hot spot in the first intron of BCL-6 is associated with increased BCL-6 expression and with longer overall survival in large B-cell lymphomas, *Am J Pathol,* **160,** 1371-80.

Ashkenazi, A. and Dixit, V. M. (1998). Death receptors: signaling and modulation, *Science,* **281,** 1305-8.

Ashton, M. J., Cook, D. C., Fenton, G., Karlsson, J. A., Palfreyman, M. N., Raeburn, D., Ratcliffe, A. J., Souness, J. E., Thurairatnam, S. and Vicker, N. (1994). Selective type IV phosphodiesterase inhibitors as antiasthmatic agents. The syntheses and biological activities of 3-(cyclopentyloxy)-4-methoxybenzamides and analogues, *J Med Chem,* **37,** 1696-703.

Avanzini, P., Vinceti, M., Ilariucci, F., Masini, L., D'Inca, M. and Vivoli, G. (1995). Serum selenium concentrations in patients with newly diagnosed lymphoid malignancies, *Haematologica,* **80,** 505-11.

Averill, L. E., Stein, R. L. and Kammer, G. M. (1988). Control of human T-lymphocyte interleukin-2 production by a cAMP-dependent pathway, *Cell Immunol,* **115,** 88-99.

Baecklund, E., Ekbom, A., Sparen, P., Feltelius, N. and Klareskog, L. (1998). Disease activity and risk of lymphoma in patients with rheumatoid arthritis: nested case-control study, *Bmj,* **317,** 180-1.

Baecklund, E., Sundstrom, C., Ekbom, A., Catrina, A. I., Biberfeld, P., Feltelius, N. and Klareskog, L. (2003). Lymphoma subtypes in patients with rheumatoid arthritis: increased proportion of diffuse large B cell lymphoma, *Arthritis Rheum,* **48,** 1543-50.

Bai, M., Tsanou, E., Agnantis, N. J., Chaidos, A., Dimou, D., Skyrlas, A., Dimou, S., Vlychou, M., Galani, V. and Kanavaros, P. (2003). Expression of cyclin D3 and cyclin E and identification of distinct clusters of proliferation and apoptosis in diffuse large B-cell lymphomas, *Histol Histopathol,* **18,** 449-57.

Bair, E. and Tibshirani, R. (2004). Semi-supervised methods to predict patient survival from gene expression data, *PLoS Biol,* **2,** E108.

Baldwin, A. S. (2001). Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB, *J Clin Invest,* **107,** 241-6.

Baris, D. and Zahm, S. H. (2000). Epidemiology of lymphomas, *Curr Opin Oncol,* **12,** 383-94.

Barrans, S. L., Carter, I., Owen, R. G., Davies, F. E., Patmore, R. D., Haynes, A. P., Morgan, G. J. and Jack, A. S. (2002a). Germinal center phenotype and bcl-2 expression combined with the International Prognostic Index improves patient risk stratification in diffuse large B-cell lymphoma, *Blood,* **99,** 1136-43.

Barrans, S. L., O'Connor, S. J., Evans, P. A., Davies, F. E., Owen, R. G., Haynes, A. P., Morgan, G. J. and Jack, A. S. (2002b). Rearrangement of the BCL6 locus at 3q27 is an independent poor prognostic factor in nodal diffuse large B-cell lymphoma, *Br J Haematol,* **117,** 322-32.

Batra, N., Karanti, S., Rai, D. and Aguiar, R. C. T. (2007). PDE4B Activity Controls Glucorticoid and Rapamycin Effects in DLBCL., *ASH Annual Meeting Abstracts,* **110,** 3461-.

Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M. and Issa, J. P. (1998). Alterations in DNA methylation: a fundamental aspect of neoplasia, *Adv Cancer Res,* **72,** 141-96.

Bea, S., Zettl, A., Wright, G., Salaverria, I., Jehn, P., Moreno, V., Burek, C., Ott, G., Puig, X., Yang, L., Lopez-Guillermo, A., Chan, W. C., Greiner, T. C., Weisenburger, D. D., Armitage, J. O., Gascoyne, R. D., Connors, J. M., Grogan, T. M., Braziel, R., Fisher, R. I., Smeland, E. B., Kvaloy, S., Holte, H., Delabie, J., Simon, R., Powell, J., Wilson, W. H., Jaffe, E. S., Montserrat, E., Muller-Hermelink, H. K., Staudt, L. M., Campo, E. and Rosenwald, A. (2005). Diffuse large B-cell lymphoma subgroups have distinct genetic profiles that influence tumor biology and improve gene-expression-based survival prediction, *Blood,* **106,** 3183-90.

Beasley, M. B., Lantuejoul, S., Abbondanzo, S., Chu, W. S., Hasleton, P. S., Travis, W. D. and Brambilla, E. (2003). The P16/cyclin D1/Rb pathway in neuroendocrine tumors of the lung, *Hum Pathol,* **34,** 136-42.

Beauparlant, P., Papp, K. and Haraoui, B. (1999). The incidence of cancer associated with the treatment of rheumatoid arthritis, *Semin Arthritis Rheum,* **29,** 148-58.

Beavo, J. A. (1995). Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms, *Physiol Rev,* **75,** 725-48.

Beavo, J. A. and Brunton, L. L. (2002). Cyclic nucleotide research -- still expanding after half a century, *Nat Rev Mol Cell Biol,* **3,** 710-8.

Beckwith, M., Ruscetti, F. W., Sing, G. K., Urba, W. J. and Longo, D. L. (1995). Anti-IgM induces transforming growth factor-beta sensitivity in a human B-lymphoma cell line: inhibition of growth is associated with a downregulation of mutant p53, *Blood,* **85,** 2461-70.

Beguin, Y., Bours, V., Delbrouck, J. M., Robaye, G., Roelandts, I., Bury, J., Fillet, G. and Weber, G. (1989). Relationship of serum selenium levels to tumor activity in acute non-lymphocytic leukemia, *Carcinogenesis,* **10,** 2089-91.

Behrmann, I., Walczak, H. and Krammer, P. H. (1994). Structure of the human APO-1 gene, *Eur J Immunol,* **24,** 3057-62.

Beiderbeck, A. B., Holly, E. A., Sturkenboom, M. C., Coebergh, J. W., Stricker, B. H. and Leufkens, H. G. (2003a). No increased risk of non-Hodgkin's lymphoma with steroids, estrogens and psychotropics (Netherlands), *Cancer Causes Control,* **14,** 639-44.

Beiderbeck, A. B., Holly, E. A., Sturkenboom, M. C., Coebergh, J. W., Stricker, B. H. and Leufkens, H. G. (2003b). Prescription medications associated with a decreased risk of non-Hodgkin's lymphoma, *Am J Epidemiol,* **157,** 510-6.

Belhadj, K., Jais, J.-P., Feugier, P., Gisselbrecht, C., Recher, C., Casasnovas, O., Coiffier, B., Sebban, C., Delmer, A., Morschhauser, F., Canioni, D., Tilly, H. and Haioun, C. (2007). Rituximab Combined to ACVBP (R-ACVBP) Supported by Pegfilgrastim as a New Inductive Treatment Followed by High-Dose Consolidative Autotransplantation (HDC) for Poor Risk Diffuse Large B-Cell Lymphoma (DLBCL) in First-Line. Results of LNH 03 39B. A GELA Study., *ASH Annual Meeting Abstracts,* **110,** 3437-.

Besson, C., Goubar, A., Gabarre, J., Rozenbaum, W., Pialoux, G., Chatelet, F. P., Katlama, C., Charlotte, F., Dupont, B., Brousse, N., Huerre, M., Mikol, J., Camparo, P., Mokhtari, K., Tulliez, M., Salmon-Ceron, D., Boue, F., Costagliola, D. and Raphael, M. (2001). Changes in AIDS-related lymphoma since the era of highly active antiretroviral therapy, *Blood,* **98,** 2339-44.

Bird, A. P. (1986). CpG-rich islands and the function of DNA methylation, *Nature,* **321,** 209-13.

Bischoff, E. (2004). Potency, selectivity, and consequences of nonselectivity of PDE inhibition, *Int J Impot Res,* **16 Suppl 1,** S11-4.

Bjornstedt, M., Kumar, S. and Holmgren, A. (1992). Selenodiglutathione is a highly efficient oxidant of reduced thioredoxin and a substrate for mammalian thioredoxin reductase, *J Biol Chem,* **267,** 8030-4.

Bleys, J., Navas-Acien, A. and Guallar, E. (2008). Serum selenium levels and all-cause, cancer, and cardiovascular mortality among US adults, *Arch Intern Med,* **168,** 404-10.

Bloom, T. J. and Beavo, J. A. (1996). Identification and tissue-specific expression of PDE7 phosphodiesterase splice variants, *Proc Natl Acad Sci U S A,* **93,** 14188-92.

Blot, W. J., Li, J. Y., Taylor, P. R., Guo, W., Dawsey, S., Wang, G. Q., Yang, C. S., Zheng, S. F., Gail, M. and Li, G. Y. (1993). Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population, *J Natl Cancer Inst,* **85,** 1483-92.

Boles, J. O., Cisneros, R. J., Weir, M. S., Odom, J. D., Villafranca, J. E. and Dunlap, R. B. (1991). Purification and characterization of selenomethionyl thymidylate synthase from Escherichia coli: comparison with the wild-type enzyme, *Biochemistry,* **30,** 11073-80.

Boswell-Smith, V., Spina, D. and Page, C. P. (2006). Phosphodiesterase inhibitors, *Br J Pharmacol,* **147 Suppl 1,** S252-7.

Bouillet, P., Purton, J. F., Godfrey, D. I., Zhang, L. C., Coultas, L., Puthalakath, H., Pellegrini, M., Cory, S., Adams, J. M. and Strasser, A. (2002). BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes, *Nature,* **415,** 922-6.

Boultwood, J. (2001). Ataxia telangiectasia gene mutations in leukaemia and lymphoma, *J Clin Pathol,* **54,** 512-6.

Braithwaite, A. W., Royds, J. A. and Jackson, P. (2005). The p53 story: layers of complexity, *Carcinogenesis,* **26,** 1161-9.

Brat, D. J., Lillemoe, K. D., Yeo, C. J., Warfield, P. B. and Hruban, R. H. (1998). Progression of pancreatic intraductal neoplasias to infiltrating adenocarcinoma of the pancreas, *Am J Surg Pathol,* **22,** 163-9.

Brooks, J. D., Metter, E. J., Chan, D. W., Sokoll, L. J., Landis, P., Nelson, W. G., Muller, D., Andres, R. and Carter, H. B. (2001). Plasma selenium level before diagnosis and the risk of prostate cancer development, *J Urol,* **166,** 2034-8.

Buell, D. N. (1983). Potential hazards of selenium as a chemopreventive agent, *Semin Oncol,* **10,** 311-21.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W. and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage, *Science,* **282,** 1497-501.

Burke, K. E., Clive, J., Combs, G. F., Jr. and Nakamura, R. M. (2003). Effects of topical L-selenomethionine with topical and oral vitamin E on pigmentation and skin cancer induced by ultraviolet irradiation in Skh:2 hairless mice, *J Am Acad Dermatol,* **49,** 458-72.

Burke, K. E., Combs, G. F., Jr., Gross, E. G., Bhuyan, K. C. and Abu-Libdeh, H. (1992). The effects of topical and oral L-selenomethionine on pigmentation and skin cancer induced by ultraviolet irradiation, *Nutr Cancer,* **17,** 123-37.

Butler, M. P., Iida, S., Capello, D., Rossi, D., Rao, P. H., Nallasivam, P., Louie, D. C., Chaganti, S., Au, T., Gascoyne, R. D., Gaidano, G., Chaganti, R. S. and Dalla-Favera, R. (2002). Alternative translocation breakpoint cluster region 5' to BCL-6 in B-cell non-Hodgkin's lymphoma, *Cancer Res,* **62,** 4089-94.

Caffrey, P. B. and Frenkel, G. D. (2000). Selenium compounds prevent the induction of drug resistance by cisplatin in human ovarian tumor xenografts in vivo, *Cancer Chemother Pharmacol,* **46,** 74-8.

Caffrey, P. B., Zhu, M. and Frenkel, G. D. (1998). Prevention of the development of melphalan resistance *in vitro* by selenite, *Biol Trace Elem Res,* **65,** 187-95.

Cai, X.-J., Block, E., Uden, P. C., Zhang, X. and Quimby, B. D. (1995). Allium chemistry: identification of selenoaminoacids in ordinary and selenium-enriched

garlic, onion and broccoliusing gas chromatography with atomic emission detection., *J Agric Food Chem,* **43,** 1754-1757.

Cairns, P., Polascik, T. J., Eby, Y., Tokino, K., Califano, J., Merlo, A., Mao, L., Herath, J., Jenkins, R. and Westra, W. (1995). Frequency of homozygous deletion at p16/CDKN2 in primary human tumours, *Nat Genet,* **11,** 210-2.

Calvo, A., Xiao, N., Kang, J., Best, C. J., Leiva, I., Emmert-Buck, M. R., Jorcyk, C. and Green, J. E. (2002). Alterations in gene expression profiles during prostate cancer progression: functional correlations to tumorigenicity and down-regulation of selenoprotein-P in mouse and human tumors, *Cancer Res,* **62,** 5325-35.

Cameron, E. E., Bachman, K. E., Myohanen, S., Herman, J. G. and Baylin, S. B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer, *Nat Genet,* **21,** 103-7.

Camidge, D. R., Gail Eckhardt, S., Gore, L., O'Bryant, C. L., Leong, S., Basche, M., Holden, S. N., Musib, L., Baldwin, J., Darstein, C., Thornton, D., Finn, R. S. and Britten, C. D. (2008). A phase I safety, tolerability, and pharmacokinetic study of enzastaurin combined with capecitabine in patients with advanced solid tumors, *Anticancer Drugs,* **19,** 77-84.

Cantor, K. P., Strickland, P. T., Brock, J. W., Bush, D., Helzlsouer, K., Needham, L. L., Zahm, S. H., Comstock, G. W. and Rothman, N. (2003). Risk of non-Hodgkin's lymphoma and prediagnostic serum organochlorines: beta-hexachlorocyclohexane, chlordane/heptachlor-related compounds, dieldrin, and hexachlorobenzene, *Environ Health Perspect,* **111,** 179-83.

Cao, S., Durrani, F. A. and Rustum, Y. M. (2004). Selective modulation of the therapeutic efficacy of anticancer drugs by selenium containing compounds against human tumor xenografts, *Clin Cancer Res,* **10,** 2561-9.

Capello, D., Vitolo, U., Pasqualucci, L., Quattrone, S., Migliaretti, G., Fassone, L., Ariatti, C., Vivenza, D., Gloghini, A., Pastore, C., Lanza, C., Nomdedeu, J., Botto, B., Freilone, R., Buonaiuto, D., Zagonel, V., Gallo, E., Palestro, G., Saglio, G., Dalla-Favera, R., Carbone, A. and Gaidano, G. (2000). Distribution and pattern of BCL-6 mutations throughout the spectrum of B-cell neoplasia, *Blood,* **95,** 651-9.

Carducci, M. A., Musib, L., Kies, M. S., Pili, R., Truong, M., Brahmer, J. R., Cole, P., Sullivan, R., Riddle, J., Schmidt, J., Enas, N., Sinha, V., Thornton, D. E. and Herbst, R. S. (2006). Phase I dose escalation and pharmacokinetic study of enzastaurin, an oral protein kinase C beta inhibitor, in patients with advanced cancer, *J Clin Oncol,* **24,** 4092-9.

Carlotti, E., Wrench, D., Iqbal, S., O'Shea, D., Davies, A., Virappane, P., Hart, J., Lai, R., Gribben, J. G., Lister, A. T. and Fitzgibbon, J. (2007). Determining the Mechanism of Transformation of Follicular Lymphoma into Diffuse Large B Cell Lymphoma., *ASH Annual Meeting Abstracts,* **110,** 181-.

Cassidy, P. B., Edes, K., Nelson, C. C., Parsawar, K., Fitzpatrick, F. A. and Moos, P. J. (2006). Thioredoxin reductase is required for the inactivation of tumor suppressor p53 and for apoptosis induced by endogenous electrophiles, *Carcinogenesis*.

Cerroni, L., Zochling, N., Putz, B. and Kerl, H. (1997). Infection by Borrelia burgdorferi and cutaneous B-cell lymphoma, *J Cutan Pathol,* **24,** 457-61.

Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T. and Korsmeyer, S. J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis, *Mol Cell,* **8,** 705-11.

Cheng, Q., Lee, H. H., Li, Y., Parks, T. P. and Cheng, G. (2000). Upregulation of Bcl-x and Bfl-1 as a potential mechanism of chemoresistance, which can be overcome by NF-kappaB inhibition, *Oncogene,* **19,** 4936-40.

Cheng, W. H., Ho, Y. S., Ross, D. A., Valentine, B. A., Combs, G. F. and Lei, X. G. (1997). Cellular glutathione peroxidase knockout mice express normal levels of selenium-dependent plasma and phospholipid hydroperoxide glutathione peroxidases in various tissues, *J Nutr,* **127,** 1445-50.

Cheng, W. H., Ho, Y. S., Valentine, B. A., Ross, D. A., Combs, G. F., Jr. and Lei, X. G. (1998). Cellular glutathione peroxidase is the mediator of body selenium to protect against paraquat lethality in transgenic mice, *J Nutr,* **128,** 1070-6.

Cherukuri, D. P., Goulet, A. C., Inoue, H. and Nelson, M. A. (2005). Selenomethionine Regulates Cyclooxygenase-2 (COX-2) Expression through Nuclear Factor-Kappa B (NF-kappaB) in Colon Cancer Cells, *Cancer Biol Ther,* **4,** 175-80.

Cheson, B. D., Horning, S. J., Coiffier, B., Shipp, M. A., Fisher, R. I., Connors, J. M., Lister, T. A., Vose, J., Grillo-Lopez, A., Hagenbeek, A., Cabanillas, F., Klippensten, D., Hiddemann, W., Castellino, R., Harris, N. L., Armitage, J. O., Carter, W., Hoppe, R. and Canellos, G. P. (1999). Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group, *J Clin Oncol,* **17,** 1244.

Chim, C. S., Liang, R. and Kwong, Y. L. (2002). Hypermethylation of gene promoters in hematological neoplasia, *Hematol Oncol,* **20,** 167-76.

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M. and Green, D. R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis, *Science,* **303,** 1010-4.

Chu, F. F., Esworthy, R. S., Chu, P. G., Longmate, J. A., Huycke, M. M., Wilczynski, S. and Doroshow, J. H. (2004). Bacteria-induced intestinal cancer in mice with disrupted Gpx1 and Gpx2 genes, *Cancer Res,* **64,** 962-8.

Chung, H. J., Yoon, S. I., Shin, S. H., Koh, Y. A., Lee, S. J., Lee, Y. S. and Bae, S. (2006). p53-Mediated enhancement of radiosensitivity by selenophosphate synthetase 1 overexpression, *J Cell Physiol,* **209,** 131-41.

Cianchi, F., Cortesini, C., Bechi, P., Fantappie, O., Messerini, L., Vannacci, A., Sardi, I., Baroni, G., Boddi, V., Mazzanti, R. and Masini, E. (2001). Up-regulation of cyclooxygenase 2 gene expression correlates with tumor angiogenesis in human colorectal cancer, *Gastroenterology,* **121,** 1339-47.

Cigudosa, J. C., Parsa, N. Z., Louie, D. C., Filippa, D. A., Jhanwar, S. C., Johansson, B., Mitelman, F. and Chaganti, R. S. (1999). Cytogenetic analysis of 363 consecutively ascertained diffuse large B-cell lymphomas, *Genes Chromosomes Cancer,* **25,** 123-33.

Clark, L. C., Cantor, K. P. and Allaway, W. H. (1991). Selenium in forage crops and cancer mortality in U.S. counties, *Arch Environ Health,* **46,** 37-42.

Clark, L. C., Combs, G. F., Jr., Turnbull, B. W., Slate, E. H., Chalker, D. K., Chow, J., Davis, L. S., Glover, R. A., Graham, G. F., Gross, E. G., Krongrad, A., Lesher, J. L., Jr., Park, H. K., Sanders, B. B., Jr., Smith, C. L. and Taylor, J. R. (1996). Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group, *Jama,* **276,** 1957-63.

Clark, L. C., Graham, G. F., Crounse, R. G., Grimson, R., Hulka, B. and Shy, C. M. (1984). Plasma selenium and skin neoplasms: a case-control study, *Nutr Cancer,* **6,** 13-21.

Clark, L. C., Hixson, L. J., Combs, G. F., Jr., Reid, M. E., Turnbull, B. W. and Sampliner, R. E. (1993). Plasma selenium concentration predicts the prevalence of colorectal adenomatous polyps, *Cancer Epidemiol Biomarkers Prev,* **2,** 41-6.

Clark, L. C. and Jacobs, E. T. (1998). Environmental selenium and cancer: risk or protection?, *Cancer Epidemiol Biomarkers Prev,* **7,** 847-8; discussion 851-2.

Claus, R. and Lubbert, M. (2003). Epigenetic targets in hematopoietic malignancies, *Oncogene,* **22,** 6489-96.

Claveau, D., Chen, S. L., O'Keefe, S., Zaller, D. M., Styhler, A., Liu, S., Huang, Z., Nicholson, D. W. and Mancini, J. A. (2004). Preferential inhibition of T helper 1, but not T helper 2, cytokines *in vitro* by L-826,141 [4-[2-(3,4-Bisdifluromethoxyphenyl)-2-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxy propan-2-yl)-phenyl]-ethyl]3-methylpyridine-1-oxide], a potent and selective phosphodiesterase 4 inhibitor, *J Pharmacol Exp Ther,* **310,** 752-60.

Cleary, M. L., Galili, N. and Sklar, J. (1986). Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas, *J Exp Med,* **164,** 315-20.

Cleaver, J. E. (1968). Defective repair replication of DNA in xeroderma pigmentosum, *Nature,* **218,** 652-6.

Clodi, K., Snell, V., Zhao, S., Cabanillas, F., Andreeff, M. and Younes, A. (1998). Unbalanced expression of Fas and CD40 in mantle cell lymphoma, *Br J Haematol,* **103,** 217-9.

Coates, R. J., Weiss, N. S., Daling, J. R., Morris, J. S. and Labbe, R. F. (1988). Serum levels of selenium and retinol and the subsequent risk of cancer, *Am J Epidemiol,* **128,** 515-23.

Coiffier, B., Gisselbrecht, C., Vose, J. M., Tilly, H., Herbrecht, R., Bosly, A. and Armitage, J. O. (1991). Prognostic factors in aggressive malignant lymphomas: description and validation of a prognostic index that could identify patients requiring a more intensive therapy. The Groupe d'Etudes des Lymphomes Agressifs, *J Clin Oncol,* **9,** 211-9.

Coiffier, B., Haioun, C., Ketterer, N., Engert, A., Tilly, H., Ma, D., Johnson, P., Lister, A., Feuring-Buske, M., Radford, J. A., Capdeville, R., Diehl, V. and Reyes, F.

(1998). Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: a multicenter phase II study, *Blood,* **92,** 1927-32.

Coiffier, B. and Lepage, E. (1989). Prognosis of aggressive lymphomas: a study of five prognostic models with patients included in the LNH-84 regimen, *Blood,* **74,** 558-64.

Coiffier, B., Lepage, E., Briere, J., Herbrecht, R., Tilly, H., Bouabdallah, R., Morel, P., Van Den Neste, E., Salles, G., Gaulard, P., Reyes, F., Lederlin, P. and Gisselbrecht, C. (2002). CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma, *N Engl J Med,* **346,** 235-42.

Comstock, G. W., Bush, T. L. and Helzlsouer, K. (1992). Serum retinol, beta-carotene, vitamin E, and selenium as related to subsequent cancer of specific sites, *Am J Epidemiol,* **135,** 115-21.

Conaway, C. C., Upadhyaya, P., Meschter, C. L., Kurtzke, C., Marcus, L. A. and el-Bayoumy, K. (1992). Subchronic toxicity of benzyl selenocyanate and 1,4-phenylenebis(methylene)selenocyanate in F344 rats, *Fundam Appl Toxicol,* **19,** 563-74.

Conlan, M. G., Armitage, J. O., Bast, M. and Weisenburger, D. D. (1991). Clinical significance of hematologic parameters in non-Hodgkin's lymphoma at diagnosis, *Cancer,* **67,** 1389-95.

Conti, M., Richter, W., Mehats, C., Livera, G., Park, J. Y. and Jin, C. (2003). Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling, *J Biol Chem,* **278,** 5493-6.

Cooper, G. M. (2002) *The Cell: A Molecular Approach,* Sinauer Associates, Sunderland.

Coultas, L. and Strasser, A. (2003). The role of the Bcl-2 protein family in cancer, *Semin Cancer Biol,* **13,** 115-23.

Cox, D. R. (1972). Regression models and life tables., *J R Stat Soc [B],* **34,** 187-220.

Cox, R. (1985). Selenite: a good inhibitor of rat-liver DNA methylase, *Biochem Int,* **10,** 63-9.

CR-UK (2003). In *Cancer Research UK Scientific Yearbook 2002-2003*.

Cross, S. H. and Bird, A. P. (1995). CpG islands and genes, *Curr Opin Genet Dev,* **5,** 309-14.

Curtis, R. E., Travis, L. B., Rowlings, P. A., Socie, G., Kingma, D. W., Banks, P. M., Jaffe, E. S., Sale, G. E., Horowitz, M. M., Witherspoon, R. P., Shriner, D. A., Weisdorf, D. J., Kolb, H. J., Sullivan, K. M., Sobocinski, K. A., Gale, R. P., Hoover, R. N., Fraumeni, J. F., Jr. and Deeg, H. J. (1999). Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study, *Blood,* **94,** 2208-16.

Dal Maso, L. and Franceschi, S. (2003). Epidemiology of non-Hodgkin lymphomas and other haemolymphopoietic neoplasms in people with AIDS, *Lancet Oncol,* **4,** 110-9.

Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R. C. and Croce, C. M. (1982). Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells, *Proc Natl Acad Sci U S A,* **79,** 7824-7.

Dalla-Favera, R., Ye, B. H., Lo Coco, F., Gaidano, G., Lista, F., Knowles, D. M., Louie, D. C., Offit, K. and Chaganti, R. S. (1994). Identification of genetic lesions associated with diffuse large-cell lymphoma, *Ann Oncol,* **5 Suppl 1,** 55-60.

Daniels, L. A. (1996). Selenium metabolism and bioavailability, *Biol Trace Elem Res,* **54,** 185-99.

Das, K. C. and White, C. W. (1997). Activation of NF-kappaB by antineoplastic agents. Role of protein kinase C, *J Biol Chem,* **272,** 14914-20.

Davis, C. D., Zeng, H. and Finley, J. W. (2002). Selenium-enriched broccoli decreases intestinal tumorigenesis in multiple intestinal neoplasia mice, *J Nutr,* **132,** 307-9.

Dawson, T. M., Starkebaum, G., Wood, B. L., Willkens, R. F. and Gown, A. M. (2001). Epstein-Barr virus, methotrexate, and lymphoma in patients with rheumatoid arthritis and primary Sjogren's syndrome: case series, *J Rheumatol,* **28,** 47-53.

De Roos, A. J., Zahm, S. H., Cantor, K. P., Weisenburger, D. D., Holmes, F. F., Burmeister, L. F. and Blair, A. (2003). Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men, *Occup Environ Med,* **60,** E11.

Deffuant, C., Celerier, P., Boiteau, H. L., Litoux, P. and Dreno, B. (1994). Serum selenium in melanoma and epidermotropic cutaneous T-cell lymphoma, *Acta Derm Venereol,* **74,** 90-2.

Degerman, E., Belfrage, P. and Manganiello, V. C. (1997). Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3), *J Biol Chem,* **272,** 6823-6.

DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., Chen, Y., Su, Y. A. and Trent, J. M. (1996). Use of a cDNA microarray to analyse gene expression patterns in human cancer, *Nat Genet,* **14,** 457-60.

Dhaliwal, H. S., Rohatiner, A. Z., Gregory, W., Richards, M. A., Johnson, P. W., Whelan, J. S., Gallagher, C. J., Matthews, J., Ganesan, T. S. and Barnett, M. J. (1993). Combination chemotherapy for intermediate and high grade non-Hodgkin's lymphoma, *Br J Cancer,* **68,** 767-74.

Dhein, J., Behrmann, I., Daniel, P. T., Debatin, K. M., Klas, C., Moller, P., Oehm, A., Trauth, B. C., Walczak, H. and Krammer, P. H. (1994). APO-I-mediated apoptosis in normal and malignant lymphocytes, *Biochem Soc Trans,* **22,** 598-600.

Diamond, I., Wrubel, B., Estrin, W. and Gordon, A. (1987). Basal and adenosine receptor-stimulated levels of cAMP are reduced in lymphocytes from alcoholic patients, *Proc Natl Acad Sci U S A,* **84,** 1413-6.

Dick, F., VanLier, S., Banks, P., Frizzera, G., Witrak, G., Gibson, R., Everett, G., Schuman, L., Isacson, P. and O'Conor, G. (1987). Use of the working formulation for non-Hodgkin's lymphoma in epidemiologic studies: agreement between reported diagnoses and a panel of experienced pathologists, *J Natl Cancer Inst,* **78,** 1137-44.

Diwadkar-Navsariwala, V., Prins, G. S., Swanson, S. M., Birch, L. A., Ray, V. H., Hedayat, S., Lantvit, D. L. and Diamond, A. M. (2006). Selenoprotein deficiency accelerates prostate carcinogenesis in a transgenic model, *Proc Natl Acad Sci U S A,* **103,** 8179-84.

Dong, Y., Ganther, H. E., Stewart, C. and Ip, C. (2002). Identification of molecular targets associated with selenium-induced growth inhibition in human breast cells using cDNA microarrays, *Cancer Res,* **62,** 708-14.

Dong, Y., Zhang, H., Hawthorn, L., Ganther, H. E. and Ip, C. (2003). Delineation of the molecular basis for selenium-induced growth arrest in human prostate cancer cells by oligonucleotide array, *Cancer Res,* **63,** 52-9.

Drake, J. W., Charlesworth, B., Charlesworth, D. and Crow, J. F. (1998). Rates of spontaneous mutation, *Genetics,* **148,** 1667-86.

Drexler, H. G. (1998). Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells, *Leukemia,* **12,** 845-59.

Drexler, H. G., Gignac, S. M., von Wasielewski, R., Werner, M. and Dirks, W. G. (2000). Pathobiology of NPM-ALK and variant fusion genes in anaplastic large cell lymphoma and other lymphomas, *Leukemia,* **14,** 1533-59.

Druker, B. J. (2004). Imatinib as a paradigm of targeted therapies, *Adv Cancer Res,* **91,** 1-30.

Duffield-Lillico, A. J., Reid, M. E., Turnbull, B. W., Combs, G. F., Jr., Slate, E. H., Fischbach, L. A., Marshall, J. R. and Clark, L. C. (2002). Baseline characteristics and the effect of selenium supplementation on cancer incidence in a randomized clinical trial: a summary report of the Nutritional Prevention of Cancer Trial, *Cancer Epidemiol Biomarkers Prev,* **11,** 630-9.

Duffield-Lillico, A. J., Slate, E. H., Reid, M. E., Turnbull, B. W., Wilkins, P. A., Combs, G. F., Jr., Park, H. K., Gross, E. G., Graham, G. F., Stratton, M. S., Marshall, J. R. and Clark, L. C. (2003). Selenium supplementation and secondary prevention of nonmelanoma skin cancer in a randomized trial, *J Natl Cancer Inst,* **95,** 1477-81.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins, *Genes Dev,* **12,** 2245-62.

Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S. and DuBois, R. N. (1994). Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas, *Gastroenterology,* **107,** 1183-8.

Eisen, M. B., Spellman, P. T., Brown, P. O. and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns, *Proc Natl Acad Sci U S A,* **95,** 14863-8.

Ejadi, S., Bhattacharya, I. D., Voss, K., Singletary, K. and Milner, J. A. (1989). *In vitro* and in vivo effects of sodium selenite on 7,12-dimethylbenz[a]anthracene--DNA adduct formation in isolated rat mammary epithelial cells, *Carcinogenesis,* **10,** 823-6.

el-Bayoumy, K. (1985). Effects of organoselenium compounds on induction of mouse forestomach tumors by benzo(a)pyrene, *Cancer Res,* **45,** 3631-5.

el-Bayoumy, K. (1994). Evaluation of chemopreventive agents against breast cancer and proposed strategies for future clinical intervention trials, *Carcinogenesis,* **15,** 2395-420.

el-Bayoumy, K., Chae, Y. H., Upadhyaya, P., Meschter, C., Cohen, L. A. and Reddy, B. S. (1992). Inhibition of 7,12-dimethylbenz(a)anthracene-induced tumors and DNA adduct formation in the mammary glands of female Sprague-Dawley rats by the synthetic organoselenium compound, 1,4-phenylenebis(methylene)selenocyanate, *Cancer Res,* **52,** 2402-7.

El-Bayoumy, K., Narayanan, B. A., Desai, D. H., Narayanan, N. K., Pittman, B., Amin, S. G., Schwartz, J. and Nixon, D. W. (2003). Elucidation of molecular targets of mammary cancer chemoprevention in the rat by organoselenium compounds using cDNA microarray, *Carcinogenesis,* **24,** 1505-14.

el-Bayoumy, K., Upadhyaya, P., Desai, D. H., Amin, S. and Hecht, S. S. (1993). Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone tumorigenicity in mouse lung by the synthetic organoselenium compound, 1,4-phenylenebis(methylene)selenocyanate, *Carcinogenesis,* **14,** 1111-3.

Elenitoba-Johnson, K. S., Gascoyne, R. D., Lim, M. S., Chhanabai, M., Jaffe, E. S. and Raffeld, M. (1998). Homozygous deletions at chromosome 9p21 involving p16 and p15 are associated with histologic progression in follicle center lymphoma, *Blood,* **91,** 4677-85.

Engels, E. A., Katki, H. A., Nielsen, N. M., Winther, J. F., Hjalgrim, H., Gjerris, F., Rosenberg, P. S. and Frisch, M. (2003). Cancer incidence in Denmark following exposure to poliovirus vaccine contaminated with simian virus 40, *J Natl Cancer Inst,* **95,** 532-9.

Engman, L., McNaughton, M., Gajewska, M., Kumar, S., Birmingham, A. and Powis, G. (2006). Thioredoxin reductase and cancer cell growth inhibition by organogold(III) compounds, *Anticancer Drugs,* **17,** 539-44.

Epstein, A. L., Levy, R., Kim, H., Henle, W., Henle, G. and Kaplan, H. S. (1978). Biology of the human malignant lymphomas. IV. Functional characterization of ten diffuse histiocytic lymphoma cell lines, *Cancer,* **42,** 2379-91.

Evan, G. I. and Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer, *Nature,* **411,** 342-8.

Evans, S. C. and Lozano, G. (1997). The Li-Fraumeni syndrome: an inherited susceptibility to cancer, *Mol Med Today,* **3,** 390-5.

Fakih, M. G., Pendyala, L., Brady, W., Smith, P. F., Ross, M. E., Creaven, P. J., Badmaev, V., Prey, J. D. and Rustum, Y. M. (2008). A Phase I and pharmacokinetic study of selenomethionine in combination with a fixed dose of irinotecan in solid tumors, *Cancer Chemother Pharmacol,* **62,** 499-508.

Fakih, M. G., Pendyala, L., Smith, P. F., Creaven, P. J., Reid, M. E., Badmaev, V., Azrak, R. G., Prey, J. D., Lawrence, D. and Rustum, Y. M. (2006). A phase I and pharmacokinetic study of fixed-dose selenomethionine and irinotecan in solid tumors, *Clin Cancer Res,* **12,** 1237-44.

Fan, A. M. and Kizer, K. W. (1990). Selenium. Nutritional, toxicologic, and clinical aspects, *West J Med,* **153,** 160-7.

Fang, N. Y., Greiner, T. C., Weisenburger, D. D., Chan, W. C., Vose, J. M., Smith, L. M., Armitage, J. O., Mayer, R. A., Pike, B. L., Collins, F. S. and Hacia, J. G. (2003). Oligonucleotide microarrays demonstrate the highest frequency of ATM mutations in the mantle cell subtype of lymphoma, *Proc Natl Acad Sci U S A,* **100,** 5372-7.

Fawcett, L., Baxendale, R., Stacey, P., McGrouther, C., Harrow, I., Soderling, S., Hetman, J., Beavo, J. A. and Phillips, S. C. (2000). Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A, *Proc Natl Acad Sci U S A,* **97,** 3702-7.

Feil, R., Lohmann, S. M., de Jonge, H., Walter, U. and Hofmann, F. (2003). Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice, *Circ Res,* **93,** 907-16.

Fenske, T. S., Hari, P., Carreras, J., Zhang, M.-J., Kamble, R., Rizzo, J. D., van Besien, K., Lazarus, H. M. and Vose, J. M. (2007). Pre-Transplant Rituximab Therapy Is Associated with Improved Progression-Free and Overall Survival in Patients Undergoing Autologous Hematopoietic Stem Cell Transplantation for Diffuse Large B-Cell Lymphoma (DLBCL). *ASH Annual Meeting Abstracts,* **110,** 19-.

Ferlay, J., Bray, F., Pisani, P. and Parkin, D. M. (2001). IARCPress, Lyon.

Ferlay, J., Bray, F., Sankila, R. and Parkin, D. M. (1998). IARCPress, Lyon.

Ferreri, A. J., Guidoboni, M., Ponzoni, M., De Conciliis, C., Dell'Oro, S., Fleischhauer, K., Caggiari, L., Lettini, A. A., Dal Cin, E., Ieri, R., Freschi, M., Villa, E., Boiocchi, M. and Dolcetti, R. (2004). Evidence for an association between Chlamydia psittaci and ocular adnexal lymphomas, *J Natl Cancer Inst,* **96,** 586-94.

Feuerhake, F., Kutok, J. L., Monti, S., Chen, W., LaCasce, A. S., Cattoretti, G., Kurtin, P., Pinkus, G. S., de Leval, L., Harris, N. L., Savage, K. J., Neuberg, D., Habermann, T. M., Dalla-Favera, R., Golub, T. R., Aster, J. C. and Shipp, M. A. (2005). NFkappaB activity, function, and target-gene signatures in primary mediastinal large B-cell lymphoma and diffuse large B-cell lymphoma subtypes, *Blood,* **106,** 1392-9.

Fiala, E. S., Joseph, C., Sohn, O. S., el-Bayoumy, K. and Reddy, B. S. (1991). Mechanism of benzylselenocyanate inhibition of azoxymethane-induced colon carcinogenesis in F344 rats, *Cancer Res,* **51,** 2826-30.

Fico, M. E., Poirier, K. A., Watrach, A. M., Watrach, M. A. and Milner, J. A. (1986). Differential effects of selenium on normal and neoplastic canine mammary cells, *Cancer Res,* **46,** 3384-8.

Filipits, M., Jaeger, U., Pohl, G., Stranzl, T., Simonitsch, I., Kaider, A., Skrabs, C. and Pirker, R. (2002). Cyclin D3 is a predictive and prognostic factor in diffuse large B-cell lymphoma, *Clin Cancer Res,* **8,** 729-33.

Fischer, J. L., Mihelc, E. M., Pollok, K. E. and Smith, M. L. (2007). Chemotherapeutic selectivity conferred by selenium: a role for p53-dependent DNA repair, *Mol Cancer Ther,* **6,** 355-61.

Fisher, R. I., Gaynor, E. R., Dahlberg, S., Oken, M. M., Grogan, T. M., Mize, E. M., Glick, J. H., Coltman, C. A., Jr. and Miller, T. P. (1994). A phase III comparison of CHOP vs. m-BACOD vs. ProMACE-CytaBOM vs. MACOP-B in patients with intermediate- or high-grade non-Hodgkin's lymphoma: results of SWOG-8516 (Intergroup 0067), the National High-Priority Lymphoma Study, *Ann Oncol,* **5 Suppl 2,** 91-5.

Fleming, J., Ghose, A. and Harrison, P. R. (2001). Molecular mechanisms of cancer prevention by selenium compounds, *Nutr Cancer,* **40,** 42-9.

Flossmann, E. and Rothwell, P. M. (2007). Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies, *Lancet,* **369,** 1603-13.

Fortuny, J., de Sanjose, S., Becker, N., Maynadie, M., Cocco, P. L., Staines, A., Foretova, L., Vornanen, M., Brennan, P., Nieters, A., Alvaro, T. and Boffetta, P. (2006). Statin use and risk of lymphoid neoplasms: results from the European Case-Control Study EPILYMPH, *Cancer Epidemiol Biomarkers Prev,* **15,** 921-5.

Foster, S. J., Kraus, R. J. and Ganther, H. E. (1986). The metabolism of selenomethionine, Se-methylselenocysteine, their selenonium derivatives, and trimethylselenonium in the rat, *Arch Biochem Biophys,* **251,** 77-86.

Frank, S. A. (2003). Somatic mutation: early cancer steps depend on tissue architecture, *Curr Biol,* **13,** R261-3.

Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S. and Vande Woude, G. F. (1996). Abnormal centrosome amplification in the absence of p53, *Science,* **271,** 1744-7.

Ganjoo, K. N., Moore, A. M., Orazi, A., Sen, J. A., Johnson, C. S. and An, C. S. (2008). The importance of angiogenesis markers in the outcome of patients with diffuse large B cell lymphoma: a retrospective study of 97 patients, *J Cancer Res Clin Oncol,* **134,** 381-7.

Ganther, H. and Lawrence, J. R. (1997). Chemical transformations of selenium in living organisms. Improved forms of selenium for cancer prevention., *Tetrahedron,* **53,** 1299-12310.

Ganther, H. E. (1968). Selenotrisulfides. Formation by the reaction of thiols with selenious acid, *Biochemistry,* **7,** 2898-905.

Ganther, H. E. (1999). Selenium metabolism, selenoproteins and mechanisms of cancer prevention: complexities with thioredoxin reductase, *Carcinogenesis,* **20,** 1657-66.

Gantner, F., Gotz, C., Gekeler, V., Schudt, C., Wendel, A. and Hatzelmann, A. (1998). Phosphodiesterase profile of human B lymphocytes from normal and atopic donors and the effects of PDE inhibition on B cell proliferation, *Br J Pharmacol,* **123,** 1031-8.

Gascoyne, R. D., Adomat, S. A., Krajewski, S., Krajewska, M., Horsman, D. E., Tolcher, A. W., O'Reilly, S. E., Hoskins, P., Coldman, A. J., Reed, J. C. and

Connors, J. M. (1997). Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma, *Blood,* **90,** 244-51.

Gebel, S. and Muller, T. (2001). The activity of NF-kappaB in Swiss 3T3 cells exposed to aqueous extracts of cigarette smoke is dependent on thioredoxin, *Toxicol Sci,* **59,** 75-81.

Ghadirian, P., Maisonneuve, P., Perret, C., Kennedy, G., Boyle, P., Krewski, D. and Lacroix, A. (2000). A case-control study of toenail selenium and cancer of the breast, colon, and prostate, *Cancer Detect Prev,* **24,** 305-13.

Ghose, A., Fleming, J., El-Bayoumy, K. and Harrison, P. R. (2001). Enhanced sensitivity of human oral carcinomas to induction of apoptosis by selenium compounds: involvement of mitogen-activated protein kinase and Fas pathways, *Cancer Res,* **61,** 7479-87.

Giaccia, A. J. and Kastan, M. B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals, *Genes Dev,* **12,** 2973-83.

Gidding, C. E., Kellie, S. J., Kamps, W. A. and de Graaf, S. S. (1999). Vincristine revisited, *Crit Rev Oncol Hematol,* **29,** 267-87.

Gillespie, P. G. and Beavo, J. A. (1989). Inhibition and stimulation of photoreceptor phosphodiesterases by dipyridamole and M&B 22,948, *Mol Pharmacol,* **36,** 773-81.

Gisbert, J. P., Garcia-Buey, L., Pajares, J. M. and Moreno-Otero, R. (2003). Prevalence of hepatitis C virus infection in B-cell non-Hodgkin's lymphoma: systematic review and meta-analysis, *Gastroenterology,* **125,** 1723-32.

Gladyshev, V. N. and Hatfield, D. L. (1999). Selenocysteine-containing proteins in mammals, *J Biomed Sci,* **6,** 151-60.

Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., Bloomfield, C. D. and Lander, E. S. (1999). Molecular classification of cancer: class discovery and class prediction by gene expression monitoring, *Science,* **286,** 531-7.

Gottlieb, A. J., Anderson, J. R., Ginsberg, S. J., Bloomfield, C. D., Norton, L., Barcos, M., Peterson, B. A., Nissen, N., Henderson, E. S. and Holland, J. F. (1990). A randomized comparison of methotrexate dose and the addition of bleomycin to

CHOP therapy for diffuse large cell lymphoma and other non-Hodgkin's lymphomas. Cancer and Leukemia Group B study 7851, *Cancer,* **66,** 1888-96.

Gouaze, V., Andrieu-Abadie, N., Cuvillier, O., Malagarie-Cazenave, S., Frisach, M. F., Mirault, M. E. and Levade, T. (2002). Glutathione peroxidase-1 protects from CD95-induced apoptosis, *J Biol Chem,* **277,** 42867-74.

Gouaze, V., Mirault, M. E., Carpentier, S., Salvayre, R., Levade, T. and Andrieu-Abadie, N. (2001). Glutathione peroxidase-1 overexpression prevents ceramide production and partially inhibits apoptosis in doxorubicin-treated human breast carcinoma cells, *Mol Pharmacol,* **60,** 488-96.

Goulet, A. C., Chigbrow, M., Frisk, P. and Nelson, M. A. (2005). Selenomethionine induces sustained ERK phosphorylation leading to cell-cycle arrest in human colon cancer cells, *Carcinogenesis,* **26,** 109-17.

Graff, J. R., Herman, J. G., Myohanen, S., Baylin, S. B. and Vertino, P. M. (1997). Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation, *J Biol Chem,* **272,** 22322-9.

Gratzinger, D., Zhao, S., Tibshirani, R. J., Hsi, E. D., Hans, C. P., Pohlman, B., Bast, M., Avigdor, A., Schiby, G., Nagler, A., Byrne, G. E., Jr., Lossos, I. S. and Natkunam, Y. (2008). Prognostic significance of VEGF, VEGF receptors, and microvessel density in diffuse large B cell lymphoma treated with anthracycline-based chemotherapy, *Lab Invest,* **88,** 38-47.

Grillo-Lopez, A. J. (2003). Rituximab (Rituxan/MabThera): the first decade (1993-2003), *Expert Rev Anticancer Ther,* **3,** 767-79.

Grogan, T. M., Lippman, S. M., Spier, C. M., Slymen, D. J., Rybski, J. A., Rangel, C. S., Richter, L. C. and Miller, T. P. (1988). Independent prognostic significance of a nuclear proliferation antigen in diffuse large cell lymphomas as determined by the monoclonal antibody Ki-67, *Blood,* **71,** 1157-60.

Gronbaek, K., de Nully Brown, P., Moller, M. B., Nedergaard, T., Ralfkiaer, E., Moller, P., Zeuthen, J. and Guldberg, P. (2000). Concurrent disruption of p16INK4a and the ARF-p53 pathway predicts poor prognosis in aggressive non-Hodgkin's lymphoma, *Leukemia,* **14,** 1727-35.

Gronbaek, K., Straten, P. T., Ralfkiaer, E., Ahrenkiel, V., Andersen, M. K., Hansen, N. E., Zeuthen, J., Hou-Jensen, K. and Guldberg, P. (1998). Somatic Fas mutations

in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity, *Blood,* **92,** 3018-24.

Gronbaek, K., Worm, J., Ralfkiaer, E., Ahrenkiel, V., Hokland, P. and Guldberg, P. (2002). ATM mutations are associated with inactivation of the ARF-TP53 tumor suppressor pathway in diffuse large B-cell lymphoma, *Blood,* **100,** 1430-7.

Gross, A., McDonnell, J. M. and Korsmeyer, S. J. (1999). BCL-2 family members and the mitochondria in apoptosis, *Genes Dev,* **13,** 1899-911.

Group, J. F. S. a. S. (1997). Ministry of Agriculture, Fisheries and Food, London.

Group, J. F. S. a. S. (1999). Ministry of Agriculture, Fisheries and Food, London.

Gruenwedel, D. W. and Cruikshank, M. K. (1979). The influence of sodium selenite on the viability and intracellular synthetic activity (DNA, RNA, and protein synthesis) of HeLa S3 cells, *Toxicol Appl Pharmacol,* **50,** 1-7.

Gui, J. and Li, H. (2005). Penalized Cox regression analysis in the high-dimensional and low-sample size settings, with applications to microarray gene expression data, *Bioinformatics,* **21,** 3001-8.

Gumy-Pause, F., Wacker, P. and Sappino, A. P. (2004). ATM gene and lymphoid malignancies, *Leukemia,* **18,** 238-42.

Guo, S. X., Taki, T., Ohnishi, H., Piao, H. Y., Tabuchi, K., Bessho, F., Hanada, R., Yanagisawa, M. and Hayashi, Y. (2000). Hypermethylation of p16 and p15 genes and RB protein expression in acute leukemia, *Leuk Res,* **24,** 39-46.

Guo, X. and Wu, L. (1998). Distribution of free seleno-amino acids in plant tissue of Melilotus indica L. grown in selenium-laden soils, *Ecotoxicol Environ Saf,* **39,** 207-14.

Gupta, R. A. and DuBois, R. N. (1998). Aspirin, NSAIDS, and colon cancer prevention: mechanisms?, *Gastroenterology,* **114,** 1095-8.

Gutcher, I., Webb, P. R. and Anderson, N. G. (2003). The isoform-specific regulation of apoptosis by protein kinase C, *Cell Mol Life Sci,* **60,** 1061-70.

Hahn, W. C. and Weinberg, R. A. (2002a). Modelling the molecular circuitry of cancer, *Nat Rev Cancer,* **2,** 331-41.

Hahn, W. C. and Weinberg, R. A. (2002b). Rules for making human tumor cells, *N Engl J Med,* **347,** 1593-603.

Han, P., Werber, J., Surana, M., Fleischer, N. and Michaeli, T. (1999). The calcium/calmodulin-dependent phosphodiesterase PDE1C down-regulates glucose-induced insulin secretion, *J Biol Chem,* **274,** 22337-44.

Hanahan, D. and Weinberg, R. A. (2000). The hallmarks of cancer, *Cell,* **100,** 57-70.

Hangaishi, A., Ogawa, S., Imamura, N., Miyawaki, S., Miura, Y., Uike, N., Shimazaki, C., Emi, N., Takeyama, K., Hirosawa, S., Kamada, N., Kobayashi, Y., Takemoto, Y., Kitani, T., Toyama, K., Ohtake, S., Yazaki, Y., Ueda, R. and Hirai, H. (1996). Inactivation of multiple tumor-suppressor genes involved in negative regulation of the cell cycle, MTS1/p16INK4A/CDKN2, MTS2/p15INK4B, p53, and Rb genes in primary lymphoid malignancies, *Blood,* **87,** 4949-58.

Hannon, G. J. and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest, *Nature,* **371,** 257-61.

Hans, C. P., Weisenburger, D. D., Greiner, T. C., Gascoyne, R. D., Delabie, J., Ott, G., Muller-Hermelink, H. K., Campo, E., Braziel, R. M., Jaffe, E. S., Pan, Z., Farinha, P., Smith, L. M., Falini, B., Banham, A. H., Rosenwald, A., Staudt, L. M., Connors, J. M., Armitage, J. O. and Chan, W. C. (2004). Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray, *Blood,* **103,** 275-82.

Harris, A. W., Strasser, A., Bath, M. L., Elefanty, A. G. and Cory, S. (1997). Lymphomas and plasmacytomas in transgenic mice involving bcl2, myc and v-abl, *Curr Top Microbiol Immunol,* **224,** 221-30.

Harris, N. L., Jaffe, E. S., Diebold, J., Flandrin, G., Muller-Hermelink, H. K., Vardiman, J., Lister, T. A. and Bloomfield, C. D. (1999). The World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November, 1997, *Ann Oncol,* **10,** 1419-32.

Harris, N. L., Jaffe, E. S., Stein, H., Banks, P. M., Chan, J. K., Cleary, M. L., Delsol, G., De Wolf-Peeters, C., Falini, B. and Gatter, K. C. (1994). A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group, *Blood,* **84,** 1361-92.

Hartwell, L. H. and Kastan, M. B. (1994). Cell cycle control and cancer, *Science,* **266,** 1821-8.

Hatzelmann, A. and Schudt, C. (2001). Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast *in vitro*, *J Pharmacol Exp Ther,* **297,** 267-79.

Haupt, Y., Maya, R., Kazaz, A. and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53, *Nature,* **387,** 296-9.

He, F., Seryshev, A. B., Cowan, C. W. and Wensel, T. G. (2000). Multiple zinc binding sites in retinal rod cGMP phosphodiesterase, PDE6alpha beta, *J Biol Chem,* **275,** 20572-7.

Hei, Y. J., Farahbakhshian, S., Chen, X., Battell, M. L. and McNeill, J. H. (1998). Stimulation of MAP kinase and S6 kinase by vanadium and selenium in rat adipocytes, *Mol Cell Biochem,* **178,** 367-75.

Helzlsouer, K. J., Huang, H. Y., Alberg, A. J., Hoffman, S., Burke, A., Norkus, E. P., Morris, J. S. and Comstock, G. W. (2000). Association between alpha-tocopherol, gamma-tocopherol, selenium, and subsequent prostate cancer, *J Natl Cancer Inst,* **92,** 2018-23.

Hercberg, S., Ezzedine, K., Guinot, C., Preziosi, P., Galan, P., Bertrais, S., Estaquio, C., Briancon, S., Favier, A., Latreille, J. and Malvy, D. (2007). Antioxidant supplementation increases the risk of skin cancers in women but not in men, *J Nutr,* **137,** 2098-105.

Hercberg, S., Galan, P., Preziosi, P., Bertrais, S., Mennen, L., Malvy, D., Roussel, A. M., Favier, A. and Briancon, S. (2004). The SU.VI.MAX Study: a randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals, *Arch Intern Med,* **164,** 2335-42.

Herigstad, R. R., Whitechair, C. K. and Olson, O. E. (1973). Inorganic and organic selenium toxicosis in young swine: comparison of pathologic changes with those in seine with vitamin E-selenium deficiency, *Am J Vet Res,* **34,** 1227-38.

Hill, K. E., Zhou, J., McMahan, W. J., Motley, A. K., Atkins, J. F., Gesteland, R. F. and Burk, R. F. (2003). Deletion of selenoprotein P alters distribution of selenium in the mouse, *J Biol Chem,* **278,** 13640-6.

Hiom, K., Melek, M. and Gellert, M. (1998). DNA transposition by the RAG1 and RAG2 proteins: a possible source of oncogenic translocations, *Cell,* **94,** 463-70.

Ho, Y. S., Magnenat, J. L., Bronson, R. T., Cao, J., Gargano, M., Sugawara, M. and Funk, C. D. (1997). Mice deficient in cellular glutathione peroxidase develop

normally and show no increased sensitivity to hyperoxia, *J Biol Chem,* **272,** 16644-51.

Hodgkin, T. (1832). On some morbid appearances of the absorbent glands and spleen, *Med Chir Soc Tr,* **17,** 68.

Hoff, P. M., Ellis, L. M. and Abbruzzese, J. L. (2004). Monoclonal antibodies: the foundation of therapy for colorectal cancer in the 21st century?, *Oncology (Huntingt),* **18,** 736-41; discussion 742, 745-6.

Hoglund, M., Sehn, L., Connors, J. M., Gascoyne, R. D., Siebert, R., Sall, T., Mitelman, F. and Horsman, D. E. (2004). Identification of cytogenetic subgroups and karyotypic pathways of clonal evolution in follicular lymphomas, *Genes Chromosomes Cancer,* **39,** 195-204.

Holly, E. A. and Bracci, P. M. (2003). Population-based study of non-Hodgkin lymphoma, histology, and medical history among human immunodeficiency virus-negative participants in San Francisco, *Am J Epidemiol,* **158,** 316-27.

Houslay, M. D. and Adams, D. R. (2003). PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization, *Biochem J,* **370,** 1-18.

Hsieh, H. S. and Ganther, H. E. (1977). Biosynthesis of dimethyl selenide from sodium selenite in rat liver and kidney cell-free systems, *Biochim Biophys Acta,* **497,** 205-17.

Hu, Y., Benya, R. V., Carroll, R. E. and Diamond, A. M. (2005). Allelic loss of the gene for the GPX1 selenium-containing protein is a common event in cancer, *J Nutr,* **135,** 3021S-3024S.

Hu, Y. J., Chen, Y., Zhang, Y. Q., Zhou, M. Z., Song, X. M., Zhang, B. Z., Luo, L., Xu, P. M., Zhao, Y. N., Zhao, Y. B. and Cheng, G. (1997). The protective role of selenium on the toxicity of cisplatin-contained chemotherapy regimen in cancer patients, *Biol Trace Elem Res,* **56,** 331-41.

Hu, Y. J. and Diamond, A. M. (2003). Role of glutathione peroxidase 1 in breast cancer: loss of heterozygosity and allelic differences in the response to selenium, *Cancer Res,* **63,** 3347-51.

Huai, Q., Wang, H., Zhang, W., Colman, R. W., Robinson, H. and Ke, H. (2004). Crystal structure of phosphodiesterase 9 shows orientation variation of inhibitor 3-isobutyl-1-methylxanthine binding, *Proc Natl Acad Sci U S A,* **101,** 9624-9.

Huang, D. C. and Strasser, A. (2000). BH3-Only proteins-essential initiators of apoptotic cell death, *Cell,* **103,** 839-42.

Huang, J. Z., Sanger, W. G., Greiner, T. C., Staudt, L. M., Weisenburger, D. D., Pickering, D. L., Lynch, J. C., Armitage, J. O., Warnke, R. A., Alizadeh, A. A., Lossos, I. S., Levy, R. and Chan, W. C. (2002). The t(14;18) defines a unique subset of diffuse large B-cell lymphoma with a germinal center B-cell gene expression profile, *Blood,* **99,** 2285-90.

Huber, R. E. and Criddle, R. S. (1967). The isolation and properties of beta-galactosidase from Escherichia coli grown on sodium selenate, *Biochim Biophys Acta,* **141,** 587-99.

Hueber, A. O., Zornig, M., Lyon, D., Suda, T., Nagata, S. and Evan, G. I. (1997). Requirement for the CD95 receptor-ligand pathway in c-Myc-induced apoptosis, *Science,* **278,** 1305-9.

Hughes, T. R., Mao, M., Jones, A. R., Burchard, J., Marton, M. J., Shannon, K. W., Lefkowitz, S. M., Ziman, M., Schelter, J. M., Meyer, M. R., Kobayashi, S., Davis, C., Dai, H., He, Y. D., Stephaniants, S. B., Cavet, G., Walker, W. L., West, A., Coffey, E., Shoemaker, D. D., Stoughton, R., Blanchard, A. P., Friend, S. H. and Linsley, P. S. (2001). Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer, *Nat Biotechnol,* **19,** 342-7.

Ichikawa, A. (2000). Prognostic and predictive significance of p53 mutation in aggressive B-cell lymphoma, *Int J Hematol,* **71,** 211-20.

Ichikawa, A., Kinoshita, T., Watanabe, T., Kato, H., Nagai, H., Tsushita, K., Saito, H. and Hotta, T. (1997). Mutations of the p53 gene as a prognostic factor in aggressive B-cell lymphoma, *N Engl J Med,* **337,** 529-34.

Ichimura, M., Eiki, R., Osawa, K., Nakanishi, S. and Kase, H. (1996). KS-505a, an isoform-selective inhibitor of calmodulin-dependent cyclic nucleotide phosphodiesterase, *Biochem J,* **316 (Pt 1),** 311-6.

Igney, F. H. and Krammer, P. H. (2002). Death and anti-death: tumour resistance to apoptosis, *Nat Rev Cancer,* **2,** 277-88.

Institute of Medicine, F. a. N. B. (2000) *Dietary reference intakes. Vitamin C, vitamin E, Selenium, and Carotenoids.,* National Academy Press, Washington, USA.

Ionov, Y., Peinado, M. A., Malkhosyan, S., Shibata, D. and Perucho, M. (1993). Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis, *Nature,* **363,** 558-61.

Ip, C. (1986). The chemopreventive role of selenium in carcinogenesis, *Adv Exp Med Biol,* **206,** 431-47.

Ip, C. (1988). Differential effect of dietary methionine on the biopotency of selenomethionine and selenite in cancer chemoprevention, *J Natl Cancer Inst,* **80,** 258-62.

Ip, C. (1998). Lessons from basic research in selenium and cancer prevention, *J Nutr,* **128,** 1845-54.

Ip, C., el-Bayoumy, K., Upadhyaya, P., Ganther, H., Vadhanavikit, S. and Thompson, H. (1994). Comparative effect of inorganic and organic selenocyanate derivatives in mammary cancer chemoprevention, *Carcinogenesis,* **15,** 187-92.

Ip, C. and Ganther, H. (1988). Efficacy of trimethylselenonium versus selenite in cancer chemoprevention and its modulation by arsenite, *Carcinogenesis,* **9,** 1481-4.

Ip, C. and Ganther, H. E. (1990). Activity of methylated forms of selenium in cancer prevention, *Cancer Res,* **50,** 1206-11.

Ip, C. and Ganther, H. E. (1992). Comparison of selenium and sulfur analogs in cancer prevention, *Carcinogenesis,* **13,** 1167-70.

Ip, C. and Hayes, C. (1989). Tissue selenium levels in selenium-supplemented rats and their relevance in mammary cancer protection, *Carcinogenesis,* **10,** 921-5.

Ip, C., Hayes, C., Budnick, R. M. and Ganther, H. E. (1991). Chemical form of selenium, critical metabolites, and cancer prevention, *Cancer Res,* **51,** 595-600.

Ip, C., Ip, M. M. and Kim, U. (1981). Dietary selenium intake and growth of the MT-W9B transplantable rat mammary tumor, *Cancer Lett,* **14,** 101-7.

Ip, C. and Lisk, D. J. (1994). Characterization of tissue selenium profiles and anticarcinogenic responses in rats fed natural sources of selenium-rich products, *Carcinogenesis,* **15,** 573-6.

Ip, C. and Lisk, D. J. (1997). Modulation of phase I and phase II xenobiotic-metabolizing enzymes by selenium-enriched garlic in rats, *Nutr Cancer,* **28,** 184-8.

Ip, C., Thompson, H. J., Zhu, Z. and Ganther, H. E. (2000). *In vitro* and in vivo studies of methylseleninic acid: evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention, *Cancer Res,* **60,** 2882-6.

Ip, C. and White, G. (1987). Mammary cancer chemoprevention by inorganic and organic selenium: single agent treatment or in combination with vitamin E and their effects on *in vitro* immune functions, *Carcinogenesis,* **8,** 1763-6.

Ip, C., Zhu, Z., Thompson, H. J., Lisk, D. and Ganther, H. E. (1999). Chemoprevention of mammary cancer with Se-allylselenocysteine and other selenoamino acids in the rat, *Anticancer Res,* **19,** 2875-80.

Iqbal, J., Sanger, W. G., Horsman, D. E., Rosenwald, A., Pickering, D. L., Dave, B., Dave, S., Xiao, L., Cao, K., Zhu, Q., Sherman, S., Hans, C. P., Weisenburger, D. D., Greiner, T. C., Gascoyne, R. D., Ott, G., Muller-Hermelink, H. K., Delabie, J., Braziel, R. M., Jaffe, E. S., Campo, E., Lynch, J. C., Connors, J. M., Vose, J. M., Armitage, J. O., Grogan, T. M., Staudt, L. M. and Chan, W. C. (2004). BCL2 translocation defines a unique tumor subset within the germinal center B-cell-like diffuse large B-cell lymphoma, *Am J Pathol,* **165,** 159-66.

Irons, R., Carlson, B. A., Hatfield, D. L. and Davis, C. D. (2006). Both selenoproteins and low molecular weight selenocompounds reduce colon cancer risk in mice with genetically impaired selenoprotein expression, *J Nutr,* **136,** 1311-7.

Izquierdo, J. L., Bateman, E. D., Villasante, c., scmid-Wurlitsch, C., Bredenbroker, D. and Wurst, W. (2003). Long term efficacy and safety over one year of once daily roflumilast, a new orally active selective phosphodiesterase 4 inhibitor in asthma., *Am J Respir Crit Care Med,* **167**.

Jacobs, M. and Forst, C. (1981a). Toxicological effects of sodium selenite in Sprague-Dawley rats, *J Toxicol Environ Health,* **8,** 575-85.

Jacobs, M. and Forst, C. (1981b). Toxicological effects of sodium selenite in Swiss mice, *J Toxicol Environ Health,* **8,** 587-98.

Jacobson, M. D., Weil, M. and Raff, M. C. (1997). Programmed cell death in animal development, *Cell,* **88,** 347-54.

Jaenisch, R. and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals, *Nat Genet,* **33 Suppl,** 245-54.

Jaffe, E. S., Harris, N. L., Stein, H. and Vardiman, J. (2001) *Pathology and Genetics of Tumours of Haemopoietic and Lymphoid Tissue,* IARC Press, Lyon.

Jager, U., Bocskor, S., Le, T., Mitterbauer, G., Bolz, I., Chott, A., Kneba, M., Mannhalter, C. and Nadel, B. (2000). Follicular lymphomas' BCL-2/IgH junctions contain templated nucleotide insertions: novel insights into the mechanism of t(14;18) translocation, *Blood,* **95,** 3520-9.

Jaiswal, S., Traver, D., Miyamoto, T., Akashi, K., Lagasse, E. and Weissman, I. L. (2003). Expression of BCR/ABL and BCL-2 in myeloid progenitors leads to myeloid leukemias, *Proc Natl Acad Sci U S A,* **100,** 10002-7.

Jazirehi, A. R., Gan, X. H., De Vos, S., Emmanouilides, C. and Bonavida, B. (2003). Rituximab (anti-CD20) selectively modifies Bcl-xL and apoptosis protease activating factor-1 (Apaf-1) expression and sensitizes human non-Hodgkin's lymphoma B cell lines to paclitaxel-induced apoptosis, *Mol Cancer Ther,* **2,** 1183-93.

Jazirehi, A. R., Huerta-Yepez, S., Cheng, G. and Bonavida, B. (2005). Rituximab (chimeric anti-CD20 monoclonal antibody) inhibits the constitutive nuclear factor-{kappą B signaling pathway in non-Hodgkin's lymphoma B-cell lines: role in sensitization to chemotherapeutic drug-induced apoptosis, *Cancer Res,* **65,** 264-76.

Jazirehi, A. R., Vega, M. I., Chatterjee, D., Goodglick, L. and Bonavida, B. (2004). Inhibition of the Raf-MEK1/2-ERK1/2 signaling pathway, Bcl-xL down-regulation, and chemosensitization of non-Hodgkin's lymphoma B cells by Rituximab, *Cancer Res,* **64,** 7117-26.

Jiang, C., Wang, Z., Ganther, H. and Lu, J. (2001). Caspases as key executors of methyl selenium-induced apoptosis (anoikis) of DU-145 prostate cancer cells, *Cancer Res,* **61,** 3062-70.

Jiang, X. H., Lam, S. K., Lin, M. C., Jiang, S. H., Kung, H. F., Slosberg, E. D., Soh, J. W., Weinstein, I. B. and Wong, B. C. (2002). Novel target for induction of apoptosis by cyclo-oxygenase-2 inhibitor SC-236 through a protein kinase C-beta(1)-dependent pathway, *Oncogene,* **21,** 6113-22.

Jiang, X. R., Macey, M. G., Lin, H. X. and Newland, A. C. (1992). The anti-leukaemic effects and the mechanism of sodium selenite, *Leuk Res,* **16,** 347-52.

Jin, D. Y., Spencer, F. and Jeang, K. T. (1998). Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1, *Cell,* **93,** 81-91.

Johannes, F. J., Prestle, J., Eis, S., Oberhagemann, P. and Pfizenmaier, K. (1994). PKCu is a novel, atypical member of the protein kinase C family, *J Biol Chem,* **269,** 6140-8.

Johansson, B., Mertens, F. and Mitelman, F. (1995). Cytogenetic evolution patterns in non-Hodgkin's lymphoma, *Blood,* **86,** 3905-14.

Johansson, B., Mertens, F. and Mitelman, F. (1996). Primary vs. secondary neoplasia-associated chromosomal abnormalities--balanced rearrangements vs. genomic imbalances?, *Genes Chromosomes Cancer,* **16,** 155-63.

Johnstone, R. W., Ruefli, A. A. and Lowe, S. W. (2002). Apoptosis: a link between cancer genetics and chemotherapy, *Cell,* **108,** 153-64.

Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J. and Wolffe, A. P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription, *Nat Genet,* **19,** 187-91.

Jones, S. E., Miller, T. P. and Connors, J. M. (1989). Long-term follow-up and analysis for prognostic factors for patients with limited-stage diffuse large-cell lymphoma treated with initial chemotherapy with or without adjuvant radiotherapy, *J Clin Oncol,* **7,** 1186-91.

Joshi, N., Johnson, L. L., Wei, W. Q., Abnet, C. C., Dong, Z. W., Taylor, P. R., Limburg, P. J., Dawsey, S. M., Hawk, E. T., Qiao, Y. L. and Kirsch, I. R. (2006). Gene expression differences in normal esophageal mucosa associated with regression and progression of mild and moderate squamous dysplasia in a high-risk Chinese population, *Cancer Res,* **66,** 6851-60.

Joyce, R. M., Regan, M., Ottaway, J., Umiel, T., Tetreault, J. C., Levine, J., McDermott, D., Hurley, D., Giallombardo, N., Smith, T., Lamontagne, D., Uhl, L. and Avigan, D. (2003). A phase I-II study of rituximab, ifosfamide, mitoxantrone and etoposide (R-IME) for B cell non-Hodgkin's lymphoma prior to and after high-dose chemotherapy and autologous stem cell transplantation (HDC-ASCT), *Ann Oncol,* **14 Suppl 1,** i21-7.

Juliger, S., Goenaga-Infante, H., Lister, T. A., Fitzgibbon, J. and Joel, S. P. (2007). Chemosensitization of B-cell lymphomas by methylseleninic acid involves

nuclear factor-kappaB inhibition and the rapid generation of other selenium species, *Cancer Res,* **67,** 10984-92.

Kaeck, M., Lu, J., Strange, R., Ip, C., Ganther, H. E. and Thompson, H. J. (1997). Differential induction of growth arrest inducible genes by selenium compounds, *Biochem Pharmacol,* **53,** 921-6.

Kajander, E. O., Harvima, R. J., Kauppinen, L., Akerman, K. K., Martikainen, H., Pajula, R. L. and Karenlampi, S. O. (1990). Effects of selenomethionine on cell growth and on S-adenosylmethionine metabolism in cultured malignant cells, *Biochem J,* **267,** 767-74.

Kamel, O. W., Holly, E. A., van de Rijn, M., Lele, C. and Sah, A. (1999). A population based, case control study of non-Hodgkin's lymphoma in patients with rheumatoid arthritis, *J Rheumatol,* **26,** 1676-80.

Kaplan, E. J. and Meier, P. (1958). Non-parametric estimation from incomplete observations., *J Am Stat Assoc,* **53,** 457-481.

Kargman, S. L., O'Neill, G. P., Vickers, P. J., Evans, J. F., Mancini, J. A. and Jothy, S. (1995). Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer, *Cancer Res,* **55,** 2556-9.

Karsan, A., Gascoyne, R. D., Coupland, R. W., Shepherd, J. D., Phillips, G. L. and Horsman, D. E. (1993). Combination of t(14;18) and a Burkitt's type translocation in B-cell malignancies, *Leuk Lymphoma,* **10,** 433-41.

Kawamata, N., Morosetti, R., Miller, C. W., Park, D., Spirin, K. S., Nakamaki, T., Takeuchi, S., Hatta, Y., Simpson, J. and Wilcyznski, S. (1995). Molecular analysis of the cyclin-dependent kinase inhibitor gene p27/Kip1 in human malignancies, *Cancer Res,* **55,** 2266-9.

Kawasaki, C., Ohshim, K., Suzumiya, J., Kanda, M., Tsuchiya, T., Tamura, K. and Kikuchi, M. (2001). Rearrangements of bcl-1, bcl-2, bcl-6, and c-myc in diffuse large B-cell lymphomas, *Leuk Lymphoma,* **42,** 1099-106.

Kerckaert, J. P., Deweindt, C., Tilly, H., Quief, S., Lecocq, G. and Bastard, C. (1993). LAZ3, a novel zinc-finger encoding gene, is disrupted by recurring chromosome 3q27 translocations in human lymphomas, *Nat Genet,* **5,** 66-70.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, *Br J Cancer,* **26,** 239-57.

Khan, J., Simon, R., Bittner, M., Chen, Y., Leighton, S. B., Pohida, T., Smith, P. D., Jiang, Y., Gooden, G. C., Trent, J. M. and Meltzer, P. S. (1998). Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays, *Cancer Res,* **58,** 5009-13.

Kim, D. H., Kim, M. and Kwon, H. J. (2003a). Histone deacetylase in carcinogenesis and its inhibitors as anti-cancer agents, *J Biochem Mol Biol,* **36,** 110-9.

Kim, T. S., Yun, B. Y. and Kim, I. Y. (2003b). Induction of the mitochondrial permeability transition by selenium compounds mediated by oxidation of the protein thiol groups and generation of the superoxide, *Biochem Pharmacol,* **66,** 2301-11.

King, L. B., Norvell, A. and Monroe, J. G. (1999). Antigen receptor-induced signal transduction imbalances associated with the negative selection of immature B cells, *J Immunol,* **162,** 2655-62.

Kinzler, K. W. and Vogelstein, B. (1996a). Lessons from hereditary colorectal cancer, *Cell,* **87,** 159-70.

Kinzler, K. W. and Vogelstein, B. (1996b). Life (and death) in a malignant tumour, *Nature,* **379,** 19-20.

Kinzler, K. W. and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers, *Nature,* **386,** 761, 763.

Kiremidjian-Schumacher, L., Roy, M., Glickman, R., Schneider, K., Rothstein, S., Cooper, J., Hochster, H., Kim, M. and Newman, R. (2000). Selenium and immunocompetence in patients with head and neck cancer, *Biol Trace Elem Res,* **73,** 97-111.

Kiremidjian-Schumacher, L., Roy, M., Wishe, H. I., Cohen, M. W. and Stotzky, G. (1994). Supplementation with selenium and human immune cell functions. II. Effect on cytotoxic lymphocytes and natural killer cells, *Biol Trace Elem Res,* **41,** 115-27.

Kirk, O., Pedersen, C., Cozzi-Lepri, A., Antunes, F., Miller, V., Gatell, J. M., Katlama, C., Lazzarin, A., Skinhoj, P. and Barton, S. E. (2001). Non-Hodgkin lymphoma in HIV-infected patients in the era of highly active antiretroviral therapy, *Blood,* **98,** 3406-12.

Kiviniemi, M., Sauroja, I., Rajamaki, A., Punnonen, K., Soderstrom, K. O. and Salminen, E. (2000). Cell cycle regulators p27 and pRb in lymphomas - correlation with histology and proliferative activity, *Br J Cancer,* **83,** 1161-7.

Kizaki, H., Suzuki, K., Tadakuma, T. and Ishimura, Y. (1990). Adenosine receptor-mediated accumulation of cyclic AMP-induced T-lymphocyte death through internucleosomal DNA cleavage, *J Biol Chem,* **265,** 5280-4.

Klangby, U., Okan, I., Magnusson, K. P., Wendland, M., Lind, P. and Wiman, K. G. (1998). p16/INK4a and p15/INK4b gene methylation and absence of p16/INK4a mRNA and protein expression in Burkitt's lymphoma, *Blood,* **91,** 1680-7.

Klein, E. A., Thompson, I. M., Lippman, S. M., Goodman, P. J., Albanes, D., Taylor, P. R. and Coltman, C. (2000). SELECT: the Selenium and Vitamin E Cancer Prevention Trial: rationale and design, *Prostate Cancer Prostatic Dis,* **3,** 145-151.

Kluin-Nelemans, H. C., Limpens, J., Meerabux, J., Beverstock, G. C., Jansen, J. H., de Jong, D. and Kluin, P. M. (1991). A new non-Hodgkin's B-cell line (DoHH2) with a chromosomal translocation t(14;18)(q32;q21), *Leukemia,* **5,** 221-4.

Knekt, P., Aromaa, A., Maatela, J., Alfthan, G., Aaran, R. K., Teppo, L. and Hakama, M. (1988). Serum vitamin E, serum selenium and the risk of gastrointestinal cancer, *Int J Cancer,* **42,** 846-50.

Knox, K. A., Johnson, G. D. and Gordon, J. (1993). Distribution of cAMP in secondary follicles and its expression in B cell apoptosis and CD40-mediated survival, *Int Immunol,* **5,** 1085-91.

Kobayashi, T., Gamanuma, M., Sasaki, T., Yamashita, Y., Yuasa, K., Kotera, J. and Omori, K. (2003). Molecular comparison of rat cyclic nucleotide phosphodiesterase 8 family: unique expression of PDE8B in rat brain, *Gene,* **319,** 21-31.

Koduru, P. R., Raju, K., Vadmal, V., Menezes, G., Shah, S., Susin, M., Kolitz, J. and Broome, J. D. (1997). Correlation between mutation in P53, p53 expression, cytogenetics, histologic type, and survival in patients with B-cell non-Hodgkin's lymphoma, *Blood,* **90,** 4078-91.

Koduru, P. R., Zariwala, M., Soni, M., Gong, J. Z., Xiong, Y. and Broome, J. D. (1995). Deletion of cyclin-dependent kinase 4 inhibitor genes P15 and P16 in non-Hodgkin's lymphoma, *Blood,* **86,** 2900-5.

Kohrle, J. (2000). The deiodinase family: selenoenzymes regulating thyroid hormone availability and action, *Cell Mol Life Sci,* **57,** 1853-63.

Kotamraju, S., Konorev, E. A., Joseph, J. and Kalyanaraman, B. (2000). Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen. Role of reactive oxygen and nitrogen species, *J Biol Chem,* **275,** 33585-92.

Kouzarides, T. (2002). Histone methylation in transcriptional control, *Curr Opin Genet Dev,* **12,** 198-209.

Kramer, M. H., Hermans, J., Parker, J., Krol, A. D., Kluin-Nelemans, J. C., Haak, H. L., van Groningen, K., van Krieken, J. H., de Jong, D. and Kluin, P. M. (1996). Clinical significance of bcl2 and p53 protein expression in diffuse large B-cell lymphoma: a population-based study, *J Clin Oncol,* **14,** 2131-8.

Kramer, M. H., Hermans, J., Wijburg, E., Philippo, K., Geelen, E., van Krieken, J. H., de Jong, D., Maartense, E., Schuuring, E. and Kluin, P. M. (1998). Clinical relevance of BCL2, BCL6, and MYC rearrangements in diffuse large B-cell lymphoma, *Blood,* **92,** 3152-62.

Krammer, P. H. (2000). CD95's deadly mission in the immune system, *Nature,* **407,** 789-95.

Kuchan, M. J. and Milner, J. A. (1991). Influence of supplemental glutathione on selenite-mediated growth inhibition of canine mammary cells, *Cancer Lett,* **57,** 181-6.

Kuchan, M. J. and Milner, J. A. (1992). Influence of intracellular glutathione on selenite-mediated growth inhibition of canine mammary tumor cells, *Cancer Res,* **52,** 1091-5.

Kuklinski, B., Buchner, M., Schweder, R. and Nagel, R. (1991). [Acute pancreatitis--a free radical disease. Decrease in fatality with sodium selenite (Na2SeO3) therapy], *Z Gesamte Inn Med,* **46,** 145-9.

Kuklinski, B., Zimmermann, T. and Schweder, R. (1995). [Decreasing mortality in acute pancreatitis with sodium selenite. Clinical results of 4 years antioxidant therapy], *Med Klin (Munich),* **90 Suppl 1,** 36-41.

Kuroda, H., Komatsu, H., Nakamura, S., Niitsu, Y., Takahashi, T., Ueda, R. and Seto, M. (1995). The positive nuclear staining observed with monoclonal antibody

against PRAD1/cyclin D1 correlates with mRNA expression in mantle cell lymphoma, *Jpn J Cancer Res,* **86,** 890-8.

Kurosu, T., Fukuda, T., Miki, T. and Miura, O. (2003). BCL6 overexpression prevents increase in reactive oxygen species and inhibits apoptosis induced by chemotherapeutic reagents in B-cell lymphoma cells, *Oncogene,* **22,** 4459-68.

Kwak, L. W., Halpern, J., Olshen, R. A. and Horning, S. J. (1990). Prognostic significance of actual dose intensity in diffuse large-cell lymphoma: results of a tree-structured survival analysis, *J Clin Oncol,* **8,** 963-77.

Lanfear, J., Fleming, J., Wu, L., Webster, G. and Harrison, P. R. (1994). The selenium metabolite selenodiglutathione induces p53 and apoptosis: relevance to the chemopreventive effects of selenium?, *Carcinogenesis,* **15,** 1387-92.

Last, K. W., Goff, L. K., Summers, K. E., Neat, M., Jenner, M., Crawley, C., Rohatiner, A. Z., Fitzgibbon, J. and Lister, T. A. (2000). Familial follicular lymphoma: a case report with molecular analysis, *Br J Haematol,* **110,** 744-5.

Lausted, C., Dahl, T., Warren, C., King, K., Smith, K., Johnson, M., Saleem, R., Aitchison, J., Hood, L. and Lasky, S. R. (2004). POSaM: a fast, flexible, open-source, inkjet oligonucleotide synthesizer and microarrayer, *Genome Biol,* **5,** R58.

Laytragoon-Lewin, N., Duhony, E., Bai, X. F. and Mellstedt, H. (1998). Downregulation of the CD95 receptor and defect CD40-mediated signal transduction in B-chronic lymphocytic leukemia cells, *Eur J Haematol,* **61,** 266-71.

Lecuit, M., Abachin, E., Martin, A., Poyart, C., Pochart, P., Suarez, F., Bengoufa, D., Feuillard, J., Lavergne, A., Gordon, J. I., Berche, P., Guillevin, L. and Lortholary, O. (2004). Immunoproliferative small intestinal disease associated with Campylobacter jejuni, *N Engl J Med,* **350,** 239-48.

Lee, S. H., Shin, M. S., Kim, H. S., Lee, H. K., Park, W. S., Kim, S. Y., Lee, J. H., Han, S. Y., Park, J. Y., Oh, R. R., Kang, C. S., Kim, K. M., Jang, J. J., Nam, S. W., Lee, J. Y. and Yoo, N. J. (2001). Somatic mutations of TRAIL-receptor 1 and TRAIL-receptor 2 genes in non-Hodgkin's lymphoma, *Oncogene,* **20,** 399-403.

Lehours, P., Menard, A., Dupouy, S., Bergey, B., Richy, F., Zerbib, F., Ruskone-Fourmestraux, A., Delchier, J. C. and Megraud, F. (2004). Evaluation of the Association of Nine Helicobacter pylori Virulence Factors with Strains Involved

in Low-Grade Gastric Mucosa-Associated Lymphoid Tissue Lymphoma, *Infect Immun,* **72,** 880-8.

Leinfelder, W., Zehelein, E., Mandrand-Berthelot, M. A. and Bock, A. (1988). Gene for a novel tRNA species that accepts L-serine and cotranslationally inserts selenocysteine, *Nature,* **331,** 723-5.

Leitges, M., Schmedt, C., Guinamard, R., Davoust, J., Schaal, S., Stabel, S. and Tarakhovsky, A. (1996). Immunodeficiency in protein kinase cbeta-deficient mice, *Science,* **273,** 788-91.

Lemercier, C., Brocard, M. P., Puvion-Dutilleul, F., Kao, H. Y., Albagli, O. and Khochbin, S. (2002). Class II histone deacetylases are directly recruited by BCL6 transcriptional repressor, *J Biol Chem,* **277,** 22045-52.

Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1997). Genetic instability in colorectal cancers, *Nature,* **386,** 623-7.

Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1998). Genetic instabilities in human cancers, *Nature,* **396,** 643-9.

Lennert, K. and Feller, A. C. (1992) *Histology of non-Hodgkin's Lymphomas,* Springer Verlag, New York.

Lenz, G., Wright, G., Dave, S., Kohlmann, A., Xiao, W., Powell, J., Zhao, H., Xu, W., Gascoyne, R. D., Connors, J. M., May, L., Weisenburger, D. D., Greiner, T., Vose, J., Armitage, J. O., Iqbal, J., Bast, M., Fu, K., Campo, E., Montserrat, E., Lopez-Guillermo, A., Jares, P., Martinez, A., Gibbs, B., Rimsza, L. M., Fisher, R. I., Braziel, R. M., Tubbs, R., Cook, J., Pohlman, B., Sweetenham, J., Troen, G., Smeland, E. B., Delabie, J., Kvaloy, S., Holte, H., Jaffe, E. S., Wilson, W. H., Grant, N., Hartmann, E., Rosenwald, A., Ott, G., Muller-Hermelink, H.-K., Lister, T. A., Williams, M., Wieczorek, L., Chan, W. C. and Staudt, L. M. (2007). Gene Expression Signatures Predict Overall Survial in Diffuse Large B Cell Lymphoma Treated with Rituximab and Chop-Like Chemotherapy., *ASH Annual Meeting Abstracts,* **110,** 348-.

Lenz, G., Wright, G. W., Emre, N. C., Kohlhammer, H., Dave, S. S., Davis, R. E., Carty, S., Lam, L. T., Shaffer, A. L., Xiao, W., Powell, J., Rosenwald, A., Ott, G., Muller-Hermelink, H. K., Gascoyne, R. D., Connors, J. M., Campo, E., Jaffe, E. S., Delabie, J., Smeland, E. B., Rimsza, L. M., Fisher, R. I., Weisenburger, D. D., Chan, W. C. and Staudt, L. M. (2008). Molecular subtypes

of diffuse large B-cell lymphoma arise by distinct genetic pathways, *Proc Natl Acad Sci U S A,* **105,** 13520-5.

Lerner, A., Kim, D. H. and Lee, R. (2000). The cAMP signaling pathway as a therapeutic target in lymphoid malignancies, *Leuk Lymphoma,* **37,** 39-51.

Leroy, K., Haioun, C., Lepage, E., Le Metayer, N., Berger, F., Labouyrie, E., Meignin, V., Petit, B., Bastard, C., Salles, G., Gisselbrecht, C., Reyes, F. and Gaulard, P. (2002). p53 gene mutations are associated with poor survival in low and low-intermediate risk diffuse large B-cell lymphomas, *Ann Oncol,* **13,** 1108-15.

Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E. and George, D. L. (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex, *Nat Cell Biol,* **6,** 443-50.

Leynadier, D., Peyrot, V., Codaccioni, F. and Briand, C. (1991). Selenium: inhibition of microtubule formation and interaction with tubulin, *Chem Biol Interact,* **79,** 91-102.

Li, J. Y., Gaillard, F., Moreau, A., Harousseau, J. L., Laboisse, C., Milpied, N., Bataille, R. and Avet-Loiseau, H. (1999). Detection of translocation t(11;14)(q13;q32) in mantle cell lymphoma by fluorescence in situ hybridization, *Am J Pathol,* **154,** 1449-52.

Li, M., Wang, X., Meintzer, M. K., Laessig, T., Birnbaum, M. J. and Heidenreich, K. A. (2000). Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3beta, *Mol Cell Biol,* **20,** 9356-63.

Li, Z., Van Calcar, S., Qu, C., Cavenee, W. K., Zhang, M. Q. and Ren, B. (2003). A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells, *Proc Natl Acad Sci U S A,* **100,** 8164-9.

Limburg, P. J., Wei, W., Ahnen, D. J., Qiao, Y., Hawk, E. T., Wang, G., Giffen, C. A., Roth, M. J., Lu, N., Korn, E. L., Ma, Y., Caldwell, K. L., Dong, Z., Taylor, P. R. and Dawsey, S. M. (2005). Randomized, placebo-controlled, esophageal squamous cell cancer chemoprevention trial of selenomethionine and celecoxib, *Gastroenterology,* **129,** 863-73.

Lincoln, D. T., Ali Emadi, E. M., Tonissen, K. F. and Clarke, F. M. (2003). The thioredoxin-thioredoxin reductase system: over-expression in human cancer, *Anticancer Res,* **23,** 2425-33.

Linden, M. A., Kirchhof, N., Carlson, C. S. and Van Ness, B. G. (2003). Targeted overexpression of Bcl-XL in B-lymphoid cells results in lymphoproliferative disease and plasma cell malignancies, *Blood*.

Lipworth, B. J. (2005). Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease, *Lancet,* **365,** 167-75.

Liu, A. Y., Robinson, R. R., Murray, E. D., Jr., Ledbetter, J. A., Hellstrom, I. and Hellstrom, K. E. (1987). Production of a mouse-human chimeric monoclonal antibody to CD20 with potent Fc-dependent biologic activity, *J Immunol,* **139,** 3521-6.

Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H. and Brown, E. L. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays, *Nat Biotechnol,* **14,** 1675-80.

Loflin, J., Lopez, N., Whanger, P. D. and Kioussi, C. (2006). Selenoprotein W during development and oxidative stress, *J Inorg Biochem,* **100,** 1679-84.

Lomo, J., Blomhoff, H. K., Beiske, K., Stokke, T. and Smeland, E. B. (1995). TGF-beta 1 and cyclic AMP promote apoptosis in resting human B lymphocytes, *J Immunol,* **154,** 1634-43.

Lopez-Rios, F., Illei, P. B., Rusch, V. and Ladanyi, M. (2004). Evidence against a role for SV40 infection in human mesotheliomas and high risk of false-positive PCR results owing to presence of SV40 sequences in common laboratory plasmids, *Lancet,* **364,** 1157-66.

Lossos, I. S., Czerwinski, D. K., Alizadeh, A. A., Wechser, M. A., Tibshirani, R., Botstein, D. and Levy, R. (2004). Prediction of survival in diffuse large-B-cell lymphoma based on the expression of six genes, *N Engl J Med,* **350,** 1828-37.

Lu, C., Qiu, F., Zhou, H., Peng, Y., Hao, W., Xu, J., Yuan, J., Wang, S., Qiang, B., Xu, C. and Peng, X. (2006a). Identification and characterization of selenoprotein K: An antioxidant in cardiomyocytes, *FEBS Lett,* **580,** 5189-97.

Lu, J., Jiang, C., Kaeck, M., Ganther, H., Vadhanavikit, S., Ip, C. and Thompson, H. (1995). Dissociation of the genotoxic and growth inhibitory effects of selenium, *Biochem Pharmacol,* **50,** 213-9.

Lu, J., Kaeck, M., Jiang, C., Wilson, A. C. and Thompson, H. J. (1994). Selenite induction of DNA strand breaks and apoptosis in mouse leukemic L1210 cells, *Biochem Pharmacol,* **47,** 1531-5.

Lu, J., Papp, L. V., Fang, J., Rodriguez-Nieto, S., Zhivotovsky, B. and Holmgren, A. (2006b). Inhibition of Mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity, *Cancer Res,* **66,** 4410-8.

Lu, Y. P., Lou, Y. R., Yen, P., Newmark, H. L., Mirochnitchenko, O. I., Inouye, M. and Huang, M. T. (1997). Enhanced skin carcinogenesis in transgenic mice with high expression of glutathione peroxidase or both glutathione peroxidase and superoxide dismutase, *Cancer Res,* **57,** 1468-74.

Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatula, S., Okimoto, R. A., Brannigan, B. W., Harris, P. L., Haserlat, S. M., Supko, J. G., Haluska, F. G., Louis, D. N., Christiani, D. C., Settleman, J. and Haber, D. A. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib, *N Engl J Med,* **350,** 2129-39.

Mabed, M., Aref, S., Fouda, M. and El-Sharawy, S. (2004). Chlorambucil plus theophylline vs chlorambucil alone as a front line therapy for B-cell chronic lymphatic leukemia, *Leuk Lymphoma,* **45,** 2029-35.

MacKenzie, J., Wilson, K. S., Perry, J., Gallagher, A. and Jarrett, R. F. (2003). Association between simian virus 40 DNA and lymphoma in the United kingdom, *J Natl Cancer Inst,* **95,** 1001-3.

Magrath, I. (1990). The pathogenesis of Burkitt's lymphoma, *Adv Cancer Res,* **55,** 133-270.

Maguire, R. T., Robins, T. S., Thorgeirsson, S. S. and Heilman, C. A. (1983). Expression of cellular myc and mos genes in undifferentiated B cell lymphomas of Burkitt and non-Burkitt types, *Proc Natl Acad Sci U S A,* **80,** 1947-50.

Maloney, D. G., Grillo-Lopez, A. J., Bodkin, D. J., White, C. A., Liles, T. M., Royston, I., Varns, C., Rosenberg, J. and Levy, R. (1997). IDEC-C2B8: results of a phase I multiple-dose trial in patients with relapsed non-Hodgkin's lymphoma, *J Clin Oncol,* **15,** 3266-74.

Malouf, M. A., Chhajed, P. N., Hopkins, P., Plit, M., Turner, J. and Glanville, A. R. (2002). Anti-viral prophylaxis reduces the incidence of lymphoproliferative disease in lung transplant recipients, *J Heart Lung Transplant,* **21,** 547-54.

Malumbres, R., Johnson, N. A., Sehn, L. H., Natkunam, Y., Tibshirani, R., Briones, J., Connors, J. M., Levy, R., Gascoyne, R. D. and Lossos, I. S. (2007). Paraffin-Based 6-Gene Model Predicts Outcome of Diffuse Large B-Cell Lymphoma Patients Treated with R-CHOP., *ASH Annual Meeting Abstracts,* **110,** 49-.

Manganiello, V. C., Taira, M., Degerman, E. and Belfrage, P. (1995). Type III cGMP-inhibited cyclic nucleotide phosphodiesterases (PDE3 gene family), *Cell Signal,* **7,** 445-55.

Manning, C. D., Burman, M., Christensen, S. B., Cieslinski, L. B., Essayan, D. M., Grous, M., Torphy, T. J. and Barnette, M. S. (1999). Suppression of human inflammatory cell function by subtype-selective PDE4 inhibitors correlates with inhibition of PDE4A and PDE4B, *Br J Pharmacol,* **128,** 1393-8.

Martin, D. A., Zheng, L., Siegel, R. M., Huang, B., Fisher, G. H., Wang, J., Jackson, C. E., Puck, J. M., Dale, J., Straus, S. E., Peter, M. E., Krammer, P. H., Fesik, S. and Lenardo, M. J. (1999). Defective CD95/APO-1/Fas signal complex formation in the human autoimmune lymphoproliferative syndrome, type Ia, *Proc Natl Acad Sci U S A,* **96,** 4552-7.

Martinez, J. C., Piris, M. A., Sanchez-Beato, M., Villuendas, R., Orradre, J. L., Algara, P., Sanchez-Verde, L. and Martinez, P. (1993). Retinoblastoma (Rb) gene product expression in lymphomas. Correlation with Ki67 growth fraction, *J Pathol,* **169,** 405-12.

Martinez-Climent, J. A., Alizadeh, A. A., Segraves, R., Blesa, D., Rubio-Moscardo, F., Albertson, D. G., Garcia-Conde, J., Dyer, M. J., Levy, R., Pinkel, D. and Lossos, I. S. (2003). Transformation of follicular lymphoma to diffuse large cell lymphoma is associated with a heterogeneous set of DNA copy number and gene expression alterations, *Blood,* **101,** 3109-17.

Matsui, S. (2006). Predicting survival outcomes using subsets of significant genes in prognostic marker studies with microarrays, *BMC Bioinformatics,* **7,** 156.

Matthews, J. N., Altman, D. G., Campbell, M. J. and Royston, P. (1990). Analysis of serial measurements in medical research, *Bmj,* **300,** 230-5.

McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn, J. P. and Korsmeyer, S. J. (1989). bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation, *Cell,* **57,** 79-88.

McDonnell, T. J. and Korsmeyer, S. J. (1991). Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18), *Nature,* **349,** 254-6.

McDuffie, H. H., Pahwa, P., McLaughlin, J. R., Spinelli, J. J., Fincham, S., Dosman, J. A., Robson, D., Skinnider, L. F. and Choi, N. W. (2001). Non-Hodgkin's lymphoma and specific pesticide exposures in men: cross-Canada study of pesticides and health, *Cancer Epidemiol Biomarkers Prev,* **10,** 1155-63.

McKeehan, W. L., Hamilton, W. G. and Ham, R. G. (1976). Selenium is an essential trace nutrient for growth of WI-38 diploid human fibroblasts, *Proc Natl Acad Sci U S A,* **73,** 2023-7.

Medina, D. (1986). Mechanisms of selenium inhibition of tumorigenesis, *Adv Exp Med Biol,* **206,** 465-72.

Medina, D. and Morrison, D. G. (1988). Current ideas on selenium as a chemopreventive agent, *Pathol Immunopathol Res,* **7,** 187-99.

Medina, D. and Oborn, C. J. (1984). Selenium inhibition of DNA synthesis in mouse mammary epithelial cell line YN-4, *Cancer Res,* **44,** 4361-5.

Medina, D., Thompson, H., Ganther, H. and Ip, C. (2001). Se-methylselenocysteine: a new compound for chemoprevention of breast cancer, *Nutr Cancer,* **40,** 12-7.

Mellor, H. and Parker, P. J. (1998). The extended protein kinase C superfamily, *Biochem J,* **332 (Pt 2),** 281-92.

Melnyk, A., Rodriguez, A., Pugh, W. C. and Cabannillas, F. (1997). Evaluation of the Revised European-American Lymphoma classification confirms the clinical relevance of immunophenotype in 560 cases of aggressive non-Hodgkin's lymphoma, *Blood,* **89,** 4514-20.

Menkes, M. S., Comstock, G. W., Vuilleumier, J. P., Helsing, K. J., Rider, A. A. and Brookmeyer, R. (1986). Serum beta-carotene, vitamins A and E, selenium, and the risk of lung cancer, *N Engl J Med,* **315,** 1250-4.

Menter, D. G., Sabichi, A. L. and Lippman, S. M. (2000). Selenium effects on prostate cell growth, *Cancer Epidemiol Biomarkers Prev,* **9,** 1171-82.

Merwin, J. R., Mustacich, D. J., Muller, E. G., Pearson, G. D. and Merrill, G. F. (2002). Reporter gene transactivation by human p53 is inhibited in thioredoxin reductase null yeast by a mechanism associated with thioredoxin oxidation and independent of changes in the redox state of glutathione, *Carcinogenesis,* **23,** 1609-15.

Mery, P. F., Pavoine, C., Pecker, F. and Fischmeister, R. (1995). Erythro-9-(2-hydroxy-3-nonyl)adenine inhibits cyclic GMP-stimulated phosphodiesterase in isolated cardiac myocytes, *Mol Pharmacol,* **48,** 121-30.

Mestre-Escorihuela, C., Rubio-Moscardo, F., Richter, J. A., Siebert, R., Climent, J., Fresquet, V., Beltran, E., Agirre, X., Marugan, I., Marin, M., Rosenwald, A., Sugimoto, K. J., Wheat, L. M., Karran, E. L., Garcia, J. F., Sanchez, L., Prosper, F., Staudt, L. M., Pinkel, D., Dyer, M. J. and Martinez-Climent, J. A. (2007). Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas, *Blood,* **109,** 271-80.

Meuillet, E. J., Mahadevan, D., Berggren, M., Coon, A. and Powis, G. (2004). Thioredoxin-1 binds to the C2 domain of PTEN inhibiting PTEN's lipid phosphatase activity and membrane binding: a mechanism for the functional loss of PTEN's tumor suppressor activity, *Arch Biochem Biophys,* **429,** 123-33.

Meyer, M. R., Angele, A., Kremmer, E., Kaupp, U. B. and Muller, F. (2000). A cGMP-signaling pathway in a subset of olfactory sensory neurons, *Proc Natl Acad Sci U S A,* **97,** 10595-600.

Michor, F., Frank, S. A., May, R. M., Iwasa, Y. and Nowak, M. A. (2003). Somatic selection for and against cancer, *J Theor Biol,* **225,** 377-82.

Migliazza, A., Martinotti, S., Chen, W., Fusco, C., Ye, B. H., Knowles, D. M., Offit, K., Chaganti, R. S. and Dalla-Favera, R. (1995). Frequent somatic hypermutation of the 5' noncoding region of the BCL6 gene in B-cell lymphoma, *Proc Natl Acad Sci U S A,* **92,** 12520-4.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P. and Moll, U. M. (2003). p53 has a direct apoptogenic role at the mitochondria, *Mol Cell,* **11,** 577-90.

Miki, T., Kawamata, N., Hirosawa, S. and Aoki, N. (1994). Gene involved in the 3q27 translocation associated with B-cell lymphoma, BCL5, encodes a Kruppel-like zinc-finger protein, *Blood,* **83,** 26-32.

Miller, T. P., Grogan, T. M., Dahlberg, S., Spier, C. M., Braziel, R. M., Banks, P. M., Foucar, K., Kjeldsberg, C. R., Levy, N. and Nathwani, B. N. (1994). Prognostic significance of the Ki-67-associated proliferative antigen in aggressive non-Hodgkin's lymphomas: a prospective Southwest Oncology Group trial, *Blood,* **83,** 1460-6.

Minerals, E. G. o. V. a. (2003). In *Safe Upper Levels for Vitamins and Minerals*, Vol. 1 Food Standards Agency, London, pp. 232-239.

Mischak, H., Kolch, W., Goodnight, J., Davidson, W. F., Rapp, U., Rose-John, S. and Mushinski, J. F. (1991). Expression of protein kinase C genes in hemopoietic cells is cell-type- and B cell-differentiation stage specific, *J Immunol,* **147,** 3981-7.

Mitelman, F., Johansson, B. and Mertens, F. (1994) *catalog of chromosomal aberrations in cancer,* Wiley-Liss, New York.

Miyamoto, A., Nakayama, K., Imaki, H., Hirose, S., Jiang, Y., Abe, M., Tsukiyama, T., Nagahama, H., Ohno, S., Hatakeyama, S. and Nakayama, K. I. (2002). Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cdelta, *Nature,* **416,** 865-9.

Moghadaszadeh, B. and Beggs, A. H. (2006). Selenoproteins and their impact on human health through diverse physiological pathways, *Physiology (Bethesda),* **21,** 307-15.

Moller, M. B., Gerdes, A. M., Skjodt, K., Mortensen, L. S. and Pedersen, N. T. (1999a). Disrupted p53 function as predictor of treatment failure and poor prognosis in B- and T-cell non-Hodgkin's lymphoma, *Clin Cancer Res,* **5,** 1085-91.

Moller, M. B., Ino, Y., Gerdes, A. M., Skjodt, K., Louis, D. N. and Pedersen, N. T. (1999b). Aberrations of the p53 pathway components p53, MDM2 and CDKN2A appear independent in diffuse large B cell lymphoma, *Leukemia,* **13,** 453-9.

Moller, M. B., Kania, P. W., Ino, Y., Gerdes, A. M., Nielsen, O., Louis, D. N., Skjodt, K. and Pedersen, N. T. (2000). Frequent disruption of the RB1 pathway in diffuse large B cell lymphoma: prognostic significance of E2F-1 and p16INK4A, *Leukemia,* **14,** 898-904.

Moller, M. B., Nielsen, O. and Pedersen, N. T. (1999c). Oncoprotein MDM2 overexpression is associated with poor prognosis in distinct non-Hodgkin's lymphoma entities, *Mod Pathol,* **12,** 1010-6.

Monni, O., Joensuu, H., Franssila, K., Klefstrom, J., Alitalo, K. and Knuutila, S. (1997). BCL2 overexpression associated with chromosomal amplification in diffuse large B-cell lymphoma, *Blood,* **90,** 1168-74.

Monni, O. and Knuutila, S. (2001). 11q deletions in hematological malignancies, *Leuk Lymphoma,* **40,** 259-66.

Monti, S., Savage, K. J., Kutok, J. L., Feuerhake, F., Kurtin, P., Mihm, M., Wu, B., Pasqualucci, L., Neuberg, D., Aguiar, R. C., Dal Cin, P., Ladd, C., Pinkus, G. S., Salles, G., Harris, N. L., Dalla-Favera, R., Habermann, T. M., Aster, J. C., Golub, T. R. and Shipp, M. A. (2005). Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response, *Blood,* **105,** 1851-61.

Moon, E., Lee, R., Near, R., Weintraub, L., Wolda, S. and Lerner, A. (2002). Inhibition of PDE3B augments PDE4 inhibitor-induced apoptosis in a subset of patients with chronic lymphocytic leukemia, *Clin Cancer Res,* **8,** 589-95.

Moon, E. Y. and Lerner, A. (2003). PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A, *Blood,* **101,** 4122-30.

Morris, S. M. (2002). A role for p53 in the frequency and mechanism of mutation, *Mutat Res,* **511,** 45-62.

Morris, V. C. and Levander, O. A. (1970). Selenium content of foods, *J Nutr,* **100,** 1383-8.

Morschhauser, F., Seymour, J. F., Kluin-Nelemans, H. C., Grigg, A., Wolf, M., Pfreundschuh, M., Tilly, H., Raemaekers, J., van 't Veer, M. B., Milpied, N., Cartron, G., Pezzutto, A., Spencer, A., Reyes, F. and Dreyling, M. (2008). A phase II study of enzastaurin, a protein kinase C beta inhibitor, in patients with relapsed or refractory mantle cell lymphoma, *Ann Oncol,* **19,** 247-53.

Mounier, N., Briere, J., Gisselbrecht, C., Emile, J. F., Lederlin, P., Sebban, C., Berger, F., Bosly, A., Morel, P., Tilly, H., Bouabdallah, R., Reyes, F., Gaulard, P. and Coiffier, B. (2003). Rituximab plus CHOP (R-CHOP) overcomes bcl-2--

associated resistance to chemotherapy in elderly patients with diffuse large B-cell lymphoma (DLBCL), *Blood,* **101,** 4279-84.

Murray, N. R., Davidson, L. A., Chapkin, R. S., Clay Gustafson, W., Schattenberg, D. G. and Fields, A. P. (1999). Overexpression of protein kinase C betaII induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis, *J Cell Biol,* **145,** 699-711.

Muschen, M., Re, D., Brauninger, A., Wolf, J., Hansmann, M. L., Diehl, V., Kuppers, R. and Rajewsky, K. (2000). Somatic mutations of the CD95 gene in Hodgkin and Reed-Sternberg cells, *Cancer Res,* **60,** 5640-3.

Naik, P., Karrim, J. and Hanahan, D. (1996). The rise and fall of apoptosis during multistage tumorigenesis: down-modulation contributes to tumor progression from angiogenic progenitors, *Genes Dev,* **10,** 2105-16.

Nakahara, Y., Shintani, S., Mihara, M., Ueyama, Y. and Matsumura, T. (2001). High frequency of homozygous deletion and methylation of p16(INK4A) gene in oral squamous cell carcinomas, *Cancer Lett,* **163,** 221-8.

Nalesnik, M. A. (2002). Clinicopathologic characteristics of post-transplant lymphoproliferative disorders, *Recent Results Cancer Res,* **159,** 9-18.

Navaratnam, S., Williams, G. J., Rubinger, M., Pettigrew, N. M., Mowat, M. R., Begleiter, A. and Johnston, J. B. (1998). Expression of p53 predicts treatment failure in aggressive non-Hodgkin's lymphomas, *Leuk Lymphoma,* **29,** 139-44.

Newton, K. and Strasser, A. (2000). Cell death control in lymphocytes, *Adv Immunol,* **76,** 179-226.

Nichol, C., Herdman, J., Sattar, N., O'Dwyer, P. J., St, J. O. R. D., Littlejohn, D. and Fell, G. (1998). Changes in the concentrations of plasma selenium and selenoproteins after minor elective surgery: further evidence for a negative acute phase response?, *Clin Chem,* **44,** 1764-6.

Nicholson, C. D. and Shahid, M. (1994). Inhibitors of cyclic nucleotide phosphodiesterase isoenzymes--their potential utility in the therapy of asthma, *Pulm Pharmacol,* **7,** 1-17.

Niitsu, N., Honma, Y., Iijima, K., Takagi, T., Higashihara, M., Sawada, U. and Okabe-Kado, J. (2003). Clinical significance of nm23-H1 proteins expressed on cell surface in non-Hodgkin's lymphoma, *Leukemia,* **17,** 196-202.

Niitsu, N., Okabe-Kado, J., Okamoto, M., Takagi, T., Yoshida, T., Aoki, S., Hirano, M. and Honma, Y. (2001a). Serum nm23-H1 protein as a prognostic factor in aggressive non-Hodgkin lymphoma, *Blood,* **97,** 1202-10.

Niitsu, N., Okamoto, M., Okabe-Kado, J., Takagi, T., Yoshida, T., Aoki, S., Honma, Y. and Hirano, M. (2001b). Serum nm23-H1 protein as a prognostic factor for indolent non-Hodgkin's lymphoma, *Leukemia,* **15,** 832-9.

Nikulina, E., Tidwell, J. L., Dai, H. N., Bregman, B. S. and Filbin, M. T. (2004). The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery, *Proc Natl Acad Sci U S A,* **101,** 8786-90.

Nomura, A., Heilbrun, L. K., Morris, J. S. and Stemmermann, G. N. (1987). Serum selenium and the risk of cancer, by specific sites: case-control analysis of prospective data, *J Natl Cancer Inst,* **79,** 103-8.

Nomura, A. M., Lee, J., Stemmermann, G. N. and Combs, G. F., Jr. (2000). Serum selenium and subsequent risk of prostate cancer, *Cancer Epidemiol Biomarkers Prev,* **9,** 883-7.

Non-Hodgkin's-Lymphoma-Classification-Project (1997). A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project, *Blood,* **89,** 3909-18.

O'Shea, D., O'Riain, C., Taylor, C., Waters, R., Carlotti, E., Macdougall, F., Gribben, J., Rosenwald, A., Ott, G., Rimsza, L. M., Smeland, E. B., Johnson, N., Campo, E., Greiner, T. C., Chan, W. C., Gascoyne, R. D., Wright, G., Staudt, L. M., Lister, T. A. and Fitzgibbon, J. (2008). The presence of TP53 mutation at diagnosis of follicular lymphoma identifies a high-risk group of patients with shortened time to disease progression and a poorer overall survival, *Blood*.

Oertel, S. H. and Riess, H. (2002). Immunosurveillance, immunodeficiency and lymphoproliferations, *Recent Results Cancer Res,* **159,** 1-8.

Offit, K., Louie, D. C., Parsa, N. Z., Roy, P., Leung, D., Lo Coco, F., Zelenetz, A., Dalla-Favera, R. and Chaganti, R. S. (1995). BCL6 gene rearrangement and other cytogenetic abnormalities in diffuse large cell lymphoma, *Leuk Lymphoma,* **20,** 85-9.

Offit, K., Parsa, N. Z., Filippa, D., Jhanwar, S. C. and Chaganti, R. S. (1992). t(9;14)(p13;q32) denotes a subset of low-grade non-Hodgkin's lymphoma with plasmacytoid differentiation, *Blood,* **80,** 2594-9.

Offit, K., Wong, G., Filippa, D. A., Tao, Y. and Chaganti, R. S. (1991). Cytogenetic analysis of 434 consecutively ascertained specimens of non-Hodgkin's lymphoma: clinical correlations, *Blood,* **77,** 1508-15.

Ogawa, R., Streiff, M. B., Bugayenko, A. and Kato, G. J. (2002). Inhibition of PDE4 phosphodiesterase activity induces growth suppression, apoptosis, glucocorticoid sensitivity, p53, and p21(WAF1/CIP1) proteins in human acute lymphoblastic leukemia cells, *Blood,* **99,** 3390-7.

Oh, S. H., Park, K. K., Kim, S. Y., Lee, K. J. and Lee, Y. H. (1995). Evaluation of chemopreventive effect of dietary selenium-rich egg on mouse skin tumor induced by 2'-(4-nitrophenoxy)oxirane and 12-O-tetradecanoylphorbol-13-acetate, *Carcinogenesis,* **16,** 2995-8.

Olejniczak, S. H., Hernandez-Ilizaliturri, F. J., Clements, J. L. and Czuczman, M. S. (2008). Acquired resistance to rituximab is associated with chemotherapy resistance resulting from decreased Bax and Bak expression, *Clin Cancer Res,* **14,** 1550-60.

Orsetti, B., Nugoli, M., Cervera, N., Lasorsa, L., Chuchana, P., Ursule, L., Nguyen, C., Redon, R., du Manoir, S., Rodriguez, C. and Theillet, C. (2004). Genomic and expression profiling of chromosome 17 in breast cancer reveals complex patterns of alterations and novel candidate genes, *Cancer Res,* **64,** 6453-60.

Oster, O., Schmiedel, G. and Prellwitz, W. (1988). The organ distribution of selenium in German adults, *Biol Trace Elem Res,* **15,** 23-45.

Otsuki, T., Yano, T., Clark, H. M., Bastard, C., Kerckaert, J. P., Jaffe, E. S. and Raffeld, M. (1995). Analysis of LAZ3 (BCL-6) status in B-cell non-Hodgkin's lymphomas: results of rearrangement and gene expression studies and a mutational analysis of coding region sequences, *Blood,* **85,** 2877-84.

Ott, G., Katzenberger, T., Greiner, A., Kalla, J., Rosenwald, A., Heinrich, U., Ott, M. M. and Muller-Hermelink, H. K. (1997). The t(11;18)(q21;q21) chromosome translocation is a frequent and specific aberration in low-grade but not high-grade malignant non-Hodgkin's lymphomas of the mucosa-associated lymphoid tissue (MALT-) type, *Cancer Res,* **57,** 3944-8.

Paez, J. G., Janne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M. J., Sellers, W. R., Johnson, B. E. and Meyerson, M. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy, *Science,* **304,** 1497-500.

Pagmantidis, V., Meplan, C., van Schothorst, E. M., Keijer, J. and Hesketh, J. E. (2008). Supplementation of healthy volunteers with nutritionally relevant amounts of selenium increases the expression of lymphocyte protein biosynthesis genes, *Am J Clin Nutr,* **87,** 181-9.

Pagnano, K. B., Vassallo, J., Lorand-Metze, I., Costa, F. F. and Saad, S. T. (2001). p53, Mdm2, and c-Myc overexpression is associated with a poor prognosis in aggressive non-Hodgkin's lymphomas, *Am J Hematol,* **67,** 84-92.

Park, H. S., Park, E., Kim, M. S., Ahn, K., Kim, I. Y. and Choi, E. J. (2000). Selenite inhibits the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) through a thiol redox mechanism, *J Biol Chem,* **275,** 2527-31.

Pasqualucci, L., Bhagat, G., Jankovic, M., Compagno, M., Smith, P., Muramatsu, M., Honjo, T., Morse, H. C., 3rd, Nussenzweig, M. C. and Dalla-Favera, R. (2008). AID is required for germinal center-derived lymphomagenesis, *Nat Genet,* **40,** 108-12.

Pasqualucci, L., Migliazza, A., Basso, K., Houldsworth, J., Chaganti, R. S. and Dalla-Favera, R. (2003). Mutations of the BCL6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma, *Blood,* **101,** 2914-23.

Pasqualucci, L., Neumeister, P., Goossens, T., Nanjangud, G., Chaganti, R. S., Kuppers, R. and Dalla-Favera, R. (2001). Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas, *Nature,* **412,** 341-6.

Patterson, B. H. and Levander, O. A. (1997). Naturally occurring selenium compounds in cancer chemoprevention trials: a workshop summary, *Cancer Epidemiol Biomarkers Prev,* **6,** 63-9.

Paulovich, A. G., Toczyski, D. P. and Hartwell, L. H. (1997). When checkpoints fail, *Cell,* **88,** 315-21.

Pearse, D. D., Pereira, F. C., Marcillo, A. E., Bates, M. L., Berrocal, Y. A., Filbin, M. T. and Bunge, M. B. (2004). cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury, *Nat Med,* **10,** 610-6.

Pease, A. C., Solas, D., Sullivan, E. J., Cronin, M. T., Holmes, C. P. and Fodor, S. P. (1994). Light-generated oligonucleotide arrays for rapid DNA sequence analysis, *Proc Natl Acad Sci U S A,* **91,** 5022-6.

Peltomaki, P. and de la Chapelle, A. (1997). Mutations predisposing to hereditary nonpolyposis colorectal cancer, *Adv Cancer Res,* **71,** 93-119.

Perucho, M. (1996). Cancer of the microsatellite mutator phenotype, *Biol Chem,* **377,** 675-84.

Pfreundschuh, M., Schubert, J., Ziepert, M., Schmits, R., Mohren, M., Lengfelder, E., Reiser, M., Nickenig, C., Clemens, M., Peter, N., Bokemeyer, C., Eimermacher, H., Ho, A., Hoffmann, M., Mertelsmann, R., Trumper, L., Balleisen, L., Liersch, R., Metzner, B., Hartmann, F., Glass, B., Poeschel, V., Schmitz, N., Ruebe, C., Feller, A. C. and Loeffler, M. (2008). Six versus eight cycles of bi-weekly CHOP-14 with or without rituximab in elderly patients with aggressive CD20+ B-cell lymphomas: a randomised controlled trial (RICOVER-60), *Lancet Oncol,* **9,** 105-16.

Pfreundschuh, M., Truemper, L., Kloess, M., Schmits, R., Feller, A. C., Rudolph, C., Reiser, M., Hossfeld, D. K., Metzner, B., Hasenclever, D., Schmitz, N., Glass, B., Ruebe, C. and Loeffler, M. (2004a). 2-weekly or 3-weekly CHOP Chemotherapy with or without etoposide for the Treatment of Young Patients with Good Prognosis (Normal LDH) Aggressive Lymphomas: Results of the NHL-B1 trial of the DSHNHL, *Blood*.

Pfreundschuh, M., Truemper, L., Kloess, M., Schmits, R., Feller, A. C., Ruebe, C., Rudolph, C., Reiser, M., Hossfeld, D. K., Eimermacher, H., Hasenclever, D., Schmitz, N. and Loeffler, M. (2004b). 2-weekly or 3-weekly CHOP Chemotherapy with or without Etoposide for the Treatment of Elderly Patients with Aggressive Lymphomas: Results of the NHL-B2 trial of the DSHNHL, *Blood*.

Pfreundschuh, M., Trumper, L., Osterborg, A., Pettengell, R., Trneny, M., Imrie, K., Ma, D., Gill, D., Walewski, J., Zinzani, P. L., Stahel, R., Kvaloy, S., Shpilberg, O., Jaeger, U., Hansen, M., Lehtinen, T., Lopez-Guillermo, A., Corrado, C., Scheliga, A., Milpied, N., Mendila, M., Rashford, M., Kuhnt, E. and Loeffler, M. (2006). CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell

lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group, *Lancet Oncol,* **7,** 379-91.

Phear, G., Bhattacharyya, N. P. and Meuth, M. (1996). Loss of heterozygosity and base substitution at the APRT locus in mismatch-repair-proficient and -deficient colorectal carcinoma cell lines, *Mol Cell Biol,* **16,** 6516-23.

Philip, T., Guglielmi, C., Hagenbeek, A., Somers, R., Van der Lelie, H., Bron, D., Sonneveld, P., Gisselbrecht, C., Cahn, J. Y. and Harousseau, J. L. (1995). Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma, *N Engl J Med,* **333,** 1540-5.

Piris, M. A., Pezzella, F., Martinez-Montero, J. C., Orradre, J. L., Villuendas, R., Sanchez-Beato, M., Cuena, R., Cruz, M. A., Martinez, B. and Pezella, F. (1994). p53 and bcl-2 expression in high-grade B-cell lymphomas: correlation with survival time, *Br J Cancer,* **69,** 337-41.

Poirier, K. A. and Milner, J. A. (1983). Factors influencing the antitumorigenic properties of selenium in mice, *J Nutr,* **113,** 2147-54.

Potzsch, C., Schaefer, H. E. and Lubbert, M. (1999). Familial and metachronous malignant lymphoma: absence of constitutional p53 mutations, *Am J Hematol,* **62,** 144-9.

Rademaker-Lakhai, J. M., Beerepoot, L. V., Mehra, N., Radema, S. A., van Maanen, R., Vermaat, J. S., Witteveen, E. O., Visseren-Grul, C. M., Musib, L., Enas, N., van Hal, G., Beijnen, J. H., Schellens, J. H. and Voest, E. E. (2007). Phase I pharmacokinetic and pharmacodynamic study of the oral protein kinase C beta-inhibitor enzastaurin in combination with gemcitabine and cisplatin in patients with advanced cancer, *Clin Cancer Res,* **13,** 4474-81.

Radford, J. A., Whelan, J. S., Rohatiner, A. Z., Deakin, D., Harris, M., Stansfeld, A. G., Swindell, R., Wilkinson, P. M., James, R. D. and Lister, T. A. (1994). Weekly VAPEC-B chemotherapy for high grade non-Hodgkin's lymphoma: results of treatment in 184 patients, *Ann Oncol,* **5,** 147-51.

Raffel, J., Bhattacharyya, A. K., Gallegos, A., Cui, H., Einspahr, J. G., Alberts, D. S. and Powis, G. (2003). Increased expression of thioredoxin-1 in human colorectal cancer is associated with decreased patient survival, *J Lab Clin Med,* **142,** 46-51.

Rana, M. P., Sardar, S. and Chatterjee, M. (1996). Selenomethionine in the inhibition of a transplantable murine lymphoma: reflection on hepatic drug metabolizing enzymes, *Tumour Biol,* **17,** 102-9.

Rao, C. V., Wang, C. Q., Simi, B., Rodriguez, J. G., Cooma, I., El-Bayoumy, K. and Reddy, B. S. (2001). Chemoprevention of colon cancer by a glutathione conjugate of 1,4-phenylenebis(methylene)selenocyanate, a novel organoselenium compound with low toxicity, *Cancer Res,* **61,** 3647-52.

Rao, P. H., Houldsworth, J., Dyomina, K., Parsa, N. Z., Cigudosa, J. C., Louie, D. C., Popplewell, L., Offit, K., Jhanwar, S. C. and Chaganti, R. S. (1998). Chromosomal and gene amplification in diffuse large B-cell lymphoma, *Blood,* **92,** 234-40.

Rathmell, J. C. and Goodnow, C. C. (1998). The in vivo balance between B cell clonal expansion and elimination is regulated by CD95 both on B cells and in their micro-environment, *Immunol Cell Biol,* **76,** 387-94.

Rayman, M. P. (2000). The importance of selenium to human health, *Lancet,* **356,** 233-41.

Rayman, M. P. and Clark, L. C. (2000). In *Trace Elements in Man and Animals 10: Proceedings of the tenth international symposium on trace elements in man and animals.*(Eds, Rousel, A. M., Favier, A. and Anderson, R. A.) Plenum Press, New York, pp. 575-80.

Reddy, B. S., Upadhyaya, P., Simi, B. and Rao, C. V. (1994). Evaluation of organoselenium compounds for potential chemopreventive properties in colon carcinogenesis, *Anticancer Res,* **14,** 2509-14.

Redman, C., Xu, M. J., Peng, Y. M., Scott, J. A., Payne, C., Clark, L. C. and Nelson, M. A. (1997). Involvement of polyamines in selenomethionine induced apoptosis and mitotic alterations in human tumor cells, *Carcinogenesis,* **18,** 1195-202.

Reeves, M. L., Leigh, B. K. and England, P. J. (1987). The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. Implications for the mechanism of action of selective phosphodiesterase inhibitors, *Biochem J,* **241,** 535-41.

Reff, M. E., Carner, K., Chambers, K. S., Chinn, P. C., Leonard, J. E., Raab, R., Newman, R. A., Hanna, N. and Anderson, D. R. (1994). Depletion of B cells in

vivo by a chimeric mouse human monoclonal antibody to CD20, *Blood,* **83,** 435-45.

Reljic, R., Wagner, S. D., Peakman, L. J. and Fearon, D. T. (2000). Suppression of signal transducer and activator of transcription 3-dependent B lymphocyte terminal differentiation by BCL-6, *J Exp Med,* **192,** 1841-8.

Remstein, E. D., James, C. D. and Kurtin, P. J. (2000). Incidence and subtype specificity of API2-MALT1 fusion translocations in extranodal, nodal, and splenic marginal zone lymphomas, *Am J Pathol,* **156,** 1183-8.

Richter, M., Weiss, M., Weinberger, I., Furstenberger, G. and Marian, B. (2001). Growth inhibition and induction of apoptosis in colorectal tumor cells by cyclooxygenase inhibitors, *Carcinogenesis,* **22,** 17-25.

Ringstad, J., Jacobsen, B. K., Tretli, S. and Thomassen, Y. (1988). Serum selenium concentration associated with risk of cancer, *J Clin Pathol,* **41,** 454-7.

Robertson, M. J., Kahl, B. S., Vose, J. M., de Vos, S., Laughlin, M., Flynn, P. J., Rowland, K., Cruz, J. C., Goldberg, S. L., Musib, L., Darstein, C., Enas, N., Kutok, J. L., Aster, J. C., Neuberg, D., Savage, K. J., LaCasce, A., Thornton, D., Slapak, C. A. and Shipp, M. A. (2007). Phase II study of enzastaurin, a protein kinase C beta inhibitor, in patients with relapsed or refractory diffuse large B-cell lymphoma, *J Clin Oncol,* **25,** 1741-6.

Roix, J. J., McQueen, P. G., Munson, P. J., Parada, L. A. and Misteli, T. (2003). Spatial proximity of translocation-prone gene loci in human lymphomas, *Nat Genet,* **34,** 287-91.

Rosenberg, B., VanCamp, L., Trosko, J. E. and Mansour, V. H. (1969). Platinum compounds: a new class of potent antitumour agents, *Nature,* **222,** 385-6.

Rosenwald, A., Wright, G., Chan, W. C., Connors, J. M., Campo, E., Fisher, R. I., Gascoyne, R. D., Muller-Hermelink, H. K., Smeland, E. B., Giltnane, J. M., Hurt, E. M., Zhao, H., Averett, L., Yang, L., Wilson, W. H., Jaffe, E. S., Simon, R., Klausner, R. D., Powell, J., Duffey, P. L., Longo, D. L., Greiner, T. C., Weisenburger, D. D., Sanger, W. G., Dave, B. J., Lynch, J. C., Vose, J., Armitage, J. O., Montserrat, E., Lopez-Guillermo, A., Grogan, T. M., Miller, T. P., LeBlanc, M., Ott, G., Kvaloy, S., Delabie, J., Holte, H., Krajci, P., Stokke, T. and Staudt, L. M. (2002). The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma, *N Engl J Med,* **346,** 1937-47.

Rosenwald, A., Wright, G., Leroy, K., Yu, X., Gaulard, P., Gascoyne, R. D., Chan, W. C., Zhao, T., Haioun, C., Greiner, T. C., Weisenburger, D. D., Lynch, J. C., Vose, J., Armitage, J. O., Smeland, E. B., Kvaloy, S., Holte, H., Delabie, J., Campo, E., Montserrat, E., Lopez-Guillermo, A., Ott, G., Muller-Hermelink, H. K., Connors, J. M., Braziel, R., Grogan, T. M., Fisher, R. I., Miller, T. P., LeBlanc, M., Chiorazzi, M., Zhao, H., Yang, L., Powell, J., Wilson, W. H., Jaffe, E. S., Simon, R., Klausner, R. D. and Staudt, L. M. (2003). Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma, *J Exp Med,* **198,** 851-62.

Ross, J. S., Fletcher, J. A., Linette, G. P., Stec, J., Clark, E., Ayers, M., Symmans, W. F., Pusztai, L. and Bloom, K. J. (2003). The Her-2/neu gene and protein in breast cancer 2003: biomarker and target of therapy, *Oncologist,* **8,** 307-25.

Roy, M., Kiremidjian-Schumacher, L., Wishe, H. I., Cohen, M. W. and Stotzky, G. (1994). Supplementation with selenium and human immune cell functions. I. Effect on lymphocyte proliferation and interleukin 2 receptor expression, *Biol Trace Elem Res,* **41,** 103-14.

Ruas, M. and Peters, G. (1998). The p16INK4a/CDKN2A tumor suppressor and its relatives, *Biochim Biophys Acta,* **1378,** F115-77.

Rubio-Moscardo, F., Blesa, D., Mestre, C., Siebert, R., Balasas, T., Benito, A., Rosenwald, A., Climent, J., Martinez, J. I., Schilhabel, M., Karran, E. L., Gesk, S., Esteller, M., deLeeuw, R., Staudt, L. M., Fernandez-Luna, J. L., Pinkel, D., Dyer, M. J. and Martinez-Climent, J. A. (2005). Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes, *Blood,* **106,** 3214-22.

Rudolf, E., Radocha, J., Cervinka, M. and Cerman, J. (2004). Combined effect of sodium selenite and campthotecin on cervical carcinoma cells, *Neoplasma,* **51,** 127-35.

Rybalkin, S. D., Yan, C., Bornfeldt, K. E. and Beavo, J. A. (2003). Cyclic GMP phosphodiesterases and regulation of smooth muscle function, *Circ Res,* **93,** 280-91.

Saez, A., Sanchez, E., Sanchez-Beato, M., Cruz, M. A., Chacon, I., Munoz, E., Camacho, F. I., Martinez-Montero, J. C., Mollejo, M., Garcia, J. F. and Piris, M.

A. (1999). p27KIP1 is abnormally expressed in Diffuse Large B-cell Lymphomas and is associated with an adverse clinical outcome, *Br J Cancer,* **80,** 1427-34.

Saez, A. I., Artiga, M. J., Romero, C., Rodriguez, S., Cigudosa, J. C., Perez-Rosado, A., Fernandez, I., Sanchez-Beato, M., Sanchez, E., Mollejo, M. and Piris, M. A. (2003). Development of a real-time reverse transcription polymerase chain reaction assay for c-myc expression that allows the identification of a subset of c-myc+ diffuse large B-cell lymphoma, *Lab Invest,* **83,** 143-52.

Saez, A. I., Saez, A. J., Artiga, M. J., Perez-Rosado, A., Camacho, F. I., Diez, A., Garcia, J. F., Fraga, M., Bosch, R., Rodriguez-Pinilla, S. M., Mollejo, M., Romero, C., Sanchez-Verde, L., Pollan, M. and Piris, M. A. (2004). Building an outcome predictor model for diffuse large B-cell lymphoma, *Am J Pathol,* **164,** 613-22.

Saito, Y., Sato, N., Hirashima, M., Takebe, G., Nagasawa, S. and Takahashi, K. (2004). Domain structure of bi-functional Selenoprotein P, *Biochem J,* **Pt**.

Salar, A., Fernandez de Sevilla, A., Romagosa, V., Domingo-Claros, A., Gonzalez-Barca, E., Pera, J., Climent, J. and Granena, A. (1998). Diffuse large B-cell lymphoma: is morphologic subdivision useful in clinical management?, *Eur J Haematol,* **60,** 202-8.

Salonen, J. T., Alfthan, G., Huttunen, J. K. and Puska, P. (1984). Association between serum selenium and the risk of cancer, *Am J Epidemiol,* **120,** 342-9.

Salonen, J. T., Salonen, R., Lappetelainen, R., Maenpaa, P. H., Alfthan, G. and Puska, P. (1985). Risk of cancer in relation to serum concentrations of selenium and vitamins A and E: matched case-control analysis of prospective data, *Br Med J (Clin Res Ed),* **290,** 417-20.

Salvesen, G. S. and Duckett, C. S. (2002). IAP proteins: blocking the road to death's door, *Nat Rev Mol Cell Biol,* **3,** 401-10.

Sanchez, E., Chacon, I., Plaza, M. M., Munoz, E., Cruz, M. A., Martinez, B., Lopez, L., Martinez-Montero, J. C., Orradre, J. L., Saez, A. I., Garcia, J. F. and Piris, M. A. (1998). Clinical outcome in diffuse large B-cell lymphoma is dependent on the relationship between different cell-cycle regulator proteins, *J Clin Oncol,* **16,** 1931-9.

Sanchez-Beato, M., Saez, A. I., Navas, I. C., Algara, P., Sol Mateo, M., Villuendas, R., Camacho, F., Sanchez-Aguilera, A., Sanchez, E. and Piris, M. A. (2001). Overall survival in aggressive B-cell lymphomas is dependent on the accumulation of alterations in p53, p16, and p27, *Am J Pathol,* **159,** 205-13.

Sandhu, C., Garbe, J., Bhattacharya, N., Daksis, J., Pan, C. H., Yaswen, P., Koh, J., Slingerland, J. M. and Stampfer, M. R. (1997). Transforming growth factor beta stabilizes p15INK4B protein, increases p15INK4B-cdk4 complexes, and inhibits cyclin D1-cdk4 association in human mammary epithelial cells, *Mol Cell Biol,* **17,** 2458-67.

Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M. and Hla, T. (1995). Expression of cyclooxygenase-1 and -2 in human colorectal cancer, *Cancer Res,* **55,** 3785-9.

Savill, J. and Fadok, V. (2000). Corpse clearance defines the meaning of cell death, *Nature,* **407,** 784-8.

Sax, J. K., Fei, P., Murphy, M. E., Bernhard, E., Korsmeyer, S. J. and El-Deiry, W. S. (2002). BID regulation by p53 contributes to chemosensitivity, *Nat Cell Biol,* **4,** 842-9.

Schena, M., Shalon, D., Davis, R. W. and Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray, *Science,* **270,** 467-70.

Schimmer, A. D., Hedley, D. W., Penn, L. Z. and Minden, M. D. (2001). Receptor- and mitochondrial-mediated apoptosis in acute leukemia: a translational view, *Blood,* **98,** 3541-53.

Schrauzer, G. N. (1992). Selenium. Mechanistic aspects of anticarcinogenic action, *Biol Trace Elem Res,* **33,** 51-62.

Schrauzer, G. N. (2000). Selenomethionine: a review of its nutritional significance, metabolism and toxicity, *J Nutr,* **130,** 1653-6.

Schrauzer, G. N. and White, D. A. (1978). Selenium in human nutrition: dietary intakes and effects of supplementation, *Bioinorg Chem,* **8,** 303-18.

Schrauzer, G. N., White, D. A. and Schneider, C. J. (1977). Cancer mortality correlation studies--III: statistical associations with dietary selenium intakes, *Bioinorg Chem,* **7,** 23-31.

Scott, A. I., Perini, A. F., Shering, P. A. and Whalley, L. J. (1991). In-patient major depression: is rolipram as effective as amitriptyline?, *Eur J Clin Pharmacol,* **40,** 127-9.

SEER and Surveillance, E. a. E. R. P. (2002). Natiional Cancer Institute, Surveillance Research Program, Cancer Statistics Branch, Bethesda.

Senftleben, U., Cao, Y., Xiao, G., Greten, F. R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S. C. and Karin, M. (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway, *Science,* **293,** 1495-9.

Seoane, J., Le, H. V. and Massague, J. (2002). Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage, *Nature,* **419,** 729-34.

Seymour, A. B., Hruban, R. H., Redston, M., Caldas, C., Powell, S. M., Kinzler, K. W., Yeo, C. J. and Kern, S. E. (1994). Allelotype of pancreatic adenocarcinoma, *Cancer Res,* **54,** 2761-4.

Shaffer, A. L., Yu, X., He, Y., Boldrick, J., Chan, E. P. and Staudt, L. M. (2000). BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control, *Immunity,* **13,** 199-212.

Shamberger, R. J. and Frost, D. V. (1969). Possible protective effect of selenium against human cancer, *Can Med Assoc J,* **100,** 682.

Shen, H. M., Ding, W. X. and Ong, C. N. (2002). Intracellular glutathione is a cofactor in methylseleninic acid-induced apoptotic cell death of human hepatoma HEPG(2) cells, *Free Radic Biol Med,* **33,** 552-61.

Shen, Q., Fan, L. and Newburger, P. E. (2006). Nuclease sensitive element binding protein 1 associates with the selenocysteine insertion sequence and functions in mammalian selenoprotein translation, *J Cell Physiol,* **207,** 775-83.

Shen, Y., Iqbal, J., Huang, J. Z., Zhou, G. and Chan, W. C. (2004). BCL2 protein expression parallels its mRNA level in normal and malignant B cells, *Blood*.

Sherr, C. J. and McCormick, F. (2002). The RB and p53 pathways in cancer, *Cancer Cell,* **2,** 103-12.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity, *Nat Rev Cancer,* **3,** 155-68.

Shipp, M. A., Ross, K. N., Tamayo, P., Weng, A. P., Kutok, J. L., Aguiar, R. C., Gaasenbeek, M., Angelo, M., Reich, M., Pinkus, G. S., Ray, T. S., Koval, M. A., Last, K. W., Norton, A., Lister, T. A., Mesirov, J., Neuberg, D. S., Lander, E. S., Aster, J. C. and Golub, T. R. (2002). Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning, *Nat Med,* **8,** 68-74.

Shipp, M. A., Yeap, B. Y., Harrington, D. P., Klatt, M. M., Pinkus, G. S., Jochelson, M. S., Rosenthal, D. S., Skarin, A. T. and Canellos, G. P. (1990). The m-BACOD combination chemotherapy regimen in large-cell lymphoma: analysis of the completed trial and comparison with the M-BACOD regimen, *J Clin Oncol,* **8,** 84-93.

Shivakumar, L. and Armitage, J. O. (2006). Bcl-2 gene expression as a predictor of outcome in diffuse large B-cell lymphoma, *Clin Lymphoma Myeloma,* **6,** 455-7.

Sieja, K. and Talerczyk, M. (2004). Selenium as an element in the treatment of ovarian cancer in women receiving chemotherapy, *Gynecol Oncol,* **93,** 320-7.

Sinha, R., Kiley, S. C., Lu, J. X., Thompson, H. J., Moraes, R., Jaken, S. and Medina, D. (1999). Effects of methylselenocysteine on PKC activity, cdk2 phosphorylation and gadd gene expression in synchronized mouse mammary epithelial tumor cells, *Cancer Lett,* **146,** 135-45.

Sinha, R., Unni, E., Ganther, H. E. and Medina, D. (2001). Methylseleninic acid, a potent growth inhibitor of synchronized mouse mammary epithelial tumor cells *in vitro*, *Biochem Pharmacol,* **61,** 311-7.

Smart, D. K., Ortiz, K. L., Mattson, D., Bradbury, C. M., Bisht, K. S., Sieck, L. K., Brechbiel, M. W. and Gius, D. (2004). Thioredoxin reductase as a potential molecular target for anticancer agents that induce oxidative stress, *Cancer Res,* **64,** 6716-24.

Smith, M. L. and Seo, Y. R. (2002). p53 regulation of DNA excision repair pathways, *Mutagenesis,* **17,** 149-56.

Smith, P. G., Wang, F., Wilkinson, K. N., Savage, K. J., Klein, U., Neuberg, D. S., Bollag, G., Shipp, M. A. and Aguiar, R. C. (2005). The phosphodiesterase PDE4B limits cAMP-associated PI3K/AKT-dependent apoptosis in diffuse large B-cell lymphoma, *Blood,* **105,** 308-16.

Smith, S. J., Cieslinski, L. B., Newton, R., Donnelly, L. E., Fenwick, P. S., Nicholson, A. G., Barnes, P. J., Barnette, M. S. and Giembycz, M. A. (2004). Discovery of BRL 50481 [3-(N,N-dimethylsulfonamido)-4-methyl-nitrobenzene], a selective inhibitor of phosphodiesterase 7: *in vitro* studies in human monocytes, lung macrophages, and CD8+ T-lymphocytes, *Mol Pharmacol,* **66,** 1679-89.

Smyth, G. K., Yang, Y. H. and Speed, T. (2003). Statistical issues in cDNA microarray data analysis, *Methods Mol Biol,* **224,** 111-36.

Soderling, S. H., Bayuga, S. J. and Beavo, J. A. (1998). Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase, *Proc Natl Acad Sci U S A,* **95,** 8991-6.

Soderling, S. H., Bayuga, S. J. and Beavo, J. A. (1999). Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A, *Proc Natl Acad Sci U S A,* **96,** 7071-6.

Soderling, S. H. and Beavo, J. A. (2000). Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions, *Curr Opin Cell Biol,* **12,** 174-9.

Sohn, S. K., Jung, J. T., Kim, D. H., Kim, J. G., Kwak, E. K., Park, T., Shin, D. G., Sohn, K. R. and Lee, K. B. (2003). Prognostic significance of bcl-2, bax, and p53 expression in diffuse large B-cell lymphoma, *Am J Hematol,* **73,** 101-7.

Soll, D. (1988). Genetic code: enter a new amino acid, *Nature,* **331,** 662-3.

Souness, J. E., Aldous, D. and Sargent, C. (2000). Immunosuppressive and anti-inflammatory effects of cyclic AMP phosphodiesterase (PDE) type 4 inhibitors, *Immunopharmacology,* **47,** 127-62.

Spallholz, J. E. (1994). On the nature of selenium toxicity and carcinostatic activity, *Free Radic Biol Med,* **17,** 45-64.

Stacey, P., Rulten, S., Dapling, A. and Phillips, S. C. (1998). Molecular cloning and expression of human cGMP-binding cGMP-specific phosphodiesterase (PDE5), *Biochem Biophys Res Commun,* **247,** 249-54.

Stadtman, T. C. (1996). Selenocysteine, *Annu Rev Biochem,* **65,** 83-100.

Stapleton, S. R. (2000). Selenium: an insulin-mimetic, *Cell Mol Life Sci,* **57,** 1874-9.

Stapleton, S. R., Garlock, G. L., Foellmi-Adams, L. and Kletzien, R. F. (1997). Selenium: potent stimulator of tyrosyl phosphorylation and activator of MAP kinase, *Biochim Biophys Acta,* **1355,** 259-69.

Starczynski, J., Simmons, W., Flavell, J. R., Byrd, P. J., Stewart, G. S., Kullar, H. S., Groom, A., Crocker, J., Moss, P. A., Reynolds, G. M., Glavina-Durdov, M., Taylor, A. M., Fegan, C., Stankovic, T. and Murray, P. G. (2003). Variations in ATM protein expression during normal lymphoid differentiation and among B-cell-derived neoplasias, *Am J Pathol,* **163,** 423-32.

Stashenko, P., Nadler, L. M., Hardy, R. and Schlossman, S. F. (1980). Characterization of a human B lymphocyte-specific antigen, *J Immunol,* **125,** 1678-85.

Staudt, L. M. and Dave, S. (2005). The biology of human lymphoid malignancies revealed by gene expression profiling, *Adv Immunol,* **87,** 163-208.

Steeg, P. S. and Zhou, Q. (1998). Cyclins and breast cancer, *Breast Cancer Res Treat,* **52,** 17-28.

Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K. and Levin, B. (2000). The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis, *N Engl J Med,* **342,** 1946-52.

Steinbrenner, H., Bilgic, E., Alili, L., Sies, H. and Brenneisen, P. (2006). Selenoprotein P protects endothelial cells from oxidative damage by stimulation of glutathione peroxidase expression and activity, *Free Radic Res,* **40,** 936-43.

Stewart, C. L., Soria, A. M. and Hamel, P. A. (2001). Integration of the pRB and p53 cell cycle control pathways, *J Neurooncol,* **51,** 183-204.

Stewart, M. S., Spallholz, J. E., Neldner, K. H. and Pence, B. C. (1999). Selenium compounds have disparate abilities to impose oxidative stress and induce apoptosis, *Free Radic Biol Med,* **26,** 42-8.

Strasser, A., Harris, A. W., Bath, M. L. and Cory, S. (1990). Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2, *Nature,* **348,** 331-3.

Strasser, A., Harris, A. W. and Cory, S. (1993). E mu-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells, *Oncogene,* **8,** 1-9.

Strasser, A., Harris, A. W., Huang, D. C., Krammer, P. H. and Cory, S. (1995). Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis, *Embo J,* **14,** 6136-47.

Strasser, A., O'Connor, L. and Dixit, V. M. (2000). Apoptosis signaling, *Annu Rev Biochem,* **69,** 217-45.

Straus, S. E., Jaffe, E. S., Puck, J. M., Dale, J. K., Elkon, K. B., Rosen-Wolff, A., Peters, A. M., Sneller, M. C., Hallahan, C. W., Wang, J., Fischer, R. E., Jackson, C. M., Lin, A. Y., Baumler, C., Siegert, E., Marx, A., Vaishnaw, A. K., Grodzicky, T., Fleisher, T. A. and Lenardo, M. J. (2001). The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis, *Blood,* **98,** 194-200.

Strauss, S. J., Higginbottom, K., Juliger, S., Maharaj, L., Allen, P., Schenkein, D., Lister, T. A. and Joel, S. P. (2007). The proteasome inhibitor bortezomib acts independently of p53 and induces cell death via apoptosis and mitotic catastrophe in B-cell lymphoma cell lines, *Cancer Res,* **67,** 2783-90.

Streubel, B., Lamprecht, A., Dierlamm, J., Cerroni, L., Stolte, M., Ott, G., Raderer, M. and Chott, A. (2003). T(14;18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma, *Blood,* **101,** 2335-9.

Su, T. T., Guo, B., Kawakami, Y., Sommer, K., Chae, K., Humphries, L. A., Kato, R. M., Kang, S., Patrone, L., Wall, R., Teitell, M., Leitges, M., Kawakami, T. and Rawlings, D. J. (2002). PKC-beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling, *Nat Immunol,* **3,** 780-6.

Sudo, T., Tachibana, K., Toga, K., Tochizawa, S., Inoue, Y., Kimura, Y. and Hidaka, H. (2000). Potent effects of novel anti-platelet aggregatory cilostamide analogues on recombinant cyclic nucleotide phosphodiesterase isozyme activity, *Biochem Pharmacol,* **59,** 347-56.

Sun, Z., Arendt, C. W., Ellmeier, W., Schaeffer, E. M., Sunshine, M. J., Gandhi, L., Annes, J., Petrzilka, D., Kupfer, A., Schwartzberg, P. L. and Littman, D. R. (2000). PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes, *Nature,* **404,** 402-7.

Swan, F., Jr., Velasquez, W. S., Tucker, S., Redman, J. R., Rodriguez, M. A., McLaughlin, P., Hagemeister, F. B. and Cabanillas, F. (1989). A new serologic staging system for large-cell lymphomas based on initial beta 2-microglobulin and lactate dehydrogenase levels, *J Clin Oncol,* **7,** 1518-27.

Takahashi, H., Feuerhake, F., Kutok, J. L., Monti, S., Dal Cin, P., Neuberg, D., Aster, J. C. and Shipp, M. A. (2006). FAS death domain deletions and cellular FADD-like interleukin 1beta converting enzyme inhibitory protein (long) overexpression: alternative mechanisms for deregulating the extrinsic apoptotic pathway in diffuse large B-cell lymphoma subtypes, *Clin Cancer Res,* **12,** 3265-71.

Tamayo, P., Slonim, D., Mesirov, J., Zhu, Q., Kitareewan, S., Dmitrovsky, E., Lander, E. S. and Golub, T. R. (1999). Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation, *Proc Natl Acad Sci U S A,* **96,** 2907-12.

Tanaka, T., Reddy, B. S. and el-Bayoumy, K. (1985). Inhibition by dietary organoselenium, p-methoxybenzene-selenol, of hepatocarcinogenesis induced by azoxymethane in rats, *Jpn J Cancer Res,* **76,** 462-7.

Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S. and Leder, P. (1982). Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells, *Proc Natl Acad Sci U S A,* **79,** 7837-41.

Tetsu, O. and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells, *Nature,* **398,** 422-6.

The-International-Non-Hodgkin's-Lymphoma-Prognostic-Factors-Project (1993). A predictive model for aggressive non-Hodgkin's lymphoma., *N Engl J Med,* **329,** 987-94.

Thompson, H. J., Meeker, L. D. and Kokoska, S. (1984). Effect of an inorganic and organic form of dietary selenium on the promotional stage of mammary carcinogenesis in the rat, *Cancer Res,* **44,** 2803-6.

Thompson, H. J., Wilson, A., Lu, J., Singh, M., Jiang, C., Upadhyaya, P., el-Bayoumy, K. and Ip, C. (1994). Comparison of the effects of an organic and an inorganic form of selenium on a mammary carcinoma cell line, *Carcinogenesis,* **15,** 183-6.

Thorley-Lawson, D. A. and Gross, A. (2004). Persistence of the Epstein-Barr virus and the origins of associated lymphomas, *N Engl J Med,* **350,** 1328-37.

Tilly, H., Lepage, E., Coiffier, B., Blanc, M., Herbrecht, R., Bosly, A., Attal, M., Fillet, G., Guettier, C., Molina, T. J., Gisselbrecht, C. and Reyes, F. (2003). Intensive

conventional chemotherapy (ACVBP regimen) compared with standard CHOP for poor-prognosis aggressive non-Hodgkin lymphoma, *Blood,* **102,** 4284-9.

Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C. and Croce, C. M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation, *Science,* **226,** 1097-9.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E. and Croce, C. M. (1985). The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining, *Science,* **229,** 1390-3.

Tsutsui, T., Kumakura, S., Yamamoto, A., Kanai, H., Tamura, Y., Kato, T., Anpo, M., Tahara, H. and Barrett, J. C. (2002). Association of p16(INK4a) and pRb inactivation with immortalization of human cells, *Carcinogenesis,* **23,** 2111-7.

Turko, I. V., Ballard, S. A., Francis, S. H. and Corbin, J. D. (1999). Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds, *Mol Pharmacol,* **56,** 124-30.

Turner, S. D., Tooze, R., Maclennan, K. and Alexander, D. R. (2003). Vav-promoter regulated oncogenic fusion protein NPM-ALK in transgenic mice causes B-cell lymphomas with hyperactive Jun kinase, *Oncogene,* **22,** 7750-61.

Tycko, B., Palmer, J. D. and Sklar, J. (1989). T cell receptor gene trans-rearrangements: chimeric gamma-delta genes in normal lymphoid tissues, *Science,* **245,** 1242-6.

Uddin, A. N., Burns, F. J. and Rossman, T. G. (2005). Vitamin E and organoselenium prevent the cocarcinogenic activity of arsenite with solar UVR in mouse skin, *Carcinogenesis,* **26,** 2179-86.

Ueda, C., Uchiyama, T. and Ohno, H. (2002). Immunoglobulin (Ig)/BCL6 versus non-Ig/BCL6 gene fusion in diffuse large B-cell lymphoma corresponds to a high- versus low-level expression of BCL6 mRNA, *Blood,* **99,** 2624-5.

Ujiie, S. and Kikuchi, H. (2002). The relation between serum selenium value and cancer in Miyagi, Japan: 5-year follow up study, *Tohoku J Exp Med,* **196,** 99-109.

Vadgama, J. V., Wu, Y., Shen, D., Hsia, S. and Block, J. (2000). Effect of selenium in combination with Adriamycin or Taxol on several different cancer cells, *Anticancer Res,* **20,** 1391-414.

Vadhanavikit, S., Ip, C. and Ganther, H. E. (1993). Metabolites of sodium selenite and methylated selenium compounds administered at cancer chemoprevention levels in the rat, *Xenobiotica,* **23,** 731-45.

Van Cutsem, E., Lang, I., D'haens, G., Moiseyenko, V., Zaluski, J., Folprecht, G., Tejpar, S., Kisker, O., Stroh, C. and Rougier, P. (2008). KRAS status and efficacy in the first-line treatment of patients with metastatic colorectal cancer (mCRC) treated with FOLFIRI with or without cetuximab: The CRYSTAL experience., *ASCO*, Chicago, J Clin Oncol 26I, abstract 2I

van den Berg, A., Maggio, E., Diepstra, A., de Jong, D., van Krieken, J. and Poppema, S. (2002). Germline FAS gene mutation in a case of ALPS and NLP Hodgkin lymphoma, *Blood,* **99,** 1492-4.

Vellenga, E., van Putten, W. L., van 't Veer, M. B., Zijlstra, J. M., Fibbe, W. E., van Oers, M. H., Verdonck, L. F., Wijermans, P. W., van Imhoff, G. W., Lugtenburg, P. J. and Huijgens, P. C. (2008). Rituximab improves the treatment results of DHAP-VIM-DHAP and ASCT in relapsed/progressive aggressive CD20+ NHL: a prospective randomized HOVON trial, *Blood,* **111,** 537-43.

Verma, A., Atten, M. J., Attar, B. M. and Holian, O. (2004). Selenomethionine stimulates MAPK (ERK) phosphorylation, protein oxidation, and DNA synthesis in gastric cancer cells, *Nutr Cancer,* **49,** 184-90.

Vilchez, R. A. and Butel, J. S. (2003). SV40 in human brain cancers and non-Hodgkin's lymphoma, *Oncogene,* **22,** 5164-72.

Vilchez, R. A., Kozinetz, C. A., Arrington, A. S., Madden, C. R. and Butel, J. S. (2003). Simian virus 40 in human cancers, *Am J Med,* **114,** 675-84.

Vilchez, R. A., Madden, C. R., Kozinetz, C. A., Halvorson, S. J., White, Z. S., Jorgensen, J. L., Finch, C. J. and Butel, J. S. (2002). Association between simian virus 40 and non-Hodgkin lymphoma, *Lancet,* **359,** 817-23.

Villuendas, R., Pezzella, F., Gatter, K., Algara, P., Sanchez-Beato, M., Martinez, P., Martinez, J. C., Munoz, K., Garcia, P., Sanchez, L., Kocialkowsky, S., Campo, E., Orradre, J. L. and Piris, M. A. (1997). p21WAF1/CIP1 and MDM2 expression in non-Hodgkin's lymphoma and their relationship to p53 status: a p53+, MDM2-, p21-immunophenotype associated with missense p53 mutations, *J Pathol,* **181,** 51-61.

Virtaneva, K., Wright, F. A., Tanner, S. M., Yuan, B., Lemon, W. J., Caligiuri, M. A., Bloomfield, C. D., de La Chapelle, A. and Krahe, R. (2001). Expression profiling reveals fundamental biological differences in acute myeloid leukemia

with isolated trisomy 8 and normal cytogenetics, *Proc Natl Acad Sci U S A,* **98,** 1124-9.

Vogelstein, B., Fearon, E. R., Kern, S. E., Hamilton, S. R., Preisinger, A. C., Nakamura, Y. and White, R. (1989). Allelotype of colorectal carcinomas, *Science,* **244,** 207-11.

Vose, J. M., Armitage, J. O., Weisenburger, D. D., Bierman, P. J., Sorensen, S., Hutchins, M., Moravec, D. F., Howe, D., Dowling, M. D. and Mailliard, J. (1988). The importance of age in survival of patients treated with chemotherapy for aggressive non-Hodgkin's lymphoma, *J Clin Oncol,* **6,** 1838-44.

Vose, J. M., Link, B. K., Grossbard, M. L., Czuczman, M., Grillo-Lopez, A., Gilman, P., Lowe, A., Kunkel, L. A. and Fisher, R. I. (2001). Phase II study of rituximab in combination with chop chemotherapy in patients with previously untreated, aggressive non-Hodgkin's lymphoma, *J Clin Oncol,* **19,** 389-97.

Wachtel, H. (1990). The second-messenger dysbalance hypothesis of affective disorders, *Pharmacopsychiatry,* **23,** 27-32.

Walczak, H. and Krammer, P. H. (2000). The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems, *Exp Cell Res,* **256,** 58-66.

Wan, X. S., Zhou, Z., Kennedy, A. R. and Kopelovich, L. (2003). *In vitro* evaluation of chemopreventive agents using cultured human prostate epithelial cells, *Oncol Rep,* **10,** 2009-14.

Wang, X. (2001). The expanding role of mitochondria in apoptosis, *Genes Dev,* **15,** 2922-33.

Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P. and McPhail, A. T. (1971). Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia, *J Am Chem Soc,* **93,** 2325-7.

Watanabe, T., Hotta, T., Ichikawa, A., Kinoshita, T., Nagai, H., Uchida, T., Murate, T. and Saito, H. (1994). The MDM2 oncogene overexpression in chronic lymphocytic leukemia and low-grade lymphoma of B-cell origin, *Blood,* **84,** 3158-65.

Waters, D. J., Shen, S., Cooley, D. M., Bostwick, D. G., Qian, J., Combs, G. F., Jr., Glickman, L. T., Oteham, C., Schlittler, D. and Morris, J. S. (2003). Effects of dietary selenium supplementation on DNA damage and apoptosis in canine prostate, *J Natl Cancer Inst,* **95,** 237-41.

Watrach, A. M., Milner, J. A. and Watrach, M. A. (1982). Effect of selenium on growth rate of canine mammary carcinoma cells in athymic nude mice, *Cancer Lett,* **15,** 137-43.

Weintraub, M., Lin, A. Y., Franklin, J., Tucker, M. A., Magrath, I. T. and Bhatia, K. G. (1996). Absence of germline p53 mutations in familial lymphoma, *Oncogene,* **12,** 687-91.

Weisenburger, D. D. (1994). Epidemiology of non-Hodgkin's lymphoma: recent findings regarding an emerging epidemic, *Ann Oncol,* **5 Suppl 1,** 19-24.

Weiss, L. M., Warnke, R. A., Sklar, J. and Cleary, M. L. (1987). Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas, *N Engl J Med,* **317,** 1185-9.

Wen, H. Y., Davis, R. L., Shi, B., Chen, J. J., Chen, L., Boylan, M. and Spallholz, J. E. (1997). Bioavailability of selenium from veal, chicken, beef, pork, lamb, flounder, tuna, selenomethionine, and sodium selenite assessed in selenium-deficient rats, *Biol Trace Elem Res,* **58,** 43-53.

Wereszczynska-Siemiatkowska, U., Mroczko, B., Siemiatkowski, A., Szmitkowski, M., Borawska, M. and Kosel, J. (2004). The importance of interleukin 18, glutathione peroxidase, and selenium concentration changes in acute pancreatitis, *Dig Dis Sci,* **49,** 642-50.

Whanger, P. D. and Butler, J. A. (1994). Can physiological responses be used to assess the chemical forms of selenium consumed by humans?, *Proceedings of the SDTA 5th International Symposium*, Brussels, Belgium, 153-156I

Wickremasinghe, R. G. and Hoffbrand, A. V. (1999). Biochemical and genetic control of apoptosis: relevance to normal hematopoiesis and hematological malignancies, *Blood,* **93,** 3587-600.

Willett, W. C., Polk, B. F., Morris, J. S., Stampfer, M. J., Pressel, S., Rosner, B., Taylor, J. O., Schneider, K. and Hames, C. G. (1983). Prediagnostic serum selenium and risk of cancer, *Lancet,* **2,** 130-4.

Wilson, K. S., Sehn, L. H., Berry, B., Chhanabhai, M., Fitzgerald, C. A., Gill, K. K., Klasa, R., Skinnider, B., Sutherland, J., Connors, J. M. and Gascoyne, R. D. (2007). CHOP-R therapy overcomes the adverse prognostic influence of BCL-2 expression in diffuse large B-cell lymphoma, *Leuk Lymphoma,* **48,** 1102-9.

Wilson, W. H., Dunleavy, K., Pittaluga, S., Hegde, U., Grant, N., Steinberg, S. M., Raffeld, M., Gutierrez, M., Chabner, B. A., Staudt, L., Jaffe, E. S. and Janik, J. E. (2008). Phase II study of dose-adjusted EPOCH and rituximab in untreated diffuse large B-cell lymphoma with analysis of germinal center and post-germinal center biomarkers, *J Clin Oncol,* **26,** 2717-24.

Wotherspoon, A. C., Pan, L. X., Diss, T. C. and Isaacson, P. G. (1992). Cytogenetic study of B-cell lymphoma of mucosa-associated lymphoid tissue, *Cancer Genet Cytogenet,* **58,** 35-8.

Wright, G., Tan, B., Rosenwald, A., Hurt, E. H., Wiestner, A. and Staudt, L. M. (2003). A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma, *Proc Natl Acad Sci U S A,* **100,** 9991-6.

Wunder, F., Tersteegen, A., Rebmann, A., Erb, C., Fahrig, T. and Hendrix, M. (2005). Characterization of the first potent and selective PDE9 inhibitor using a cGMP reporter cell line, *Mol Pharmacol,* **68,** 1775-81.

Xu, X. M., Mix, H., Carlson, B. A., Grabowski, P. J., Gladyshev, V. N., Berry, M. J. and Hatfield, D. L. (2005). Evidence for direct roles of two additional factors, SECp43 and soluble liver antigen, in the selenoprotein synthesis machinery, *J Biol Chem,* **280,** 41568-75.

Xue, S. A., Labrecque, L. G., Lu, Q. L., Ong, S. K., Lampert, I. A., Kazembe, P., Molyneux, E., Broadhead, R. L., Borgstein, E. and Griffin, B. E. (2002). Promiscuous expression of Epstein-Barr virus genes in Burkitt's lymphoma from the central African country Malawi, *Int J Cancer,* **99,** 635-43.

Yamada, M., Tomida, A., Yoshikawa, H., Taketani, Y. and Tsuruo, T. (1996). Increased expression of thioredoxin/adult T-cell leukemia-derived factor in cisplatin-resistant human cancer cell lines, *Clin Cancer Res,* **2,** 427-32.

Yamasaki, L. (2003). Role of the RB tumor suppressor in cancer, *Cancer Treat Res,* **115,** 209-39.

Yamochi, T., Kaneita, Y., Akiyama, T., Mori, S. and Moriyama, M. (1999). Adenovirus-mediated high expression of BCL-6 in CV-1 cells induces apoptotic cell death accompanied by down-regulation of BCL-2 and BCL-X(L), *Oncogene,* **18,** 487-94.

Yan, L. and Frenkel, G. D. (1992). Inhibition of cell attachment by selenite, *Cancer Res,* **52,** 5803-7.

Yan, L. and Spallholz, J. E. (1993). Generation of reactive oxygen species from the reaction of selenium compounds with thiols and mammary tumor cells, *Biochem Pharmacol,* **45,** 429-37.

Yan, L., Yee, J. A., McGuire, M. H. and Graef, G. L. (1997). Effect of dietary supplementation of selenite on pulmonary metastasis of melanoma cells in mice, *Nutr Cancer,* **28,** 165-9.

Yang, G., Yin, S., Zhou, R., Gu, L., Yan, B. and Liu, Y. (1989a). Studies of safe maximal daily dietary Se-intake in a seleniferous area in China. Part II: Relation between Se-intake and the manifestation of clinical signs and certain biochemical alterations in blood and urine, *J Trace Elem Electrolytes Health Dis,* **3,** 123-30.

Yang, G. and Zhou, R. (1994). Further observations on the human maximum safe dietary selenium intake in a seleniferous area of China, *J Trace Elem Electrolytes Health Dis,* **8,** 159-65.

Yang, G., Zhou, R., Yin, S., Gu, L., Yan, B., Liu, Y. and Li, X. (1989b). Studies of safe maximal daily dietary selenium intake in a seleniferous area in China. I. Selenium intake and tissue selenium levels of the inhabitants, *J Trace Elem Electrolytes Health Dis,* **3,** 77-87.

Yang, X., Tian, Y., Ha, P. and Gu, L. (1997). [Determination of the selenomethionine content in grain and human blood], *Wei Sheng Yan Jiu,* **26,** 113-6.

Yano, T., Jaffe, E. S., Longo, D. L. and Raffeld, M. (1992). MYC rearrangements in histologically progressed follicular lymphomas, *Blood,* **80,** 758-67.

Ye, B. H., Cattoretti, G., Shen, Q., Zhang, J., Hawe, N., de Waard, R., Leung, C., Nouri-Shirazi, M., Orazi, A., Chaganti, R. S., Rothman, P., Stall, A. M., Pandolfi, P. P. and Dalla-Favera, R. (1997). The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation, *Nat Genet,* **16,** 161-70.

Ye, B. H., Rao, P. H., Chaganti, R. S. and Dalla-Favera, R. (1993). Cloning of bcl-6, the locus involved in chromosome translocations affecting band 3q27 in B-cell lymphoma, *Cancer Res,* **53,** 2732-5.

Yi, X., Yin, X. M. and Dong, Z. (2003). Inhibition of Bid-induced apoptosis by Bcl-2. tBid insertion, Bax translocation, and Bax/Bak oligomerization suppressed, *J Biol Chem,* **278,** 16992-9.

Yoo, M. H., Xu, X. M., Carlson, B. A., Gladyshev, V. N. and Hatfield, D. L. (2006). Thioredoxin reductase 1 deficiency reverses tumor phenotype and tumorigenicity of lung carcinoma cells, *J Biol Chem,* **281,** 13005-8.

Yoshida, M., Iizuka, K., Terada, A., Hara, M., Nishijima, H., Akinori, Shimada, Nakada, K., Satoh, Y. and Akama, Y. (2000). Prevention of nephrotoxicity of cisplatin by repeated oral administration of ebselen in rats, *Tohoku J Exp Med,* **191,** 209-20.

Yoshizawa, K., Willett, W. C., Morris, S. J., Stampfer, M. J., Spiegelman, D., Rimm, E. B. and Giovannucci, E. (1998). Study of prediagnostic selenium level in toenails and the risk of advanced prostate cancer, *J Natl Cancer Inst,* **90,** 1219-24.

Young, K. H., Weisenburger, D. D., Dave, B. J., Smith, L., Sanger, W., Iqbal, J., Campo, E., Delabie, J., Gascoyne, R. D., Ott, G., Rimsza, L., Muller-Hermelink, H. K., Jaffe, E. S., Rosenwald, A., Staudt, L. M., Chan, W. C. and Greiner, T. C. (2007). Mutations in the DNA-binding codons of TP53, which are associated with decreased expression of TRAILreceptor-2, predict for poor survival in diffuse large B-cell lymphoma, *Blood,* **110,** 4396-405.

Yu, S. Y., Zhu, Y. J. and Li, W. G. (1997). Protective role of selenium against hepatitis B virus and primary liver cancer in Qidong, *Biol Trace Elem Res,* **56,** 117-24.

Yu, W., Murray, N. R., Weems, C., Chen, L., Guo, H., Ethridge, R., Ceci, J. D., Evers, B. M., Thompson, E. A. and Fields, A. P. (2003). Role of cyclooxygenase 2 in protein kinase C beta II-mediated colon carcinogenesis, *J Biol Chem,* **278,** 11167-74.

Zech, L., Haglund, U., Nilsson, K. and Klein, G. (1976). Characteristic chromosomal abnormalities in biopsies and lymphoid-cell lines from patients with Burkitt and non-Burkitt lymphomas, *Int J Cancer,* **17,** 47-56.

Zeller, E., Stief, H. J., Pflug, B. and Sastre-y-Hernandez, M. (1984). Results of a phase II study of the antidepressant effect of rolipram, *Pharmacopsychiatry,* **17,** 188-90.

Zeng, H. (2002). Selenite and selenomethionine promote HL-60 cell cycle progression, *J Nutr,* **132,** 674-9.

Zhang, A., Ohshima, K., Sato, K., Kanda, M., Suzumiya, J., Shimazaki, K., Kawasaki, C. and Kikuchi, M. (1999). Prognostic clinicopathologic factors, including

immunologic expression in diffuse large B-cell lymphomas, *Pathol Int,* **49,** 1043-52.

Zhang, H., Dong, Y., Zhao, H., Brooks, J. D., Hawthorn, L., Nowak, N., Marshall, J. R., Gao, A. C. and Ip, C. (2005a). Microarray Data Mining for Potential Selenium Targets in Chemoprevention of Prostate Cancer, *Cancer Genomics Proteomics,* **2,** 97-114.

Zhang, J., Anastasiadis, P. Z., Liu, Y., Thompson, E. A. and Fields, A. P. (2004a). Protein kinase C (PKC) betaII induces cell invasion through a Ras/Mek-, PKC iota/Rac 1-dependent signaling pathway, *J Biol Chem,* **279,** 22118-23.

Zhang, X., Feng, Q. and Cote, R. H. (2005b). Efficacy and selectivity of phosphodiesterase-targeted drugs in inhibiting photoreceptor phosphodiesterase (PDE6) in retinal photoreceptors, *Invest Ophthalmol Vis Sci,* **46,** 3060-6.

Zhang, Y., Holford, T. R., Leaderer, B., Boyle, P., Zahm, S. H., Flynn, S., Tallini, G., Owens, P. H. and Zheng, T. (2004b). Hair-coloring product use and risk of non-Hodgkin's lymphoma: a population-based case-control study in Connecticut, *Am J Epidemiol,* **159,** 148-54.

Zhao, H., Whitfield, M. L., Xu, T., Botstein, D. and Brooks, J. D. (2004). Diverse effects of methylseleninic acid on the transcriptional program of human prostate cancer cells, *Mol Biol Cell,* **15,** 506-19.

Zhou, N., Xiao, H., Li, T. K., Nur, E. K. A. and Liu, L. F. (2003). DNA damage-mediated apoptosis induced by selenium compounds, *J Biol Chem,* **278,** 29532-7.

Zhou, P., Levy, N. B., Xie, H., Qian, L., Lee, C. Y., Gascoyne, R. D. and Craig, R. W. (2001). MCL1 transgenic mice exhibit a high incidence of B-cell lymphoma manifested as a spectrum of histologic subtypes, *Blood,* **97,** 3902-9.

Zimmermann, K. C., Bonzon, C. and Green, D. R. (2001). The machinery of programmed cell death, *Pharmacol Ther,* **92,** 57-70.

Zong, W. X., Lindsten, T., Ross, A. J., MacGregor, G. R. and Thompson, C. B. (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak, *Genes Dev,* **15,** 1481-6.

Zu, K., Bihani, T., Lin, A., Park, Y. M., Mori, K. and Ip, C. (2006). Enhanced selenium effect on growth arrest by BiP/GRP78 knockdown in p53-null human prostate cancer cells, *Oncogene,* **25,** 546-54.

Zu, K. and Ip, C. (2003). Synergy between selenium and vitamin E in apoptosis induction is associated with activation of distinctive initiator caspases in human prostate cancer cells, *Cancer Res,* **63,** 6988-95.