

Multidrug resistance in solid tumours

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Abstract

Introduction: Most cancers show heterogeneity of response to chemotherapy. This may be due in part to the differential expression of drug resistance proteins and the molecular targets of the drugs concerned.

Methods: An *ex vivo* ATP-based Tumour Chemosensitivity Assay (ATP-TCA), immunohistochemistry and quantitative RT-PCR have been used to assess the chemosensitivity and resistance of a variety of solid tumours and cell lines.

Results:

(a) Melanoma cell lines showed higher chemosensitivity than tumour-derived cells, partially reversible by lowering the serum concentration, and hence the proliferation rate of the cells.

(b) Studies of retinoblastoma samples confirmed that this malignancy is susceptible to cytotoxic drugs of all types, though multidrug resistance may occur in some cases.

(c) The ATP-TCA was used to study the activity of high-dose doxorubicin in combination with other cytotoxic agents in ovarian adenocarcinoma samples. The combination of liposomal doxorubicin + vinorelbine was selected for further development.

(d) A number of experimental drugs with varying sensitivity to resistance mechanisms were also assessed. One drug, XR5944, has entered phase I/II clinical trials during the course of this project, and the data have provided clinical indications.

(e) An inhibitor of multi-drug resistance, tariquidar, has been tested in combination with doxorubicin, vinorelbine or paclitaxel, and has been shown to reverse this resistance.

(f) Molecular studies have determined the expression of topoisomerases and drug transporters in tumour cells before and after exposure to chemotherapeutic agents. P-gp expression has been found to be a determinant of sensitivity to a certain number of drugs.

Conclusion: The results suggest that drug resistance contributes to heterogeneity of chemosensitivity in many solid tumour types, as well as other mechanisms. Reversal of such resistance may benefit a subset of patients undergoing chemotherapy.

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Abbreviations

4-HC	4-hydroxycyclophosphamide
5-FU	5-fluorouracil
Ab	Antibody
AGAT	O ⁶ -alkylguanine-DNA alkyltransferase
AIC	aminoimidazole carboxamide
ALL	Acute lymphocytic leukaemia
AML	Acute myelocytic leukaemia
AMP	Adenosine 5'-monophosphate
AMV	Avian Myeloblastosis Virus
ANLL	Acute non lymphocytic leukaemia
Apaf-1	apoptotic protease activating factor 1
APES	3-aminopropyltriethoxysilane
AraC	cytosine arabinoside
ASK1	Apoptosis signal-regulating kinase
ATCC	American Type Culture Collection
ATM	ataxia-telangiectasia mutated
ATP	Adenosine 5'-triphosphate
ATP-TCA	ATP-tumour chemosensitivity assay
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated x protein
Bcl-2	B-cell leukaemia/lymphoma 2
BCNU	1,3bis(2-chloroethyl)-1-nitrosourea; carmustine
BCRP	breast cancer resistance protein
BER	base excision repair
bFGF	basic Fibroblast Growth Factor
bp	base pairs
BSO	buthionine-(S,R)-sulfoxime
CAM	complete assay media
CASPASE	cysteinyl aspartic acid-protease
CAVE	cyclophosphamide, doxorubicin, vincristine, etoposide
CCNU	1-(2-chloroethyl)3-cyclohexyl-1-nitrosourea; lomustine
CDDP	cis-diamminedichloroplatinum II; cisplatin
CDK	cyclin dependent kinase
cDNA	complementary DNA
cFLIP	cellular-FLICE inhibitory protein
Chk2	checkpoint kinase 2
CLL	Chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
cMOAT	Multispecific organic anion transporter; MRP-2
CoV	Coefficient of Variance
COX-2	cyclooxygenase 2
CR	Complete response
CYP	Cytochrome P450
Ct	Threshold cycle
DACA	N-[2-(Dimethylamino)ethyl]acridine-4-carboxamide
DIABLO	direct IAP-binding protein with low pI

DiSC	differential staining cytotoxicity assay
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy-Nucleotide 5'-triphosphate
Doxo	Doxorubicin
DPD	dihydropyrimidine dehydrogenase
DPX	dibutylphthalate xylene
DTIC	5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide; Dacarbazine
dTMP	deoxythymidine 5'-triphosphate
ECACC	European Collection of Cell Cultures
ECF	Epirubicin + Cisplatin + 5-Fluorouracil
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	Enzyme Linked Immunosorbant Assay
ERCC1	excision repair cross-complementing 1
FACS	Fluorescence-activated cell sorter
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FdUMP	Fluoro-deoxyuridine 5'-monophosphate
FITC	Fluorescein Isothiocyanate
FMCA	Fluorometric Microculture Cytotoxicity Assay
GAPDH	glyceraldehyde-3 phosphate dehydrogenase
GDP	guanine diphosphate
Gem	gemcitabine
GI	Gastro-intestinal
GST	glutathione S-transferases
GST- π	glutathione-S-transferase isoform π
GTIC	guanidine isothiocyanate
GTP	guanine triphosphate
hCNT	human concentrative nucleoside transporter
hENT	human equilibrative nucleoside transporter
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HER2	Human Epidermal Growth Factor Receptor 2
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HMMTIC	5-[3-hydroxy-methyl-3-methyl-triazen-1-yl]-imidazole-4- carboxamide
HPRT1	hypoxanthine phosphoribosyltransferase 1
HtRA2	High temperature Requirement Protein 2
IAP	inhibitor of apoptosis protein
IC	inhibition concentration (followed by a percentage)
IFN α	interferon alpha
IFN α 2b	interferon alpha 2b
IFN β	interferon beta
IFN γ	interferon gamma
IgG	Immunoglobulin G
IL-2	interleukin-2
IL-4	interleukin-4

IHC	Immunohistochemistry
Irino	irinotecan
JNK	c-Jun-N-terminal kinase 1
LREC	Local Regional Ethics Committee
LRP	Lung resistance-related protein
MAP	Mitogen-activated protein
MDM2	mouse double minute 2
MDR	multidrug resistance
MDR1	multi-drug resistance gene 1
MGMT	O ⁶ -methylguanine-DNA methyltransferase
MLH1	mutL homologue 1
MI	Maximum inhibitor
MO	No drug Inhibitor
MREC	multi-regional ethics committee
mRNA	messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
MRP1	multi-drug resistance related protein 1
MSH2	mutS homolog 2
MSH6	mutS homolog 6
MT II	metallothionein II
MTIC	5(3-methyltriazeno)imidazole-4-carboxamide
MTT	3-[4,5-dimethyl (thiazol-2-yl)-3,5-diphenyl]tetrazolium bromide
MVP	major vault protein
NAD ⁺	Nicotinamide adenine dinucleotide
NBMPR	nitrobenzylmercaptapurine ribonucleoside
NCI	National Cancer Institute
NER	Nucleotide excision repair
NGS	normal goat serum
Noxa	phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1); adult T cell leukemia-derived PMA-responsive; ATL-derived PMA-responsive gene
NSCLC	non-Small Cell Lung Cancer
NT	Nucleoside transporter
OS	Overall Survival
P450	cytochrome P450
PARP	poly(ADP-ribose) polymerase-1
PBGD	human porphobilinogen deaminase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG-DOX	liposomal doxorubicin
PMS2	postmeiotic segregation increased 2t
PPC	peak plasma concentration
PR	Partial response
PUMA	p53-upregulated modulator of apoptosis ; Bcl-2 binding component 3 (BBC3)
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase PCR
Ref-1/Ape	Redox effector factor-1/apurinic/aprimidinic endonuclease
RB	retinoblastoma

RNA	ribonucleic acid
RT	room temperature
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase-PCR
SCLC	Small Cell Lung Cancer
SD	Standard deviation
SDHA	succinate dehydrogenase complex-subunit A
SEM	Standard error of the mean
Smac	second mitochondria-derived activator of caspases
SWOG	South-west Oncology Group
TARDIS	Trapped in Agarose DNA Immunostaining
Tax	paclitaxel
TBP	TATA box binding protein
TBS	Tris-buffered saline
TCER	Tumour cell extraction reagent
TDC	Test Drug Concentration
TDE	Tumour Dissociating Enzyme
Temo	temozolomide
T _m	Melting temperature
TNF α	Tumour Necrosis Factor alpha
TOPO I	topoisomerase I
TOPO II α	topoisomerase II α
TOPO II β	topoisomerase II β
TP	thymidine phosphorylase
TRAIL	TNF-related apoptosis-inducing ligand
Treo	treosulfan
Treo + Gem	treosulfan <i>plus</i> gemcitabine
TS	thymidylate synthase
UNG	uracil-N-glycosylase
UV	Ultra violet (light)
VAD	Vincristine, doxorubicin, dexamethasone
VEGF	Vascular endothelial growth factor
VinB	vinblastine
VinR	vinorelbine
XTT	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2 <i>H</i> -tetrazolium hydroxide

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Declaration

I, the undersigned declare that no portion of this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Federica Di Nicolantonio

Chapter 1 - INTRODUCTION

1.1 Chemotherapy

Chemotherapy is the application of selective toxicity. The goal of chemotherapeutic treatment is to selectively attenuate or destroy pathogenic microorganisms or cells with minimal side effects to the host. These targeted cells or organisms may be bacteria, viruses, protozoans, fungi, helminths, or neoplastic cells. In order to achieve selective toxicity, the target for chemotherapeutic agents may be unique to the target population, may be structurally different in the target population from the form in the host population, or may be more essential in the target population than in the host population. The latter applies to classical cancer chemotherapeutic agents, which are aimed either to kill cancer cells (a cytotoxic effect), or to halt proliferation (a cytostatic effect). Tumour cells are highly metabolically active cells, which are continually duplicating their DNA, RNA and proteins providing a large number of therapeutic targets. Most cancer chemotherapy has selective toxicity based on the premise that tumour cells are dividing more rapidly than non-malignant host cells. However, some host cell populations also divide rapidly such as those in bone marrow, gut and reproductive/germ cells. These cells are damaged by chemotherapeutic agents as well as cancer cells, leading to side effects such as neutropaenia, nausea and sterility.

Cytotoxic drugs fall into four broad categories according to their mode of action: antimetabolites; topoisomerase inhibitors, microtubule interfering agents (vinca alkaloids and taxanes) and DNA damaging agents (which may be further sub-classified according to their chemical structure as nitrogen mustards and derivatives; nitrosoureas; busulfan and derivatives; dacarbazine and derivatives; platinum-based compounds);

Table 1.1 contains a list of commonly used chemotherapeutic agents, along with a brief description of their mechanisms of action.

Table 1.1 Mechanism of action of common chemotherapeutic agents.

Cytotoxic agent	Mechanism of action
Platinum-based compounds	Bind to individual bases of DNA disrupting its function. Protein binding may also occur resulting in toxicity
Carmustine (BCNU)	Alkylates DNA <i>via</i> the production of reactive intermediates that attack nucleophilic sites
Cytosine arabinoside (AraC, Cytarabine)	Inhibits DNA polymerases involved in repairing damaged DNA and is also incorporated into DNA (and presumably RNA) resulting in chain termination
Dacarbazine (DTIC)	Requires bioactivation by CYP 450, acts <i>via</i> direct methylation of nucleic acids
Doxorubicin (Adriamycin) or Caelyx® or Doxil® (liposomal doxorubicin) Epirubicin	Pleiotropic effects including: Inhibition of DNA topoisomerase II Activation of protein kinase C signal transduction cascades Generation of reactive oxygen intermediates Stimulation of apoptosis
Etoposide (VP-16)	Inhibition of DNA topoisomerase II
5-Fluorouracil (5-FU)	Three main mechanisms of action: Incorporation of fluorouridine triphosphate (a metabolite of 5-FU) into the RNA interferes with synthesis and function of RNA Inhibition of thymidylate synthase (TS) by fluorodeoxyuridine monophosphate (a metabolite of 5-FU) leads to depletion of deoxythymidine phosphates Incorporation of fluorodeoxyuridine triphosphate and deoxyuridine triphosphate into DNA may affect the stability of the DNA
Fotemustine	Alkylates DNA <i>via</i> the production of reactive intermediates that attack nucleophilic sites
Gemcitabine	Cytidine analogue: binds to and inhibits DNA polymerases involved in repairing damaged DNA and is also incorporated into DNA (and presumably RNA) resulting in chain termination

Table 1.1 (continued). Mechanism of action of common chemotherapeutic agents.

Cytotoxic agent	Mechanism of action
Cyclophosphamide	Alkylates DNA <i>via</i> the production of reactive intermediates that attack nucleophilic sites
Interferon α 2b	Direct anti-proliferative effects on the tumour cells, via reduction in functional capacity and/or induction of differentiation Indirect induction of host anti-tumour mechanisms
Methotrexate (MTX)	Folic acid analogue, inhibits dihydrofolate reductase leading to the partial depletion of reduced folates, required for deoxythymidine monophosphate (dTMP) synthesis
Mitoxantrone	Binds with high affinity directly to nucleic acids and inhibits DNA and RNA synthesis, also inhibits topoisomerase II
Mitomycin C	Active after reduction, binds to DNA via mono- or bifunctional alkylation and inhibits DNA synthesis
Taxanes	Inhibition of microtubule depolymerisation, causing the inhibition of mitosis and probably intracellular transport
Temozolomide	Undergoes rapid non-enzymatic conversion under physiological conditions to give the reactive compound MTIC; the cytotoxicity of MTIC is thought to be primarily due to methylation of DNA which occurs mainly at the O6 and N7 positions of guanine.
Topotecan and Irinotecan	Potent inhibitors of DNA topoisomerase I
Treosulfan	Bifunctional alkylating agent which can cross-link DNA
Vinca alkaloids	Inhibit the polymerisation of microtubules therefore inhibiting mitosis

Over the last decade there has been a trend in the pharmaceutical industry to design and develop new anti-cancer agents, which target molecular abnormalities differentially expressed in tumours. These new drugs may provide a more specific and less toxic way of treating cancer. One example is the development of chimeric and humanised antibodies (Abs) for the treatment of haematological malignancies and directed towards

antigens present on the surface of lymphocytes, such as CD20, CD33 and CD52. Some of these drugs (rituximab, alemtuzumab) act via Ab-dependent cell-mediated cytotoxicity and lysis by complement, while others have been conjugated with toxins or radionuclides (ibritumomab tiuxetan, tositumomab, gemtuzumab ozogamicin) in an attempt to increase their efficacy and exploit their specificity against targeted cells. In the field of solid tumours, the monoclonal Ab trastuzumab has been approved for use in the treatment of metastatic breast tumours that overexpress HER-2. HER-2 is a receptor tyrosine kinase (RTK) with no known natural ligand and is overexpressed in approximately 25% of breast tumours (Ross *et al.*, 2003).

Inhibition of the tyrosine kinase activity of RTKs, as opposed to using monoclonal antibodies against the extracellular portion of these receptors, provides another target. These inhibitors are able to exert their activity in cells that do not over-express the receptor or that have mutant, truncated forms of the receptor that are constitutively activated (Lal *et al.*, 2002). The first agent in this class to become clinically available was imatinib mesylate, which is an inhibitor of the tyrosine kinase activity of BCR-ABL (Druker *et al.*, 1996), the fusion protein that results from the translocation between chromosome 9 and 22 (also known as the Philadelphia chromosome), seen in over 95% of cases of chronic myeloid leukaemia (CML) and in a small percentage of cases of acute lymphoblastic and myeloid leukaemias (reviewed in Laurent *et al.*, 2001). Another drug in clinical use is gefitinib, an inhibitor of the epithelial growth factor receptor (EGFR) tyrosine kinase activity, which gained FDA approval in 2003 as a second-line agent for NSCLC.

Another cellular mechanism that may serve as target for therapeutic intervention includes the intracellular pathway that hydrolyses proteins marked for destruction by the ubiquitin enzyme cascade (proteasome). The clinical development of bortezomib followed preclinical observations of its ability to inhibit reversibly the proteolytic (chymotryptic) activity of the proteasome complex in mammalian cells. Inhibition of the intracellular protein degradation pathway alters the levels of numerous intracellular signaling and regulatory proteins as well as other proteins and, in some fashion, then alters the regulation of cellular processes that may lead to growth arrest or apoptosis (Mitsiades *et al.*, 2002).

Table 1.2 Some of the targeted anti-cancer agents that were approved by the FDA in the period January 1997-December 2003 (Source: FDA website at www.fda.gov).

Trade name (Generic name)	Molecular Targets	Clinical condition	Year of FDA approval
Rituxan® / Mabthera® (rituximab)	First monoclonal antibody (Ab) directed towards CD20 molecules present on B lymphocytes	CD20 positive low-grade or follicular B-cell lymphomas	1997
Herceptin® (trastuzumab)	Ab directed towards HER-2, RTK present on surface of breast cancer cells	HER-2 positive breast cancer	1998
Mylotarg® (gemtuzumab ozogamicin)	DNA targeted agent (calicheamicin derivative) conjugated to Ab directed towards CD33 antigen present on myeloid cells	Acute myeloid leukaemia (AML)	2000
Campath® (alemtuzumab)	Ab directed towards CD52 antigen present on lymphocytes	B-cell chronic lymphocytic leukaemia	2001
Gleevec® (imatinib mesylate)	Inhibitor of tyrosine kinase activity of BCR-ABL (fusion protein in CML) and kit	Chronic myeloid leukemia (CML), advanced GIST (gastrointestinal stromal tumour)	2001- 2002
Zevalin® (ibritumomab tiuxetan)	⁹⁰ Y-labelled Ab directed towards CD20 antigen present on B cells	CD20 positive low-grade non-Hodgkin's lymphoma	2002
Bexxar® (tositumomab)	¹³¹ I-labelled Ab directed towards CD20 antigen present on B cells	CD20 positive low-grade non-Hodgkin's lymphoma	2003
Velcade® (bortezomib)	Inhibitor of the proteolytic activity of the proteasome (intracellular protein degradation pathway) complex in mammalian cells	Multiple myeloma (3 rd line)	2003
Iressa® (gefitinib)	Inhibitor of EGFR tyrosine kinase activity	NSCLC (2 nd line)	2003

1.2 Resistance to anti-cancer drugs

1.2.1 Introduction

Although the design of anti-cancer drugs has become increasingly sophisticated, there is no treatment that is 100% effective against cancer. Failure of a patient's cancer to respond to a specific therapy can result from one of two general causes: host factors and specific genetic or molecular alterations in the cancer cells. Host factors include poor absorption or rapid metabolism or excretion of a drug, resulting in low serum levels; poor tolerance to effects of a drug, especially in elderly patients, resulting in a need to reduce doses below optimal levels; inability to deliver a drug to the site of a tumour, as could occur with bulky tumours or with biological agents of high molecular weight and low tissue penetration such as monoclonal antibodies and immunotoxins (Pluen *et al.*, 2001); various alterations in the host-tumour environment that affect response of the tumour including local metabolism of a drug by non-tumour cells, unusual features of the tumour blood supply that may affect transit time of drugs within tumours and the way in which cells in a cancer interact with each other and with interstitial cells from the host (Kobayashi *et al.*, 1993; Green *et al.*, 1999).

Resistance is often intrinsic to the cancer, but as therapy becomes more and more effective, acquired resistance has also become frequent. According to an early hypothesis formulated by Goldie and Coldman (1979), chemotherapy can select drug-resistant clones that develop by spontaneous mutation and the probability of a resistant phenotype increases with the mutation rate. However, recently, a few studies have suggested that drug resistance can develop not only by mutation but also through cellular adaptation. It appears that slow-growing tumours can become chemoresistant by altering their molecular phenotype in response to a specific cytotoxic agent (Matsumoto *et al.*, 1997; see chapter 11). The most common reason for acquisition of resistance to a broad range of anti-cancer drugs is probably expression of one or more energy-dependent transporters that eject anticancer drugs from cells. However other mechanisms of resistance, including insensitivity to drug induced apoptosis and induction of drug-detoxifying enzymes, probably play an important role in acquired anticancer drug resistance. Cellular mechanisms of drug resistance have been intensively studied, as experimental models can be easily generated by *in vitro* selection with cytotoxic agents. Cancer cells in culture can become resistant to a single drug, or a class of drugs with a similar mechanism of action, and may also show cross-resistance

to other structurally and mechanistically unrelated drugs - a phenomenon that is known as multidrug resistance (MDR).

1.2.2 Transport-mediated drug resistance

Transport mediated drug resistance is probably the most studied form of MDR in human tumours. Resistance to natural-product hydrophobic drugs, sometimes known as classical MDR, generally results from expression of ATP-dependent efflux pumps with broad drug specificity. These pumps belong to a family of ATP-binding cassette (ABC) transporters that share sequence and structural homology. So far, 49 human ABC genes have been identified and divided into seven distinct subfamilies (ABCA-ABCG) on the basis of their sequence homology and domain organization (Müller, 2003). Resistance results because increased drug efflux lowers intracellular drug concentrations. Drugs that are affected by classical multidrug resistance include the vinca alkaloids, the anthracyclines, the RNA transcription inhibitor actinomycin-D, and the microtubule-stabilising drug paclitaxel (Gottesman *et al.*, 2002).

There are other drug transporters that do not belong to the large family of ABC transporters described above: for example, Major Vault Proteins (MVP) are able to pump toxins out of the nucleus and other intracellular compartments, and ATP7B has recently been shown to transport cisplatin. Nucleoside Transporters (NT), although included in this section, represent a different class of membrane proteins, and are responsible for the active transport of cytotoxic nucleoside drugs.

Tables 1.3 and 1.4 list the most studied drug transporters, their physiological substrates, chemotherapeutic substrates and inhibitors.

Table 1.3 Tissue localisation, substrates and inhibitors of ABC transporters (Gottesman *et al.*, 2002; Hamada *et al.*, 2003; Kruh & Belinsky, 2003; Lage, 2003; Thomas & Coley, 2003; Asakura *et al.*, 2004)

Common Name	Gene name	Localisation	Non-chemotherapeutic and physiological substrates	Chemotherapeutic substrates (known and suspected)	Inhibitors
P-gp/MDR1	ABCB1	Intestine, liver, kidney, placenta, blood–brain barrier	Cortisol, aldosterone, sphingolipids, IL-2, IL-4, interferon- γ neutral and cationic organic compounds, many commonly used drugs	Doxorubicin, daunorubicin, epirubicin, vincristine, vinblastine, actinomycin-D, paclitaxel, docetaxel, etoposide, teniposide, bisantrene, homoharringtonine (STI-571), imatinib mesylate ^b , gemtuzumab, ozogamicin	Verapamil, nifedipin, azodipin, quinine, flupentixol, progesterone, megestrol acetate, tamoxifen, azythromycin ^a , cyclosporin A, valspodar (PSC 833), biricodar (VX-710), ONT-093, tariquidar (XR9576), zosuquidar (LY335979), laniquidar (R101933), elacridar (GF120918)
TAP1	ABCB2		Peptides	Mitoxantrone, epipodophyllotoxins	
TAP2	ABCB3		Peptides	Mitoxantrone, epipodophyllotoxins	
MDR2	ABCB4	Liver	Phosphatidylcholine	Paclitaxel, vinblastine	
SPGP (BSEP)	ABCB11	Liver	Bile salts	Paclitaxel	
BCRP (MXR)	ABCG2	Placenta, intestine, breast, liver	Sulfated estrogens, prazosin	Doxorubicin, daunorubicin, mitoxantrone, topotecan, SN-38	Fumitremorgin C, Ko-143, CI1033, elacridar (GF120918/GG918), novobiocin
ABCA2	ABCA2	Brain, monocytes	Steroids derivatives, lipids	Estramustine	
MRP1	ABCC1	All tissues	Glutathione and other conjugates, organic anions, leukotriene C4	Doxorubicin, epirubicin, etoposide, vincristine, methotrexate	Genistein, probenecid, MK-571, PAK-104P, indomethacin, BSO, verapamil, cyclosporin A, valspodar (PSC 833)

Table 1.3 (continued). Tissue localisation, substrates and inhibitors of ABC transporters (Gottesman *et al.*, 2002; Hamada *et al.*, 2003; Kruh & Belinsky, 2003; Lage, 2003; Thomas & Coley, 2003; Asakura *et al.*, 2004)

Common Name	Gene name	Tissue	Non-chemotherapeutic and physiological substrates	Chemotherapeutic substrates (known and suspected)	Inhibitors
MRP2 (cMOAT)	ABCC2	Liver, kidney, intestine	Similar to MRP1, non-bile salt organic anions	Methotrexate, etoposide, doxorubicin, cisplatin, vincristine, mitoxantrone, platinum	MK-571
MRP3	ABCC3	Pancreas, kidney, liver, adrenal glands, intestine	Glucuronate and glutathione conjugates, bile acids	Etoposide, teniposide, methotrexate, cisplatin, vincristine, doxorubicin	
MRP4	ABCC4	Prostate, testis, ovary, intestine, pancreas, lung	Organic anions, nucleotide analogues	Methotrexate, thiopurines	
MRP5	ABCC5	Most tissues	Cyclic nucleotides, nucleotide analogues, organic anions	6-Mercaptopurine, 6-Thioguanine	
MRP6	ABCC6	Kidney, liver, lung, colon	?	Anthracyclines, etoposide, platinum	
MRP8	ABCC11	Testis, prostate, liver	Cyclic nucleotides	5-Fluorouracil (5-FdUMP)	

Table 1.4 Tissue localisation, substrates and inhibitors of non-ABC transporters (data from Komatsu *et al.*, 2000; Lu *et al.*, 2002; Damaraju *et al.*, 2003)

Transporter Name	Tissue	Non-chemotherapy and physiological substrates	Chemotherapy substrates (known and suspected)	Inhibitors
ATP7B	liver, kidney, placenta; breast, ovarian and GI tumours	copper	cisplatin	
MVP (LRP)	lung, digestive tract, renal proximal tubules, keratinocytes, macrophages, adrenal cortex	nucleocytoplasmic transport	doxorubicin, mitoxantrone, methotrexate, etoposide, vincristine, cytarabine, cisplatin	PAK-104P
CNT1	Heart, skeletal muscle, placenta, pancreas, lung	Pyrimidine nucleosides, adenosine	gemcitabine, cytarabine, cladribine, idoxuridine	
CNT2	Heart, placenta, pancreas, lung	Purine nucleosides, uridine	cladribine	
CNT3	Heart, placenta, pancreas, lung	Purine and pyrimidine nucleosides	fludarabine, zebularine, cladribine, gemcitabine, 5-fluorouridine, floxidine	
ENT1	All tissues	Purine and pyrimidine nucleosides	gemcitabine, cladribine, AraA, cytarabine, fludarabine, 5-fluorouridine	NBMPR
ENT2	All tissues	Purine and pyrimidine nucleosides	gemcitabine	NBMPR
NCBT1	Kidney, intestine, choroidal plexus, placenta	Purine and pyrimidine nucleobases, hypoxanthine	5-FU	
NCBT2	Kidney, intestine, choroidal plexus, placenta	Purine and pyrimidine nucleobases, uracil and 5-methyluracil	5-FU	

1.2.2.1 P-glycoprotein (P-gp)

The first ABC transporter to be identified was P-glycoprotein (P-gp), the product of the human MDR1 gene, localised to chromosome 7q21. This ATP-dependent transmembrane efflux pump is responsible for detoxifying normal cells as well as rendering tumour cells resistant to chemotherapy. Its role as a cellular efflux pump, controlling intracellular concentrations of harmful substances, is reflected by its cell and organ specific distribution, as well as its ability to recognise and transport a wide range of compounds. MDR1 is mainly expressed on the surface of luminal epithelial cells of the lower gastrointestinal tract where it influences intestinal drug absorption. Significant amounts of P-gp can be found in biliary canalicular membranes of hepatocytes and in the membrane of the proximal tubules in the kidney confirming the importance of P-gp for the excretion of xenobiotics and endogenous metabolites. P-gp is also expressed at the luminal surface of capillary endothelial cells. In the blood brain barrier this expression may have a protective function. The expression of P-gp in untreated human cancers is highly variable, being almost universal in colon, hepatic, renal and adrenal carcinomas; while it is less common in human haematological malignancies, breast, ovarian, lung and gastric carcinomas, skin cancers, certain germ cell tumours and sarcomas (Cordon-Cardo *et al.*, 1990). At least part of the heterogeneity is attributable to different definitions of positivity, even when a common method of detection is used (Beck *et al.*, 1996).

1.2.2.2 Methods of P-gp detection

Expression of MDR1/P-gp can be detected in human cancers by reverse transcription-polymerase chain reaction (RT-PCR) for mRNA expression and by immunohistochemistry (IHC) for protein expression. Imperfections in these methods include errors due to normal tissue contamination of tumour tissue (for RNA methods), poor sensitivity and specificity, and difficulties in quantitation (for IHC methods). Most studies of P-gp have used IHC. A comparison of several studies reveals that there are inter-laboratory differences due to differing tissue fixation, processing, and staining technique, experience of the observer in selecting hot spots, and the technique of counting positive cells (Beck *et al.*, 1996).

As the activity of P-gp can be affected by different factors, for example the intracellular concentration of ATP (Broxterman *et al.*, 1995), another approach has been to

determine its activity; based on the fluorescent properties of the dye rhodamine 123 (Rh123), which is transported by P-gp, and a functional flow cytometric assay has been developed for the detection of multidrug-resistant (MDR) cells (Ludescher *et al.*, 1992).

Functional assays of P-gp based on flow cytometry have been widely employed to study cell lines and haematological malignancies, but only occasionally in cells derived from solid tumours (Mechetner *et al.*, 1998). For these, *in vivo* radioimaging techniques have been developed (Del Vecchio *et al.*, 1997; Ciarmiello *et al.*, 1998). The use of ^{99m}Tc-MIBI and analogous ^{99m}Tc-labeled agents (which have been shown to be P-gp substrates) allows the clinical assessment of P-gp function in cancer patients (Agrawal *et al.*, 2003), but so far no large prospective clinical trials have been performed to investigate their potential as predictive tests (Palmedo, 2002). A current prospective study in patients with locally advanced breast cancer correlating functional imaging, IHC, and clinical response to chemotherapy may determine the role of functional imaging of MDR in chemotherapy response and prognosis (Tan *et al.*, 2000).

The plurality of methods for diagnosis of P-gp expression reflects the imperfection of currently available diagnostic procedures. In particular, the lack of standardisation has been a major concern, because conflicting definitions of assay end points, scarcity or diversity in control samples, differing assay reagents, and variable methods of sample preparation, data acquisition, analysis, and quality control contribute to inconsistencies in P-gp detection. To address these problems the St. Jude MDR Workshop on ‘Methods to Detect P-Glycoprotein-associated Multidrug Resistance’ in 1996 reached a set of consensus recommendations for standardisation of P-gp assays (Beck *et al.*, 1996). This was intended to improve the reliability of the most common P-gp assays in clinical samples (immunocytochemistry, flow cytometry, functional test, RT-PCR). The recommendations specified sample handling, assay procedures, data analyses, and quality control in detail, but no advice was given with respect to choice of diagnostic procedure. Whether the assay parameter should be MDR1 mRNA, MDR1 gene product, or P-gp substrate transport ability remains unresolved. However, multiparameter assays provide an adequate compromise and should be considered whenever possible. Dual parameter flow cytometry is a less cumbersome and attractive approach due to the combination of protein detection and functional test in one single assay.

1.2.2.3 Clinical relevance of P-gp

Having outlined the difficulties in detecting MDR1/P-gp in cancer cells, it is not surprising that our knowledge of its clinical relevance may be at least partially incomplete. Despite this, there is little doubt that levels of expression of P-gp in many different tumours are high enough to confer significant drug resistance, and the presence of P-gp correlates with drug resistance in several different cancers. In fact malignancies considered to be primarily chemoresistant, such as renal cell, adrenocortical, colon, and hepatocellular cancers, have been shown to consistently demonstrate expression of MDR1 (Fojo *et al.*, 1987; Goldstein *et al.*, 1989).

The acquisition of drug resistance after chemotherapy is associated with increased P-gp levels *in vitro* (Goldstein *et al.*, 1989; Chaudhary & Roninson, 1993), and this increased expression occurs *via* specific molecular mechanisms, such as gene rearrangement and selection of cells showing these rearrangements (Mickley *et al.*, 1998), suggesting that the P-gp-expressing cells have a selective advantage.

Tumours with low levels of P-gp expression at baseline, such as leukaemia, breast cancer, and SCLC, show an increase in expression on relapse, following chemotherapy. Whether this represents selection and repopulation of resistant clones or upregulation due to cytotoxic therapy exposure is unclear. However, recent studies have shown that the expression of P-gp may be rapidly up-regulated in response to chemotherapeutic agents *in vivo* (Abolhoda *et al.*, 1999; Hu *et al.*, 1999; Stein *et al.*, 2002; Tada *et al.*, 2002), and therefore the latest advances favour the second hypothesis.

Several studies have shown an inverse correlation between P-gp expression and chemosensitivity or survival in a variety of tumour types, including leukaemia (Marie *et al.*, 1991; Campos *et al.*, 1992; Damiani *et al.*, 1998), lymphomas (Yuen & Sikic, 1994), osteogenic sarcoma (Baldini *et al.*, 1995; Serra *et al.*, 2003), small-cell lung cancer (Savaraj *et al.*, 1997; Hsia *et al.*, 2002), breast cancer (Linn *et al.*, 1995; Gregorcyk *et al.*, 1996; Burger *et al.*, 2003), advanced ovarian cancer (Baekelandt *et al.*, 2000; Raspollini *et al.*, 2002) and paediatric solid tumours (Chan *et al.*, 1990, 1991a-b). The most reproducible studies on expression in human cancers involve leukaemic cells. P-gp is expressed in acute myelocytic leukaemia (AML) cells in approximately 30% of patients at diagnosis, but in over 50% at relapse (Leith *et al.*, 1999). A lower expression

rate (17%) of P-gp is found in leukaemic cells from patients less than 35 years of age, compared with rates of expression in the elderly (39%), and this may partly explain the better response to therapy seen in younger patients (Leith *et al.*, 1999). The suggestion that P-gp is a marker of poor prognosis due to its mediation of drug resistance in affected cells is supported by studies showing that the prognostic value of P-gp can be mitigated if treatment consists of agents that are not substrates for P-gp-mediated drug efflux (Broxterman *et al.*, 2000).

The results of studies in solid tumours are more heterogeneous. For example a review of ten studies carried out in ovarian cancer showed that only four were able to demonstrate a correlation between MDR1/P-gp expression and response/clinical outcome (table 1.5). A marked variation in the reported detection rates can be seen, depending on the method of detection, previous therapy, the type of tumour, and the tumour grade. A breast cancer meta-analysis concluded that P-gp expression could be detected in 41% of patients with breast cancer, with increased levels post-treatment (Trock *et al.*, 1997). However, an incidence range of 0%–80% for expression of P-gp was reported in the assembled studies.

Table 1.5 Predictive and prognostic role of MDR1/P-gp expression in ovarian cancer, where *n* indicates the number of samples tested.

Study	<i>n</i>	Test	Correlation with response to chemotherapy	Correlation with survival
Holzmayr <i>et al.</i> , 1993	60	RT-PCR	Yes	ND
Izquierdo <i>et al.</i> , 1995	57	IHC	No	No
Schneider <i>et al.</i> , 1998	95	IHC	No	Yes
Arts <i>et al.</i> , 1999	115	IHC	No	No
Yokoyama <i>et al.</i> , 1999	58	IHC	No	No
Baekelandt <i>et al.</i> , 2000	73	IHC	Yes	Yes
Kamazawa <i>et al.</i> , 2002	27	RT-PCR	Yes (paclitaxel-based regimen)	ND
Nakayama <i>et al.</i> , 2002	82	RT-PCR	ND	No
Raspollini <i>et al.</i> , 2003	83	IHC	Yes	Yes
Ikeda <i>et al.</i> , 2003	93	IHC	ND	No

Studies that have shown a change in expression in treated populations relative to baseline are more easily interpretable (Chung *et al.*, 1997). Table 1.6 lists selected studies examining tumour expression levels of P-gp; studies were chosen where data were available on pre-and-post treatment expression levels of P-gp.

Table 1.6 Studies that investigated the expression of P-gp before and after treatment, where *n* indicates the number of samples tested.

Disease	Study	<i>n</i>	Pre or post treatment	MDR1/P-gp test	Expression of P-gp
AML	Van den Heuvel-Eibrink <i>et al.</i> (2002)	20	De novo	qRT-PCR	0.11 (*)
		20	Relapse/refractory	qRT-PCR	0.09 (*)
	Han <i>et al.</i> (2000)	109	De novo	Flow cytometry	27%
		36	Relapse/refractory		64%
	Chauncey <i>et al.</i> (2000)	20	De novo	Flow cytometry	65%
Zhou <i>et al.</i> (1995)	10	Secondary		80%	
	51	De novo	RT-PCR	18%	
Myeloma	Dalton <i>et al.</i> (1995)	41	Pretreatment	IHC	29%
		2	Posttreatment		50%
	Grogan <i>et al.</i> (1993)	47	Pretreatment	IHC	6%
		49	Posttreatment		43%
Breast cancer	Chevillard <i>et al.</i> (1996)	63	Pretreatment	IHC	14%
			Posttreatment		43%
Mechetner <i>et al.</i> (1998)	244	Pretreatment	IHC	11%	
			Posttreatment		30%
Ovarian cancer	van der Zee <i>et al.</i> (1995)	89	Pretreatment	IHC	15%
		38	Posttreatment		48%
Sarcoma	Oda <i>et al.</i> (1997)	5	Pretreatment	RT-PCR	40%
		5	Posttreatment		20%
Bladder cancer	Park <i>et al.</i> (1994)	29	Pretreatment	IHC	75%
		5	Posttreatment		80%
	Nakagawa <i>et al.</i> (1997)	33	Pretreatment	IHC	67%
		28	Posttreatment		86%
Glioma	Abe <i>et al.</i> (1998)	23	Pretreatment	IHC	18%
		7	Posttreatment		57%
Cervical cancer	Riou <i>et al.</i> (1990)	84	Pretreatment	Slot blotting	39%
		8	Posttreatment		88%
	Schneider <i>et al.</i> (1992)	9	Pretreatment	IHC	88%
		6	Posttreatment		83%

(*) As MDR1 mRNA was found in all 20 samples, results are expressed as arbitrary units ($2^{-\Delta Ct}$; see section 2.4)

1.2.2.4 Reversal of P-gp mediated MDR by chemosensitising agents

Chemosensitisation strategies use the administration of one drug or agent to render cancer cells more susceptible to a second agent (Cree *et al.*, 2002a). A broad range of compounds that interact with P-gp and block drug efflux have been reported to reverse the MDR phenotype. The first generation modulators consisted of calcium channel blockers, calmodulin inhibitors, hormonal/steroidal derivatives, antibiotics, cardiovascular drugs and the cyclosporins (Ford, 1996). These compounds were developed for pharmacological uses other than the reversal of MDR and were relatively non-specific and weak inhibitors. The requirement for more selective and potent agents as resistance modifiers has led to the development of several “second-generation” modulators such as the non immunosuppressive cyclosporin D analogue, PSC 833 (valdospar), and VX-710 (biricodar). Second-generation P-gp modulators have a better pharmacologic profile than the first-generation compounds, but they also retain some characteristics that limit their clinical usefulness. In particular, these compounds significantly inhibit the metabolism and excretion of cytotoxic agents, thus leading to unacceptable toxicity that has necessitated chemotherapy dose reductions in clinical trials. In response to cytotoxic agents, cytochrome P450 enzymes are often induced along with members of the ABC transporter family, and it is thought that the genes of these families share overlapping regulatory elements. In fact, many of the cytotoxic agents that are substrates for P-gp are also substrates for the cytochrome P450 isoenzyme 3A4. It is not surprising, then, that the agents that are affected by the development of MDR are also metabolised by cytochrome P450 3A4 (Wandel *et al.*, 1999). Several of the second-generation P-gp modulators, including valspodar and biricodar, are substrates for this enzyme. The competition between cytotoxic agents and these P-gp modulators for cytochrome P450 3A4 activity has resulted in unpredictable pharmacokinetic interactions. For example, valspodar inhibits the cytochrome P450 3A4-mediated metabolism of paclitaxel and vinblastine (Fischer *et al.*, 1998) resulting in increased serum concentrations of the cytotoxic agents and potential risk of cytotoxic drug overexposure in patients (Bates *et al.*, 2001). Similarly, in a pharmacokinetic study in patients with solid tumours, biricodar administered in a 24-hour intravenous infusion decreased the clearance of paclitaxel in a dose-dependent manner (Rowinsky *et al.*, 1998). It has been suggested that this interaction may be due in part to the inhibition of cytochrome P450 3A4 by biricodar, thereby interfering with the metabolism of paclitaxel. The most common response of clinical researchers to this drug interaction

has been to reduce the dose of the cytotoxic agent. However, it should be noted that since the pharmacokinetic interactions between chemosensitisers and cytotoxic agents are unpredictable and cannot be determined in advance, reducing the dose of a cytotoxic agent by a set percentage may result in under- or over-dosing in a significant number of patients.

In addition to inhibiting P-gp, many second-generation modulators also function as substrates for other ABC transporters involved in the elimination of xenobiotics; therefore the inhibition of transporters other than P-gp could lessen the ability of normal cells and tissues to protect themselves from cytotoxic agents. For instance, valspodar and biricodar are not specific solely to P-gp; both of these agents affect MRP1 (Rowinsky *et al.*, 1998; Krishna *et al.*, 2000). It is possible that this inhibition of non-target transporters may lead to greater adverse effects of anticancer drugs, including neutropenia and other myelotoxic effects. For example, the ABC transporter BCRP is a functional regulator of hematopoietic stem cells (Bunting *et al.*, 2002) and its inhibition may contribute to these effects.

Further studies led to the development of third-generation molecules such as XR9576 (tariquidar), GF-120918 (elacridar), LY335979 (zosuquidar), R101933 (laniquidar) and ONT-093 (Thomas & Coley, 2003). These agents have high potency and specificity for P-gp and their clinical efficacy is currently under investigation.

Several phase II studies with first and second generation P-gp inhibitors have been conducted with encouraging results. However, these have not been confirmed in phase III trials. Table 1.7 summarises the randomised clinical trials conducted with P-gp inhibitors up to August 2003. It must be noted that in at least 3 studies the negative results of phase III trials are not clear-cut. The study with PSC 833 in AML by Baer *et al.* (2002) administered cytarabine, doxorubicin, and etoposide chemotherapy with or without the inhibitor to 120 previously untreated patients. The PSC 833 arm was closed early due to excessive mortality, but available data demonstrated no difference in disease-free or overall survival rates. However, functional studies in the leukaemic cells allowed an analysis of subsets based on P-gp expression. In patients with PSC 833-inhibitable efflux (indicating P-gp expression), the median disease-free survival was greater at 14 *versus* 5 months ($p = 0.07$) with the addition of PSC 833. Similarly,

patients treated with chemotherapy alone had a lower complete remission rate (41% versus 91%), a higher non-response rate (41% versus 9%), and a higher death rate (18% versus 0%) when *in vitro* studies exhibited PSC 833-modulated dye efflux, compared with those patients whose cells did not demonstrate efflux ($p = 0.03$). Similarly, another randomised study by Solary *et al.* (2003) found no difference in response rate or survival when a multi-sequential regimen of cytarabine, amsacrine, etoposide and mitoxantrone was administered with or without the inhibitor, quinine. However, in the subset of 160 patients who could be studied, 54 demonstrated rhodamine 123 efflux, and in these patients, quinine significantly improved the CR rate from 12/25 (48.0%) to 24/29 (82.8%) ($p = 0.01$).

The findings from this subset analyses suggest that careful selection of patients is essential in facilitating positive outcomes, as patients deriving benefit from MDR inhibitors may be obscured in trials failing to identify the appropriate target population.

Another little explored field is the ability of P-gp modulators to prevent the development of MDR mediated resistance. Cocker *et al.* (2001) failed in several attempts to obtain cell lines resistant to vincristine when the cells were grown in the presence of the MDR modulators tariquidar, PSC833, and VX710. In early clinical trials testing P-gp modulation in acute leukaemia, cells that survived chemotherapy in the presence of modulators, resulting in clinical relapse, had reduced expression of P-gp (List *et al.*, 1993; Marie *et al.*, 1993). The hypothesis that the development of MDR may be preventable using P-gp modulators therefore warrants further clinical studies.

Table 1.7 Randomised trials of P-gp inhibitors, where n indicates the number of patients.

Disease	Study	n	Chemotherapy	Response rate ±inhibitor		Median overall survival ± inhibitor	
				-	+	-	+
Breast cancer	Wishart <i>et al.</i> , 1994	233	Epirubicin/quinidine	44%	43%	59 weeks	47 weeks
Breast cancer	Belpomme <i>et al.</i> , 2000	99	Vindesine + 5-fluorouracil/verapamil	11%	27%	209 days	323 days*
NSCLC	Millward <i>et al.</i> , 1993	68	Vindesine + ifosfamide/verapamil	18%	41%	22 weeks	41 weeks*
SCLC	Milroy, 1993	220	CAVE/verapamil	80%	85%	44 weeks	41 weeks
Multiple myeloma	Dalton <i>et al.</i> , 1995	59	VAD/verapamil	41%	36%	10 months	13 months
Multiple myeloma	Sonneveld <i>et al.</i> , 2001	75	VAD/cyclosporin A	53%	49%	13 months	14.6 months
MDS (\$)	Wattel <i>et al.</i> , 1998	131	Mitoxantrone + Ara-C/quinine	41%	47%	11 months	13 months
Acute Leukaemia	Solary <i>et al.</i> , 1996	315	Mitoxantrone + Ara-C/quinine	45.5%	52.8%	11.5 months	11.5 months
AML	List <i>et al.</i> , 2001	226	Daunorubicin + cytarabine/cyclosporinA	33%	39%	12% (2-year)	22%* (2-year)
AML	Liu Yin <i>et al.</i> , 2001	213	ADE (cytarabine, daunorubicin, etoposide) /cyclosporin A	41%	45%	7%	8%
AML (\$)	Baer <i>et al.</i> , 2002	120	ADE/PSC 833	46%	39%	7 months	2 months
AML (\$)	Solary <i>et al.</i> , 2003	425	AraC+Idarubicin/amsacrine/mitoxantrone/etoposide +/- quinine	80.6%	81.2%	22 months	30 months

*Statistically significant difference in favour of the modulator.

\$ These studies found statistically significant differences in favour of the modulator when the subset of P-gp positive tumours was analysed.

Abbreviations

VAD = vincristine, doxorubicin, dexamethasone

CAVE = cyclophosphamide, doxorubicin, vincristine, etoposide

ADE = cytarabine, daunorubicin, etoposide

MDS = myelodysplastic syndromes

1.2.2.5 Multidrug resistance associated proteins - the MRP family

After the discovery of P-gp and the demonstration of its widespread expression in many human cancers, it was found that many multidrug-resistant cancers, such as lung cancers, rarely express P-gp. Using a doxorubicin-selected multidrug-resistant lung cancer cell line as a model system, Deeley and Cole and colleagues (1992) cloned another ABC family member, known as *MRP* (for multidrug resistance associated protein). Due to the identification of various homologous proteins to MRP (Borst *et al.*, 2000), it is now designated as MRP1. Together with the cystic fibrosis trans-membrane conductance regulator CFTR, the MRPs constitute the 13 transporter containing human ABC-transporter subfamily C. MRP1 shares only between 34% and 58% sequence identity with the other MRPs (MRP2-MRP6), but the overall membrane topology is similar in all members of subfamily C. MRP1 is widely expressed in many human tissues and cancers (Borst *et al.*, 2000). MRP1, unlike MDR1, transports negatively charged natural-product drugs. Non-anionic compounds may be transported as glutathione, glucuronide, or sulfate conjugates. In some cases cotransport of glutathione (without conjugation) with positively charged drugs such as vincristine can occur (Loe *et al.*, 1998). There is generally some overlapping between the drug resistance spectra of P-gp and MRP1, although taxanes are a notable exception as they are usually poor substrates for MRP1 (Leonard *et al.*, 2003). The specificity of MRP1 for organic anions has also been found in other MRPs. The second member of the MRP (ABCC) family, MRP2, or canalicular multispecific organic anion transporter (cMOAT), has been found to be associated with bilirubin glucuronide transport, with defects resulting in the Dubin-Johnson syndrome. It is also a transporter of MRP1 substrates and cisplatin, with the potential to confer resistance to these agents (Kool *et al.*, 1997). MRP3 is expressed at high levels in the liver and may be involved in the efflux of organic anions from the liver to the blood in the presence of biliary obstruction (Zeng *et al.*, 1999). MRP4 and MRP5 have been found to transport nucleoside analogues. Both confer resistance to thiopurines, and MRP4 has been found to confer resistance to antiretroviral nucleoside analogues (Schuetz *et al.*, 1999; Kruh *et al.*, 2001). MRP6 appears to be a lipophilic anion pump with a wide spectrum of chemotherapy resistance, and mutations in this protein are linked to pseudoxanthoma elasticum (Belinsky *et al.*, 2002).

To date, the only evidence of a link between the MRP family and clinical drug resistance is for MRP1. As for P-gp the different studies are difficult to compare

because of heterogeneity of patient populations, techniques, and because of different criteria with regard to the definition of overexpression. In several studies of haematological malignancies, no or only minor differences have been found in MRP1 expression before and after treatment; however other studies found higher levels of MRP mRNA at time of relapse in AML (reviewed in van den Heuvel-Eibrink *et al.*, 2000). The clinical role of MRP1 in solid tumours is even less clear, due to the almost ubiquitous expression of this protein in normal tissues. For example, whether the MRP1 expression levels associated with breast cancer are enough to confer drug resistance is not yet resolved (Filipits *et al.*, 1996; Dexter *et al.*, 1998). As MRP1 is expressed ubiquitously, it is not surprising that using RT-PCR, MRP1 mRNA can be detected in all breast cancer samples at levels comparable to that in normal tissues. However, one immunohistochemical analysis of a series of resected invasive primary breast carcinomas has reported a correlation between relapse-free survival and MRP1 expression (Nooter *et al.*, 1997). The incidence of MRP1 expression is much higher (about 80%) in small-cell lung cancer (SCLC) samples. MRP1 expression was detected in 100% of non-small cell lung cancers (NSCLC), with higher levels noted in 30% of the samples, though this is not surprising, given its ubiquitous expression in normal lung tissue (Nooter *et al.*, 1996; Young *et al.*, 1999).

1.2.2.6 Breast cancer resistance protein (BCRP)

Observations of functional adenosine triphosphate (ATP)-dependent drug efflux in certain multidrug-resistant cancer cell lines without overexpression of P-glycoprotein or multidrug resistance protein (MRP) family members suggested the existence of another ATP-binding cassette (ABC) transporter capable of causing cancer drug resistance. Overexpression of a novel member of the G subfamily of ABC transporters was found in a subline of the MCF-7 human breast carcinoma cells, and this was therefore termed the Breast Cancer Resistance Protein (BCRP) (Doyle *et al.*, 1998). Owing to tissue localisation in the placenta, bile canaliculi, colon, small bowel, and brain microvessel endothelium, BCRP may play a role in protecting the organism from potentially harmful xenobiotics (Jonker *et al.*, 2000). The spectrum of anticancer drugs pumped by BCRP includes mitoxantrone, camptothecin-derived and indolocarbazole topoisomerase I inhibitors, methotrexate, flavopiridol, and quinazoline ErbB1 inhibitors (reviewed in Doyle & Ross, 2003). Transport of anthracyclines is variable and appears to depend on the presence of a BCRP mutation at codon 482 (Honjo *et al.*, 2001). Potent inhibitors of

BCRP are now being developed (Maliepaard *et al.*, 2001), opening the door to clinical applications of BCRP inhibition. Studies are emerging on the role of BCRP expression in drug resistance in clinical tumour specimens, although none of them has so far prospectively investigated the contribution of BCRP to drug resistance in human cancers.

As for the other transporters, no method of BCRP detection has so far become the 'gold standard'. IHC studies have employed two different mouse monoclonal antibodies against BCRP, BXP34 and BXP21, and have reported differing results (Diestra *et al.*, 2002). A functional assay for the detection of BCRP has recently been described (Robey *et al.*, 2001): flow cytometric measurement of FTC-inhibitable mitoxantrone efflux was found to be a sensitive and specific method in both selected and unselected cell lines.

BCRP has been studied in tumour samples from patients with acute myeloid leukaemia (AML) both at the mRNA level (Ross *et al.*, 2000; van Den Heuvel-Eibrink *et al.*, 2002) and at the protein level by immunocytochemistry (Sargent *et al.*, 2001) or flow cytometry (van der Kolk *et al.*, 2002). Ross *et al.* (2000) found relatively high expression of BCRP mRNA in 33% AML samples: BCRP expression did not correlate with P-gp expression and the authors suggested that BCRP could be responsible for resistance to certain antileukaemic drugs in P-gp negative cases. Sargent *et al.* (2001) found that BCRP detected by immunocytochemistry in the cells of AML patients correlated with the LC50 of daunorubicin determined by the MTT *in vitro* chemosensitivity assay, but not with the IC50 values of mitoxantrone, topotecan or doxorubicin. Steinbach *et al.* (2002) were able to associate BCRP gene expression measured using TaqMan qRT-PCR with a poor response to remission induction therapy in 59 children affected with AML. One study (van der Heuvel-Eibrink *et al.*, 2002) found that BCRP mRNA levels (determined by qRT-PCR) in 20 paired AML clinical samples were increased after chemotherapy, while another study (van der Kolk *et al.*, 2002) failed to find an increased expression of the protein (determined by flow cytometry) in relapsed/refractory patients.

There is less information about solid tumours. Nakayama *et al.* (2002) were able to detect BCRP expression in ovarian carcinomas using standard RT-PCR, but did not find

any prognostic relevance. Kanzaki *et al.* (2001) found that primary breast carcinomas had low mRNA levels (determined by semi-quantitative RT-PCR) of several MDR related transporters, including BCRP. Scheffer *et al.* (2000) found that IHC expression of BCRP was undetectable or very low in a panel of human solid tumours. However, this series included only a few carcinomas for each tumour type, and most were from untreated patients. Furthermore IHC was found to be a poorly sensitive method to detect BCRP (Faneyte *et al.*, 2002), compared to other techniques, such as real time RT-PCR, which allow detection of the target molecule mRNA even from a single cell. More recently Faneyte *et al.* (2002) studied the expression of BCRP in breast cancer specimens from 25 primary carcinomas and 27 anthracycline-exposed patients applying a variety of methods, including immunostaining with mouse monoclonal antibodies against BCRP, BXP34 and BXP21. The authors were not able to observe specific tumour cell staining, but were able to detect BCRP mRNA levels by sensitive qRT-PCR in both chemotherapy-naïve and anthracycline treated patients. Positive immunostaining of vascular endothelium and of a part of the normal epithelium in large mammary ducts occurred with both antibodies, suggesting that antibody sensitivity was not an issue, but it should be noted that RNA was extracted from 20 µm slices of the cryopreserved tissue samples and not from a pure isolated cancer cell population. Endothelial cells in the tumour may therefore be responsible for the qPCR results. Burger *et al.* (2003) used qRT-PCR to determine the relevance of BCRP mRNA expression in breast specimens. It is notable that all three studies conducted on breast tumours failed to find any correlation between BCRP expression and response to doxorubicin-based chemotherapy (Kanzaki *et al.*, 2001; Burger *et al.*, 2003) or patient survival (Faneyte *et al.*, 2002; Burger *et al.*, 2003).

1.2.2.7 ATP 7B

There is evidence to indicate that reduced drug accumulation is a significant mechanism of cisplatin resistance (Kelland, 1993; Chau & Stewart 1999). The cause of the reduced cisplatin accumulation in resistant cells may be ascribed to either an inhibition of drug uptake, an increase of drug efflux, or both. A defect in the uptake process has been investigated, but the mechanism for this remains unclear and has been attributed to changes in the non-saturable process of passive drug diffusion (Yoshida *et al.*, 1994; Kelland, 2000) or other forms of active transport (Andrews *et al.*, 1988; Gately and Howell, 1993).

Development of resistance as a result of increased cisplatin efflux was largely discounted in earlier studies involving only P-gp (Teicher *et al.*, 1987; Andrews *et al.*, 1988). More recently, MRP2 has been associated with cisplatin resistance (Kool *et al.*, 1997; Koike *et al.*, 1998; Cui *et al.*, 1999), although some controversy still exists (Shen *et al.*, 2000; Nakayama *et al.*, 2002).

A second important area of investigation involving cisplatin efflux has centered on ATP7B. This protein is a member of a class of heavy metal-transporting, P-type ATPases that pump copper, cadmium, zinc, silver or lead (Solioz *et al.*, 1996; Gupta *et al.*, 1999). The induction of the ATP7B gene was observed by exposure to cisplatin in cell lines, and human tumour cells transfected with ATP7B acquired significant resistance to cisplatin (nine-fold), primarily as a consequence of its enhanced efflux (Komatsu *et al.*, 2000).

In human tumours the expression of ATP7B has been studied mainly by IHC and has been detected with variable frequency in breast (22%; Kanzaki *et al.*, 2002), ovarian (44%; Nakayama *et al.*, 2002), oesophageal (76%; Higashimoto *et al.*, 2003) and gastric (41%; Ohbu *et al.*, 2003) carcinomas and in 55% of primary oral squamous cell carcinomas (SCC) (Miyashita *et al.*, 2003). The expression of ATP7B is generally higher in poorly differentiated carcinomas than in well-differentiated tumours (Kanzaki *et al.*, 2002; Nakayama *et al.*, 2002; Ohbu *et al.*, 2003). Recently, two studies, the first in ovarian cancer (Nakayama *et al.*, 2002), and the second in oral SCC (Miyashita *et al.*, 2003) have shown that ATP7B positivity in tumour samples at diagnosis is a negative prognostic factor in patients who had received cisplatin-containing chemotherapy.

1.2.2.8 Major vault protein - Lung resistance protein

Although frequently included in discussions of transporter-mediated resistance, lung resistance protein (LRP) is not an ABC transporter, but is a major vault protein (MVP) (Scheffer *et al.*, 1995) found in the cytoplasm and nuclear membrane. MVP is broadly distributed in normal and malignant cells. High MVP expression is seen in bronchus, digestive tract, renal proximal tubules, keratinocytes, macrophages and adrenal cortex whereas varying levels were observed in other organs (Kedersha *et al.*, 1990; Izquierdo

et al., 1996; Schroeijers *et al.*, 2002). The localisation of MVP suggests a role in the defence of these organs and cells against toxic compounds.

The expression of MVP closely reflects the chemoresistance profile of many tumour cell lines and untreated cancers (Scheper *et al.*, 1993; Izquierdo *et al.*, 1996a; Kickhoefer *et al.*, 1998; Schroeijers *et al.*, 2000; Siva *et al.*, 2001). Elevated MVP levels have been observed in cell lines resistant to various classes of cytotoxic agents including doxorubicin, mitoxantrone, methotrexate, etoposide, vincristine, cytarabine and cisplatin (Scheper *et al.*, 1993; Versantvoort *et al.*, 1995; Verovski *et al.*, 1996; Laurençot *et al.*, 1997; Moran *et al.*, 1997; Wyler *et al.*, 1997; Komarov *et al.*, 1998). In non-small-cell lung cancer cells, MVP expression levels, determined by protein and mRNA, correlated with resistance to cisplatin (Berger *et al.*, 2000). However, in this study, no correlation was observed with resistance to daunomycin, bleomycin, doxorubicin, etoposide and vinblastine. In contrast, in pharyngeal carcinoma KB-3-1 cells, increased MVP levels were found to correlate with decreased accumulation of doxorubicin in the nuclei of these cells (Cheng *et al.*, 2000). Another recent study performed in U-937 leukaemia cells reports that cells selected by doxorubicin exposure upregulated vault levels and acquired resistance against doxorubicin, etoposide and mitoxantrone. This resistance seemed to be independent of P-gp (MDR1), multidrug-resistance-related protein (MRP1), MRP2 and breast cancer resistance protein (BCRP) (Hu *et al.*, 2002).

It is thought that MVP drives drugs out of the nucleus. Recently, the group of Akiyama reported on experiments supporting a role for vaults of extrusion of anthracyclines from the nuclei of resistant cells (Kitazono *et al.*, 1999 & 2001; Ohno *et al.*, 2001). Treatment of the colon carcinoma cell line SW620 with sodium butyrate led to a strong induction of MVP and made the cells significantly less sensitive to doxorubicin, etoposide (VP-16), vincristine, paclitaxel and the transport antibiotic gramicidin D. The stable expression of two unrelated MVP-specific ribozymes reversed the observed resistant phenotype. The molecular mechanism of vault-mediated resistance against doxorubicin was investigated more closely. The drug, which accumulated in the nuclei of untreated cells, was shown to efflux more rapidly from the nuclei of sodium butyrate-treated cells. The efflux of doxorubicin, from the nuclei in intact cells or isolated nuclei, could be inhibited by the expression of the anti-MVP ribozymes or the addition of polyclonal

anti-MVP antibodies (Kitazono *et al.*, 1999). In a subsequent study, the pyridine analog PAK-104P was introduced as specific inhibitor of vault-mediated efflux (Kitazono *et al.*, 2001). Taken together, these findings provide evidence for the hypothetical model in which vaults function in nuclear drug export and as a consequence may cause drug resistance.

The hypothesis that MVP expression may reflect a novel pathway of multidrug resistance has prompted several clinical studies to determine the expression of this molecule in human tumours. These studies have mainly focused on whether the level of MVP expression predicts clinical outcome after chemotherapy. The majority of the studies have been performed in haematological malignancies, but some solid tumours have been examined. Various detection techniques have been used for MVP, including immunohistochemistry and RNA expression as determined by RT-PCR. The results obtained with these detection assays are variable, mainly because a functional assay of MVP activity has not yet been developed. Thus far, the evidence that MVP expression correlates with clinical response is weak, due partly to the small numbers of patients investigated. Another limitation is that most studies have been founded on univariate analysis without evaluating other prognostic parameters. Therefore compelling evidence that MVP expression correlates with the clinical response and prognosis is still lacking and no prospective trials using a functional assay and multivariate analysis of risk factors have been performed.

The expression of MVP in human cancers is variable; low rates of MVP positivity are seen in testicular cancer, neuroblastoma, and AML; intermediate in ovarian cancer; and high in colon, renal, and pancreatic carcinomas (Izquierdo *et al.*, 1996a). Several studies have investigated the role of MVP in haematological malignancies. Some investigators have proposed that MVP expression negatively correlates with either response or survival in AML (Goasguen *et al.*, 1996; List *et al.*, 1996; Hart *et al.*, 1997; Borg *et al.*, 1998; Filipits *et al.*, 1998 & 2000; Xu *et al.*, 1999). In contrast, other studies have not pointed towards a predictive or prognostic significance for MVP (Damiani *et al.*, 1998; Legrand *et al.*, 1998; Leith *et al.*, 1999; Michieli *et al.*, 1999; Pallis *et al.*, 1999).

Relatively few studies have addressed the role of MVP in solid tumours. The strongest expression of MVP is found in colorectal tumours. In this tumour, the expression

increases from pre-malignant lesions such as colonic adenoma to aggressive colon carcinoma, which indicates that MVP may be associated with more aggressive disease (Izquierdo *et al.*, 1996a; Meijer *et al.*, 1999). Other tumours in which expression of MVP has been reported include melanoma, osteosarcoma and neuroblastoma (Ramani and Dewchand, 1995). In uveal melanoma, high expression is observed, which seems to correlate with aggressive behaviour of the tumour, (Schadendorf *et al.*, 1995; van der Pol *et al.*, 1997). In osteosarcoma, MVP expression was reported to correlate with failure to respond to chemotherapy and poor survival (Uozaki *et al.*, 1997). Two studies from the same group have investigated the expression of MVP in ovarian cancer (Izquierdo *et al.*, 1995; Arts *et al.*, 1999). In advanced ovarian cancer FIGO stage III/IV, 77% of the patients express MVP at diagnosis. In localised cancer FIGO stage I/II, a similar figure is observed. In advanced ovarian cancer, a correlation was found between MVP expression and lack of response and/or shorter OS, but this was not found in early-stage ovarian cancer. In contrast, in early-stage ovarian cancer, MVP expression was associated with favourable prognostic variables. Two studies were performed in breast cancer using immunohistochemistry to investigate MVP expression. In one study, the expression ranged from 69 to 75% without significant differences between samples obtained at diagnosis or at relapse after chemotherapy (Linn *et al.*, 1997). The second study found 68% of patients with intermediate or high MVP expression (Pohl *et al.*, 1999). In neither study, was a clear correlation with clinical outcome observed. Similarly, Burger *et al.* (2003) failed to correlate the expression of MVP mRNA with response or overall survival in a series of 59 breast tumour specimens. However this study found only a very modest correlation between high expression levels of *LRP* and poor progression-free survival in a sub-group of patients who had received an anthracycline-based regimen.

1.2.2.9 Antimetabolite transporters

Drugs that disrupt nucleotide synthesis are important anticancer therapeutics. Synthetic nucleoside and nucleobase analogues, such as 5-fluorouracil, 6-mercaptopurine, 6-thioguanine, cytarabine, cladribine, fludarabine and gemcitabine (table 1.1) have been developed as clinically useful drugs in the chemotherapy of a variety of human tumours. Drugs inhibiting folate metabolism, such as methotrexate and drugs inhibiting ribonucleotide reductase, such as the hydroxyurea and triapine, also target nucleotide metabolism by inhibiting nucleotide synthesis. Other antimetabolites inhibit phosphor-

ylation of nucleotides, such as tiazofurin. The cytotoxicity of all of these drugs may depend on metabolic activation and uptake/efflux of the drugs themselves as well as their active metabolites and endogenous or exogenous rescue agents.

Under physiologic conditions, nucleosides and nucleobases and their analogues are hydrophilic and they do not readily cross the plasma membrane by diffusion. Efficient cellular uptake therefore requires the presence of specialised plasma membrane proteins, called nucleoside transporters (NTs) (Baldwin *et al.*, 1999) and nucleobase transporters (NCBTs). The NTs consist of two structurally unrelated protein families that are designated ENT and CNT, depending on whether they mediate, respectively, equilibrative (E) or concentrative (C) NT processes (reviewed in Damaraju *et al.*, 2003). Concentrative nucleoside transporters (CNTs) have been found in heart, skeletal muscle, placenta, pancreas and lung, while equilibrative nucleoside transporter (ENTs) expression is ubiquitous.

The NCBTs are expressed in kidney, intestine, choroid plexus and placenta and have been demonstrated to be the route of entry for nucleobases and nucleobase analogues used in cancer chemotherapy (Griffith *et al.*, 1996; table 1.4).

As mechanisms of uptake, increased expression of these transporters may therefore result in increased sensitivity to their respective substrates. Several *in vitro* studies have suggested that there may be a relation between transporter expression and antimetabolite drug sensitivity. Transduction of ENT2 into bone marrow cells resulted in resistance to anti-folate drugs in murine bone marrow cells (Patel *et al.*, 2000). Decreased or deficient ENT1 activity has been shown to contribute to the *in vitro* resistance to gemcitabine and the active capecitabine metabolite 5'-deoxy-5-fluorouridine (Mackey *et al.*, 1998 & 2002).

The first assays available for the quantitation of NT proteins in clinical samples relied on radiolabeled or fluorescently labeled binding analysis of the nucleoside transport inhibitor nitrobenzylmercaptapurine ribonucleoside (NBMPR; Gati *et al.*, 1997). Such assays, although technically demanding and requiring large numbers of homogeneous viable cells, demonstrated that hENT1 deficiency in myeloblasts correlated with clinical cytarabine resistance (Gati *et al.*, 1997). The relevance of hNTs deficiency in clinical

resistance to nucleoside drugs in solid tumours remains speculative. It is hoped that new detection methods for NTs recently developed, such as immunohistochemistry (Mackey *et al.*, 2002) and real time PCR (Lu *et al.*, 2002), would clarify the role of these transporters in solid malignancies.

On the other hand, increased expression of NTs may also cause increased resistance to nucleoside analogues by importing competing substrates that can rescue cells from toxicity.

1.2.3 Increased detoxification

1.2.3.1. Glutathione S-transferases

Glutathione S-transferases (GSTs) are a family of phase II detoxification enzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds. GSH conjugates are then pumped out of the cells by the MRP series of ABC transporter molecules (section 1.2.1). GSTs are divided into two distinct super-family members: the membrane bound microsomal and cytosolic family members. Cytosolic GSTs are subject to significant genetic polymorphisms in human populations. They are divided into six classes, which are designated by Greek letters α , μ , θ , π , ψ , and ζ (Barone & Tew 1996).

Recent studies have demonstrated a regulatory role for the π and μ classes of GSTs in the mitogen-activated protein (MAP) kinase pathway that participates in cellular survival and death signaling (reviewed in Townsend & Tew, 2003). Specifically, ASK1 (apoptosis signal-regulating kinase) is a MAP kinase that activates the c-Jun N-terminal kinase (JNK) and p38 pathways leading to cytokine- and stress-induced apoptosis (Ichijo *et al.*, 1997). The activity of ASK1 is low in non-stressed cells due to its sequestration via protein: protein interactions with GST μ 1 (GST μ 1: ASK1 complex), and/or thioredoxin (Trx: ASK1 complex) (Cho *et al.*, 2001; Saitoh *et al.*, 1998). Dissociation of the GST μ 1: ASK1 complex is heat shock dependent (Saitoh *et al.*, 1998). Forced expression of GSTM1 blocked ASK1 oligomerisation and repressed ASK1-dependent apoptotic cell death (Cho *et al.*, 2001). GST- π was shown to be an endogenous inhibitor of JNK1, a kinase involved in stress response, apoptosis, and cellular proliferation (Adler *et al.*, 1999). In non-stressed cells, low JNK activity is

observed due to the sequestration of the protein in a GST- π : JNK complex (Adler *et al.*, 1999). However, suppression of JNK activity is reversed by conditions of oxidative stress (UV irradiation or hydrogen peroxide treatment), resulting in the dissociation of the GST- π : JNK complex, oligomerisation of GST- π , and induction of apoptosis (Adler *et al.*, 1999). This novel, non-enzymatic role for GST- π has direct relevance to the GST-overexpressing phenotypes of many drug-resistant tumors. As an endogenous switch for the control of signaling cascade pathways, elevated expression of GST- π can alter the balance of regulation of kinase pathways during drug treatment, thereby conferring a potential selective advantage (Townsend & Tew 2003). This process also provides a plausible explanation for the numerous examples of drug resistance linking GST overexpression with agents that are not substrates for these enzymes (table 1.8).

Several studies have investigated whether the effects of chemotherapy can be potentiated by inhibiting GSH detoxification pathway. The main approaches to inhibiting the GSH system are either to deplete cellular glutathione or to inhibit the GSTs. The commonest way to deplete cellular GSH is to inhibit its synthesis. Inhibition of γ -glutamylcysteine synthetase, an enzyme required for GSH synthesis, has been found to be the most effective method of inhibition (Barone & Tew 1996). Buthionine sulfoximine acts by inhibiting γ -glutamylcysteine synthetase (Campbell *et al.*, 1991) and it has been shown to enhance the activity of a number of varied drugs, including treosulfan (Reber *et al.*, 1998) and cisplatin (Pendyala *et al.*, 1997, Perez *et al.*, 1998).

Inhibition of GSTs has also been studied as a method to increase the efficacy of a number of chemotherapeutic agents. Ethancrynic acid (Caffrey *et al.*, 1999) and glutathione derivatives (Nakanishi *et al.*, 1997) have all been used to inhibit GSTs and to enhance the activity of a wide range of chemotherapeutic agents.

Table 1.8 Compounds associated with GST mediated resistance (Barrone & Tew, 1996; Townsend & Tew, 2003)

Substrates of GST	Not characterised as substrates
Chlorambucil	Antimetabolites*
Melphalan	Antimicrotubule drugs*
Nitrogen mustard	Topoisomerase I & II inhibitors*
Phosphoramidate mustard	Bleomycin
Acrolein	Hepsulfam
Carmustine	Mitomycin C*
Hydroxyalkenals	Doxorubicin*
Ethacrynic acid	Cisplatin*
Steroids	Carboplatin

*Require JNK activation for cytotoxicity

1.2.3.2 Metallothioneins

Metallothioneins (MTs) are a group of low-molecular weight, cysteine rich intracellular proteins, which are encoded by a family of genes containing at least 10 functional isoforms in human. Although a number of biological functions have been proposed for MTs, most of them are related to its metal-binding property (reviewed in Cherian *et al.*, 2003). Thus, MTs may protect against certain metal toxicity, and may donate zinc/copper to certain metallo-enzymes and transcription factors. MTs may also protect against oxidative stress because of its high cysteine content. MTs are mainly cytoplasmic proteins in adult tissues, although they are also detected in the nucleus of cells in fetal/early neonatal period (Chan & Cherian 1993). A transient localisation of MTs into nucleus under certain conditions such as cell proliferation and differentiation has been reported (Apostolova & Cherian 2000).

Previous studies have demonstrated that overexpression of MTs can confer resistance to radiation and antineoplastic drugs such as cisplatin (Satoh *et al.*, 1994). It has been proposed that the sequestration of free radicals, drugs or their metabolites by MTs, may inhibit the direct interaction of antineoplastic agents with their intracellular targets, thereby decreasing the efficacy of their chemotherapeutic effects. A role in modulation of apoptosis has also recently been suggested, as cadmium-induced MT levels were negatively correlated with sensitivity to etoposide-induced apoptosis (Shimoda *et al.*, 2003).

The role of MT in the development of chemoresistance in the clinical setting is still controversial. Studies in human tumours such as ovarian, testicular and colon tumours, appear to suggest that overexpression of MT may have a protective effect against antineoplastic agents, whereas there are other reports which do not support this perception (Chin *et al.*, 1993; Ioachim *et al.*, 1999).

In breast cancer, two separate studies have concluded that MT overexpression is associated with significantly poorer prognosis and increased recurrence in pT2 invasive ductal breast carcinomas (Schmid *et al.*, 1993; Goulding *et al.*, 1995). However Ioachim *et al.* (2003) have recently shown that MT expression is a positive prognostic factor in primary invasive breast cancer.

In ovarian cancer, previous studies do not point towards a clinical relevant role of MT (Murphy *et al.*, 1991), although there is marginal and discordant evidence that platinum-based chemotherapy may increase MT expression in paired tumour biopsies (McGown *et al.*, 1994; Wrigley *et al.*, 2000).

MT gene overexpression has been found in a number of bladder tumours that had failed cisplatin chemotherapy (Wood *et al.*, 1993), and MT was found to be a negative predictive factor for cisplatin-based chemotherapy in various urological cancers (Bahnson *et al.*, 1994; Kotoh *et al.*, 1994; Siu *et al.*, 1998).

The role of MT in gastrointestinal (GI) malignancies is also controversial. Two studies failed to correlate MT expression with tumour progression in colorectal adenocarcinoma (Ioachim *et al.*, 1999). However, others have demonstrated that GI cancer is accompanied by a decrease in MT expression, but that the most malignant phenotypes show the highest MT levels (Janssen *et al.*, 2002). In particular, Janssen *et al.* (2002) found that more than 74% of GI carcinomas had a lower MT level than their corresponding normal mucosa. In colorectal cancer patients, but not in gastric cancer patients, a high MT level in both the carcinomas and normal mucosa was, however, significantly associated with a poor overall survival, independently from clinicopathological features. In gastric cancer, however, MT expression in the gastric mucosa was not of prognostic significance. MT expression was not found to correlate with survival also in another series of 61 oesophageal tumours (44 adeno- and 17

squamous cell carcinomas) treated only with surgical resection, while low-level P-gp expression was a prognostic factor (Aloia *et al.*, 2001). Similarly, another study found no association between MT expression and survival in a group of 118 oesophageal cancer patients who were treated with a cisplatin-based regimen (Harpole *et al.*, 2001); while high-level expression of GST- π , P-gp, and TS were negative prognostic factors. However, in two studies, MT-positive oesophageal squamous cell carcinomas had a poor response to chemoradiation therapy (Yamamoto *et al.*, 1999; Kishi *et al.*, 2002).

1.2.3.3 Cytochrome P450 enzymes

Several cytochrome P450 (CYP) enzymes are involved in the metabolism of a range of anticancer drugs, including dacarbazine, cyclophosphamide, paclitaxel, and docetaxel (Kivisto *et al.*, 1995; Marre *et al.*, 1996; Crommentuyn *et al.*, 1998). CYP-mediated metabolism usually results in reduced activity or inactivation of the anticancer drugs, but in some cases bioactivation to a more cytotoxic metabolite occurs.

One example of detoxification of anticancer drugs is shown by the taxanes. The major pathway of metabolism of paclitaxel is catalysed by CYP2C8 and involves the hydroxylation of position 6 on the taxane ring (Rahman *et al.*, 1994). The metabolite 6-hydroxytaxol is 30-fold less cytotoxic than the parent compound paclitaxel (Harris *et al.*, 1994), and this metabolite is further metabolised by CYP3A4 (Crommentuyn *et al.*, 1998). Docetaxel is metabolised by CYP3A to apparently less cytotoxic metabolites (Marre *et al.*, 1996; Crommentuyn *et al.*, 1998), and a role for CYP1B1 has also been recently demonstrated (Rochat *et al.*, 2001; Mc Fadyen *et al.*, 2001).

Other anticancer drugs are metabolically activated by CYPs, such as DTIC and cyclophosphamide (Chang *et al.*, 1993; Reid *et al.*, 1999). In particular, cyclophosphamide must first undergo a 4-hydroxylation reaction to 4-hydroxycyclophosphamide by CYP enzymes (CYP2B6 and CYP3A4) (Chang *et al.*, 1993) before becoming cytotoxic (Bohnenstengel *et al.*, 1996). CYP3A4 has also been shown to be involved in the metabolic activation of doxorubicin to the more cytotoxic morpholino-doxorubicin (Lewis *et al.*, 1992; Goepfert *et al.*, 1994).

1.2.4 Topoisomerase-mediated resistance

Topoisomerases are essential enzymes, which play an important role during cell division. Topoisomerases I and II unravel DNA during transcription, DNA replication, and DNA repair. While topoisomerase I (TOPO I) cleaves single strands of duplex DNA, topoisomerases II (TOPO II) introduce double strand breaks that allow relaxation, unknotting and decatenation (Champoux 2001).

TOPO I is involved in DNA replication, RNA transcription, as well as in chromosome condensation and segregation. Inhibitors of this enzyme, camptothecin and its derivatives, topotecan and irinotecan, are currently used to treat solid tumours such as ovarian and colorectal cancer (Takimoto *et al.*, 1998). Mutations, decreased expression and/or activity of TOPO I have all been associated with camptothecin resistance in cell line experiments (Saleem *et al.*, 2000). Multiple mechanisms contribute to the regulation of TOPO I levels in the cell. In tumours demonstrating increased levels of enzyme expression, increased levels of mRNA have also been observed (Husain *et al.*, 1994). This would indicate either increased transcription or increased mRNA stability. On the other hand, cell lines with reduced expression were found to have hypermethylation of one allele of the TOPO I gene, leading to decreased transcription and thus reduced enzymatic production of TOPO I (Tan *et al.*, 1989).

Very little is known about the role played by TOPO I levels in determining chemotherapy response in human cancers. Preliminary data suggest that gene expression levels of TOPO I may be predictive of response to therapy with irinotecan in colorectal cancer patients (Iqbal & Lenz 2001), but further dynamic studies evaluating the change of TOPO I expression in cancer patients during a treatment period are warranted.

Two isoforms of TOPO II exist, α and β . TOPO II α is essential for cell growth and is a cell proliferation and tumour marker, while TOPO II β is apparently nonessential for growth and its function remains unclear (Errington *et al.*, 1999). The two isoforms are expressed at different points in the cell cycle. TOPO II α reaches its highest level in the late S/G2 phase (figure 1.1), whereas the β isoform is expressed at a constant level throughout the cell cycle (Kimura *et al.*, 1994). In addition, the level of TOPO II α is

strongly decreased (Larsen & Skladanowski 1998) in growth-arrested cells. Inhibitors of TOPO II α use multiple mechanisms. Early studies demonstrated that certain compounds, including AMSA, doxorubicin and mitoxantrone, act primarily by intercalating into the DNA between the bases while other drugs, such as etoposide, bind to the actual enzyme (Pommier *et al.*, 1996). All of these drugs poison the enzyme by increasing the steady-state concentration of its covalent DNA cleavage complexes. This action converts TOPO II into a physiological toxin that creates double-stranded DNA breaks in the genome of treated cells and induces mutagenic and lethal events (Burden & Osheroff 1998).

TOPO II α targeted drugs are considerably more lethal to cells that contain high levels of the enzyme and are undergoing high rates of DNA replication. Several studies in patient specimens have shown a relation between TOPO II α expression and the proliferative state of the tumour, higher TOPO II α levels being seen in more highly proliferating tumour types (Kellner *et al.*, 2002). Therefore clinically aggressive cancers such as acute leukaemia appear to be most responsive to TOPO II inhibitors (Hande 1998).

In vitro resistance to TOPO II inhibitors can be mediated by efflux pumps (section 1.2, table 1.3), but is also seen when the activity and sensitivity of the target enzyme topoisomerase TOPO II α itself are decreased by down-regulation or mutation ('atypical' MDR) (Kellner *et al.*, 2002). Mutations in TOPO II α do not seem to have a major role in clinical resistance; the few studies conducted so far found no mutations (Kaufmann *et al.*, 1994) or a low frequency (Kubo *et al.*, 1996). The relevance of TOPO II α expression in predicting response to chemotherapy is uncertain. No relation was observed between expression or activity of TOPO II α and response to chemotherapy in a number of studies (Dingemans *et al.*, 1999 & 2001; Jarvinen *et al.*, 1998); nonetheless one group reported a correlation between complete response to anthracycline-based chemotherapy and TOPO II α gene amplification and protein overexpression in breast cancer where TOPO II α may be co-amplified with HER2 (Cardoso *et al.*, 2004). Moreover, in one study a trend of reduced TOPO II α levels was seen in samples taken after chemotherapy treatment, as compared with specimens prior to treatment (Kaufmann *et al.*, 1994). While the predictive role of TOPO II α overexpression is unclear, its negative prognostic significance is well established in several malignancies

(Dingemans *et al.*, 1999 & 2001; Jarvinen *et al.*, 1998; Provencio *et al.*, 2003; Skotheim *et al.*, 2003), though this probably reflects tumour aggressiveness rather than a parameter determining response to chemotherapy.

An observation of considerable clinical interest is that the expression of TOPO II α can be stimulated by TOPO I inhibitors. Treatment of patients with camptothecins led to a transient increase in the expression of TOPO II α protein in peripheral blood mononuclear cells (Rubin *et al.*, 1995; Nicklee *et al.*, 1996; Gupta *et al.*, 1998). Similarly, topotecan or irinotecan treatment resulted in an increase in TOPO II α levels and sensitivity to etoposide in human colon cancer xenografts (Whitacre *et al.*, 1997; Eder *et al.*, 1998). These observations led to phase I trials to evaluate the sequential administration of topotecan followed by etoposide, but these have been rather disappointing and hampered by severe toxicity (Hammond *et al.*, 1998; Crump *et al.*, 1999). However, a recent phase I study in patients with advanced malignancies found acceptable toxicity and significant activity when the combination of cisplatin and irinotecan was followed by etoposide (Licitra *et al.*, 2003).

An alternative approach to sequential administration studies has been to combine in one molecule the properties of both TOPO I and TOPO II inhibitors (Denny & Baguley 2003). Given the differing roles and expression of the two types of enzymes within the cell cycle, inhibitors of both TOPO I and II ('dual inhibitors') would be expected to have significant therapeutic advantage over agents targeting one type of topoisomerase alone. In addition, dual inhibitors may circumvent mechanisms of drug resistance attributable to alteration (e.g. by mutation or down-regulation) of a single target enzyme. Dual inhibitors may also be expected to have a broader spectrum of activity, as the expression levels of the two enzymes are variable between different types of cancers (McLeod *et al.*, 1994). Recently several joint inhibitors of TOPO I and TOPO II have been described: DACA/XR5000 (Finlay *et al.*, 1996), TAS-103 (Utsugi *et al.*, 1997), intoplicine (Riou *et al.*, 1993), F 11782 (Perrin *et al.*, 2000), XR11576 (Mistry *et al.*, 2002), and XR5944 (Stewart *et al.*, 2001), although the last of these has recently been found to act mainly via a non-TOPO mediated mechanism of action (Sappal *et al.*, 2004).

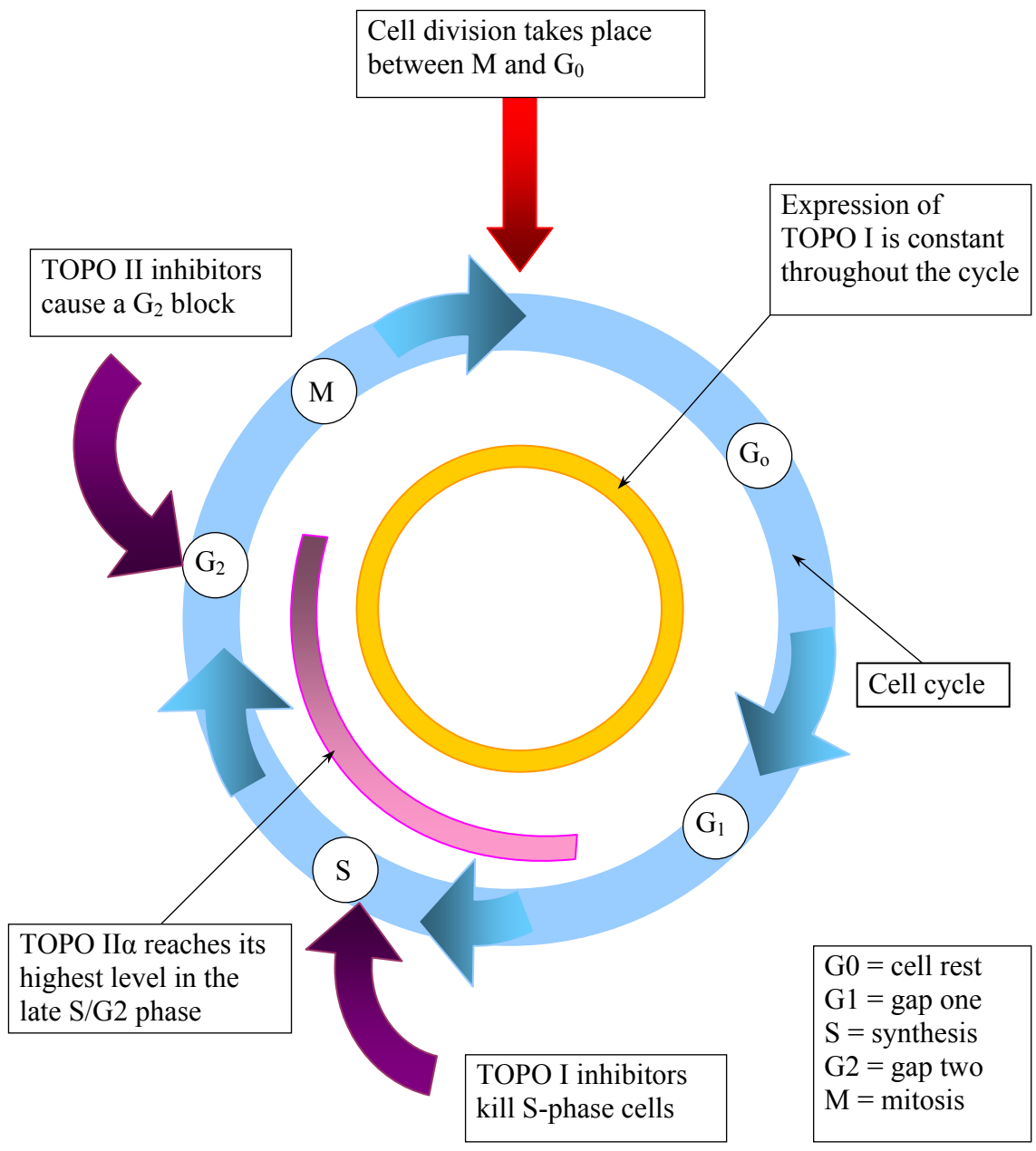


Figure 1.1 Cell cycle and topoisomerase expression.

1.2.5 Mechanisms involved in resistance to 5-FU

5-FU is an antimetabolite widely used in the treatment of breast, colorectal and upper GI malignancies. More than 80% of administered 5-FU is normally catabolised by hepatic dihydropyrimidine dehydrogenase (DPD) to dihydrofluorouracil (DHFU) (figure 1.2). The remaining 5-FU rapidly enters the cell using the same nucleobase transport mechanism as uracil (section 1.2.2.9). 5-FU is converted intracellularly to several active metabolites (figure 1.2) that disrupt RNA synthesis and the action of thymidylate synthase (TS).

TS catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), and this reaction provides the sole *de novo* source of thymidylate, which is necessary for DNA replication and repair. Preclinical studies have demonstrated that TS expression is a key determinant of 5-FU sensitivity. Gene amplification of TS with consequent increases in TS mRNA and protein has been observed in cell lines that are resistant to 5-FU and fluorodeoxyuridine (FUDR) (Johnston *et al.*, 1992; Copur *et al.*, 1995). Treatment with 5-FU has been shown to acutely induce TS expression in cell lines, animal models and human tumours (Swain *et al.*, 1989; Chu *et al.*, 1993; Peters *et al.*, 1995; Welsh *et al.*, 2000). This induction seems to be due to inhibition of a negative- feedback mechanism in which ligand-free TS protein binds to and inhibits the translation of TS mRNA (Chu *et al.*, 1994). When stably bound by FdUMP, TS is no longer able to bind to its mRNA and suppress its own translation, resulting in increased protein expression (Chu *et al.*, 1994). This constitutes a potential mechanism of resistance, as the acute increase in TS protein levels would facilitate recovery of enzymatic activity.

High TS levels generally predict for lack of response to 5-FU based therapy, while lower levels are correlated with response in GI cancers (Johnston *et al.*, 1995; Lenz *et al.*, 1996; Leichman *et al.*, 1997; Salonga *et al.*, 2000; Ichikawa *et al.*, 2003; Hu *et al.*, 2003). A recent systematic review and meta-analysis of TS expression has shown that colorectal tumours expressing high levels of TS appeared to have a poorer overall survival (OS) compared with tumours expressing low levels (Popat *et al.*, 2004). Several groups have reported that TS levels generally predict for OS also in upper GI malignancies (Kuniyasu *et al.*, 1998; Suda *et al.*, 1999; Harpole *et al.*, 2001), although some discrepancies exist (Tsujitani *et al.*, 2000; Hironaka *et al.*, 2002). Generally,

mRNA levels of TS have proven to be as good predictors of response as well as protein levels determined by IHC (Johnston *et al.*, 1995; Lenz *et al.*, 1996; Leichman *et al.*, 1997; Lenz *et al.*, 1998; Aschele *et al.*, 1999; Salonga *et al.*, 2000).

Thymidine phosphorylase (TP) is an enzyme that catalyses the reversible phosphorolysis of thymidine and its analogues to their respective bases and 2-deoxyribose-1-phosphate. Hence 5-FU is reversibly converted to fluorodeoxyuridine (FdUMP), which in turn can be converted to the active metabolite FdUMP (figure 1.2). Characterisation of the role of TP in modulating 5-FU responsiveness has been confusing due to contradictory pre-clinical and clinical data. TP overexpression in cell culture and xenograft models has been shown to increase sensitivity to 5-FU and its prodrugs, presumably due to enhanced formation of FdUMP (Patterson *et al.*, 1995; Kato *et al.*, 1997; Evrard *et al.*, 1999). However, retrospective analysis of TP mRNA expression in colorectal tumours indicated that tumours with high TP expression were actually less likely to respond to 5-FU (Metzger *et al.*, 1998; Salonga *et al.*, 2000). These contradictory findings might be explained by the fact that TP is also an angiogenic endothelial-cell growth factor and high TP expression in colorectal oesophageal tumours has been correlated with worse prognosis (Takebayashi *et al.*, 1996; Koide *et al.*, 1999; van Triest *et al.*, 2000). It is possible that high TP expression might be a marker for a more invasive and aggressive tumour phenotype that is less responsive to chemotherapy. However, as tumour cells do not benefit from increased angiogenic potential in tissue culture, TP-mediated activation of 5-FU might predominate in this setting.

The rate-limiting enzyme in 5-FU catabolism is DPD and patients who are deficient in DPD experience profound systemic toxicity in response to 5-FU due to prolonged drug over-exposure (Johnson *et al.*, 1999). Recent genetic studies have started to define the mutations in the DPD gene that are responsible for the DPD-deficient phenotype (Johnson *et al.*, 2002). *In vitro* studies have also shown that DPD expression in the NCI 60 cancer cell line panel is correlated with 5-FU chemosensitivity (Scherf *et al.*, 2000). Furthermore, high levels of DPD mRNA expression in colorectal tumours have been shown to correlate with resistance to 5-FU chemotherapy in patients (Salonga *et al.*, 2000; Ichikawa *et al.*, 2003) and have also been associated with poorer survival

(Kornmann *et al.*, 2003). These findings presumably reflect higher DPD-mediated degradation of 5-FU in these tumours.

In general there is strong evidence that the expression of DPD and TS in GI cancers is predictive of response to 5-FU. It should be noted that the concomitant measurement of both these markers markedly enhanced the ability to predict tumour response to 5-FU-based chemotherapy in a number of studies (Salonga *et al.*, 2000; Ishikawa *et al.*, 2000; Ichikawa *et al.*, 2003; Kornmann *et al.*, 2003).

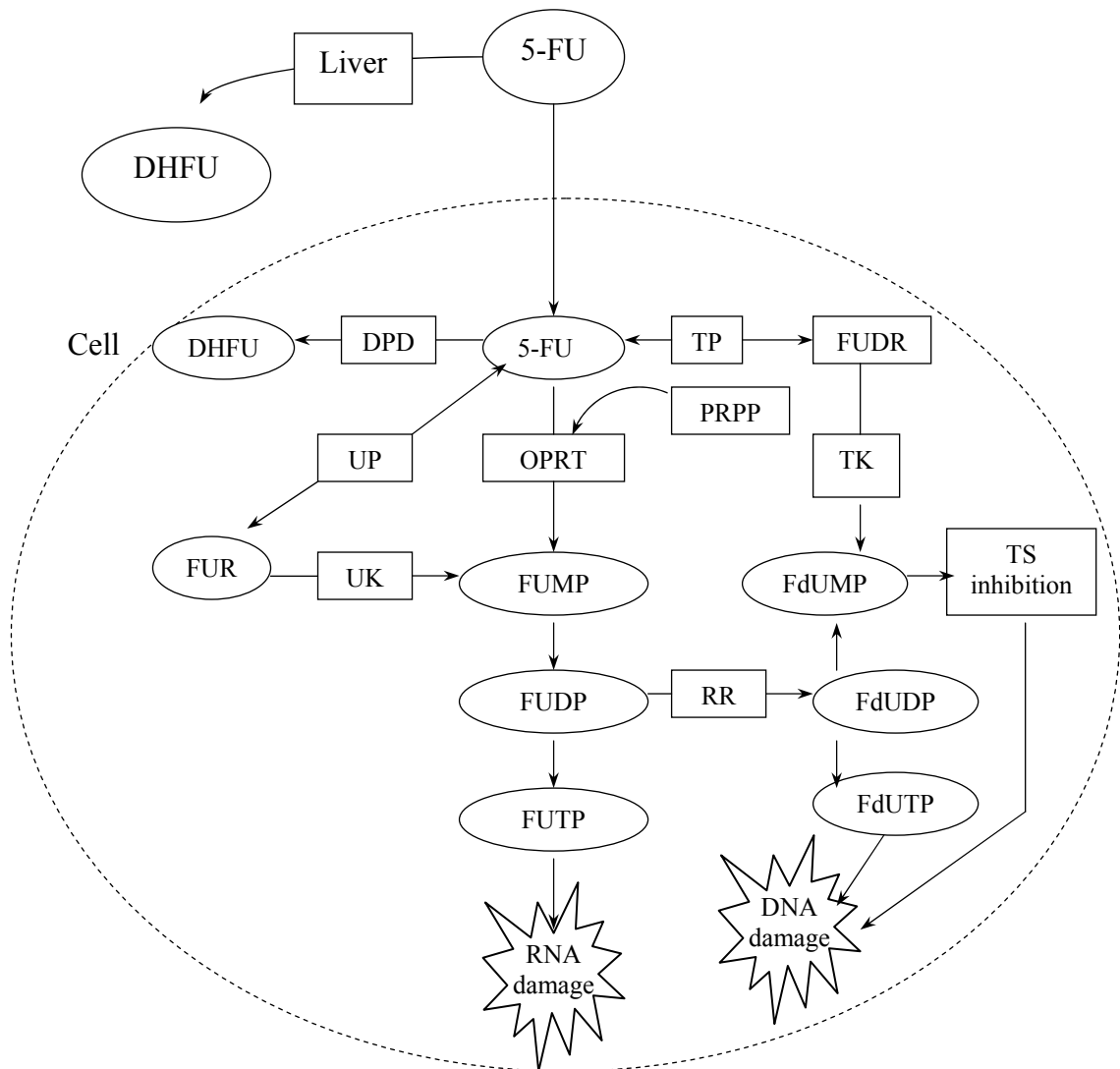


Figure 1.2. Schematic representation of 5-FU metabolism. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumour cells. Up to 80% of administered 5-FU is broken down by DPD in the liver. 5-FU is converted to three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The main mechanism of 5-FU activation is conversion to fluorouridine monophosphate (FUMP), either directly by orotate phosphoribosyltransferase (OPRT) with phosphoribosyl pyrophosphate (PRPP) as the cofactor, or indirectly via fluorouridine (FUR) through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK). FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites FdUTP and FdUMP, respectively. An alternative activation pathway involves the thymidine phosphorylase catalysed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated by thymidine kinase (TK) to FdUMP.

1.2.6 Other mechanisms of resistance: an overview

Most chemotherapeutic agents act by damaging DNA either directly or indirectly (table 1.1). When the cellular DNA is damaged, the cell has two main options, either to growth arrest and repair the damage, or to enter the apoptosis pathway.

The cell possesses several mechanisms to repair damage to its DNA. These DNA repair mechanisms either act directly on the damaged bases (direct repair), or they remove a section of DNA containing the damaged bases (indirect repair). Mechanisms which repair individually damaged bases usually require only a single enzyme. DNA repair mechanisms that remove a section of DNA require numerous different classes of enzymes, including DNA polymerases and DNA ligase (Barret & Hill 1998).

The best example of direct repair is by O⁶-alkylguanine-DNA alkyltransferase (AGAT), an enzyme also called O⁶-methylguanine-DNA methyltransferase (MGMT). MGMT removes methyl groups from the O⁶ position on guanine residues of DNA. Many alkylating agents act by alkylating DNA at the O⁶ position on guanine residues, including DTIC, temozolomide, and the nitrosoureas (Friedman *et al.*, 1996, Tew *et al.*, 1996). High MGMT levels have been shown to cause resistance to DTIC (Friedman *et al.*, 1996), temozolomide and the nitrosoureas (Wedge *et al.*, 1996), while low expression of this gene is a favourable prognostic factor (Gerson 2002). Chen *et al.*, (1994) found a positive correlation between high tumour MGMT activity and poor initial response to platinum containing chemotherapy. Other studies in patients with gliomas confirmed the silencing of MGMT gene caused by hypermethylation correlated with response and survival after treatment with BCNU (Esteller *et al.*, 2000) or temozolomide (Paz *et al.*, 2004).

Nucleotide excision repair (NER), an example of indirect repair, is a complex process that requires the co-ordinated effects of a series of enzymes to firstly cut-out the damaged section of DNA, and secondly to fill in the gap left (Araújo & Wood 1999). Enzymes involved in NER include, DNA polymerase, DNA ligase, endonucleases, topoisomerases, and several replication factors such as PCNA (Araújo & Wood 1999). NER, and above all ERCC1 which has a crucial role in the incision process, is thought to be partly responsible for resistance to a number of drugs which damage DNA including treosulfan, cisplatin, and many of the other alkylating agents (Barret & Hill

1998; Rosell *et al.*, 2003). ERCC1 mRNA levels have been correlated with clinical resistance to platinum based therapy in NSCLC (Lord *et al.*, 2002) in ovarian (Dabholkar *et al.*, 1994) and gastric cancer (Metzger *et al.*, 1998), and also with oxaliplatin resistance in colorectal cancer patients (Shirota *et al.*, 2001).

In addition to NER, there are two other major types of DNA repair in human cells: base excision repair (BER) and mismatch repair (MMR), both of which may also be involved in drug resistance. In common with NER these repair mechanisms both require DNA polymerases, DNA ligase and endonucleases in order to repair damaged DNA (Kolodner 1995, Seeberg *et al.*, 1995).

BER is similar to NER in that it is a group of proteins that remove the damaged DNA and then fill in the gap, however BER only removes the damaged base and not a section of DNA either side of the damage (Seeberg *et al.*, 1995). The key proteins in BER are redox effector factor-1/apurinic/apyrimidinic endonuclease (Ref-1/Ape) and poly(ADP-ribose) polymerase-1 (PARP). In both prostate and ovarian cancer the level of Ref-1/Ape is elevated suggesting that BER provides a growth advantage for tumours (Kelley *et al.*, 2001; Tanner *et al.*, 2004). PARP is activated immediately in response to DNA damage by alkylating agents. It seems that at lower levels of DNA damage, PARP protects against apoptosis. When DNA damage is greater, excessive PARP consumes NAD^+ , thereby depleting the cell of ATP, leading to either necrosis or apoptosis (Bernstein *et al.*, 2002). As yet there are no human disorders related to BER deficiencies, however inactivation of BER core proteins produces embryonic lethality, which underlines the importance of the mechanism (Hoeijmakers 2001).

Unlike NER and BER which repair damaged DNA producing drug resistance, loss of MMR is thought to confer resistance upon the cell to a number of different chemotherapeutic agents, including methylating agents and cisplatin (Lin & Howell 1999, Colella *et al.*, 1999, Lage & Dietel 1999). The MMR system is used to correct bases mispaired by DNA polymerases and insertion/deletion loops (from 1-10 bases) that arise from slippage during replication of repetitive sequences during recombination (Jacob *et al.*, 2001). The main components involved are the *E. coli* prototype factors MutS and MutL (Harfe & Jinks-Robertson 2000). In humans more enzymes are involved (MLH1, MSH2, PMS2, and MSH6), but serve similar functions. The

consequence of MMR deficiency in tumours is a high frequency of mutation both in noncoding and coding regions of the genome. Alterations in coding regions enhance genetic diversity and facilitate the rapid evolution of clones with a more malignant or drug-resistance phenotype. Cells exhibiting mutations or deficiency in their MMR system often show *in vitro* resistance to DNA damaging agents (Aquilina *et al.*, 1999). Loss of MMR expression has also been shown to confer clinical resistance in a number of malignancies. Samimi *et al.* (2000) investigated MLH1 and MSH2 expression in paired ovarian tumor sections from 54 ovarian cancer patients before and after platinum-based therapy and found that the immunostaining for both proteins decreased significantly after platinum-based therapy. In this study staining of either protein did not correlate with survival, but MSH2 staining at diagnosis was shown to be an independent prognostic indicator in another study (Geisler *et al.*, 2000). The prognostic significance of MLH1 staining has been shown also in breast cancer (Mackay *et al.*, 2000). In this study loss of MLH1 expression after combination chemotherapy was shown in paired biopsies from some patients and proved to be an independent prognostic marker. In addition, malignant gliomas with low immunocytochemical expression of MSH2 and MLH1 in combination with high expression of MGMT may identify the majority of patients resistant to temozolomide (Friedman *et al.*, 1998).

A cell with damaged DNA may enter apoptosis. Apoptosis or programmed cell death is a highly regulated process by which unwanted cells are removed from tissue physiology; it is characterised by the following processes: nuclear chromatin condensation; cytoplasmic shrinking; dilated endoplasmic reticulum and membrane blebbing (Kastan & Skapek 2001). Most of the proteolytic cleavages during apoptosis result from the action of a unique family of cysteine dependent proteases called caspases (Earnshaw *et al.*, 1999). Caspases 8, 9 and 10 are the major initiator caspases identified to date. Upon activation, they acquire the ability to cleave and activate effector Caspases 3, 6, and 7, which are the main enzymes capable of cleaving the vast majority of polypeptides that undergo proteolysis in apoptotic cells. Initiation of the caspase cascade can be mediated by two different pathways: the extrinsic and the intrinsic pathways (Green 2000; Wang 2001). The extrinsic pathway is initiated by ligation of transmembrane death receptors (CD95, TNF receptor, and TRAIL receptor) to activate membrane-proximal (activator) caspases 8 and 10. This pathway can be regulated by c-FLIP, which inhibits upstream activator caspases, and inhibitor of

apoptosis proteins (IAPs), which affect both activator and effector caspases. The intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins including Smac/DIABLO, HtrA2, and cytochrome c. Cytochrome c functions with Apaf-1 to induce activation of caspase 9, thereby initiating the apoptotic caspase cascade, while Smac/DIABLO and HtrA2 bind to and antagonise IAPs (Wang, 2001; Suzuki *et al.*, 2001). Oncoproteins, direct DNA damage, hypoxia, and survival factor deprivation, can activate the intrinsic apoptotic pathway. As a sensor of cellular stress, p53 is a critical initiator of this pathway (Lowe & Lin 2000). For example, proteins that sense DNA damage, such as ATM and Chk2, phosphorylate and stabilise p53 directly, and inhibit MDM2-mediated ubiquitination of p53 (Khanna & Jackson 2001). p53 can initiate apoptosis by transcriptionally activating proapoptotic Bcl-2 family members (e.g. Bax, Bak, PUMA and Noxa) and repressing antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-X_L) and IAPs (survivin) (Hanahan and Weinberg 2000; Bartke *et al.*, 2001; Hoffman *et al.*, 2001; Ryan *et al.*, 2001; Wu *et al.*, 2001). p53 was the second tumour-suppressor gene to be identified after the retinoblastoma (Rb); both copies of the p53 gene are inactivated in over 50% of human cancers (Vogelstein *et al.*, 2000; Evan & Vousden 2001). The normal state of p53 is the 'off' position, only becoming activated when cells are stressed or damaged; the result of activated p53 is the arrest of progression from the G₁ to the S phase of the cell cycle and in many cases it will induce apoptosis. This process therefore prevents the replication of mutagenic DNA, which could otherwise contribute to carcinogenesis (Hickman & Samson 1999).

Drugs of differing structure and specificity induce the characteristic morphological changes associated with apoptosis, and it is now believed that apoptotic pathways contribute to the cytotoxic action of most chemotherapeutic drugs (Lowe & Lin 2000). Anticancer drugs commonly activate the intrinsic apoptotic pathway. Mutations in *p53* or in the p53 pathway can produce multidrug resistance *in vitro* and *in vivo*, and reintroduction of wild-type *p53* into *p53* null tumor cells can re-establish chemosensitivity (Wallace-Brodeur & Lowe 1999). However, *p53* status is not a universal predictor of treatment response, in part because not all drugs absolutely require p53 for their apoptotic function (Herr & Debatin 2001) and, in some settings, p53 loss can enhance drug-induced cell death (Bunz *et al.*, 1999). Still, loss of p53

function correlates with multidrug resistance in many tumour types (Wallace-Brodeur & Lowe 1999).

Mutations or altered expression of Bcl-2-related proteins can drastically alter drug sensitivity in experimental models (Reed 1999; Schmitt *et al.*, 2000; Wei *et al.*, 2001; Zhang *et al.*, 2000), and are associated with multidrug resistance in human cancers (Reed 1999).

The contribution of the death receptor pathway in chemotherapeutic drug-induced cell death is controversial (Herr & Debatin 2001). Treatment of tumor cells with drugs can induce CD95 and TRAIL receptors, and downregulate c-FLIP and the IAPs (Asselin *et al.*, 2001; Chatterjee *et al.*, 2001; Herr & Debatin 2001). However, experiments using cells derived from mice with functional mutations in the death receptor pathway indicate that this pathway is dispensable for the cytotoxic action of chemotherapeutic agents (Los *et al.*, 1999). It has been argued that certain cell types require both the death receptor and mitochondrial pathways for drug-induced death, while others require only the mitochondrial pathway (Fulda *et al.*, 2001).

Previous studies have demonstrated that a wide range of anticancer agents induce apoptosis in malignant cells *in vitro* (Mesner *et al.*, 1997). It is important to emphasise that treatment-induced apoptosis is not merely a tissue culture phenomenon. Serial examination of peripheral blood mononuclear cells from acute leukaemia patients undergoing induction therapy has demonstrated that various agents, including cytarabine, mitoxantrone, etoposide, paclitaxel, and topotecan, cause a marked increase in the number of apoptotic blasts (Li *et al.*, 1994). Characteristic apoptotic changes have also been described in solid tumors after treatment of mice with various cytotoxic drugs, including cytarabine, 5-FU, fludarabine, doxorubicin, cyclophosphamide, cisplatin, etoposide, dactinomycin, and camptothecin (reviewed in Mesner *et al.*, 1997). The occurrence of apoptosis after treatment of solid tumors is not as well documented in the clinical setting (Borst *et al.*, 2001).

1.3 Tumour chemosensitivity assays

1.3.1 Introduction

The prediction of tumour sensitivity to anticancer agents has been intensively investigated for the purpose of optimising the therapy for each individual patient. There have been two approaches to predict the efficacy of drugs against individual tumours: drug-sensitivity tests and molecular assessment of marker genes. Although some of these tests, such as the determination of Her-2 status as a marker of sensitivity to Herceptin (section 1.1), are already widely available in the clinic, the prediction of chemosensitivity remains a goal to be achieved. So far cancer treatment has been determined predominantly by histological findings and cancer type. However, in the vast majority of cases, histology cannot identify drugs to which the cancer will be sensitive or resistant. Therefore, assessing the chemosensitivity of individual tumours could be greatly beneficial to patient survival, as only drugs that are likely to be active against the tumour can be identified and administered to the patient. On the other hand, drugs to which the tumour is resistant can also be identified, so avoiding the administration of any drugs likely to be unsuccessful and their consequent side effects.

The assessment of the chemosensitivity of individual tumours in the laboratory suffers from the obvious limitations common to most *in vitro* tests: it by-passes *in vivo* metabolism; it cannot recapitulate the complex interactions between stromal and tumour cells; there is no implication of the immune system; it cannot recreate the conditions of oxygen and nutrient supply found *in vivo*; importantly, also toxicity to normal tissues is not assessed.

Tumour chemosensitivity assays are laboratory tests in which fresh biopsy specimens of human tumours are cultured in the presence and absence of anticancer drugs. At the end of a period cell culture, measurements are made to determine whether or not the drugs were effective in either killing or in preventing the growth of the tumour cells. Proponents of these tests maintain that this information correlates with drug effects in the patient and can therefore be used to assist the clinical oncologist in selecting the most appropriate drugs to be used in the treatment of individual patients.

There has been a proliferation of names/terms applying to this testing. It should be noted that the terms ‘chemosensitivity assay’, ‘drug response assay’ and ‘tumour

response assay' can be used interchangeably to indicate tests aimed at predicting the anti-cancer agents that are most likely to be effective for an individual tumour. Similarly the terms 'chemoresistance assay' and 'drug resistance assay' are synonyms that indicate tests aimed at identifying inactive drugs. However from this point onwards the generic term 'tumour chemosensitivity assay' (TCA) will be employed to indicate all those cell-based assays that were developed to predict sensitivity and/or resistance to anti-cancer drugs; in fact 'chemosensitivity' assays give information about the ineffective chemotherapy as well as drugs which are likely to be effective, while 'chemoresistance' assays are more restricted and do not give information about sensitivity.

Likewise, the terms "*in vitro* assay" and "*ex vivo* assay" will be used interchangeably in this context, although the latter should only be used for primary cultures, e.g. fresh cells isolated from tumour samples and not exposed to longer-term artificial culture, such as that as used in the ChemoFx® Assay (Precision Therapeutics, Pittsburgh, PA), in which the tissue explants are pre-incubated in culture medium to grow a living population of cancer cells.

All chemosensitivity assays have four essential stages (Fruehauf & Bosanquet, 1993). Firstly, the cancer cells must be isolated. When a solid tumour specimen arrives in the laboratory, it is finely minced and cells extracted by enzymatic or mechanical means. Liquid samples are centrifuged to isolate cells. Assay requirements vary regarding the size of a specimen, but the specimen should have a sufficient percentage of viable cancer cells and an absence of bacterial and yeast contamination.

Secondly, in all of these assays, cancer cells are exposed to the desired chemotherapeutic agents. The incubation time varies from one hour to fourteen days, depending on the assay. The concentrations of drugs used in the exposure also can vary, depending on the assay, from using a combination of sub-clinical, clinical, and supra-clinical concentrations to using a single supra-clinical concentration. In addition to single drugs, some of the assays can test combinations and sequences of chemotherapeutic agents. All of the assays use control samples of tumour cells that are cultured under identical conditions but are not treated with the chemotherapeutic agents.

Thirdly, all chemosensitivity assays must determine the effects of the drugs on the cancer cells. This is called the assay endpoint, which is either a measurement of cell proliferation or cell death, although biosensor chips are currently being developed to monitor several cellular metabolic parameters continuously for several days (Henning *et al.*, 2001; Otto *et al.*, 2003; Mestres-Ventura, 2003).

Lastly, a report of the assay results is issued. There is great heterogeneity in this field, as different investigators using different assays have defined various indices of chemosensitivity/chemoresistance.

There is a clear divide between TCAs based on cell proliferation as an endpoint (clonogenic assays) and TCAs based on cell death as an endpoint. Historically, the cell proliferation endpoint received great attention, as a result of studies by Salmon, Von Hoff, and others during the late 1970s and early 1980s (Salmon *et al.*, 1978; Von Hoff *et al.*, 1981). At that time cancer was most prominently considered to be a disease of disordered cell growth. In contrast, the concept of apoptosis (programmed cell death) had yet to become widely recognised. Also unrecognised were the concepts that cancer may be a disease of disordered apoptosis/cell death and that the mechanisms of action of most if not all available anticancer drugs may be mediated through apoptosis (Hickman *et al.*, 1992; Zunino *et al.*, 1997; Jaffrezou *et al.*, 1998). When problems with the first proliferation-based assays emerged (Selby *et al.*, 1983), there was relatively little enthusiasm for studying cell death as an alternative endpoint.

1.3.2 Cell proliferation assays

1.3.2.1 Clonogenic assays

The first tests to be developed to assess the chemosensitivity of tumour material, the clonogenic assays, were based on the assessment of cell proliferation. Also known as the human tumour stem cell assay, clonogenic assays were developed from antibiotic sensitivity testing in microbiology (Hamburger & Salmon 1977, Selby *et al.*, 1983). The test specimen is subjected to a mild digestion to give a single cell suspension. The cell suspension is divided into two parts, one for treatment with the test drug and one as control. The cells are incubated with or without test drug at 37°C for one hour.

Following incubation, the cells are washed, plated out on a two-layer soft agar system,

and incubated for up to 14 days at 37°C. In the original assays the cells were only exposed to a single concentration of the test drug for one hour. Alternatively the drug is incorporated into one of the agar layers, thus keeping the cells in contact with the drug for the total time of the assay (Kern & Weisenthal 1990). The number of colonies formed after incubation can be counted by an automated image analysis system, or by eye. The number of colonies is compared with the control plates of cells that were not exposed to test drug and sensitivity or resistance to test agent can then be assessed.

The clonogenic assay has been regarded as something of a “gold standard” since it was first used for assessing chemosensitivity of tumour material, but it has a number of difficulties that have limited its clinical utility (Selby *et al.*, 1983). The major problem was an inability to grow sufficient cancer cells for the assay. Only 40%–70% of the tumor specimens would grow into colonies, and those that did grow took a very long time, reducing the clinical utility of the assay (reviewed in Kochli *et al.*, 1994). On the other hand, if too many cells were cultured in agar, the cells could not proliferate sufficiently and visible colonies were not seen. As with other methods, a single cell suspension was required; in fact if the enzymatic digestion was not complete and cell clumping occurred, the number of cells plated out could not be determined accurately leading to a considerable experimental variation. In addition, determination of whether the cells being tested were actually neoplastic or normal cells was difficult.

There have been many clinical studies comparing the *in vitro* and clinical sensitivity of tumours using the clonogenic assay. A review of 54 different correlation trials found that a 69% true positive rate (samples sensitive *in vitro* were also sensitive *in vivo*) and a 91% true negative rate (samples were resistant *in vitro* and *in vivo*) (Von Hoff 1990). The Southwest Oncology Group (SWOG) conducted a study using the tumour clonogenic assay to predict responses in ovarian cancer patients and to direct therapy in a group of patients (Von Hoff *et al.*, 1991). The patients treated on the basis of their sensitivity using the assay had an increased response rate (28% versus 11% for patients treated by physician’s choice; $p=0.03$) but did not have an improved overall survival. This study also found a 100% true positive and a 100% true negative rate, although this was only in seven evaluable patients. A third study on a range of tumour types, using a modified methodology, found that patients treated on the basis of assay results had a

better response rate, but once again there was no difference in overall survival (Von Hoff *et al.*, 1990).

A new version of the clonogenic assay has been developed using a collagen gel droplet embedded culture system (Inaba *et al.*, 1996). Although the technical feasibility of this test had already been demonstrated by Jason *et al.* in 1979, its clinical relevance has only been reported in more recent years (Kobayashi 2003). After enzymatic dissociation from solid specimens, tumour-derived cells are incubated in collagen gel coated flask for 12-24 hours. Living cells are then collected, placed in collagen gel droplets (3,000 cells/droplet) and further incubated for other 24 hours. Cytotoxic drugs are then added to the droplets at a single clinically relevant concentration. After incubation for 24 hours, the drugs are removed, and the cells in the droplets are incubated for further 7 days in a proprietary serum-free medium (Nitta Gelatin Inc., Osaka, Japan) that inhibits fibroblast overgrowth. At the end of this assay, the collagen gel droplets are stained with neutral red, fixed and quantified by image analysis (Koezuka *et al.*, 1993). Cell growth is assessed calculating the ratio of image density measured on day 7 and day 1 after the addition of drugs. The cloning efficiency of lung, breast, colon and gastric tumour cells cultured in collagen gel droplets is overwhelmingly higher than the efficiency in soft agar culture (Kobayashi, 2003). Kobayashi (2003) has reported an overall 83% evaluability rate of the assay in over 1,000 specimens tested (including 659 NSCLC, 163 breast, 107 colorectal cancers). The same author has also reported a predictive accuracy of 84.1% in 183 varied solid malignancies; in particular the assay was able to predict resistance in 79/89 (88.8%) cases, while the predicted sensitivity in 75/94 (79.8%).

1.3.2.2 Thymidine incorporation assay/Kern assay

For the last decade, the cell proliferation assay which has been most heavily promoted and provided as a service to patients in the USA (Oncotech and Impath) is the radioactive thymidine incorporation assay, or Kern assay, as it was originally described by Tanigawa and Kern in 1982. In this assay, applied only to solid malignancies and not to haematological neoplasms, tumour cells are suspended in soft agarose and cultured for 3 - 4 days in the continuous presence of single supra-clinical concentrations of chemotherapy agents. At the end of the culture period, radiolabelled thymidine ($[^3\text{H}]$ thymidine) is added to the medium to label the proliferating cancer cells. It is

assumed that the [³H]thymidine is incorporated into the dividing cells replicating their DNA, whereas non-proliferating cells and dead cells fail to incorporate the label. After further 2 days incubation, the radioactivity is measured using a liquid scintillation counter and differences in putative thymidine incorporation into DNA are compared between control and drug-treated cultures. Cancer cells that can grow and proliferate in such extreme conditions are considered resistant to the chemotherapy agent. On the basis of this information, it can be deduced that these assays do not provide any information about chemosensitivity but excel at predicting chemoresistance.

Indeed, eight years after developing the assay, Kern and Weisenthal correlated the thymidine incorporation assay results with clinical data and defined the concept of 'extreme drug resistance' or EDR (1990). This was defined as an assay result that was one standard deviation more resistant than the median result calculated from a database of assays. Patients treated with single agents showing EDR in the assay virtually never had a partial or complete response. Kern and Weisenthal also defined "low drug resistance" (LDR) as a result less resistant than the median and "intermediate drug resistance" (IDR) as a result more resistant than the median but less resistant than EDR (in other words, between the median and one standard deviation more resistant than the median).

The principles and clinical correlation data with the thymidine "EDR" assay were reviewed over 10 years ago (Weisenthal and Kern, 1992). Based on the publication validating the assay (Kern and Weisenthal, 1990), this test has a very high specificity (>98%) for the identification of inactive single agents, but a low sensitivity (<40%). In other words, a drug with assay-defined "EDR" is predicted to be almost certain to be inactive as a single agent (high specificity for identifying inactive drugs), but many drugs without "EDR" will also be inactive (low sensitivity for identifying inactive drugs).

Only a few follow-up studies have been published since the 1992 review (Weisenthal & Kern, 1992). One such study showed that EDR to one or more of the single agents used in a two drug combination is not apparently associated with a lower probability of response to the two drug combination in the setting of intraperitoneal chemotherapy of appendiceal and colon cancers (Fernandez-Trigo *et al.*, 1995). It is, however, possible

that response to the high drug concentrations achievable with intraperitoneal chemotherapy may be more closely associated with drug penetration into the tumour than to intrinsic drug resistance of the tumour cells. Eltabbakh *et al.*, (1998 & 2000) reported that EDR to paclitaxel did not appear to be a prognostic factor in ovarian cancer patients or in patients with primary peritoneal carcinoma treated with paclitaxel plus platinum. However, it has been recently reported that EDR to platinum alone in ovarian cancer may have prognostic implications (Fruehauf *et al.*, 2001). Previously untreated breast cancer patients with tumours showing LDR (defined above) had superior times to progression and overall survivals than patients with tumours showing either IDR or EDR (Mehta *et al.*, 2001).

1.3.2.3 Other cell proliferation assays

In recent studies Bachrach and Wang have proposed a new chemosensitivity assay (1999, 2003) using ornithine decarboxylase as a marker for cell proliferation. This enzyme catalyses the conversion of ornithine into the diamine putrescine, which is the precursor for the synthesis of the naturally occurring polyamines. The polyamines spermidine and spermine play an essential role in growth and proliferation processes (Russell 1985; Cohen 1998). Ornithine decarboxylase is ubiquitous, it is expressed early in the cell cycle and has an extremely short half-life (15-20 minutes), so that it decays rapidly when cell proliferation is arrested (Bachrach & Wang 2003). Although the Bachrach group originally reported chemosensitivity testing of cell lines using the enzymatic activity of ornithine decarboxylase (Assaraf *et al.*, 1994), more recently they have employed immunocytochemical detection of this enzyme in lymphocytes from patients with haematological malignancies (Wang *et al.*, 1999). The lymphocytes are grown for 24 hours in culture medium containing 15% autologous plasma and up to five different concentrations of each cytotoxic. At the end of the incubation period, the cells are harvested by centrifugation and dropped on slides coated with polylysine. Slides are incubated with a primary antibody directed towards ornithine decarboxylase and examined with a confocal laser microscope after being exposed to a secondary FITC-conjugated antibody. Limited data is available on the clinical usefulness of this method; drug resistance was detected in five leukaemic patients who subsequently died; while the lymphocytes of other 33 chemo-responders affected with mild haematological malignancies showed sensitivity to various drugs (Bachrach & Wang 2003).

Another type of cell proliferation assay currently in clinical use (NuOncology Labs, Houston, TX) is the adhesive tumour cell culture system, based on comparing monolayer growth of cells over a proprietary ‘cell adhesive matrix’ (Ajani *et al.*, 1987). Positive clinical correlations were described with this system in 1987 (Ajani *et al.*, 1987), but confirmatory and follow-up studies have not been reported.

1.3.3 Total cell kill/cell death assays

As opposed to measuring cell proliferation, there is a closely-related family of assays based on the concept of *total cell kill*, or, in other words, *cell death* occurring in the entire population of tumour cells (as opposed to only in a small fraction of the tumour cells, such as the proliferating fraction or clonogenic fraction) (Weisenthal *et al.*, 1984 & 1985). The concepts underlying cell death assays are relatively simple, even though the technical features and data interpretation can be very complex. There has been considerable work based on these assays reported during the past fifteen years.

The basic technology concepts are straightforward. After the cells are incubated with the drugs for a variable period (3-7 days), a measurement is made of cell injury, which correlates directly with cell death. There is evidence that the majority of available anticancer drugs work by causing sufficient damage to trigger “programmed cell death”, more properly known as apoptosis (Hickman, 1992; Zunino *et al.*, 1997). Although there are methods for specifically measuring apoptosis, *per se*, there are practical difficulties in applying these methods to mixed (and clumpy) populations of tumour cells and normal cells.

Thus, more general measurements of cell death have been applied. These include:

- (1) loss of protein content, as measured in the sulphorhodamine B assay (Skehan *et al.*, 1990, Monks *et al.*, 1991);
- (2) delayed loss of cell membrane integrity (which has been found to be a useful surrogate for apoptosis), as measured by differential staining in the DISC assay method (Weisenthal *et al.*, 1992 & 1993);
- (3) loss of cell membrane esterase activity and cell membrane integrity, as measured by the fluorescein diacetate assay (Rotman *et al.*, 1988; Larsson *et al.*, 1990; Nygren *et al.*, 1992);

- (4) loss of mitochondrial Krebs cycle activity, as measured in the *MTT assay* (Mosmann 1983; Carmichael *et al.*, 1987);
- (5) loss of cellular ATP, as measured in the *ATP assay* (Kangas *et al.*, 1984; Garewal *et al.*, 1986; Sevin *et al.*, 1988; Andreotti *et al.*, 1995).

1.3.3.1 The Sulphorhodamine B assay

The Sulphorhodamine B assay determines the protein content of a sample and uses this as an indicator of cell growth and viability. Tumour cells are grown in 96-well plates and cultured in the presence of drug for a set period of time. After the incubation period, the cells are fixed *in situ* and stained using the long lasting stain sulphorhodamine B, which is quantified using a spectrophotometer. There are concerns that debris from dead cells being stained by the dye may produce false results (Keepers *et al.*, 1991) but these seem to be minimised if readings are taken 24 hours after staining in cell line culture.

The sulphorhodamine B assay can be automated and currently forms the basis of the NCI drug screening program (Rubinstein *et al.*, 1990, Skehan *et al.*, 1990, Monks *et al.*, 1991).

1.3.3.2 Dye Exclusion Methods / DiSC Assay

Cell viability testing relies on the phenomenon that living cells exclude various dyes whilst dead or dying cells do not. An example of this is trypan blue exclusion, which is commonly used in cell culture to assess cell number (as detailed in methods section 2.2.7). A major drawback of this method of assessment is the poor correlation between the ability of a cell to divide and its ability to exclude dye.

The differential staining cytotoxicity (DiSC) assay is a modification on earlier dye exclusion methods (Weisenthal *et al.*, 1983, Weisenthal & Kern 1991). Biopsies are dissociated to obtain a single cell suspension, which are generally cultured for 4 days in liquid media with or without the test drug. Typically, three drug concentrations are tested, of which the intermediate concentration represents clinically achievable plasma levels, and the remaining two concentrations represent ten-fold higher and ten-fold lower plasma levels. After incubation the cells are stained with either a fast green or a fast green/nigrosin dye, which stains dead or dying cells bright green, while the live, viable cells remain unstained. The cells are counter-stained with haematoxylin and eosin

to differentiate between tumour and non-tumour cells. The cells can then be assessed as either tumour or non-tumour cells by cytology (which is difficult for entire cell populations, as in most cases this requires immunocytochemistry) and as either live/viable or as dead/dying cells, to determine the sensitivity or resistance to the tested agents. As the state and type of cell needs to be determined by eye, the results are subject to the bias of the individual assessor and the procedure is labour-intensive. Another disadvantage is that for biological reasons 5-FU cannot be tested in the DiSC assay. One major advantage of this assay is that it is completed in a relatively short period of time (maximum 6 days). In clinical studies more than 70% of specimens have been tested for sensitivity to at least one drug or combination (Weisenthal *et al.*, 1983).

In 1991, Bosanquet published a relatively large number of correlations between clinical response and DiSC assay results, chiefly in CLL (Bosanquet 1991). More recently the same group reported the predictive value of this assay to determine sensitivity to fludarabine in 243 CLL patients (Bosanquet *et al.*, 1999). Correlations between DiSC assay results and patient survival in ANLL were first published by a Swedish group in 1989 (Tidefelt *et al.*, 1989). These results were recently confirmed and extended by a group at the University of Cologne (Staib *et al.*, 1999).

1.3.3.3 Fluorescein-based assays

The Fluorometric Microculture Cytotoxicity Assay (FMCA) is another cytotoxicity assay that relies on the metabolic activity of live cells after incubation with test drug. The tumour cells are incubated with or without cytotoxics in 96-well plates for a set period of time, normally 72 hours. At the end of this time the media is removed from the cells and fluorescein diacetate is added to the cells. Fluorescein diacetate is a lipid soluble material that readily penetrates cell membranes. Viable cells contain a membrane esterase that cleaves the dye to non-lipid soluble fluorescein, which is concentrated in cells containing a functionally intact membrane; the fluorescence emitted by free fluorescein is measured by a spectrofluorimeter and is proportional to the number of cells present (Larsson & Nygren 1993). The proportion of live cells present in relation to untreated controls can be calculated.

The FMCA is conceptually similar to the DiSC assay, which measures the ability of cells with functionally intact membranes to exclude non-lipid soluble dyes, and indeed

the results obtained with these two assays correlate very well with each other (Nygren *et al.*, 1992, 1994; Csoka *et al.*, 1994).

The FMCA has been evaluated in a number of different tumour types including ovarian carcinoma (Csoka *et al.*, 1994), AML (Nygren *et al.*, 1992, Larsson *et al.*, 1992, Kristensen *et al.*, 1999), and kidney and urinary carcinomas (Nygren *et al.*, 1999). The FMCA has also been used to evaluate new agents and combinations such as vinorelbine in relation to current drugs (Fridborg *et al.*, 1996) or topotecan in combination with other cytotoxics (Jonsson *et al.*, 1998).

A related test is the fluorescent cytoprint assay (FCPA or FCA), which measures the activity of cytosolic esterases by monitoring the hydrolysis of fluorescein monoacetate to fluorescein. In the FCPA tumours are disaggregated into small clumps, or 'microorgans', which are immobilised between sheets of cellulose-collagen; these sheets are then placed onto a grid in tissue culture flasks containing medium and a baseline 'fluorescent cytoprint' is recorded by digital photography. The cells are then cultured for 24 hours, exposed to the drugs for 48 hours, rinsed and grown for an additional 48 hours in drug-free medium. Fluorescein monoacetate is then added, the second 'cytoprint' is obtained and the difference in fluorescence between the two images is calculated by image analysis software (Leone *et al.*, 1991). Although the 'microorgan' structure has the advantage of recreating the *in vivo* tumour environment, it requires samples with a large number of cancer cells, as the assay end point does not distinguish between the fluorescent signal produced by the malignant cells and the stromal component of each organoid.

The FCPA has been shown good predictive efficacy (~70%) in ovarian and breast cancers (Blackman *et al.*, 1994).

1.3.3.4 MTT assay

In the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, tumours are either tested as small fragments grown on collagen gel sponges or as disaggregated cells cultured in monolayer adherent conditions. Tumour cells or organoids are incubated with or without the test drugs for 4 to 5 days. The MTT assay detects mitochondrial succinate dehydrogenase (SDH) activity as a determinant of

mitochondrial function and cell viability (Mosmann 1983). SDH is a component of the citric acid cycle, and it generates FADH₂ and fumarate from succinate and FAD. SDH activity resides on the mitochondrial inner membrane and requires a functioning electron transport system. SDH activity is therefore a measure of mitochondrial and cellular viability. SDH activity is measured by its capacity to convert the yellow compound MTT into a blue crystallized formazan derivative that is dissolved in DMSO at the end of the assay. The amount of formazan formation is determined by measuring the optical density of the tissue culture well using a spectrophotometer that measures absorbance at the wavelength absorbed by the blue solution (Carmichael *et al.*, 1987).

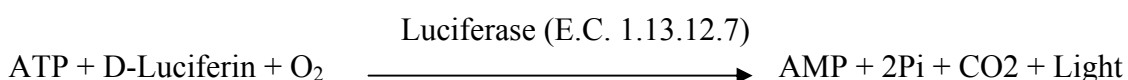
A variation of the MTT assay is the XTT assay, which uses XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide) as the tetrazolium reagent. The XTT assay has the advantage that the formazan product is soluble in aqueous solution and so DMSO extraction is not required before the results are obtained. One disadvantage of the XTT assay over the MTT assay is that several cell lines do not metabolise XTT as well as they do MTT (Scuderio *et al.*, 1988), so the MTT assay remains the assay of choice in this situation.

The results obtained for cell lines with the MTT assay correlate well with the other methods of assessing viability such as protein measurements, clonogenic assays and the dye exclusion assays (Pieters *et al.*, 1989; Kirkpatrick *et al.*, 1990; Rubinstein *et al.*, 1990; Keepers *et al.*, 1991). However there are several disadvantages of the MTT assay. Firstly, the colour of the formazan product fades within a short time of forming so the results need to be read immediately after solubilisation of the formazan in DMSO. Secondly, the reduction of the MTT is affected by several factors such as the pH of the media and chemical interference or chemical reduction of MTT by factors in the growth media, including some drugs. For this reason paclitaxel and docetaxel cannot be tested in this assay. Thirdly, the metabolic condition of the cells affects the reduction of MTT. For example, if the cells are lacking a vital metabolite, such as glucose, they may not be able to produce reducing agents capable of reducing the MTT.

Despite these difficulties, the MTT assay has been used to predict the response of patients to chemotherapy in solid and haematological malignancies (Yamaue *et al.*, 1992, 1996; Stute *et al.*, 1999; Xu *et al.*, 1999; Taylor *et al.*, 2001).

1.3.3.5 ATP assays

As the name suggests, the ATP assays are based on measurement of ATP as an endpoint. ATP is the major intracellular source of energy required for metabolism, and it is therefore considered a valid indicator of living cells (Lundin *et al.*, 1986). When cells die their ATP is rapidly degraded by ATPases. Firefly luciferin-luciferase has been used as a means of quantifying amounts of ATP. The hydrolysis of ATP to AMP by luciferase in the presence of the substrate D-luciferin produces light according to the reaction described below.



The light emitted is proportional to the amount of ATP hydrolysed, so the concentration of ATP can be determined for comparison between samples.

This phenomenon forms the basis of the ATP bioluminescence assay which has been used in various forms by several groups to determine *in vitro* and *ex vivo* chemosensitivity of different tumours (Kangas *et al.*, 1984; Maehara *et al.*, 1986; Kumitz *et al.*, 1986; Crouch *et al.*, 1993; Andreotti *et al.*, 1995; Möllgård *et al.*, 2000).

The method of testing chemosensitivity is similar to that of the MTT assay, although considerable technical variations of this method exist between different groups. While all of the ATP chemosensitivity assays measure ATP as an endpoint, major differences exist in culture media, assay formats, drug concentrations and incubation time. The method described here is that of Andreotti *et al.* (1995), currently sold as a kit (TCA-100) by DCS Innovative Diagnostik Systeme, Hamburg, Germany (see 'Materials and Methods' Chapter 2). The tumour sample is subjected to gentle enzymatic dissociation to produce a single cell suspension. The cells are then cultured in liquid media in 96 well polypropylene plates with or without the test drugs. Two controls are included; a maximum inhibitor (MI) is added to kill all the cells present giving a zero ATP count and a medium-only control (MO), which equates to the 100% ATP level achievable with that sample. The cells are cultured for 6 days at 37°C with 5% CO₂.

A commercial extraction reagent is then used to lyse the cells and inhibit the ATPases contained within the cells, which prevents ATP degradation. ATP quantification takes

place by adding luciferin-luciferase to the cell lysate. The amount of light produced is measured with a luminometer. By comparing the readings obtained for the controls, with the results for the cells cultured with drugs, the effects of the drugs on the tumour can be assessed. If the ATP levels for the drug treated cells are lower than the 100% ATP control then the drug is showing an inhibitory effect on the growth of the tumour cells, i.e. the tumour is sensitive to the drug. The degree of sensitivity to the drugs can be quantified by comparing the readings of drug treated cells to the controls. Indices of efficacy such as IC50 and IC90 can be calculated from the data.

The ATP assay is more sensitive than any of the other assays described above, requiring plating densities of as few as 1000 tumour cells per well to obtain an evaluable reading. This compares favourably with the MTT assay, which, for example, requires at least 25,000 cells to give evaluable readings (Petty *et al.*, 1995). The ability of the ATP assay to measure the presence of <5 viable cells remaining in one well after the assay means that drugs can be compared for their ability to kill all of the tumour cells present (Andreotti *et al.*, 1995, Cree & Kurbacher 1997). The sensitivity of the ATP assay translates into high evaluability rates, which are usually greater than 90% (table 1.9).

A further advantage of the ATP assay is that interference from non-tumour cells is minimised by the use of round-bottomed polypropylene plates, which inhibit cell adherence. Fibroblasts, mesothelial cells and other stromal cells can proliferate in adherence-based culture systems and therefore add 'noise' to most assay types when the cancer cell growth/death signals are measured. The growth signal of disaggregated cells obtained from tumour biopsies grown in non-adherent culture conditions is therefore more cancer-cell specific than growth in adherent culture systems. The use of a serum-free medium in another technique employed to suppress non-transformed cell proliferation. Andreotti *et al.* performed immunocytochemical analysis to assess the ratio of malignant to non-malignant cells before and after culture in a specialised media (Complete Assay Medium; CAM; DCS, Hamburg, Germany). They found that the mean proportion of malignant cells increased from the initial 54% to 83% by the end of the 6- to 7-day assay period, with a significant expansion of the malignant population in 98% of the cases evaluated.

One drawback of the ATP assay is that the observed reduction of ATP in response to drug treatment may be due not only to a decrease of the number of cells but also to a decrease in the ATP content per cell. As already stated, ATP is essential for cell viability and DNA replication. Metabolically active cells will produce more ATP, as will cells metabolising glucose through the citric acid cycle linked to aerobic mitochondrial function. *In vitro* drug effects that inhibit glucose metabolism or mitochondrial function result in decreased intracellular ATP concentrations.

The ATP tumour chemosensitivity assay (ATP-TCA) has shown good correlation between *ex vivo* sensitivity and clinical response in breast and ovarian carcinomas (table 1.10). A phase III randomised, multi-institutional, international trial was concluded in 2003 to compare assay-directed therapy to physician's choice treatment in patients with recurrent platinum-resistant ovarian adenocarcinoma (Kurbacher *et al.*, 1997b). Follow-up data are still being collected and no data analysis has been published at the time of writing.

The ATP-TCA described above has been employed to predict the *e -vivo* activity of novel drug combinations such as mitoxantrone plus paclitaxel (Kurbacher *et al.*, 1997a), treosulfan plus gemcitabine (Neale *et al.*, 1999), liposomal doxorubicin plus vinorelbine (Di Nicolantonio *et al.*, 2002; see chapter 8), mitomycin C plus gemcitabine (Whitehouse *et al.*, 2003); the activity of the first two combinations translated well *in vivo*, while the remaining two are currently being assessed in the clinic.

Finally the ATP assay has also shown its potential usefulness in evaluating new cytotoxic agents (Neale *et al.*, 2000; see chapters 8 and 9) and chemosensitizers (see chapter 10).

Table 1.9 Evaluability rates of the ATP tumour chemosensitivity assay performed according to TCA100 protocol (DCS, Hamburg, Germany).

<i>Tumour site</i>	Assay evaluability %	Reference
Breast	97%	Cree <i>et al.</i> , 1996
	94.5%	Kurbacher <i>et al.</i> , 1996
Colorectal	87%	Whitehouse <i>et al.</i> , 2003
Choroidal melanoma	84%	Neale <i>et al.</i> , 1999
Cutaneous melanoma	96%	Cree <i>et al.</i> , 2000
Oesophageal	73%	Mercer <i>et al.</i> , 2003
Ovarian	93%	Kurbacher <i>et al.</i> , 1998
	89%	Konecny <i>et al.</i> , 2000
Retinoblastoma	70%	Di Nicolantonio <i>et al.</i> , 2003

Table 1.10 A summary of recent studies with clinical correlation using the ATP tumour chemosensitivity assay.

Tumour site	Number of samples	Findings	Reference
Breast	29	76% positive predictive value	Cree <i>et al.</i> , 1996
Ovarian	114	Greater than 90 % accuracy for predicting cisplatin resistance	Andreotti <i>et al.</i> , 1995
Ovarian	25	64% overall response rate compared to 37% in control group with increased progression free survival and overall survival	Kurbacher <i>et al.</i> , 1998
Ovarian	38	76% of patients were classed as chemosensitive, and had significantly longer progression free-survival (28.5 vs 12.6 months, $P < 0.033$) and overall survival (46.1 vs 17.6, $P < 0.03$) than patients predicted to be resistant	Konecny <i>et al.</i> , 2000
Ovarian	54	61% overall response rate in patients that were treated with assay-selected therapy	Sharma <i>et al.</i> , 2003

1.4 Correlation of mRNA expression data from DNA microarrays with drug response

Another approach to predict sensitivity in cancer chemotherapy takes advantage of the latest genomic technologies, and DNA microarrays in particular (Macgregor & Squire 2002), which enable the study of the expression levels of several thousand genes simultaneously. This is attractive, as resistance to anticancer drugs is multi-factorial (see section 1.2) and considering the effect of single factors in isolation may therefore be insufficient to predict chemosensitivity.

1.4.1 DNA microarray technology

The two most commonly used DNA microarrays are based on oligonucleotide and cDNA probes. Short oligonucleotide probes (~25mers), first introduced by Affymetrix (<http://www.affymetrix.com>), are synthesised on the array *in situ* using photolithography (Lipshutz *et al.*, 1999). The newest Affymetrix array (HG-U133) contains approximately 45,000 probe sets representing >39,000 transcripts derived from about 33,000 well-substantiated human genes (Macgregor 2003). Alternatively, longer oligonucleotide probes (50–80 bases) can be spotted onto solid supports such as glass slides using robotic printers. cDNA microarrays are produced by robotically printing PCR products or plasmids onto glass slides (DeRisi *et al.*, 1996).

A commonly used approach to identify genes involved in chemosensitivity is to correlate gene expression profiles in multiple cancer tissues with drug response. However the accuracy of this approach depends on the selectivity, precision and accuracy of the mRNA expression data obtained from microarray experiments. The results from microarrays can vary considerably in quality, especially when these rely only on single measurements for each gene. Most spotted oligonucleotide and cDNA array experiments are performed using two RNA samples (a reference and an experimental sample) that are labelled with different fluorescent dyes. Quantitation of absolute mRNA levels is problematic and thus only ratios between the two samples can be assessed. By contrast, Affymetrix arrays permit the measurement of absolute mRNA levels with the use of analysis metrics for multiple probes and controls for each transcript (perfect match and mismatch probes). However, accurate measurement of expression may vary among genes, and furthermore, comparison between Affymetrix arrays and ratio arrays is difficult (Macgregor & Squire 2002). Proper normalisation

procedures are critical to obtain reliable data. Therefore, links between anticancer drugs and important chemosensitivity genes might be difficult to assess. In addition, microarrays are inherently less sensitive than other quantitative methods, such as quantitative RT–PCR, so less abundant transcripts can be missed.

1.4.2 Chemosensitivity studies using untreated cell lines

Scherf and coworkers have pioneered the use of cDNA microarrays for profiling chemosensitivity (Scherf *et al.*, 2000). These researchers analysed the gene expression patterns of the NCI60 panel. Gene expression was then correlated with the growth inhibitory activity of a subset of ~70,000 compounds that had been previously tested against the NCI60 panel. Increased expression of a given gene in cell lines sensitive to a given drug yields a positive correlation between gene expression and drug activity, whereas increased expression in resistant cells results in a negative correlation. The NCI study focused on 1376 genes (out of a total of 8000) that showed the strongest patterns of variation across the cell lines, and 118 drugs with established mechanisms of action. Two previously known drug–gene relationships revealed by the correlation analysis served to validate this approach. First, DPD expression was negatively correlated with 5-FU activity, which is consistent with the finding that high levels of DPD, which is involved in the catabolism of pyrimidines and 5-FU, decrease exposure of cells to the active phosphorylated forms of 5-FU (see section 1.2.5). Second, the expression of ASNS (asparagine synthetase) was negatively correlated with sensitivity to L-asparaginase in the NCI60 panel. Because L-asparaginase is used in the treatment of leukaemia, this correlation was tested in a sub-panel of leukaemic cells, resulting in an even stronger negative correlation. This is consistent with the notion that leukaemia cells lacking ASNS are more sensitive to L-asparaginase because it depletes extracellular L-asparagine.

Affymetrix oligonucleotide microarrays have also been used to measure expression levels of approximately 6800 genes in the NCI60 panel in a second study (Staunton *et al.*, 2001). The gene expression data were similarly applied to the NCI database of cancer susceptibility to anticancer drugs. Staunton and coworkers generated a panel of genes, the expression of which was predictive of sensitivity or resistance to 232 drugs. The authors suggested that for some drugs prediction of chemosensitivity in clinical samples might become feasible. However, none of the genes in the predictive panel was

directly associated with known mechanisms of drug resistance, and may represent characteristics of the tumour cell, such as proliferative ability. Moreover, correlation between gene expression in the NCI60 panel measured by Affymetrix arrays and that measured by cDNA arrays was only moderate (Scherf *et al.*, 2000). Therefore, array results must be validated before conclusions can be drawn concerning the mechanism of the interaction and the utility for clinical applications.

Owing to the complex nature of the array data, several studies have applied advanced computational models for mining the NCI60 databases. One study used relevance networks to deduce a stringent correlation coefficient threshold for extracting significant gene–drug relationships from the noise associated with mRNA expression data in the NCI60 (Butte *et al.*, 2000). Another study developed a refined strategy to identify the gene–drug, gene–gene and drug–drug associations that might exist within a cell line subset, but not in the complete NCI60 set (Bao *et al.*, 2002). Wallqvist and coworkers assessed the reproducibility of the gene expression data within the different array datasets and found statistically significant results for no more than 36% of those cases where at least one replicate of a gene appears in each dataset (Wallqvist *et al.*, 2002).

An independent study similar to that of Scherf and coworkers involved the development of an integrated chemosensitivity database of 55 anticancer drugs and the gene expression profiles of 39 human cancer cell lines (Dan *et al.*, 2002). This study identified several genes with expression patterns that showed significant correlation to patterns of drug response. Some of these genes were correlated with entire classes of drugs. For example, AKR1B1 (aldose reductase) and DDB2 (damage-specific DNA binding protein 2) showed positive correlation with multiple drugs, indicating that they are common predictive markers of chemosensitivity. They also identified genes that were correlated only with specific drugs that had similar mechanisms of action. For example, BIRC5 (survivin) and BIRC2 (apoptosis inhibitor 1), genes involved in apoptosis, were negatively correlated with the activity of 5-FU derivatives.

Another group evaluated the relationship between chemosensitivity to eight anticancer drugs and gene expression profiles in eight human hepatoma cell lines using cDNA microarray analysis, and analysed the data by constructing relevance networks (Moriyama *et al.*, 2003). They identified 42 genes that showed significant correlation.

Nearly 20% of these represented various types of membrane transporters, most of which were negatively correlated with chemosensitivity. For example, TAP1 (transporter associated with antigen processing 1) was associated with resistance to mitoxantrone, consistent with previous reports (Lage *et al.*, 2001).

These studies demonstrate the utility of correlating gene expression and drug activity in transformed human cell lines. Because gene expression was measured in untreated cells, these studies focused on preexisting sensitivity to drug treatment rather than on the consequences of treatment.

1.4.3 Gene expression changes as a result of drug exposure

A number of recent studies have focused on the molecular consequences of exposure to anticancer drugs or have addressed differential expression patterns between drug-sensitive cell lines and those with acquired resistance. Kudoh and colleagues used cDNA microarrays to monitor the expression profiles of MCF-7 cells that were either transiently treated with doxorubicin or selected for resistance to doxorubicin (Kudoh *et al.*, 2000). These researchers identified a set of genes with altered expression that overlap between doxorubicin-induced and -selected cells. The authors suggested that these genes represent a profile indicative of putative doxorubicin resistance.

Furthermore, gene expression profiles of doxorubicin- and cisplatin-resistant cells were found to differ – as one would expect for drugs with different mechanisms of action.

A similar study used cDNA microarrays to search for differentially expressed genes between a human multiple myeloma cell line and doxorubicin-selected sub-clones that express ABCB1 (MDR1) and show multi-drug resistance (Watts *et al.*, 2001). This study identified many differentially expressed genes, including ABCB1 and genes involved in apoptotic signaling.

Wittig *et al.* (2002) determined the difference in gene expression between a drug-sensitive melanoma cell line and three derived sub-lines with acquired resistance to the DNA-damaging agents cisplatin, etoposide and fotemustine. They found that 110 genes were transiently or permanently deregulated in at least two resistant sublines, and 14 genes were differentially expressed in all three drug-resistant sub-lines. In addition, chromosomal aberrations (such as deletions and duplications) were investigated by

comparative genomic hybridization and compared with gene expression, which was used as a criterion for the selection of promising candidate genes.

Another study (Maxwell *et al.*, 2003) determined the difference in gene expression between the MCF-7 breast cancer cell line and a 5-FU induced sub-clone. Genes that were consistently found to be up-regulated were spermine/spermidine acetyl transferase (SSAT), annexin II, thymosin-beta-10, chaperonin-10, and MAT-8. However, it must be noted that the majority of genes identified in these studies have no known role in chemosensitivity.

1.4.4 *In vivo* chemosensitivity studies

Zembutsu and coworkers extended the method of correlation analysis to *in vivo* animal models (Zembutsu *et al.*, 2002). They used a cDNA microarray representing 23,040 genes to analyze expression profiles in a panel of 85 cancer xenografts derived from nine human organs. Furthermore, the xenografts were examined for sensitivity to nine anticancer drugs and 1578 genes displaying expression levels that were correlated with chemosensitivity were identified.

Drug efficacy in mouse models might differ from that in humans. To avoid this confounding factor, gene expression profiles of clinical samples were compared with drug response. The expression profiles of 20 oesophageal cancer tissues from patients who were treated with the same chemotherapy regimen after tumour removal by surgery were examined using cDNA microarray analysis of 9216 genes (Kihara *et al.*, 2001). Comparison of these expression profiles with the duration of survival identified 52 genes having expression profiles correlated with prognosis, and probably with sensitivity and/or resistance to drugs. However, these candidate genes could affect tumour aggressiveness rather than chemosensitivity, which cannot be separated in this study. Surprisingly, the correlation between ABCB1 expression and response was moderate at best, even though the chemotherapy regimen used includes MDR1 substrates. The controversy over whether MDR1 represents a clinically important chemoresistance factor in oesophageal cancer remains unresolved.

Sotiriou *et al.* (2002) used cDNA microarrays to study gene expression profiles obtained from fine-needle aspirations of primary breast tumours before and after

systemic chemotherapy. This study identified candidate genes that might distinguish tumours with complete response to chemotherapy from tumours that do not respond. However, it remains unclear whether gene expression-based predictors reflect sensitivity to treatment or more fundamental aspects of tumour cell biology that are important to disease progression (Ramaswamy *et al.*, 2003).

Although several studies claim that clinical outcome of individual cancer patients can be predicted using gene expression profiles of primary tumours at diagnosis (for review see Ntzani & Ioannidis 2003), further work needs to be done to confirm the role of these genes in chemosensitivity and to validate the microarray technology.

Table 1.11 Representative studies that correlated gene expression with chemosensitivity.

Array	Samples	Analytic method	Representative gene-drug pairs	Refs
cDNA array	NCI60	Correlation	<i>DPYD</i> /5-FU, <i>ASNS</i> /L-asparaginase	Scherf <i>et al.</i> , 2000
Affymetrix array	NCI60	Prediction / correlation	Keratin 8/cytochalasin D	Staunton <i>et al.</i> , 2001
Affymetrix array	NCI60	Relevance networks	<i>LCPI</i> /4-thiazolidinecarboxylic acid	Butte <i>et al.</i> , 2000
cDNA array	NCI60	Multi-scale clustering	Caveolin-2/thaliblastine	Bao <i>et al.</i> , 2002
cDNA and Affymetrix	NCI60	Correlation	<i>CAMK1</i> /STO	Wallqvist <i>et al.</i> , 2002
cDNA array	NCI60	Correlation	Melanoma-specific genes / benzodithiophenedione-containing compounds	Blower <i>et al.</i> , 2002
cDNA array	Cell lines	Correlation	<i>AKR1B1</i> /24 drugs, <i>BIRC5</i> /5-FU	Dan <i>et al.</i> , 2002
cDNA array	Cell lines	Relevance networks	<i>TAP1</i> /mitoxantrone	Moriyama <i>et al.</i> , 2003
cDNA array	Cell lines	Differential expression	<i>XRCC1</i> /doxorubicin	Kudoh <i>et al.</i> , 2000
cDNA array	Cell lines	Differential expression	Apoptosis genes/doxorubicin	Watts <i>et al.</i> , 2001
cDNA array	Cell lines	Differential expression	<i>MPP1</i> /cisplatin	Wittig <i>et al.</i> , 2002
Affymetrix array	Cell lines	Differential expression	<i>ABCC1</i> /doxorubicin	Kang <i>et al.</i> , 2004
cDNA array	Xenografts	Correlation	<i>GPX2</i> /CPM	Zembutsu <i>et al.</i> , 2002
cDNA array	Tissues (oesophageal)	Prediction	<i>GSTA3</i>	Kihara <i>et al.</i> , 2001
CDNA array	Blood (ALL)	Correlation/ Prediction	<i>BTK</i> and <i>BAK1</i> /imatinib	Hofmann <i>et al.</i> , 2002
cDNA array	Tissues (breast)	Correlation / prediction	<i>HMG1</i>	Sotiriou <i>et al.</i> , 2002

1.5 Experimental Hypothesis and Aims

1.5.1 Hypothesis

This thesis examines the hypothesis that changes in molecular phenotype, particularly with respect to multidrug resistance, underlie resistance and sensitivity of human tumour-derived cancer cells to TOPO inhibitors and other anti-cancer agents.

1.5.2 Aims:

1. To develop methods to determine the degree of correlation between the molecular phenotype of tumour cells, cell lines and chemosensitivity to chemotherapeutic agents.
2. To determine the effect of combined inhibition of TOPO I and II on tumour-derived cells.
3. To determine the effect of the combination of TOPO inhibitors with other cytotoxic drugs.
4. To determine the importance of known mechanisms of sensitivity and resistance to chemotherapeutic agents.

Chapter 2 - MATERIALS and METHODS

2.1 Introduction

This chapter describes the various methods that have been used during the course of this Ph.D. The main method used was the ATP based chemosensitivity assay (ATP-TCA, which is described in section 1.3. The ATP-TCA was used to assess the *ex vivo* chemosensitivity of retinoblastoma, cutaneous melanoma, ovarian, breast and GI carcinoma samples, as well as various cell lines. The ATP-TCA was also used to evaluate new drug combinations and the use of tariquidar as a chemosensitiser in human cancer specimens.

qRT-PCR (section 1.4) has been employed to investigate molecular changes that occur after exposure to anticancer agents used in the ATP-TCA and *in vivo* in oesophageal cancer patients.

IHC has been used for several different purposes throughout this study; to determine cell type, and to investigate expression levels of different proteins during the course of an ATP-TCA and exposure to different drugs. All of the IHC used here was carried out by the staff of the Histopathology laboratory, Queen Alexandra Hospital, Portsmouth, and of the Histology laboratory, Institute of Ophthalmology. The method used is included here for completeness.

2.2 Materials and Methods for the ATP-TCA

The ATP based Tumour Chemosensitivity Assay (ATP-TCA) was performed as previously described (Andreotti *et al.*, 1995) and according to the protocol supplied by DCS Innovative Diagnostik Systeme, Hamburg Germany for the TCA-100 kit. The method is described below.

2.2.1 Cell Culture media

All chemosensitivity assays for human specimens were performed in Complete Assay Medium (CAM) (DCS Innovative Diagnostik Systeme, Hamburg, Germany). CAM is serum free and does not contain growth factors. This media was supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma Chemical CO Ltd, Poole, Dorset, Cat No. P0781) and 10 mM HEPES (Sigma H9136).

2.2.2 Transportation of tumour material

Tumour material was transported in sterile polystyrene tubes containing 10ml Dulbecco's Modified Eagle's Medium (DMEM) (Sigma D5671) to which 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma P0781) and 10 mM HEPES (Sigma H9136) had been added. Tumour material from contaminated sources was transported in the same medium, with added 2.5µg/ml amphotericin B (Fungizone, A2942; Sigma) and 1µg/ml metronidazole (Flagyl®; Rhône Poulenc Roreer Limited, Eastbourne, UK).

Up to 225 ml of ascites and pleural fluid were transported in 250 ml bottles, containing 25 ml (DMEM) (Sigma D5671) to which 250 IU/ml penicillin and 250 µg/ml streptomycin (Sigma P0781) and 250 mM HEPES (Sigma H9136). Heparin sodium 5000 IU (Monoparin®, CP Pharmaceuticals Ltd, Wales) was added as a precaution to prevent blood clots forming whilst in transit. Tumours that required overnight delivery were packed in a polystyrene box containing an ice pack, with the specimen separated from the ice pack by paper towels to prevent freezing of the specimen, which results in cell death and an inadequate sample for assay.

2.2.3 Source of tumour material

Tissue was sent under sterile conditions to the laboratory, at either the Institute of Ophthalmology (before April 2001) or the Translational Oncology Research Centre in Portsmouth (after April 2001). Fresh tumour material was obtained with patient consent for research use of the tissue from several hospitals and cancer centres. Enucleations from retinoblastoma patients were obtained from Moorfield's Eye Hospital and St Bartholomew's Hospital, London. Cutaneous melanoma biopsies from metastatic deposits were sent from Southend General Hospital, Westcliff-on-Sea, Essex and St Mary's Hospital, Portsmouth, Hampshire. Gastro-intestinal and breast carcinomas were obtained from Queen Alexandra Hospital, Portsmouth, Hampshire. Ovarian carcinoma samples (ascites and solid tumours) were submitted by Southend General Hospital, Westcliff-on-Sea, Essex; Royal Preston Hospital, Preston, Lancashire; Airedale General Hospital, Keighly, West Yorkshire; Dorset Cancer Centre, Poole Hospital, Dorset; Mount Vernon Centre for Cancer treatment, Northwood, Middlesex; New Cross Hospital, Wolverhampton, West Midlands; Western General Infirmary, Edinburgh, Scotland; St Mary's Hospital, Portsmouth, Hampshire.

Many of the ovarian adenocarcinoma specimens were obtained from platinum-refractory patients requiring second line chemotherapy as part of a randomised trial comparing physician's choice chemotherapy against chemosensitivity assay directed therapy (Kurbacher *et al.*, 1997b).

Ethics committee approval was granted from Moorfields Eye Hospital and Portsmouth Hospitals NHS Trust Ethics Committees for the use of tissue not required for diagnosis. Individual trials received approval from the Local Regional Ethics Committee (LREC) for the areas submitting tissue. The TCA Ovarian Cancer trial had Multi-centre Regional Ethics Committee (MREC) approval.

2.2.4 Initial preparation of solid tumour specimens

Upon receipt of solid tumour specimens, a small piece of tumour material was cut from the biopsy and placed into 10% buffered formaldehyde for histopathological examination to ensure that the specimen contained neoplastic cells. The hospital sending the sample kept the majority of the sample so that a pathology report could be obtained for patient management and only material not required for diagnosis was sent for testing. The remaining tumour material was digested as described in section 2.2.6.

When the number of cells allowed, drop-preparations of ascites and pleural effusion samples and cytoclots were prepared so that an immunohistochemical diagnosis could be made to confirm that neoplastic cells were present.

2.2.5 Enzymatic tumour dissociation

Two different enzymes were used to digest the tumour specimens: collagenase (Sigma C8051) or Tumour Dissociation Reagent (TDE) (DCS, Innovative Diagnostik Systeme, Hamburg Germany). Depending on tumour type (table 2.1), the appropriate dissociation enzyme was reconstituted with 10 ml complete assay medium (CAM, DCS Innovative Diagnostik Systeme, Hamburg, Germany) and diluted to the concentrations listed in table 2.1. The enzyme solution was then filter sterilised into a 30ml sterile universal using a 0.22 µm filter and 10 ml syringe.

Table 2.1. Enzymes used for the dissociation of tumour samples. Collagenase was made up in DMEM containing 100 IU/ml penicillin and 100 µg/ml streptomycin. Amphotericin B (2.5 µg/ml) and metronidazole (1 µg/ml) were included if the tumour was thought to be contaminated (i.e. GI tumours).

Dissociation enzyme	Dissociation time	Tumour
Collagenase 0.075 mg/ml	2 hours	Retinoblastoma
Collagenase 0.50-0.75 mg/ml	6 hours - O/N	Cutaneous melanoma
Collagenase 0.75 – 1.0 mg/ml	6 hours - O/N	Ovarian and breast carcinoma
Collagenase 1.0-1.5 mg/ml	6 hours - O/N	GI tumours
Tumour Dissociation Reagent* 1:4 dilution	6 hours - O/N	Ovarian adenocarcinoma (clinical trial specimens only)

*TDE, DCS Innovative Diagnostik Systeme GmbH, Hamburg, Germany

2.2.6 Preparation of solid tumour specimens

The tumour specimen was placed in a 100 x 15 mm Petri dish and dissected within a class II safety cabinet. Using sterile scalpels, excess fat and connective tissue (and skin in the case of some cutaneous melanoma metastases) was excised. The specimens were then minced using surgical scalpels into 0.5 - 2.0 mm³ pieces and transferred into the universal containing the previously prepared enzyme solution. If the tumour sample was large, i.e. greater than 5 x 10 x 10 mm, then more than one container was set up with equal amounts of tumour material going into each digest. The tumour and enzyme solution was then incubated for a minimum of 2 hours, but generally overnight (table 2.1), at 37° C. The mixture was shaken / inverted at intervals to disrupt the specimen and encourage the dissociation.

Once a cell suspension was evident, the preparation was centrifuged at 300g for 10 minutes at room temperature (RT) and the supernatant discarded. The cells were then washed twice by resuspending in 10 ml CAM and centrifuging at 300g for 10 minutes. The supernatant was kept separate from the sample after each wash until the cells had been counted. After the second wash, the cells were resuspended in 10 ml CAM (less if the specimen was small) and the universal stood vertically to allow any undigested material to sediment out.

The cell viability and concentration were then assessed using the trypan blue exclusion method described in section 2.2.7. If the cell viability was below 50%, if there was a large amount of debris, or blood cells were present, the viable cells were separated using Ficoll-Hypaque density gradient centrifugation (see section 2.2.9).

In the case of ascitic or pleural fluid samples, the specimen was centrifuged at 300g for 10 minutes and resuspended in CAM before assessing cell number and viability. The majority of ascites specimen contained a large amount of blood cells that were removed by Ficoll - Hypaque density gradient centrifugation (see section 2.2.9).

2.2.7 Trypan Blue exclusion method

Cell number and viability were routinely assessed using a haemocytometer and the trypan blue exclusion method. Equal volumes of cell suspension and a 0.4% solution of trypan blue (Sigma T8154), normally 15 μ l, were mixed in a polypropylene Eppendorf container and pipetted onto the haemocytometer. Dead or dying cells are unable to pump trypan blue out of the cytoplasm and so appear blue under the microscope with viable cells remaining clear (Kaltenbach *et al.*, 1958).

After counting the number of viable cells (N) present in the grid of the haemocytometer, the number of cells per millilitre can be calculated using the following formula: $N * 20,000$.

This method also allowed the number of red blood cells to be estimated, as these are much smaller than other cells. When the number of erythrocytes represented more than 10% of the total number of cells, excess red blood cells were removed by Ficoll-Hypaque density gradient centrifugation (see section 2.2.9). The specialised media (CAM) and the culture plates ensured that lymphocytes and other non-malignant cells did not survive in culture and therefore did not interfere with the assay results (see Chapter 1.3).

2.2.8 Ficoll-Hypaque density gradient separation

Ficoll-Hypaque density gradient (Boyum, 1968) separation was performed following manufacturers instructions as described below. 10 ml of Ficoll-Hypaque (Histopaque

Sigma 1077-1) was transferred into a sterile polystyrene 30 ml universal container. An equal volume of the digested specimen was layered on top of the Ficoll-Hypaque using a sterile Pasteur pipette, taking care not to mix the two layers. The sample was then centrifuged at 400g for 30 minutes at room temperature. After this period the blood cells should have formed a pellet, leaving the tumour derived cells forming an interface between the Ficoll-Hypaque and CAM. The interface was transferred using a sterile Pasteur pipette into another sterile 30 ml universal. The cells were then washed twice with 10 ml CAM by centrifugation at 300g for 10 minutes. The cell number and viability were then reassessed using the trypan blue exclusion method.

2.2.9 Preparation of chemotherapeutic agents

The cytotoxic drugs used in the assay were obtained as vials for injection either from the Pharmacy Departments at Southend General Hospital or at Queen Alexandra Hospital in Portsmouth. All of the chemotherapeutic drugs or combinations were tested in triplicate at 6 dilutions, corresponding to 200%, 100%, 50%, 25%, 12.5% and 6.25% of the estimated Test Drug Concentration (TDC). The TDC is based on the peak plasma concentration and the protein binding for the individual drugs (Andreotti *et al.*, 1995), and in some cases has been adjusted to provide correlation with clinical response rates. In each 96-microwell culture plates four drugs could be tested. The remaining wells in the plate were used for maximum inhibitor (MI) and no drug (MO) controls. The Maximum Inhibitor (MI) reagent consists of 0.02% v/v Triton X-100 (Sigma T8787) in DMEM (Sigma D5671); Triton X-100 is a detergent that kills all the cells, and is used as a negative control. The plate design used is shown in Figure 2.1.

All the chemotherapeutic drugs were prepared following manufacturers instructions and divided into aliquots which were then stored at room temperature, 4°C, -20°C or -70°C (Hunter *et al.*, 1994). A list of all the drugs used including the stock and test drug concentrations and the storage conditions can be found in Table 2.2.

An 800% TDC solution of each drug to be tested was prepared by diluting the stock solution into either 5 or 10ml CAM. Table 2.2 shows the volume that needs to be added to 5ml CAM to obtain an 800% TDC solution, as well as the TDC for all the drugs used. CAM contains Penicillin, Streptomycin and Gentamicin: Amphotericin B and

Metronidazole were added if the tumour sample was not sterile, as previously published (Whitehouse *et al.*, 2003). This has no effect on the sensitivities observed (Dr Pauline Whitehouse, personal communication).

Table 2.2 Storage and TDC for drugs used in the ATP-TCA. Room temperature has been abbreviated as room temp.

DRUG	TDC μM	TDC μg/ml	Stock mM	Stock mg/ml	μL added to 5 ml CAM to give 800% TDC	Storage
Cisplatin	10	3.0	3.3	1.0	120	room temp
Doxorubicin	0.86	0.50	3.4	2.0	10	-20°C
Liposomal Doxorubicin	2.6	0.50	10	2.0	30	-20°C
DTIC	110	20	55	10	80	-20°C
Epirubicin	0.86	0.50	3.4	2.0	10	-20°C
Etoposide	81	16	34	20	96	-20°C
5-FU	346	45	192	25	72	room temp
Gemcitabine	40	12	133	40	12	-20°C
Irinotecan	148	100	29	20	200	-20°C
Mitomycin C	2.0	0.70	2.9	1.0	28	-20°C
Mitoxantrone	1.2	0.60	3.9	2.0	12	room temp
Paclitaxel	16	14	7.0	6.0	91	room temp
Temozolomide	52	10	103	20	20	-20°C
Topotecan	1.6	0.75	2.2	1.0	30	-20°C
Treosulfan	72	20	180	50	16	-20°C
Vinblastine	0.55	0.50	1.1	1.0	20	-20°C
Vinorelbine	11	12	9.3	10	48	+4°C

2.2.10 Choice of drugs for ATP-TCA

The panel of drugs used for each assay were chosen by Prof Cree in consultation with the referring Oncologist to represent different mechanisms of action and the drugs normally used for the particular tumour type. The drugs tested in the ovarian, breast, GI and cutaneous melanoma samples formed part of a set drug list due to their inclusion in clinical trials. Whenever the number of cells allowed, additional plates were also set up with experimental agents. The set drug lists are shown below.

Breast Cancer

Plate A

1. Epirubicin / doxorubicin
2. 5-fluorouracil (5-FU)
3. Paclitaxel
4. Cisplatin

Colorectal Cancer

Plate A

1. 5-FU
2. Irinotecan
3. Oxaliplatin
4. Oxaliplatin + 5-FU

Plate B

5. Mitomycin C + 5-FU
6. Mitomycin C
7. 5-FU + irinotecan
8. Mitomycin C + gemcitabine

Plate C (from 1st August 2002 only)

9. Celecoxib
10. Celecoxib + 5-FU
11. Celecoxib + irinotecan
12. Celecoxib + 5-FU + irinotecan

Cutaneous melanoma

Plate A

1. Cisplatin
2. Paclitaxel
3. Cisplatin + paclitaxel
4. Vinorelbine

Plate B

5. DTIC
6. Treosulfan
7. Gemcitabine
8. Treosulfan + gemcitabine

Oesophageal Adenocarcinoma

Plate A

1. ECF (Epirubicin + cisplatin + 5-FU)
2. 5-FU
3. Epirubicin
4. Cisplatin

Plate B

5. Gemcitabine
6. Cisplatin + gemcitabine
7. Mitomycin C (MMC)
8. MMC + 5-FU

Plate C

9. Irinotecan
10. Irinotecan + 5-FU
11. Irinotecan + cisplatin
12. Paclitaxel

Recurrent Ovarian Cancer - Phase III trial (drug list from protocol)

Plate A

1. Cisplatin
2. Gemcitabine
3. Cisplatin + Gemcitabine
4. Doxorubicin x3 (Caelyx)

Plate B

5. Paclitaxel
6. Mitoxantrone
7. Paclitaxel + Mitoxantrone
8. Topotecan

Plate C

9. Treosulfan
10. Treosulfan + Gemcitabine
11. Cisplatin x2 + Etoposide
12. Etoposide

Plate D

13. Vinorelbine
14. Doxorubicin x3 + Vinorelbine

Retinoblastoma

Plate A

1. Cisplatin
2. Etoposide
3. Vinblastine
4. Doxorubicin

Plate B

5. VAC (cisplatin + doxorubicin + vinblastine)
6. VEC (cisplatin + etoposide + vinblastine)
7. 5-FU
8. Gemcitabine

	1	2	3	4	5	6	7	8	9	10	11	12
A	MI	MI	MI	MI	MI	MI	MI	MI	MI	MI	MI	MI
B	DRUG 1 200% TDC	DRUG 1 200% TDC	DRUG 1 200% TDC	DRUG 2 200% TDC	DRUG 2 200% TDC	DRUG 2 200% TDC	DRUG 3 200% TDC	DRUG 3 200% TDC	DRUG 3 200% TDC	DRUG 4 200% TDC	DRUG 4 200% TDC	DRUG 4 200% TDC
C	DRUG 1 100% TDC	DRUG 1 100% TDC	DRUG 1 100% TDC	DRUG 2 100% TDC	DRUG 2 100% TDC	DRUG 2 100% TDC	DRUG 3 100% TDC	DRUG 3 100% TDC	DRUG 3 100% TDC	DRUG 4 100% TDC	DRUG 4 100% TDC	DRUG 4 100% TDC
D	DRUG 1 50% TDC	DRUG 1 50% TDC	DRUG 1 50% TDC	DRUG 2 50% TDC	DRUG 2 50% TDC	DRUG 2 50% TDC	DRUG 3 50% TDC	DRUG 3 50% TDC	DRUG 3 50% TDC	DRUG 4 50% TDC	DRUG 4 50% TDC	DRUG 4 50% TDC
E	DRUG 1 25% TDC	DRUG 1 25% TDC	DRUG 1 25% TDC	DRUG 2 25% TDC	DRUG 2 25% TDC	DRUG 2 25% TDC	DRUG 3 25% TDC	DRUG 3 25% TDC	DRUG 3 25% TDC	DRUG 4 25% TDC	DRUG 4 25% TDC	DRUG 4 25% TDC
F	DRUG 1 12.5% TDC	DRUG 1 12.5% TDC	DRUG 1 12.5% TDC	DRUG 2 12.5% TDC	DRUG 2 12.5% TDC	DRUG 2 12.5% TDC	DRUG 3 12.5% TDC	DRUG 3 12.5% TDC	DRUG 3 12.5% TDC	DRUG 4 12.5% TDC	DRUG 4 12.5% TDC	DRUG 4 12.5% TDC
G	DRUG 1 6.25% TDC	DRUG 1 6.25% TDC	DRUG 1 6.25% TDC	DRUG 2 6.25% TDC	DRUG 2 6.25% TDC	DRUG 2 6.25% TDC	DRUG 3 6.25% TDC	DRUG 3 6.25% TDC	DRUG 3 6.25% TDC	DRUG 4 6.25% TDC	DRUG 4 6.25% TDC	DRUG 4 6.25% TDC
H	MO	MO	MO	MO	MO	MO	MO	MO	MO	MO	MO	MO

Figure 2.1 96-well-plate layout used for the ATP-TCA. Four drugs are tested at six concentrations ranging from 6.25% up to 200% of the TDC. The plate has 12 no drug controls (MO) and 12 maximum inhibitor wells (MI) to allow for percentage growth inhibition values for each drug at each concentration to be calculated at the end of the assay.

2.2.11 Preparation of 96-well microculture plates for addition of tumour cells

All ATP-TCAs with tumour-derived cells were performed in round bottomed polypropylene culture plates (Corning-Costar, High Wycombe, UK, cat no.3790), unless specified in chapter 3. The maximum inhibitor reagent (MI) was either purchased from DCS Innovative Diagnostik Systeme, Hamburg, Germany, or freshly prepared using 0.02% v/v Triton X-100 in DMEM. An aliquot (100 µl) of MI solution was added to the wells in the top row of the plate as illustrated in Figure 2.1. To the remaining wells of the plate, including the MO wells (e.g. positive control, no-drug) in row H, 100µl of CAM was added.

An aliquot (100 µl) of the 800% TDC solution of each drug was added in triplicate to row B of the 96-well plate. Using a multichannel pipette (or a robot, Kemble mod SPI) the drugs were serially diluted down the plate from row B to row G, while the excess 100µl remaining after the serial diluting was discarded. The plate was now ready for addition of the tumour cells.

2.2.12 Addition of tumour cells to the 96-well plate

The tumour cells were diluted in CAM to give a final concentration of 200,000 cells per millilitre. For ovarian ascites and pleural fluid the cells were diluted to 100,000 cells per millilitre. Aliquots (100 µl) of cells were then added to each well of the 96 well plate. The final cell concentration in each well was 20,000 for solid tumour specimens and 10,000 for the ascites and pleural fluid. The plates were incubated in a 95% air humidified, 37°C, 5% CO₂ incubator for 5-7 days. The plates were checked periodically for overgrowth and infection.

Any cells that were not required for ATP-TCA were cryopreserved in DMEM supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin, 10% foetal calf serum (FCS) (Labtech International, East Sussex, UK, cat. 4-101-500) and 10% DMSO (Sigma D5879), as detailed in section 2.3.3.

After the incubation period the cells were lysed and the ATP was quantitated.

2.2.13 ATP extraction

A reagent for the extraction of ATP was either purchased from DCS Innovative Diagnostik Systeme ('ATP extractant', Hamburg, Germany) or prepared according to the recipe herein described. HEPES buffer (101.7 mg/ml; Sigma H-4034) ammonium meta-vanadate (1.267 mg/ml; Sigma A-1183) and Triton X-100 (0.5% v/v; Sigma T-8787) were dissolved in sterile water (B.P. Water for Irrigation, Pharmacy, Queen Alexandra hospital) and the solution was titrated to pH=7.8 with 5.0 N sodium hydroxide. The solution was then let stand overnight in a safety cabinet uncovered until the colour of the liquid changed from yellow to clear. The solution was then filter sterilised using a 0.22 µm filter and 20ml syringe and aliquoted into 30ml sterile universal containers, which were stored in the refrigerator.

The ATP was released from the cells by the addition of 50 µl of ATP extraction reagent to each of the wells of the 96 well plate. The cells were immediately mixed by gentle pipetting 4-6 times using a multichannel pipette. Pipette tips were discarded after each drug triplicate. Plates were incubated at room temperature for a minimum of 20 minutes and a maximum of one hour before the ATP was measured.

2.2.14 Preparation of Luciferin-Luciferase Counting Reagent

The ATP in the wells was quantitated using a luciferin-luciferase 'counting' reagent, which was obtained from DCS Innovative Diagnostik Systeme (Hamburg, Germany) until the first half of 2001. A bottle of lyophilised luciferin-luciferase counting reagent was reconstituted with 21ml of Dilution buffer (DCS Innovative Diagnostik Systeme, Hamburg, Germany), which allowed for four 96 well plates to be read as well as an ATP standard curve (figure 2.2). The bottle was inverted several times to mix and incubated at room temperature for at least 30 minutes before use. The vial containing the reconstituted luciferin-luciferase was wrapped in foil to protect the reagent from light.

Alternatively (from July 2001 onwards), D-luciferin (Cat. 800-LN) and recombinant luciferase (Cat. 700-LF) were purchased from R&D systems (Abingdon, UK) and a luciferin-luciferase counting reagent was prepared as described below. Firstly, a 10x HEPES buffer was prepared mixing 4.766mg/ml HEPES powder (Sigma H-0887) and

1.221mg/ml magnesium sulphate heptahydrate (Sigma M-9397). The solution was adjusted to pH 7.7 with 5.0 N sodium hydroxide, filter sterilised and used to dissolve D-luciferin (0.732mg/ml buffer). Secondly, a 0.5 M Tris-succinate buffer containing 1% BSA was prepared dissolving 0.226 mg/ml TRIZMA®-succinate (Sigma T-9632) and 25.9 µl/ml bovine albumin 35% solution (Sigma A-7409) and adjusted to pH to 7.5 with sodium hydroxide. This buffer was used to reconstitute the recombinant luciferase (1.0mg/ml); the solution was then held on ice for 1.0 hour to let the luciferase dissolve completely. The final reagent was then prepared combining 102.5 ml of 10x Hepes buffer containing D-Luciferin with 24.75 ml of recombinant luciferase (1.0mg/ml) in 0.5 M Tris succinate buffer and 41.25 ml of 3% BSA. The solution was stored at -20°C in 2.0 ml aliquots. Before use each 2.0 ml aliquot was diluted with 18 ml of dilution buffer, which consisted of a 1 in 10 dilution of the HEPES buffer that was used to dissolve the D-luciferin.

The ATP was measured using a Berthold Diagnostic Systems MPLX luminometer (Berthold Diagnostic Systems, Pforzheim, Germany). All luminescence measurements were performed following the manufacturer's instructions.

2.2.15 ATP standard curve

An ATP standard curve was generated prior to reading any ATP-TCA plates as a quality control to ensure that all reagents and equipment were working properly. A vial of lyophilised ATP standard (DCS Innovative Diagnostik Systeme, Hamburg, Germany) was reconstituted with 2 ml of dilution buffer to give a stock ATP concentration of 250 ng/ml. Alternatively, a vial of ATP suitable for luminescence application was purchased from Sigma (Cat. FLAAS), and a stock solution of 100 µg/ml was prepared in dilution buffer (HEPES powder 0.4766mg/ml; magnesium sulphate heptahydrate 0.1221 mg/ml; pH 7.7). The ATP solution was then filter sterilised using a 0.22 µm sterile disposable filter and a disposable 10 ml plastic syringe, and stored in 25 µl aliquots at -20°C for a maximum of six months. For each calibration curve 10 µl of the stock solution were added to 4 ml of dilution buffer to give a final concentration of 250 ng/ml.

A serial dilution of the ATP standard was performed in the first 9 wells of a white polystyrene 96 well plate (Dynatech, Cat no 7905), with each well containing 50 µl of a

1:3 dilution of the ATP from the previous well to give ATP concentrations of 151, 50.4, 16.8, 5.60, 1.86, 0.620, 0.207, 0.0689 and 0.0218 nM. An aliquot (50 μ l) of the previously prepared luciferin-luciferase counting reagent was added to each well and then the luminescence was read using the luminometer. Plotting the log of ATP concentration against the log of luminescence counts produced a straight standard curve (figure 2.2).

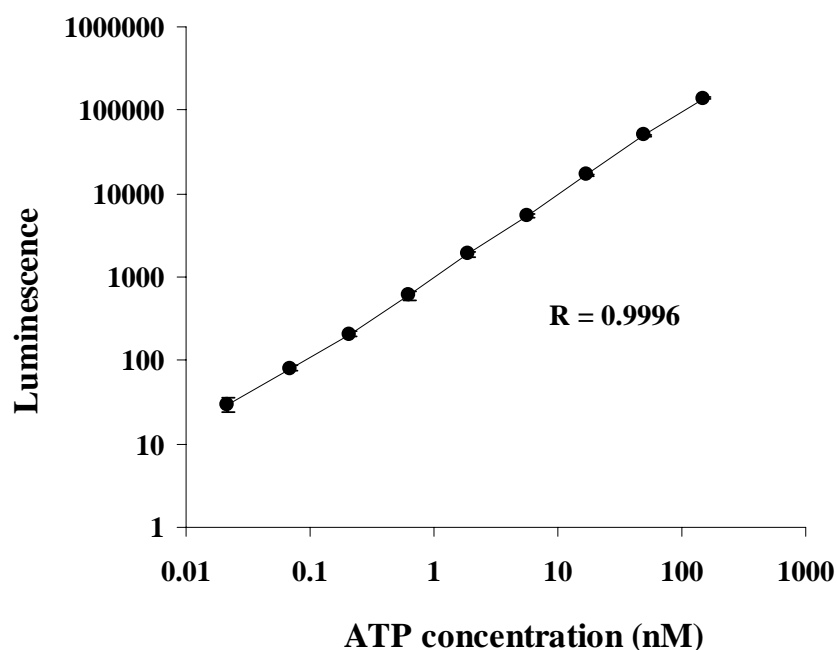


Figure 2.2 ATP standard curve produced on a Berthold MPLX plate luminometer. Results are shown as mean (\pm SD) of triplicate values. Here and elsewhere in this thesis, when error bars are not shown for some data points, this is because they are smaller than the markers on the graph.

2.2.16 Evaluability criteria

For an assay to be evaluable the average positive control luminescence had to be at least as high as the luminescence produced by 1.86 nM (equivalent to 1.028 ng/ml) ATP in the standard curve. The luminescence produced by 1.86 nM ATP varied with the age of the ATP standard, and the luciferin-luciferase counting reagent. This also varies with the luminometer used, as all such instruments express their measurements as relative light units. The evaluability cut-off for the ATP was chosen according to previous data published by Andreotti *et al.* (1995), as low cell numbers can produce spurious results.

Also a cut-off at 1.86 nM provides a good discrimination between the luminescence measured in the MI and MO wells.

2.2.17 Reading ATP levels from assay plates

To read the ATP levels in the ATP-TCA plates following cell lysis (ATP extraction), 50 µl from each well of the 96 well culture plate was transferred into a white 96 well plate, using fresh pipette tips for each drug. To each well, 50 µl of the previously prepared luciferin-luciferase counting reagent was added and then the luminescence was read using the luminometer, following the manufacturer's operating instructions. After reading, the remaining extracted cells were stored in the 96 well culture plate at -80°C until the data had been analysed confirming that the plate did not need to be read again, or to permit RNA to be extracted for further experiments.

2.2.18 Alternative procedure of ATP extraction for qRT-PCR studies

The material left over after ATP extraction and reading did not prove suitable for RNA studies. The cell lysis buffer (section 2.2.13) was not found able to prevent RNA degradation by RNAses. Therefore, an alternative procedure of ATP extraction and quantification was set up for tumour-derived cells that were employed in further qRT-PCR experiments. Prior to cells lysis with the ATP extracting reagent, 150 µl of cell suspension were removed from each well, centrifuged, washed with phosphate buffered saline (PBS) and stored at -80°C in 350 µl GTIC-containing solution that prevented RNA degradation for at least six months (lysis buffer RA1, Macherey-Nagel, Germany, Cat. N.o 740961). The remaining 50 µl cell suspension was then lysed by adding 12.5 µl of ATP extracting reagent. After 20 minutes 50 µl of cell lysate were transferred to a white plate and the ATP was measured following addition of 50 µl luciferin-luciferase.

2.2.19 Calculation and interpretation of luminometry results

The data produced from each ATP-TCA plate was entered into an Excel (Microsoft) spreadsheet that calculated the percentage tumour growth inhibition at each concentration, the IC₅₀, IC₉₀ and the area under the concentration *versus* inhibition graph (Index_{AUC}) for each drug (see Appendix). The data calculated in the Excel spreadsheets was then entered into an Access (Microsoft) database. The % inhibition at each drug concentration was used to plot % inhibition curves for each drug or

combination. As the variation between the wells that were averaged to calculate % inhibition is small (typical coefficient of variance of less than 10%), error bars representing the standard deviation cannot be seen in most graphs shown in this thesis, as they are often smaller than the markers on the graphs.

The percentage tumour growth inhibition was calculated as follows:

$$\left[1 - \frac{(TEST) - (MI)}{(MO) - (MI)} \right] \cdot 100 = \text{Percent tumour inhibition}$$

(TEST) = mean counts for test drug wells

(MI) = mean counts for maximum inhibitor wells

(MO) = mean counts for no drug controls

For ease of comparison between different tumours, a sensitivity index $\text{Index}_{\text{SUM}}$ for each drug in each tumour was calculated. This involved summing the percentage tumour growth inhibition and subtracting this figure from 600 ($\text{Index}_{\text{SUM}} = 600 - \text{Sum}[\text{Inhibition}_{6,25,\dots,200}]$). The figure 600 derives from the fact that 6 drug concentrations are tested in the ATP-TCA and the maximum % inhibition caused by each drug dilution is 100. Therefore a hypothetical drug able to kill the totality of the cells at all concentrations tested would have an $\text{Index}_{\text{SUM}}$ equal to zero. The Excel spreadsheet calculated the sensitivity index automatically for each drug or combination.

A second sensitivity index, $\text{Index}_{\text{AUC}}$, was calculated using the trapezoidal rule to calculate the area under the % inhibition curve for each drug (see Appendix). The effects of combinations of drugs compared to the constituent single agents are also analysed in the Excel spreadsheet using the methods determined by Poch *et al.* (Poch *et al.*, 1990; 1995), which compares the expected (additive) effect with the achieved effect at each drug concentration. The results are presented graphically (Chapter 6, figure 6.3). As suggested by Neale *et al.* (1999), the methods of Poch *et al.* are better suited to the data produced by the ATP-TCA than other methods commonly used to evaluate combination effects such as those of Chou and Talalay (Chou & Talalay 1984). The methods of Poch *et al.* propose a corrective factor for dose response curves having a slope different from 1, such as those commonly shown by vinorelbine in the ATP-TCA

(see chapters 6 and 8). A Chou and Talalay analysis was also performed when the Poch method suggested at least additivity between two agents and the results were compared. In detail the combination indices (CI) were determined at 50% and 90% cell death, and were defined as follows:

$$CI_{A+B} = [(D_{A/A+B})/D_A] + [(D_{B/A+B})/D_B] + [\alpha(D_{A/A+B} \times D_{B/A+B})/D_A D_B]$$

where CI_{A+B} = CI for a fixed effect (F=50% or 90%) for the combination of cytotoxic A and cytotoxic B; $D_{A/A+B}$ = concentration of cytotoxic A in the combination A + B giving an effect F; $D_{B/A+B}$ = concentration of cytotoxic B in the combination A + B giving an effect F; D_A = concentration of cytotoxic A alone giving an effect F; D_B = concentration of cytotoxic B alone giving an effect F. α = parameter with value 0 when A and B are mutually exclusive and 1 when A and B are mutually non-exclusive. According to Greco *et al.* (1995), the combination index CI indicated: synergism <0.8; 0.8< additivity <1.2; antagonism >1.2.

2.3 Cell line assays

2.3.1 Cell lines

The M14, MALME-3M, RPMI-7951, UACC-62 and SK-MEL-2 melanoma cell lines and the acute lymphoblastic leukaemia CCRF-CEM cell line were purchased from the ATCC (LGC Promochem, Teddington, Middlesex, UK). The SK-MEL-28 melanoma cell line was kindly supplied by David Jackson, ICRF, Leeds, England. The SK-MEL-28 cell lines were supplied as growing cultures. All the remaining melanoma cell lines and CCRF-CEM cell line were supplied as frozen aliquots. All cell lines grew as adherent cultures with the exception of the CCRF-CEM cells, which grew in suspension.

2.3.2 Cell Culture Media

All melanoma cell lines were grown in standard RPMI-1640 medium (Sigma R7638) supplemented with 10% foetal calf serum (FCS) (Labtech International, East Sussex, UK, cat. 4-101-500), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma P0781), and 2 mM glutamine (Sigma G7513). The cells were incubated at 37°C in a humidified 5% CO₂ incubator until they were confluent. Ficoll-Hypaque density gradient centrifugation was used to separate pure cell populations from debris or dead cells that

might adversely affect the ATP-TCA. Following viability estimation and cell counting by Trypan blue exclusion, cells were plated (seeding density 2,000 cells/well) in 96 well polystyrene microplates (Falcon, BD Biosciences, Oxford, UK, Cat 35 3072) containing drugs that had been diluted as previously described above (section 2.2).

For the experiments described in Chapter 4, the cells were seeded in the plates and incubated for 24 hours at 37°C in a humidified 5% CO₂ atmosphere; before adding the drugs, the FCS in the wells was diluted to 2% by replacing 160 µl of the RPMI medium containing 10% FCS with 110 µl of serum free standard RPMI medium.

The CCRF-CEM cell line was grown in RPMI-1640 Dutch Modification medium (Sigma R7638) containing 2 mM L-glutamine, 100 IU/ml Penicillin, 100 µg/ml Streptomycin and 20% FCS.

Cell lines, obtained from the supplier as frozen aliquots, were initially passaged into 25 cm² flasks and cultured at 37°C, 5% CO₂. Cultures were grown to confluence and passaged into either 75 cm² or 175 cm² (Falcon) flasks. Cultures were passaged regularly when between 80-95% confluent. Cell lines were regularly screened for Mycoplasma by the technicians at the Institute of Ophthalmology using the VenorGeM® Mycoplasma-PCR-Detection Kit (Minerva Biolabs, Berlin, Germany).

2.3.3 Storage of cell lines in liquid nitrogen

Early passage number cells were divided into aliquots of about 10⁶ cells per ml growth media and stored in a liquid nitrogen dewar. The method used was that suggested by the European Collection of Cell Cultures (ECACC, Centre for Applied Microbiology & Research, Salisbury, Wiltshire, England). Briefly, the adherent cells were trypsinised using a 1:10 dilution of a 10x trypsin-EDTA solution (5.0 g/L trypsin in 0.2% EDTA; obtained either from Gibco BRL, Cat. 35400-027, or from Sigma, Cat T4174). After the cells were completely detached from the flask, the trypsin activity was inhibited by the addition of growth media containing foetal calf serum, and the cells pelleted by centrifugation at 300g for 10 minutes. After two washes in growth media, the cells were counted using the trypan blue exclusion method and were adjusted to 1x10⁶ cells per ml in growth media containing 10% v/v dimethylsulphoxide (DMSO) (Sigma D5879). The

cells were then divided into aliquots of 1 ml in polypropylene cryovials (Corning Costar cat no.430658 or 430659) and placed in a 'Mr Frosty' cryo-container (Nalgene cat no. 5100-0001) and then immediately placed in a -70°C freezer. After 24 hours in a -70°C freezer the cryovials containing cells were transferred to a rack in a liquid nitrogen dewar.

2.3.4 Selection of CCRF-CEM cells with XR5944

The bis-mesylate salt of the experimental agent XR5944 was supplied by Xenova Ltd (Slough, Berkshire, UK) as powder. It was dissolved in DMSO to give a stock solution of 1.28 mM (equivalent to 1.0 mg/ml) and aliquots were stored at -80°C. CCRF-CEM lines were grown in RPMI-1640 (Sigma, R7638) supplemented with 2mM L-Glutamine (Sigma, G7513) and 10% FCS. A resistant subline was obtained by step-wise treatment with XR5944 up to 10 nM. In brief, cells were initially exposed to 0.1nM XR5944 for 7 days, after which the cells were spun, washed and fed with fresh medium containing no drug. After resting for 7 days cells were treated again with XR5944 at a concentration 0.25 nM for a further week. Rest-treatment cycles were repeated for several weeks exposing the cells to the following concentrations of XR5944: 0.50 nM, 1.0 nM, 2.0 nM, 4.0 nM, 6.0 nM, 8.0 nM, 10nM. Resting time was increased to 14 days when the cells were treated with the top three concentrations. After drug selection, the cell response to XR5944 was tested in the ATP-TCA. The IC₅₀ values for XR5944 were 0.27±0.05 nM and 4.24 ±0.25 nM in the CCRFCEM parental and XR5944-resistant sub-lines, respectively. These values were essentially unchanged when the assay was repeated two months after the end of the exposure to XR5944.

2.3.5 External Quality Assurance (EQA) for ovarian trial

To ensure that intra-laboratory results were comparable, an EQA scheme was started between the three laboratories involved in the TCA Ovarian Carcinoma Trial. Two 25 cm² flasks of CCRF-CEM cells and 100 ml of culture media were sent out to each of the laboratories taking part in the trial. Each laboratory upon receipt of the cells set up an ATP chemosensitivity assay with the cells, using the above standard protocol with some modifications. Instead of CAM the assays were performed in CCRF-CEM media with 10% FCS. Cells were plated out at 2,000 cells per well.

Only one plate was set up for the quality assurance; the cytotoxics needed to be tested at lower concentrations (table 2.3) than those employed for clinical samples as the CCRF-CEM cells are extremely chemosensitive.

Table 2.3. List of drugs used for the ATP-TCA ovarian cancer trial EQA.

DRUG	TDC μ M	TDC μ g/ml	Stock mM	Stock mg/ml	μ L added to 5 ml medium to give 800% TDC
Cisplatin	1.7	0.50	3.3	1.0	20
Doxorubicin	0.17	0.10	3.4	2.0	2.0
5-FU	38	5.0	192	25	8.0
Vinblastine	0.11	0.10	1.1	1.0	4.0

The results from each laboratory were sent to Prof. Ian Cree for comparison.

2.4 Materials and Methods for the Immunohistochemistry (IHC)

2.4.1 Introduction

All the IHC technical work described in this section has been carried by the biomedical scientists working with Prof Cree's research team in Portsmouth and London, namely Mrs Lisa Mills, Mrs Alison Parker and Mrs Penny Johnson.

IHC techniques have been in use for the recognition of cells in tissue sections for at least 50 years. The first method reported use of fluorescent labels, while enzymatic labels, such as horseradish peroxidase (HRP) and alkaline phosphatase (AP), were introduced later. The IHC techniques employed in this thesis are based on a dual antibody (Ab) system using the Avidin-Biotin Complex method. The primary Ab binds to specific antigens present in the specimen and any excess is removed by washing. Next, the secondary Ab-conjugated to biotin is added and reacts with the primary Ab present on the tissue. Unbound Ab is removed by washing. The sample is then incubated with a solution containing the enzymatic label conjugated to avidin, which binds to the biotin-labelled secondary Ab present on the tissue. As avidin has an extraordinarily high affinity for biotin, their binding (unlike antibody-antigen interactions) is essentially irreversible. Finally, chromogenic substrates are added which, in the presence of the enzymatic label, deposit a coloured insoluble precipitate at those antigenic sites recognised by the primary Ab.

Two methods have been used to stain the samples studied in this thesis, the Vectastain® Universal Alkaline phosphatase kit (Vector laboratories, LTD, Peterborough. UK, Cat# AK-5200) until February 2002 for MDR1, TOPO I and TOPO II α ; while the Chemicon IHC Select™ - Immuno Peroxidase secondary detection system (Chemicon International, Chandlers Ford, Southampton, UK, Cat# Det-HP1000) was employed from March 2002 onwards for all Abs. Table 2.4 provides a list of the Abs used, their source together with the appropriate antigen retrieval method for each. The concentration of each Ab was determined by titration on positive control material and was made up to its optimal dilution in Tris buffered saline (TBS) pH 7.6 (table 2.4). A positive control section was run with each batch of staining (table 2.4). A duplicate of each test section was included as a negative control by omitting the primary Ab and replacing it with TBS.

2.4.2 Preparation of cytoclots

Cells were centrifuged at 400g and washed with PBS. Pellets were resuspended with an equal volume of human plasma using a Pasteur pipette. A few drops of human thrombin were added to the cellular suspension and mixed vigorously. The solution was allowed to clot for about 1 minute and the clot was transferred into 10% buffered formalin. Clots were fixed for 24-48 hours, processed as standard solid specimens and embedded in paraffin wax.

2.4.3 Specimen preparation and pre-treatment

Sections from paraffin-embedded blocks were cut on to Surgipath® positively charged slides and dried at 37° C for 24 hours. Wax was removed from the sections prior to staining by immersion in several changes of xylene followed by several changes of alcohol and rinsed in running tap water.

The appropriate antigen retrieval method for each Ab (pressure cooking, trypsination, microwaving; table 2.4) was used to reveal the antigen presenting sites blocked by formalin fixation. The sections were rapidly cooled and washed in running tap water.

2.4.4 Alkaline Phosphatase (AP) method

The sections were stained manually according to the protocol described below. Slides were incubated for 20 minutes in normal serum. This was followed by incubation at room temperature for 30 minutes with the primary Ab for TOPO I and II α . To block endogenous avidin binding, sections for the MDR-1 Ab were treated with separate blocking stages of 20 minutes in avidin and 20 minutes in biotin, prior to overnight incubation in the primary Ab at 4°C in a humid incubation chamber.

Following incubation in the primary Ab and rinsing in TBS, the slides were incubated for 30 minutes with diluted biotinylated universal secondary solution, rinsed and then incubated for 30 minutes with VECTASTAIN® ABC-AP reagent (Vector laboratories, AK-5200). To visualise the reaction the slides were incubated for 20 minutes in Vector® Red alkaline phosphate substrate kit (Vector laboratories, SK-5100).

Levamisole was also included to inhibit endogenous AP activity (Vector laboratories, SP-5000). AP catalyses the hydrolysis of a variety of phosphate-containing substances in the alkaline pH range. The enzymatic activity of AP can be localised by coupling a soluble product generated during the hydrolytic reaction with a chromogenic substrate, such as Vector® Red, producing a coloured insoluble precipitate.

The slides were counterstained with Gill's Haematoxylin, dehydrated and cleared using the Leica® XL slide staining machine. The sections were mounted in Vector® Mount (Vector laboratories, H5000), which produces a permanently mounted section, and has an optimal refractive index to retain the colour intensity of Vector® Red substrate reaction product.

2.4.5 Horseradish Peroxidase (HRP) method

The slides were placed on a Dako Autostainer instrument (Dako, Ely, UK) and were stained according to an automated protocol. Reagents were added in the following order and incubation times for each are indicated in brackets: normal goat serum (20 minutes); primary Ab (30 minutes; overnight; table 2.4); secondary Ab (15 minutes); streptavidin HRP (15 minutes); chromogenic reagent (3,3' diaminobenzidine, DAB, HD

Supplies, Aylesbury, UK, Cat. 4170) (5 minutes). DAB produces a brown colour during the enzymatic degradation of hydrogen peroxide (H₂O₂) by HRP.

Rinses (5 minutes) with TBS (pH 7.6) were performed between each step. 50µl avidin/ml goat serum and 50µl biotin/ml primary Ab were used to block endogenous avidin binding.

The slides were counterstained with Gill's Haematoxylin, dehydrated and cleared using the Leica © XL slide staining machine. The sections were mounted in Styrolite® mounting medium (BDH, Cat# 361704Y), which produces a permanently mounted section.

2.4.6 Assessment of IHC slides.

Assessment of slides was performed by a histopathologist (either Dr Silvana Di Palma or Prof Ian A Cree) using the H-score. Staining intensity (none, 0 points; weak, 1 point; moderate, 2 points; strong, 3 points) and percentage of positive tumour cells were multiplied to achieve a score between 0 and 300. An H-score of 100 or more was chosen as an arbitrary cut-off and regarded as positive by the histopathologist.

Table 2.4 List of Abs used for immunohistochemical studies. When sections required microwaving a Matsui MIOM microwave was used at 800W power. Pressure cooking was performed with a Tefal Clipso Pressure Cooker using 70P power.

Ab	Pre-Treatment	Dilution	Incubation	Cat No.	Source	Control Tissue
COX-2 (Clone CX229)	Pressure Cook 2 min pH 6.0	1:100	30 min; room temperature	CAY-160112	Cayman Chemicals (Distributor: Alexis – Nottingham, UK)	Ca Colon
P-glycoprotein (MDR-1) (Clone JSB-1)	Pressure Cook 2 min pH 6.0	1:100	Overnight; 4° C	NCL-JSB1	Novo Castra Newcastle-upon-Tyne, UK	Kidney
Topoisomerase I TOPO-I (Clone 1D6)	Pressure Cook 2 min pH 6.0	1:50	30 min; room temperature	NCL-TOPO1	Novo Castra Newcastle-upon-Tyne, UK	Tonsil
Topoisomerase II Alpha TOPO-II α (Clone 3F6)	Pressure Cook 2 min pH 6.0	1:40	30 min; room temperature	NCL-TOPOIIA	Novo Castra Newcastle-upon-Tyne, UK	Tonsil

2.5 Materials and methods for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

2.5.1 Introduction

Total mRNA was extracted from tumour-derived cells, reverse transcribed and the resulting c-DNA amplified by qRT-PCR. All the steps were performed using filter-barrier sterile tips and plasticware that was certified DNase- and RNase-free by the manufacturer (Fisher Scientific, UK).

The polymerase chain reaction (PCR) is an *in vitro* method for enzymatic synthesis and amplification of defined nucleic acid sequences, such as a particular known DNA sequence. The cDNA product (amplicon) formed by a thermostable DNA polymerase in one cycle can serve as a template in the next cycle so in an ideal, fully optimised PCR reaction, the number of amplicons doubles every cycle. Ten cycles will yield 1024 amplicons; 20 cycles more than a million. After a fixed number of cycles, the amplified cDNA can be analysed for size and quantity, usually by electrophoresis on agarose gels, and sequenced.

In recent years there has been intensive use of the newly developed technique of qRT-PCR for the investigation of gene expression. qPCR enables the monitoring of the progress of the PCR as it occurs. This is achieved including SYBR Green in the reaction mixture, an intercalating dye that emits a strong fluorescent signal when it binds to double-stranded nucleic acid; the fluorescence is proportional to the amount of product formed. Alternatively, in addition to the primers, a probe labelled with two dyes can be added to the reaction mixture. These molecular probes consist of a short oligonucleotide sequence (usually between 20 and 30 bases) which is encompassed in the region amplified by the primers. These probes rely on the close proximity of a quenching dye to the reporter dye and disruption of this quenching interaction by the action of the polymerase causes an increase in fluorescence that is proportional to the product formation. Compared to SYBR Green method such probes are more specific, and give lower background fluorescence, but they are more expensive and require greater optimisation.

During a real time PCR, data are collected after every cycle, rather than allowing completion of a fixed number of cycles before analysis of the products. In the initial

cycles of PCR there is little change in the fluorescent signal, and this defines the baseline for the amplification plot. Detection of an accumulating product is indicated by an increase in fluorescence above this baseline. The parameter 'threshold cycle' (Ct) is defined as the fractional cycle number at which the fluorescence crosses a fixed threshold. This method has many advantages over conventional PCR, having a greater accuracy and sensitivity, a wider dynamic range, a capacity for high throughput of samples, and no requirement for post-PCR manipulations, with their potential for contamination of other samples. In qRT-PCR, reactions are characterised by the point in time during cycling when amplification of a target is first detected, rather than the amount of target accumulated after a fixed number of cycles. The higher the starting number of the RNA target, the sooner a significant increase in fluorescence is detected.

qRT-PCR is a relatively new technique, and different research groups have used different methods of experimental design and data analysis. For the work in this thesis, various techniques and systems were trialled, and eventually a methodology was selected that allowed repeated, high-throughput of biological samples with a high degree of reliability, reproducibility and robustness. Chapter 3 will describe the technical development of the real time methodology used in this thesis, while the remaining part of this section will focus more in detail on the single steps performed to investigate the changes of gene expression caused by exposure of cancer cells to chemotherapeutic agents.

2.5.2 Total RNA extraction

The first step in the measurement of gene expression is the isolation of RNA from the sample. This was done using one of a variety of commercially available kits, based on the nucleic acid binding properties of silica membranes, the NucleoSpin® RNA II mini kit, that was purchased from Macherey-Nagel, Germany (Cat. #740955). The instructions provided in the kit manufacturer's protocol were essentially followed.

Cells that survived after drug exposure in the ATP-TCA were pooled from at least 6 wells to increase nucleic acid yield and stored as previously described in section 2.2.18. Ice-cold 70% ethanol (350 µl) was added to the cell lysate, and the mixture was vortexed. For each preparation, one NucleoSpin® RNA II column was placed in a 2ml centrifuge tube, and the lysate was added. The column was centrifuged for 1 minute at

8,000g, after which the eluate was discarded. A Membrane Desalting Buffer (MBD) (350 μ l) was added, and the column was centrifuged at 11,000g for 1 minute to dry the silica membrane of the spin column. At this stage the protocol included a DNase digestion step to prevent carry-over of genomic DNA in further analysis. A DNase reaction mixture was prepared in a microcentrifuge tube; for each sample, 10 μ l reconstituted DNase I were added to 90 μ l DNase reaction buffer and mixed by flicking the tube. The DNase reaction mixture (95 μ l) was then applied directly onto the centre of the membrane of the column, and incubated at room temperature for 15 minutes. At the end of this period 200 μ l buffer RA2 were added to the column to inactivate DNase. The sample was then centrifuged for 1 minute at 8,000g, after which the eluate was discarded, and the spin column placed in a new 2 ml microcentrifuge tube. 600 μ l wash buffer RA3 were then added to the column and centrifuged for 1 minute at 8,000g. The eluate was discarded and another wash step was performed with 250 μ l buffer RA3 and centrifuging the sample for 2 minutes at 11,000g. The eluate was discarded, and the column was centrifuged again for 1 minute at 11,000g to completely dry the membrane. Finally, total RNA was eluted in 60 μ l RNase-free water by further centrifugation at 11,000g for 1 minute. The eluate was collected in a RNase-free Eppendorf tube and immediately frozen at -80°C.

2.5.3 Reverse transcription

Prior to starting the PCR process, target RNA is subjected to reverse transcription, a process during which the enzyme reverse transcriptase synthesises a complementary strand for each strand of RNA. The resulting single stranded product is known as complementary DNA, or cDNA.

Two basic techniques of RT-PCR are described in the literature: one-step and two-step RT-PCR. In one-step RT-PCR, all reagents for both reverse transcription and the PCR process are added to the same tube, and a thermal cycling protocol is utilised that allows these reactions to proceed sequentially. Two-step RT-PCR initially involves a first-strand cDNA synthesis reaction that is followed by inactivation of RT-enzyme and dilution of the mixture. The second step involves addition of the newly-synthesised cDNA to a PCR mix containing DNA polymerase. Each method has its benefits and drawbacks. One-step RT-PCR reduces hands-on time and the likelihood of introducing contaminants into the reaction, and is quicker. Two-step RT-PCR allows dilution of the

newly-synthesised cDNA, so that more reactions can be performed, and reduces the formation of non-specific PCR products. Throughout the work in this thesis, in which limited amounts of RNA were available, the two-step process was employed.

Total RNA was reverse-transcribed using the Promega ‘reverse transcription system’ (Promega, Southampton, UK, Cat # A3500) according to the manufacturer’s protocol. A master mix solution for the required number of reactions was prepared pipetting all the reagents in the sequence listed in table 2.5, except the Avian Myeloblastosis Virus (AMV) reverse transcriptase enzyme and RNA. As a negative control, 11.4µl were transferred to a thin-wall 0.2 ml PCR tube, to which 0.6µl of water had been previously pipetted. After preparing the negative control, the AMV enzyme was added, the master mix solution was vortexed and aliquoted into thin-wall 0.2 ml PCR tubes. 8µl RNA sample was added to each reaction, and the tubes were incubated for 10 minutes at RT to allow extension of the primers.

Table 2.5 List of reagents, volumes and concentrations for each RT reaction

Reagent	Volume / sample	Master Mix Final Concentration
Sterile nuclease-free water	1.9 µl	
MgCl ₂ 25mM	4.0 µl	5 mM
Reverse transcription 10X buffer	2.0 µl	1X
dNTP Mixture – 10 mM each	2.0 µl	1 mM
Random primers 0.5 µg/µl	1.0 µl	25 ng/µl
Recombinant RNasin® ribonuclease inhibitor (50 U/µl)	0.5 µl	25 U
AMV reverse transcriptase (25 U/µl)	0.6 µl	15 U
RNA sample	8.0 µl	
TOTAL	20.0 µl	

The samples were then transferred to a thermal cycler (ThermoHybaid, Ashford, UK) and incubated at 42°C for 60 minutes to improve the efficiency of the reaction, as recommended by the manufacturer. At the end of this period, samples were heated at 95°C for 5 minutes, then incubated at 4°C for 5 minutes to inactivate the AMV Reverse Transcriptase and prevent it from binding to the cDNA. The c-DNA samples were then stored at -80°C.

2.5.4 qPCR

The c-DNA was amplified by real-time quantitative PCR in 96 well plates (BioRad Cat. 2239441) on a Biorad iCycler instrument (BioRad Laboratories, Hemel Hempstead, UK). All reagents were obtained from Applied Biosystems (Warrington, UK), and used in accordance with the manufacturer's instructions. The final constituents of each PCR (25 µl) were 1 µl of cDNA, 200-500 nM of each primer (table 2.6), 200µM each dATP, dCTP, dGTP, 400µM dUTP, 3.0-5.0 mM MgCl₂, 0.625 units of AmpliTaq Gold DNA polymerase, 1x SYBR Green PCR buffer (SYBR Green PCR Core Reagents, Applied Biosystems, P/N 4304886) and 0.125 units AMPErase® uracil-N-glycosylase (UNG).

UNG was added to the reaction to prevent carry-over contamination (Longo *et al.*, 1990). During PCR, abundant amplification products are synthesised. Contamination of new PCRs with trace amounts of these previously amplified products (carry-over contamination) yields false positive results. Carry-over contamination can be controlled by incorporating dUTP in all PCR products and treating all subsequent PCRs with UNG. UNG hydrolyses the N-glycosidic bond between the deoxyribose sugar and uracil in DNA that contains deoxyuridine in place of thymidine (Lindahl *et al.*, 1977). UNG is thermolabile, being fully active at 50°C, and being inactivated by ten minutes incubation at 95°C. UNG is active on both single- and double stranded DNA that contains uracil, but has no activity on thymine-containing DNA. The resulting apyrimidinic sites block replication by DNA polymerases, and are very labile to acid/base hydrolysis. Because UNG does not react with dUTP, and is also inactivated by heat denaturation prior to the actual PCR, carry-over contamination of PCRs can be controlled effectively if the contaminants contain uracils in place of thymines.

The reagents were prepared in the following order. Firstly, a master mix was made up containing the following - SYBR Green dye in a 10x buffer solution, dNTP mixture, AMPErase®UNG, DNA polymerase, an appropriate volume of 25mM MgCl₂, and DNase-free water. The final volume of the master mix was enough to aliquot 12.5µl into each reaction well. Next, a mixture was made up containing an appropriate concentration of the forward and reverse primers, and 7.5 µl was added to each reaction well. Finally, 5µl of 1:5 diluted cDNA template was added to each reaction well, taking the final volume to 25 µl. Negative control wells were incorporated into the plate design (figure 2.1); instead of template, these contained 5 µl water only (no template controls)

or 5µl of appropriately diluted RNA that had undergone reverse transcription reaction in the absence of the AMV enzyme (RT-negative controls). All negative control wells contained primers and DNA polymerase.

Product amplification was performed up to 50 PCR cycles, after uracil removal by AMPErase®UNG for 2 minutes at 50°C and DNA polymerase activation for 10 minutes at 95°C. Each two-step PCR cycle comprised denaturing (15 s at 95°C), annealing, and extending (1 min at 60°C). An amplification reaction includes the target complementary DNA (cDNA), a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), and reaction buffer containing magnesium. In the initial step of a cycle, heating to 95°C for 15 seconds denatures the target cDNA; this separates the two strands from each other, providing a single-stranded cDNA template for the thermostable polymerase. In the next step, the temperature is cooled to 60°C for 1 minute, encouraging annealing of the oligonucleotide primers with the separated strands of cDNA. This allows DNA synthesis by the thermostable DNA polymerase, extending the oligonucleotide primers to copy the target cDNA. At the end of each run a final melt curve cycle (cooling to 50°C and then increasing stepwise 1 °C for 10 seconds up to 95 °C) was performed to exclude the presence of non-specific products. During amplification cycles, fluorescence measurements were made in each cycle during the last 15 seconds of the 60°C phase. During the subsequent melt curve analysis, they were made in the last 2 seconds at each new temperature.

At least two housekeeping (HK) genes were used as internal references for each experiment chosen among the following: glyceraldehyde-3 phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), human porphobilinogen deaminase (PBGD), succinate dehydrogenase complex-subunit A (SDHA) and TATA box binding protein (TBP). The internal reference genes were selected due to their relative low abundance in normal tissue (Vandesompele *et al.*, 2002). The HK genes were amplified in parallel to the target genes in separate wells. The target genes were different for each series of experiments and were chosen among a number of genes known or suspected to confer resistance to the anti-cancer agent tested, or generally associated with cell growth and proliferation. Figure 2.3 shows the layouts that were used for each case.

When possible, primers were chosen to have similar melting temperatures (T_m ; this can be estimated using the following simplified formula $T_m = 2^\circ\text{C} \times (A+T) + 4^\circ\text{C} \times (G+C)$) values and, whenever possible, to span exon-exon boundaries in the target sequences (table 2.6). Negative controls with no template and RT-negative as template were added in every experiment (figure 2.3). All assays were run in triplicate. Validation experiments were run to show that the efficiencies of the target and reference genes amplifications were approximately equal, and in the range 95-105% (see chapter 3). The PCR cycle number that generated a fluorescence signal above a set threshold (threshold cycle, C_t ; 10 standard deviations above the mean fluorescence generated during the baseline cycles) was determined, and a comparative C_t method was then used to measure relative gene expression (ABI PRISM 7700 User Bulletin #2, 2001 update). The following formula was used to calculate the relative amount of the transcript in the sample: $2^{-\Delta\Delta C_t}$, where ΔC_t is the difference in C_t between the gene of interest and the mean of at least two reference genes, and $\Delta\Delta C_t = \Delta C_t$ of drug exposed cells *minus* ΔC_t of control cells (exposed to medium only), for the *ex vivo* experiments, or $\Delta\Delta C_t = \Delta C_t$ of post-chemotherapy sample *minus* ΔC_t of pre-chemotherapy sample, for paired tumour biopsies.

Real time RT-PCR is a relatively new technique, and there is not yet a universally applicable method of data analysis, nor is there a means by which statistical significance can be estimated. Various groups have analysed their data in different ways, with differing levels of robustness. The data analysis methods used in this thesis were developed within our group, primarily by Surgeon Lt Cmdr SJ Mercer and myself, following assessment of methods recommended by real time PCR instruments, such Applied Biosystems and BioRad, and assisted by a critical appraisal of the literature. The details are described in Chapter 3.

Table 2.6 Sequence of primers (forward and reverse) used for qPCR experiments. GenBank accession numbers for each gene are indicated in brackets. When no reference is indicated, the primers were designed using an old version of the software Primer 3.0, available at the following website: <http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>. T_m values were calculated using a demo version of the Beacon Designer Software (PREMIER Biosoft International, Palo Alto, USA).

Name (GenBank num.)	Sequence 5'-3' (forward and reverse primer)	Product size(bp)	Product T_m (°C)	Primer conc nM	Primer T_m (°C)	Mg ²⁺ conc mM	Reference
GAPDH (NM002046)	5'-GAA GGT GAA GGT CGG AGT C-3'	226	86	200	62.3	4	
	5'-GAA GAT GGT GAT GGG ATT TC-3'			200	58.3		
HPRT1 (NM000194)	5'-TCA GGC AGT ATA ATC CAA AGA TGG T-3'	84	84	400	61.3	4	Moniotte <i>et al.</i> , 2001
	5'-AGT CTG GCT TAT ATC CAA CAC TTC G-3'			400	62.9		
PBGD (NM000190)	5'-CTG CAC GAT CCC GAG ACT CT-3'	98	89	400	64.5	4	Van den Heuvel- Eibrink, <i>et al.</i> , 2002
	5'-GCT GTA TGC ACG GCT ACT GG-3'			400	64.5		
SDHA (NM004168)	5'-TGG GAA CAA GAG GGC ATC TG-3'	86	81	400	62.4	4	Vandesompele <i>et al.</i> , 2002
	5'-CCA CCA CTG CAT CAA ATT CAT G-3'			400	60.8		
TBP (X54993)	5'-CAC GAA CCA CGG CAC TGA TT-3'	89	85	400	62.4	4	Bieche <i>et al.</i> , 1999
	5'-TTT TCT TGC TGC CAG TCT GGA C-3'			400	62.6		
BCRP (AF098951)	5'-CAC AAC CAT TGC ATC TTG GC-3'	74	82	400	60.4	4	Faneyte <i>et al.</i> , 2002
	5'-GCT GCA AAG CCG TAA ATC CA-3'			400	60.4		
COX-2 (M90100)	5'-CCT TCC TCC TGT GCC TGA TG-3'	81	83	400	64.5	4	Sales <i>et al.</i> , 2002
	5'-ACA ATC TCA TTT GAA TCA GGA AGC T-3'			400	59.7		
DPD (NM000110)	5'-CCA AAG GCA GTA AAG CAG GAA-3'	66	84	400	60.6	4	Blanquicett <i>et al.</i> , 2002
	5'-TCA CGA CTC CCC GTA TCG A-3'			400	62.3		
EGFR (NM005228)	5'-TGG TCA AGT GCT GGA TGA TAG A-3'	156	86	400	60.8	4	
	5'-GGT AGA AGT TGG AGT CTG TAG GA-3'			400	62.7		
ERCC-1 (NM001983)	5'-GGG AAT TTG GCG ACG TAA TTC-3'	71	87	400	60.6	3	Lord <i>et al.</i> , 2002
	5'-GCG GAG GCT GAG GAA CAG-3'			400	64.5		
GST- π (NM000852)	5'-CGG AGA CCT CAC CCT GTA-3'	169	90	400	62.2	5	
	5'-CGC CTC ATA GTT GGT GTA GA-3'			400	60.4		

Table 2.6 (continued). Sequence of primers (forward and reverse) used for qPCR experiments. GenBank accession numbers for each gene are indicated in brackets. When no reference is indicated, the primers were designed using an old version of the software Primer 3.0, available at the following website: <http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>. T_m values were calculated using a demo version of the Beacon Designer Software (PREMIER Biosoft International, Palo Alto, USA).

Name (GenBank num.)	Sequence 5'-3' (forward and reverse primer)	Product size(bp)	Product T _m (°C)	Primer conc nM	Primer T _m (°C)	Mg ²⁺ conc mM	Reference
MDR1 (AF016535)	5'-TGG TTC AGG TGG CTC TGG AT-3'	72	83	300	62.5	4	Yague <i>et al.</i> , 2003
	5'-CTG TAG ACA AAC GAT GAG CTA TCA CA-3'			300	63.0		
MLH-1 (NM000249)	5'-GGC ACA GCA TCA AAC CAA GT-3'	147	84	400	60.4	4	
	5'-GCA AGC ATG GCA AGG TCA A-3'			400	60.2		
MRP1 (L05628)	5'-CAA TGC TGT GAT GGC GAT G-3'	70	83	400	60.2	4	Van den Heuvel- Eibrink, <i>et al.</i> , 2002
	5'-GAT CCG ATT GTC TTT GCT CTT CA-3'			400	61.0		
MRP2 (NM000392)	5'-TGC AGC CTC CAT AAC CAT GAG-3'	80	84	400	62.6	3	Taipalensuu <i>et al.</i> , 2001
	5'-GAT GCC TGC CAT TGG ACC TA-3'			400	62.5		
MT-II (NM005953)	5'-GAT CCC AAC TGC TCC TGC-3'	127	90	400	62.2	4	
	5'-ACT TGG CAC AGC CCA CAG-3'			400	62.2		
MVP (NM017458)	5'-CAG CTG GCC ATC GAG ATC A-3'	68	87	400	62.3	4	Van den Heuvel- Eibrink, <i>et al.</i> , 2002
	5'-TCC AGT CTC TGA GCC TCA TGC-3'			400	64.5		
TP (NM001953)	5'-CCT TGG ATA AGC TGG AGT CT-3'	107	87	400	60.4	4	
	5'-CCT ACT CTG ACC CAC GAT AC-3'			400	62.4		
TS (NM001071)	5'-CCA GAG ATC GGG AGA CAT GG-3'	66	86	400	64.5	4	
	5'-TAC GTG AGC AGG GCG TAG CT-3'			400	64.5		
TOPO I (J03250)	5'-CTC CAC AAC GAT TCC CAG AT-3'	149	86	400	60.4	3	
	5'-TTA TGT TCA CTG TTG CTA TGC TT-3'			400	57.4		
TOPO II α (NM001067)	5'-GTA ATT TTG ATG TCC CTC CAC GA-3'	223	85	400	61.0	3	
	5'-TCA AGG TCT GAC ACG ACA CTT-3'			400	60.6		
TOPO II β (NM001068)	5'-GCA GCC GAA AGA CCT AAA TA-3'	85	81	400	58.4	3	
	5'-AAT CAT TAT TGT CAT CAT CAT C-3'			400	56.4		

	1	2	3	4	5	6	7	8	9	10	11	12
No-drug cells	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	MVP	TOPO I	TOPO II α	TOPO II β
	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	MVP	TOPO I	TOPO II α	TOPO II β
	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	MVP	TOPO I	TOPO II α	TOPO II β
Water	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers
RNA template	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg
Doxorubicin treated cells	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	MVP	TOPO I	TOPO II α	TOPO II β
	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	MVP	TOPO I	TOPO II α	TOPO II β
	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	MVP	TOPO I	TOPO II α	TOPO II β

Figure 2.3 (a) Plate layout employed to study changes in gene expression after *ex vivo* exposure to doxorubicin in breast- and ovarian-tumour derived cells.

	1	2	3	4	5	6	7	8	9	10	11	12
No-drug cells	HPRT1	PBGD	SDHA	TBP	BCRP	COX-2	EGFR	ERCC1	MDR1	MRP1	TOPO I	TOPO II α
	HPRT1	PBGD	SDHA	TBP	BCRP	COX-2	EGFR	ERCC1	MDR1	MRP1	TOPO I	TOPO II α
	HPRT1	PBGD	SDHA	TBP	BCRP	COX-2	EGFR	ERCC1	MDR1	MRP1	TOPO I	TOPO II α
Water	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers
RNA template	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg
Topotecan treated cells	HPRT1	PBGD	SDHA	TBP	BCRP	COX-2	EGFR	ERCC1	MDR1	MRP1	TOPO I	TOPO II α
	HPRT1	PBGD	SDHA	TBP	BCRP	COX-2	EGFR	ERCC1	MDR1	MRP1	TOPO I	TOPO II α
	HPRT1	PBGD	SDHA	TBP	BCRP	COX-2	EGFR	ERCC1	MDR1	MRP1	TOPO I	TOPO II α

Figure 2.3 continued. (b) Plate layout employed to study changes in gene expression after *ex vivo* exposure to topotecan in ovarian-tumour derived cells.

	1	2	3	4	5	6	7	8	9	10	11	12
No-drug cells	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	TOPO I	TOPO II α	TOPO II β	
	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	TOPO I	TOPO II α	TOPO II β	
	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	TOPO I	TOPO II α	TOPO II β	
Water RNA template	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	
	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	
Irinotecan treated cells	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	TOPO I	TOPO II α	TOPO II β	
	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	TOPO I	TOPO II α	TOPO II β	
	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	TOPO I	TOPO II α	TOPO II β	

Figure 2.3 continued. (c) Plate layout employed to study changes in gene expression after *ex vivo* exposure to irinotecan in colorectal-tumour derived cells.

No-drug cells			5-FU treated cells			Primers	RT neg				
1	2	3	4	5	6	7	8	9	10	11	12
GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH				
HPRT1	HPRT1	HPRT1	HPRT1	HPRT1	HPRT1	HPRT1	HPRT1				
PBGD	PBGD	PBGD	PBGD	PBGD	PBGD	PBGD	PBGD				
TBP	TBP	TBP	TBP	TBP	TBP	TBP	TBP				
COX-2	COX-2	COX-2	COX-2	COX-2	COX-2	COX-2	COX-2				
DPD	DPD	DPD	DPD	DPD	DPD	DPD	DPD				
TP	TP	TP	TP	TP	TP	TP	TP				
TS	TS	TS	TS	TS	TS	TS	TS				

Figure 2.3 continued. (d) Plate layout employed to study changes in gene expression after *ex vivo* exposure to 5-FU in breast, upper GI and colorectal-tumour derived cells.

	1	2	3	4	5	6	7	8	9	10	11	12
Pre-ECF (control)	HPRT1	PBGD	TBP	COX-2	DPD	GST- π	MDR1	MRP1	MT	MVP	TP	TS
	HPRT1	PBGD	TBP	COX-2	DPD	GST- π	MDR1	MRP1	MT	MVP	TP	TS
	HPRT1	PBGD	TBP	COX-2	DPD	GST- π	MDR1	MRP1	MT	MVP	TP	TS
Water	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers	Primers
RNA template	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg	RT neg
Post-ECF (treated cells)	HPRT1	PBGD	TBP	COX-2	DPD	GST- π	MDR1	MRP1	MT	MVP	TP	TS
	HPRT1	PBGD	TBP	COX-2	DPD	GST- π	MDR1	MRP1	MT	MVP	TP	TS
	HPRT1	PBGD	TBP	COX-2	DPD	GST- π	MDR1	MRP1	MT	MVP	TP	TS

Figure 2.3 continued. (e) Plate layout employed to study changes in gene expression in paired upper GI biopsies after *in vivo* administration of Epirubicin+Cisplatin+5-FU (ECF). The same layout was also employed when oesophageal tumour derived cells were exposed to ECF in the ATP-TCA.

Chapter 3 - Technical development

3.1 Introduction

A considerable amount of work has been carried out to establish or improve the methodologies described in chapter 2. This chapter summarises these efforts.

3.2 Further development of the ATP-TCA

Despite having great advantages over the other TCA methods, the ATP-TCA still suffers from some limitations, including: the number of cells required (a minimum of 10,000 cells/well which means a total of 180,000 cells is required to test each drug in triplicate at 6 different concentrations); the cost of reagents; the time to prepare the drugs and set up the plates.

A translation of the ATP-TCA from the current 96-well format to a 384-well format would have the advantage that it would reduce the amount of tumour material needed for the test and also the quantity (and costs) of reagents employed.

This approach was explored during the first part of my studies. The initial experiments were performed in 384 well polypropylene plates with flat bottom wells (Corning Costar, High Wycombe, UK) or V-shaped wells (Falcon, BD Biosciences, Oxford, UK). The first assays were carried out by plating tumour cells (usually spare cells from ovarian tumour ascites) without the drugs to establish the ideal number of cells under the new conditions. The results showed that the amount of ATP detected after 6 days incubation at 37°C and 5% CO₂ was dependent on the number of cells originally plated. An example can be viewed in figure 3.1.

On the basis of such preliminary data the number of cells for 384 well plates was set up at 3,000 cells/well for ascites samples and at 5,000 cells/well for solid samples. This represents a quarter of the number of cells needed for the corresponding 96-well plate. The final incubation volume in each well of the 384-well plate was set up at 40 µl, consisting of 20 µl of cell suspension with the remaining 20 µl with medium only (MO or positive control), or medium containing drug(s) at the appropriate dilution, or maximum ATP-inhibitor (MI or negative control). The volumes of reagents required were therefore only 20% of those required to perform the assay in the 96-well format.

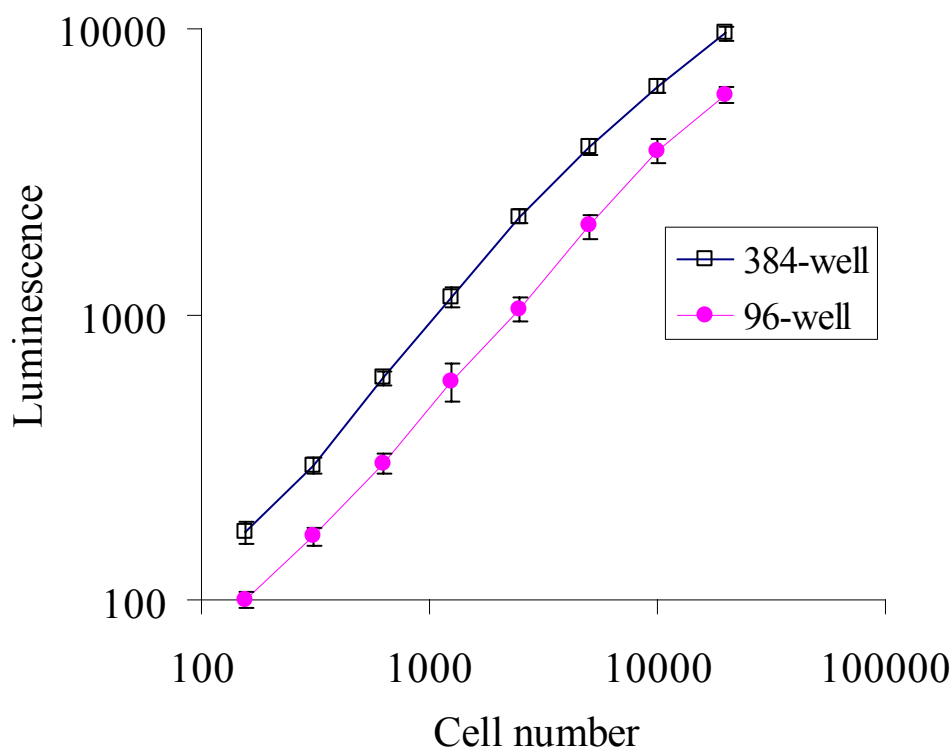


Figure 3.1 ATP content of untreated ovarian tumour derived cells cultured for 6 days in polypropylene 96-well and 384-well plates.

To test the system, 4 ovarian adenocarcinoma samples were then tested in parallel in both plate formats (96 and 384-well) adding the drugs. These experiments generally produced erratic results in the 384-well format (figure 3.2). Furthermore, the percentage of variation among triplicates was higher in the new format, as shown in table 3.1.

Table 3.1 Difference in the coefficient of variation (CV) among triplicates in 96-well and 384-well plates (the results are shown in figure 3.2); * $p < 0.01$ t student test

Mean values of % CV		
	96-well plate	384-well plate
Control (12 wells)	5.84%	23.4%
Cisplatin	9.04%	24.4%
Gemcitabine	7.13%	15.5%
Cisplatin + Gemcitabine	5.39%	17.6%
Doxorubicin x 5	7.40%	33.8%
MEAN	6.96%*	23.0%*

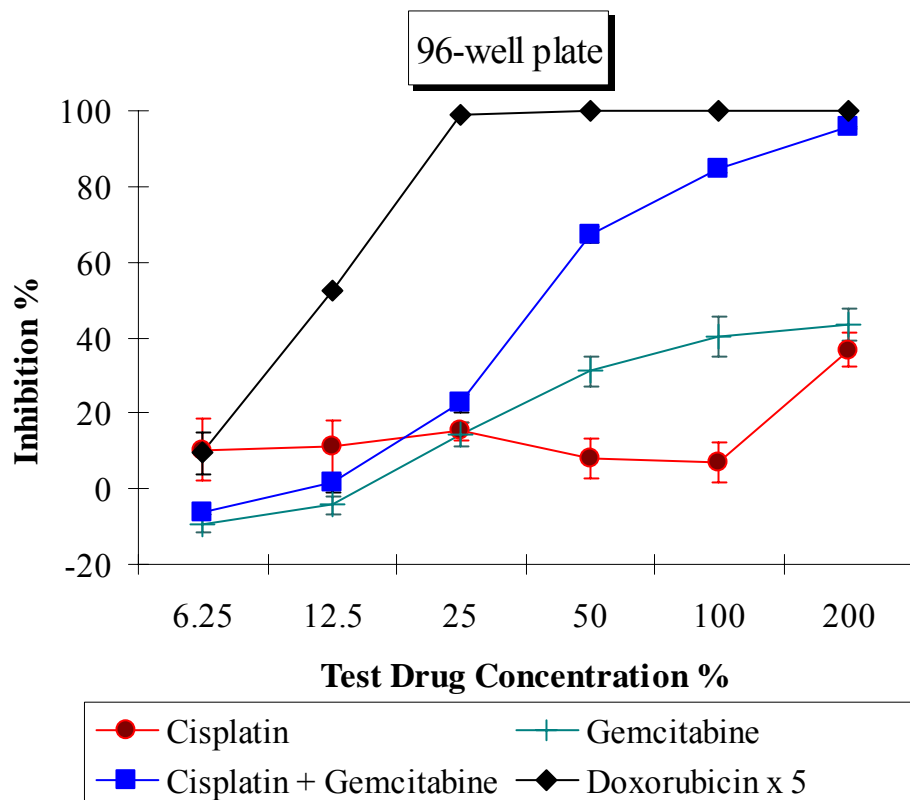
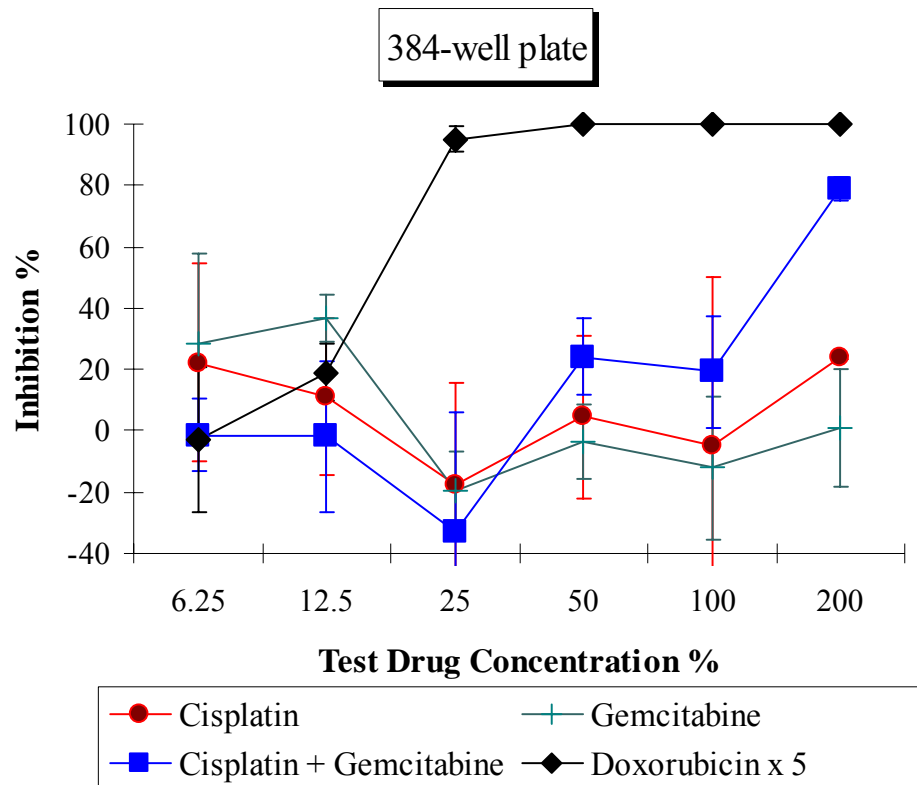


Figure 3.2 ATP-TCA results obtained culturing ovarian tumour derived cells in a 384-well plate. Results shown are means (\pm SD) of triplicate values. Erratic results were obtained for this sample when the ATP-TCA was performed in a flat-bottomed 384-well plate compared to a standard 96 well plate.

A robot suitable for application to 384-well plates was also employed (Kemble instruments, mod. SPI) and the cells derived from 3 ovarian adenocarcinoma ascites were simultaneously tested both in 96-well and 384-well plates, either by hand or by the machine. The use of the robot did not improve the accuracy of the assay in the 384-well format, giving CVs even higher than expected (table 3.2).

Table 3.2 Untreated tumour-derived cells were plated both in 96-well and 384-well plates, either by hand or by robot. The ATP content was inferred by measurement of light output, expressed as Relative light units (RLUs). Data represented are mean values of 12 replicates.

Plate format	96 Hand	96 Robot	384 Hand	384 Robot
RLUs mean	17572	13811	12349	15931
CV	4.20%	4.82%	22.7%	39.2%

During the second year of my PhD studies, better data was obtained by growing the cells in a new brand of 384-well plates that were introduced on the market (Corning Costar, Cat. N. 3656). These plates have rounded bottoms and rounded square-well geometry (Costar). The second feature, in particular, was a determining factor that improved the accuracy of pipetting. Figure 3.3 shows an example of ATP-TCA results obtained in a single 384-well plate for 12 agents or combinations. In this instance, the cells were obtained from a solid ovarian tumour by enzymatic digestion and plated at 5,000 cells/well.

The cross-talk between wells was found to be generally greater than usually observed in the 96-well plates. To minimise this effect a new plate layout was designed (Figure 3.4) which included three consecutive rows of positive controls (MO), with the first and third one adjacent to the wells containing the lowest concentrations of cytotoxics.

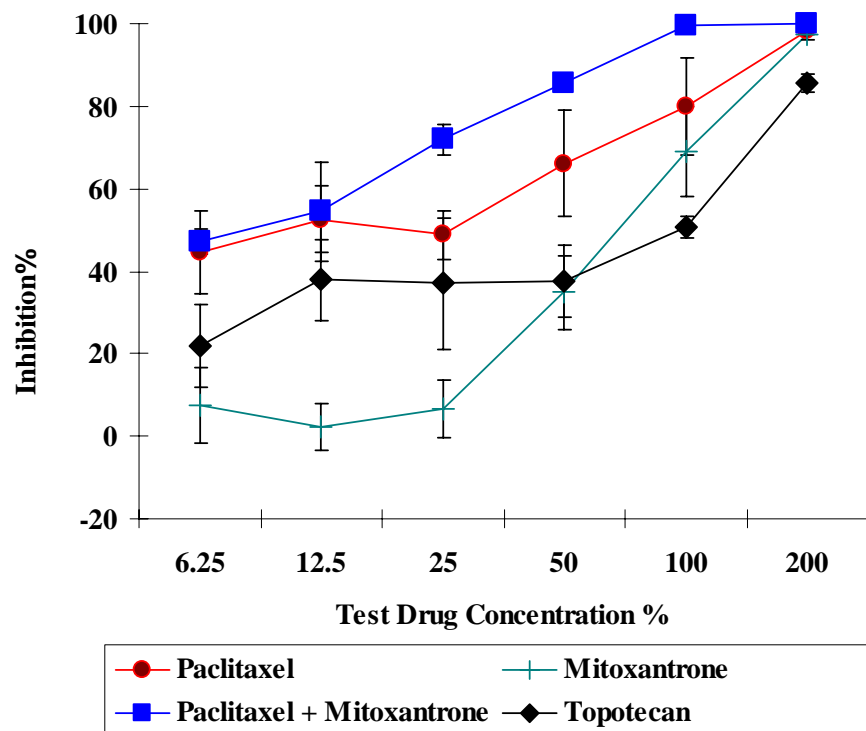
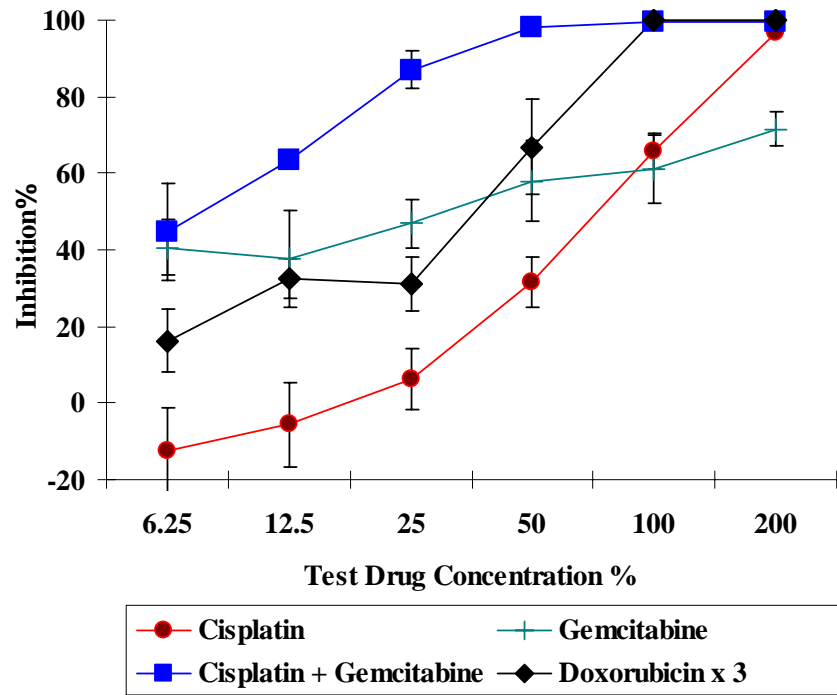


Figure 3.3 ATP-TCA results obtained culturing cells in a 384-well plate. Results shown are means of triplicate values. Error bars indicate SD, however in some cases these are not visible as they were smaller than the symbols on the graph.

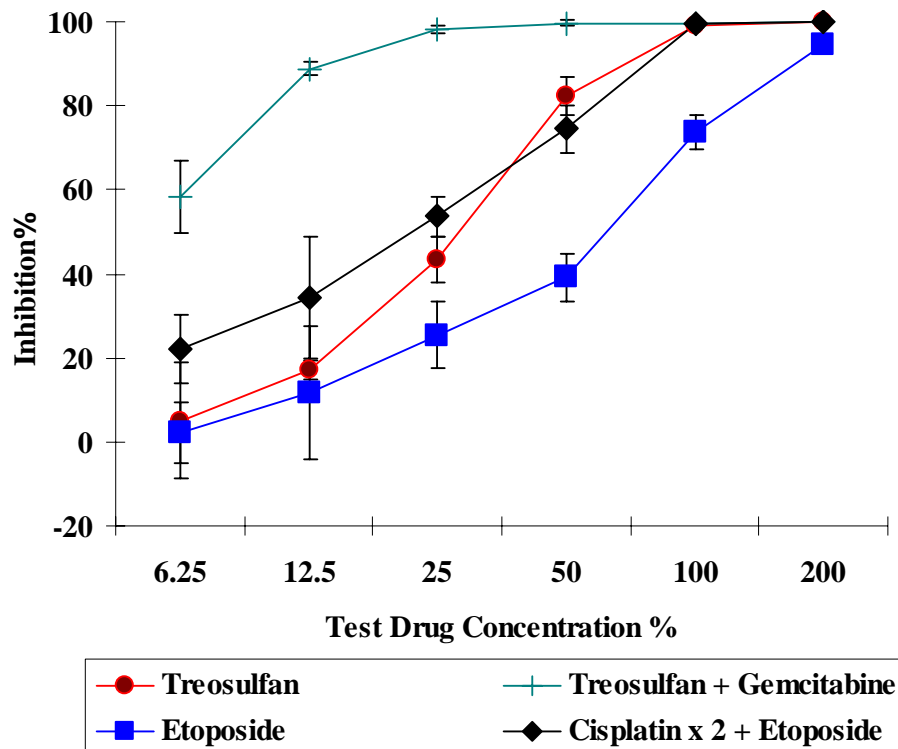


Figure 3.3 (continued). ATP-TCA results obtained after culturing the cells in a 384-well plate. Results shown are means of triplicate values. Error bars indicate SD, however in some cases these are not visible as they were smaller than the symbols on the graph.

The 384-well format has been employed to assess the chemosensitivity of 12 samples (6 uveal melanomas, 2 ovarian ascites, 2 ovarian biopsies, 1 unknown primary and 1 oesophageal) and evaluable results were obtained in 11/12 cases (92%). The only failure was attributed to insufficient cell growth of the oesophageal biopsy, a specimen type which was found to produce evaluable results only in 60% cases when plated in the standard 96-well format (Mercer *et al.*, 2003).

In general, the tumours grown in the successful assays were very well digested, with little, if any, cell aggregates. A source of variability between the 96- and 384-well plates could be the seeding density (Crouch & Slater, 2001). This might have affected my results, as well as the choice of the plate. Tumour derived cells are often aggregated in clumps, and the larger volume of cell suspension added to the 96-well plates compared to the 384-well plates (100 μ l *versus* 20 μ l) is likely to minimise the variation in cell

number when the cells are plated. This was confirmed by the fact that in our laboratory, assays with the leukaemic CCRF-CEM cells produced highly reproducible results. Dissociation of tumour to a single cell suspension would involve greater loss of cells during their preparation, and would be counter-productive.

Another factor that increases variability of the assay could be the sensitivity of the detection system, but this is not likely to have influenced the results, as the ATP bioluminescence assay gave a CV of <3% for the replicate experiments using an ATP standard of 1 μ M (Crouch and Slater 2001), and similar results are consistently seen in our laboratory when ATP standard curves are performed (chapter 2).

In conclusion, although partially positive results were obtained, the 384-well format is not recommended for routine use in the ATP-TCA with tumour-derived cells.

		1 2 3	4 5 6	7 8 9	10 11 12	13 14 15	16 17 18	19 20 21	22 23 24		
		Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Drug 6	Drug 7	Drug 8		
A	MI	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		A
B	200% TDC	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		B
C	100% TDC	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		C
D	50% TDC	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		D
E	25% TDC	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○		E
F	12% TDC	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○		F
G	6% TDC	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○		G
H	MO	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		H
I	MO	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		I
J	MO	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		J
K	6% TDC	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○		K
L	12% TDC	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○		L
M	25% TDC	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○		M
N	50% TDC	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		N
C	100% TDC	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		C
P	200% TDC	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●		P
		Drug 9	Drug 10	Drug 11	Drug 12	Drug 13	Drug 14	Drug 15	Drug 16		

Figure 3.4 A diagram representing the 384-well plate layout.

3.3 Real time PCR development

3.3.1 Relative *versus* absolute quantitation of gene expression

Gene expression can be quantitated on a relative basis or an absolute basis. Absolute quantitation yields gene expression in terms of nanograms of mRNA, and requires that the absolute quantities of a standard be obtained by some independent means, such as the use of plasmid DNA or *in vitro* transcribed RNA. The concentration of the absolute standard is obtained by means of an ultraviolet spectrophotometer, and converted to the number of copies using the molecular weight of the RNA. Absolute mRNA quantitation is more expensive and more complicated than relative quantitation, and inaccuracy is introduced by the instability of RNA in solution.

Relative quantitation compares the expression of a target gene in a sample with the expression of a reference gene in that same sample (also known as a housekeeping gene or an internal control) and is particularly suited to RT-PCR which allows greater sensitivity by amplification of small amounts of mRNA. A reference gene is a gene whose expression is constant under all cellular conditions; its expression must not be affected by differences in temperature, pH or drug exposure. Use of a reliable reference gene allows normalisation of a number of variations that would otherwise introduce inaccuracies into the system (for instance, the amount of starting material, and differences between cells in overall transcriptional activity). It also avoids the requirement for post-PCR analysis of data to determine the exact mass of mRNA in the starting material.

In the experiments detailed in this thesis, relative quantitation of mRNA was performed using the standard curve method, in which target quantity is calculated from a standard curve graph and divided by the target quantity of the calibrator. This method requires the preparation and maintenance of an accurately diluted stock RNA solution; also, standard curves must be prepared for the reference gene and all target genes, with regular revalidation of all standards.

A development of this method for qRT-PCR is the use of the comparative C_t method (threshold cycle), in which an arithmetic formula is used to achieve the same result. This means that, so long as the appropriate validation experiments have been carried out, the use of standard curves can be eliminated, and the amount of a target can be

measured by the use of an equation (chapter 2), which includes the difference in Ct between target and housekeeping gene. Before using this method for quantitation of gene expression, a validation experiment must be performed to demonstrate that the efficiencies of the PCR reaction for target and reference are approximately equal. Once this has been proven, relative quantitation can be calculated without the requirement to run standard curves on the same plate.

3.3.2 Selection of appropriate reference genes

The selection of appropriate reference genes is critical to the accuracy of qRT-PCR when the comparative Ct method described above is used. If varying cellular conditions (such as pH or drug exposure) alter the expression of the reference gene, then any calculations made using that reference gene are invalid. Also, if the initial starting concentration of the reference gene is of a different order of magnitude to the target genes, this makes it less precise for the quantitation of those target genes. This is due to the mathematics of PCR; indeed, the BioRad iCycler instruction manual include tables that show how the different efficiency of the reaction can affect the accurate measurement of gene expression. For example, if a reference gene is detected 10 cycles earlier than the target gene, then even a 5% difference in efficiency between the amplification of the reference gene and the target gene would lead to an inaccuracy of 0.36 cycles, equating to a 30% apparent difference in expression.

Investigators using qRT-PCR techniques have employed a number of different reference genes. One of the most commonly used is GAPDH (glyceraldehyde-3-phosphate dehydrogenase), an enzyme involved in glycolysis and gluconeogenesis. This gene has been used in a large proportion of studies using relative methods of quantitation, and is recommended for the optimisation of qRT-PCR techniques by some manufacturers (ABI PRISM 7700 User Bulletin #2, 2001 update). However, there is increasing evidence that the expression of GAPDH is altered by a number of conditions, including drug exposure and mitogenic stimulation. GAPDH is also expressed to a relatively high level in the majority of cells, which makes it less accurate for the quantitation of genes with low expression levels (Vandensompele *et al.*, 2002).

Other groups have used the gene β -actin as a reference gene. However, this gene is expressed at an extremely high level in most cells, with a Ct of between 3 and 10, and

as already demonstrated, large differences in Ct lead to inaccuracy in quantitation of low levels of target gene expression.

A possible solution has been published by Vandesompele *et al.* (2002). This involves the use of multiple reference genes to increase the accuracy and reliability of relative quantitation. They studied 10 different reference genes across a range of tissues, and assessed the constancy with which each was expressed. As expected, they demonstrated that the conventional use of a single gene for normalisation could lead to relatively large errors in a significant proportion of samples tested. However, by taking the geometric mean of multiple carefully selected reference genes, they were able to calculate an accurate normalisation factor. For these reasons, we initially selected three reference genes from the panel tested by Vandesompele *et al.* (2002): HPRT1 (Hypoxanthine Phosphoribosyl-Transferase-I); PBGD (Porphobilinogen Deaminase) and TBP (TATA-box Binding Protein). These three reference genes were included in every PCR performed for the work in this thesis. For some experiments (chapter 2), GAPDH or SDHA (succinate dehydrogenase complex, subunit A) were also included.

Primers for each of these reference genes were obtained and optimised (see Primer selection and optimisation, section 3.2.3). Further analysis of results from qRT-PCR enabled refinement of the selection. PBGD expression sometimes varied markedly from sample to sample, particularly following exposure to chemotherapeutic agents. Thus PBGD was eventually excluded from the data analysis, and the geometric mean of the remaining two reference genes was used as a normalisation factor (see data analysis, section 3.2.5).

3.3.3 Use of SYBR Green vs TaqMan™ probes

In qRT-PCR, target RNA is measured by the fluorescence of a DNA-binding dye or a sequence-specific fluorescence-labelled probe. Both methods were considered during the early stages of the experiments described in this thesis, and in particular the use of the SYBR Green intercalating dye or TaqMan™ fluorogenic labelled probes (chapter 2) was evaluated.

Each of these methods has its advantages and disadvantages. The SYBR Green method does not require the use of labelled probes, so assay set-up and running costs are

reduced. Because it is non-specific, it measures the amplification of any double-stranded DNA sequence. However, a TaqMan™ labelled probe only allows for specific hybridisation between the target sequence and the probe, which increases the specificity of the reaction and eliminates the requirement for post-PCR analysis and sequencing of the product. A further advantage of TaqMan™ methodology is the ability to label probes with different, distinguishable dyes, allowing amplification and measurement of two or more distinct target sequences in one reaction tube (multiplex PCR). The primary disadvantage of TaqMan™ methodology is that a different probe is required for the detection of each target sequence. Also, TaqMan™ is less sensitive than SYBR Green; this is because multiple DNA binding dyes can bind to a single amplicon in direct proportion to its length, whereas with a fluorogenic probe a single reporter dye is released from quenching for each amplicon synthesised, whatever its length.

Although the lack of specificity of SYBR Green represents a drawback, at the same time it has also the advantage of detecting non-specific amplification products, which can seriously affect the accuracy of measurements made using TaqMan probes. At the end of a fixed number of cycles of qRT-PCR, the reaction mixture contains a quantity of amplified cDNA product. If an intercalating dye such as SYBR Green is the detection dye, then a melt curve can be constructed. The thermal cycler is programmed to cool the reaction mixture to 50°C, which allows annealing of all complementary cDNA strands to each other; SYBR Green binds to double-stranded DNA and fluoresces, so at 50°C the SYBR Green fluoresces in proportion to its concentration. The temperature of the mixture is then raised by 1°C every 10 seconds, and the fluorescence measured again. As the temperature increases, there comes a point at which the cDNA strands start to separate; strand separation prevents binding of SYBR Green dye, and the fluorescence reduces in proportion. The point at which 50% of strands are separated is the melting temperature (T_m), and at around this temperature there is a marked reduction in fluorescence as there is a high rate of strand separation. The temperature is raised in stepwise fashion to 95°C, by which time all strands will have separated, so fluorescence will be almost zero. A graph is then plotted of the first differential of fluorescence *versus* temperature; this gives the rate of change in fluorescence. A peak in this curve represents a temperature at which marked strand separation is occurring; usually, the peak representing the target amplicon will be at the predicted T_m of the PCR product. If there are other peaks in the melt curve, then other, non-specific sequences were also

amplified in the reaction mixture. Thus the melt curve gives useful information about the specificity of the qRT-PCR reaction. Melt curves cannot be obtained using fluorogenic-labelled probes, because separation of the reporter dye from the TaqMan™ probe is an irreversible reaction.

The experiments in this thesis were performed using SYBR Green as the fluorogenic probe; TaqMan™ probes were used occasionally due to their specificity, mainly as a means of confirming the identity of the product amplified by a primer pair.

3.3.4 Primer selection and optimisation

Primer design was carried out using one of a number of computerised primer-design programs; Beacon designer 2.0 demo version (Primer BioSoft, Ca, USA) and an old version of the software Primer 3.0, (available at the following website: <http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/>) were used for the development of primers in this thesis. The peer-reviewed literature was another source of primer sequences (Chapter 2, table 2.6). The set parameters for primer design were: length between 18-25 bases; similar annealing temperature, usually between 55°C and 60°C; content of guanine (G) and cytosine (C) between 40% and 60%. The size of the amplified target sequence was usually 70-200 base pairs, although longer amplicons have occasionally been used (Chapter 2, table 2.6). Whenever possible, primers were designed to cross exon boundaries; this makes the primers specific for cDNA rather than also annealing to genomic DNA. This was done by forcing the primer design software to find suitable primers in a specified region of the target sequence.

Once primers were designed, they were checked against the entire human DNA genome, using the BLAST computer program. If marked homology existed between the primers or the target amplicon and more than one site in the genome, this suggested a lack of specificity, and new primers were designed.

For each target, at least two primer pairs were designed, obtained and tested against pooled cDNA from a variety of human tumours, including breast, ovarian, colorectal and oesophageal carcinoma. Analysis of the peer-reviewed literature suggested that each of the target genes (table 2.6) might be reasonably expected to appear in each of

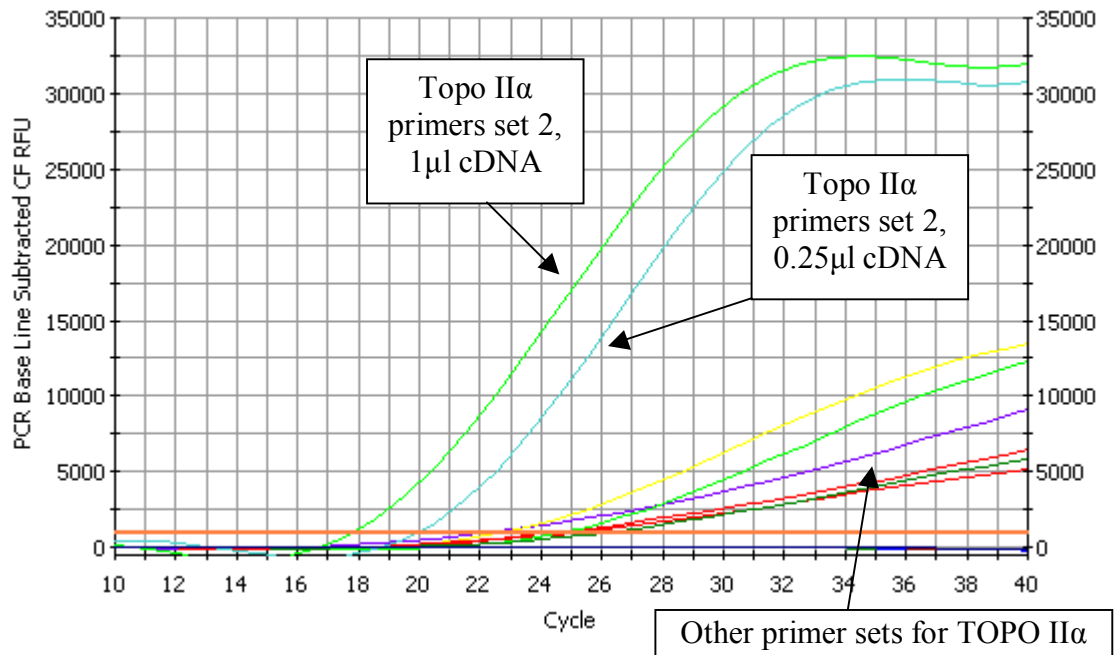
these tissues, and the use of pooled cDNA from different tissue types made this assumption even more likely.

Salt-free purified primers were purchased from Qiagen Operon (Cologne, Germany) and reconstituted with DNase free water to give a 100 μM solution, which was stored at -80°C . Prior to experiments, primers were diluted to 1:10, usually mixing 15 μl of the 100 μM solution with 135 μl DNase free water. The resulting diluted primers (10 μM) were stored at 4°C for a maximum of 2 weeks, after which they were discarded and fresh dilutions were prepared from the stock solution.

New primers were initially tested at a final concentration of 400nM in the PCR, which also contained 4.0mM MgCl_2 , 200 μM each of dATP, dCTP, dGTP, 400 μM dUTP, 0.125 units AMPErase® uracil-N-glycosylase (UNG), 0.625 units of AmpliTaq Gold DNA polymerase and 1x SYBR Green PCR buffer (SYBR Green PCR Core Reagents, Applied Biosystems, P/N 4304886). For each new pair of primers four reactions were performed, two wells containing respectively 1 μl and 0.25 μl cDNA (previously diluted), and two negative control wells containing water or RT-negative as a template. Due to the exponential amplification of DNA during the PCR, the tested 1:4 dilution should theoretically be detected 2 cycles later than the concentrated sample, while no fluorescence indicating the absence of non-specific products should be produced in the negative controls.

As an example, four different pairs of primers were designed for TOPO II α and tested as described above.

(a)



(b)

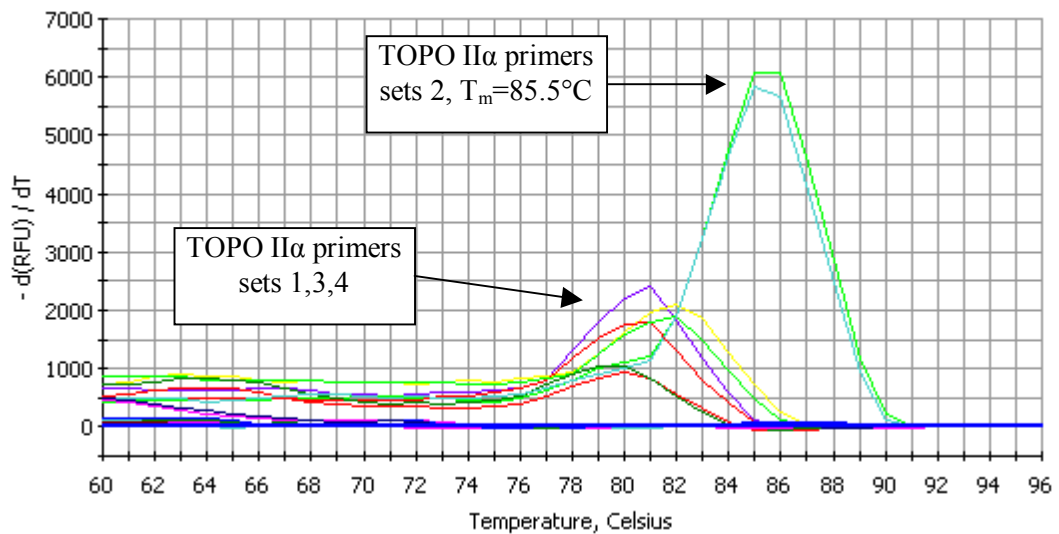


Figure 3.5 (a) Amplification curve of new primer pairs for TOPO II α . The presence of an amplification curve indicates that the primers have detected the target; the slope of the curve is an indication of the efficiency of amplification. (b) The shape of the melt curve demonstrates specificity of the primer pair, while the peak of the melt curve indicates the T_m of the product, which should correlate with that predicted by the design program.

Table 3.3 Threshold cycle (Ct) values obtained during the evaluation of four different primer pairs for TOPO II α . N/A indicates no amplification.

Primers for TOPO II α	c-DNA 1 μ l	c-DNA 0.25 μ l	Negative (water)	RT-negative
Set 1	22.9	25.0	N/A	N/A
Set 2	18.3	20.3	N/A	N/A
Set 3	22.1	24.1	N/A	N/A
Set 4	25.6	26.1	N/A	N/A

Of the four set of primers tested for TOPO II α , only one pair was selected, set 2, which amplified the target sequence earlier and more efficiently than the others (table 3.3; figure 3.5). None of the primer pairs designed for TOPO II α resulted in primer-primer interactions as shown by single peaks measured during the melt curve analysis (figure 3.5 (b)). However, some of the primers designed for other genes were discarded because of the formation of non specific products detected by a typical double-peak in the melt curve.

The selected primer pairs for each target were then tested in other experiments to find their optimal concentration for the amplification reaction. This was done concurrently with other experiments aimed at determining the optimal Mg²⁺ concentration.

Mg²⁺ concentration is a crucial factor affecting the performance of PCR, because DNA polymerase is inactive in the absence of adequate Mg²⁺. Many of the reaction components can bind free magnesium, reducing the amount of free Mg²⁺; however, excess free Mg²⁺ decreases DNA polymerase fidelity and increases the level of non-specific amplification. Thus there is an optimal Mg²⁺ concentration for each primer pair.

The primer concentrations are also critical for the optimum efficiency of qRT-PCR. Inadequate primer concentration leads to low efficiency reactions, and a certain excess of primers is required for optimum efficiency. However, too high a concentration increases the incidence of non-specific annealing and primer-primer interactions, reducing the accuracy of the system.

For each functioning primer pair, a qRT-PCR experiment was performed to find the optimum Mg^{2+} concentration and the optimum concentration of each complementary primer. This was done using a checkerboard design, in which 3 different concentrations of each variable were tested in every possible combination, using the same known quantity of pooled cDNA in each reaction well (table 3.4).

Table 3.4. Plate layout for the optimisation of primers. Each forward (+) and reverse (-) primers were tested at 100nM, 200nM or 400nM with 3 different concentrations of Mg^{2+} .

	1	2	3	4	5	6	7	8	9	10
	3mM Mg^{2+}			4mM Mg^{2+}			5mM Mg^{2+}			
A	100nM + 100 nM -	100nM + 200nM -	100nM + 400nM -	100nM + 100 nM -	100nM + 200nM -	100nM + 400nM -	100nM + 100 nM -	100nM + 200nM -	100nM + 400nM -	-ve control
B	200nM + 100 nM -	200nM + 200nM -	200nM + 400nM -	200nM + 100 nM -	200nM + 200nM -	200nM + 400nM -	200nM + 100 nM -	200nM + 200nM -	200nM + 400nM -	-ve control
C	400nM + 100 nM -	400nM + 200nM -	400nM + 400nM -	400nM + 100 nM -	400nM + 200nM -	400nM + 400nM -	400nM + 100 nM -	400nM + 200nM -	400nM + 400nM -	

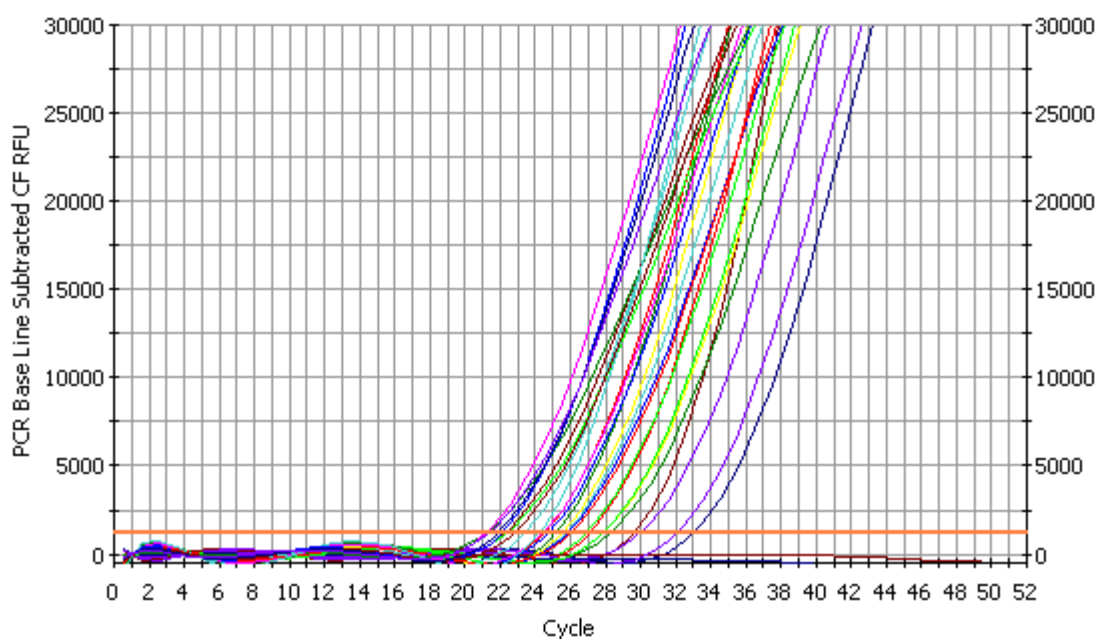


Figure 3.6 Amplification plot recorded during the optimisation of TOPO II α primers. There is considerable variation in Ct for each of these options, and the checkerboard analysis allows the best to be chosen. The inset shows the corresponding melt curve.

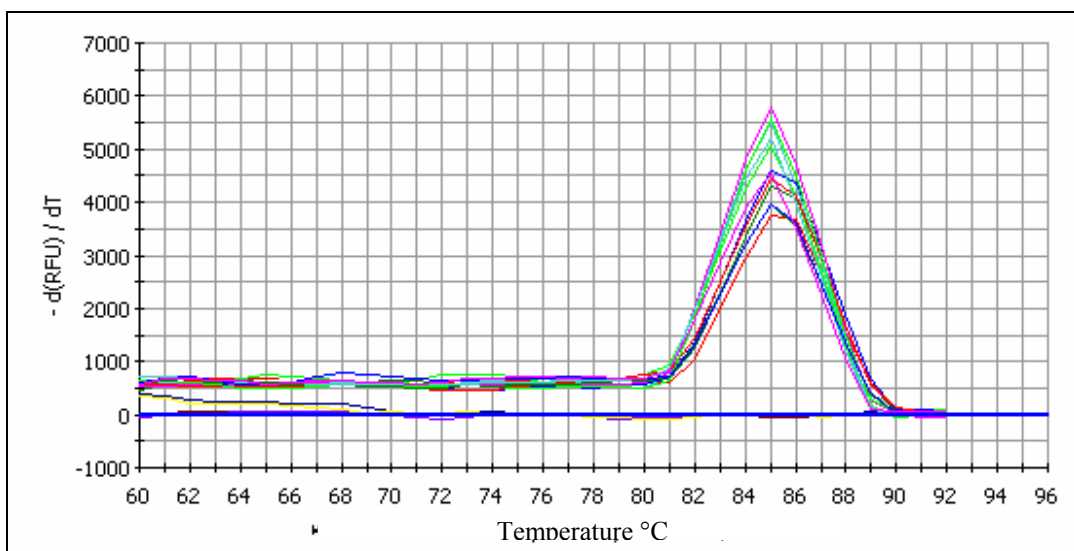


Figure 3.7 Melt curve recorded during the optimisation of TOPO II α primers.

The PCR amplification plot and the melt curves (figures 3.6 and 3.7) were examined to determine which combination of primer concentrations and Mg²⁺ concentration demonstrated the highest efficiency. Figures 3.6 and table 3.5 show the amplification plot and the Ct results from one of such experiments using TOPO II α primers set 2. It was concluded that a Mg²⁺ concentration of 3 mM, with 5'- and 3'-primer concentrations of 400 nM, had the highest efficiency in this system.

Table 3.5 C_t values of the TOPO II α primer optimisation experiment performed according the plate layout shown in table 3.4. An optimal concentration of 3 mM was chosen for Mg²⁺ and both primers were used at a final concentration of 400 nM in all following assays.

	1	2	3	4	5	6	7	8	9	10
	3mM Mg ²⁺			4mM Mg ²⁺			5mM Mg ²⁺			
A	29.6	33.0	30.0	27.9	27.1	26.9	28.3	25.8	25.8	N/A
B	32	25.5	24.5	27.8	25.2	23.4	24.7	24.5	22.2	N/A
C	25.9	22.4	21.2	24.2	22.1	21.2	22.8	21.8	21.4	

3.3.5 Construction of a standard curve for each primer pair

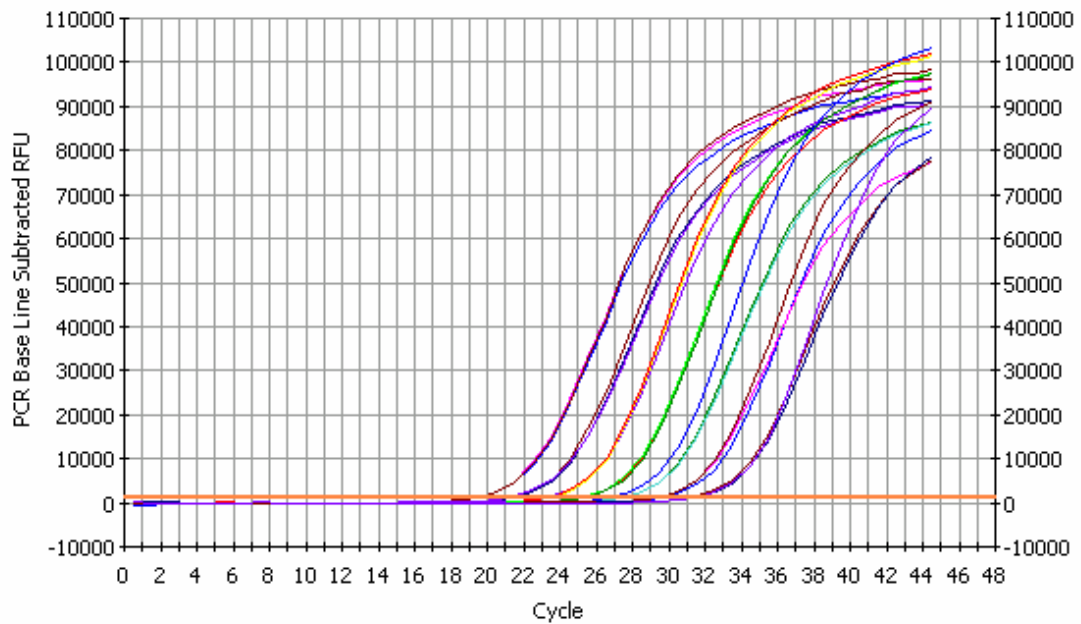
Once the optimum reaction conditions for each amplification have been established, the efficiency of the primer pair must be calculated. This is done by making a series of dilutions of pooled cDNA, and testing them under the optimised reaction conditions. The resulting amplification curves yield a series of Cts that are plotted against the logarithm of the pooled cDNA concentration to give a standard curve for that particular primer pair. The ideal primer pair, with an efficiency of 100%, would lead to a doubling of the amplicon quantity during each PCR cycle; application of a mathematical formula to the slope of the standard curve yields a value for the efficiency of the primer pair.

Table 3.6 shows the PCR plate layout for an experiment to determine the efficiency of TOPO II α primers; figure 3.8 (a) shows the resulting amplification plot, and figure 3.8 (b) is a graph of the resulting standard curve. The slope of the curve is -3.333, which equates to an efficiency of 99.6% for this primer pair across a 4,000-fold dilution series.

Table 3.6 PCR plate layout employed when primers calibration curves were performed.

	1	2	3	4	5	6	7	8
A	1 μ l	1:4	1:16	1:64	1:256	1:1024	1:4096	-ve
B	1 μ l	1:4	1:16	1:64	1:256	1:1024	1:4096	-ve
C	1 μ l	1:4	1:16	1:64	1:256	1:1024	1:4096	

(a)



(b)

Correlation Coefficient: 0.999 Slope: -3.333 Intercept: 19.014 $Y = -3.333 X + 19.014$
PCR Efficiency: 99.6 %

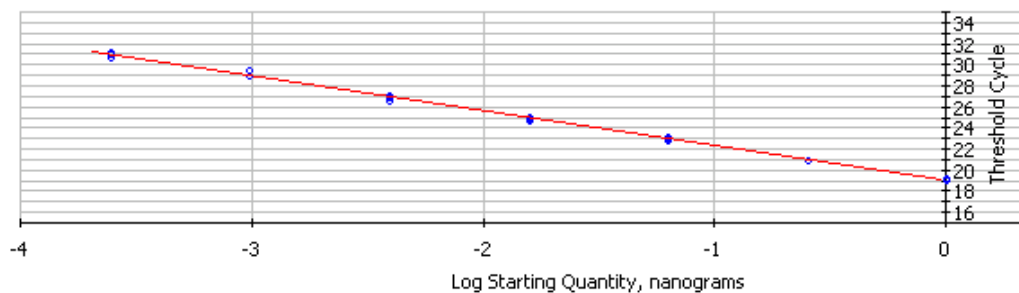


Figure 3.8 (a) Amplification curve recorded during the amplification of 1:4 serial dilutions of 1 μ l c-DNA using TOPO II α primers. (b) A calibration curve in generated by a plot of the Ct values *versus* the amount of c-DNA (on a logarithmic scale).

Standard curves were generated for all the genes studied in this thesis (table 2.6) and the following efficiencies have been calculated: GAPDH 99%; HPRT1 98%; PBGD 101%; SDHA 100%; TBP 98%; BCRP 101%; COX-2 97%; DPD 100%; EGFR 100%; ERCC-1 101%; GST-pi 102%; MDR1 100%; MLH-1 106%; MRP1 99%; MRP2 95%; MTII 98%; MVP 97%; TOPO I 100%; TOPO II α 100%; TOPO II β 102%; TP 96%; TS 95%.

3.3.6 Intra-assay and inter-assay variability

All qRT-PCR in this thesis were carried out in triplicate. This allows detection of the majority of pipetting errors and malfunctions in a single well in the qRT-PCR machine. For data analysis, the geometric mean value of the triplicate measurements was taken.

Tests of intra-assay and inter-assay consistency are an essential control to confirm the reliability and accuracy of qRT-PCR for the quantitation of mRNA expression. The aim of the intra-assay control was to confirm accuracy of pipetting of the microlitre volumes involved and to confirm consistency of measurement between wells in the PCR machine. The aim of the inter-assay control is to confirm reproducibility from day to day. Combined, these controls validate the technique for the reliable, accurate and reproducible measurement of mRNA expression on different samples on different days, as long as consistent conditions are employed throughout.

The qRT-PCR plate layout used for these experiments is shown in table 3.7. All 96 wells of a plate were used. Twenty-one replicates of 4 reference genes were used as the targets, with equal concentrations of primers in each well. The remaining wells of the plate were used as template-negative controls.

Table 3.8 shows the results of these experiments. Exactly the same experiment was performed on 3 different days, and statistical estimates of intra-assay and inter-assay variability were calculated. There is minimal variation between points within each experiment. This indicates that taking the mean value of 3 triplicate measurements in experiments using tumour cells is an appropriately precise means of obtaining an accurate reading of mRNA expression in this system.

	1	2	3	4	5	6	7	8	9	10	11	12
A	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
B	GAPDH	GAPDH	GAPDH	HPRT1	HPRT1	HPRT1	PBGD	PBGD	PBGD	TBP	TBP	TBP
C	GAPDH	GAPDH	GAPDH	HPRT1	HPRT1	HPRT1	PBGD	PBGD	PBGD	TBP	TBP	TBP
D	GAPDH	GAPDH	GAPDH	HPRT1	HPRT1	HPRT1	PBGD	PBGD	PBGD	TBP	TBP	TBP
E	GAPDH	GAPDH	GAPDH	HPRT1	HPRT1	HPRT1	PBGD	PBGD	PBGD	TBP	TBP	TBP
F	GAPDH	GAPDH	GAPDH	HPRT1	HPRT1	HPRT1	PBGD	PBGD	PBGD	TBP	TBP	TBP
G	GAPDH	GAPDH	GAPDH	HPRT1	HPRT1	HPRT1	PBGD	PBGD	PBGD	TBP	TBP	TBP
H	GAPDH	GAPDH	GAPDH	HPRT1	HPRT1	HPRT1	PBGD	PBGD	PBGD	TBP	TBP	TBP

Table 3.8 Intra-assay variability was estimated by measuring the expression levels of each reference gene in 21 reactions. Inter-assay variability was assessed by 3 repeats of each experiment performed on 3 different days. The parameter ΔC_t indicates the difference in threshold cycles between the C_t of GAPDH and the C_t of the other reference genes.

		GAPDH	HPRT1	PBGD	TBP
Exp 1	Mean	22.7	29.2	31.0	30.9
	SD	0.19	0.31	0.44	0.29
	CoV	0.85%	1.0%	1.4%	0.94%
	ΔC_t	0.00	6.52	8.34	8.19
Exp 2	Mean	22.5	29.0	30.8	30.7
	SD	0.13	0.34	0.29	0.33
	CoV	0.57%	1.2%	0.96%	1.1%
	ΔC_t	0.00	6.59	8.29	8.21
Exp 3	Mean	22.4	29.0	30.9	30.8
	SD	0.16	0.32	0.40	0.36
	CoV	0.70%	1.12%	1.29%	1.16%
	ΔC_t	0.00	6.60	8.53	8.40

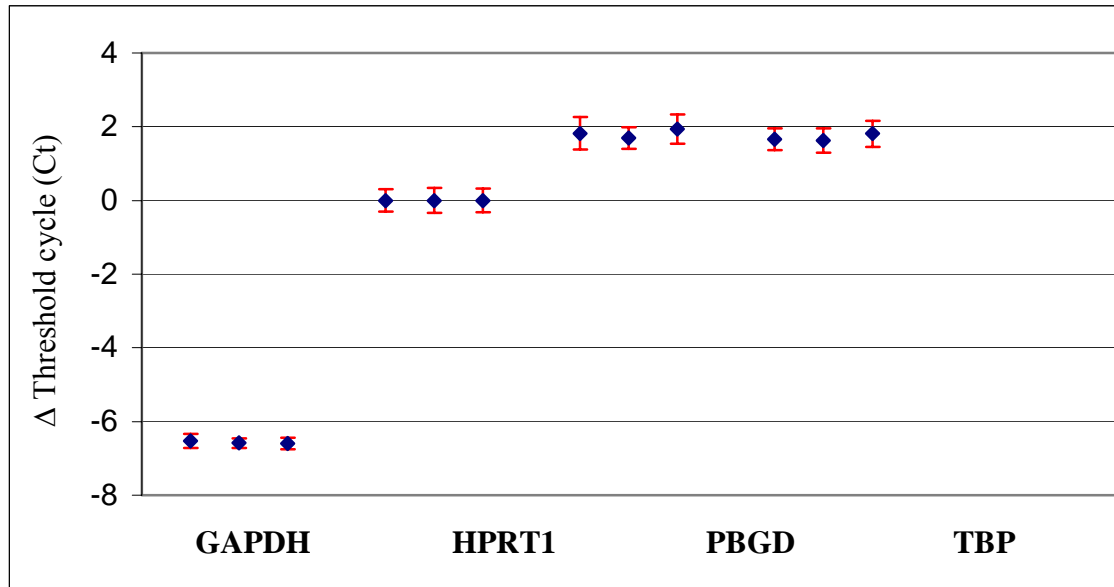


Figure 3.9 Intra- and inter-assay variability of qPCR. Each of the points represents the mean of 63 reactions performed in 3 different days; the error bars represent 2 standard deviations on each side. The parameter on the y axis represents the difference in Ct between HPRT1 and the other reference genes.

3.3.7 Data analysis

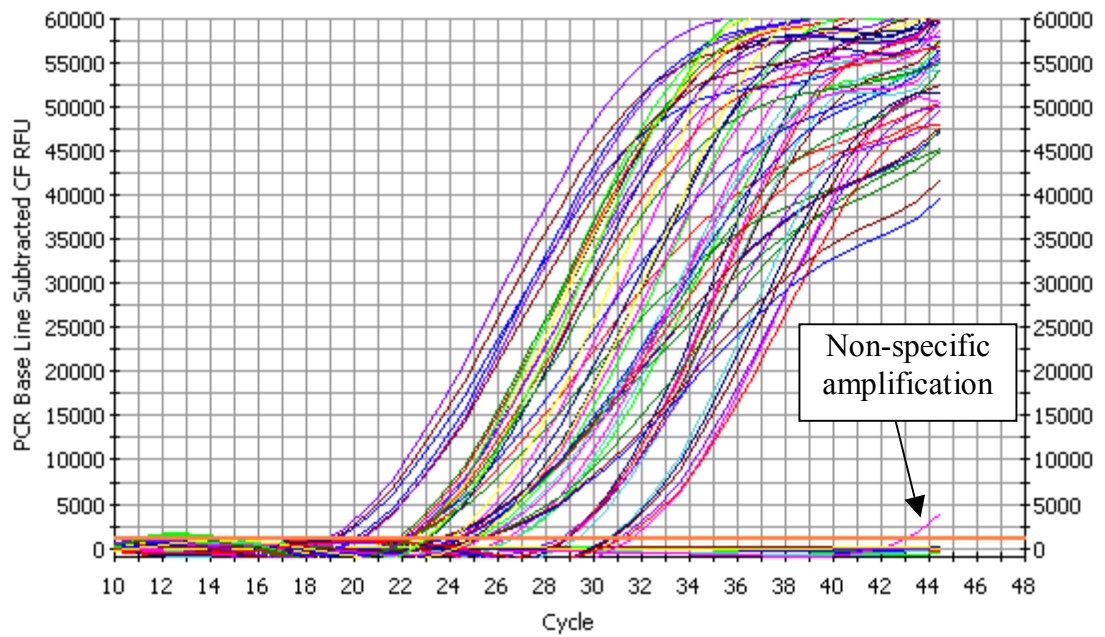
The technique of qRT-PCR is relatively new, and there are not yet any universally recognised methods of analysing the data, nor have tests of statistical significance been appropriately applied in the field. The peer-reviewed literature includes a number of papers that attempt to use qRT-PCR data to compare gene expression between multiple samples; some of the methods used are questionable, and none include appropriate statistical analysis of the results. Therefore, in order to use the data generated by the experiments in this thesis, an in-house method of analysis was developed.

Before any data analysis was performed, the PCR amplification curves were inspected to confirm that the traces for the reference and target genes appeared as expected. The melt curves were also inspected to ensure that there was no non-specific amplification and that the T_m of each amplicon corresponded to that predicted by the primer design software. Figure 3.10 shows the traces of a single experiment that compared gene expression in breast tumour derived cells exposed to medium only (control) or doxorubicin. The plate layout has been shown previously in chapter 2 (figure 2.3, panel a). The amplification plot demonstrates non-specific amplification or possible minimal

contamination in one of the 24 negative control wells, and in particular the RT negative control for the MRP1 gene; however this signal appeared about 20 cycles later than the corresponding MRP1 traces in the sample wells (figure 3.11); moreover, the second negative control for this gene, the PCR negative (containing all the reagents, including primers and DNA polymerase, except cDNA) did not produce any trace, and therefore the results relative to MRP1 in this instance were still considered evaluable.

The final output of the BioRad iCycler PCR machine is a series of 96 numbers, accurate to 1 decimal place, corresponding to the Ct of the sample of each individual well. A template worksheet was created in Excel 2000 (Microsoft) to speed up the analysis of the qPCR data (figure 3.11). The PCR results can be copied directly from the PCR machine analysis software into the Excel template, which calculates the mean, the standard deviation and the coefficient of variation for each set of triplicate values.

(a)



(b)

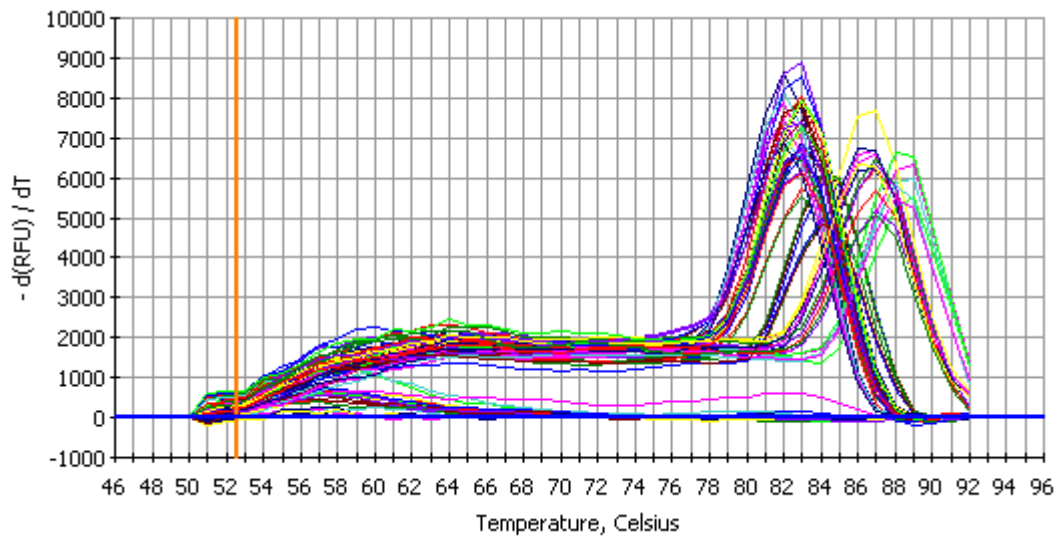


Figure 3.10 (a) Amplification plot and (b) melt curve analysis of a single 96-well experiment that compared gene expression in breast tumour derived cells exposed to medium only (control) or doxorubicin.

										TOPO	TOPO	TOPO
	HPRT1	PBGD	TBP	BCRP	COX-2	ERCC1	MDR1	MRP1	MVP	I	IIA	IIB
	1	2	3	4	5	6	7	8	9	10	11	12
Control TCA cells	22.2	26.7	23.8	29.2	19.3	24.3	28.7	22.2	21.9	19.4	22.7	19.8
	22.3	26.9	24	28.8	19	24.6	28.5	22.1	21.8	19	22.6	19.7
	21.9	26.2	23.9	28.7	18.8	23.9	28.7	22.2	21.6	19	22.2	19.6
primers	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
RT neg	N/A	N/A	N/A	N/A	N/A	N/A	N/A	43.1	N/A	N/A	N/A	N/A
Doxo TCA cells	24.4	24.8	25.3	30.7	19.7	25	30.9	22.8	21.8	22.1	30.1	22
	24.5	25.3	25.6	31.9	20	24.7	31.4	22.7	22	22.2	30.6	22.1
	24.6	25.5	25.7	30.8	20	25	31.5	22.8	22.1	22.3	30.6	22.3

Control	22.13	26.60	23.90									
	0.21	0.36	0.10									
	0.94%	1.36%	0.42%									
Mean	24.21			28.90	19.03	24.27	28.63	22.17	21.77	19.13	22.50	19.70
SD				0.26	0.25	0.35	0.12	0.06	0.15	0.23	0.26	0.10
CoV				0.92%	1.32%	1.45%	0.40%	0.26%	0.70%	1.21%	1.18%	0.51%

Doxo	24.50	25.20	25.53									
	0.10	0.36	0.21									
	0.41%	1.43%	0.82%									
Mean	25.08			31.13	19.90	24.90	31.27	22.77	21.97	22.20	30.43	22.13
SD				0.67	0.17	0.17	0.32	0.06	0.15	0.10	0.29	0.15
CoV				2.14%	0.87%	0.70%	1.03%	0.25%	0.70%	0.45%	0.95%	0.69%

Calculations

$\Delta Ct = Ct \text{ target} - Ct [(HPRT1+PBGD+TBP)/3]$												
$\Delta\Delta Ct = \Delta Ct [\text{Doxo}] - \Delta Ct [\text{Control}]$												
Control	0.00			4.69	-5.18	0.06	4.42	-2.04	-2.44	-5.08	-1.71	-4.51
Doxo	0.00			6.06	-5.18	-0.18	6.19	-2.31	-3.11	-2.88	5.36	-2.94
$\Delta\Delta Ct$	0.00			1.37	0.00	-0.23	1.77	-0.27	-0.67	2.20	7.07	1.57
$2^{-\Delta\Delta Ct}$	1			0.39	1.00	1.18	0.29	1.20	1.59	0.22	0.01	0.34

Figure 3.11 Template worksheet for qPCR data analysis. The data generated from the reaction shown in figure 3.10 were copied into an Excel 2000 spreadsheet which calculated the mean, SD and CoV for each set of triplicate values.

The second step in data analysis was the inspection of each triplicate in turn. If the coefficient of variation (CoV) of the mean of a triplicate was greater than 1%, the raw data were examined for outliers; this allowed either exclusion of an outlier or acceptance of a CoV of greater than 1% for that particular triplicate. For instance, in the example shown in figure 3.11, one of the replicate wells for BCRP is judged to be an outlier and excluded from the final analysis.

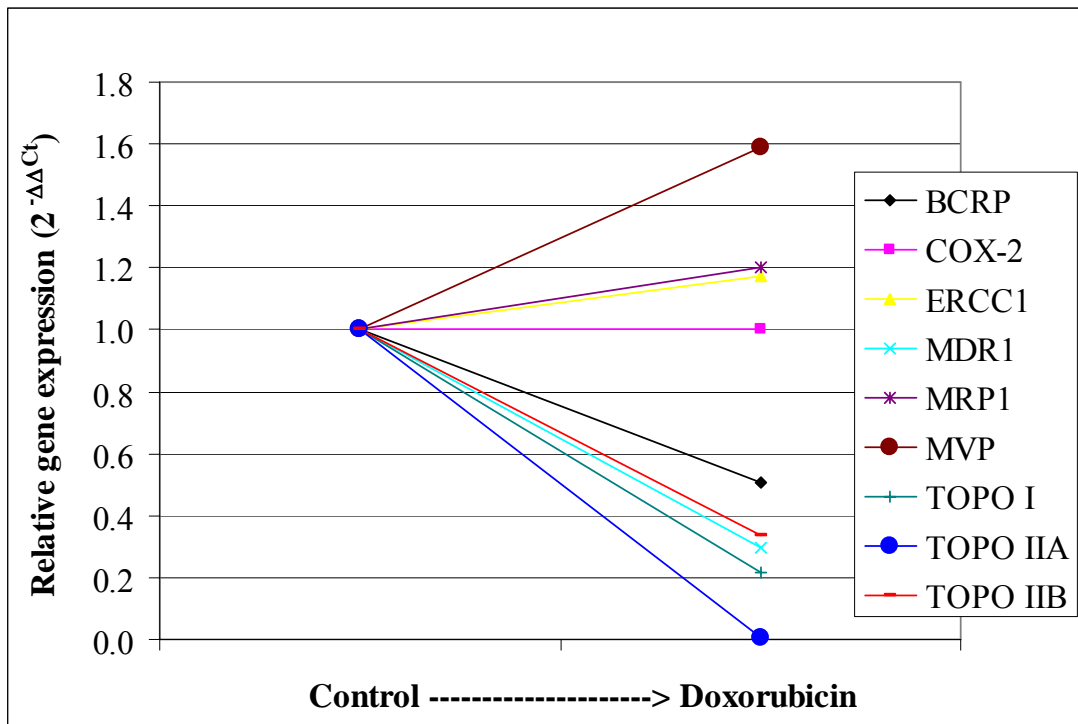
Next, the mean Ct of the reference genes was calculated and normalised, and the mean Ct of each target gene was subtracted from this to give a ΔCt for each target gene (figure 3.11). This is a measure of the expression of each target gene relative to that of the reference gene, and can be represented graphically for each sample. Finally, the relative target gene expression in drug treated cells was calculated according to the equation $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}$ of drug exposed cells $-\Delta\text{Ct}$ of control cells (exposed to medium only).

These relative values can then be represented graphically. There is currently no established way of representing real time PCR data graphically. While some published studies represented simple ΔCt values (van den Heuvel-Eibrink *et al.*, 2002), others used undefined arbitrary units (Burger *et al.*, 2003) and many preferred a numerical description. As the comparative Ct method is used for the determination of relative gene expression, no absolute units can be given on the Y axis, and this is probably the reason underlying the lack of a conventional graphical representation of real time PCR data.

In figure 3.12 two graphics have been drawn to present the results of the reaction shown in figures 3.10 and 3.11. These graphics show the actual $2^{-\Delta\Delta\text{Ct}}$ values, respectively plotted on a linear (a) or a Log10 scale (b).

In this thesis the results of the PCR assays that measured gene expression changes induced by cytotoxic drug treatment in each individual sample are represented by plotting the actual $2^{-\Delta\Delta\text{Ct}}$ values on a logarithmic scale, as shown in figure 3.12-b.

(a)



(b)

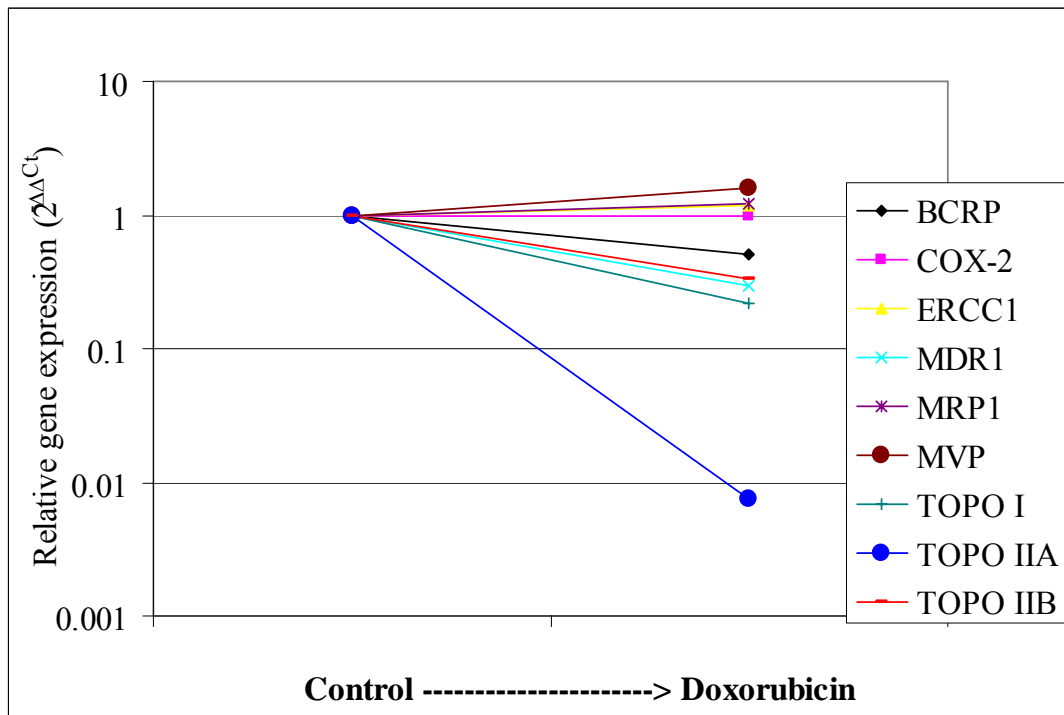


Figure 3.12 Graphic representations of $\Delta\Delta C_t$ values calculated in the worksheet shown in figure 3.11. Both graphs show the actual $2^{-\Delta\Delta C_t}$ values, plotted either on a linear (a) or on a logarithmic scale (b).

When drug-induced changes are studied in more than one sample, the analysis can be more complicated. In most published studies, the measurement of gene expression levels among different samples is performed within one or few experiments. This is because most studies have investigated one or two target genes in reference to a single house-keeping gene, and therefore several samples could be processed in a handful of reactions. For the work described in this thesis only one sample was tested at any one time to obtain a molecular profile for multiple gene changes, rather than a single target. This fact, combined with the demonstration that the conventional use of a single gene for normalisation in relative real time PCR could lead to relatively large errors in a significant proportion of samples tested (Vandesompele *et al.*, 2002), persuaded us to use up to four house-keeping genes in any single reaction. To further increase the accuracy and reliability of relative quantitation, we decided to analyse the changes induced by anticancer drug exposure on housekeeping gene expression levels. During the course of the experiments it was noted that cytotoxic exposure did occasionally affect the expression of some reference genes. The reference gene most commonly affected by chemotherapeutic agents appeared to be PGBD, but some mathematical basis was required to confirm this. The literature contains no such search for the most reliable reference genes, the majority of papers simply rely on GAPDH or another gene, without validating its constancy in their system. This renders their results questionable.

The procedure employed to validate the use of housekeeping genes in tumour-derived cells exposed to doxorubicin is described below.

At the end of the PCR experiments shown in this thesis, all the results were entered into an Access 2000 database for further analysis. A list of all the mean Ct values of the house-keeping genes was obtained and a plot of these values recorded in 14 breast samples was drawn (figure 3.13; a). If the expression levels of each housekeeping gene in different tumour samples were constant, this plot would show three parallel lines. In the case of the 14 breast tumours analysed the yellow line indicating PGBD was not parallel to the other two housekeeping genes in at least two samples. Another graph was drawn, this time comparing the Ct values of control cells versus the Ct values of drug exposed cells for each individual housekeeping gene (figure 3.13; b). The data was then normalised to the control cells and the graph re-plotted (figure 3.13; c). Finally, the

mean change in reference gene expression was calculated and plotted in a separate graph (figure 3.13; d), where the error bars represent 2 standard deviations on each side.

This demonstrates that although the expression of reference genes is sometimes affected by exposure to doxorubicin, HPRT1 and TBP exhibit less variation with respect to each other. Use of the mean value of HPRT1 and TBP as the normalising factor will minimise the errors introduced by inconsistency of the reference genes.

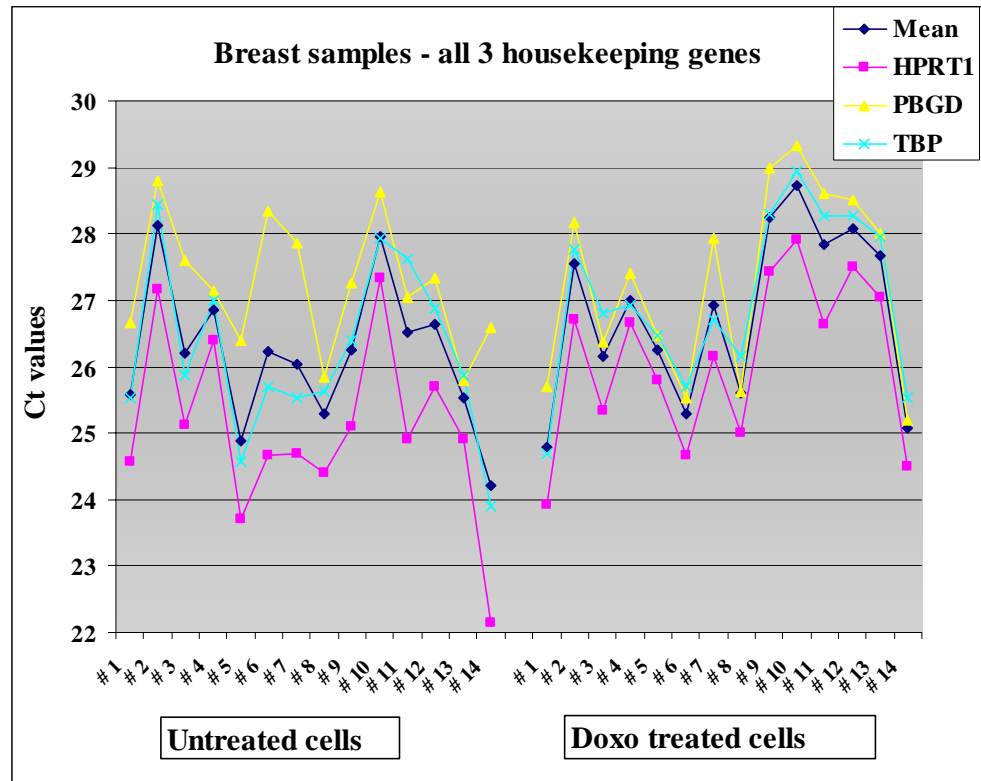
Similar plots of variation in housekeeping gene expression were obtained for the other cytotoxics. Table 3.9 lists the reference genes that produced the least variation for each drug in each tumour type; mean values (± 2 SD) of the differences in the housekeeping gene expression between control and drug treated cells are also given.

Table 3.9 Housekeeping genes selected for the analysis of gene expression in tumour derived cells from different human cancers.

Drug	Tissue (n= number of samples)	Selected HK genes	Variation Mean ± 2 SD	Discarded HK gene(s)
5-FU	Breast (n=13)	HPRT1 & TBP	-0.29 \pm 1.35	GAPDH, PBGD
5-FU	Colorectal (n=10)	HPRT1 & PBGD	-0.17 \pm 1.00	TBP
Doxorubicin	Breast (n=14)	HPRT1 & TBP	0.04 \pm 0.89	GAPDH
Doxorubicin	Ovarian (n=12)	HPRT1 & TBP	-0.32 \pm 0.86	PBGD
ECF(*)	Oesophageal (n=12)	HPRT1 & TBP	0.53 \pm 2.94	PBGD
Irinotecan	Colorectal (n=7)	HPRT1 & TBP	-0.31 \pm 0.58	PBGD
Topotecan	Ovarian (n=10)	SDHA & TBP	-0.18 \pm 0.98	HPRT1, TBP

(*) ECF chemotherapy was administered in oesophageal cancer patients and 12 paired biopsies - 6 before and 6 after treatment - were obtained from 6 patients.

(a)



(b)

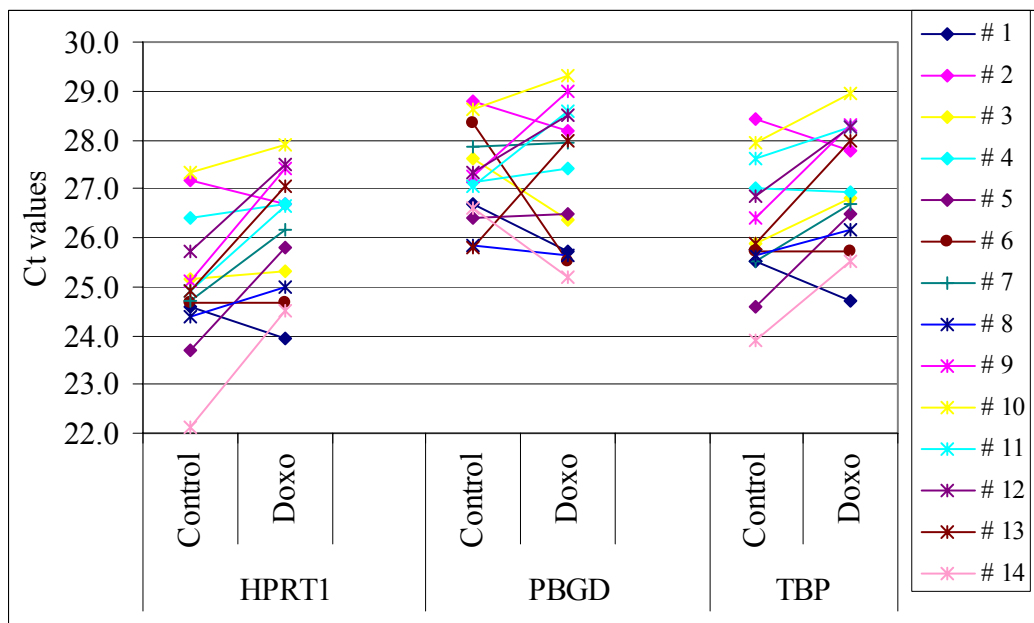


Figure 3.13 Analysis of house keeping gene expression levels in breast cancer cells exposed to doxorubicin in the ATP-TCA. The combination of HPRT1 and TBP was more consistently expressed than the other two. Panel d) shows the mean values ($\pm 2SD$) of the differences in the housekeeping gene expression between control and doxorubicin treated cells.

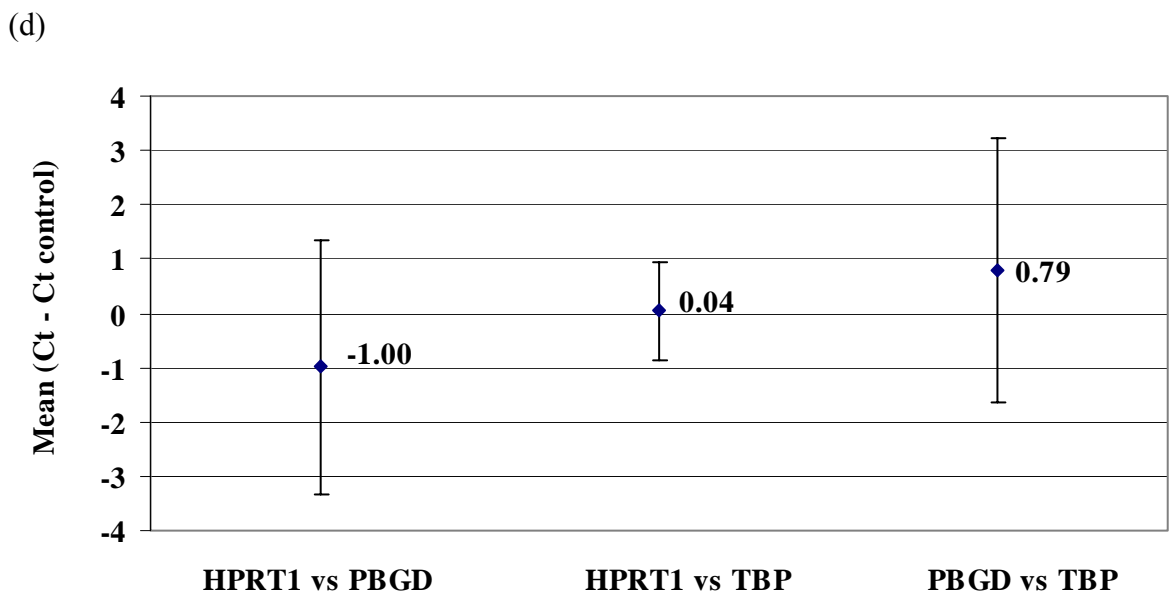
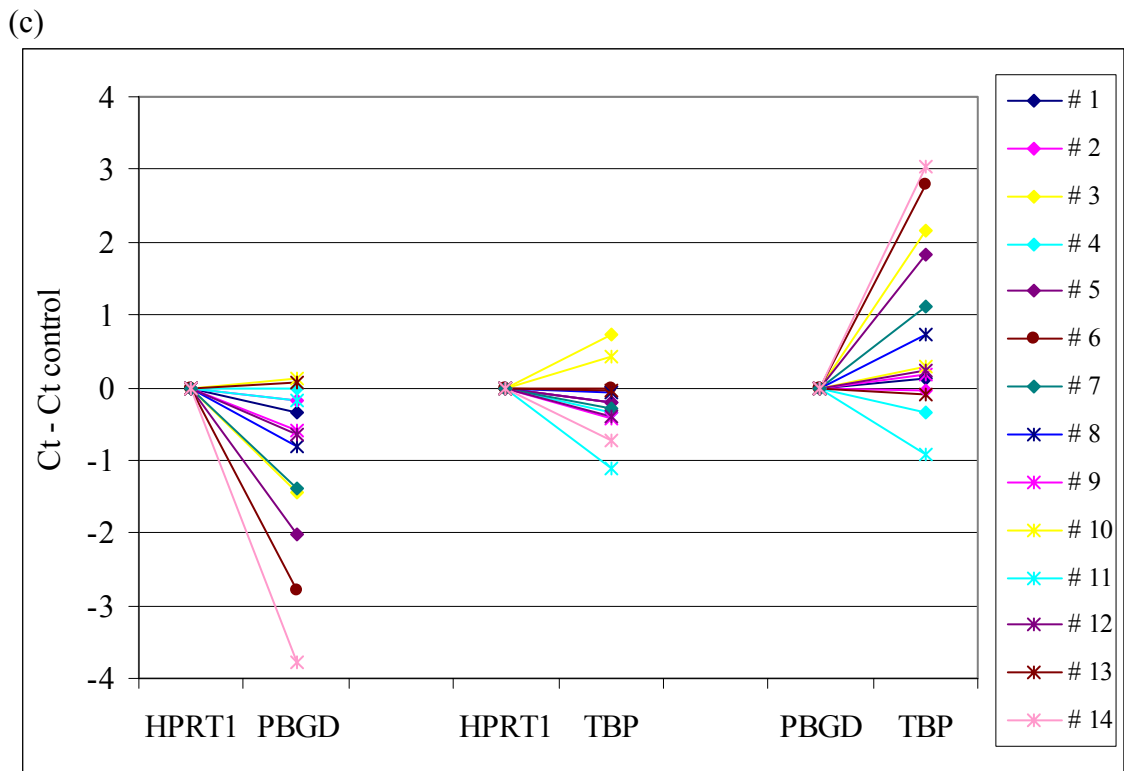


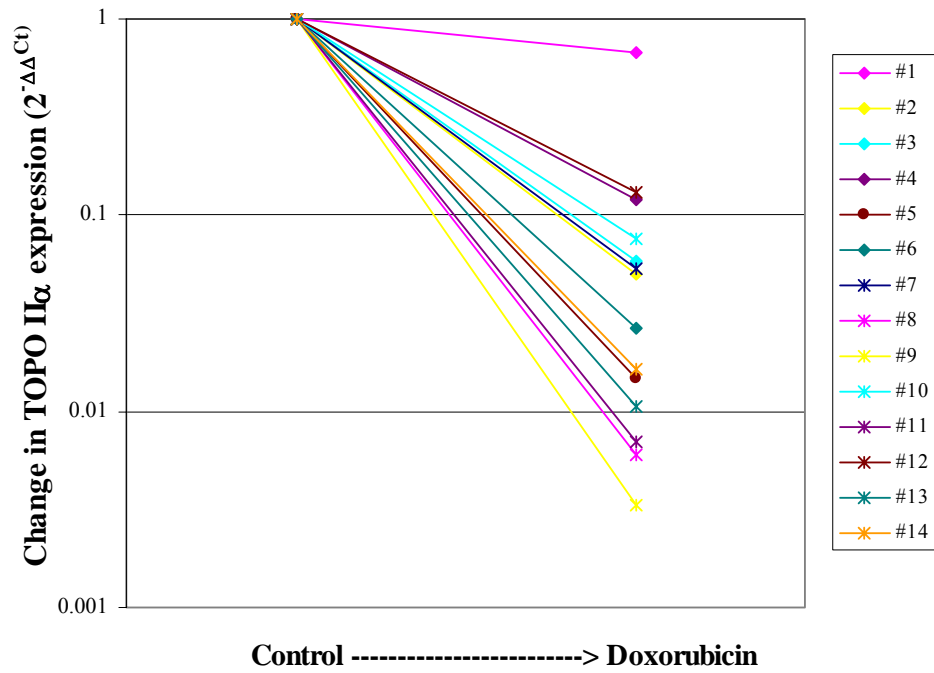
Figure 3.13 (continued). Analysis of house keeping gene expression levels in breast cancer cells exposed to doxorubicin in the ATP-TCA. The combination of HPRT1 and TBP was more consistently expressed than the other two. Panel d) shows the mean values ($\pm 2SD$) of the differences in the housekeeping gene expression between control and doxorubicin treated cells.

Following housekeeping gene analysis, all the ΔCt and $\Delta\Delta\text{Ct}$ values were recalculated by using the mean only of the two reference genes that were shown to be less affected by a particular treatment in a determined tumour type. The data was entered into a new Access database and analysed on the basis of the variation of each target gene. As considerable heterogeneity of expression was found for most targets in all tumour types, the results were expressed as median values, rather than using means, which have statistical meaning only when the data follow a normal distribution.

As in the case of the individual experiments, a widely recognised way of displaying the data does not exist. The most straightforward graphic can be obtained by plotting the values of $2^{-\Delta\Delta\text{Ct}}$ calculated for each tumour for the gene of interest, as shown in figure 3.14 (a). However, this way of representing the data results in a plot that only shows how the levels of a certain gene are affected by a particular treatment. No information is given about the differential expression of that gene in the various tumour samples. This information can be displayed only when the actual ΔCt values are plotted (figure 3.14, b), as any other method that employs $\Delta\Delta\text{Ct}$ values will always show normalized data (i.e. the expression of the target gene is always '0' or '1'). To allow a more comprehensive visualisation of the results in this thesis the parameter ΔCt has been plotted in those graphs that included more than one tumour sample (figure 3.14, b).

In conclusion, the new technique of qRT-PCR is extremely sensitive, highly specific and repeatable, has a wide functional range, and is able to provide robust quantitative data. These attributes make it the ideal investigative tool for quantitating low levels of expression of putative chemoresistance genes, and for detecting differences in expression caused by chemotherapy treatment *ex vivo* and *in vivo*. However, it should be noted that the sensitivity and quantitative capabilities of this technique can only be fully utilised following a considerable effort to optimise reagents, methodology and data analysis.

(a)



(b)

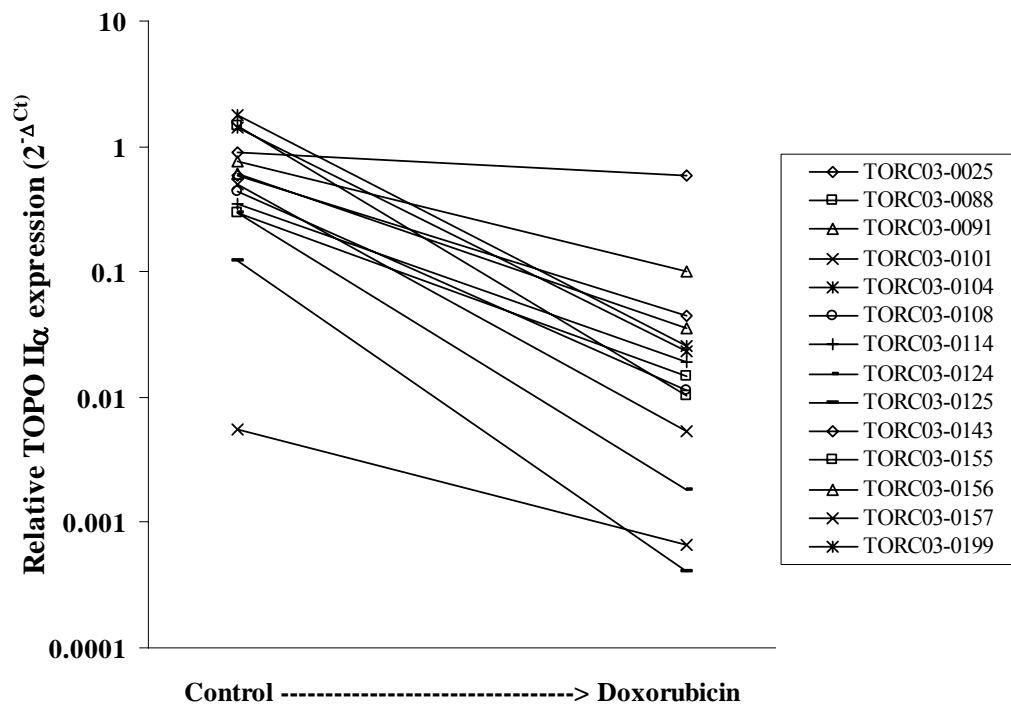


Figure 3.14 Effect of doxorubicin on the levels of TOPO II α in breast cancer cells tested in the ATP-TCA (n=14 samples). (a) Actual $2^{-\Delta\Delta C_t}$ values; (b) $2^{-\Delta C_t}$, where ΔC_t is the difference in Ct between TOPO II α and the mean of two reference genes (HPRT1 and TBP, in this instance).

Chapter 4 - Differential chemosensitivity of cutaneous melanoma cell lines and tumour derived cells

4.1 Introduction

4.1.1 Preclinical screening of anti-cancer drugs

Most anticancer agents that enter clinical trials are never approved for use, and in the majority of cases their development is aborted by insufficient anti-tumour activity in Phase II clinical studies rather than intolerable or unpredictable toxicity. The current *in vitro* drug screening panels used by the NCI (Monks *et al.*, 1991) and by most pharmaceutical companies are composed of human tumour cell lines derived from multiple sequential *in vitro* subcultures of human tumour explants. In the NCI screening programme, the candidate drugs are tested for efficacy on 3 cell lines from a panel of 60, representing all the major human cancers. Active compounds progress to be tested against a panel of human tumour xenografts in nude mice. If successful, the drugs enter phase I and phase II clinical trials.

The NCI-60 cell line panel is well characterised from a molecular standpoint and can be useful in identifying molecular determinants of *in vitro* sensitivity (Scherf *et al.*, 2000; Staunton *et al.*, 2001) or confirming putative molecular mechanisms of action of the compounds screened. The use of cell lines has also many other advantages. It is relatively inexpensive, and gives rapid, reproducible results. However, there are some disadvantages. Anticancer drug screening programmes work on the premise that *in vitro* drug sensitivity will correlate with *in vivo* performance (Phillips *et al.*, 1990).

However, as is the case for all *in vitro* systems, these assays cannot account for the *in vivo* pharmacological determinants of antitumour activity. In contrast to human solid tumours, which often show low growth rates, cell lines consist of rapidly proliferating cells that grow reproducibly in culture. The convenience of rapidly growing cells in culture may have implications for their sensitivity to chemotherapy, as most anti-cancer agents preferentially target dividing cells. Even normal proliferating cells show enhanced sensitivity to anti-cancer drugs (Drewinko *et al.*, 1981; Dewys *et al.*, 1972). This is reflected in the response rates of rapidly proliferating cancers to anti-cancer drugs (Valeriote & van Putten 1975), but also applies to cell lines derived from tumours by selection for cells which grow rapidly in serum-containing cell culture. In addition to this, cell lines consist of a homogenous population, while most human tumours have many accumulated genetic and molecular abnormalities and display a high degree of phenotypic heterogeneity.

Before the initiation of clinical trials new anticancer agents which are found to be active *in vitro* are then screened *in vivo* in human tumour xenografts grown subcutaneously in nude mice. However, most of these studies use tumour growth inhibition, not tumour shrinkage, as sufficient evidence of antitumour activity. These xenografts are not representative of the heterogeneous population of tumour cells of the human tumour from which they were derived. Considerable changes in chemosensitivity may occur when cells are brought from *in vitro* to *in vivo* conditions and *vice versa* (Tveit *et al.*, 1981). It is well known that the morphological and cell kinetic characteristics of the tumours change with subsequent passages (Kruczynski & Kiss 1993). In many cases, xenografts are selected to suit the putative molecular mechanism of the agent tested, the approach being one of proof of principle in an *in vivo* model rather than screening the new agent in a panel of clinically relevant and predictive models.

The use of *ex vivo* chemosensitivity testing of fresh tumour cells derived from clinical specimens has recently been suggested as an aid to anticancer drug development (Cree 2003; Neale *et al.*, 2000; see chapters 6,7, 10). Tumour-derived cells have the distinct advantage of preserving the heterogeneity observed in human cancers, but this method has its drawbacks too. It is technically difficult and costly to obtain large numbers of tumour-derived cells. Cells derived from different tumours vary in their chemosensitivity (Hunter *et al.*, 1993; Cree *et al.*, 1999; Neale *et al.*, 2001) and it is therefore difficult to compare experiments between tumours. The approach has some merit when considering which tumours may respond to a new drug, but is not suitable for screening large numbers of potential anti-cancer agents for activity (Cree & Kurbacher 1999).

4.1.2 The chemosensitivity of cutaneous melanoma

Advanced malignant melanoma has a very poor prognosis. Systemic therapy, both adjuvant and treatment of disseminated (stage IV) disease, remains unsatisfactory, with response rates lower than 30% (Helmbach *et al.*, 2003). Chemotherapy is mainly ineffective and unsatisfactory in this tumour type because of the drug resistance that is characteristic of this disease and is either intrinsic or develops during chemotherapy (Helmbach *et al.*, 2001). Alternative treatment methods based on immunological principles are currently being developed but have shown limited efficacy. Thus chemotherapy continues to be the primary treatment method for disseminated melanoma

and urgently needs to be improved. Regimens including treatment with DTIC are still widely used in malignant melanoma, though only a minority of patients achieve a long-lasting response and the overall survival is poor (Middleton *et al.*, 2000).

Chemosensitivity testing studies with fresh melanoma samples have been undertaken to identify the minority of patients who are most likely to benefit from chemotherapy. The first reports using the clonogenic assay found cutaneous melanoma to be highly chemoresistant *in vitro*, in keeping with the poor chemotherapeutic response observed *in vivo* (Salmon, 1984; Tveit *et al.*, 1988). Some correlation of *in vitro* data with clinical response was also provided by Schadendorf *et al.* (1994) in a small group of 19 samples tested with a clonogenic assay. Another study used an agarose-based cell culture system and measured [³H]-thymidine incorporation as an indicator of cell growth (Marshall *et al.*, 1992). In this paper the authors did not provide any clinical correlation, but showed considerable heterogeneity of chemosensitivity among different melanoma samples. This heterogeneity of chemosensitivity and general chemoresistance were essentially confirmed in later studies performed with the ATP-TCA in both uveal (Myatt *et al.*, 1997) and cutaneous melanoma (Cree *et al.*, 1999; Ugurel *et al.*, 2003). Neale *et al.* (1999) employed the ATP-TCA to develop and test the *ex vivo* activity of a novel drug combination, treosulfan plus gemcitabine, in uveal melanoma (Pfohler *et al.*, 2003); this regimen is currently being clinically evaluated in cutaneous melanoma and ovarian cancer.

More effective chemotherapeutic regimens are needed for the treatment for metastatic melanoma. The *ex vivo* ATP-TCA has already shown the ability to predict the clinical activity of new combinations (Kurbacher *et al.*, 1997), but the availability of fresh tumour cells limits the number of drugs and combinations that can be tested. Therefore we started a pilot study aimed at investigating the chemosensitivity of a panel of human melanoma cell lines in the ATP-TCA, in comparison to that observed in human tumour samples. The ultimate goal was to select one or more cell lines, which - cultured under growth-limiting conditions - could be used as a model of human melanoma in molecular and chemosensitivity studies.

4.2 Materials and Methods

Cell lines and tumours. Six melanoma cell lines (M14, MALME-3M, RPMI-7951, UACC-62, SK-MEL-2 and SK-MEL-28) were grown in RPMI-1640 medium (Sigma R7638) supplemented with 10% FCS (Labtech International, East Sussex, UK, cat. 4-101-500), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma P0781), and 2 mM glutamine (Sigma G7513), as detailed in section 2.3. The chemosensitivity of the cell lines was assessed and compared to previous data available from chemosensitivity tests performed on 56 skin melanomas (31M:25F, patient median age 54 years, range 30-87).

ATP-TCA. Chemosensitivity testing of cell lines was performed in RPMI-1640 medium supplemented with either 10% FCS or 2% FCS (section 2.3.2) according to the standard protocol described in section 2. At least three experiments for each cell line were carried out in media containing 2% FCS and four cell lines (MALME-3M, RPMI-7951, SK-MEL-2 and SK-MEL-28) were also tested in medium containing 10% FCS. Finally, three experiments for the cell line SK-MEL-28 were also performed in CAM (DCS). Nine single agents were tested for each cell line, CDDP, DTIC, gemcitabine (Gem), interferon α -2b (IFN α -2b), paclitaxel (Tax), temozolomide (Temo), treosulfan (Treo), vinblastine (VinB), vinorelbine (VinR), and one combination, treosulfan plus gemcitabine (Treo+Gem) (Neale *et al.*, 1999). The TDC of IFN α -2b was 1000 IU/ml, while all other cytotoxics were tested at the concentrations previously indicated (table 2.2, chapter 2).

Data analysis. The percentage cell growth inhibition in the presence of various concentrations of FCS was calculated as follows:

$$\left[1 - \frac{(X\%FCS) - (MI)}{(10\%FCS) - (MI)} \right] \cdot 100 = \text{Percent cells growth inhibition}$$

(X % FCS) = mean counts for wells containing cells grown with X % FCS

(MI) = mean counts for maximum inhibitor wells

(10% FCS) = mean counts for wells containing cells grown with 10% FCS

Chemosensitivity results were expressed as Index_{SUM} (MEAN \pm SD) calculated by the direct addition of the % survival at each concentration (section 2.2.19); this parameter

was preferred to other values, such as IC90 and IC50, as it was previously found that a natural logarithmic sum index provides the best discrimination between drugs and tumour samples to show the heterogeneity of the activity observed (Hunter *et al.*, 1993; Andreotti *et al.*, 1995; Konecny *et al.*, 2000). For the purposes of comparison between drugs and tumours, an Index_{SUM} of <300 was taken as *ex-vivo* sensitivity and >350 as resistance, as previously published (Hunter *et al.*, 1993; Cree *et al.*, 1999). Values between these two points were regarded as equivocal. An arbitrary cumulative chemoresistance index was also calculated for each cell line summing the means of the Index_{SUM} values for the twelve agents tested; the higher the index, the more resistant is the cell line in question.

4.3 Results

4.3.1 Measurement of cell line growth in different FCS concentrations

The first series of assays were carried out by growing the cell lines in RPMI-1640 supplemented with 10% FCS. The cell lines were found to be extremely chemosensitive under these conditions, and no typical drug response curves were obtained for most agents, the curves having a flat rather than a sigmoidal shape.

The hypothesis that the high chemosensitivity was due to a rapid growth rate of the cell lines in the presence of 10% FCS led me to perform experiments with various FCS concentrations without drugs to establish the minimum concentration of serum required for the growth of each cell line (figure 4.1).

A second series of experiments was carried out culturing the cells in RPMI-1640 supplemented with 2% FCS, as at this level cell growth rate was substantially diminished, but evaluable readings (luminescence equal to or greater than the luminescence produced by 1.86 nM ATP in the standard curve; section 2.2.16) were still obtained.

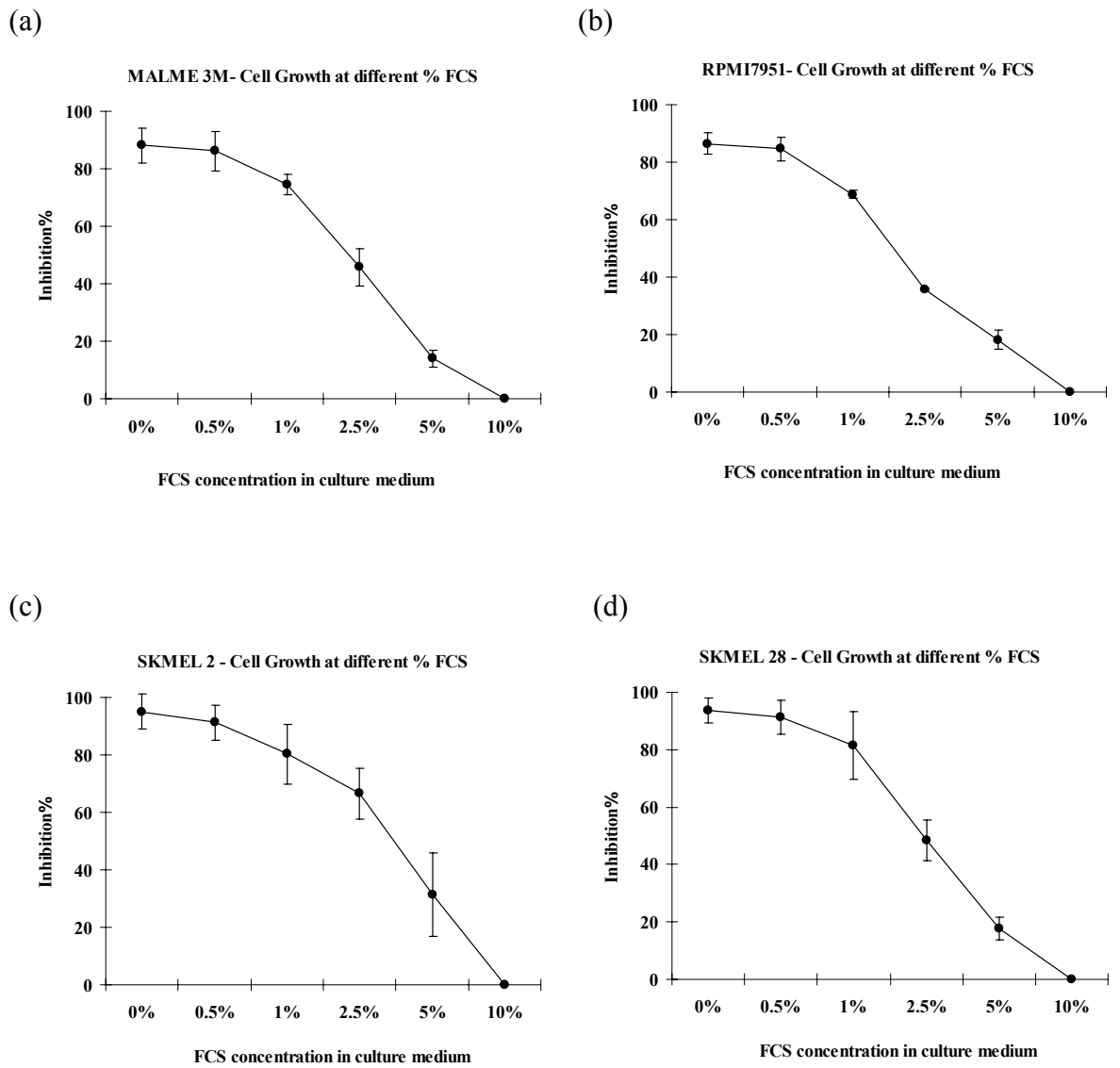


Figure 4.1 Cell growth inhibition was evaluated in the presence of various concentrations of FCS in the (a) MALME-3M, (b) RPMI-7951, (c) SK-MEL-2 and (d) SK-MEL-28 cell lines. Data is represented as Mean±SD of at least three experiments for each cell line.

4.3.2 Chemosensitivity of melanoma cell lines

As expected, the cell lines showed heterogeneity of sensitivity to the agents tested (table 4.1). One cell line, RPMI-7951, appeared to be particularly sensitive to all the cytotoxics and therefore had the lowest cumulative index (table 1.4; figure 4.2). Based on this parameter, the most resistant cell line was SK-MEL-2, followed by the MALME-3M and the SK-MEL-28 lines (figure 4.2).

The most effective single agents in all cell lines were paclitaxel and gemcitabine, while in tumour-derived cells, both these drugs showed a mean $\text{Index}_{\text{SUM}}$ in the resistance range (>350). The combination of treosulfan with gemcitabine was extremely active in all the cell lines and at both concentrations of FCS tested (figure 4.3); it also showed good cytotoxicity in the series of melanomas studied. However, while all cell lines grown in serum showed an $\text{Index}_{\text{SUM}}$ less than 100, the mean $\text{Index}_{\text{SUM}}$ value for this combination in tumour samples was 226 ± 125 , suggesting considerable heterogeneity. It is notable that sensitivity indices similar to those found for both gemcitabine and Treo+Gem in tumour-derived cells were obtained when the SK-MEL-28 line was grown in serum-free CAM ($\text{Index}_{\text{SUM}} = 312 \pm 32$ and 219 ± 15 , for GEM and TREO+GEM, respectively) (figure 4.4).

The *vinca* alkaloids were extremely active in all cell lines, while tumour samples were usually resistant, in particular to vinblastine. It must be noted that considerable heterogeneity was found in melanoma biopsies, and a number of tumours were found to be sensitive to the microtubule-active agents. The decrease of serum in the culture medium did not significantly affect the chemosensitivity to the *vinca* alkaloids in the SK-MEL-28, MALME-3M and RPMI-7951 lines. However, a moderate increase in resistance was observed in the SK-MEL-2 cells when the concentration of FCS was reduced to 2% (figure 4.3).

The activity shown by the alkylating agents DTIC and temozolomide was rather disappointing. DTIC was active only in the UACC-62 cell line (table 4.1); however the MALME-3M and RPMI-7951 also exhibited sensitivity to this agent when cultured with 10% FCS (figure 4.3). The M14 were the most resistant cells (table 4.1) with a mean sensitivity index a little lower than the corresponding value calculated in the

tumour series. Temozolomide was consistently the least active drug in both the series of tumour samples and the panel of cell lines (table 4.1).

Variable sensitivity was observed with the other two DNA damaging agents tested, CDDP and treosulfan. In the group of skin melanomas, treosulfan was on average more active than CDDP, while in the cell lines the activity of CDDP was similar, if not superior, to that of treosulfan. Interestingly, the line SK-MEL-2 was rendered more resistant to both agents by lowering the serum concentration (figure 4.3). The SK-MEL-28 cells, in contrast, showed little or no change in sensitivity to either CDDP or treosulfan when tested in 2% FCS (figure 4.4). However a significant increase in resistance to these drugs was found when the SK-MEL-28 cells were grown in serum-free CAM (figure 4.4). Thus the $\text{Index}_{\text{SUM}}$ for CDDP increased from a mean value of 210 ± 23 to 456 ± 20 ($p < 0.0005$, unpaired t test), and the $\text{Index}_{\text{SUM}}$ for treosulfan from 344 ± 23 to 456 ± 38 ($p < 0.02$, unpaired t test).

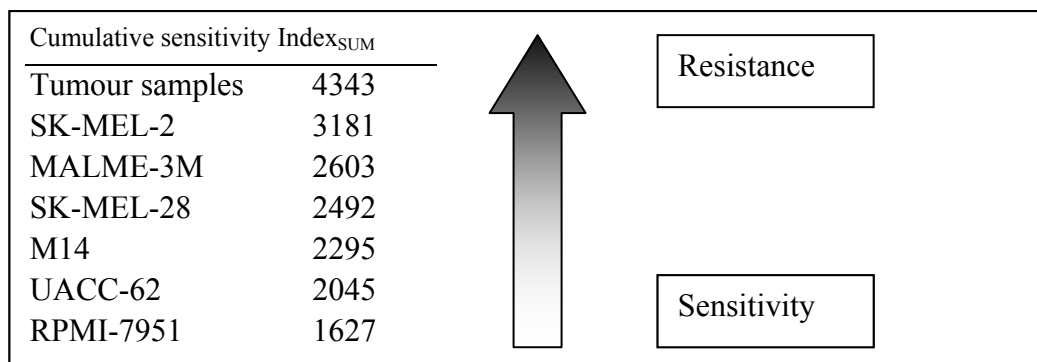
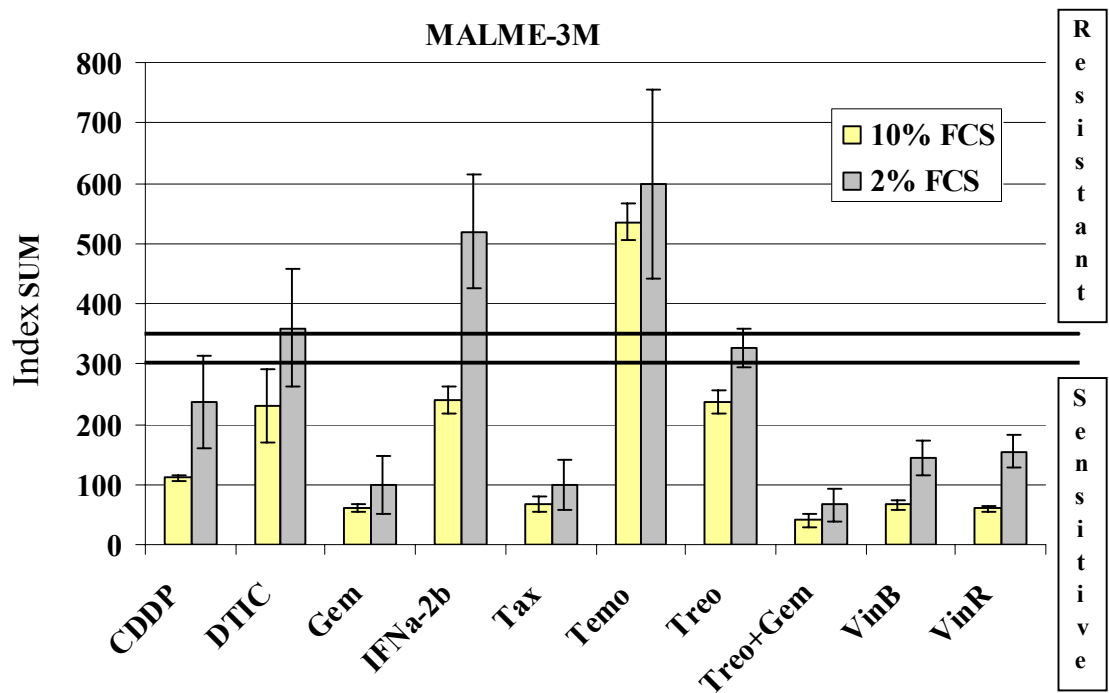


Figure 4.2. Cumulative sensitivity $\text{Index}_{\text{SUM}}$ values that were calculated summing the means of the $\text{Index}_{\text{SUM}}$ for all the agents tested in each cell line in the presence of 2% FCS.

Table 4.1 Summary of the chemosensitivity Index_{SUM} values expressed as mean±SD. Cell lines were grown in standard RPMI-1640 containing 2% FCS; while tumour samples were cultured in serum-free CAM. Abbreviations are listed in section 4.2.

Sample (Number of assays)	DRUGS									
	CDDP	DTIC	Gem	IFN α -2b	Tax	Temo	Treo	Treo+Gem	VinB	VinR
M14 (n=3)	138 (±47)	499 (±69)	19 (±12)	434 (±282)	34 (±12)	832 (±146)	153 (±78)	11 (±5)	72 (±31)	103 (±56)
MALME-3M (n=6)	236 (±77)	360 (±99)	99 (±49)	519 (±94)	100 (±41)	598 (±156)	327 (±33)	66 (±27)	144 (±30)	154 (±27)
RPMI-7951 (n=4)	45 (±39)	313 (±111)	11 (±3)	438 (±42)	6 (±3)	667 (±140)	101 (±18)	5 (±2)	27 (±28)	14 (±3)
SK-MEL-2 (n=5)	424 (±48)	384 (±20)	162 (±23)	458 (±63)	50 (±14)	742 (±107)	402 (±39)	72 (±26)	248 (±66)	239 (±64)
SK-MEL-28 (n=3)	210 (±23)	318 (±34)	128 (±22)	485 (±22)	100 (±17)	516 (±10)	344 (±39)	91 (±12)	147 (±41)	153 (±56)
UACC-62 (n=3)	220 (±146)	266 (±22)	51 (±46)	404 (±164)	15 (±12)	454 (±66)	260 (±99)	47 (±37)	162 (±165)	166 (±142)
Tumour samples (n=56)	429 (±126)	563 (±96)	403 (±176)	502 (±139)	358 (±131)	614 (±195)	347 (±139)	226 (±125)	527 (±205)	374 (±182)

(a)



(b)

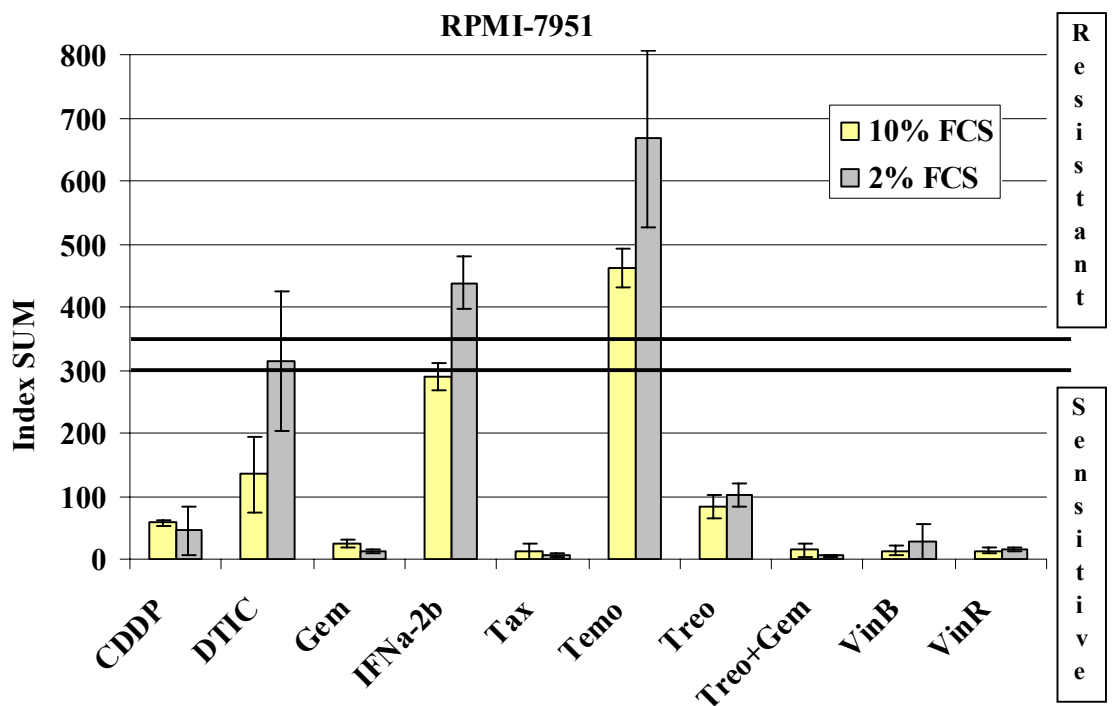
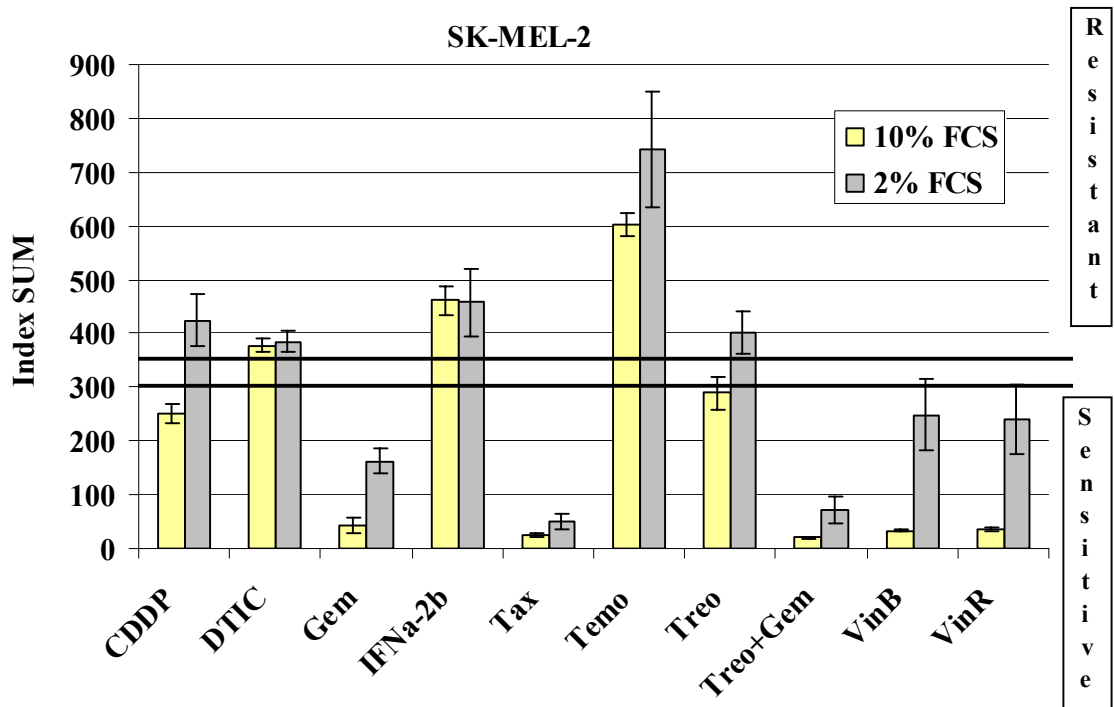


Figure 4.3. Chemosensitivity indices calculated from the ATP-TCA performed in RPMI-1640 medium containing 10% or 2% FCS. Data is represented as mean \pm SD of at least three experiments for each cell line, MALME-3M (a) and RPMI-7951 (b).

(c)



(d)

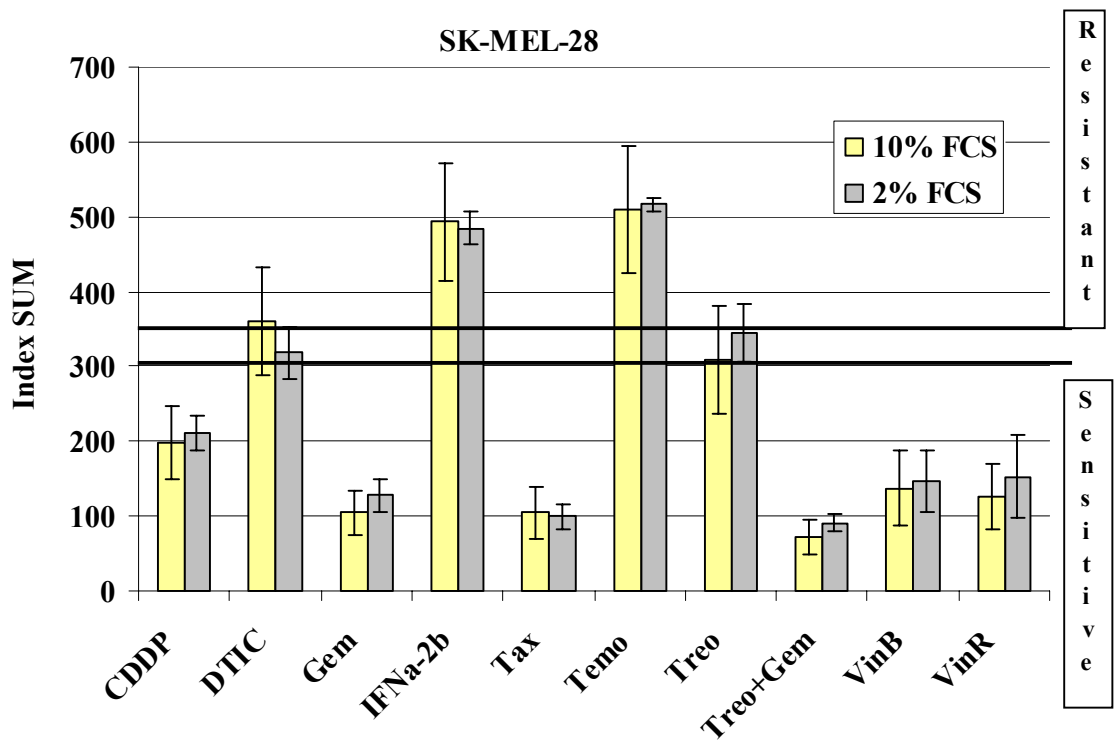


Figure 4.3 (continued). Chemosensitivity indices calculated from the ATP-TCA performed in RPMI-1640 medium containing 10% or 2% FCS. Data is represented as mean \pm SD of at least three experiments for each cell line, SK-MEL-2 (c) and SK-MEL-28 (d).

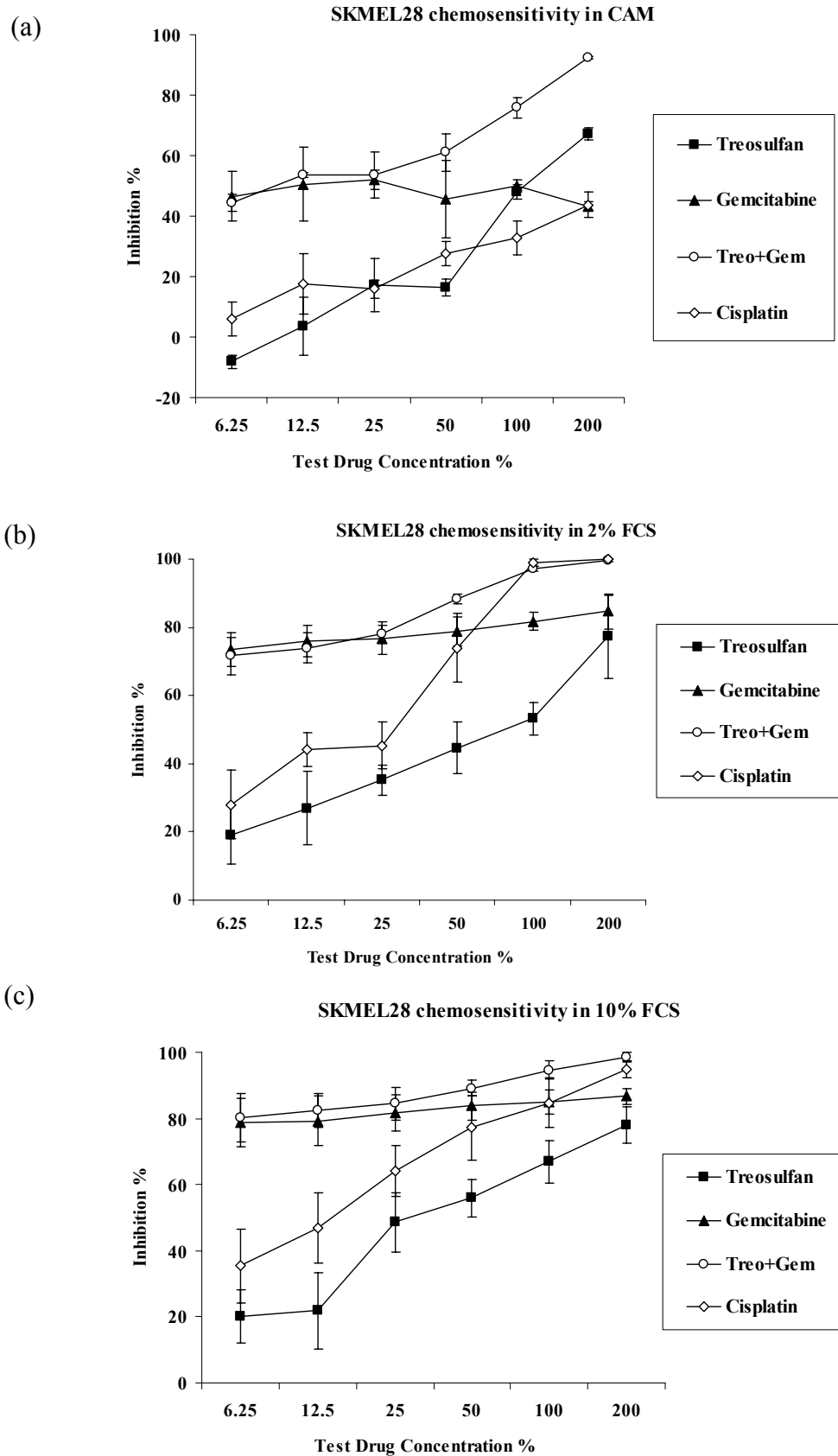


Figure 4.4. Concentration-inhibition curves for the SK-MEL-28 cell line grown in serum-free CAM (a); 2% FCS – RPMI-1640 medium (b); 10% FCS – RPMI-1640 medium (c). Data are represented as mean \pm SD of at least three experiments.

4.4 Discussion

The results show that melanoma cell lines tested against cytotoxic drugs at standard 10% FBS concentrations are much more sensitive to certain classes of anti-cancer agent than tumour-derived cells. One cell line, RPMI-7951, appeared to be particularly sensitive to all the cytotoxics. This is reflected by the rapid growth rate of these cells that required passaging every two days and remained viable even in serum-free culture media. Three cell lines, namely SK-MEL-2, SK-MEL-28 and MALME-3M, grown in the presence of reduced serum levels, exhibited a lower chemosensitivity that, at least for certain drugs, resembled the chemosensitivity of clinical samples. In particular, pilot experiments with the SK-MEL-28 in serum-free CAM suggested that this might provide even better congruity between cell lines and tumour-derived cells than reduced serum.

A literature search revealed only one previous study on the same subject (Marshall *et al.*, 1992), which however used the [³H]-thymidine incorporation method and not a cell-death based assay. Although the results are not directly comparable, these investigators also found that the chemosensitivity of two established melanoma cell lines (MM-96 and FME) was comparable to that exhibited by at least some fresh tumour biopsies. The results presented in this chapter also partially confirm data that were published over 20 years ago by Drewinko *et al.* (1981) using the clonogenic assay. This group found that several anticancer drugs, including doxorubicin, 5-FU and vincristine, were more cytotoxic to S-phase colon carcinoma cells compared to cells in a phase of stationary growth. However, this early study did not use a cell death based assay, and did not provide any comparison with the chemosensitivity of fresh tumour derived cells.

As expected, some heterogeneity of chemosensitivity was noted between the cell lines, although this was not true for some of the drugs tested.

Among the single agents, the activity of DTIC and temozolomide was rather disappointing in all the cell lines. DTIC requires metabolism by cytochrome P450 (CYP450), primarily by the isoforms 1A1 (extrahepatic) and 1A2 (hepatic) (Reid *et al.*, 1999), to form the reactive N-hydroxylated metabolite, 5-[3-hydroxymethyl-3-methyl-triazene-1-yl]-imidazole-4-carboxamide (HMMTIC), ultimately forming the methylating species 5-(3-methyltriazene-1-yl)imidazole-4-carboxamide (MTIC) which is responsible for cytotoxicity. In this process, MTIC itself is decomposed to the major

plasma and urine metabolite 5(4)-aminoimidazole-4(5)-carboxamide (AIC) and the reactive species methane diazohydroxide, which produces molecular nitrogen and a methyl cation believed to be the methylating species (Tsang *et al.*, 1991; Long & Dolan 2001). The cytotoxicity of MTIC is thought to be primarily due to alkylation at the O⁶ position of guanine and N³ position of adenine. An early study (Tsang *et al.*, 1991) that investigated the *in vitro* cytotoxicity of DTIC, MTIC and temozolomide in TLX5 murine lymphoma cells found that DTIC was only active in the presence of exogenous CYP450; whereas MTIC and temozolomide were cytotoxic even in the absence of mouse liver microsomes. This raises the possibility that a lack of bioactivation in melanoma cells could be responsible for the poor activity observed by DTIC. However, over the last decade, most human tumours were found to express several isoforms of CYP450, namely 1A, 1B, 2C, 3A, 2D (Patterson & Murray 2002). CYP450 enzyme expression patterns were also determined for the NCI cell 60 panel (Yu *et al.*, 2001) using a series of enzymatic assays. This study found that the melanoma cell line SK-MEL-2 was the most active of the entire NCI60 panel in the assay that determined CYP1A1 activity; the SK-MEL-28 and UACC-62 cells were also found to be active; while the enzyme activity was below the limits of detection in the M14 cell line, which was also the least sensitive line to DTIC in our panel. Therefore it seems likely that most tumour cells have enough CYP450 to activate DTIC; in the case of M14 cells, the undetectable CYP1A1 activity might be one factor explaining the poor activity of DTIC, though this hypothesis needs to be explored in further studies.

Temozolomide has been proposed as an alternative agent to DTIC in the treatment of advanced melanoma (Agarwala & Kirkwood 2000) due to its penetration into CNS, and therefore its potential activity in treating brain metastases. Temozolomide was the least active drug in both the series of tumour samples and the panel of cell lines. No heterogeneity of chemosensitivity to temozolomide was noted in the melanoma specimens that were all extremely resistant to this drug (INDEX_{SUM}>400).

Temozolomide is an imidazotetrazine alkylating agent which is stable at acid pH, but undergoes chemical conversion at physiological pH to the active species 5-(3-methyltriazene-1-yl) imidazole-4-carboxamide (MTIC). The instability of temozolomide and its active metabolite at physiological pH could account for the poor cytotoxicity observed in our samples. Previous studies have shown that MTIC is unstable in human plasma at 25°C with a half life less than 30 minutes (Kim *et al.*,

1997). In another study the same group found that almost all of the temozolomide was converted to AIC after 45 minutes of incubation at 37°C in human plasma (Kim *et al.*, 2001); while at 4°C the decomposition rate was slower, a substantial 30% of temozolomide was converted to AIC after 120 minutes. Although every precaution was taken while preparing the solutions of the cytotoxic drugs (diluting temozolomide last in ice-cold medium), the time required to set up the plates before the addition of the cells often exceeded 30 minutes. Thus it is possible that the cells were only exposed to a minimal fraction of the initial drug concentration, and for an extremely short time.

Most importantly, it can be suggested that temozolomide and DTIC showed reduced cytotoxicity due to DNA repair mechanisms present in these melanoma cell lines. The first cellular line of defense to the cytotoxicity elicited by these two agents is provided by the enzyme MGMT (see chapter 1.2.6), that removes O⁶-methylguanine adducts caused by DTIC and temozolomide. The level of MGMT varies depending on cell type (Irving & Hall, 2001). It is possible that the melanoma cell lines used in this study show a particularly high expression of this enzyme able to confer resistance to methylating agents. This might be the case of the SK-MEL-28 line, as recently reported by Pepponi *et al.* (2003). However the same study also reported a high sensitivity of M14 cells to temozolomide in the MTT assay which correlated with their modest MGMT enzymatic activity. This is in contrast with our findings that M14 cells were the least sensitive to both DTIC and temozolomide.

A second possible mechanism of resistance to DTIC and temozolomide is represented by loss of MMR (Liu *et al.*, 1999). In fact, after saturation of MGMT, any remaining O⁶-methylguanine adducts may then be detected by the MMR system, leading to cell death. For cells with proficient MMR, high MGMT levels prevent the cytotoxic effect of methylating agents by removing all O⁶-methylguanine adducts, and inhibition of MGMT sensitizes the cells (Middleton *et al.*, 2000; Pegg, 2000). Loss of a functional MMR system and its detector function allows the cell to resist the cytotoxic barrage and continue to divide whilst accumulating O⁶-methylguanine residues. These can in turn accelerate the rate of accumulation of mutations as they can result in GC→AT transitions. Of the cell lines used in this study, data on the MMR status have been found only for the SK-MEL-28 and M14 cells, both of which were shown to have a proficient

MMR (Pepponi *et al.*, 2003); therefore, at least for these two lines, our results cannot be explained by a loss of MMR.

The largest differences between cell lines and tumour-derived cells were seen with spindle-active agents (paclitaxel, vinblastine) and with gemcitabine. The apparent high activity of gemcitabine was also been noted in some tumour samples, and in these cases the dose-response curve appeared as a flat line. Some recent work by Peters' group in Amsterdam (Van Moorsel *et al.*, 2000 & 2003) has shown that gemcitabine could alter the ribonucleotide pools in various cell lines; however a similar range of gemcitabine concentrations to those used in the ATP-TCA, produced an increase of ATP, rather than diminishing its levels. Therefore the decreased amounts of ATP measured in the ATP-TCA after gemcitabine exposure are unlikely to be caused by this ribonucleotide perturbation effect.

The results show that melanoma cell lines tested against cytotoxic drugs at standard 10% FCS concentrations are much more sensitive to certain classes of anti-cancer agent than tumour-derived cells. To some extent this lack of relevance can be reversed by lowering the serum concentration, and hence the proliferation rate of the cells. While this produces greater apparent relevance for most of the cell lines examined, some appear more suitable than others for chemosensitivity studies. On the basis of the results presented in this chapter, the SK-MEL-2, MALME-3M and SK-MEL-28 lines should be included in an optimised panel of cell lines used for anti-cancer drug screening.

Chapter 5 - The *ex vivo* chemosensitivity profile of retinoblastoma

5.1 Introduction

Retinoblastoma (RB) is the most common primary intraocular tumour in children, with an incidence of 1 in 15,000-20,000 live births (Finger *et al.*, 1999). More than 90% of cases are diagnosed before 5 years of age, and presentation of RB in adults is rare (Biswas *et al.*, 2000).

Successful treatment of RB has traditionally depended on surgery and external beam radiation therapy, which is associated with significant short and long-term morbidity (reviewed in Wilson *et al.*, 2001). Recently, multiple studies have been published reporting initial experiences with an association of systemic chemotherapy and focal methods as primary treatments for this type of cancer (Shields & Shields, 1999; Friedman *et al.*, 2000).

Chemotherapy is used in RB as adjuvant therapy to control intra-ocular tumour growth with or without radiation, to prevent the growth of metastases and to treat metastatic disease once this has become clinically apparent (Gallie *et al.*, 1996; Kingston *et al.*, 1996; Gunduz *et al.*, 1998). Current regimens are based on empirical drug combinations derived from neuroblastoma treatments (Gobie *et al.*, 1990; Kingston *et al.*, 1996). Few clinical trials have been conducted due to the rarity of this tumour and the difficulty of funding such trials. The current regimens in use tend to use carboplatin, vincristine, thiotepa, etoposide or doxorubicin.

Metastasis is rare following adjuvant therapy, but potentially devastating. There is a need to design less toxic, but equally or more effective regimens with a low risk of mutagenesis that might avoid the development of second malignancies (Tucker *et al.*, 1987). This is a particular problem for some alkylating agents, such as cyclophosphamide, (which has been used in RB) and etoposide, which is associated with an increased risk of leukaemia (Felix, 1998). Etoposide is still widely used in RB, though at a dose not expected to cause problems (Shields *et al.*, 1997). Although there is a feeling that more retinal tumours may develop in patients treated with chemotherapy (Shields CL, personal communication), there is as yet no evidence of an increase in chemotherapy induced malignancies in RB patients.

A small number of studies have investigated *in vitro* drug resistance in RB, and these have mainly used cell lines (in particular the Y79 and WeriRb1 cells) (Chan *et al.*, 1989 & 1991; Giuliano *et al.*, 1998; Di Felice *et al.*, 1998). The isolation of tumour-derived cells from RB samples is technically challenging, as these tumours tend to be rather small and necrotic or calcified. Until 2003, there were only two reports of chemosensitivity testing in human RB tumours (Chan *et al.*, 1989; Inomata *et al.*, 1997). Both studies used the clonogenic assay, which measures colony forming capacity and has therefore a cell growth endpoint (section 1.3). Chan *et al.* did not test fresh tumour cells; cells were passaged through immunodeficient mice before the assay, probably to generate the large number of cells required to perform this type of assay. This study found resistance to methotrexate in nearly all samples, while good sensitivity was noted for the other cytotoxics tested, which included etoposide, vincristine, cyclophosphamide, melphalan, DTIC, cisplatin, doxorubicin and cytarabine. The second study by Inomata *et al.* employed fresh tumour-derived cells, and did not confirm the findings from the previous report. All samples in the Inomata series were sensitive to melphalan, and a proportion was sensitive to doxorubicin and cisplatin, while vincristine, 5-FU, DTIC, methotrexate and cytarabine did not influence cell growth.

In the period 2001-2003 two new studies appeared on the subject (Schouten-van Meeteren, *et al.*, 2001; Di Nicolantonio *et al.*, 2003), both of which employed cell death assays, the MTT and ATP assays respectively, to investigate the chemosensitivity profile in fresh RB cells. The results of the study based on the ATP assay are reported here.

5.2 Materials and Methods

Tumour. Material from ten untreated primary RBs (2M:8F, patient median age 5 months, range 2-36 months) and one skin metastasis from a primary tumour that was not required for diagnosis and further genetics research at St Bartholomew's hospital was obtained. Of these samples only 6 primary tumours and the metastasis contained sufficient viable cells.

ATP-TCA. Cells were obtained from the tumours either by mechanical dissociation with a Pasteur pipette or by a gentle enzymatic digestion, usually 75 µg/ml collagenase for 2 hours. Viable tumour-derived cells were separated from dead cells and debris by

density centrifugation (Histopaque 1077-1, Sigma), washed, counted and resuspended to 100,000 cells/ml. The cells were used to set up ATP-TCA plates as described in section 2.1 (Andreotti *et al.*, 1995). Not all drugs were tested in all samples.

5.3 Results

The ATP-TCA was performed successfully in 7/11 samples giving an assay evaluability rate of 64%. In two cases no viable cells were obtained after collagenase digestion, and in the remaining two the cells did not survive the incubation period.

The results showed very high sensitivity to single agents, particularly cisplatin, doxorubicin and vinca alkaloids (table 5.1). The shape of the inhibition curves is interesting. In most cases there was a plateau effect with etoposide and vinblastine, which was not present with cisplatin (figure 5.1, panels a,b,e).

Of the anti-metabolites tested, 5-FU was relatively disappointing (though still active), and gemcitabine showed considerable activity consistent with a cytotoxic effect (figure 5.1, panels c-d).

One tumour, indicated as case 3, was much more resistant than the others tested particularly to vinblastine, but also to the topoisomerase inhibitors which failed to achieve complete kill at any concentration tested. This may represent overexpression of MDR1 by a proportion of the cells present, but no further tissue has been available to check this using immunohistochemistry.

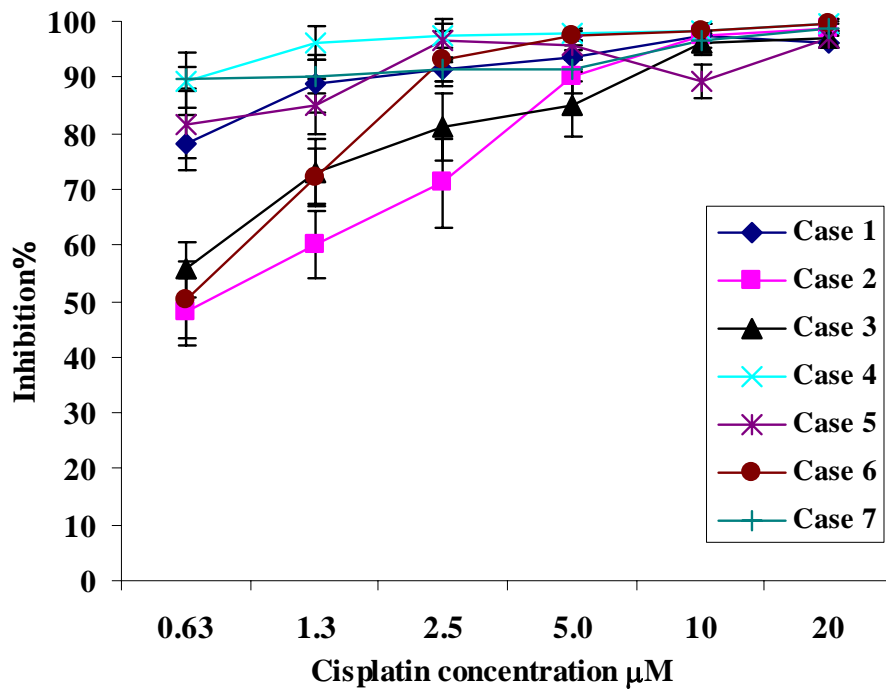
Of the combinations (VAC and VEC), the VAC regimen looks marginally more active in the more resistant of the two cases tested.

Table 5.1 Median values for AUC, Index_{SUM}, IC₉₀ and IC₅₀ calculated for the cytotoxics tested in 7 RB samples. For the combinations the values marked with (*) are expressed as percentage of TDC. Where the inhibition exceeded 50% even at the lowest concentration of drug used, the IC₅₀ was extrapolated using the formulas indicated in the Appendix.

Abbreviations are VEC = vinblastine + etoposide + cisplatin; VAC = vinblastine + doxorubicin + cisplatin.

Drug	IndexAUC	IC₉₀ μM	IC₅₀ μM	Index_{SUM}
Cisplatin (n=7)	18409 (17626-19021)	1.8 (0.60-7.2)	0.40 (0.30-0.70)	55 (21-135)
Etoposide (n=5)	17939 (17312-18399)	38 (4.1-77)	3.2 (2.4-4.1)	51 (29-99)
Doxorubicin (n=4)	16963 (16697-17621)	0.22 (0.086-0.81)	0.052 (0.034-0.14)	77 (4-127)
Vinblastine (n=5)	18769 (11739-19041)	0.061 (0.033-1.6)	0.022 (0.016-0.050)	50 (14-256)
5-FU (n=2)	14153 (13915-14931)	649 (623-675)	41 (38-45)	218 (212-223)
Gemcitabine (n=3)	18386 (15308-19287)	8.0 (2.3-87)	1.6 (1.3-2.0)	50 (4-148)
VEC (n=3)	18806 (17904-19207)	6* (6-53)	3* (3-4)	21 (9-78)
VAC (n=2)	19033 (18841-19225)	6* (-)	3* (-)	17 (7-26)

(a)



(b)

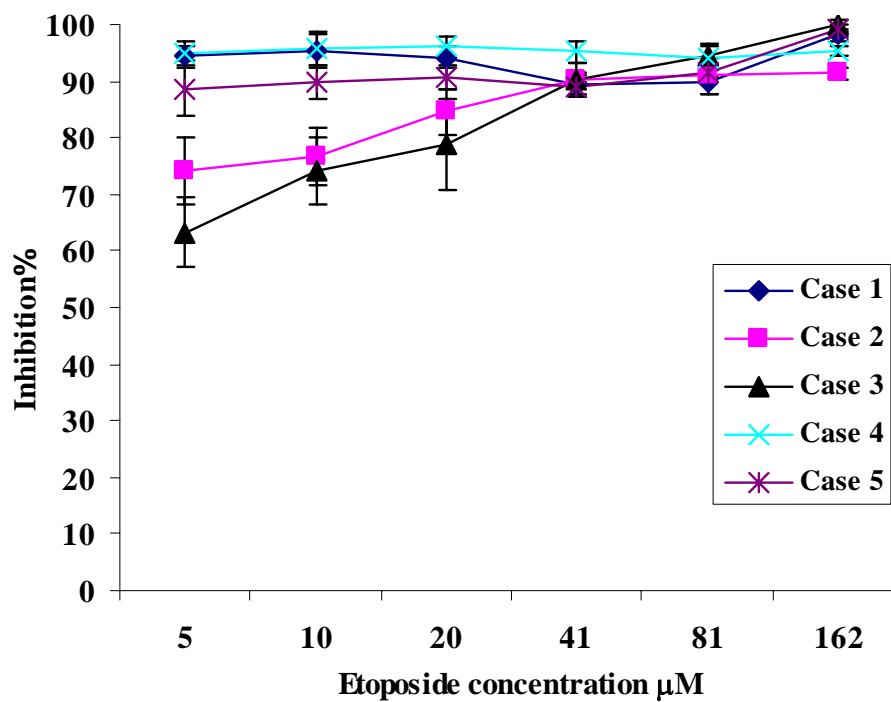
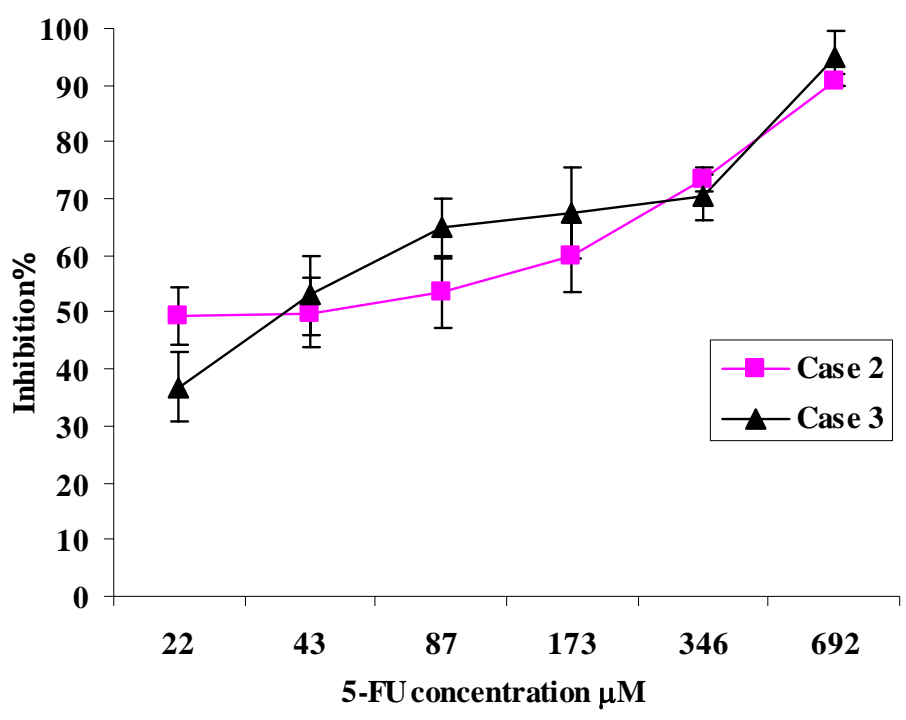


Figure 5.1 Concentration - inhibition curves in RB samples tested with cisplatin (a) and etoposide (b). Data are represented as mean \pm SD of triplicate values.

(c)



(d)

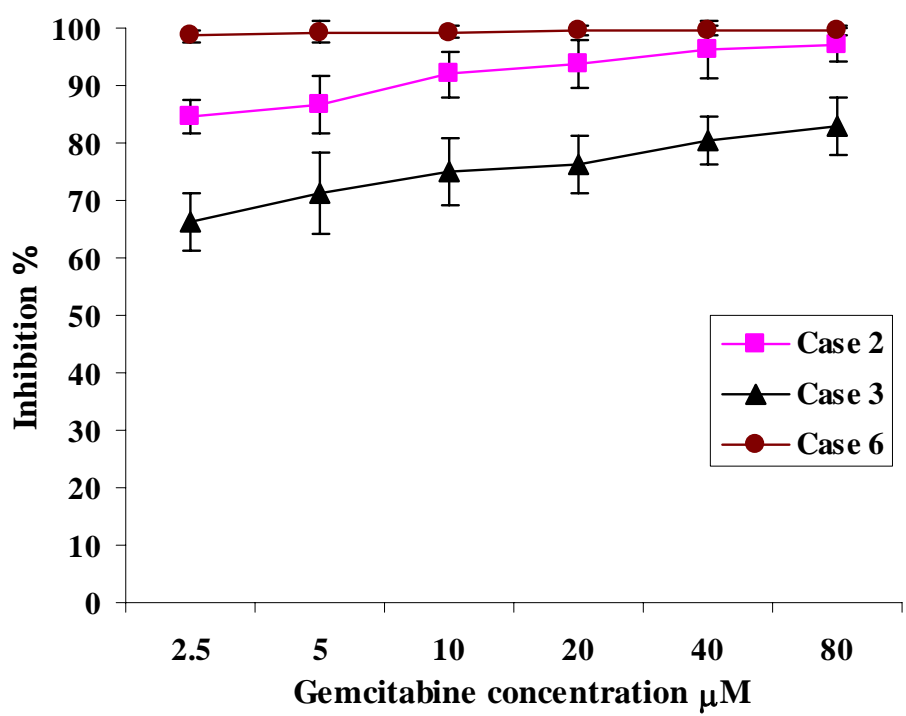
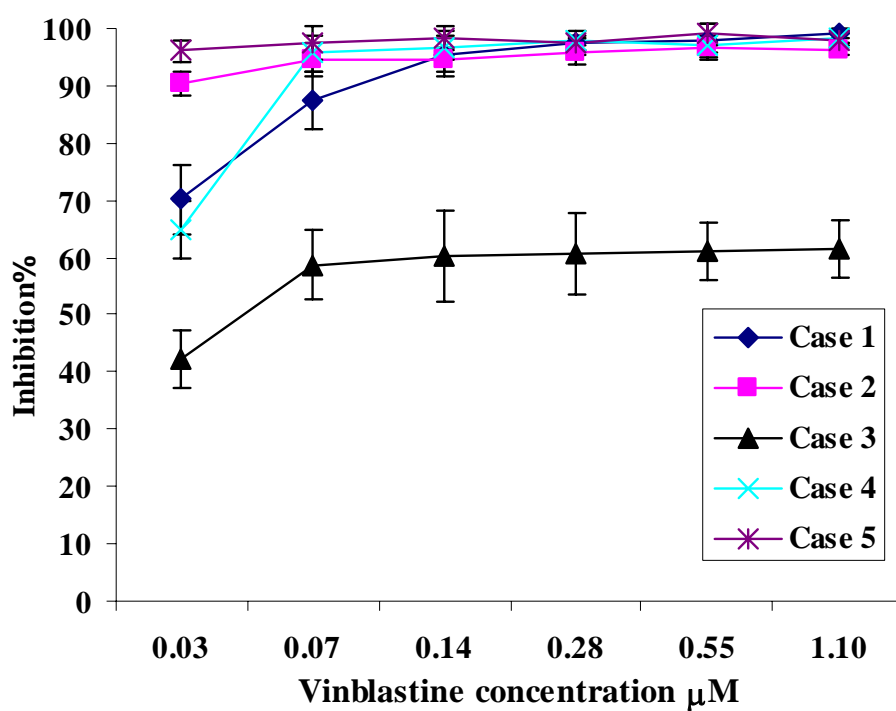


Figure 5.1 (continued). Concentration - inhibition curves in RB samples tested with 5-FU (c) and gemcitabine (d). Data are represented as mean \pm SD of triplicate values.

(e)



(f)

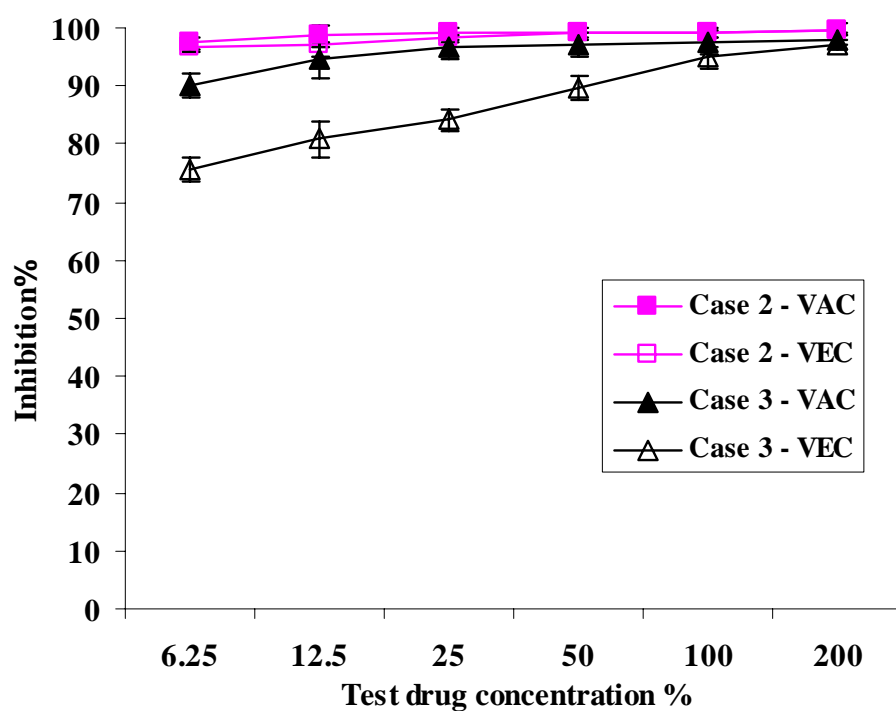


Figure 5.1 (continued). Concentration - inhibition curves in RB samples tested with vinblastine (e) and the combinations VAC and VEC (f). Data are represented as mean \pm SD of triplicate values.

5.4 Discussion

In this study the ATP-TCA has been used to investigate the chemosensitivity profile of RB samples, despite the small numbers of cells available. This is the first report of using this assay for such tumour, and one of the very few studies available on the subject. The evaluability rate of the ATP-TCA in our series was 64%, which is lower than that achievable with other tumours (see table 1.9). However, this is comparable to the 70% success rate obtained by Schouten-van Meeteren (2003) with the MTT assay. In both studies an extremely low concentration of collagenase during the digestion step was found to be a critical factor to obtain viable cells. In two successful cases in our series that presented as solid tiny fragments, the enzymatic digestion was omitted and replaced by mechanical disruption by simply pipetting the suspension several times. Another factor that could have affected our low evaluability rate is the size of the tumour samples received, as part of the spare tissue not required for diagnosis was shared for genetic studies with Dr Zerrin Onadim's group at St Bartholomew's hospital.

The results confirm that this is a very chemosensitive tumour, in keeping with its high growth fraction and the observed clinical efficacy of the drugs tested. Similar data has been produced by Gertjan Kaspers' group in Amsterdam using the MTT assay (Schouten-van Meeteren *et al.*, 2003).

Based on other tumour types, all these results show significant activity of at least one of the cytotoxic drugs tested, although in one case none produce complete ATP inhibition consistent with 100% cell kill. The best dose response effect was seen with cisplatin, which probably indicates that platinum is the most effective drug in this tumour. The combination with doxorubicin is likely to be as active as the three-drug combination VAC and the availability of liposomal preparations (Doxil/Caelyx) should allow high concentrations to be achieved with a lower risk from MDR positive tumours. Addition of an MDR inhibitor in such patients may be more rational than the use of the current three drug regimen incorporating vincristine (Chan *et al.*, 1996).

The major risk to survival in these patients is second malignancy. Etoposide use is associated with haematogenous malignancy (Felix, 1998) and could probably be avoided. The evidence of this study suggests that doxorubicin is equally effective and it does carry lower risk.

Drug resistance can occur, and may arise in several different ways. There is relatively little information on the relative importance of these mechanisms in RB, although as a tumour with a relatively simple molecular pathogenesis, it may have much to teach oncology in general. The MDR1/PgP mechanism has been studied in some detail by the Toronto group. Up to 15% of RBs express the MDR1 drug efflux protein and there is evidence that resistant cases express MRP (Chan *et al.*, 1997). Cyclosporin, an inhibitor of MDR1, can augment the control of RB (Chan *et al.*, 1996) and new inhibitors are in development. Other mechanisms are less well studied: BCRP and MVP are alternative drug efflux pump molecules similar to P-gp in their function, resistance to DNA damaging agents could be influenced anti-apoptotic mechanisms. In RB, p53 inactivation and p21^{Waf1} expression have recently been implicated in resistance to apoptosis, as could BCL-2 expression, which also occurs in RB (Divan *et al.*, 2001).

Although chemotherapy for RB is already very successful, several newer cytotoxic drugs are available with improved side effect profiles and anti-neoplastic activity, including different mechanisms of action. While it would be difficult to justify clinical trials of the large number of different options in RB, it would be sensible to explore these options pre-clinically and then conduct a trial against existing practice.

In conclusion the results show that RB is highly chemosensitive, in keeping with the highly proliferative nature of this tumour and the observed clinical efficacy of the drugs tested. Chemosensitivity testing of RB is feasible and can be used to improve understanding of the sensitivity/resistance of RB to chemotherapy, and to develop new drug combinations.

Chapter 6 - The *ex vivo* activity of XR11576, a novel dual topoisomerase inhibitor

6.1 Introduction

It has been proposed that the ATP-TCA can be used in the development of new agents and combinations for use in cancer patients (Cree & Kurbacher, 1999). As an example, this method has been previously employed to assess the *ex vivo* activity of a novel dual topoisomerase I and II inhibitor, XR5000 (Neale *et al.*, 2000b): the assay showed that this new drug was active against melanoma as well as ovarian cancer, but at concentrations which were unlikely to be achieved in patients (Cree *et al.*, 2003).

Several dual inhibitors of topoisomerase I and II have been described in addition to XR5000/DACA (Finlay *et al.*, 1996; Neale *et al.*, 2000b). These are intoplicine (Riou *et al.*, 1993), F 11782 (Perrin *et al.*, 2000), XR5944 (Stewart *et al.*, 2001), XR11576 (Mistry *et al.*, 2002), XR11612 (Mistry *et al.*, 2001), TAS-103 (Utsugi *et al.*, 1997; figure 6.1), although the latter predominantly inhibits topoisomerase II α (Byl *et al.*, 1999). Most of these compounds are currently undergoing clinical trials (Denny & Baguley 2003). During the course of my PhD I have had access to a number of such experimental agents, as a part of collaboration with Xenova plc (Slough, Berkshire, UK). These compounds were XR11576 (MLN576; Millennium Pharmaceuticals) and its isomer XR11612. XR5000/DACA and TAS-103 were also supplied to enable comparative testing.

XR11576 demonstrated potent cytotoxic activity against a panel of human and murine tumour cell lines (IC_{50} = 6-47nM). This activity was unaffected by multidrug resistance (MDR) mediated by overexpression of either P-glycoprotein or MDR-associated protein (MRP), or by down regulation of topoisomerase II ((Mistry *et al.*, 2002). Importantly, XR11576 also showed marked efficacy against a number of human tumours including sensitive (H69/P) and multidrug-resistant (H69/LX4) small cell lung cancer and HT29 colon carcinoma xenografts (Mistry *et al.*, 2002). XR11576 was proposed as a dual inhibitor of topoisomerase I and II based on its ability to stabilise cleavable complexes for both topoisomerase I and II *in vitro* in a dose dependent fashion (Mistry *et al.*, 2002). Cleavable complex formation by XR11576 was also analysed in human leukaemic K562 cells using the 'Trapped in Agarose DNA Immunostaining' (TARDIS) assay (Jobson *et al.*, 2002 & 2003). In this assay equal volumes of drug-exposed cells and an agarose solution in PBS are mixed together and spread evenly across a microscope slide. The slides are cooled to 0°C to allow for agarose gelification and

lysed with a buffer containing sodium dodecyl sulfate. This traps DNA with covalently bound TOPO molecules. The slides are then exposed to a primary Ab directed towards a specific isoform of TOPO and a fluorescein isothiocyanate (FITC)-conjugated secondary Ab, which binds to the primary Ab. Areas occupied by DNA are defined using Hoechst dye blue fluorescence, and FITC green immunofluorescence within each defined DNA area is quantified using an imaging analysis software (Willmore *et al.*, 1998). Using this technique, Jobson *et al.* (2002, 2003) demonstrated XR11576-induced cleavable complex formation for topoisomerase I, II α and II β in a dose and time dependent manner. These observations, however, do not exclude additional or alternative mechanisms of action for the cytotoxic activity of XR11576. XR11612 is an isomer of XR11576, which has shown promising cytotoxic activity in tumour cell lines (Mistry *et al.*, 2001).

As XR11576 has recently entered clinical trials, it is important to demonstrate that the compound is effective against cells derived from clinical tumour samples. A broad *ex vivo* activity profile would strengthen further clinical development and generate information to help design Phase II trials.

In the present study we aimed to determine the *ex vivo* activity of XR11576 in a variety of solid tumours and compare its activity with other dual topoisomerase I/II inhibitors, including XR5000 and TAS-103, as well as other cytotoxics currently in clinical use.

6.2 Materials and Methods

Patients and Samples. A total of 89 tumours (44 solid tumours, 37 ascites, 8 pleural effusion) were tested with XR11576 (table 6.1). The ovarian cancer patients sub-group were all previously treated with carboplatin alone, or carboplatin plus taxane first line, followed in 5 cases by an anthracycline-containing regimen, in 4 cases by the combination treosulfan plus gemcitabine, in 3 cases by carboplatin plus gemcitabine and in 3 cases by etoposide.

ATP-TCA. The ATP-TCA was performed as detailed in chapter 2. XR11576 hydrochloride salt, XR11612, XR5000 and TAS-103 were supplied by Xenova Ltd (Slough, Berkshire, UK) as powder. They were dissolved in DMSO to give a stock solution of 1 mg/ml and aliquots were stored at -20 °C. TDCs were 145nM for XR11576, 290nM for XR11612, 1.45 µM for TAS-103 and 2.9 µM for XR5000. The TDCs for the experimental compounds were determined on the basis of cell lines data provided by Xenova Ltd. The other cytotoxics were tested at the concentrations listed in table 2.2. XR11576 was tested in every case, while the other compounds were tested only when number of cells allowed. We evaluated the activity of XR11576 in combination with paclitaxel or vinorelbine in a subgroup of tumours (n = 43). These samples consisted of 22 ovarian carcinomas (of which 12 had previous taxane exposure), 7 unknown primaries, 5 skin melanomas, 4 colorectal carcinomas, 3 breast carcinomas, 1 oesophageal carcinoma, and 1 sarcoma.

Combinations were made up by adding both drugs concurrently at their 200% TDC to the wells at the beginning of the assay and diluted in a constant ratio: sequential studies were not performed.

IHC. Of the 89 tumour samples studied, material for IHC was available for 40 tumours (12 skin melanomas, 10 ovarian carcinomas, 9 colorectal carcinomas, 3 oesophageal carcinomas, 3 unknown primaries, 2 breast carcinoma and 1 sarcoma). These were stained for MDR1, TOPO I and TOPO II α as described in Chapter 2.4.

Data analysis. Non-parametric statistical analysis was performed, the Wilcoxon two-tailed rank sum test for paired observations or the Mann-Whitney U test for unpaired data, as appropriate (Statsdirect). IC50 and IC90 values for XR11576 were compared to

those for other drugs using Spearman's rank correlation coefficient, with a Bonferroni correction: statistical significance was taken as $p < 0.005$.

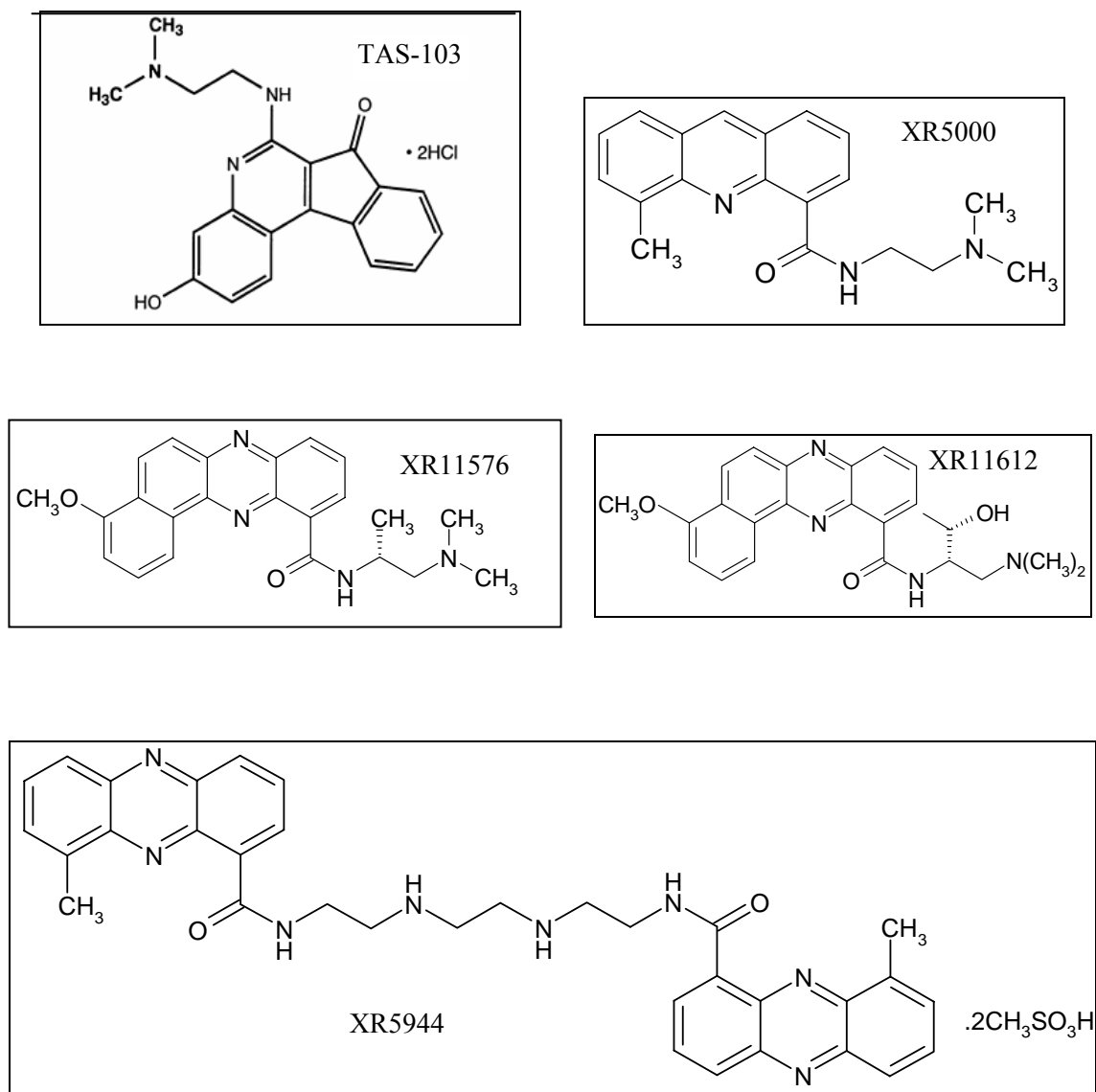


Figure 6.1 Chemical structures of the proposed dual topoisomerase inhibitors TAS-103, XR5000, XR5944, XR11576 and XR11612.

Table 6.1 Tumours tested with XR11576, showing the average age (range), sex ratio, and previous treatment.

Tumour type	n	Age	Sex	Previous treatment
Ovarian Carcinoma	42	58 (38-78)	0M:42F	Primary: platinum + taxane (n=25), platinum (n=17) Second-line: anthracycline (n=5), treosulfan+gemcitabine (n=4), etoposide (n=3), carboplatin+gemcitabine (n=3), mitoxantrone+paclitaxel (n=1)
Cutaneous Melanoma	14	58 (35-78)	7M:7F	Primary: melphalan (n=3), vindesine (n=2), cyclophosphamide (n=1)
Unknown primary	10	63 (45-78)	3M:7F	Primary: carboplatin (n=1)
Colorectal Carcinoma	9	71 (39-88)	4M:5F	Primary: irinotecan (n=1)
Breast Carcinoma	5	57 (39-61)	0M:5F	Primary: cyclophosphamide + anthracycline (n=3), antihormonal agent (n=2)
Oesophageal Carcinoma	3	55 (52-72)	3M:0F	Primary: Epirubicin + Cisplatin + 5-FU (n=2)
Endometrial Carcinoma	2	60 (55-66)	0M:2F	Primary: carboplatin (n=2) Second-line: paclitaxel (n=1)
Sarcoma	2	70 (57-82)	0M:2F	None
Lung (NSCLC)	1	58	0M:1F	Primary: cisplatin+vinorelbine (n=1)
Uveal Melanoma	1	67	0M:1F	None
TOTAL	89	60 (35-88)	17M:72F	

6.3 Results

6.3.1 Activity of XR11576 against tumour-derived cells

XR11576 showed a steep concentration response curve in most tumours. The effect of XR11576 varied both between tumour types and markedly between patients within the same tumour type (figure 6.2). Overall, XR11576 had median IC₅₀ and IC₉₀ values of 110nM and 242nM, respectively. The median IC₅₀ and IC₉₀ values calculated for each tumour type are shown in table 6.2. The IC₉₀ values well correlated with the IC₅₀ values ($r=0.893$, $p<0.0001$, non parametric Spearman correlation). In all samples XR11576 was more potent than other experimental or clinical chemotherapeutic agents, such as XR5000, TAS-103, paclitaxel, doxorubicin and topotecan (table 6.3).

XR11576 showed activity against a wide variety of different tumours. Unknown primaries, breast and gynaecological malignancies proved highly sensitive to this new agent, though GI tumours were less sensitive (figure 6.2). In particular, 37/42 (88%) ovarian cancer samples had an IC₅₀ value below 200nM and an IC₉₀ below 300nM. When compared to other agents used in second-line for ovarian cancer, XR11576 was at least 10-fold more potent than doxorubicin or topotecan (figure 6.1, table 6.3). The median IC₉₀ value for XR11576 in those samples from ovarian cancer patients who had received a taxane-based regimen ($n=26$) was 242nM compared to 155nM of samples from the small number of patients who had not been pre-exposed to taxanes ($n=16$), but this difference was not statistical significant ($p=0.108$, Mann-Whitney U-test).

The best activity (e.g. the lowest median IC₉₀ value) was observed in the small group of breast tumours tested ($n=5$). In these tumours, XR11576 had an IC₉₀ of 173nM, which was 170-fold more potent than the test results for paclitaxel. XR11576 showed remarkable activity against skin melanoma, a tumour type that is usually poorly sensitive to chemotherapy. In 11/14 (79%) melanoma samples XR11576 had an IC₅₀ value below 200nM and an IC₉₀ value below 300nM.

XR11576 showed decreased potency in colorectal and oesophageal samples. When all 12 GI tumours were analysed together, the median IC₉₀ and IC₅₀ were 308nM and 212nM (figure 6.2), and these values were significantly higher than the corresponding values in ovarian samples ($p<0.0005$, Mann-Whitney test). 5/9 (56%) colorectal samples had IC₅₀ values below 200nM, while all three oesophageal specimens had an

IC50 slightly over 200nM. However, GI samples were also very resistant to other drugs: in this tumour type we recorded the highest median IC50 and IC90 values for most drugs.

Table 6.2 Median IC90 and IC50 values (range) for XR11576 tested in the ATP-TCA in n=number of samples for each tumour type.

Tumour type	<i>n</i>	IC ₅₀ nM	IC ₉₀ nM
Ovarian carcinoma	42	97.0 (7.20-675)	212 (63.5-1215)
Skin melanoma	14	125 (5.80-229)	215 (42.0-400)
Unknown primary	10	111 (46.4-252)	244 (122-410)
Colorectal carcinoma	9	200 (33.4-338)	306 (242-608)
Breast carcinoma	5	109 (42.1-184)	173 (137-356)
Oesophageal carcinoma	3	213 (212-231)	310 (299-380)
Sarcoma	2	118 (14.8-222)	176 (65.2-287)
Endometrial carcinoma	2	125 (83.0-167)	237 (175-300)
Lung (NSCLC)	1	97.3	137
Uveal Melanoma	1	181	325
TOTAL	89	110 (5.80-675)	242 (42.0-1215)

Table 6.3 Median IC90 and IC50 values (range) for the cytotoxics tested in the ATP-TCA in n=number of samples of all tumour types.

Drug	<i>n</i>	IC ₅₀ µM	IC ₉₀ µM
XR11576 (MLN576)	89	0.110 (0.00580-0.675)	0.242 (0.0420-1.215)
XR11612	30	0.250 (0.0145-0.499)	0.505 (0.113-0.911)
XR5000	73	1.94 (0.174-5.13)	5.05 (0.870-7.26)
TAS-103	27	0.991 (0.0590-5.54)	2.79 (1.38-9.98)
Cisplatin	79	13.3 (0.430-80.4)	25.9 (7.57-145)
Treosulfan	76	39.4 (2.88-391)	116 (23.9-703)
Etoposide	47	74.2 (3.27-933)	177 (16.8-1679)
Topotecan	51	1.02 (0.0656-10.0)	3.60 (0.293-18.0)
Doxorubicin	73	1.10 (0.103-45.7)	2.47 (0.296-82.3)
Paclitaxel	82	8.78 (0.554-32.8)	25.4 (3.50-59.0)
Vinorelbine	58	2.17 (0.371-34.5)	16.8 (0.668-62.1)

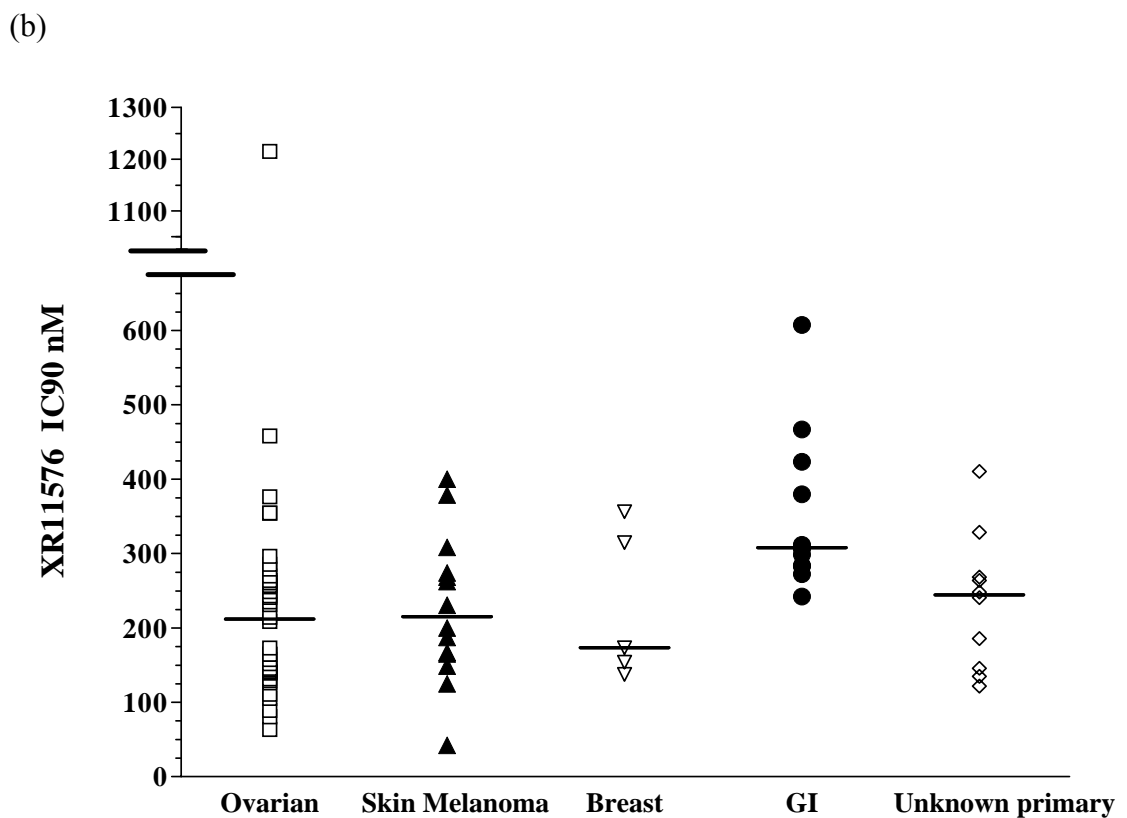
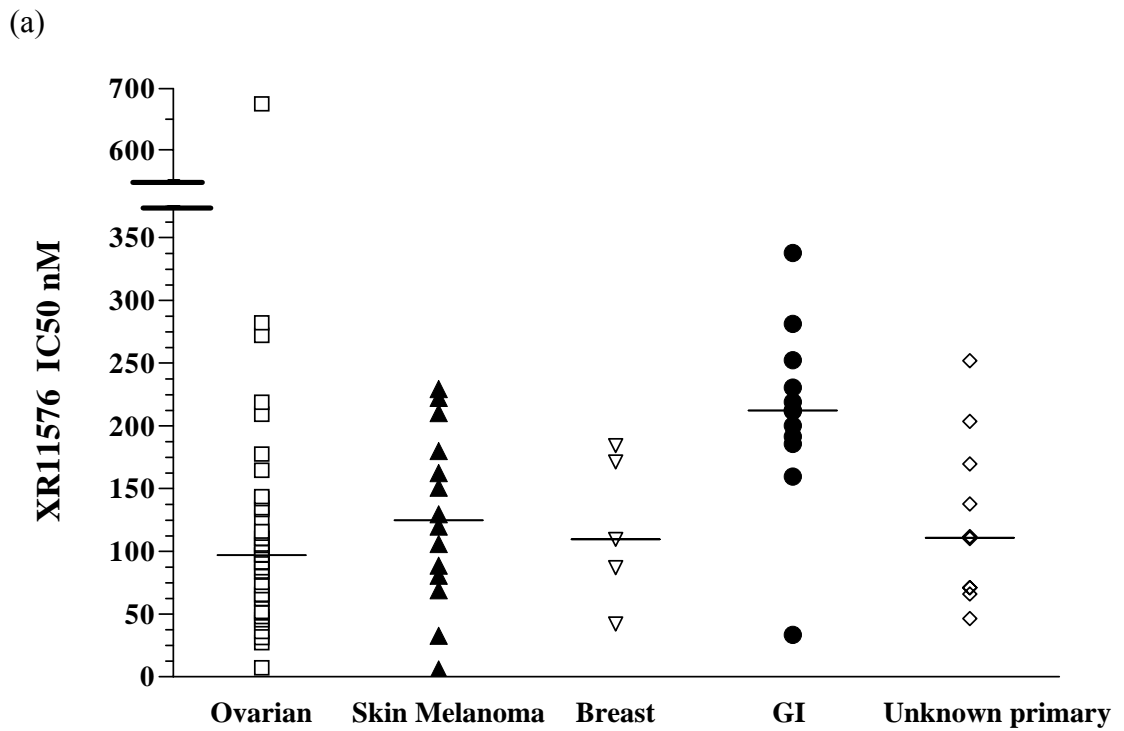


Figure 6.2 Heterogeneity of XR11576 sensitivity in ovarian samples (n=42), skin melanomas (n=14), breast samples (n=5), gastro-intestinal tumours (n=12) and tumours of unknown origin (n=10). Each symbol represents the IC50 (a) or IC90 (b) value for an individual patient. The horizontal lines represent median values.

6.3.2 Cross-resistance of XR11576 with other anticancer agents

When IC₅₀ values were analysed using Spearman's rank correlation coefficient (non-parametric method for paired data), cross-resistance was seen between XR11576 and the other experimental agents XR5000 ($r=0.609$, $p<0.0001$), XR11612 ($r=0.484$, $p<0.005$) and TAS-103 ($r=0.724$, $p<0.0001$). A significant correlation was also found with the other topoisomerase II inhibitors doxorubicin ($r=0.407$, $p<0.0005$) and etoposide ($r=0.428$, $p<0.005$). Some cross-resistance was seen with paclitaxel ($r=0.330$, $p<0.005$), while no correlation was observed between the IC₅₀ values for XR11576 and vinorelbine ($r=0.285$, NS), treosulfan ($r=0.258$, NS) and topotecan ($r=0.250$, NS) and cisplatin ($r=0.148$, NS).

The IC₉₀ values confirmed the cross-resistance pattern described above. A significant correlation was found between XR11576 and the topoisomerase inhibitors XR5000 ($r=0.582$, $p<0.0001$), XR11612 ($r=0.588$, $p<0.005$), TAS-103 ($r=0.556$, $p<0.005$), doxorubicin ($r=0.441$, $p<0.0001$) and etoposide ($r=0.389$, $p<0.005$). Some cross-resistance was still seen with paclitaxel ($r=0.325$, $p<0.005$), while no correlation was observed between the IC₉₀ values for XR11576 and vinorelbine ($r=0.240$, NS), cisplatin ($r=0.135$, NS), treosulfan ($r=0.106$, NS) or topotecan ($r=0.100$, NS).

6.3.3 Activity of XR11576 in combination with other anticancer agents

The effect of XR11576 in combination with other cytotoxics was studied by the method of Poch *et al.*, 1995 in which the observed effect at each concentration tested is compared with the expected effect. A median-effect analysis was also performed (Chou & Talalay 1984) and combination indices calculated for each sample tested with XR11576 combinations at 90% cell death (CI₉₀) and at 50% cell death (CI₅₀). Combination indices were also calculated for the median concentration-inhibition curve produced by each combination.

6.3.3.1 XR11576 and doxorubicin

The combination of XR11576 with doxorubicin showed a median CI₅₀ value of 1.03, therefore suggesting an additive effect (figure 6.3). Considerable heterogeneity was found among samples as shown in tables 4a and 4b. Synergism (CI<0.8) was identified in 6/38 (16%) and in 12/38 (32%) samples when CI₉₀ and CI₅₀ values were considered, respectively; while antagonism (CI₅₀>1.2) was found in 13/38 (34%) samples tested

with this combination, although this percentage increased to 47% when CI_{90} values were calculated. Additivity was identified in 13/38 (34%) samples on the basis of the CI_{50} and in 14/38 (37%) samples on the basis on the CI_{90} .

6.3.3.2 XR11576 and DNA damaging agents

The median CI_{50} values for the combinations of XR11576 with cisplatin or treosulfan were 1.19 and 1.37, while the median CI_{90} values were 1.22 and 1.38, respectively. As suggested by these high median CI values, antagonism between XR11576 and these two DNA damaging agents was identified in a large proportion of cases: CI_{50} values >1.2 were calculated in 28/40 (70%) and in 20/42 (48%) samples for the combinations with treosulfan or cisplatin, respectively. Synergism was found only in a minority of cases. When CI_{90} values for each individual tumour were considered, the combination of XR11576 with treosulfan showed synergism in only 1/41 (2%) samples ($CI_{90}=0.76$), while the addition of cisplatin to XR11576 did not produce any synergistic effect. On the basis of the CI_{50} values, the combinations of XR11576 with treosulfan or cisplatin showed synergism in 3/40 (8%) and in 5/42 (12%) samples, respectively.

6.3.3.3 XR11576 and microtubule-interfering agents

The median CI_{50} values for the combinations of XR11576 with vinorelbine or paclitaxel were 1.11 and 2.12, while the median CI_{90} values were 1.11 and 1.51, respectively. The best effect was obtained with the combination of XR11576 + vinorelbine, which was better than XR11576 alone in 30/31 paired observations ($p<0.0001$ on IC_{50} or IC_{90} , Wilcoxon matched-pairs signed-ranks test). Figure 6.4a shows the advantage of the combination XR11576 and vinorelbine over the individual agents in terms of inhibition; when analysed by the method of Poch et al., 1995, some synergism was seen at the lower concentrations, while additivity was observed at the highest dose (figure 6.4b). This is reflected by the combination indices calculated by the Chou and Talalay method on the median dose-response curve, which were 0.45 (synergy) and 0.86 (additivity) for an effect of 50% and 90% inhibition, respectively. CI_{50} and CI_{90} values >1.2 (antagonism) were found, respectively, in 38/42 (90%) and in 31/43 (72%) samples tested with the combination of XR11576 with paclitaxel. According to Poch, a median antagonistic effect at the lower concentrations between these 2 drugs was also found (figure 6.5). Some additivity was found at the higher concentrations as shown by the Poch method (figure 6.5) and by the analysis of the CI_{90} values (table 6.4b).

Table 6.4 Effect of combining XR11576 with other cytotoxics using the Chou and Talalay Combination Index (a) for 50% cell death (CI₅₀) and (b) for 90% cell death (CI₉₀) calculated in *n*= number of cases.

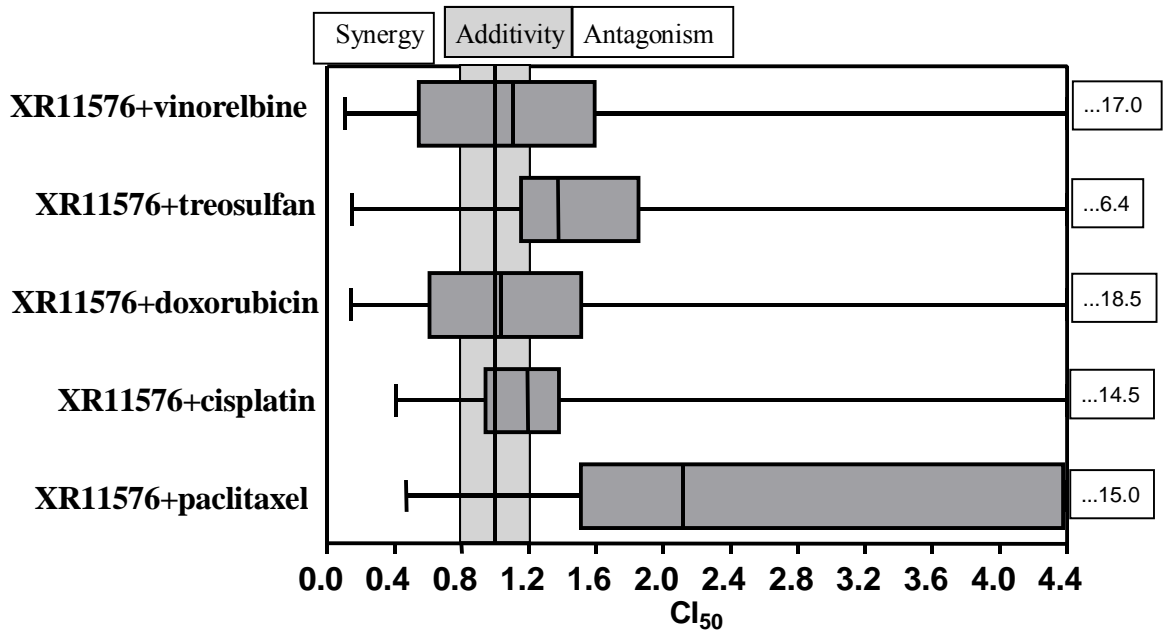
(a)

Tumour type	XR11576 and	Number of samples tested	Combination Index (<i>n</i>) CI ₅₀		
			<0.8	0.8-1.2	>1.2
All tumours	paclitaxel	42	3	1	38
All tumours	vinorelbine	30	9	9	12
All tumours	treosulfan	40	3	9	28
All tumours	cisplatin	42	5	17	20
All tumours	doxorubicin	38	12	13	13
Ovarian	paclitaxel	22	0	1	21
Ovarian	vinorelbine	17	4	5	7
Ovarian	treosulfan	21	1	2	18
Ovarian	cisplatin	22	2	6	14
Ovarian	doxorubicin	22	7	8	7
Non-ovarian	paclitaxel	20	3	0	17
Non-ovarian	vinorelbine	14	5	4	5
Non-ovarian	treosulfan	19	2	7	10
Non-ovarian	cisplatin	20	3	11	6
Non-ovarian	doxorubicin	16	5	5	6

(b)

Tumour type	XR11576 and	Number of samples tested	Combination Index (<i>n</i>) CI ₉₀		
			<0.8	0.8-1.2	>1.2
All tumours	paclitaxel	43	2	10	31
All tumours	vinorelbine	31	7	15	9
All tumours	treosulfan	41	1	17	23
All tumours	cisplatin	42	0	19	23
All tumours	doxorubicin	38	6	14	18
Ovarian	paclitaxel	22	1	4	17
Ovarian	vinorelbine	17	5	9	3
Ovarian	treosulfan	22	0	7	15
Ovarian	cisplatin	22	0	8	14
Ovarian	doxorubicin	22	5	6	11
Non-ovarian	paclitaxel	21	1	6	14
Non-ovarian	vinorelbine	14	2	6	6
Non-ovarian	treosulfan	19	1	10	8
Non-ovarian	cisplatin	20	0	11	9
Non-ovarian	doxorubicin	16	1	8	7

(a)



(b)

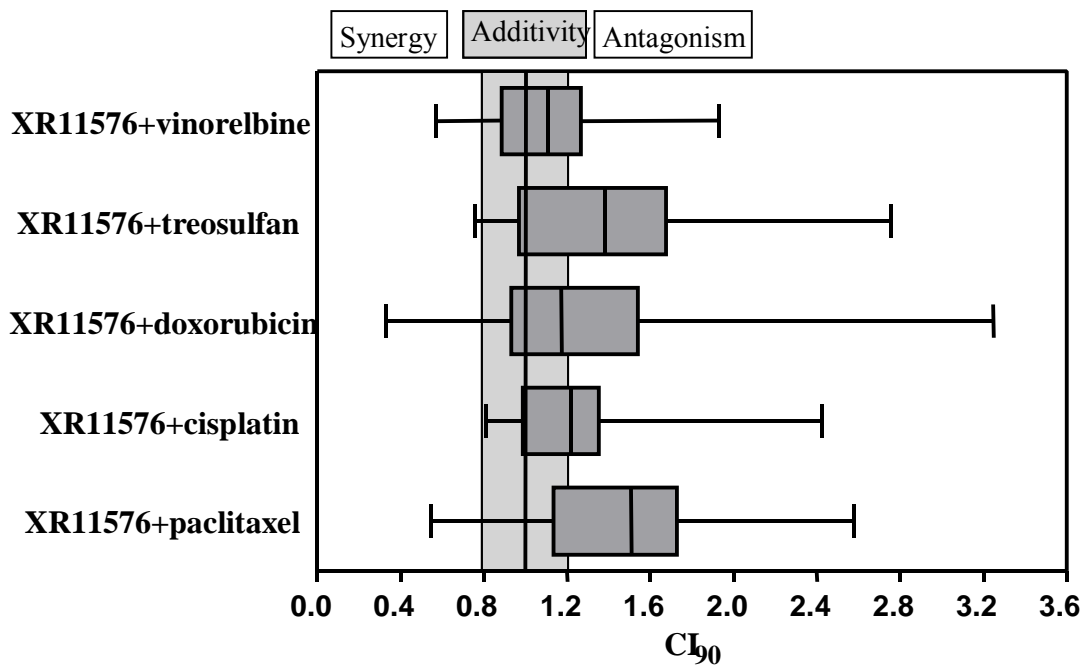
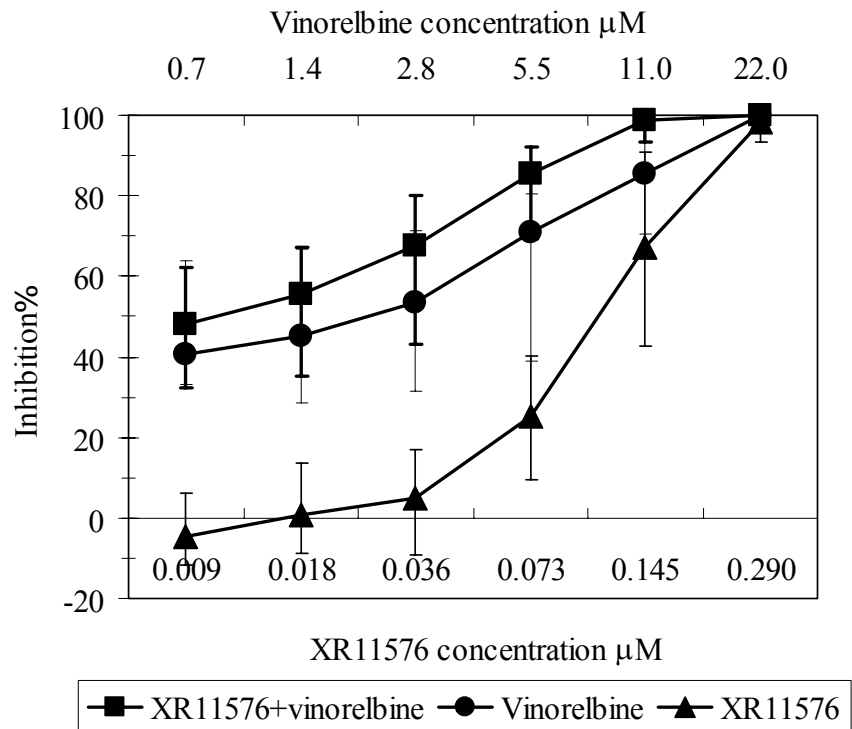


Figure 6.3 Summary of the combination indices values calculated for (a) 50% (CI₅₀) and (b) 90% cytotoxicity (CI₉₀) in tumour derived cells treated with the combinations shown. For each combinations the interquartile range (grey histograms), the median and the range are shown. In graph a) the figures to the right indicate off-scale values.

(a)



(b)

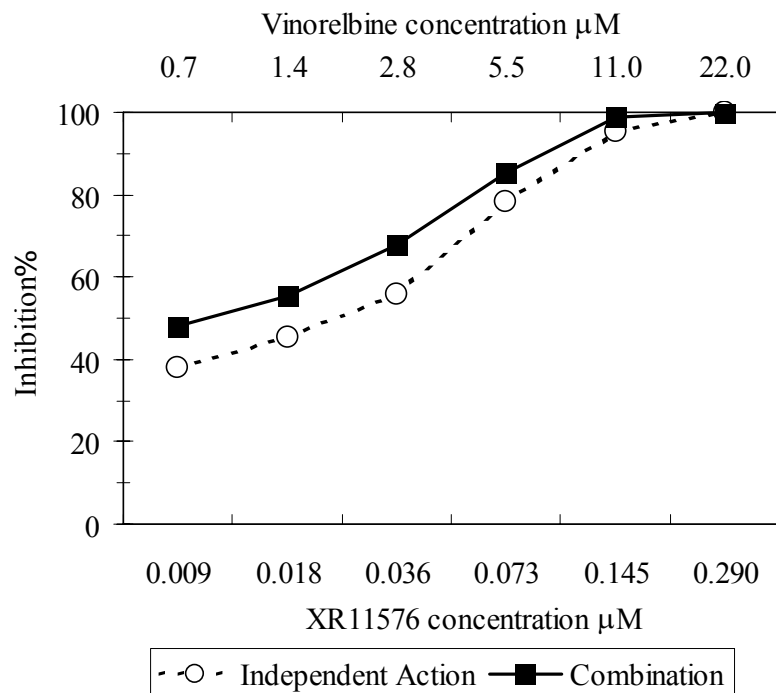
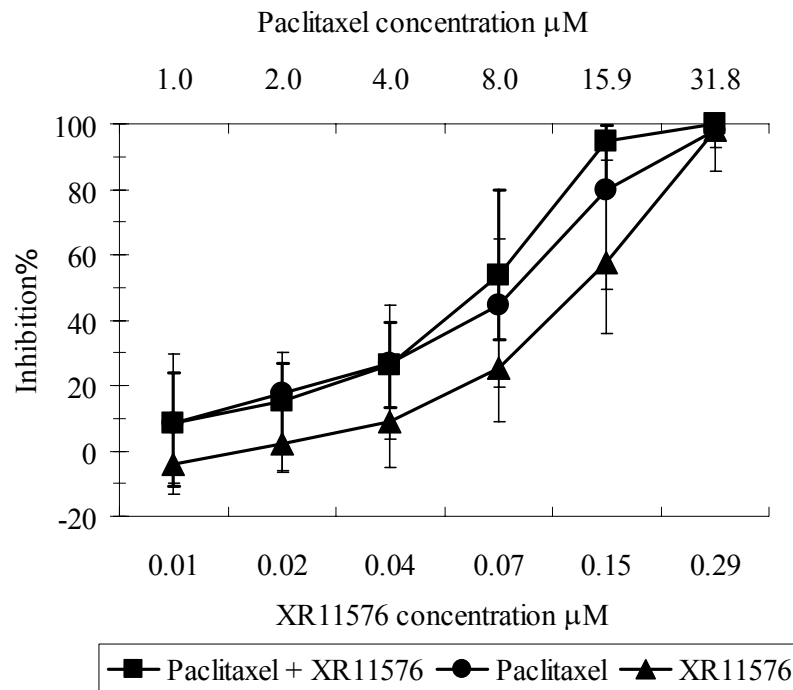


Figure 6.4 (a) Median activity (\pm interquartile range) of XR11576 in combination with vinorelbine in $n=31$ samples. (b) Combination analysis (Poch *et al.*, 1995) showing a greater than additive effect. When analysed by the Chou and Talalay method (1984) a $CI_{50}=0.45$ (synergy) and a $CI_{90}=0.86$ (additivity) were calculated.

(a)



(b)

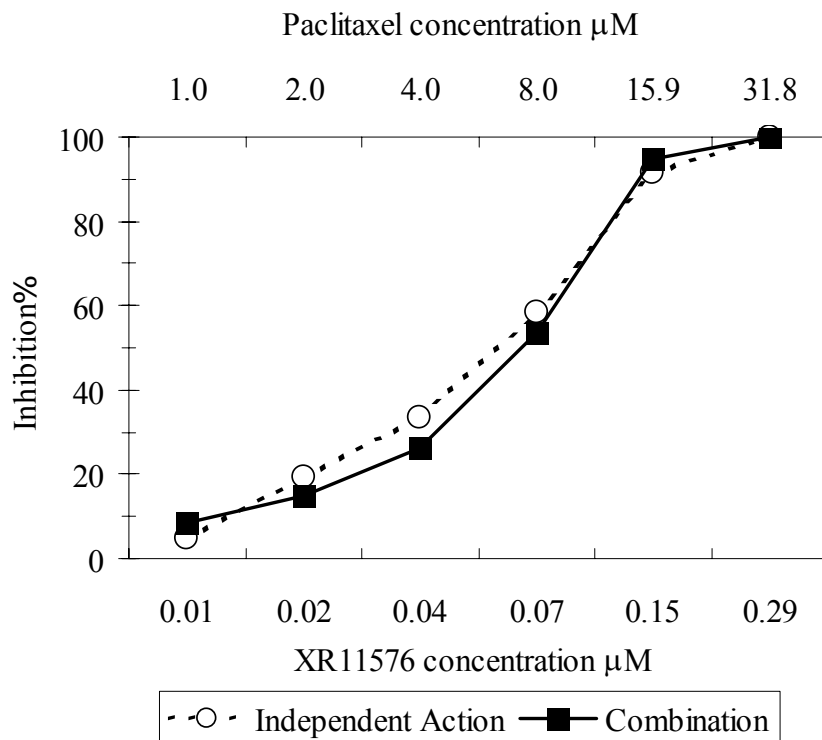


Figure 6.5 (a) Median activity (\pm interquartile range) of XR11576 in combination with paclitaxel in $n=43$ samples. (b) Combination analysis (Poch *et al.*, 1995) showing a slightly antagonistic effect. When analysed by the Chou and Talalay method (1984) a $CI_{50}=2.26$ (antagonism) and a $CI_{90}=1.09$ (additivity) were found.

6.3.3.4 Correlation of XR11576 activity with immunohistochemistry

Immunostaining for P-gp was positive in 14/40 (35%) samples tested with XR11576. The P-gp expressing samples consisted of 4 ovarian tumours, 3 skin melanomas, 3 oesophageal cancers, 2 colorectal tumours and 2 unknown primary carcinomas. The median XR11576 IC₅₀ values for P-gp negative and positive samples were 140nM and 209nM, respectively (p=0.0488, Mann-Whitney U test), while the median IC₉₀ values were 263 and 308 nM, respectively (p=0.098, ns, Mann-Whitney U test). No correlation by linear regression analysis was found between the IC₅₀ of XR11576 or the IC₉₀ and the intensity of P-gp staining.

Immunostaining for topoisomerase I and II α was positive in 35/40 (87%) and 9/40 (22%) samples tested with XR11576, respectively. Those samples that were positive for topoisomerase I included 13 ovarian tumours, 8 skin melanomas, 6 colorectal tumours, 3 unknown primary carcinomas, 2 oesophageal cancers, 2 breast tumours and 1 sarcoma. The topoisomerase II α expressing samples consisted of 6 colorectal tumours, 2 ovarian tumours and 1 skin melanoma. The median XR11576 IC₅₀ values for topoisomerase I negative and positive samples were 162nM and 135nM, respectively (p=0.7185, NS, Mann-Whitney U test), while the median IC₉₀ values were 274 and 262 nM, respectively (p=0.8368, NS, Mann-Whitney U test). The median XR11576 IC₅₀ values for topoisomerase II α negative and positive samples were 133nM and 191nM, respectively (p=0.154, NS, Mann-Whitney U test), while the median IC₉₀ values were 261nM and 283nM, respectively (p=0.3226, NS, Mann-Whitney U test). No correlation by linear regression analysis was found between the IC₅₀ or IC₉₀ values for XR11576 and either the topoisomerase I or the topoisomerase II α immunohistochemistry indices (p=NS).

6.4 Discussion

The *ex vivo* profile of XR11576 in human solid tumours has confirmed it to be an exceptionally potent cytotoxic agent. In all samples tested, XR11576 was more active than all other compounds tested, including topotecan, doxorubicin, paclitaxel and the experimental agents XR5000 and TAS-103. As expected, the potency of XR11576 was less in tumour-derived cells compared with cell lines, which probably reflects the homogeneity and the fast-growing status of the cell lines used in previous studies

(Mistry *et al.*, 2002). This is true of most cytotoxic drugs (Andreotti *et al.*, 1994; see chapter 4). The effect of XR11576 varied considerably between tumour types as well as against samples from the same tumour type derived from different patients. This is consistent with both the situation found in the clinic and also other previous findings involving the testing of conventional cytotoxics (Cree *et al.*, 1999; Whitehouse *et al.*, 2003; Mercer *et al.*, 2003).

XR11576 showed considerable activity in the subset of ovarian cancer samples. The fact that a large proportion of samples obtained from heavily pre-treated ovarian cancer patients do not appear resistant to XR11576 augurs well for its use as second-line therapy in this clinical condition.

Furthermore, XR11576 activity is only slightly reduced in GI tumours that are well known to overexpress MDR1 and other pump proteins (Cordon-Cardo *et al.*, 1990), and are generally refractory to chemotherapy (Gottesman *et al.*, 2002). This is in accordance with previous studies that have shown XR11576 is not a substrate for either P-gp or MRP efflux pumps (Mistry *et al.*, 2002). Data from this study suggest that P-gp plays little, if any, role in resistance to XR11576.

Previous chemosensitivity studies have reported reduced levels of Topoisomerase II α to correlate with *in vitro* resistance to topoisomerase inhibitors such as doxorubicin and etoposide (Matsumoto *et al.*, 1997; Koshiyama *et al.*, 2001). However our data showed that XR11576 activity does not correlate with the expression of either topoisomerase I or II α . On the other hand, there is cross-resistance with topoisomerase inhibitors, including doxorubicin, TAS-103, and XR5000. These findings are therefore consistent with inhibition of both enzymes, though we cannot exclude an alternative mechanism of action, as recently suggested (Fleming *et al.*, 2003).

Previous *in vitro* and *ex vivo* studies have reported enhanced cell kill when topoisomerase inhibitors were combined with vinca alkaloids (Bahadori *et al.*, 2001; Barret *et al.*, 2002; Di Nicolantonio *et al.*, 2002; see chapter 8). While an increased effect was not found for all samples tested, individual cases that displayed true synergism between XR11576 and vinorelbine were apparent in our series. Despite some cross-resistance between XR11576 and doxorubicin, we found synergism between these

two drugs in a proportion of samples, suggesting that non-topoisomerase mediated effects of the anthracycline may be important for cytotoxicity. Antagonism was found between XR11576 and cisplatin or treosulfan; this is in contrast with other *ex vivo* studies that reported some synergism between cisplatin and topoisomerase inhibitors (Jonsson *et al.*, 1998; Neale *et al.*, 2000; Sargent *et al.*, 2003). Substantial antagonism was noted between XR11576 and paclitaxel. These results are consistent with several *in vitro* studies that have shown an antagonistic effect when paclitaxel is combined with certain non-anthracycline topoisomerase inhibitors, such as etoposide, topotecan, SN38 and tafluposide (Kaufmann *et al.*, 1996; de Jonge *et al.*, 1998; Kano *et al.*, 1998; Ma *et al.*, 1998; Perez *et al.*, 1998; Barret *et al.*, 2002), although others reported additive or synergistic effects in different cell lines (Chou *et al.*, 1994; Jonsson *et al.*, 1998; Bahadori *et al.*, 2001).

Chemosensitivity testing using fresh tumour cells taken directly from patients is not commonly used for pre-clinical studies at present. It has been proposed, nevertheless, that it provides useful information on the cellular sensitivity of novel compounds before they reach more expensive phase I/II trials (Cree, 2003). The results of this study may assist the further clinical development of XR11576. If phase I studies prove the safety of this compound, then these results suggest that phase II trials should target those clinical conditions (breast, ovarian cancer, and cutaneous melanoma) where this new agent is likely to produce the greatest benefit.

Chapter 7 - The *ex vivo* characterisation of XR5944 (MLN944) against a panel of human clinical tumour samples

7.1 Introduction

The novel bis-phenazine XR5944 (MLN944) is an extremely potent cytotoxic agent both *in vitro* and *in vivo* (Stewart *et al.*, 2001). Against a panel of human cell lines *in vitro* the IC₅₀ of XR5944 was 0.04-0.4 nM and this potency translated well to human xenograft models *in vivo* where XR5944 induced complete tumour regression in the H69 SCLC model (Stewart *et al.*, 2001). Although XR5944 originated from a programme to generate dual topoisomerase (TOPO) I and II inhibitors (Gamage *et al.*, 2001) recent data suggest that cell death is not mediated *via* TOPO inhibition, but the precise mechanism of action is still being elucidated. XR5944 has been reported to bind strongly and intercalate into DNA (Gamage *et al.*, 2001) and can stabilise TOPO-dependent cleavage complexes as visualised by electrophoresis using linearised labelled plasmid DNA and purified TOPO I and II. XR5944 also induced cleavage complex formation for TOPO I and II (α and β) in human leukaemic K562 cells visualised using the TARDIS assay (Jobson *et al.*, 2002). Although these observations suggested a TOPO-mediated mechanism for XR5944, the increase in enzyme-mediated DNA cleavage required relatively high concentrations of XR5944 and, in the K562 cells, long incubation times. Furthermore, data have been presented recently demonstrating a TOPO-independent mechanism of action for XR5944. In yeast models, the cytotoxicity of XR5944 was not dependent on the presence of either TOPO I or II and the potency was not attenuated in mutant strains unable to repair double strand DNA breaks (Fleming *et al.*, 2003). Cell cycle analysis also differentiated XR5944 from both TOPO I and II inhibitors as XR5944 treatment induced a G1 and G2 arrest in contrast to the G2/M arrest noted with either doxorubicin (a TOPO II inhibitor) or camptothecin (a TOPO I inhibitor) (Freathy *et al.*, 2003). Finally, functional genomic data has also differentiated XR5944 from known TOPO inhibitors. Transcript profiling of XR5944 in yeast cells indicated up-regulated expression of RNA polymerase subunits, as well as genes involved in rRNA processing, but importantly, DNA damage response genes appeared to be unaffected (Fleming *et al.*, 2003; Sappal *et al.*, 2004).

Previous *in vitro* studies showed that XR5944 is probably a substrate for both the multidrug resistance-associated protein (MRP) and P-glycoprotein (P-gp) (Stewart *et al.*, 2001), the product of the multidrug resistance gene (MDR1). Although the potency of XR5944 is somewhat attenuated in cell lines overexpressing these drug efflux

proteins, XR5944 remains a very active cytotoxic with IC50 values similar to or better than other chemotherapeutic agents such as topotecan and paclitaxel in drug resistant cells.

As part of ongoing collaboration with Xenova plc (Slough, Berkshire, UK), during the course of my PhD studies, I had access to XR5944. As this compound has very recently entered clinical trials, it is important to demonstrate that the compound is effective against cells derived from clinical tumour samples. Therefore in the present study I aimed to determine the *ex vivo* activity of XR5944 in a variety of solid tumours tested in the ATP-TCA.

7.2 Materials and Methods

Patients and Samples. A total of 90 tumours (44 ovarian, 9 colorectal, 5 breast, 3 oesophageal and 2 endometrial carcinomas, 14 cutaneous melanomas, 1 sarcoma, 1 NSCLC and 10 carcinomas of unknown primary origin) were tested with XR5944, with local ethics committee approval for the use of tissue or cells not required for diagnosis. The median age of the patients was 59 years (range 35-88; 17M:73F). The ovarian cancer subgroup patients were all previously treated with carboplatin alone, or carboplatin plus taxanes first line, followed in 4 cases by an anthracycline-containing regimen and in 2 cases by etoposide. A drug resistant CCRF-CEM sub-line was obtained by step-wise treatment with XR5944 up to 10 nM (for details see section 2.3.4), and was also tested.

ATP-TCA. The ATP-TCA was performed as described in chapter 2. XR5944 bis-mesylate salt was supplied by Xenova Ltd (Slough, Berkshire, UK) as powder. It was dissolved in DMSO to give a stock solution of 1 mg/ml and aliquots were stored at -20 °C. The TDC for XR5944 was 72.5nM, while all other cytotoxics were tested at the concentrations listed in chapter 2. XR5944 was tested in every case, while not all the other compounds were tested for all samples, as preference to clinically relevant drugs for each tumour type was given.

Immunohistochemistry (IHC). Of the 90 tumour samples studied, material for IHC was available for 32 tumours (12 skin melanomas, 10 ovarian carcinomas, 3 colorectal carcinomas, 3 oesophageal carcinomas, 2 unknown primaries, 1 breast carcinoma and 1

sarcoma). These were stained for MDR1, TOPO I and TOPO II α as described in Chapter 2.4.

qRT-PCR. Total RNA was extracted from at least 10⁷ CCRF-CEM cells and reverse-transcribed as described in chapter 2.5. The expression levels of BCRP, MDR1, MRP1, MRP2, MVP, TOPO I, TOPO II α and TOPO II β were measured in reference to HPRT1, PBGD and TBP in both the parental and the drug selected sub-line. The following formula was used to calculate the relative amount of the transcript in the sample: $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in Ct between the gene of interest and the mean of the 3 reference genes, and $\Delta\Delta Ct = \Delta Ct$ of the parental CCRFCEM line - ΔCt of XR5944-resistant subline.

Data analysis. IC50 and IC90 values for XR5944 were compared to those for other drugs using Spearman's rank correlation coefficient, with a Bonferroni correction: statistical significance was taken as $p < 0.005$.

7.3 Results

7.3.1 Activity of XR5944 against tumour-derived cells

The concentration response curve of XR5944 was steep in most tumours, as displayed in the ovarian cancer sample shown in figure 7.1. XR5944 exhibited potent cytotoxic activity in the nanomolar range for the vast majority of samples tested (table 7.1). Overall, XR5944 had median IC50 and IC90 values of 26 nM and 68 nM, respectively (table 7.1). Notably, in all samples XR5944 was more potent than other common chemotherapeutic agents currently used in the clinic (table 7.1 and figure 7.1). The IC90 of XR5944 was above the 1.0 μ M threshold in only 1/90 sample (table 7.1): this ovarian tumour was relatively refractory to chemotherapy, as shown also by the extremely high IC90 and IC50 values for doxorubicin (53.8 μ M and 29.9 μ M, respectively, table 7.1).

XR5944 demonstrated its activity on a wide variety of different tumours. Unknown primaries, breast and gynaecological malignancies proved highly sensitive to this new agent. In particular, an IC50 value below 50nM was calculated in 37/44 (84%) ovarian cancer samples and an IC90 below 100nM in 33/44 (75%) specimens. When compared to other agents used in second-line therapy for ovarian cancer, XR5944 was at least 40-fold more potent than doxorubicin or topotecan.

The best median activity was observed in the small group of breast tumours tested (n=5), in which XR5944 had an IC₅₀ of 23 nM and an IC₉₀ of 55 nM. In this tumour type XR5944 had an IC₅₀ value 340-fold better than paclitaxel.

XR5944 retained excellent activity in skin melanoma, a tumour that is usually poorly sensitive to chemotherapy. In 11/14 (79%) melanoma samples we found an IC₅₀ value below 50nM and in 10/14 (71%) an IC₉₀ value below 100nM.

XR5944 had slightly decreased potency in colorectal and oesophageal samples, in which the median IC₅₀ values were, respectively, 126 nM and 66 nM. Only 2/9 (22%) colorectal samples had an IC₅₀ value below 50nM, and, none had IC₉₀ values below 100 nM. However, in colorectal samples we recorded the highest median IC₅₀ and IC₉₀ values of most of the tested drugs (table 7.1).

Table 7.1(a). Median IC90 and IC50 values (range) for XR5944 tested in the ATP-TCA in n=number of samples for each tumour type.

Tumour type	n	IC50 nM	IC90 nM
Ovarian carcinoma	44	22.9 (2.90-253)	59.6 (16.0-1315)
Skin melanoma	14	31.2 (2.18-64.3)	71.0 (15.2-157)
Unknown primary	10	29.0 (7.25-54.3)	65.7 (29.0-125)
Colorectal carcinoma	9	126 (8.70-221)	249 (160-399)
Breast carcinoma	5	23.3 (3.97-76.0)	55.5 (35.5-164)
Oesophageal carcinoma	3	65.9 (16.7-97.2)	178 (69.6-197)
Sarcoma	2	34.4 (11.6-57.3)	115 (110-120)
Endometrial carcinoma	2	25.1 (24.2-25.9)	97.0 (43.6-150)
Lung (NSCLC)	1	17.1	33.1
TOTAL	90	26.5 (2.18-253)	68.2 (15.2-1315)

Table 7.1 (b). Median IC90 and IC50 values (range) for XR5944 and other cytotoxics tested in the ATP-TCA in n=number of samples of all tumour types.

Drug	n	IC50 μ M	IC90 μ M
XR5944 (MLN944)	90	0.0265 (0.00218-0.253)	0.0682 (0.0152-1.32)
Cisplatin	81	13.4 (0.430-80.4)	25.9 (7.57-145)
Treosulfan	77	39.6 (2.9-390.8)	116.4 (23.9-703.4)
Etoposide	49	74.2 (3.27-932)	177 (16.8-1678)
Topotecan	53	1.00 (0.0656-10.0)	3.62 (0.293-18.0)
Doxorubicin	74	1.20 (0.103-45.7)	2.49 (0.296-82.3)
Paclitaxel	84	8.99 (0.554-32.8)	25.4 (3.50-59.0)
Vinorelbine	58	2.17 (0.371-34.5)	16.8 (0.668-62.1)

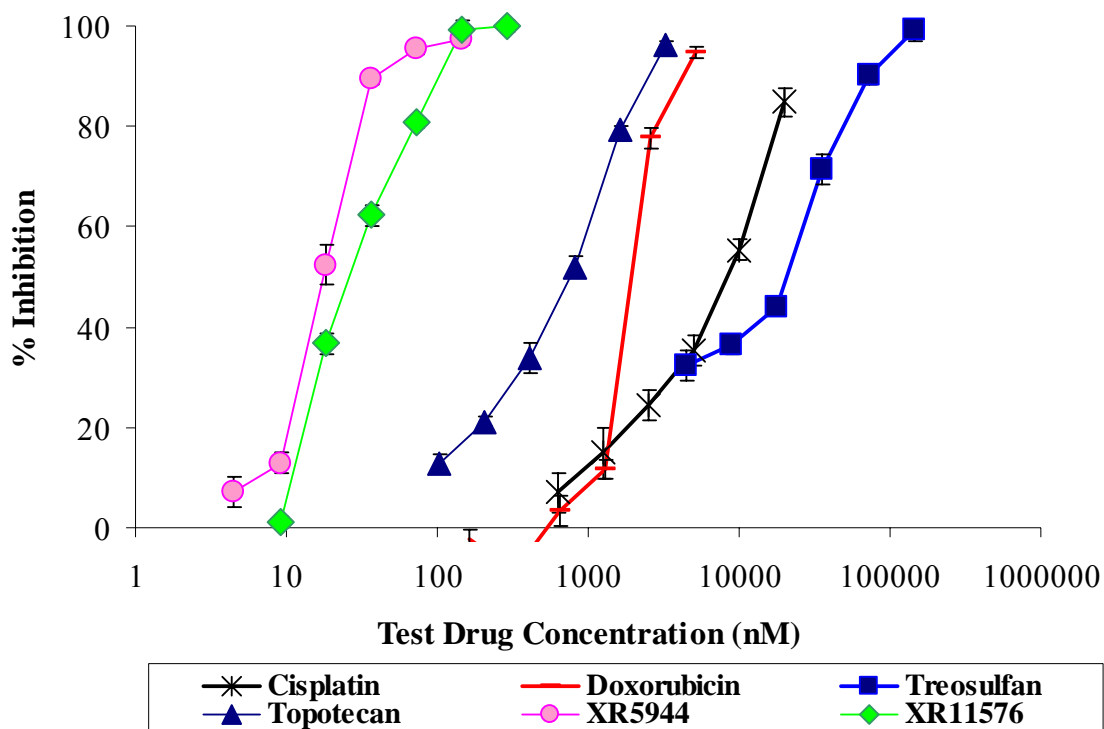


Figure 7.1 Representative example of ATP-TCA results in an ovarian tumour. XR5944 shows a steep concentration-inhibition curve and is at least 10-fold more potent than the other conventional cytotoxics tested.

7.3.2 Cross-resistance of XR5944 with other anticancer agents

When IC₅₀ and IC₉₀ values were analysed using Spearman's rank correlation coefficient (table 7.2) cross-resistance was clearly seen only between XR5944 and doxorubicin ($r=0.426$ and $r=0.468$, respectively, both $p<0.0001$). A modest correlation was also found between the IC₉₀ values of XR5944 and those of vinorelbine ($r=0.352$, $p<0.0034$), although not between the IC₅₀ values (table 7.2). No statistically significant correlation was seen between XR5944 and cisplatin, treosulfan, paclitaxel, etoposide or topotecan (table 7.2).

Table 7.2 Cross-resistance patterns between XR5944 and other cytotoxics. The IC₉₀ and IC₅₀ values for XR5944 were compared to those for other drugs using Spearman's rank correlation coefficient (non-parametric method for paired data) with a Bonferroni's correction for the number of correlations ($n=14$). Statistical significance was taken at $p<0.0036$.

Drugs XR5944 vs	Samples tested (n)	IC ₉₀ values		IC ₅₀ values	
		Spearman's correlation coefficient R	p	Spearman's correlation coefficient R	p
Cisplatin	81	0.1022	ns (0.1819)	0.1524	ns (0.0914)
Treosulfan	77	0.1816	ns (0.0570)	0.2518	ns (0.0136)
Doxorubicin	74	0.4676	<0.0001	0.4256	<0.0001
Etoposide	49	0.1070	ns (0.2322)	0.08017	ns (0.2920)
Topotecan	53	0.2500	ns (0.0355)	0.2469	ns (0.0388)
Paclitaxel	84	0.2068	ns (0.0296)	0.1211	ns (0.1392)
Vinorelbine	58	0.3515	<0.0034	0.2802	ns (0.0182)

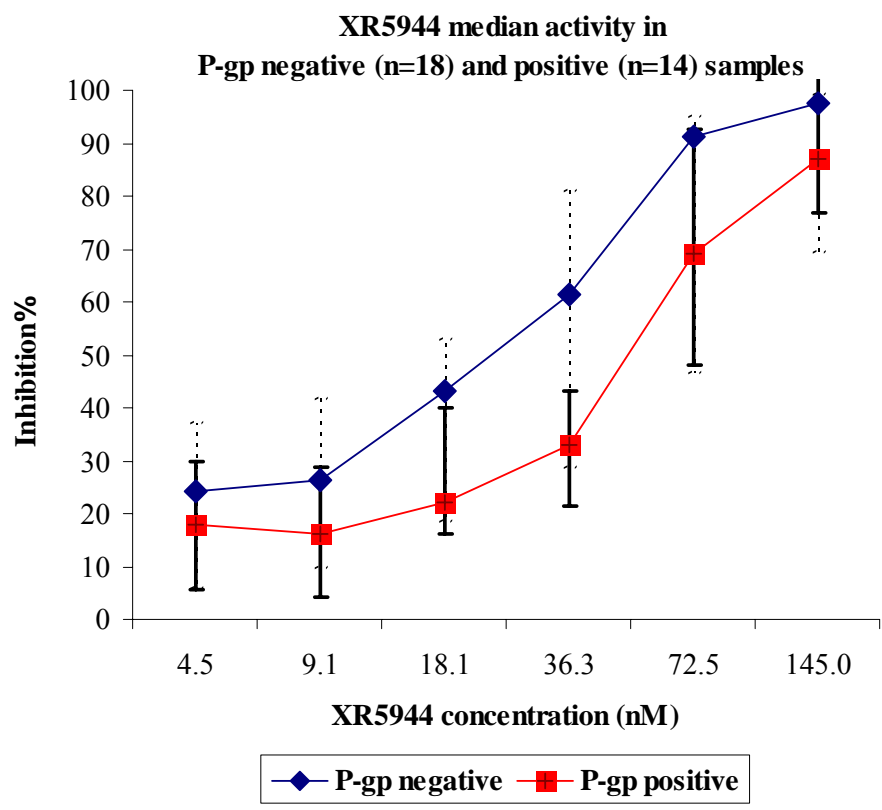
7.3.3 Correlation of XR5944 activity and immunohistochemical data

Immunostaining for P-gp was positive in 14/32 (44%) samples tested with XR5944. The P-gp expressing samples consisted of 5 ovarian tumours, 3 skin melanomas, 3 oesophageal cancers, 2 colorectal tumours and 1 unknown primary carcinoma. The median XR5944 IC₅₀ values for P-gp negative and positive samples were 23 and 58 nM, respectively ($p < 0.05$, Mann-Whitney U test, $p = 0.0143$), while the median IC₉₀ values were 68 and 142 nM, respectively ($p < 0.05$, Mann-Whitney U test, $p = 0.0113$). The expression of P-gp shifted to the right the drug-response curve of XR5944 (figure 7.2-a). A positive correlation by linear regression analysis was found between P-gp staining and the IC₅₀ ($r = 0.65$, $p < 0.0001$) and the IC₉₀ ($r = 0.54$, $p < 0.005$) for XR5944 (figure 7.2-b), but not with either the TOPO I or II α IHC indices ($p = \text{NS}$; data not shown). It must be pointed that the highly statistically significant correlation in Fig. 7.2b ($p < 0.0001$) might have been biased by the presence of three P-gp strongly positive tumours which showed high IC₉₀ and IC₅₀ values for XR5944.

7.3.4 Activity of XR5944 against CCRF-CEM cell lines

In the ATP-TCA the IC₅₀ values for XR5944 were 0.27 ± 0.05 nM and 4.24 ± 0.25 nM in the CCRFCEM parental and XR5944-resistant sub-lines, respectively. The changes in gene expression profile of various MDR transporters and the TOPO enzymes between the parental and the resistant sub-line were investigated by qRT-PCR in three experiments. The qRT-PCR technique produced extremely reproducible results with an intra- and inter-assay coefficient of variation of $< 1.5\%$ and $< 5\%$, respectively. The XR5944-line showed a 10-fold and a greater than 100-fold increase in mRNA levels of MVP and MDR1, respectively, when compared to the parental line (Figure 7.3). qRT-PCR also showed a modest increase in the expression of MRP-1 ($2^{-\Delta\Delta C_t} = 2.0$) in the resistant line. The levels of BCRP, MRP2 and the TOPO isoforms were essentially unchanged in the resistant sub-line.

(a)



(b)

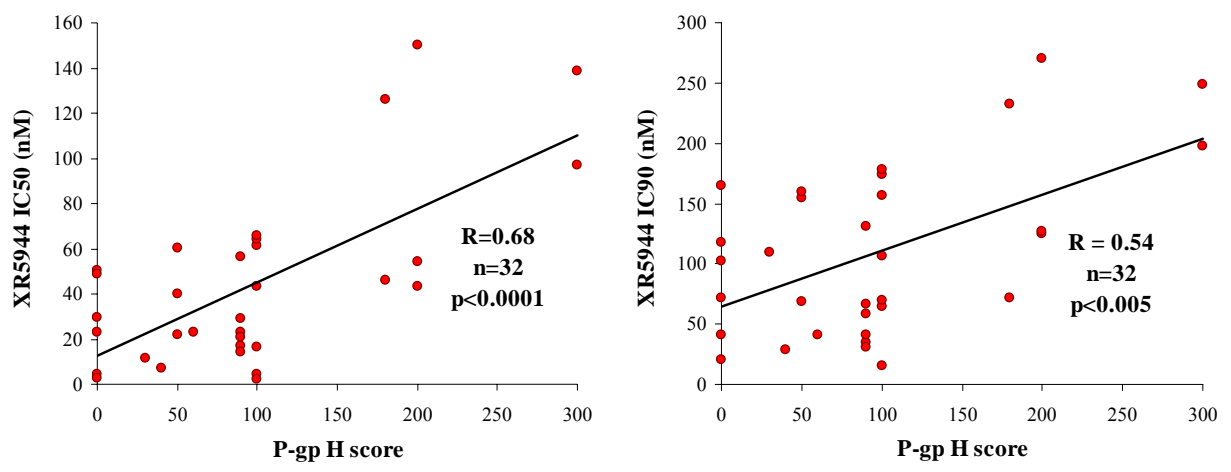


Figure 7.2 (a) Influence of P-gp status on XR5944 activity (median \pm interquartile range); (b) correlation between XR5944 IC50/IC90 (nM) and the intensity of P-gp immunostaining.

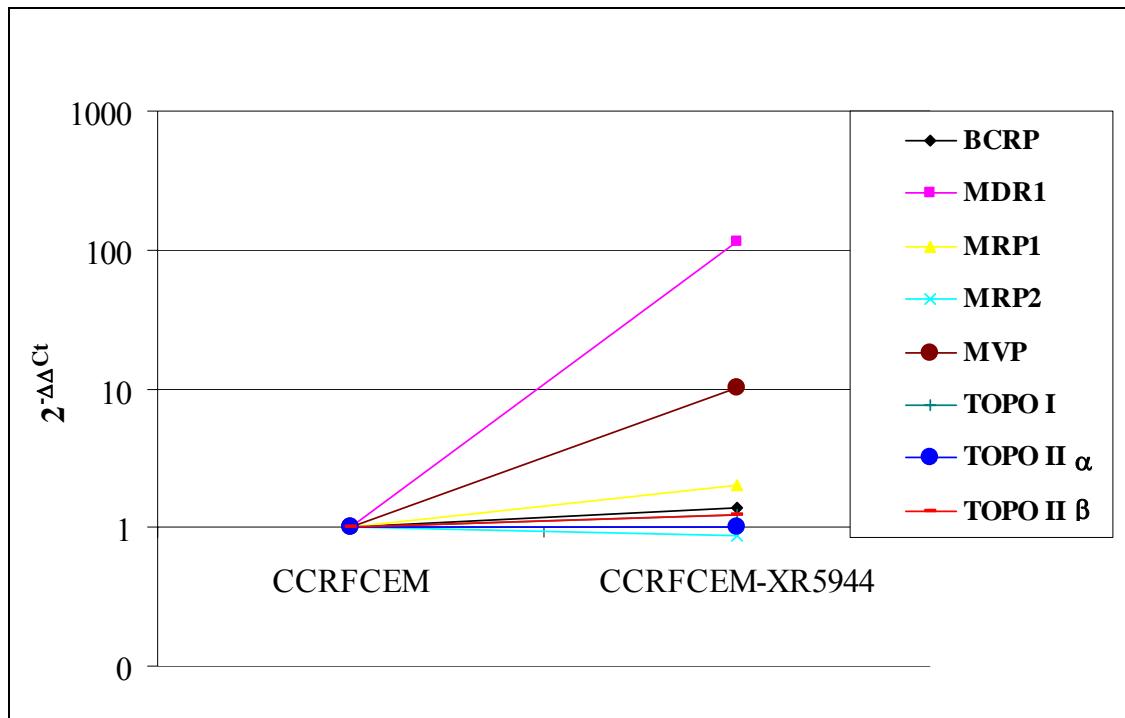


Figure 7.3 Difference in gene expression between CCRFCEM parental and XR5944-selected subline determined by qRT-PCR. Results are represented as mean of 3 replicate observations. Standard deviation error bars are not shown as they were smaller than the marks on the graph, in keeping with the minimal intra- (<1.5%) and inter-assay (<5%) coefficient of variation showed by the qRT-PCR technique.

7.4 Discussion

The *ex-vivo* profile of XR5944 in human solid tumours showed it to be an exceptionally potent cytotoxic agent. In all samples tested, XR5944 was several-fold more active than all other compounds tested, including topotecan, doxorubicin and paclitaxel. In keeping with the results of many other drugs, the potency of XR5944 is attenuated in tumour-derived cells compared to cell lines (chapter 4, *vide infra*), but this probably reflects the homogeneity and the fast-growing status of the CCRF-CEM cells used here and the cell lines used in previous studies (Stewart *et al.*, 2001).

Although the expression levels of MDR1 were increased at least 100-fold after prolonged and gradual XR5944 selection of the CCRF-CEM cells, the IC₅₀ value in the resistant line showed a modest 15-fold increase when compared to the IC₅₀ of the parental line. The relevance of this resistance model in the clinical setting may be questionable. In fact, it should be pointed that resistant lines generated *in vitro* after prolonged treatment might not reflect the *in vivo* situation, as factors that allow cell survival following acute cytotoxic drug exposure *in vivo* may differ from mechanisms selected by chronic drug exposure *in vitro*. Furthermore, a recent study (Yague *et al.*, 2003) has shown that in K562 myelogenous leukaemic cells, only long-term, and not short-term- drug exposure, was able to overcome a translational block so that MDR1 mRNA was translated and P-gp overexpressed.

It is remarkable that XR5944 showed its greatest activity in the subset of ovarian cancer samples, as all of the ovarian patients had relapsed after receiving a platinum-based regimen, which might have resulted in a gradual enrichment of resistant cells in those samples. Platinum treatment may cause upregulation of drug detoxifying enzymes, such as the glutathione-S-transferases (GST) (Cheng *et al.*, 1997), and loss of mismatch repair mechanisms (MMR) in a proportion of patients (Samimi *et al.*, 2000), therefore rendering the cells more resistant to several chemotherapeutic agents (Irving & Hall, 2001). Our data therefore suggest that XR5944 may not be sensitive to the common resistance mechanisms affecting cisplatin in ovarian cancer patients.

The median IC₉₀ and IC₅₀ values of XR5944 in colon carcinoma samples were 4-fold greater than the median for all tumour samples. All but 1 of these patients were chemotherapy-naïve, excluding the possibility that previous treatment could have

altered tumour response in these cases. On the other hand, colorectal carcinoma is well known to overexpress MDR1 and other pump proteins (Cordon-Cardo *et al.*, 1990) and is generally refractory to chemotherapy. However, it should be noted that even in those samples overexpressing P-gp, XR5944 showed better activity than the other cytotoxic agents tested.

Although the expression of P-gp could play a role in conferring resistance to XR5944, resistance is very often multifactorial and other mechanisms may be important, too. Our data on the CCRF-CEM and other cell lines (Stewart *et al.*, 2001) suggest that other MDR transporters, such as MVP and MRP-1, may mediate chemoresistance to this new agent. No immunostaining for these proteins was performed in this study, and further investigation is needed to evaluate the importance of these newly recognised pumps to XR5944 resistance in the clinical setting.

Previous experiments of sub-lines selected using TOPO inhibitors reported alteration in the expression levels of the targeted enzyme (reviewed in Larsen & Skladanowski 1998). The data in this chapter showed that XR5944 activity does not correlate with the expression of either TOPO I or II α , and that the levels of these enzymes are unaltered in drug-selected leukaemic CCRF-CEM cells. Although these findings could be explained by inhibition of both enzymes, it is probable that XR5944 acts through an alternative mechanism of action, distinct from TOPO inhibition, as has recently been suggested (Fleming *et al.*, 2003; Sappal *et al.*, 2004).

Multiple resistance mechanisms (including those not mentioned here or as yet undiscovered) may explain the heterogeneity of chemosensitivity between tumour types and individual tumours within the same tumour type observed in our series. However, in this study, the cell line data, the decreased potency in gastrointestinal tumours, and the better correlation of P-gp with the IC₅₀ of XR5944 than with the IC₉₀, all suggest that P-gp may be a mechanism of resistance relevant to very low concentrations of XR5944, and that higher levels of the drug may overcome such resistance. The results of this study may help further clinical development of XR5944. If phase I studies prove the safety of this compound, then our results suggest that phase II trials should target those clinical conditions (breast, ovarian cancer, and possibly metastatic skin melanomas) where the new agent is likely to produce the greatest benefit.

Chapter 8 - The *ex vivo* effect of high dose doxorubicin combinations in ovarian cancer

8.1 Introduction

Anthracyclines are active against a wide variety of human cancers, but their toxicities, such as bone marrow suppression and cardiac damage, have largely prevented dose intensification. Cardiovascular damage may represent a potentially life-threatening and dose-limiting toxicity. In the last decade, several efforts have been made to improve the therapeutic index of doxorubicin. The doxorubicin 4'-epimer, epirubicin, has a more favourable toxicity profile than doxorubicin since at equimolar doses is associated with identical clinical activity and less cardiotoxicity and myelosuppression (Bonadonna *et al.*, 1993; Plosker & Faulds 1993). More recently doxorubicin has been encapsulated in polyethylene-glycol (PEG) coated 'stealth' liposomes that confer to the drug a significantly longer half-life and an altered tissue distribution, with a high concentration of the active agent in the tumour (Gabizon *et al.*, 1994; Vaage *et al.*, 1994; Lasic, 1996; Gabizon & Martin 1997; Gabizon *et al.*, 1998; Symon *et al.*, 1999; Gabizon 2001). Pegylated liposomal doxorubicin (PEG-DOX; Caelyx/Doxil; Schering Plough/Sequus) has been demonstrated to be active against Kaposi's sarcoma, and ovarian, breast, and head/neck carcinomas (Muggia 1997; Stewart *et al.*, 1998; Caponigro *et al.*, 2000; Safra *et al.*, 2001; Muggia & Hamilton 2001). In clinical trials PEG-DOX has been reported to induce less cardiac toxicity and myelosuppression than free doxorubicin (Alberts & Garcia 1997; Berry *et al.*, 1998; Lyass *et al.*, 2000; Safra *et al.*, 2001), but its use has been associated with mucositis and the development of a peculiar side-effect, palmar-plantar erythrodysesthesia (hand-foot syndrome), which may represent a dose-limiting toxicity (Vail *et al.*, 1998; Amantea *et al.*, 1999).

Doxorubicin is not a new drug and has been widely used in combination with alkylating agents, antimetabolites and spindle-active agents. There is less data on the combination of liposomal preparations with other drugs, but current results indicate that similar combinations are likely to be effective (Sparano *et al.*, 2001; Gebbia *et al.*, 2002).

The ATP-TCA has been previously employed to assess the chemosensitivity of 21 ovarian carcinoma samples to the high concentrations of doxorubicin achievable with PEG-DOX (Neale *et al.*, 2000). During the course of this PhD it has been possible to use excess cells from many of the ovarian adenocarcinomas that were sent for the Phase III trial (chapters 1-2) to extend the original study by Neale *et al.* (2000). The ATP-TCA has again been used to determine the responsiveness of tumour-derived cells to concentrations of doxorubicin

achievable with liposomal preparations, but also to look at the addition of other drugs in combination with doxorubicin (Di Nicolantonio *et al.*, 2002). Treosulfan, vinorelbine and cisplatin were tested in combination with doxorubicin, which was used at five times (x5) or three times (x3) the standard TDC (see Chapter 2) to mimic the concentrations that have been obtained from *in vivo* studies with PEG-DOX (Gabizon *et al.*, 1994, Vaage *et al.*, 1994, Gabizon *et al.*, 1998, Symon *et al.*, 1999). It is not possible to test PEG-DOX directly in the ATP-TCA as serum-free media do not hydrolyse pegylated liposomes, and a previous study has shown no effect of PEG-DOX in cell lines maintained in such media (Wang *et al.*, 1999). However, *in vivo*, the intratumoral concentration of doxorubicin can range from 4-16 fold greater than standard preparations of doxorubicin when using PEG-DOX (Gabizon *et al.*, 1994, Vaage *et al.*, 1994, Gabizon *et al.*, 1998, Symon *et al.*, 1999). Therefore to achieve similar concentrations of doxorubicin *in vitro* the TDC of doxorubicin was increased x3 or x5, as originally suggested by Neale *et al.*, 2000.

8.2 Materials and Methods

Tumours. A total of 200 recurrent ovarian tumours were tested. These were all previously treated with carboplatin alone, or carboplatin + taxanes first line, followed in 21 cases by an anthracycline-containing regimen. The median age of the patients was 59 years (range 35 -81). Tumour histological type was stated on the submission form in 78 cases: 52 were of serous histological type, 9 endometrioid, 4 clear cell, 3 mucinous, and 10 poorly differentiated.

ATP-TCA. The ATP-TCA was performed as previously described in Chapter 2.

In the first series of samples studied the TDC of doxorubicin was 4.31 μM , which represented x5 the concentration normally employed for free doxorubicin (x1) in the ATP-TCA. For the second group of experiments (from October 2001 onwards) the TDC of doxorubicin was decreased to 2.59 μM (Doxorubicin x3). Combinations were made up by adding both drugs at their 200% TDC to the wells at the beginning of the assay: sequential studies were not performed. Not all drugs or combinations were tested in every case, as the number of cells available varied and clinical needs took precedence.

8.3 Results

8.3.1 *Ex vivo* activity of single agents

For the purposes of comparison between drugs and tumours, an $\text{Index}_{\text{SUM}}$ of <300 was taken as *ex vivo* sensitivity and >350 as resistance, as previously published (Hunter *et al.*, 1993; Cree *et al.*, 1999). Values between these two points were regarded as equivocal. On this basis, 76% (90/118) of the ovarian tumours tested showed resistance to cisplatin, with only 16% (19/118) showing sensitivity. In contrast, 84% (103/123) showed sensitivity to doxorubicin x5, 37% (37/106) to treosulfan, and 77% (41/53) to vinorelbine (table 8.1). Only six tumours were tested with 5-FU, though four showed sensitivity.

Doxorubicin x5 achieved $>95\%$ inhibition at 100% TDC (4.31 μM) in 102/123 (83%) of the ovarian tumours tested (table 8.1); and $>99\%$ inhibition was only observed in 61/123 (49.6%) of the samples tested. Ten ovarian tumours showed complete resistance. Of these, only three had previous exposure to anthracyclines.

8.3.2 *Ex vivo* activity of doxorubicin x5 in combination with other cytotoxics

The combinations tested were only slightly better than doxorubicin x5 alone, as shown in table 8.1 and in figures 8.1, 8.2 and 8.3. All produced $>90\%$ sensitivity based on an $\text{Index}_{\text{SUM}}$ of <300 (doxorubicin x5 with cisplatin = 98%, with treosulfan = 98%, with 5-FU = 90%, with vinorelbine = 100%). The best effect was obtained with the combination of doxorubicin x5 plus vinorelbine (figure 8.3), which was better than doxorubicin x5 alone in 51/53 paired observations (Wilcoxon matched pairs test: $p < 0.0001$ on $\text{Index}_{\text{SUM}}$), and better than doxorubicin x5 plus treosulfan in 39/41 paired observations ($p < 0.0001$). When analysed by the method of Poch *et al.* (1995), in which the observed effect at each concentration tested is compared with the expected effect, the combinations of doxorubicin x5 with cisplatin, treosulfan and vinorelbine all produced a greater than additive effect at the lower concentrations tested (figures 8.1, 8.2, 8.3).

8.3.3 *Ex vivo* activity of doxorubicin x3 with vinorelbine

The proportion of tumours apparently sensitive to doxorubicin x5 exceeded the response rates obtained in clinical trials of PEG-DOX in recurrent ovarian cancer (Muggia *et al.*, 1997; Campos *et al.*, 2001; Gabizon, 2001; Gordon *et al.*, 2001; Hensley *et al.*, 2001; Muggia & Hamilton, 2001). The largest of these obtained a 20% response rate (Gordon *et*

al., 2001). Therefore during the course of this study, the concentration of doxorubicin was decreased to x3 in order to improve the predictive accuracy of the ATP-TCA.

In the second group of 77 ovarian tumours tested, doxorubicin x3 achieved >95% inhibition at 100% TDC (2.59 μM) in 31/77 (40%) of the samples (table 8.2); and >99% inhibition was observed in 16/77 (21%) cases. On the basis of $\text{Index}_{\text{SUM}}$, 45% (35/77) of the ovarian tumours tested showed sensitivity to doxorubicin x3, as opposed to 84% samples which were found sensitive to x5 in the first part of the study (tables 8.1 and 8.2), in line with the concentration response curves previously obtained.

The second group of tumour biopsies was found to be generally more resistant to doxorubicin when the actual IC90 and IC50 values were considered and compared to those calculated in the first series of samples. In the first series, tested with x5, the median IC90 and IC50 values were 1.57 μM and 0.523 μM , while they were respectively 2.66 μM and 1.57 μM in the second group tested with x3 (tables 8.1 and 8.2). These differences were found to be statistically significant for both parameters ($p < 0.0001$, Mann Whitney U test). However, it should be noted that the shape of the median concentration-inhibition curve for doxorubicin x3 is sigmoidal (figure 8.4), while that for doxorubicin x5 is somewhat flatter (figure 8.3), and this may have influenced the extrapolation of parameters such as IC90 and IC50.

The second group of samples appeared to be also slightly more resistant to vinorelbine when compared to the first series, although no significant differences were found when the various sensitivity indices were analysed (NS for $\text{Index}_{\text{SUM}}$, IC90 and IC50, Mann Whitney U test).

The combination of doxorubicin x3 and vinorelbine tested at 100% TDC produced >95% inhibition in 65/77 (84%) samples (table 8.2). On the basis of $\text{Index}_{\text{SUM}}$, 68/77 samples (88%) showed sensitivity to this combination, while only 4/77 (5%) were found to be resistant. The combination was better than doxorubicin x3 alone in 72/77 paired observations ($p < 0.0001$ on $\text{Index}_{\text{SUM}}$, Wilcoxon matched pairs test:), and better than vinorelbine alone in 73/77 paired observations ($p < 0.0001$).

When analysed by the method of Poch *et al.* (1995), in which the observed effect at each concentration tested is compared with the expected effect, the combination of doxorubicin x3 with vinorelbine produced a median additive effect (figure 8.4).

A median-effect analysis was also performed (Chou & Talalay 1984) and combination indices calculated for each sample tested with doxorubicin x3 and vinorelbine at 90% cell death (CI_{90} ; figure 8.5, panel a) and at 50% cell death (CI_{50} ; figure 8.5, panel b). The median of all CI_{90} values was 0.97 (range 0.27-2.95; figure 8.5) and the median of the CI_{50} values was 1.08 (range 0.11-7.82; figure 8.5). Considerable heterogeneity was found among samples as shown in figure 8.5. Synergism ($CI < 0.8$) was identified in 21/77 (27%) and in 13/77 (32%) samples when CI_{90} and CI_{50} values were considered, respectively; while antagonism ($CI > 1.2$) was found in 26/77 (34%) samples both for CI_{90} and CI_{50} values. Additivity was identified in 30/77 (39%) samples on the basis of the CI_{90} although this percentage increased to 49% (38/77) when CI_{50} values were calculated.

Combination indices were also calculated for the median concentration-inhibition curve produced by doxorubicin x3 and vinorelbine shown in panel a) of figure 8.4. In this instance the CI_{90} and CI_{50} values were respectively 0.85 and 0.79, in keeping with the additive effect observed with the Poch analysis in panel b) of figure 8.4.

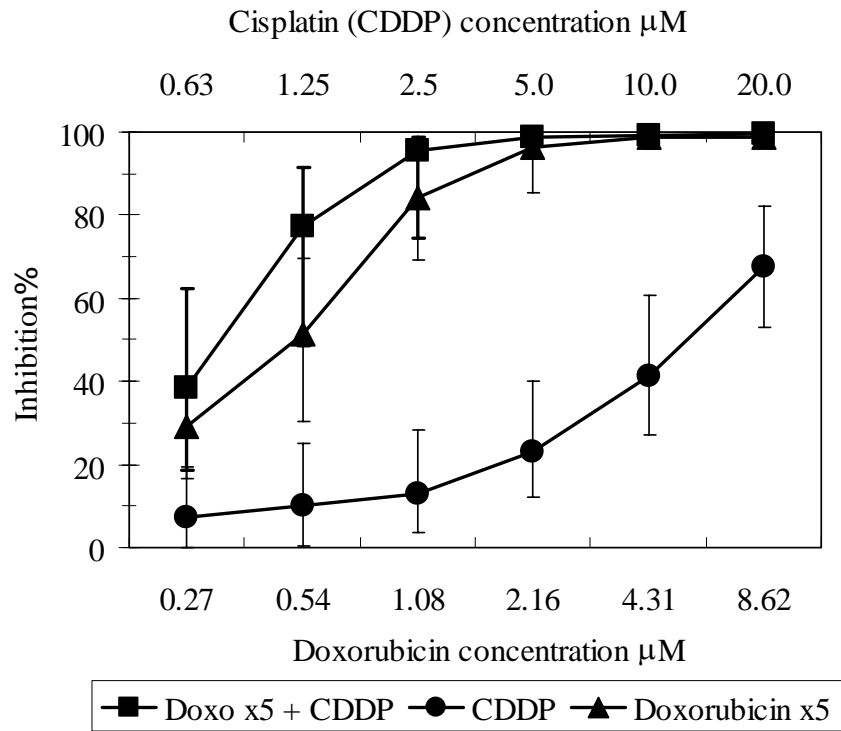
Table 8.1 Median values and range (in brackets) for AUC, Index_{SUM}, IC90 and IC50 measured in n samples. For the combination the values marked with (*) are expressed as percentage of TDC. An Index_{SUM} of > 600 represents no activity and < 300 is taken as sensitivity for most single agents. An IndexAUC of 19,000 would represent complete inhibition at all doses tested, while zero would equal no effect. The percentage of tumours showing >95% inhibition at 100% concentration tested is also shown.

Drug/Combination	n	Age	Index AUC	IC90 μM	IC50 μM	Index_{SUM}	> 95% Inh
Doxorubicin x5	123	58 (35 - 80)	18014 (7085-19565)	1.57 (0.259 – 12.8)	0.523 (0.144 – 7.10)	139 (3 - 505)	83% (102/123)
Cisplatin (CDDP)	118	58 (35 - 80)	7686 (266 - 17144)	25.7 (7.61 – 143)	13.5 (0.400 – 79.4)	445 (113 - 940)	4% (5/118)
Treosulfan (TREO)	106	59 (35 - 80)	12657 (433- 18121)	133 (23.9 - 703)	44.9 (3.39 - 391)	343 (91 - 846)	44% (47/106)
5-Fluorouracil (5-FU)	6	51 (48 - 64)	16672 (0 - 18936)	294 (62.0-931)	52.6 (12.7 - 574)	151 (46 - 1338)	67% (4/6)
Vinorelbine (VinR)	53	61 (40 - 80)	15508 (6145 - 19158)	16.2 (0.660 – 26.9)	0.639 (0.367– 15.5)	183 (15 - 502)	72% (38/53)
Doxorubicin x5 + CDDP	52	60 (36 - 80)	18615 (12267 - 19488)	21* (6 - 246)	9* (3 - 52)	92 (8 - 314)	88% (46/52)
Doxorubicin x5 + TREO	41	61 (44 - 80)	18725 (12857 - 19491)	21* (6 - 205)	8* (4 - 72)	92 (8 - 346)	98% (40/41)
Doxorubicin x5 + 5-FU	11	58 (48 - 69)	17455 (10696 - 19234)	40* (10 - 292)	9* (4 - 46)	119 (21 - 361)	55% (6/11)
Doxorubicin x5 + VinR	53	61 (36 - 80)	19013 (16746 - 19488)	12* (6 - 86)	4* (3 - 28)	37 (0 - 209)	98% (52/53)

Table 8.2 Median values and range (in brackets) for AUC, Index_{SUM}, IC₉₀ and IC₅₀ measured in n samples. For the combination the values marked with (*) are expressed as percentage of TDC. An Index_{SUM} of > 600 represents no activity and < 300 is taken as sensitivity for most single agents. An IndexAUC of 19,000 would represent complete inhibition at all doses tested, while zero would equal no effect. The percentage of tumours showing >95% and >99% inhibition at 100% concentration tested is also shown.

Drug/Combination	n	Age	Index AUC	IC₉₀ µM	IC₅₀ µM	Index_{SUM}	> 95% Inh	> 99% Inh
Doxorubicin x3	77	61 (38 - 81)	14084 (600-18788)	2.66 (0.543 – 31.9)	1.215 (0.129 – 17.7)	319 (101 - 684)	40% (31/77)	21% (16/77)
Vinorelbine (VinR)	77	61 (38 - 81)	15712 (2391 - 18883)	13.0 (1.56 – 56.4)	1.67 (0.429 – 31.3)	199 (35 - 643)	31% (24/77)	16% (12/77)
Doxorubicin x3 + VinR	77	61 (38 - 81)	17947 (5581 - 19300)	48* (6 - 381)	9* (3 - 211)	131 (13 - 469)	84% (65/77)	66% (51/77)

(a)



(b)

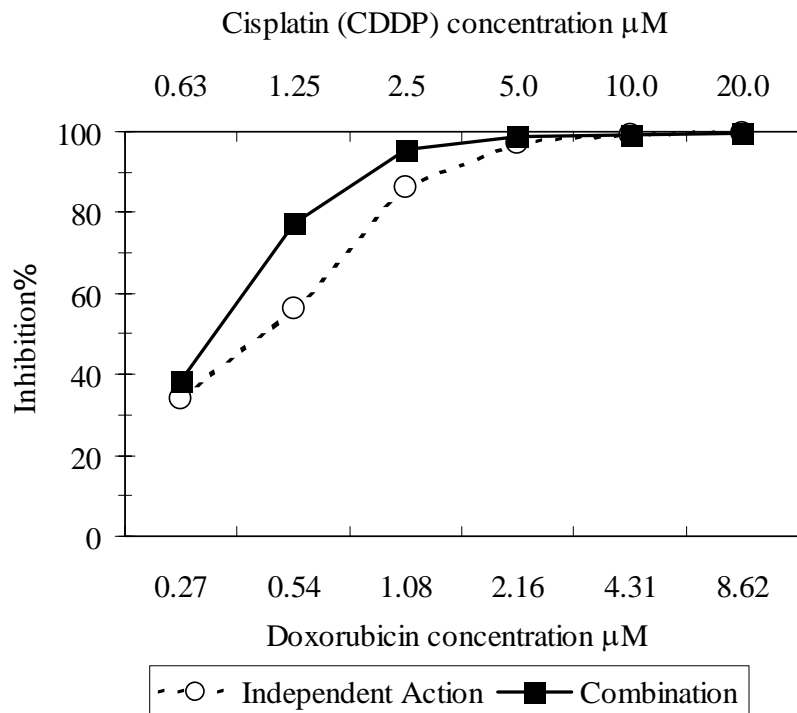
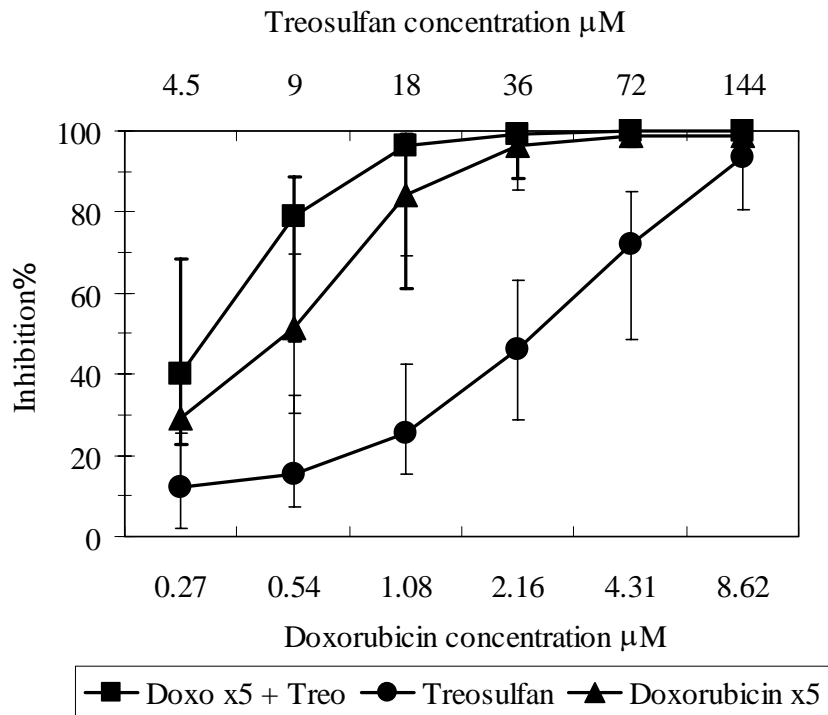


Figure 8.1 (a) Median (\pm interquartile range) activity of doxorubicin x5 in combination with cisplatin (CDDP) in $n=52$ ovarian cancer samples. (b) Combination analysis (Poch *et al.*, 1995) showing at least an additive effect of the two drugs in this series of samples.

(a)



(b)

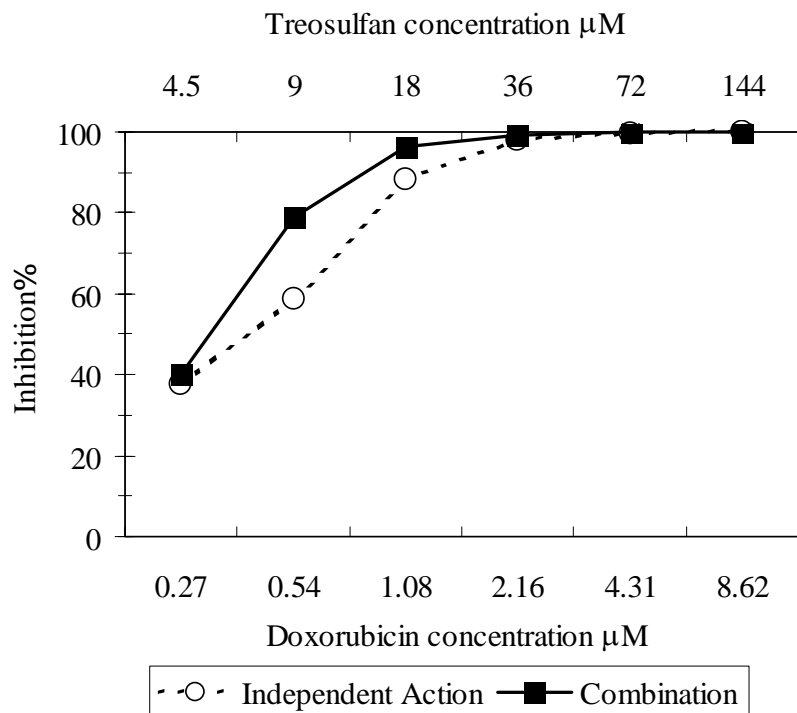
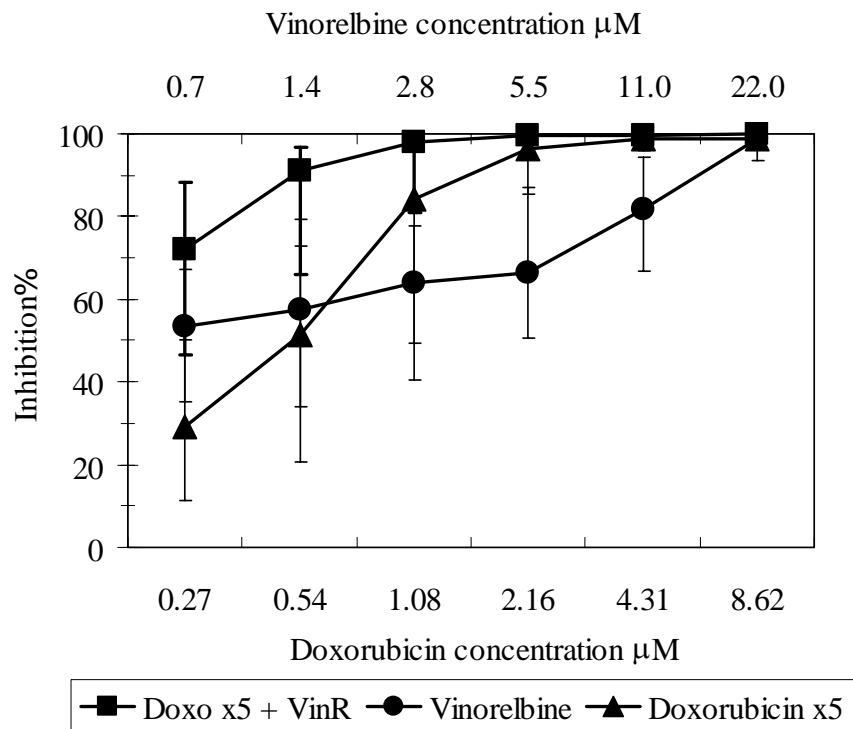


Figure 8.2 (a) Median (\pm interquartile range) activity of doxorubicin x5 in combination with treosulfan (Treo) in $n=41$ ovarian cancer samples. (b) Combination analysis (Poch *et al.*, 1995) showing at least an additive effect of the two drugs in this series of samples.

(a)



(b)

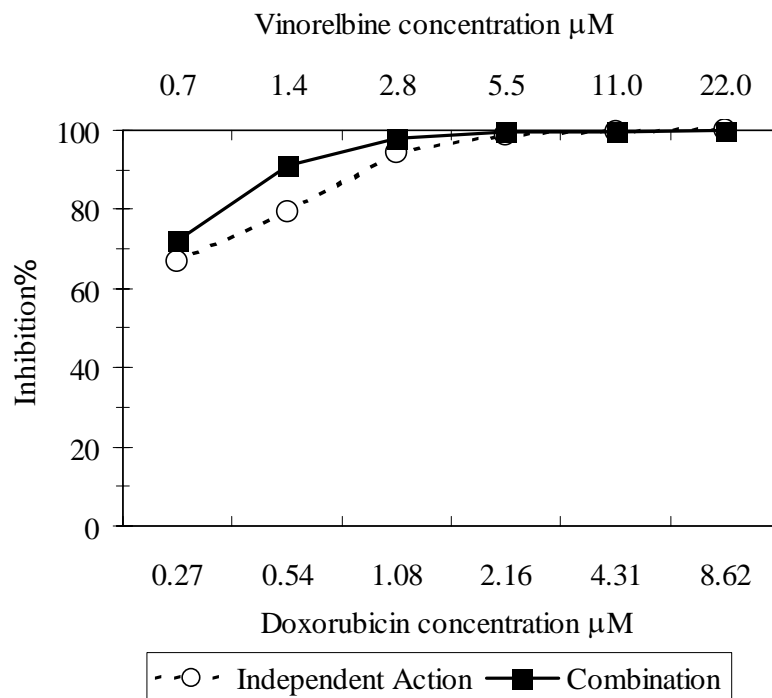
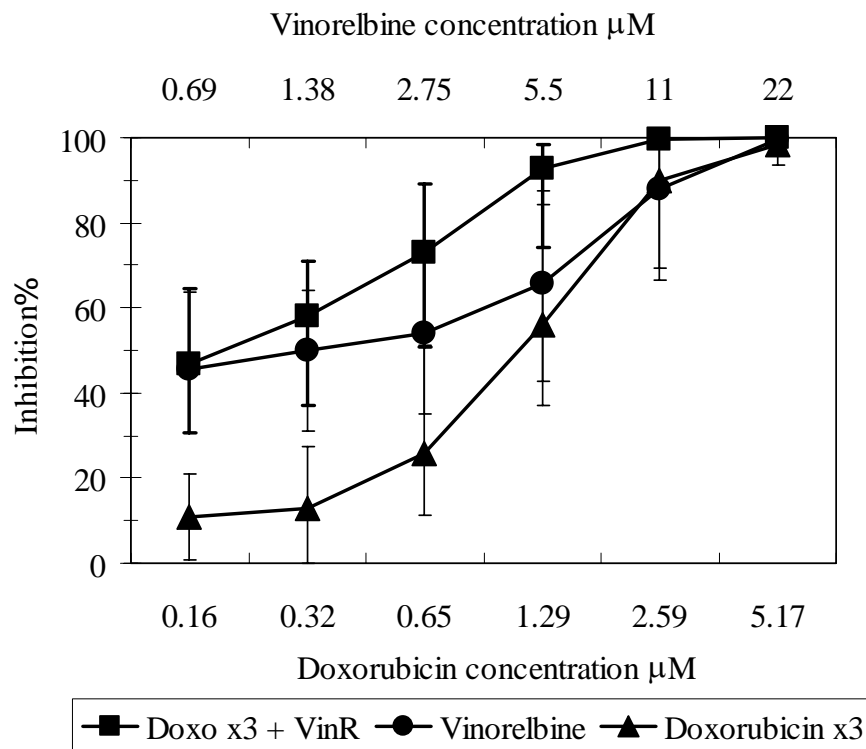


Figure 8.3 (a) Median (\pm interquartile range) activity of doxorubicin x5 in combination with vinorelbine (VinR) in n=53 ovarian cancer samples. (b) Combination analysis (Poch *et al.*, 1995) confirming at least an additive effect of the two drugs in this series of samples.

(a)



(b)

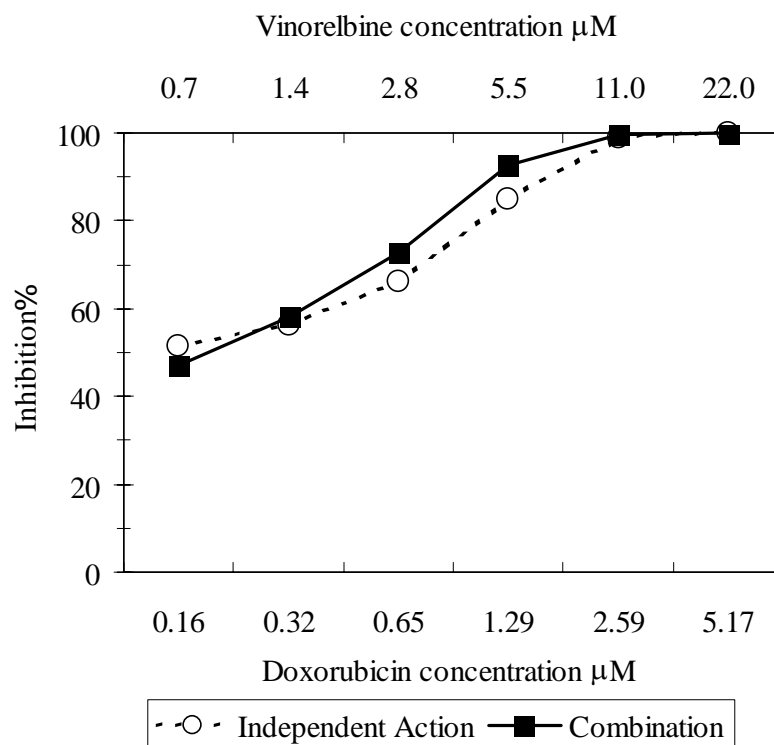
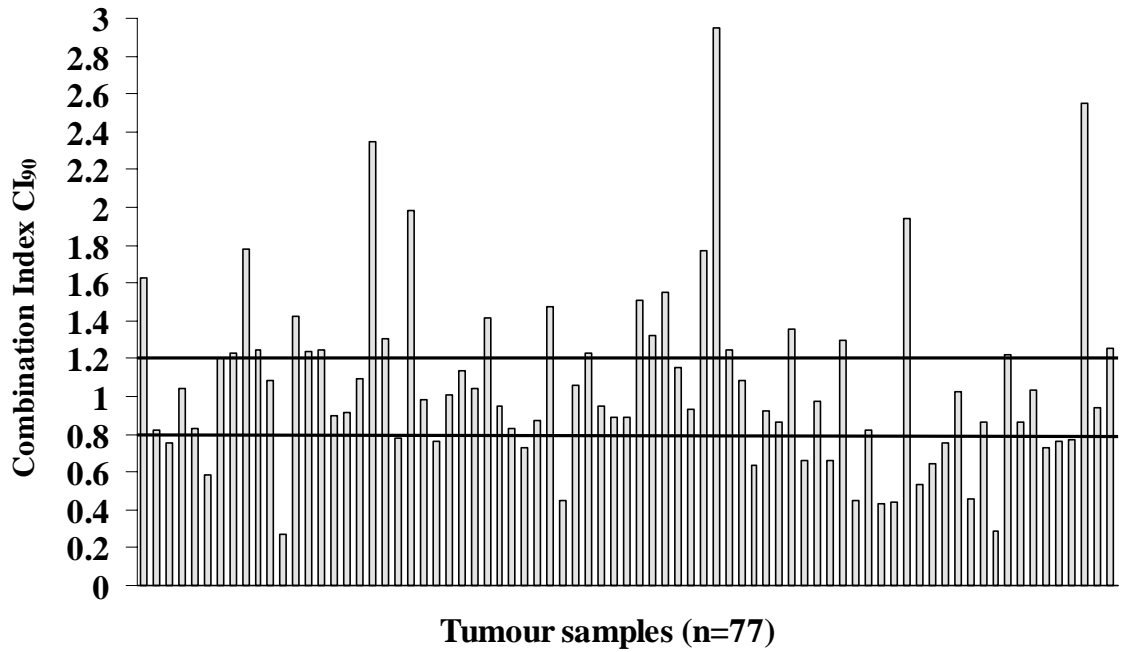


Figure 8.4 (a) Median (\pm interquartile range) activity of doxorubicin x3 in combination with vinorelbine (VinR) in n=77 ovarian cancer samples. (b) Combination analysis (Poch *et al.*, 1995) showing an additive effect of the two drugs in this series of samples.

(a)



(b)

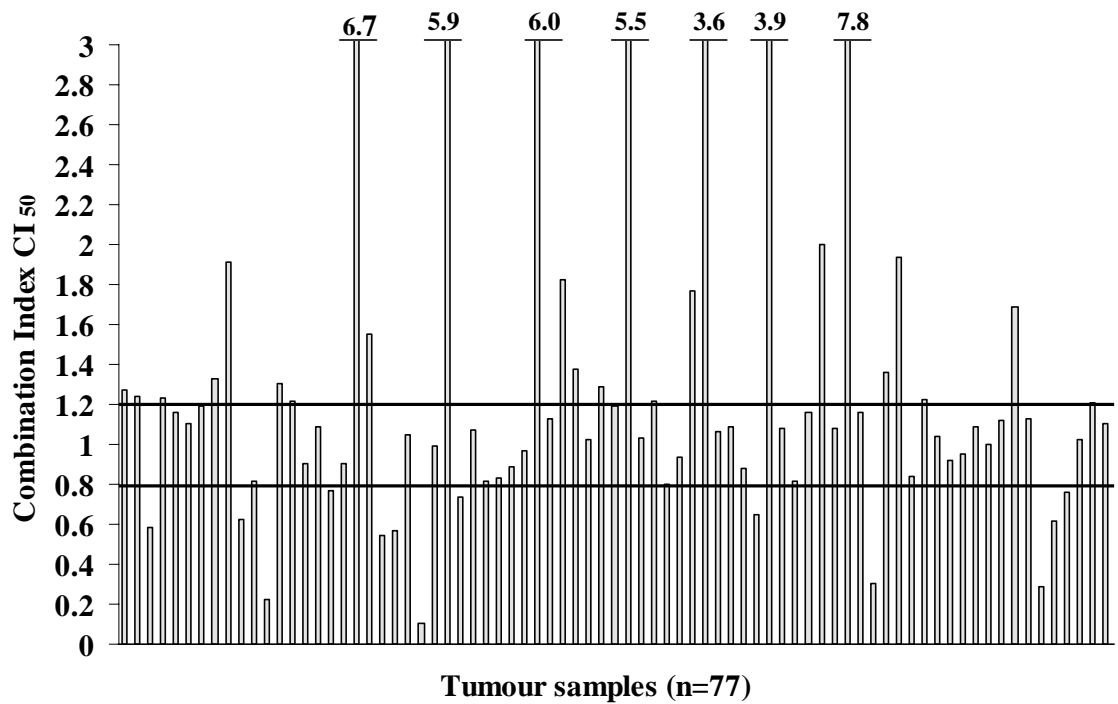


Figure 8.5 Combination indices calculated for each sample tested with doxorubicin x3 and vinorelbine at 90% cell death (CI_{90} ; panel a) and at 50% cell death (CI_{50} ; panel b). CI values <0.8 suggest synergy; CI values >1.2 indicate antagonism; and values between 0.8 and 1.2 indicate additivity (Chou & Talalay 1984, Greco *et al.*, 1995).

8.4 Discussion

PEG-DOX has proved somewhat useful as a single agent in recurrent ovarian cancer (Muggia *et al.*, 1997; Campos *et al.*, 2001; Gabizon, 2001; Gordon *et al.*, 2001; Hensley *et al.*, 2001; Muggia & Hamilton, 2001). Therefore it was decided to investigate its activity in this tumour type using the ATP-TCA. As it was not possible to test PEG-DOX directly in the assay (Wang *et al.*, 1999), high concentrations of free doxorubicin (x5) (Neale *et al.*, 2000a) were used to take into account the enhanced intratumoral concentrations apparently achievable with PEG-DOX (Gabizon *et al.*, 1994; Vaage *et al.*, 1994; Lasic, 1996; Gabizon *et al.*, 1998; Symon *et al.*, 1999; Gabizon, 2001). The proportion of tumours apparently sensitive to doxorubicin x5 exceeded the response rates obtained in clinical trials of PEG-DOX in recurrent ovarian cancer (Muggia *et al.*, 1997; Campos *et al.*, 2001; Gabizon, 2001; Gordon *et al.*, 2001; Hensley *et al.*, 2001; Muggia & Hamilton, 2001). The largest of these obtained a 20% response rate (Gordon *et al.*, 2001). The reason for this discrepancy may be overestimation of the likely concentration of free doxorubicin to which tumour cells are actually exposed. Some of the doxorubicin measured in the previous pharmacokinetics studies of PEG-DOX may have been sequestered within liposomes. Gabizon *et al.* (1994) measured the pharmacokinetics of doxorubicin and/or liposome-associated doxorubicin in seven patients after injections of equivalent doses (50 mg / m²) of free doxorubicin and PEG-DOX. The plasma elimination of PEG-DOX followed a biexponential curve with median half-lives of 2 and 45 hours, and nearly 100% of the drug detected in plasma after PEG-DOX injection was in liposome-encapsulated form (Gabizon *et al.*, 1994). In the ATP assay free doxorubicin is incubated directly with the tumour cells for 144 hours, bypassing the distribution phase, and also excluding renal and hepatic clearance; therefore in light of the differences in pharmacokinetics, the results presented in this chapter are a very vague approximation of the activity of PEG-DOX.

The initial use of x5 the standard TDC for doxorubicin was based on available data from animal studies (Vaage *et al.*, 1994; reviewed in Gabizon *et al.*, 2003). Forty hours after treatment with PEG-DOX the levels of doxorubicin in mice prostate carcinoma xenografts were greater than 7.0 µg / mg tumour tissue; the intratumour concentration then decreased following a biphasic pharmacokinetics, with a remarkable level of 1.5 µg drug / mg tissue still present 160 hours after treatment (Vaage *et al.*, 1994). Northfelt *et al.* (1996) reported localisation in Kaposi's sarcoma lesions of 5.2- to 11.4-fold higher

concentrations of doxorubicin after treatment with PEG-DOX compared to treatment with an equivalent dose of free doxorubicin. Seventy-two hours after 10 mg/m² PEG-DOX was administered, the levels of doxorubicin in sarcoma biopsies were 2.06 ± 0.42 µg / mg tissue. This concentration is in the range tested in the ATP-TCA (1.5 µg/ml for Doxo x3 and 2.5 µg/ml for Doxo x5), although in the assay the cells were incubated for a longer period, 144 hours, with a likely increase of AUC. Another report measured doxorubicin concentration in bone tumour metastases from two breast carcinoma patients (Symon *et al.*, 1999): in the first case, 6 days after administration of 50 mg/m² PEG-DOX the levels were 6.5 µg/g of tissue; the intratumour doxorubicin concentration was 1.4 µg/g of tissue in the second patient who had received 35 mg/m² PEG-DOX twelve days prior obtaining the biopsy. Another study of human subjects presented at the 2001 San Antonio Breast Cancer Symposium also suggested a higher and more sustained enhancement of doxorubicin levels in tumours from breast cancer patients treated with PEG-DOX (Schueller *et al.*, 2001), however these observations have not been fully published.

On the basis of the data above described, the concentration tested in the ATP-TCA for liposomal doxorubicin was lowered to x3 the normal TDC for free doxorubicin. The reduced concentration was tested in a group of 77 ovarian cancer specimens, and, as expected, the proportion of samples sensitive to the drug was decreased when compared to that observed in the series tested with x5. However, the second group of samples was found to be in general more resistant to doxorubicin, as demonstrated by the increased median IC₅₀ and IC₉₀ values. While this can be attributed to heterogeneity of chemosensitivity in different human cancers, other technical factors may be important. The shape of the median concentration-inhibition curve for doxorubicin x3 was sigmoid, while that for x5 was somewhat flatter. This could have produced inaccurate calculations of the IC₉₀ and IC₅₀ parameters, which are extrapolated with a mathematical algorithm from the dose-inhibition curve. In addition, the high concentrations employed when testing doxorubicin at the x5 concentration could have produced a general increase in chemosensitivity due to the volatility of the drug itself. Although cross-talk between wells in the plate does not generally represent a problem in the ATP-TCA, occasional cases of enhanced cytotoxicity due to drug volatility of certain compounds have been observed (Andreotti *et al.*, 1995). Thus it is possible that the levels of doxorubicin present in the wells containing the 100% TDC and 200%

TDC, by evaporation, have increased the actual levels of drug exposure also in those wells containing the lower concentrations. While these are only speculations, correlation data comparing ATP-TCA results with clinical outcome from follow-up of those patients in this series treated with PEG-DOX are awaited.

The previous study that tested high concentrations of doxorubicin in the ATP-TCA did not explore combinations in any detail, though it was noted that the addition of gemcitabine was unable to augment the response to any great extent (Neale *et al.*, 2000a), a finding that has now been explored clinically in a small phase I study (Rivera *et al.*, 2001). While this study did show 9/27 responses, it should be noted that 6 patients had not received prior chemotherapy. Equally, I did not try to combine TOPO I and II inhibitors, as topotecan and PEG-DOX was found to be toxic (Ryan *et al.*, 2000).

As most of the patients had had previous exposure to taxanes, I did not test this in combination with doxorubicin x5, though it should be noted that recent results combining liposomal doxorubicin with docetaxel are encouraging (Sparano *et al.*, 2001). I chose instead to examine the effect of doxorubicin on cisplatin resistance and in combination with another alkylating agent to which resistance is less common in this setting, treosulfan. The results for the combination of doxorubicin x5 with treosulfan, 5-FU and cisplatin are relatively disappointing, with little improvement in efficacy compared with single agent doxorubicin x5, though these combinations are still active (table 8.1).

The combination of vinorelbine and doxorubicin x5 or x3 showed consistently additive effects in both group of samples studied. In a few cases strong antagonism was found, while at the same time in a number of cases true synergism was identified. This combination was tried in the clinic in metastatic breast cancer and was found to be safe, with acceptable toxicity (Burstein *et al.*, 1999; Gebbia *et al.*, 2002). It also showed a good response rate (68%) in the small number of patients (n = 18) treated (Gebbia *et al.*, 2002). Recently a phase I trial conducted in refractory or resistant ovarian cancer recorded a response in 6/29 patients (Tambaro *et al.*, 2003), which is somewhat lower than expected from the data presented above, but still suggests that this combination is worthy of further clinical consideration.

There are a number of other combinations that need to be studied. Some of these are already being tested in the clinic on an empirical basis, for example the combination of PEG-DOX with Herceptin (Winer & Burstein 2001). It would be particularly interesting to examine the role of Caelyx/Doxil in combination with taxanes in ovarian cancer samples from previously untreated patients, as combinations of anthracyclines with taxanes have previously been shown to be very active in this tumour type (Kurbacher *et al.*, 1997 & 1998; Konecny *et al.*, 2000). It is clear from this and previous studies that the use of ATP-based chemosensitivity testing can assist the development of new regimens and has the potential to speed up their introduction to the clinic (Cree & Kurbacher 1999). The combination vinorelbine and PEG-DOX is clearly of considerable interest and further clinical data are awaited.

Chapter 9 - Molecular determinants of sensitivity to doxorubicin

9.1 Introduction

Resistance to doxorubicin is thought to be mediated via a number of different mechanisms (see chapter 2), which include mutation or alteration of its target enzyme, TOPO II α ; up-regulation of drug export proteins, such as BCRP, MRP1, MVP and MDR-1; and other complex mechanisms, which are related to the multiple pathways leading to cell death, including apoptosis, cell cycle regulation and checkpoints, and DNA repair (Larsen & Skladanowski, 1998; Beck *et al.*, 1999; Gottesman *et al.*, 2002). Most of these mechanisms have been identified in cell lines studies, while their clinical relevance in relation to doxorubicin chemotherapy has been less investigated.

A number of recent studies have tried to correlate the molecular phenotype with *ex vivo* chemosensitivity of tumour-derived cells to anthracyclines (Satherley *et al.*, 2000; Koshiyama *et al.*, 2001a & 2001b; Coley *et al.*, 2002; Lewandowicz *et al.*, 2002). Koshiyama *et al.* (2001) used the MTT assay to assess the *ex vivo* chemosensitivity of 24 endometrial and 19 ovarian carcinoma samples; this study found that the immunohistochemical detection of TOPO II α well correlated with sensitivity to topoisomerase II α inhibitors, including doxorubicin. Another study by Coley *et al.* (2002) also employed the MTT assay to study doxorubicin resistance in ovarian tumour derived cells; but they did not find any significant relationship between P-gp expression determined by immunocytochemistry and sensitivity to doxorubicin or the ability of P-gp inhibitor PSC-833 to modulate such sensitivity. Similarly another study from the same group (Lewandowicz *et al.*, 2002) failed to correlate MRP1 expression with doxorubicin sensitivity in a group of 20 ovarian cancer samples tested with the MTT assay. Satherley *et al.* (2000) correlated the ATP-TCA chemosensitivity data of 29 choroidal melanoma samples with the immunohistochemical expression of MDR1, MRP, MVP, TOPO II α and TOPO II β . Again, this study did not find any significant difference in sensitivity to anthracyclines with any of the molecular markers examined. However of the 13 tumours found to show some anthracycline sensitivity *ex vivo*, six expressed TOPO II α ; while at least one of the classical drug resistance molecules LRP, MDR1 or MRP was expressed in all but one of the tumours showing weak anthracycline sensitivity.

While studies exist on the relationship between drug resistance gene expression in ovarian cancer and sensitivity to doxorubicin in the MTT assay, no such data has been

published for the ATP-TCA in any cancers other than choroidal melanoma (Satherley *et al.*, 2000). Therefore I decided to determine the immunohistochemical expression of MDR1, TOPO I and TOPO II α in a series of ovarian cancer tumours for which chemosensitivity testing results were available. In addition I also investigated the relevance of P-gp and other putative chemoresistance genes by measuring the mRNA levels in breast and ovarian tumour derived cells using qRT-PCR. I also aimed to test the hypothesis that the lack of correlation between gene expression and doxorubicin resistance is due to rapid up- or down-regulation of genes involved in sensitivity to this drug.

9.2 Materials and Methods

IHC. IHC for P-gp, TOPO I and TOPO II α was performed on 39 ovarian cancer specimens as detailed in Chapter 2. The median age of the patients was 58 years (range 38 -76). These were all previously treated with carboplatin alone, or carboplatin + taxane first line, followed in 9 cases by an anthracycline-containing regimen.

qRT-PCR samples. Tumour derived cells were obtained from 14 breast cancer patients (median age 58; range 37-94; previous treatment included paclitaxel plus mitoxantrone for one patient; epirubicin plus cyclophosphamide for another patient; anti-hormonal treatment for a third patient) and 12 ovarian cancer patients (median age 58; range 42-72; 3 primaries, and 9 from recurrent ovarian cancer patients who had been previously treated with platinum alone (n=4) or platinum plus taxane (n=5), followed by treosulfan and gemcitabine in 2 cases). Cells were grown for 6 days in CAM with or without doxorubicin, before RNA extraction and further PCR analysis (chapters 2 & 3).

9.3 Results

9.3.1 IHC expression of P-gp, TOPO I / II α and sensitivity to doxorubicin

Immunostaining for P-gp was positive in 11/39 (28%) samples tested with doxorubicin. The median doxorubicin IC₉₀ values for P-gp negative and positive samples were 2.37 and 3.71 μ M, respectively (p<0.041, Mann-Whitney U test), while the median IC₅₀ values were respectively 1.10 μ M and 1.54 μ M (p=NS, Mann-Whitney). A modest correlation was observed by linear regression analysis (figure 9.1) between the IC₉₀ values of doxorubicin and P-gp (R=0.357; p<0.0257), while there was no correlation with the IC₅₀ values. A modest correlation was found when the data was analysed using

non-parametric Spearman's correlation (table 9.1). P-gp positivity shifted the concentration-inhibition curve of doxorubicin to the right (figure 9.2).

Immunostaining for TOPO I and II α was positive in 32/39 (82%) and 8/39 (20%) samples tested with doxorubicin respectively. No correlation by linear regression analysis or Spearman rank test was found between the IC₅₀ or IC₉₀ values for doxorubicin and either the TOPO I or the TOPO II α IHC indices (table 9.1).

Table 9.1 Spearman rank correlation analysis for comparison between the IC₉₀ and IC₅₀ values of doxorubicin in the ATP-TCA and the IHC expression of P-gp, TOPO I and TOPO II α .

Protein	IC ₉₀ μ M		IC ₅₀ μ M	
	R	p	R	p
P-gp	0.348	<0.0301	0.343	<0.0325
TOPO I	-0.016	0.9245	0.074	0.6547
TOPO II α	0.178	0.2794	0.218	0.1825

9.3.2 Effect of *in vivo* chemotherapy on doxorubicin sensitivity

I was able to compare the activity of doxorubicin x3 in 18 paired ovarian samples that were obtained from 9 patients before and after administration of an anthracycline-based regimen (mitoxantrone and paclitaxel, n=7; liposomal doxorubicin, n=2). An increase in resistance was seen in 7/9 cases (figure 9.3a). The mean IC₅₀ values (\pm SD) of doxorubicin in the pre- and post-treatment samples were 0.65 \pm 0.46 and 1.44 \pm 1.03, respectively (p<0.0243; paired t test), confirming that resistance developed following treatment. No material from patients was available for PCR analysis both before and after treatment with PEG-DOX. However, I was able to compare the molecular profile of one pair of ovarian tumours obtained from another patient who received 6 cycles of mitoxantrone plus paclitaxel, rather than doxorubicin. The mRNA expression of 7 chemosensitivity genes implicated in resistance to anthracyclines was measured by qRT-PCR as described previously (chapters 2 and 3) in two different experiments which produced similar results. The mean values of the calculated differences in gene expression are presented in figure 9.3 (b). A remarkable 114-fold increase of MDR1 mRNA was found in the post-chemotherapy specimen when compared to the pre-chemotherapy sample. Among the other MDR genes studied, no changes were observed

for BCRP and MVP, while a marginal decrease of MRP1 expression was noted in both experiments. The expression of TOPO II α appeared essentially similar between the two biopsies. A significant up-regulation of TOPO I mRNA levels (>3-fold) was detected, while TOPO II β expression was markedly decreased in the post-chemotherapy specimen.

9.3.3 Correlation between gene expression in control cells and chemosensitivity

Univariate analysis was performed to correlate the results obtained in the ATP-TCA with the gene expression profile measured in tumour derived cells (cultured for six days in CAM only) by qRT-PCR. The results indicated a correlation ($R=0.7204$, $p<0.0124$) between the expression of ERCC1 and the IC50 values determined for doxorubicin in 11 breast samples obtained from chemotherapy naïve patients (figure 9.4). No other statistically significant correlations were identified for the other genes tested.

9.3.4 Doxorubicin effects in short-term cell culture

To pursue the changes in gene expression after doxorubicin exposure further, we used RT-PCR for the genes as the endpoint in plates set up alongside the ATP-TCA using both breast and ovarian tumour derived cells. This allowed us to examine the changes in gene expression after 6 days of drug exposure.

An example of an ovarian tumour is shown in figure 9.5 (a); in this case doxorubicin exposure induced at least a 4-fold up-regulation of both MDR1 and BCRP genes, while at the same time markedly reducing the expression levels of Topo II α . The ATP-TCA results (figure 9.5 b) for this sample showed resistance to doxorubicin ($IC_{90} = 4.92\mu\text{M}$; $IC_{50} = 2.20\mu\text{M}$; $\text{Index}_{\text{SUM}} = 472$), which was partly reversed in the assay by the addition of tariquidar, a P-gp specific inhibitor. Notably, IHC staining for P-gp in this sample (cytocolot) was negative, as shown in figure 9.5 (c).

When the results of the qRT-PCR experiments were grouped together for each target gene in each tumour type, a significant up-regulation of MRP-1 ($p<0.0001$, Wilcoxon matched pairs test; table 9.2) and of MVP ($p<0.0023$, Wilcoxon matched pairs test) was observed, but this was limited to the breast cancer subgroup. In the ovarian cancer group the levels of MRP-1 and MVP increased over 0.5 fold in only 4/12 and 3/12 samples, respectively. No significant changes were found in the median expression of MDR-1

and BCRP, although it should be noted that there was substantial heterogeneity among individual tumours (figure 9.6- a,d). For example, I measured a greater than 2-fold increase of MDR-1 levels in 2/12 ovarian samples (none of whom had previously received a MDR-1 pumped chemotherapeutic agent), and a significant up-regulation of BCRP in 3/12 ovarian samples.

Doxorubicin exposure also induced significant down-regulation of TOPO II α (table 9.2); its median levels decreased from 0.546 to 0.017 relative units in a group of 14 breast samples ($p < 0.0001$, Wilcoxon matched pairs test) and from 1.491 to 0.089 relative units in the ovarian cancer subgroup ($p < 0.0015$, Wilcoxon). The down-regulation of TOPO II α levels was greater than 2-fold in 13/14 breast samples and in 9/12 ovarian samples (figure 9.6-h). We observed a slight increase of TOPO I only in 1 breast tumour, while we noted a general trend of diminished levels in the remaining samples, particularly in the ovarian subgroup.

No significant changes were found in the median expression of ERCC1 (table 9.2; figure 9.6-c). However it must be noted that the expression levels of this gene increased more than 0.5 fold in 3/11 ovarian and 3/14 breast samples.

Doxorubicin exposure decreased the expression of COX-2 in ovarian cells from 0.445 to 0.144 units (table 9.2). Although this difference was not statistically significant, a moderate down-regulation was noted in 11/12 ovarian samples. The level of COX-2 in breast cancer cells, instead, increased after doxorubicin exposure in 6/14 samples.

Table 9.2 Relative expression of mRNA levels in tumour samples after *ex vivo* exposure to doxorubicin (Doxo). The p values have been calculated using non parametric statistics, i.e. the Wilcoxon matched pairs test for paired samples with a Bonferroni's correction (statistical significance was taken at $p < 0.005$).

Target gene	Number of samples	Breast tumour samples		p	Number of samples	Ovarian tumour samples		p
		Control	Doxo			Control	Doxo	
BCRP	14	0.039	0.044	0.8077	12	0.010	0.027	0.042
COX-2	14	7.72	12.5	0.2958	12	0.445	0.144	0.0269
ERCC1	14	0.711	0.826	0.2412	11	0.440	0.506	0.4922
MDR1	14	0.0698	0.0567	0.0494	12	0.00550	0.00493	0.2036
MRP1	14	3.89	5.69	0.0001	12	1.89	2.32	0.083
MVP	14	4.99	6.92	0.0023	12	2.25	2.69	0.042
TOPO I	14	5.46	5.13	0.0419	12	6.11	3.36	0.001
TOPO II α	14	0.546	0.017	0.0001	12	1.49	0.089	0.0015
TOPO II β	14	6.62	6.46	0.0785	12	5.91	5.25	0.0322

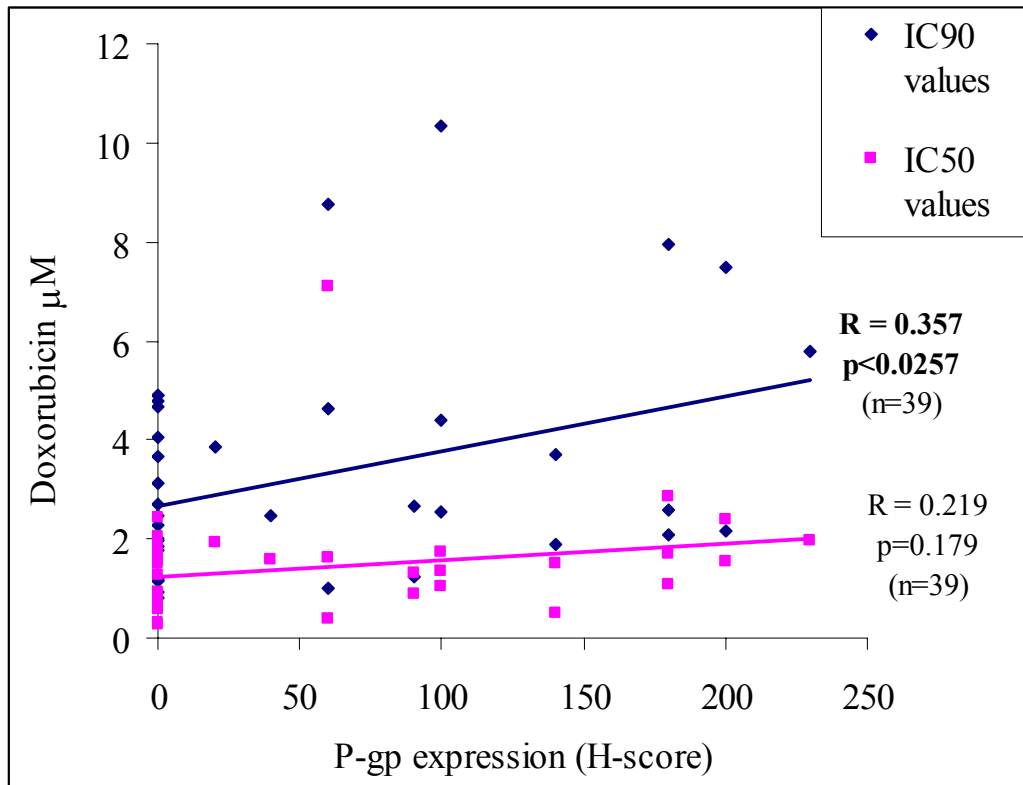


Figure 9.1 Correlation between sensitivity to doxorubicin (expressed as IC90 and IC50) and P-gp expression determined by immunohistochemistry in 39 ovarian cancer samples.

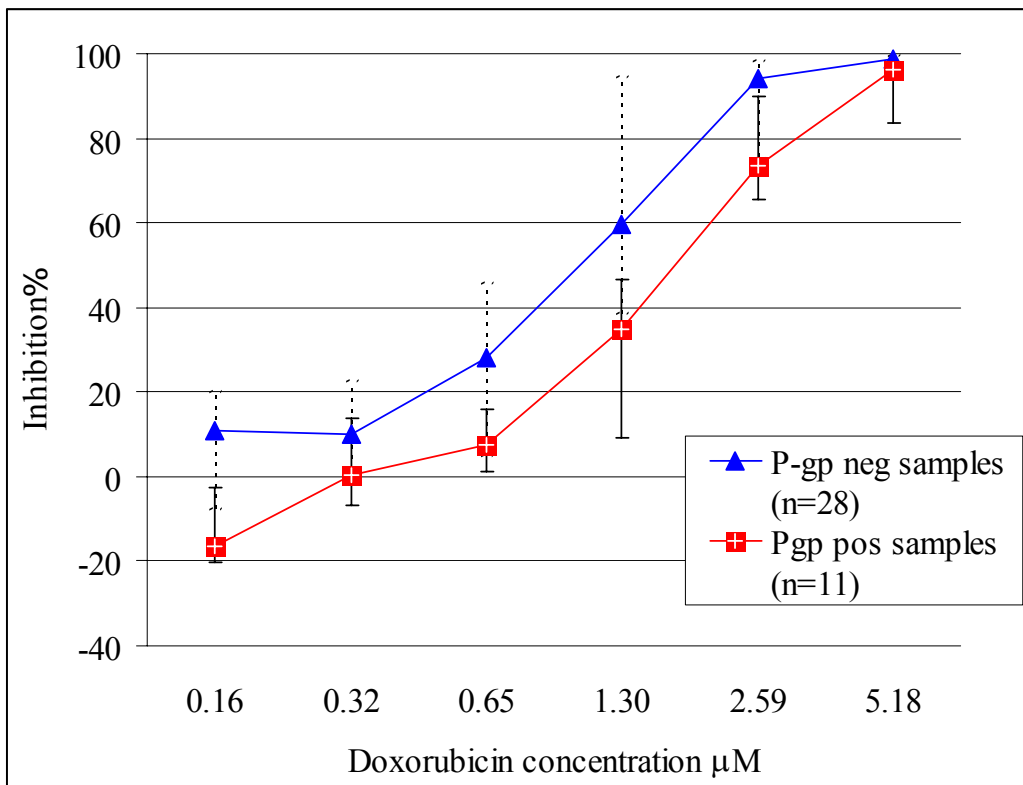
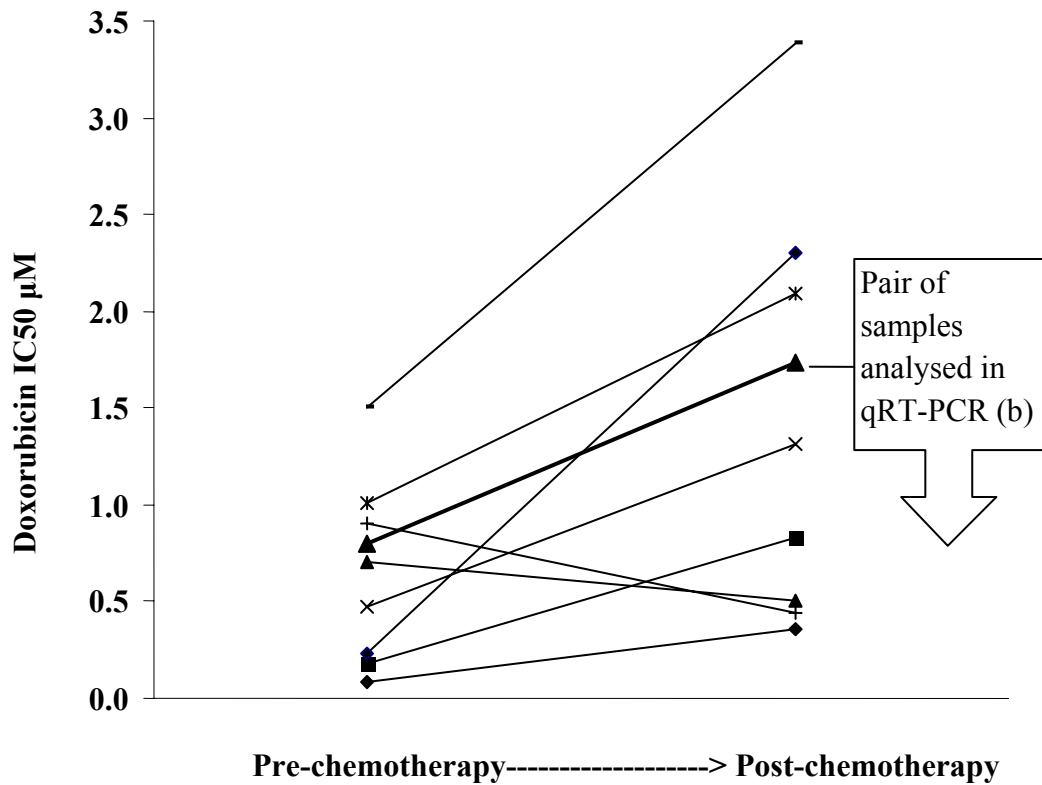


Figure 9.2 Concentration-inhibition curves for doxorubicin in P-gp negative (n=28) and P-gp positive (n=11) samples. The error bars show the interquartile range.

(a)



(b)

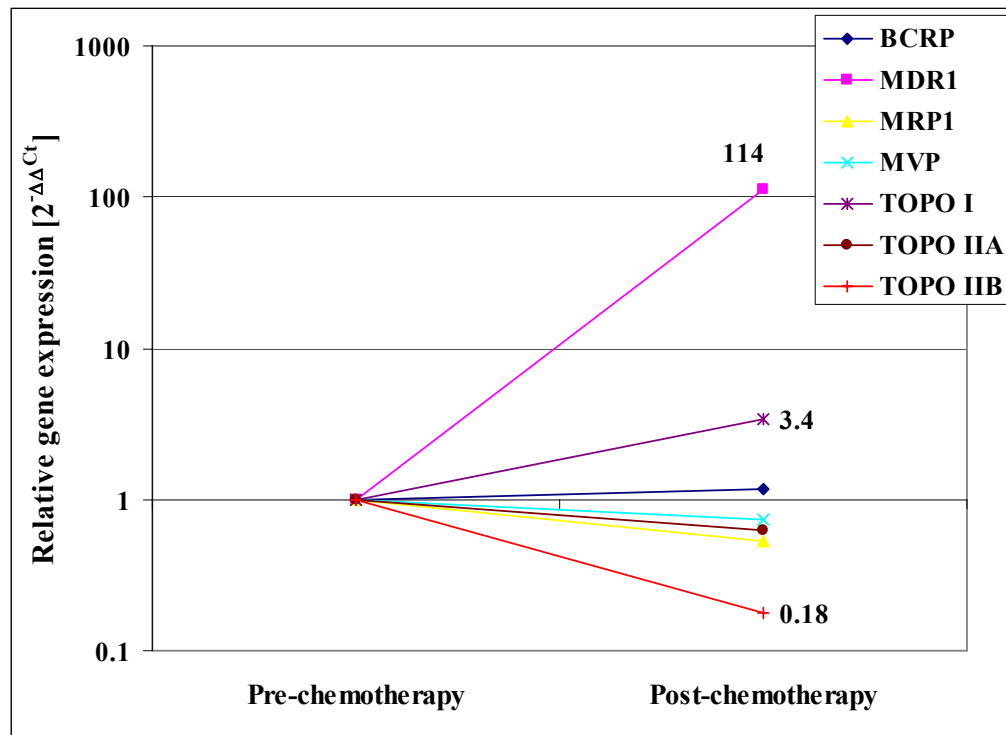


Figure 9.3 (a) Doxorubicin activity (IC₅₀) in the ATP-TCA in paired samples obtained from 9 ovarian cancer patients before and after they were treated with an anthracycline containing regimen (paclitaxel+mitoxantrone n=7; liposomal doxorubicin n=2). Gene expression changes in one of these pairs of samples were analysed by qRT-PCR (b).

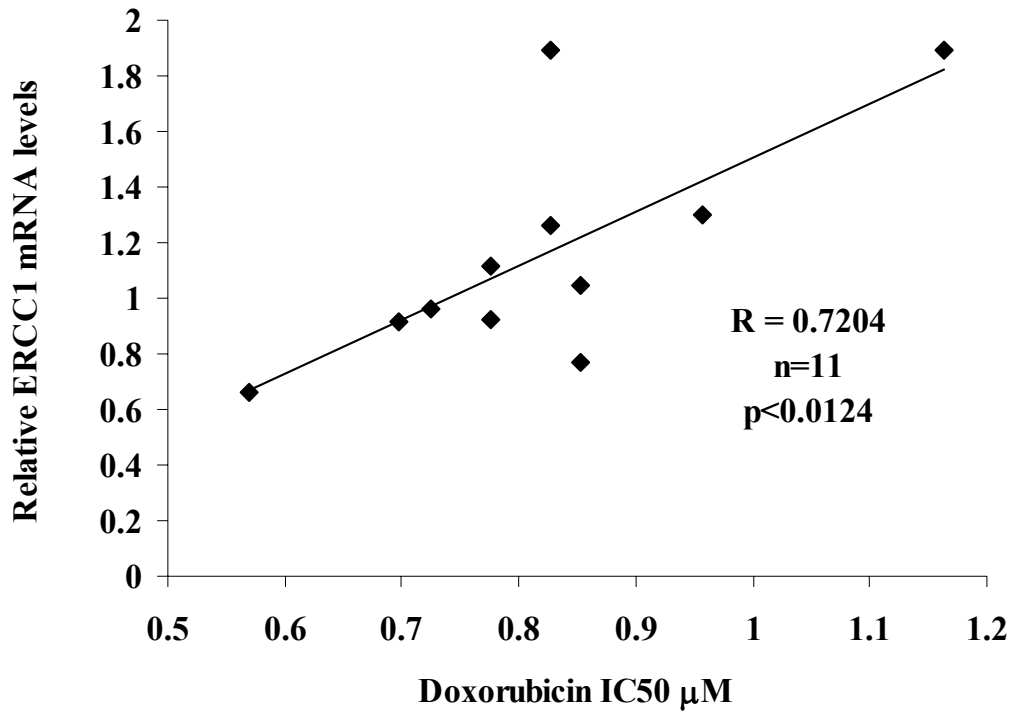


Figure 9.4 Correlation between the sensitivity to doxorubicin (expressed as IC50µM) and the relative ERCC1 mRNA levels measured by qRT-PCR in breast tumour derived cells not exposed to previous chemotherapy (n=11).

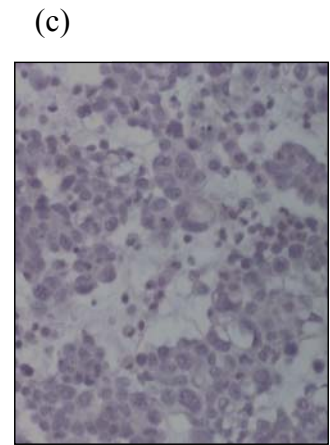
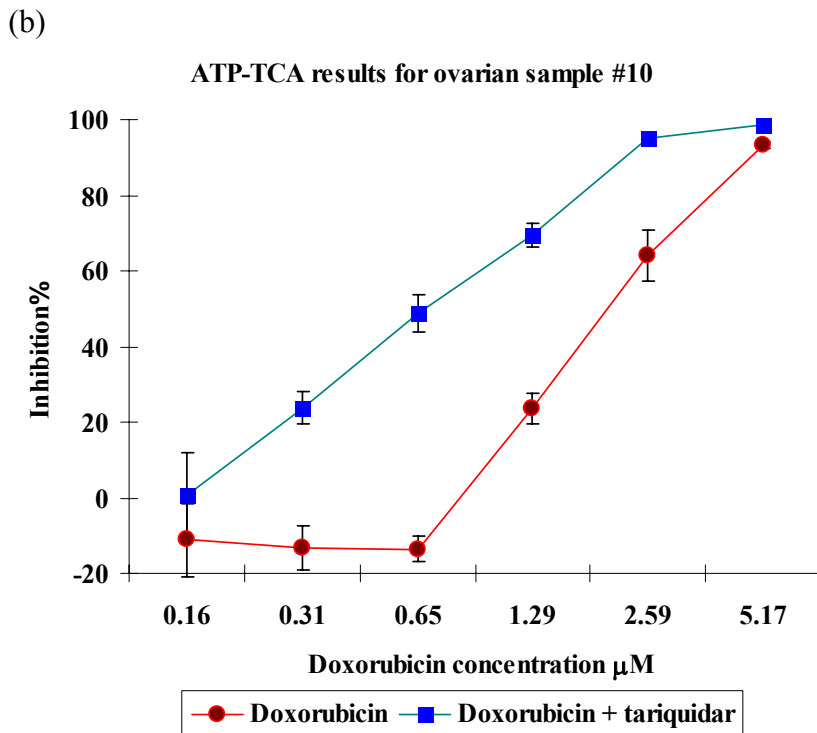
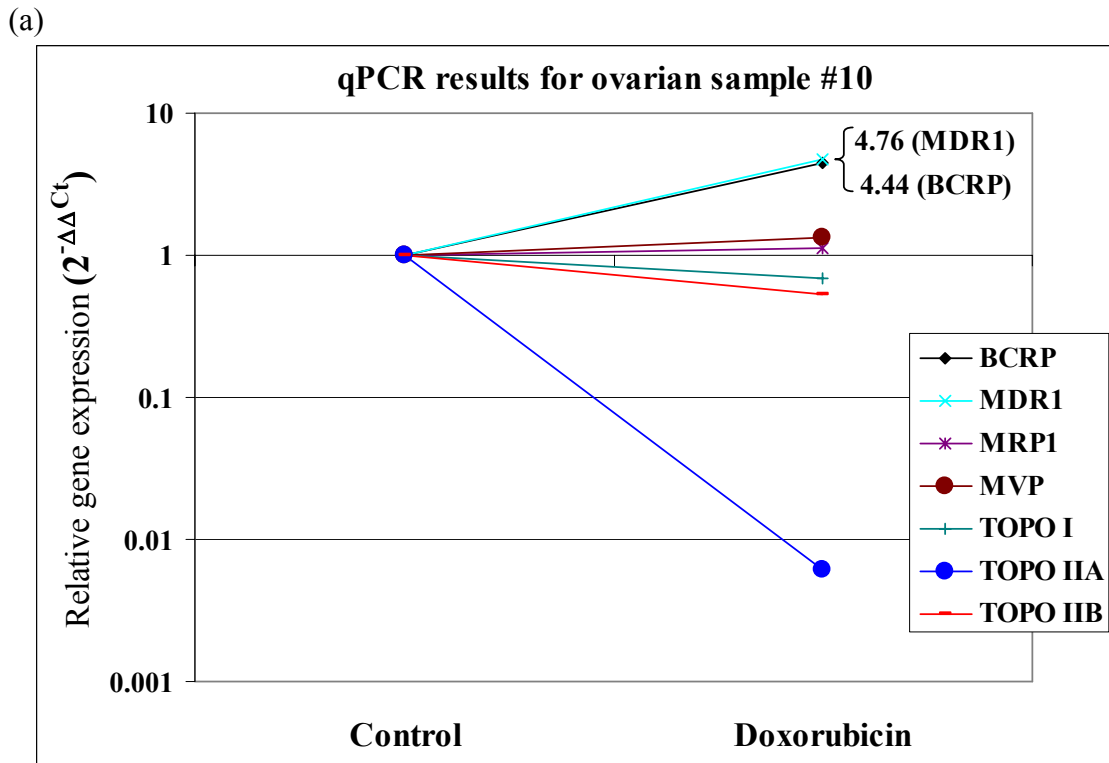


Figure 9.5 (a) Changes in gene expression after doxorubicin exposure in the ATP-TCA in ovarian tumour derived cells from patient #10. Panel (b) shows the ATP-TCA results (mean \pm SD) for the same sample; in this case doxorubicin resistance is partly reversed in the assay by the addition of tariquidar, a P-gp specific inhibitor. IHC staining for P-gp in this sample (cytocolot) was negative (c).

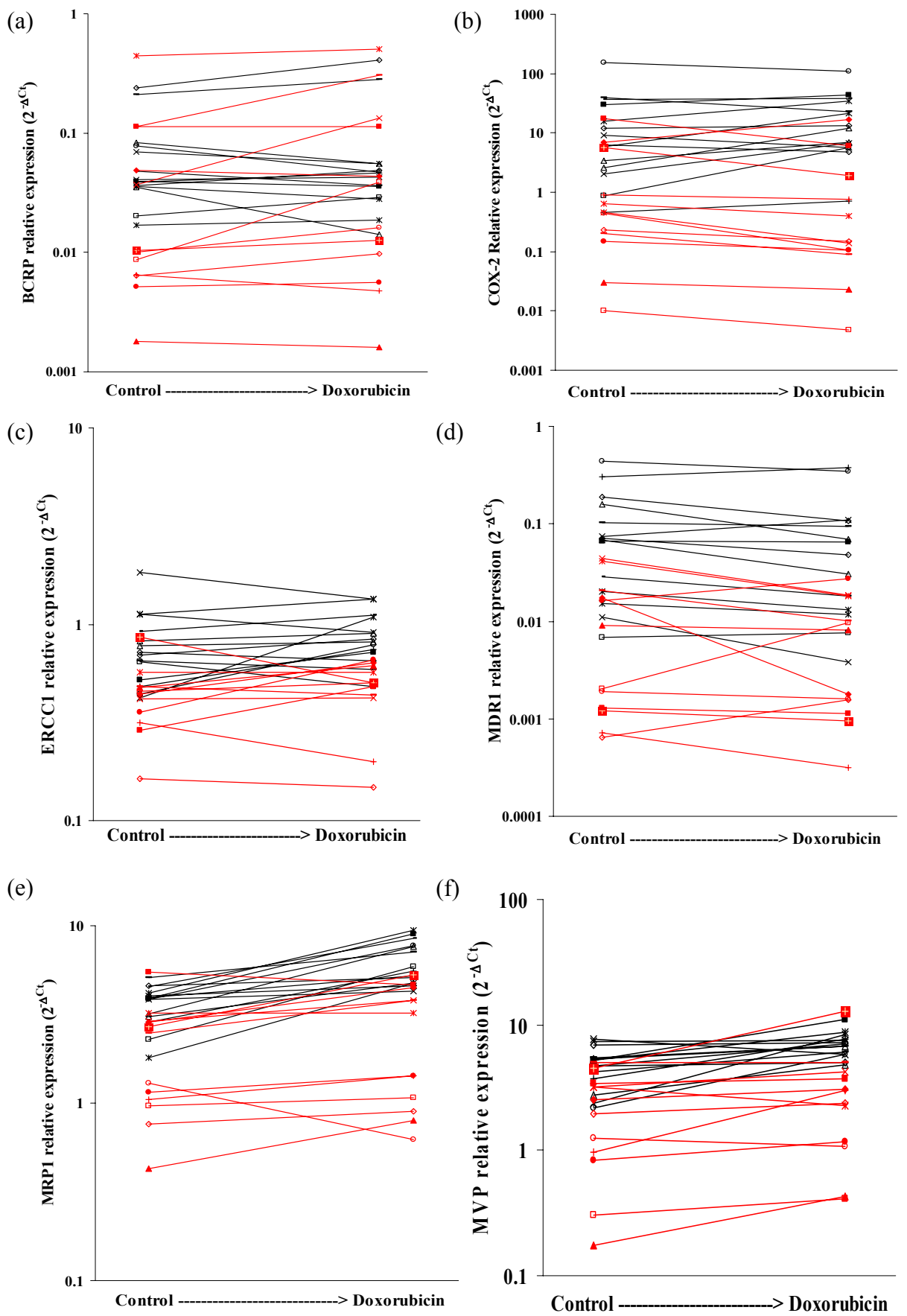


Figure 9.6 Changes in (a) BCRP, (b) COX-2, (c) ERCC1, (d) MDR1, (e) MRP1, (f) MVP gene expression after doxorubicin exposure in the ATP-TCA in tumour derived cells from 12 ovarian (in red) and 14 breast (in black) cancer patients.

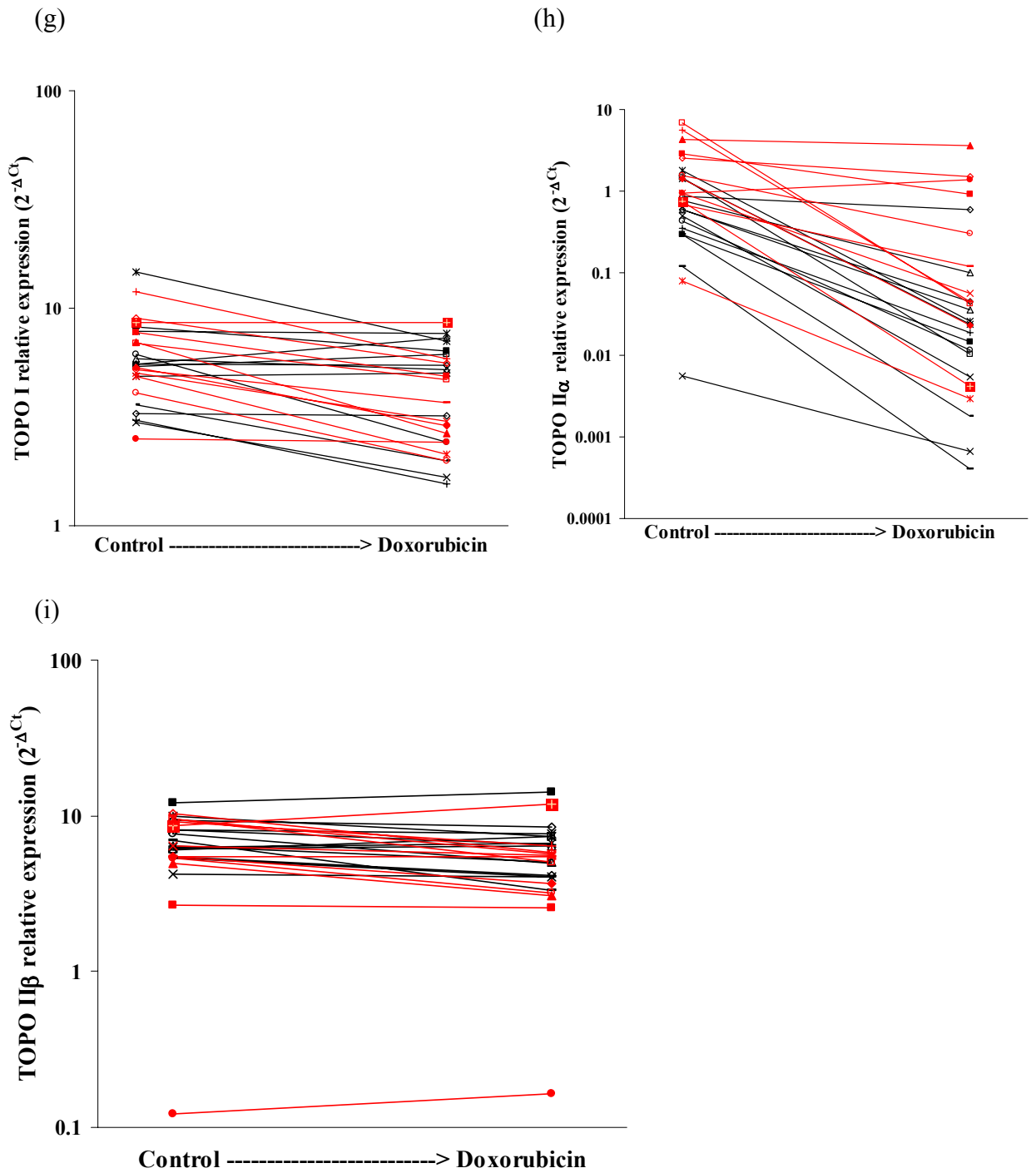


Figure 9.6 (continued). Changes in (g) TOPO I, (h) TOPO II α and (i) TOPO II β gene expression after doxorubicin exposure in the ATP-TCA in tumour derived cells from 12 ovarian (in red) and 14 breast (in black) cancer patients.

9.4 Discussion

The heterogeneity of chemosensitivity to doxorubicin observed in chapter 8 has been investigated further in this chapter and correlated with gene expression profiling for P-gp and TOPO I/II α determined by both IHC and qRT-PCR. This study failed to correlate strongly any of the investigated genes with doxorubicin resistance, although evidence was found supporting a role of P-gp, especially in a sub-group of the ovarian cancer patients. These findings are consistent with previous reports from various groups that were not able to correlate molecular expression with chemosensitivity data (Satherley *et al.*, 2000; Coley *et al.*, 2002; Lewandowicz *et al.*, 2002).

In this chapter it was also showed that ovarian patients who relapse after being treated with an anthracycline based regimen are usually more resistant to doxorubicin in the ATP-TCA when the results of the assay are compared with the results of the test before chemotherapy. In the only case in which material was available, the expression levels of MDR1 mRNA were increased over 100-fold in the biopsy obtained after anthracycline plus taxane treatment. This is no surprise as the acquisition of a multidrug resistance phenotype in relapsed ovarian cancer is well documented (Baekelandt *et al.*, 2000). According to the model proposed by Goldie and Coldman (1979) drug resistance in a cancer cell population arises from spontaneous mutations which confer a selective survival advantage. The growth of selected clones requires several cell divisions and the development of drug resistance becomes apparent after several cycles of chemotherapy (Duhem *et al.*, 1996). However, a number of recent studies have shown that the expression of P-gp may be rapidly up-regulated shortly (i.e. within hours) after the patients are administered chemotherapeutic agents such as anthracyclines (Abolhoda *et al.*, 1999; Hu *et al.*, 1999; Stein *et al.*, 2002; Tada *et al.*, 2002). The short timeframe does not support a role for mutations driven resistance. On the other hand, if this was a general pattern in cancer behaviour, then it could explain, at least in part, the inconsistent results obtained when gene expression measured at diagnosis is compared with the *in vivo* or *ex vivo* response to chemotherapy.

To test this hypothesis I measured the changes in gene expression induced by doxorubicin exposure for 6 days in primary breast and ovarian cells. The length of exposure was chosen so that tumour derived cells from the ATP-TCA could be used for qRT-PCR studies. While a period of 6 days is longer in relation to the half life of free

doxorubicin (Northfelt *et al.*, 1996), it is considerably shorter when compared to the several months employed by other groups to select doxorubicin resistant sublines (Kudoh *et al.*, 2000; Watts *et al.*, 2001; Kang *et al.*, 2004).

A marked and consistent decrease in the expression levels of TOPO II α was observed after doxorubicin exposure. This was expected, as TOPO II α inhibitors preferentially kill cells with high levels of this enzyme (Hande 1998).

A significant up-regulation of drug transporter genes, in particular of MRP1 and MVP in breast cancer cells, was also noted. However, not all tumours up-regulated these genes, and considerable variation was found for all transporters studied.

This is the first study that has coupled a well standardised *ex vivo* chemosensitivity assay to qRT-PCR technology to measure gene expression changes induced by drug exposure. The only comparable data existing in literature are provided by cDNA microarray studies. Kudoh *et al.* (2000) used cDNA microarrays to monitor the expression profiles of MCF-7 cells that were either transiently treated with doxorubicin or selected for resistance to doxorubicin. These researchers identified a set of genes with altered expression that overlap between doxorubicin-induced and -selected cells, and among them XRCC1. In our experiments, another enzyme involved in similar NER processes, ERCC1 was found to correlate with doxorubicin sensitivity in tumour derived cells from untreated breast cancer patients, but not in ovarian cancer cells. Up-regulation of ERCC1 in response to doxorubicin treatment was noted in approximately 25% samples, suggesting a role of for NER in repairing the DNA damage indirectly induced by topoisomerase inhibitors (Champoux 2001).

Another study used cDNA microarrays to search for differentially expressed genes between a human multiple myeloma cell line and doxorubicin-selected sub-clones that express ABCB1 and are multidrug resistant (Watts *et al.*, 2001); these authors found that sensitivity to doxorubicin was correlated with the expression of a number of apoptotic genes, most of which have never been associated with anthracycline resistance.

While cDNA microarray studies offer the potential advantage of assessing thousands of target genes, the poor reproducibility of the data produced and the background noise (Macgregor & Squire 2002) suggests the need for a cautious approach when interpreting their predictive value, particularly when assessing small differences in gene expression. Moreover microarrays are inherently less sensitive than real time RT-PCR, so, as suggested by Macgregor & Squire (2002), less abundant transcripts (such as MDR1 or BCRP in breast and ovarian cancer cells) may be and almost certainly will be missed.

In conclusion this study has confirmed that the resistance to doxorubicin observed in the ATP-TCA is multifactorial and cannot be predicted by the expression of a single gene such as MDR1/P-gp or TOPO II α . The results have also shown that the substantial heterogeneity of chemosensitivity among samples of the same tumour type is reflected by heterogeneity of resistance/sensitivity molecular determinants induced by doxorubicin at the mRNA level. The clinical relevance of these findings are partly corroborated by the observed rapid up-regulation of MDR1/P-gp in patients undergoing anthracycline-based chemotherapy (Abolhoda *et al.*, 1999; Hu *et al.*, 1999; Stein *et al.*, 2002; Tada *et al.*, 2002). More clinical studies are needed to confirm the importance of such rapid changes in the molecular phenotype in conferring resistance to anthracyclines.

Chapter 10 – The effect of tariquidar on sensitivity to doxorubicin, vinorelbine and paclitaxel

10.1 Introduction

New specific P-gp inhibitors are currently being developed to overcome MDR-1 mediated resistance (see Chapter 1.2.1), among them tariquidar (XR9576; figure 10.1). This is a highly potent anthranilic acid derivative that has been demonstrated to reverse P-gp dependent multidrug resistance in various preclinical models. Tariquidar is a non-competitive P-gp inhibitor, it has a high affinity for P-gp ($K_d=2.5\pm 0.7\text{nM}$; Martin *et al.*, 1999) and, importantly, has no effect on the related transporter multidrug-resistance-associated protein (Stewart *et al.*, 1998). The binding site of tariquidar on P-gp has not clearly been defined, but it inhibits the ATPase activity of P-gp (Martin *et al.*, 1999). The inhibitory effects of tariquidar on the P-gp transporter pump greatly exceed those of first- and second-generation P-gp modulators with respect to potency and duration of action. In an *in vitro* study, P-gp pump transport remained blocked for more than 22 hours after tariquidar had been removed from the culture medium; in the same assay, the clearance time for cyclosporin was 60 minutes (Mistry *et al.*, 2001). Tariquidar was found to potentiate the activity of a number of MDR-associated cytotoxics in cell lines overexpressing P-gp with a typical IC_{50} of approximately 30 nM (Mistry *et al.*, 2001). This *in vitro* activity translates to tumour bearing animals where tariquidar has been shown to reverse the resistance of multidrug-resistant human xenografts at well-tolerated doses (Mistry *et al.*, 2001).

Phase I clinical trials of tariquidar commenced in May 1998 in healthy volunteers and were single dose, dose escalation studies to establish its safety and tolerance and to evaluate the plasma concentrations. In these studies, doses of tariquidar up to 2mg/Kg i.v. and up to 750mg/volunteer p.o. were very well tolerated. These trials also included the use of a surrogate marker of efficacy to establish inhibition of P-gp as a pharmacological end point. As reported by Witherspoon *et al.* (1996), CD56+ lymphocytes constitutively express significant levels of P-gp. Thus the measurement of inhibition of P-gp in CD56+ lymphocytes is a surrogate for the inhibition of P-gp in tumours. In healthy volunteers tariquidar effectively inhibited P-gp in CD56+ lymphocytes, and 100% blockade was seen at tariquidar plasma concentration around 240 nM (Stewart *et al.*, 2000). In phase IIa trials, the pharmacokinetic behaviour of tariquidar was studied in combination therapy with a range of P-gp pumped cytotoxic agents, namely vinorelbine, doxorubicin and paclitaxel. The primary purpose of these trials was to assess the degree of interaction, if any, between tariquidar and the cytotoxic

agent. The successful conclusion of the first phase IIa trial involving tariquidar and paclitaxel in ovarian cancer patients was announced in March 2000. Although not designed as an efficacy study, a significant response was observed in 6 (3CR+3PR) of the 12 cases in this cohort of patients who had recurred more than six months after first (n=11) or second-line (n=1) treatment (Thomas *et al.*, 2001). Further positive data was also announced for the second of the Phase IIa trials, in which tariquidar was administered in combination with doxorubicin (Ferry *et al.*, 2001). In the third and final Phase IIa trial tariquidar was given in combination therapy with vinorelbine and one patient each with breast cancer and renal cancer responded to chemotherapy. In the last study administration of tariquidar resulted in a 15% increase in vinorelbine AUC and reduced clearance, but these differences were not statistically significant (Abraham *et al.*, 2001). The results of these three studies demonstrated that tariquidar is a potent P-gp inhibitor, without significant side effects and with less pharmacokinetic interaction than other inhibitors used previously (Boniface *et al.*, 2002). This allowed the use of standard doses of these chemotherapeutic agents without the need for dose reduction. A randomised placebo-controlled phase III clinical trial in patients with non-small-cell lung cancer (NSCLC) started in 2002, using tariquidar as an adjunctive treatment in combination with first-line chemotherapy. After enrolling 304 patients this trial was stopped in May 2003 following a recommendation from the independent Data Safety Monitoring Committee (DSMC). The review of the data arising from the Phase III NSCLC trial has recently been completed. This review has shown that the levels of toxicities associated with the cytotoxic drugs administered to patients in the trial were increased in those individuals receiving tariquidar and cytotoxic compared with those receiving placebo and cytotoxic.

The aim of this study was to determine the ability of tariquidar to alter the chemosensitivity of various solid tumours to commonly used chemotherapeutic agents. It was chosen to study doxorubicin, paclitaxel, and vinorelbine as examples of anthracyclines, taxanes and vinca alkaloids, and have related the results to the expression of P-gp by the tumours studied, though this was limited by the nature of the material available.

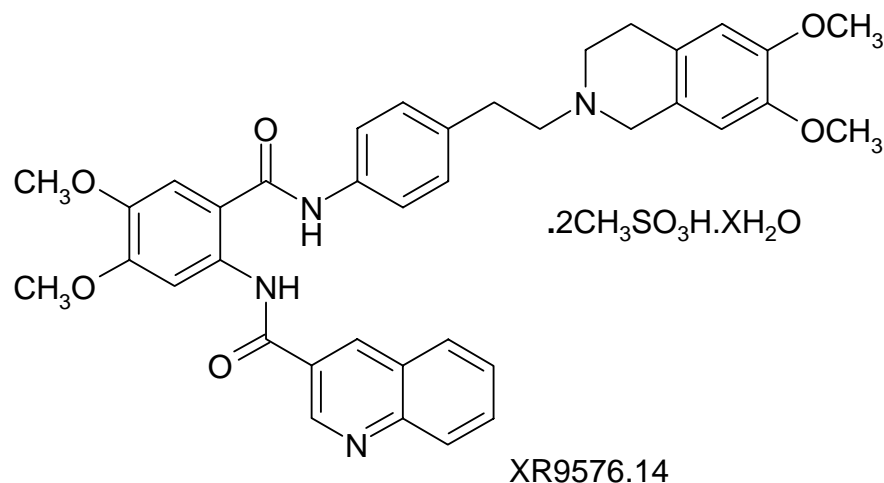


Figure 10.1 Chemical structure of tariquidar bis mesylate salt that was used in the *ex-vivo* studies.

10.2 Materials and methods

Tissue Samples. Of the 37 solid tumour samples studied, 21 were ovarian carcinomas, 5 were unknown primaries, 5 were skin melanomas, 2 were breast carcinomas, 2 were oesophageal, 1 colon and 1 lung (NSCLC) carcinomas. Patients consisted of 32 females and 5 males, having a median age of 58 (range 36-76). Several patients received one or more chemotherapy regimens, while 10/37 had no previous treatment (table 10.1).

Drugs. Tariquidar was provided by Xenova plc (Slough, Berkshire) as powder. A stock solution of tariquidar (XR9576.14 bis-mesylate salt; figure 10.1) was prepared in DMSO at a concentration of 1 mg/ml and stored as aliquots at -20°C . For cell treatments, the stock solution was further diluted in culture medium to give a tariquidar TDC $1\mu\text{M}$ with the final DMSO concentration less than 0.1%. The other drugs used in the assay were prepared as described in chapter 2.2.9. Only one plate was prepared with the combinations of tariquidar with vinorelbine, paclitaxel and doxorubicin. Combinations were made up by adding both drugs at their 200% TDC to the wells at the beginning of the assay: sequential studies were not performed. Not all drugs or combinations were tested in every case.

IHC. When solid tissue or cytoclots were available (n=23), MDR1/P-gp monoclonal antibody (NCL-JSB1) from Novacastra Laboratories Ltd was visualised using the Vectastain® Universal Alkaline Phosphatase kit (Vector Laboratories Ltd., Peterborough, UK), as detailed in chapter 2.4. All of the IHC shown here was carried out by the biomedical scientists working with Prof Cree's research team in Portsmouth and London.

Table 10.1 Patient and sample characteristics.

Tumour Type	No of samples	Patient age (median)	Sex	Sample type	Previous Treatment (1)
Ovarian carcinoma	21	58 (38-76)	21F	5 solid, 2 pleural fluid, 14 ascites	Carboplatin (n=13), Platinum+Taxane (n=10), Liposomal Doxorubicin (n=3), Treosulfan+Gemcitabine (n=2), Carboplatin+Gemcitabine (n=1), Mitoxantrone+Paclitaxel (n=1), Topotecan (n=1), Etoposide (n=1)
Skin Melanoma	5	48 (36-66)	3M:2F	5 solid	Melphalan (n=1), Cyclophosphamide (n=1)
Unknown Primary	5	61 (45-68)	5F	3 solid, 2 pleural fluid	Carboplatin (n=1)
Breast carcinoma	2	49 (39-59)	2F	1 solid, 1 pleural fluid	Anastrozole (n=1)
Oesophageal carcinoma	2	62 (52-72)	2M	2 solid	Epirubicin+Cisplatin+5-Fluorouracil (n=1)
Colon carcinoma	1	39	1F	solid	Irinotecan (n=1)
Lung (NSCLC)	1	58	1F	pleural fluid	Cisplatin+Vinorelbine (n=1)
Total	37	58 (36-76)	5M:32F	17 solid, 20 fluid	

(1) The numbers in brackets indicates the number of patients who received the previously listed treatment. In the ovarian carcinoma subset several patients received more than one chemotherapeutic treatment.

10.3 Results

10.3.1 Anti-tumour activity of single agents

As expected, tariquidar alone showed no activity over a wide range of concentrations up to and including 2 μM (n=14) (figure 10.2). Each of the cytotoxics tested produced a wide range of tumour inhibition, as can be seen in table 10.2. This is consistent with considerable heterogeneity of chemosensitivity, as previously published (Cree *et al.*, 1999; Whitehouse *et al.*, 2003; Mercer *et al.*, 2003).

The calculated IC₉₀ values were used to assess the activity of each cytotoxic. Values of IC₉₀ \geq 100 %TDC were considered to suggest resistance, while values $<$ 100 %TDC were interpreted to suggest probable clinical sensitivity to the agent tested. When the addition of tariquidar decreased the cytotoxic IC₉₀ to $<$ 50 % TDC, this was interpreted as a complete reversal of resistance.

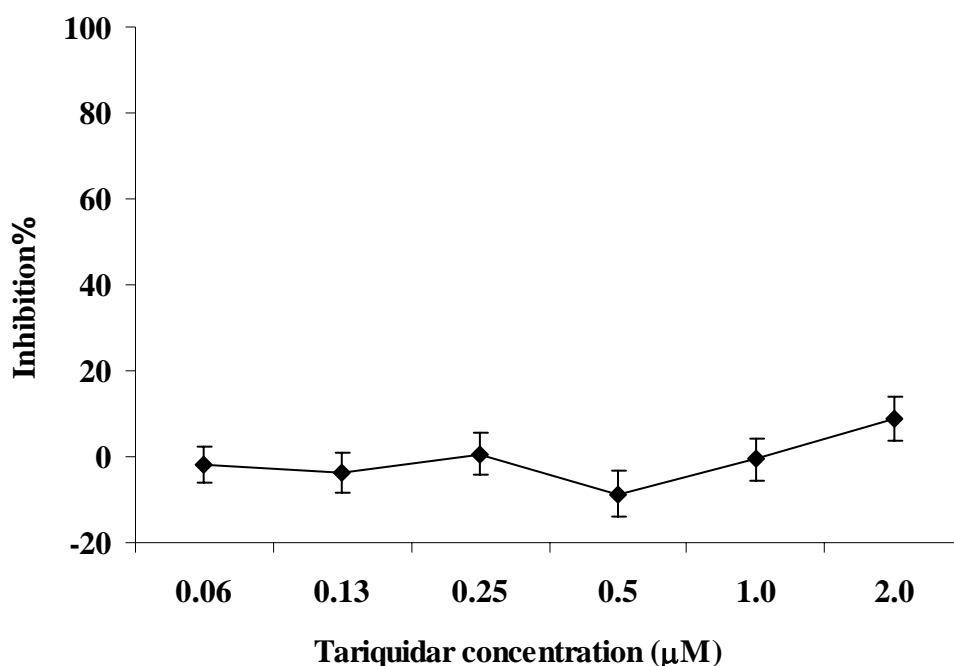


Figure 10.2. Tumour cell growth inhibition by tariquidar as a single agent (n=14). The results are expressed as mean \pm SEM of the values calculated for each tumour for each drug concentration.

Table 10.2. Summary of ATP-TCA results (median and range) of tariquidar activity in combination with vinorelbine, doxorubicin and paclitaxel. Statistical analysis was performed using the two-tailed Wilcoxon paired signed rank test; n indicates the number of samples tested for each drug combination.

Patients n= 31 (4M:27F)	Age	Vinorelbine IC90 μM	Vinorelbine + tariquidar IC90 μM	p	Vinorelbine IC50 μM	Vinorelbine + tariquidar IC50 μM	p
Median	59	15.51	9.51	0.0003	2.27	0.77	0.0739
Range	(36-76)	(0.66-61.41)	(0.67-20.01)		(0.37-34.11)	(0.37-11.96)	
Patients n=37 (5M:32F)	Age	Doxorubicin IC90 μM	Doxorubicin + tariquidar IC90 μM	p	Doxorubicin IC50 μM	Doxorubicin + tariquidar IC50 μM	p
Median	58	2.57	1.67	<0.0001	1.57	0.92	0.0028
Range	(36-76)	(0.39-21.49)	(0.05-16.16)		(0.10-11.95)	(0.03-8.98)	
Patients n=34 (4M:30F)	Age	Paclitaxel IC90 μM	Paclitaxel + tariquidar IC90 μM	p	Paclitaxel IC50 μM	Paclitaxel + tariquidar IC50 μM	p
Median	58	27.42	20.65	0.0201	9.72	7.73	0.0425
Range	(36-76)	(5.73-59.02)	(6.53-38.17)		(0.80-32.81)	(0.80-25.89)	

10.3.2 Effect of tariquidar on doxorubicin activity

The median doxorubicin IC₉₀ and IC₅₀ were 2.57 μ M and 1.57 μ M, respectively. The IC₉₀ of doxorubicin was >100%TDC (2.5 μ M) in 20/37 (54%) samples. For the ovarian cancer subset, *in vitro* resistance, according to IC₉₀, was seen in 12/21 (57%) samples.

Immunostaining for P-gp was positive in 9/22 (41%) samples tested with doxorubicin. It is notable that 5/13 P-gp negative samples were obtained from chemotherapy naïve patients, while 8/9 P-gp positive samples were from patients who had received previous treatment. The median doxorubicin IC₅₀ values for P-gp negative and positive samples were 0.90 and 2.00 μ M, respectively, and this difference was statistically significant ($p=0.0065$, Mann-Whitney U test). The median doxorubicin IC₉₀ values for P-gp negative and positive samples were 2.05 and 5.08 μ M, respectively ($p=0.0071$, Mann-Whitney U test). A correlation was also observed by linear regression analysis between the IC₉₀ of doxorubicin and P-gp staining ($R=0.705$; $p=0.0002$), and between the IC₅₀ and P-gp expression ($R=0.815$; $p < 0.0001$; Figure 10.3). It should be noted that this statistically significant correlation might be biased by the presence of three P-gp strongly positive tumours which showed high IC₉₀ and IC₅₀ values for doxorubicin. Indeed, on the basis of IC₉₀, only 22 % (2/9) of the P-gp positive samples showed sensitivity to doxorubicin, while 85% (11/13) of the P-gp negative samples showed sensitivity.

The addition of tariquidar to doxorubicin decreased the median IC₉₀ of doxorubicin from 2.57 to 1.67 μ M ($p<0.0001$) and the IC₅₀ from 1.57 to 0.92 μ M ($p=0.0028$, Wilcoxon). An example of ATP-TCA results in which tariquidar modulates sensitivity to doxorubicin is shown in figure 10.4. Overall, tariquidar showed some reversal of resistance to doxorubicin in 14/20 (70%) samples classified as *in vitro* resistant, and in 2 cases (10%) converted the sensitivity to an IC₉₀ below 1.25 μ M, which represents 50% TDC. Both these patients had recurrent ovarian carcinoma and none had previous anthracycline exposure. In the ovarian cancer subset, tariquidar showed some reversal of resistance to doxorubicin in 11/12 (92%) samples classified as *in vitro* resistant (figure 10.5). However, in one case that was considered sensitive to doxorubicin as a single agent, the addition of tariquidar increased the IC₉₀ of doxorubicin from 1.24 to 3.37 μ M. In all 9 P-gp positive cases the addition of tariquidar improved the activity of

doxorubicin in the ATP-TCA. In 4/9 cases (44%), the IC₉₀ of doxorubicin decreased below 2.5 μ M, and in 1/9 below 1.25 μ M (figure 10.4).

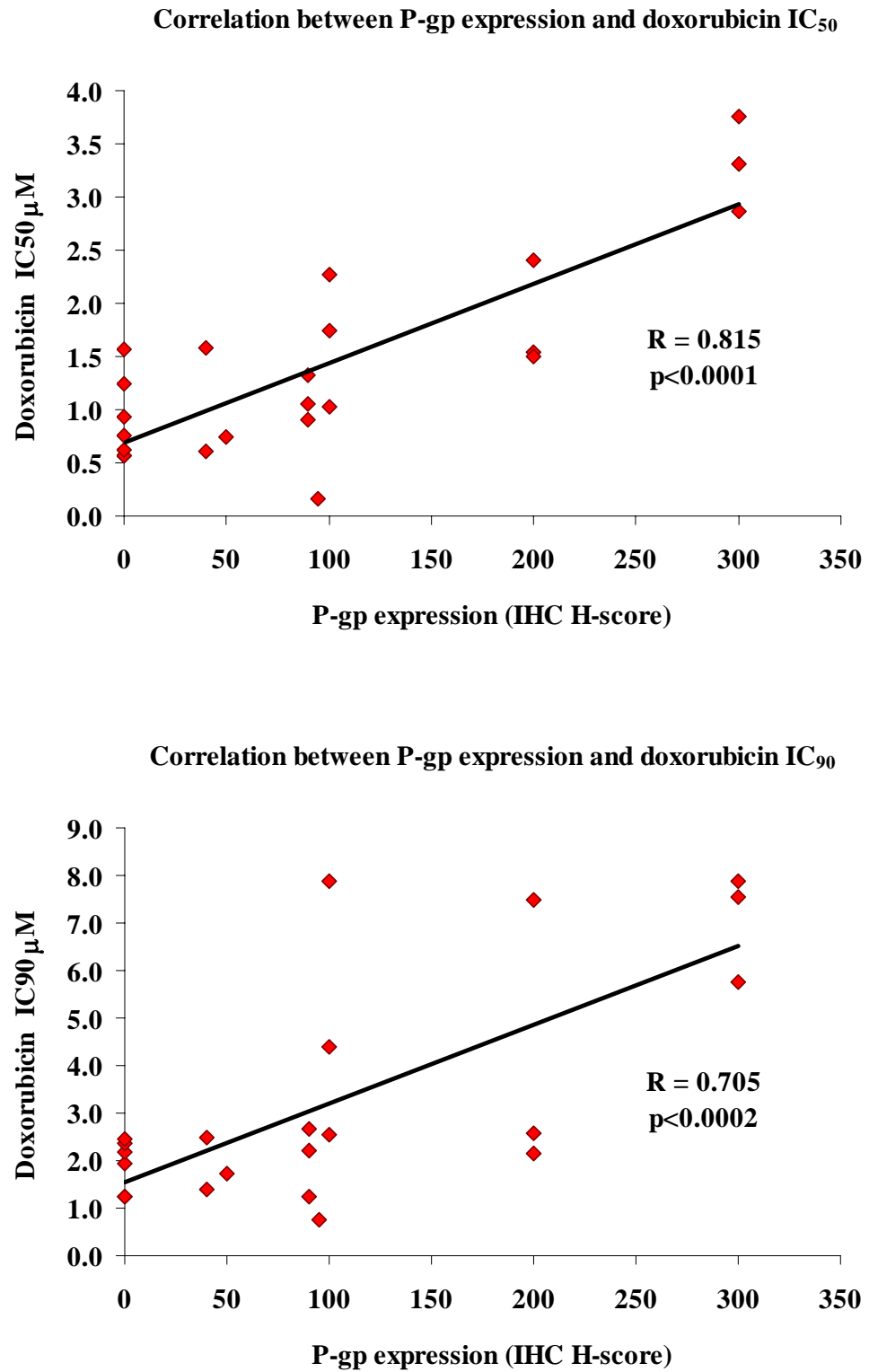
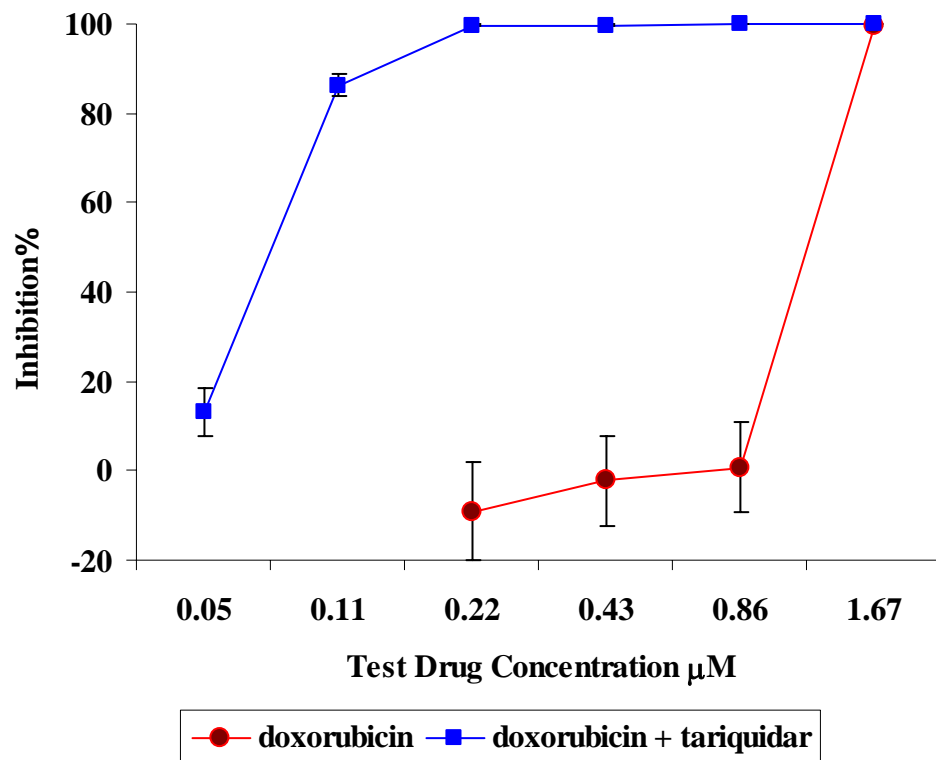


Figure 10.3 Linear regression analyses showing a correlation between P-gp staining in tumour samples (n=22) and sensitivity to doxorubicin in the ATP-TCA.

(a)



(b)

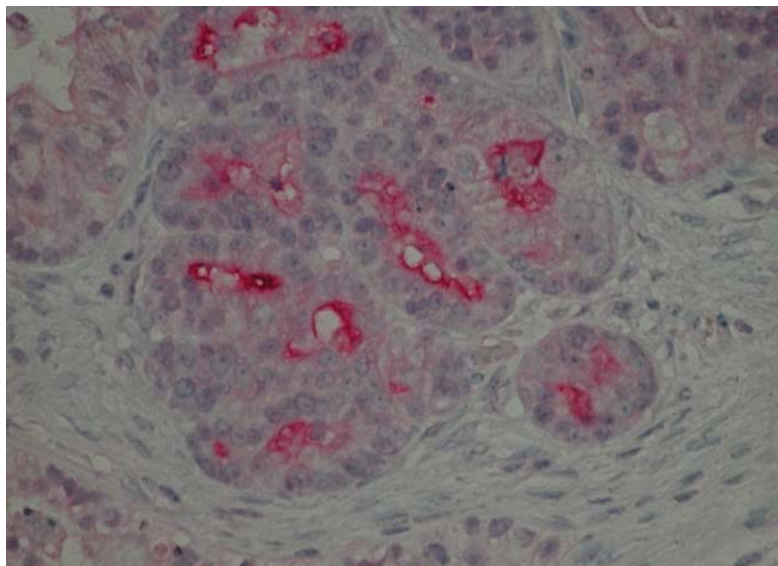
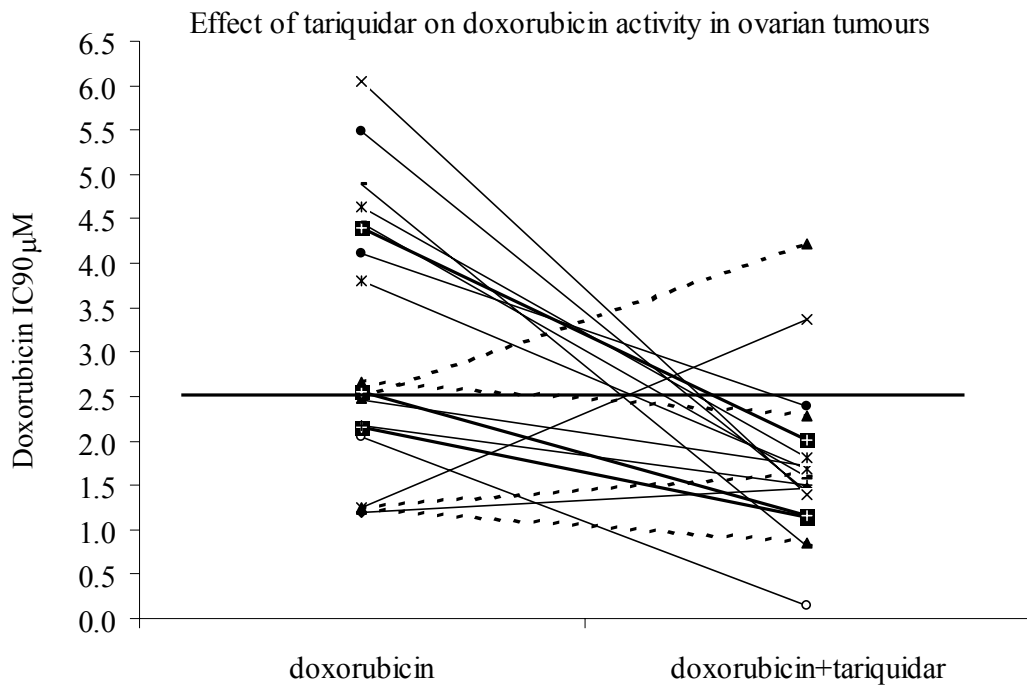


Figure 10.4 An example of the ATP-TCA results for an ovarian adenocarcinoma (a). Results are shown as mean (\pm SD) of triplicate values for each drug concentration. Error bars are not shown for some data points, as they are smaller than the symbols on the graph. (b) P-gp IHC for this sample.

(a)



(b)

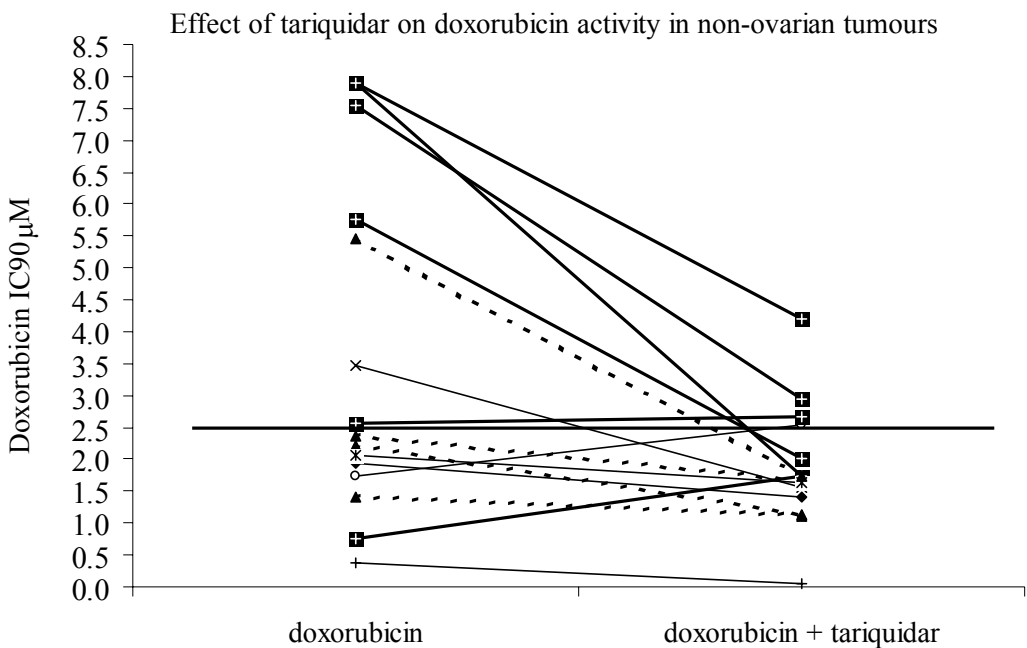


Figure 10.5 Effect of the addition of tariquidar on the activity of doxorubicin expressed as IC90 μM in ovarian (a) and non-ovarian tumour samples (b). The samples labelled with a 'plus' in a black square are P-gp positive tumours, while the samples labelled with a triangle and a dashed line are P-gp negative tumours; P-gp staining was not performed in samples labelled with the other symbols. A line has been drawn in each graph to indicate 100% TDC of doxorubicin.

10.3.3 Effect of tariquidar on vinorelbine activity

The median IC₉₀ and IC₅₀ for vinorelbine were 15.5 μM and 2.3 μM. The IC₉₀ of vinorelbine was >100% TDC (11 μM) in 17/31 samples, indicating probable *in vitro* resistance in 55% of the tumours tested. For the ovarian cancer subset, *in vitro* resistance according to IC₉₀ was seen in 8/17 (47%) tumours. Immunohistochemical staining for P-gp was positive in 7/22 (32%) samples tested with vinorelbine. The median IC₉₀ values for P-gp negative and positive samples were 12.5 and 20.8 μM, respectively, but this difference did not reach statistical significance (p=0.0556, Mann-Whitney U test). Semiquantitative visual assessment of the degree of staining showed no correlation with sensitivity to vinorelbine (IC₉₀ and IC₅₀) by linear regression analysis (data not shown). However, it should be noted that only 1/7 (14%) of the P-gp positive samples showed sensitivity to vinorelbine on the basis of IC₉₀, while 8/15 (53%) of the P-gp negative samples showed sensitivity to vinorelbine.

The addition of tariquidar decreased the median IC₉₀ of vinorelbine from 15.5 μM to 9.5 μM (p<0.001, Wilcoxon). Tariquidar showed some reversal of resistance to vinorelbine in 6/17 (35%) samples classified as resistant and in 1 case (6%) converted the sensitivity to an IC₉₀ below 5.5 μM, which represents 50% TDC (figure 10.6). In the ovarian cancer subset, tariquidar showed some reversal of resistance to vinorelbine in 3/8 (37%) samples classified as resistant (figure 10.6). Examples of tariquidar effects on individual tumours tested with vinorelbine are shown in figure 10.7.

Tariquidar produced little or no effect on vinorelbine activity in P-gp negative samples (Figure 10.8). In all 7 P-gp positive cases the addition of tariquidar improved the activity of the vinca alkaloid in the ATP-TCA. In 4/7 P-gp positive cases (57%) the IC₉₀ of vinorelbine decreased below 11 μM and in 1/7 below 5.5 μM.

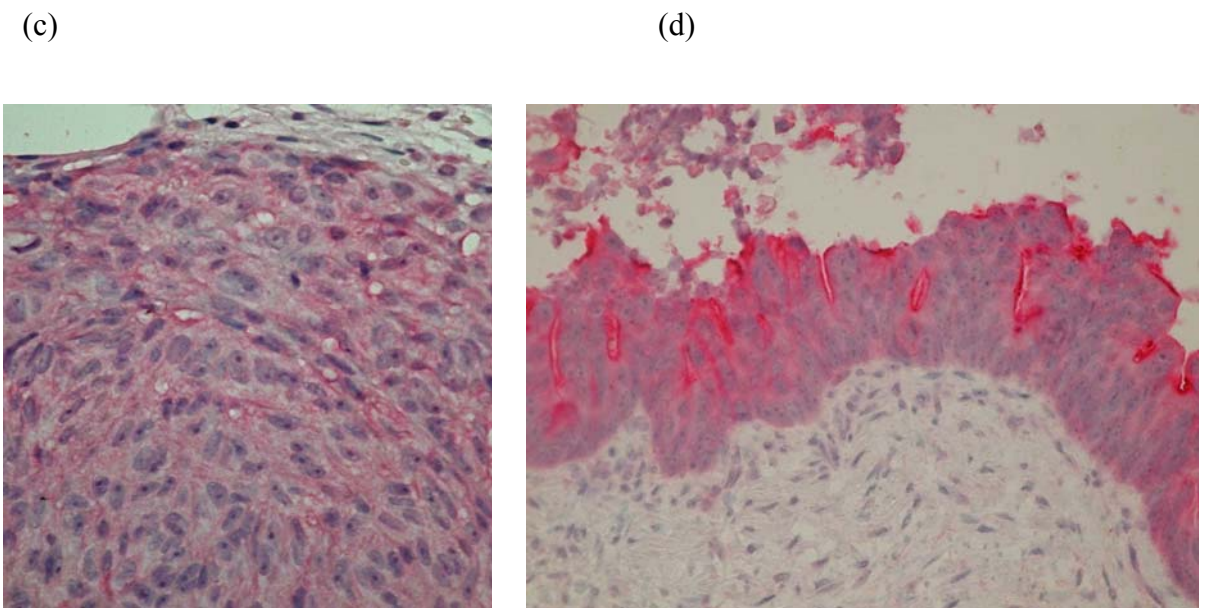
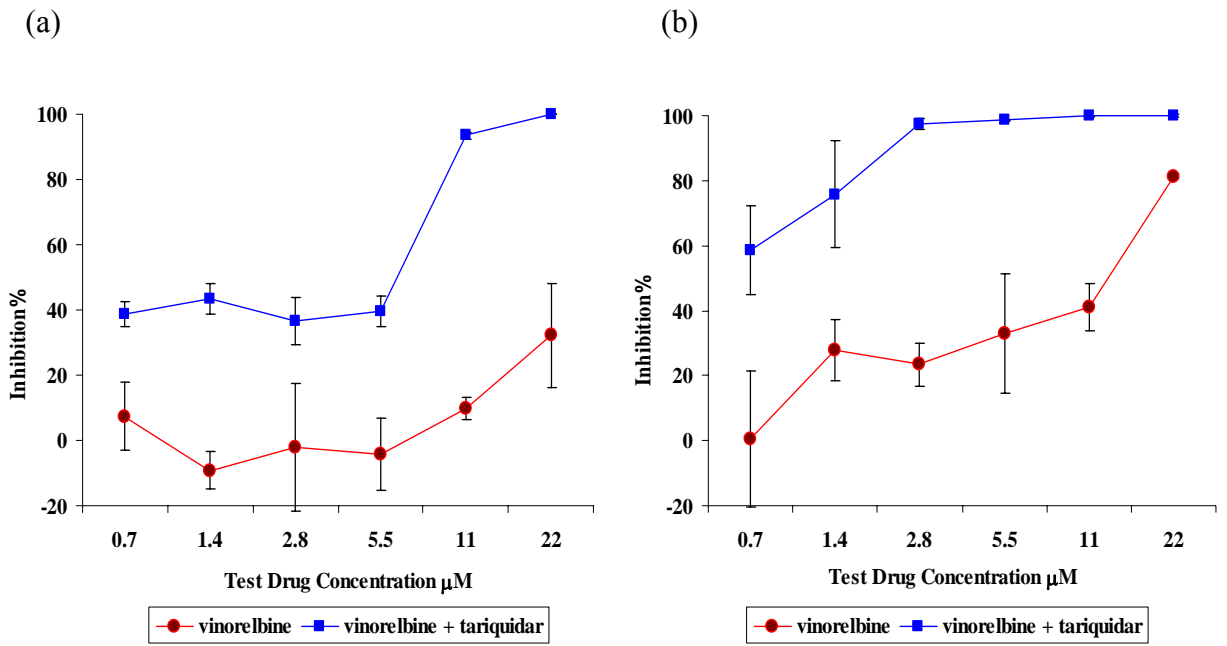
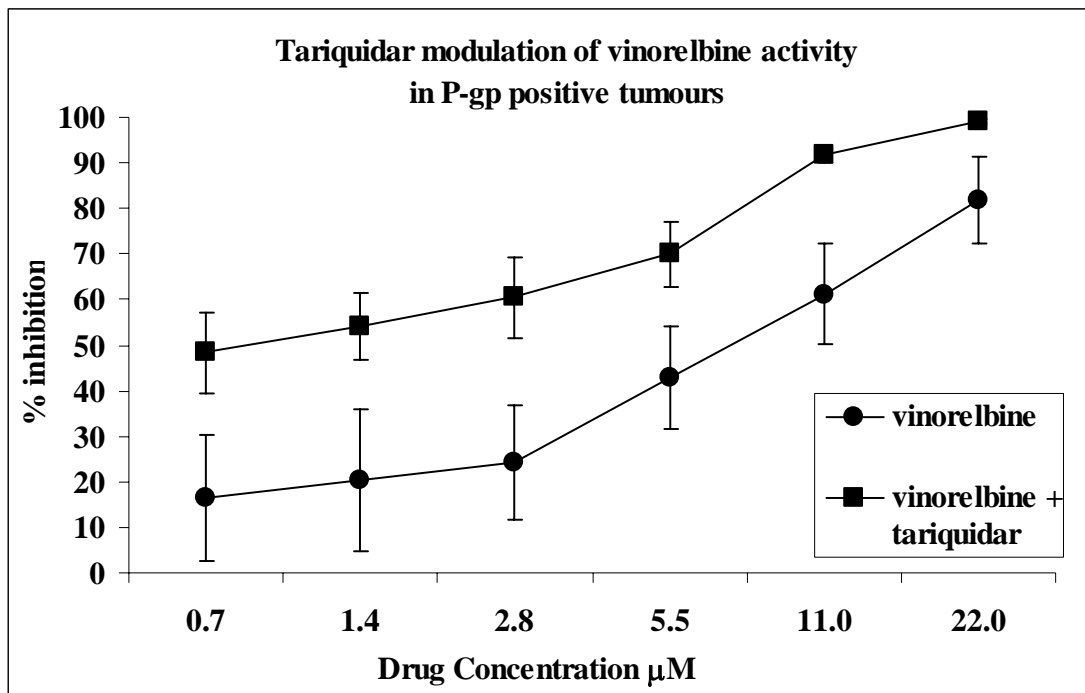


Figure 10.7 Examples of the ATP-TCA results for (a) a cutaneous melanoma and (b) a colorectal adenocarcinoma. Results are shown as mean ($\pm\text{SD}$) of triplicate values for each drug concentration. Error bars are not shown for some data points, as they are smaller than the symbols on the graph. P-gp immunostaining for each sample is shown in panels (c) and (d).

(a)



(b)

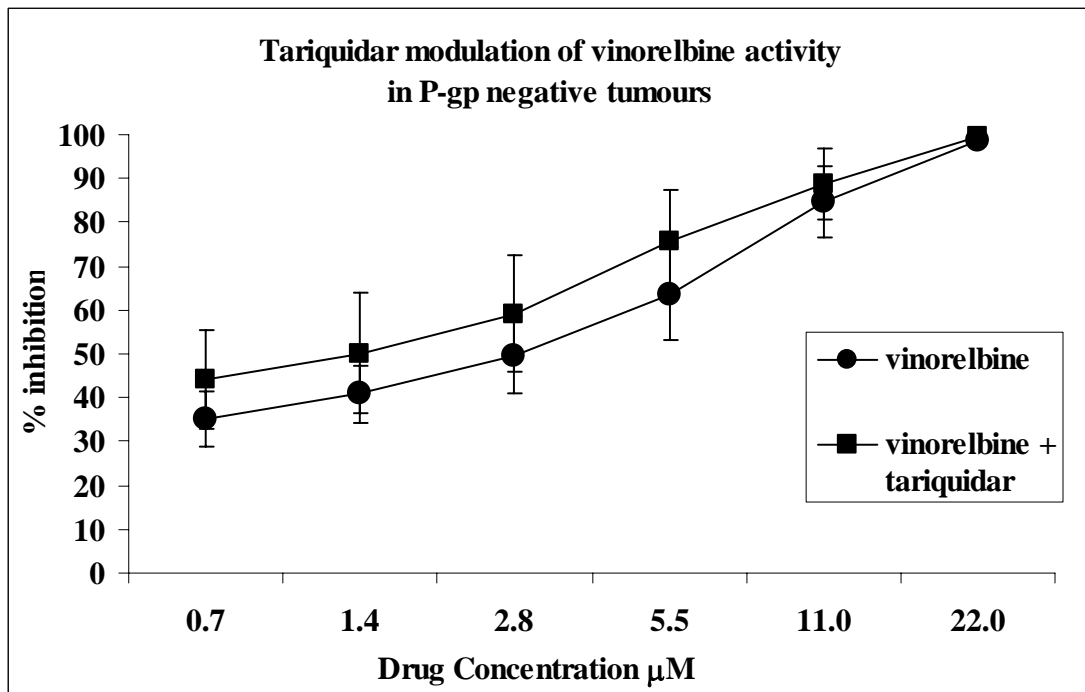


Figure 10.8 Effect of tariquidar on vinorelbine activity (mean \pm SEM) in (a) P-gp positive (n=7) and (b) P-gp negative (n=15) tumours.

10.3.4 Effect of tariquidar on paclitaxel activity

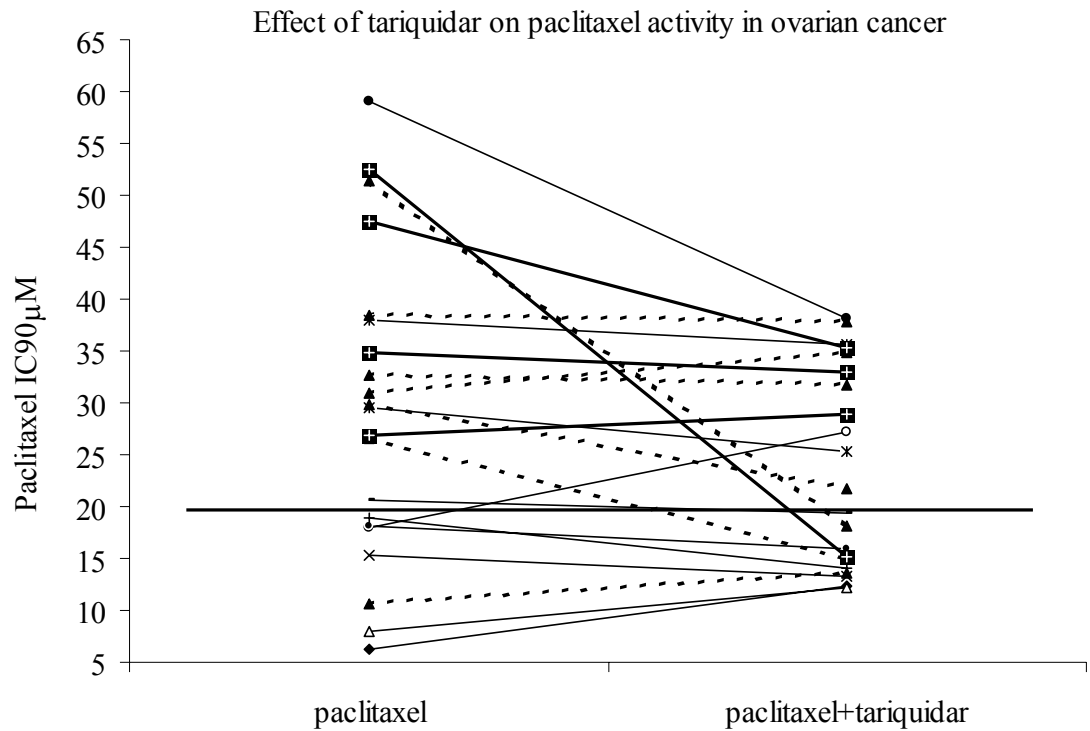
The median IC90 and IC50 for paclitaxel were 27.4 μM and 9.7 μM respectively. *In vitro* resistance to paclitaxel according to IC90 was seen in 27/34 (79%) samples. In 9/27 cases classified as resistant *in vitro*, the patients had been previously exposed to a taxane-based regimen. In this particular subset the median IC90 for paclitaxel was 38.4 μM , while in the remaining 18/27 *in vitro* resistant cases the median IC90 decreased to 29.0 μM ($p < 0.05$, Mann-Whitney). For the ovarian cancer subset, *in vitro* resistance to paclitaxel according to IC90 was seen in 17/21 (81%) samples

Immunohistochemical staining for P-gp was positive in 9/21 (43%) samples.

Semiquantitative visual assessment of the degree of staining in this relatively small number of cases showed no correlation with sensitivity (IC90 and IC50) by linear regression analysis (data not shown). It should be noted that only 1/9 (11%) of the P-gp positive samples showed sensitivity to paclitaxel on the basis of IC90, and only 2/12 (17%) of the P-gp negative samples showed sensitivity to paclitaxel. Of those patients with P-gp positive samples, 8/9 had received previous chemotherapy, and 3 had been treated with a paclitaxel based regimen. Of those patients with P-gp negative samples, 5/12 were chemotherapy naïve and 4/12 had previously received a taxane-based regimen.

The addition of tariquidar decreased the median IC90 of paclitaxel from 27.4 μM to 20.6 μM ($p < 0.05$, Wilcoxon). Tariquidar showed some reversal of *in vitro* resistance to paclitaxel in 6/27 (22%) samples classified as resistant, of which 2/17 (12%) were ovarian cancer samples (figure 10.9). An example of tariquidar effects on paclitaxel activity in a P-gp positive ovarian carcinoma is shown in figure 10.10.

(a)



(b)

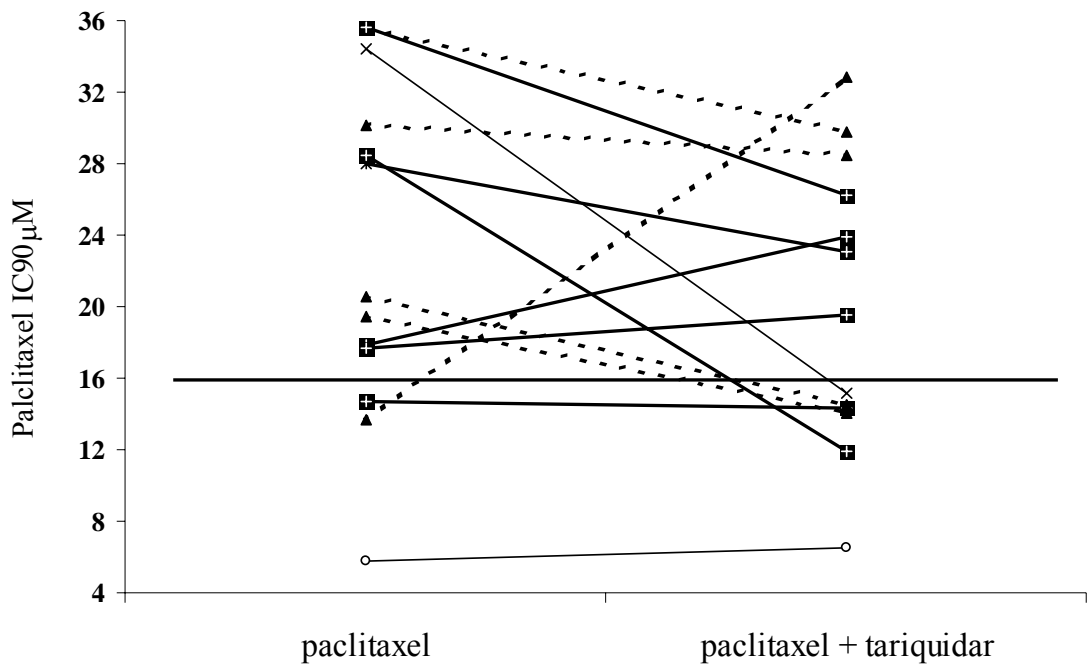
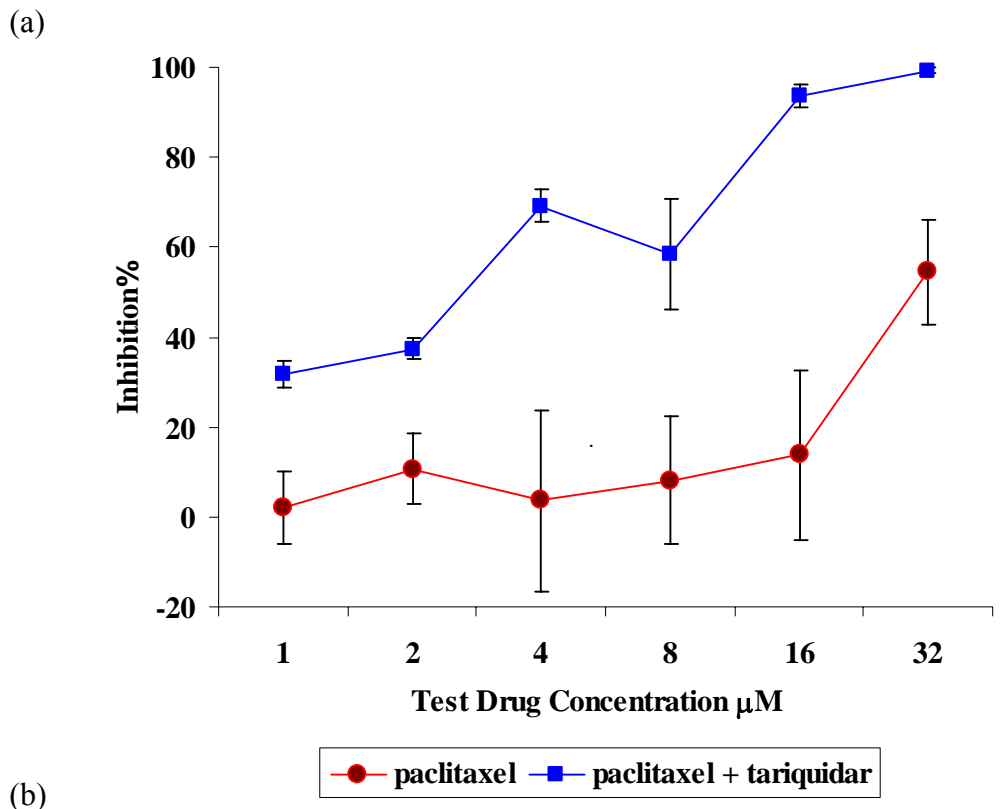


Figure 10.9 Effect of the addition of tariquidar on the activity of paclitaxel expressed as IC90 μ M in (a) ovarian and (b) non-ovarian tumour samples. The samples labelled with a 'plus' in a black square are P-gp positive tumours, while the samples labelled with a triangle and a dashed line are P-gp negative tumours; P-gp staining was not performed in samples labelled with the other symbols. A line has been drawn in each graph to indicate 100% TDC of paclitaxel.



(b)

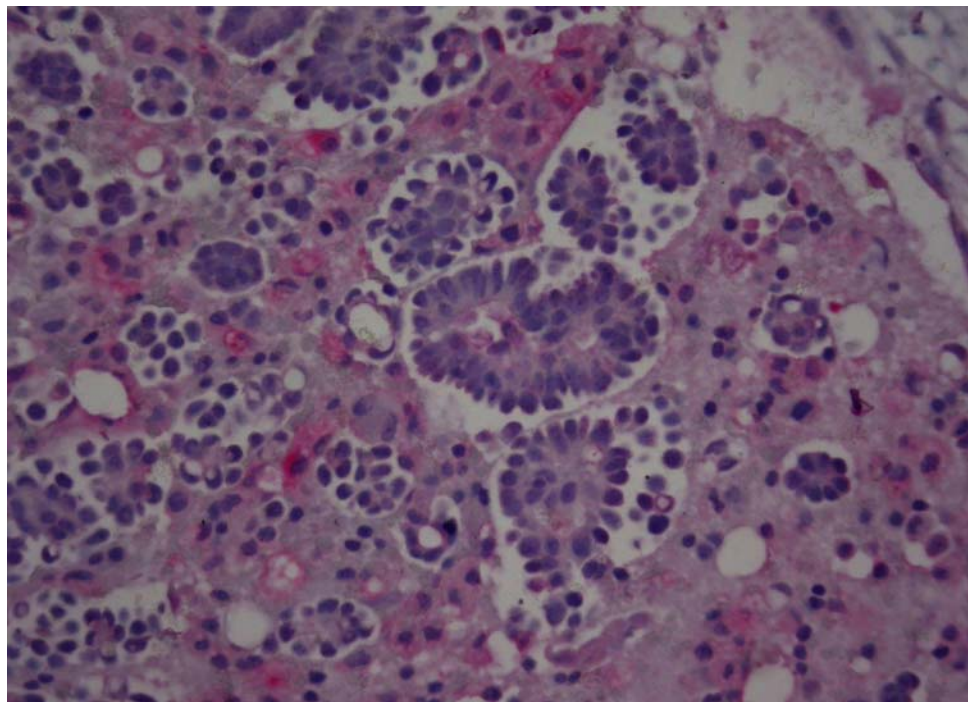


Figure 10.10 (a) Example of the ATP-TCA results for a P-gp positive ovarian adenocarcinoma. Results are shown as mean (\pm SD) of triplicate values for each drug concentration. Error bars are not shown for some data points, as they are smaller than the symbols on the graph. The bottom panel (b) shows the results of the P-gp IHC for this sample.

10.4 Discussion

The results of this study show that in the ATP-TCA, tariquidar is able to modulate the chemosensitivity in a number of solid tumours that are *in vitro* resistant to cytotoxic drugs known to be P-gp substrates. However, not all tumours showed such potentially beneficial effects, nor all drugs, and these results suggest that patients must be carefully selected.

Most tumours that showed some increased sensitivity after the addition of tariquidar were P-gp positive by immunohistochemistry, but an effect was also seen in a proportion of P-gp negative samples. Thus, there was a distinct lack of correlation with estimates of the degree of expression present. This may simply reflect the subjective and at best semi-quantitative nature of immunohistochemistry. Further, it is possible that P-gp negative samples up-regulated P-gp in the presence of the cytotoxic, and tariquidar was able to reverse P-gp mediated resistance. It has been clearly shown that expression of P-gp may be rapidly up-regulated in response to chemotherapeutic agents both *in vitro* and *in vivo* (Chaudhary & Roninson, 1993; Abolhoda *et al.*, 1999; Hu *et al.*, 1999; Stein *et al.*, 2002; Tada *et al.*, 2002), so it might be possible that up-regulation of MDR1/P-gp occurred within the six days of the assay.

In three cases, two ovarian samples tested with doxorubicin, and one unknown primary tested with paclitaxel, the addition of tariquidar caused a marked increase in the IC₉₀ of the cytotoxic. It should be noted that at least two of these samples were P-gp negative, and the hypothesis that tariquidar prevented the development of classic MDR, and therefore favoured the onset of other alternative and more effective mechanisms of resistance, might be considered. In a previous *in vitro* study (Cocker *et al.*, 2001) the investigators failed in several attempts to obtain cell lines resistant to vincristine when the cells were grown in the presence of tariquidar; while classic MDR developed when the cells were grown in the presence of the cytotoxic alone. In early clinical trials testing P-gp modulation in acute leukaemia, cells that survived chemotherapy in the presence of modulators, resulting in clinical relapse, had reduced expression of P-gp (List *et al.*, 1993; Marie *et al.*, 1993). The hypothesis that the development of MDR may be preventable using P-gp modulators therefore warrants further *in vitro* and clinical studies.

The data suggest that while P-gp expressing tumours are nearly always resistant to pumped drugs, those that do not express P-gp are not always sensitive. Although the MDR phenotype mediated by P-gp appears to be an important mechanism of resistance to anthracyclines, taxanes and vinca alkaloids, other mechanisms may be of greater importance in many patients. It should be noted that many of our patients had been previously exposed to paclitaxel and there may have been induction of non-MDR mechanisms of resistance in these cases. Other ATP-dependent pumps such as MRP1 and BCRP have been shown to confer resistance to doxorubicin (Grant *et al.*, 1994; Doyle *et al.*, 1998), though it should be noted that the role of MRP in paclitaxel and vinorelbine resistance is controversial and probably marginal (Vanhoefer *et al.*, 1997; Dantzig *et al.*, 2002; Shepard *et al.*, 2003; Thomas & Coley, 2003). Non-classical mechanisms of resistance are also likely to be involved. For microtubule-interfering agents, altered microtubule dynamics, alterations in α - and β -tubulins, and/or altered binding sites may confer resistance. Altered metabolism and/or subcellular distribution, altered interaction of antitubulin agents with microtubules and inadequate induction of apoptotic signals are among the possible non-MDR mechanisms of resistance to tubulin-binding agents (Dumontet & Sikic, 1999). For anthracyclines such as doxorubicin, decreased levels of topoisomerase II α have been associated with resistance, and changes in DNA repair may also be important (Fedier *et al.*, 2001).

In this study, immunohistochemical detection of P-gp alone was not sufficient to predict an *ex vivo* response to tariquidar. Pluralities of techniques have been used for diagnosis of P-gp expression in clinical samples, such as immunocytochemistry, flow cytometry, functional test, RT-PCR (see section 1.2.1). Imperfections exist in all methods, and the lack of standardisation renders it virtually impossible to compare studies from different laboratories. Whether the assay parameter should be MDR1 mRNA, protein, or P-gp substrate transport ability remains unresolved.

The ATP-TCA is a well-standardised assay capable of determining the activity of cytotoxic drugs *ex vivo* in tumour-derived cells and shows excellent correlation with outcome in breast and ovarian cancer (Cree *et al.*, 1996; Konecny *et al.*, 2000; Sharma *et al.*, 2003). It could be used to select patients for tariquidar therapy, and has the distinct advantage that it measures the effect of P-gp blocking agents in the context of

other resistance mechanisms that may be present, but it is only applicable to those from whom sufficient tumour tissue can be obtained for testing.

In summary, the results of this study show that tariquidar is able to significantly reduce the median IC90s for doxorubicin, paclitaxel and vinorelbine in a panel of human tumours. Considerable translational research will be required to determine which are the most cost-effective predictive methods to employ, but it seems unlikely that immunohistochemistry alone will be sufficient and the use of functional assays showing *ex vivo* efficacy of tariquidar should be considered.

Chapter 11 – Cellular adaptation to chemotherapy

11.1 Introduction

In chapter 9 it was noted a general increase in resistance to doxorubicin in the ATP-TCA in tumour-derived cells from paired biopsies collected from ovarian cancer patients before and after administration of anthracycline-based regimens. Another study had previously shown a similar effect in tumour derived cells from paired biopsies collected from breast cancer patients before and after administration of doxorubicin-containing chemotherapy (Cree *et al.*, 1996). In the study by Cree *et al.* (1996) the loss of ATP was measured as a marker of chemosensitivity in tumour-derived cells, and the authors were able to show that tumour cells adapt pre- and post-chemotherapy to the drugs that they are given, but show variable or no change in cytotoxicity in response to drugs with unrelated mechanisms of resistance.

In chapter 9 the same endpoint, i.e. loss of ATP, was used, but I was also able to correlate chemosensitivity with changes in gene expression caused by doxorubicin exposure in tumour-derived cells from ovarian and breast cancer patients.

I have extended the initial observations to other tumour types such as GI carcinomas and other classes of cytotoxic agents, including the TOPO I inhibitors topotecan and irinotecan, and the anti-metabolite 5-FU. In this chapter the first data of these experiments are reported and in the case of oesophageal cancer specimens the results are correlated with the changes in gene expression observed in biopsies taken before and after *in vivo* chemotherapy.

11.2 Materials and Methods

Patients and tissue samples for *ex vivo* studies. Tumour derived cells were obtained from 13 breast cancer patients (12 primaries; 1 pre-treated with mitoxantrone and paclitaxel), 10 ovarian cancer patients (all pre-treated with a cisplatin-based regimen), 10 colorectal cancer patients (all primaries) and 7 oesophageal cancer patients (3 untreated; 4 treated with ECF). Cells were grown for 6 days in CAM with or without camptothecins, 5-FU or ECF, before RNA extraction and further PCR analysis (chapters 2 & 3).

Patients and tissue samples for *in vivo* study. Twelve paired oesophageal adenocarcinoma biopsies were obtained from 6 patients before and after administration

of 2 cycles of ECF chemotherapy. The median age of the patients was 60 years old (range 42-77; 6M:0F). After enzymatic digestion, tumour derived cells were centrifuged over Ficoll -Hypaque (Sigma 1077-1) to remove blood contaminating cells, washed in PBS, and stored in RNA later (Ambion) at -80°C until further molecular analysis was performed (chapters 2 and 3). Tissue sections from these samples were stained for P-gp and COX-2 as detailed in chapter 2.

ATP-TCA and qRT-PCR methods. These were performed as detailed in chapters 2-3.

11.3 Results

11.3.1 Gene expression changes induced by camptothecins in short-term cell culture

The effects of *ex vivo* exposure to two camptothecin derivatives, irinotecan and topotecan, were studied on colorectal and ovarian tumour cells, respectively. As both compounds act *via* TOPO I inhibition, we firstly measured the levels of the mRNA encoding their target enzyme. We noted a trend towards down-regulation of TOPO I in treated cells (table 11.1): exposure to irinotecan decreased TOPO I levels >2-fold in 3/7 colorectal tumours, while topotecan caused down-regulation in 4/10 ovarian samples (figures 11.1g and 11.2f). The decrease of TOPO I was accompanied by a concomitant reduction of TOPO II α expression, which was particularly pronounced (>4-fold) in 6/7 colorectal tumours and 8/10 ovarian tumours.

No significant changes were observed in the expression of the drug efflux molecules, MDR1, BCRP and MRP1 (table 11.1), though, considerable heterogeneity was noted. For example, we observed an increase of BCRP levels after irinotecan or topotecan exposure in 2/7 colorectal samples and 3/9 ovarian samples (figures 11.1a and 11.2a).

Among the genes implicated in DNA repair, the modest down-regulation of MLH1 by topotecan exposure was not found statistically significant, although it was noted in 7/10 ovarian samples. Up-regulation of ERCC1 expression was found in all 10 ovarian cancer samples exposed to topotecan ($p < 0.002$, Wilcoxon matched pairs test), and in all 7 colorectal specimens treated with irinotecan, although in this group the increase was modest and the median difference did not reach a statistically significant value ($p = 0.016$, Wilcoxon) when a Bonferroni's correction was applied.

Finally, I examined COX-2 and EGFR as markers of tumour growth and progression. Following treatment of ovarian-tumour derived cells with topotecan, the levels of COX-2 increased in 4/10 samples, decreased in 4/10 and remained essentially unaltered in the remaining 2/10 tumours analysed. After irinotecan treatment, it was observed a down-regulation of COX-2 in all 7 colorectal samples analysed (figure 11.2b), with the median level decreasing from 105 to 38.5 units ($p=0.0156$, Wilcoxon matched pairs test; NS, with Bonferroni). Changes in the expression of EGFR were only studied after topotecan exposure: we found a decrease (more than 2-fold) in the expression of this growth factor in 9/10 ovarian samples (figure 11.1c), with the median levels decreasing from 0.470 to 0.163 units ($p<0.0039$, Wilcoxon matched pairs test).

Table 11.1 Relative expression of mRNA levels ($2^{-\Delta Ct}$) in tumour samples after *ex-vivo* exposure to camptothecins. The p values have been calculated using non parametric statistics (Wilcoxon matched pairs test for paired samples with a Bonferroni's correction; statistical significance was taken at $p<0.006$).

Target gene	n	Ovarian samples		p	n	Colorectal samples		p
		Control	Topotecan			Control	Irinotecan	
BCRP	9	0.0156	0.0125	>0.999	7	0.0354	0.0515	0.688
COX-2	10	0.253	0.475	0.770	7	105	38.5	0.016
EGFR	10	0.470	0.163	0.004	-	-	-	-
ERCC1	10	0.252	0.399	0.002	7	1.40	2.47	0.016
MDR1	9	0.00897	0.00340	0.570	7	0.225	0.294	>0.999
MLH1	10	0.263	0.227	0.037	-	-	-	-
MRP1	-	-	-	-	7	5.13	3.78	0.016
TOPO I	10	4.49	2.45	0.037	7	1.47	1.12	0.109
TOPO II α	10	1.09	0.0670	0.002	6	0.285	0.0733	0.219
TOPO II β	-	-	-	-	7	0.740	0.506	0.031

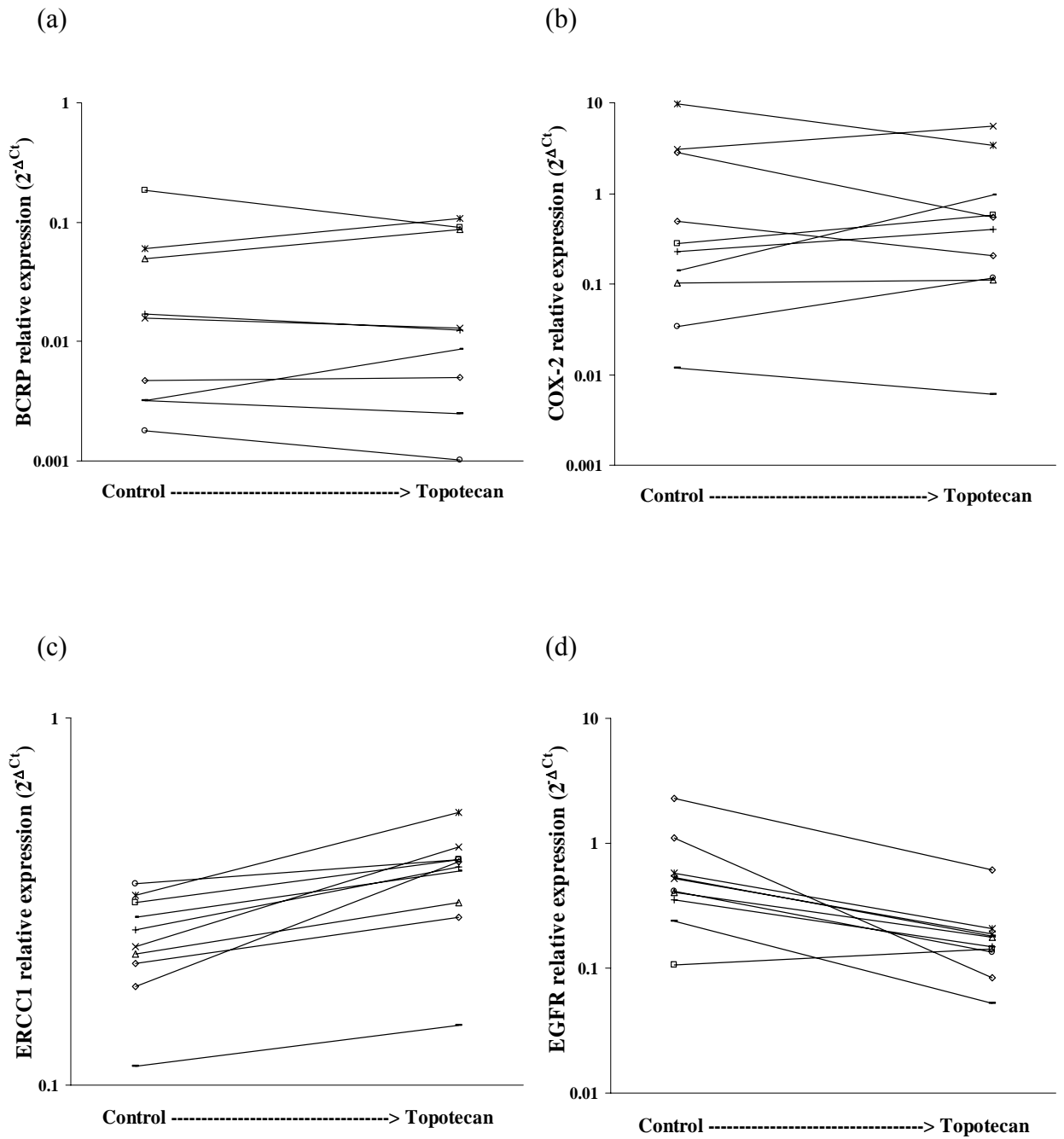


Figure 11.1 Changes in BCRP (a), COX-2 (b), ERCC1 (c) and EGFR (d) gene expression after topotecan exposure in the ATP-TCA in tumour derived cells from 10 ovarian cancer patients.

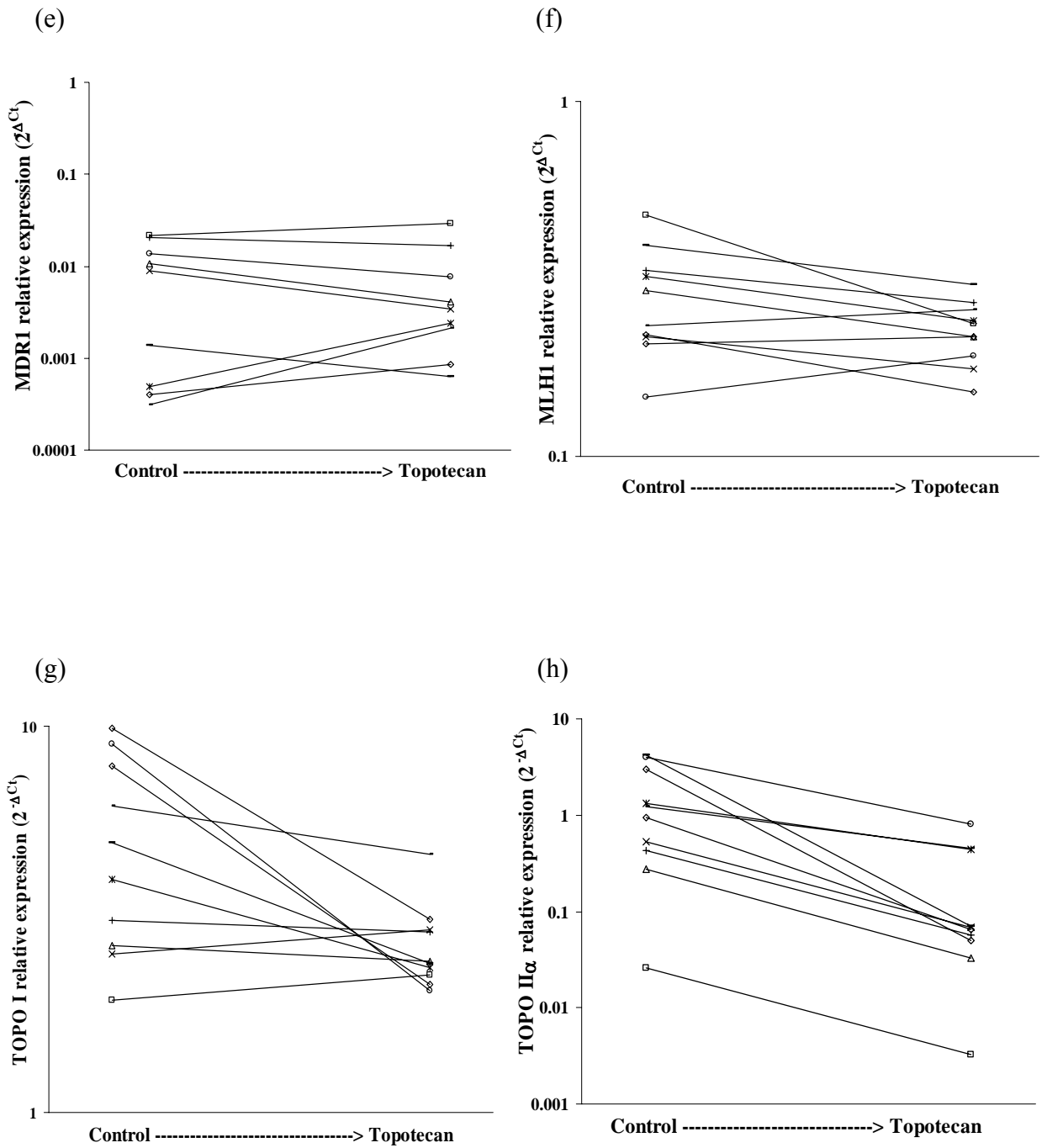


Figure 11.1 (continued). Changes in MDR1 (e), MLH1 (f), TOPO I (g) and TOPO II α (h) gene expression after topotecan exposure in the ATP-TCA in tumour derived cells from 10 ovarian cancer patients.

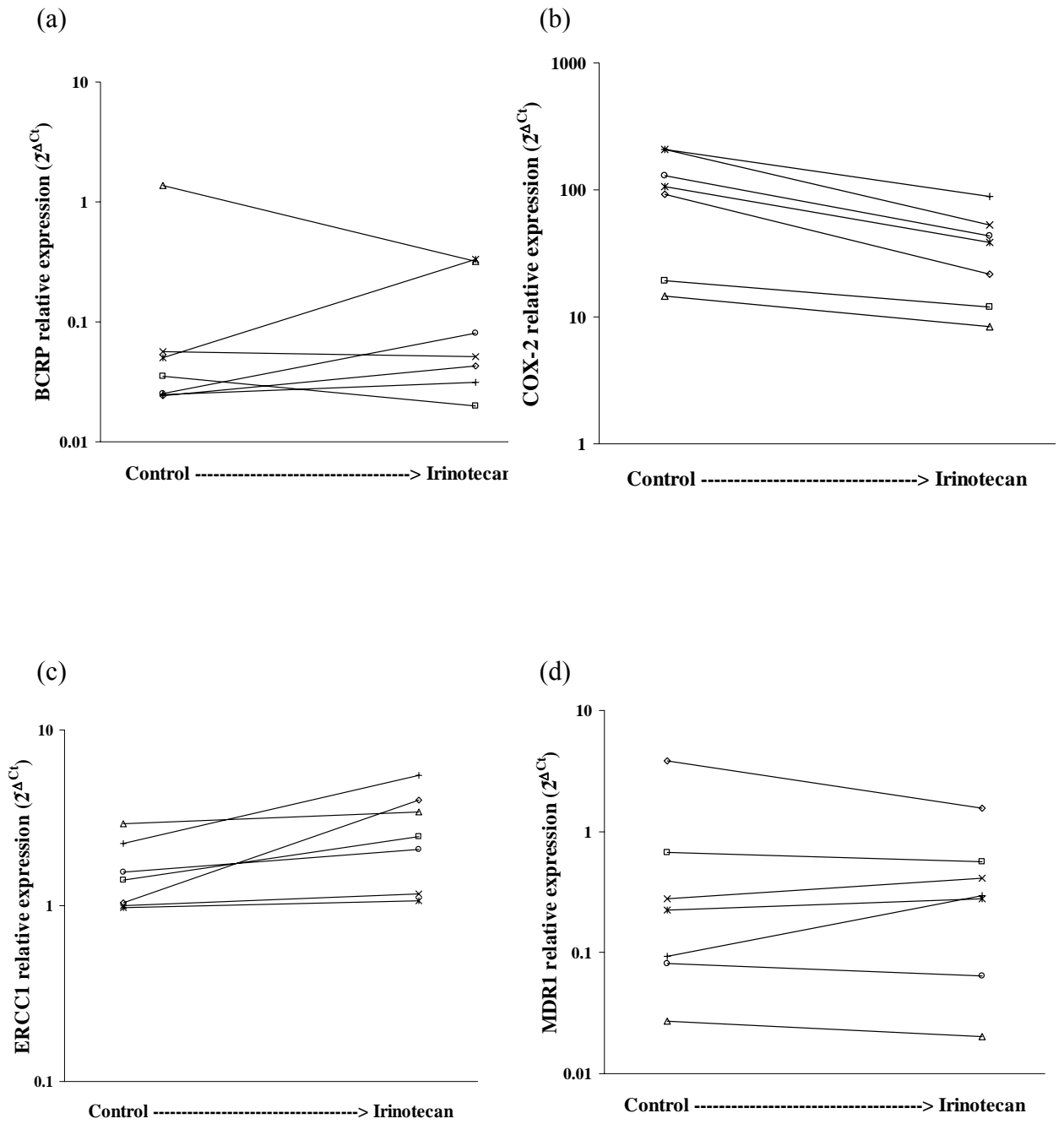


Figure 11.2 Changes in BCRP (a), COX-2 (b), ERCC1 (c) and MDR1 (d) gene expression after irinotecan exposure in the ATP-TCA in tumour derived cells from 7 colorectal cancer patients.

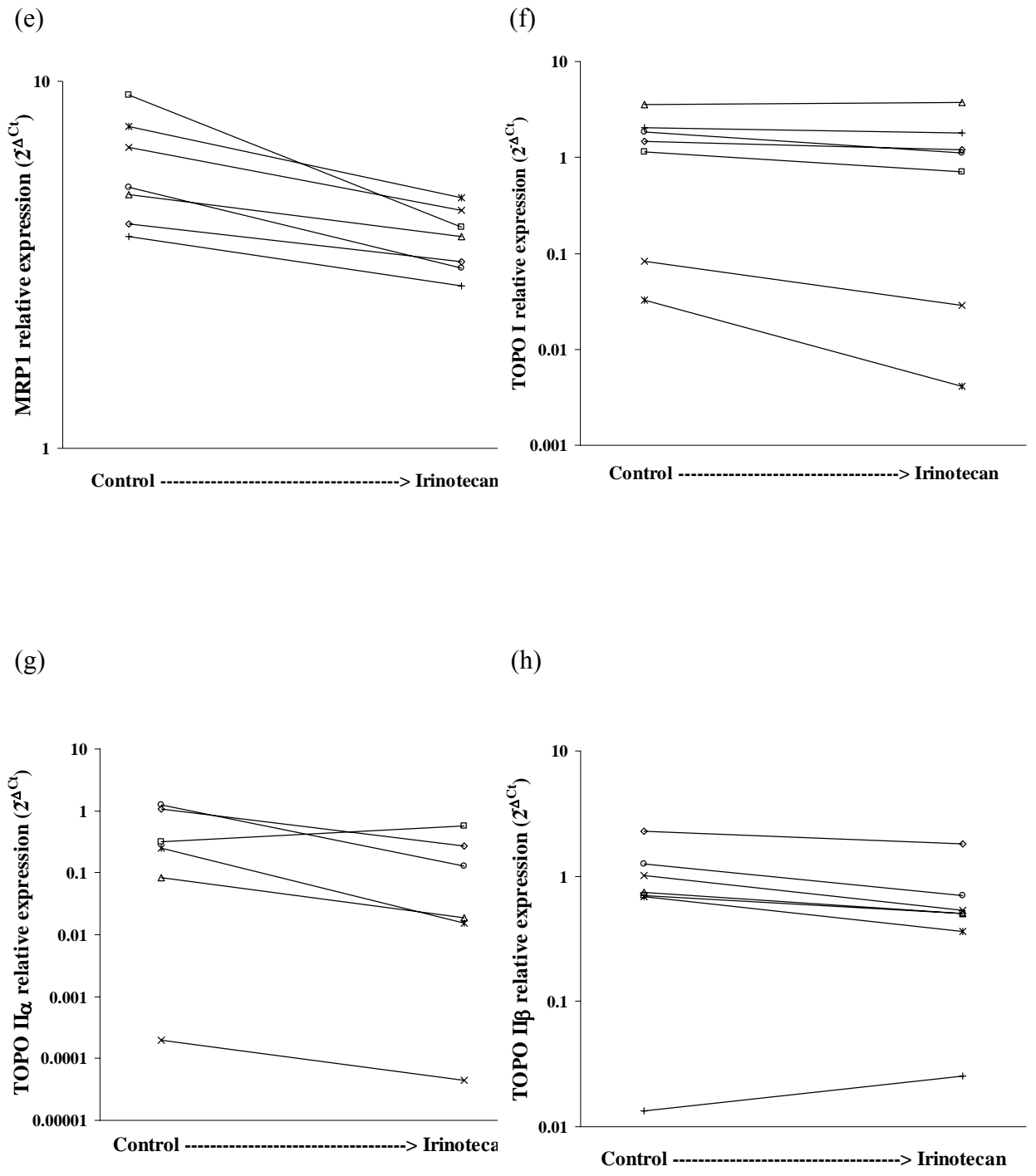


Figure 11.2 (continued). Changes in MRP1 (e), TOPO I (f), TOPO II α (g) and TOPO II β (h) gene expression after irinotecan exposure in the ATP-TCA in tumour derived cells from 7 colorectal cancer patients.

11.3.2 Gene expression changes induced by 5-FU in short-term cell culture

The effects of *ex vivo* exposure to 5-FU were studied on material derived from 10 colorectal tumour samples and 13 breast tumour samples. Among the genes known to be involved in 5-FU metabolism, we found an increase of TS levels in 12/13 breast samples and in all colorectal specimens (figures 11.3d and 11.4d). The results also indicated a general trend towards DPD down-regulation in the cells that had been exposed to 5-FU *ex vivo* (table 11.2; figures 11.3b and 11.4b), this effect being more pronounced in breast (10/13 samples) than in colorectal cells (6/10). It should also be noted that in 3/10 colorectal samples 5-FU treatment caused >2-fold up-regulation (figure 11.4b). Heterogeneity was found in the expression of TP levels; after 5-FU exposure TP levels increased in 6/10 colorectal samples, decreased in 2/10 and remained unaltered in other 2 cases (figure 11.4c). Similarly, in breast no changes were seen in 9/13 samples (figure 11.3c).

The results showed a significant up-regulation of the median mRNA levels of COX-2, which increased from 7.91 to 171 units in the breast subgroup ($p < 0.0002$, Wilcoxon matched pairs test) and from 122 to 392 units in colorectal samples ($p < 0.004$, Wilcoxon matched pairs test). Notably, there was a COX-2 increase greater than 2-fold in 11/13 breast samples (figure 11.3a) and 9/10 colorectal samples (figure 11.4a).

Table 11.2 Relative expression of mRNA levels ($2^{-\Delta C_t}$) in n tumour samples after *ex vivo* exposure to 5-FU. The p values have been calculated using non parametric statistics (Wilcoxon matched pairs test for paired samples with a Bonferroni's correction; statistical significance was taken at $p < 0.0125$).

Target gene	n	Breast samples		p	n	Colorectal samples		p
		Control	5-FU			Control	5-FU	
COX-2	13	7.91	171	0.0002	10	122	392	0.0039
DPD	13	1.29	0.494	0.0012	10	0.503	0.448	0.4922
TP	13	8.88	10.9	0.6848	10	4.62	5.96	0.0840
TS	13	1.14	3.82	0.0005	10	2.16	7.95	0.002

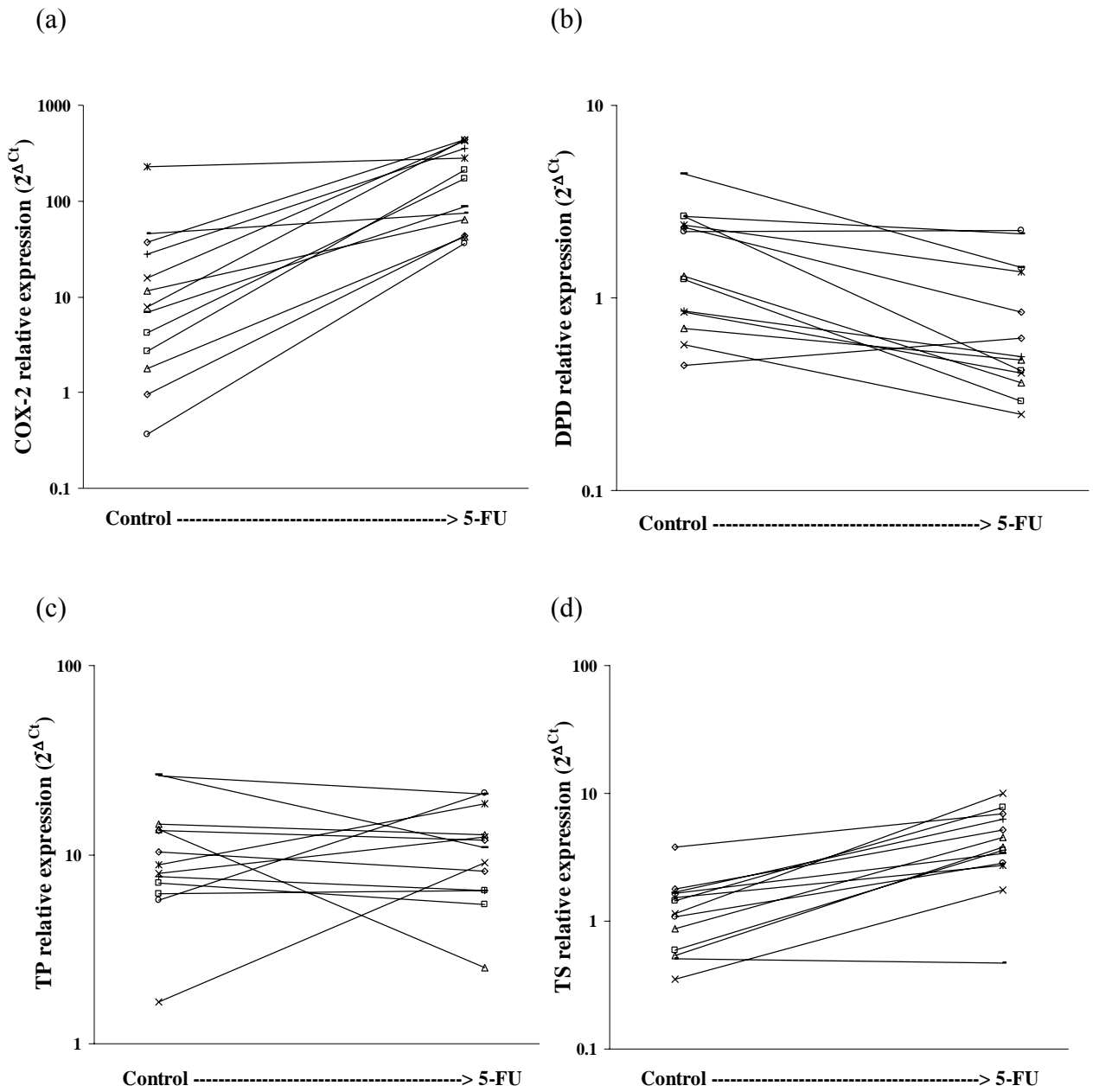


Figure 11.3 Changes in COX-2 (a), DPD (b), TP (c) and TS (d) gene expression after 5-FU exposure in the ATP-TCA in tumour derived cells from 13 breast cancer patients.

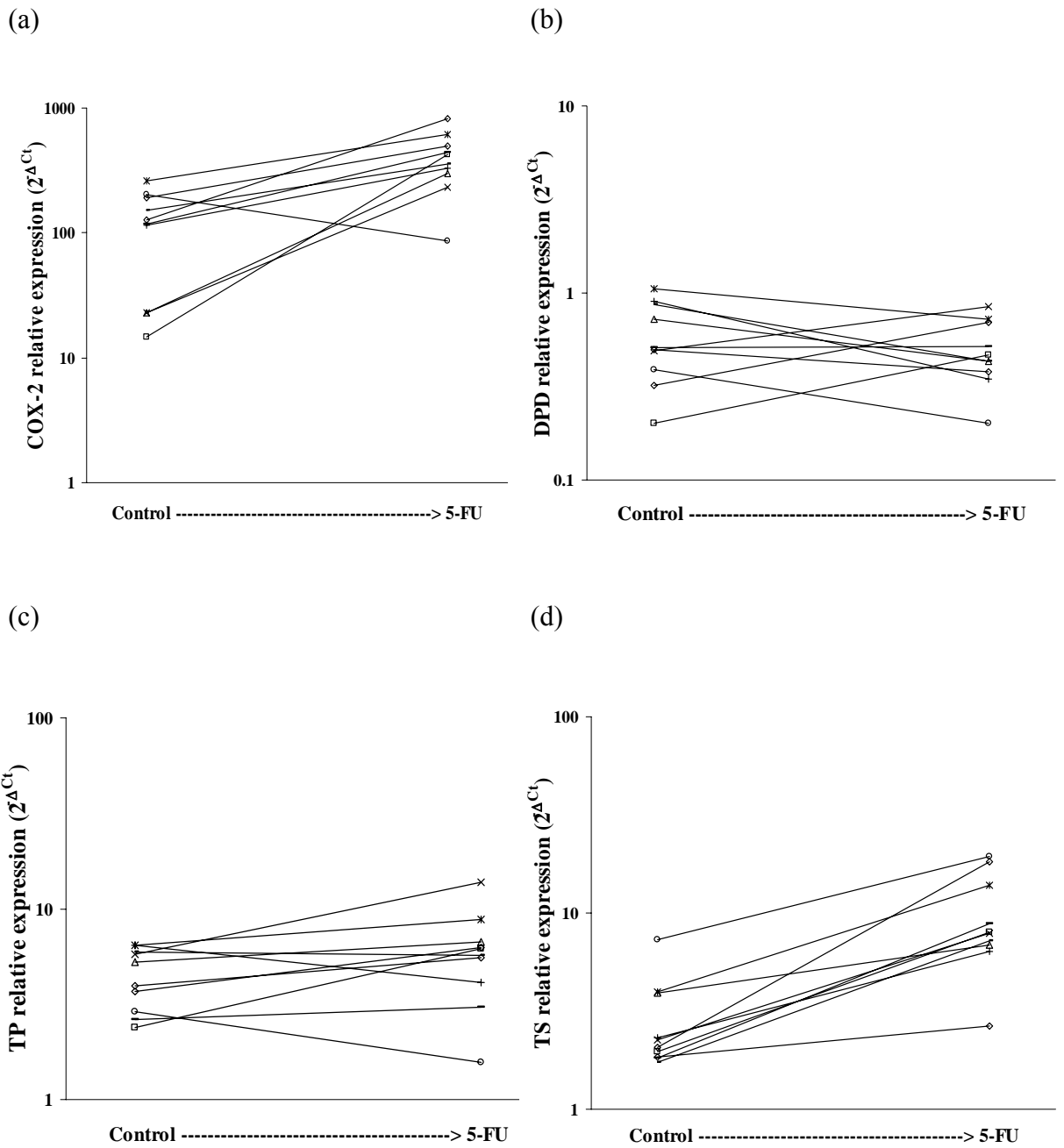


Figure 11.4 Changes in COX-2 (a), DPD (b), TP (c) and TS (d) gene expression after 5-FU exposure in the ATP-TCA in tumour derived cells from 10 colorectal cancer patients.

11.3.3 Gene expression changes induced by ECF in short-term cell culture

I was able to study the *ex vivo* effect of the combination of epirubicin, cisplatin and 5-FU (ECF) in tumour-derived cells from 7 oesophageal cancer patients, 4 of which had already been previously treated with ECF. Few genes could be tested in 2 cases due to limited amounts of RNA.

No statistically significant differences were observed in any of the genes studied when a Bonferroni's correction was applied to the Wilcoxon test performed in such a small number of cases (table 11.3). However, a marked increased expression of TS was found in all 6 samples after ECF exposure (figure 11.5 h). A general trend of increased expression of COX-2 was also noted, being more pronounced (>4-fold) in 3 cases. Among the pump proteins, up-regulation of MDR1 was detected in 3/7 samples, while the levels of MRP1 and MVP were unchanged or slightly decreased (figure 11.5 c,d,f).

When genes possibly implicated in cisplatin resistance were considered, there was marked up-regulation of Metallothionein IIA (MTII) in 1 sample, while in all the others a decrease in expression was noted.

Table 11.3 Relative expression of mRNA levels ($2^{-\Delta Ct}$) in n oesophageal tumour samples after *ex vivo* exposure to ECF. The p values have been calculated using non parametric statistics (Wilcoxon matched pairs test for paired samples with a Bonferroni's correction; statistical significance was taken at $p < 0.006$).

Target gene	n	Oesophageal samples		p
		Control	ECF	
COX-2	7	106	197	0.0313
GST- π	7	0.769	0.686	0.1563
MDR1	7	0.171	0.195	> 0.999
MRP1	6	9.37	5.61	0.0313
MTII	6	24.5	13.5	0.4375
MVP	5	9.55	8.21	0.625
TP	6	5.56	2.46	0.0313
TS	6	3.50	16.4	0.0313

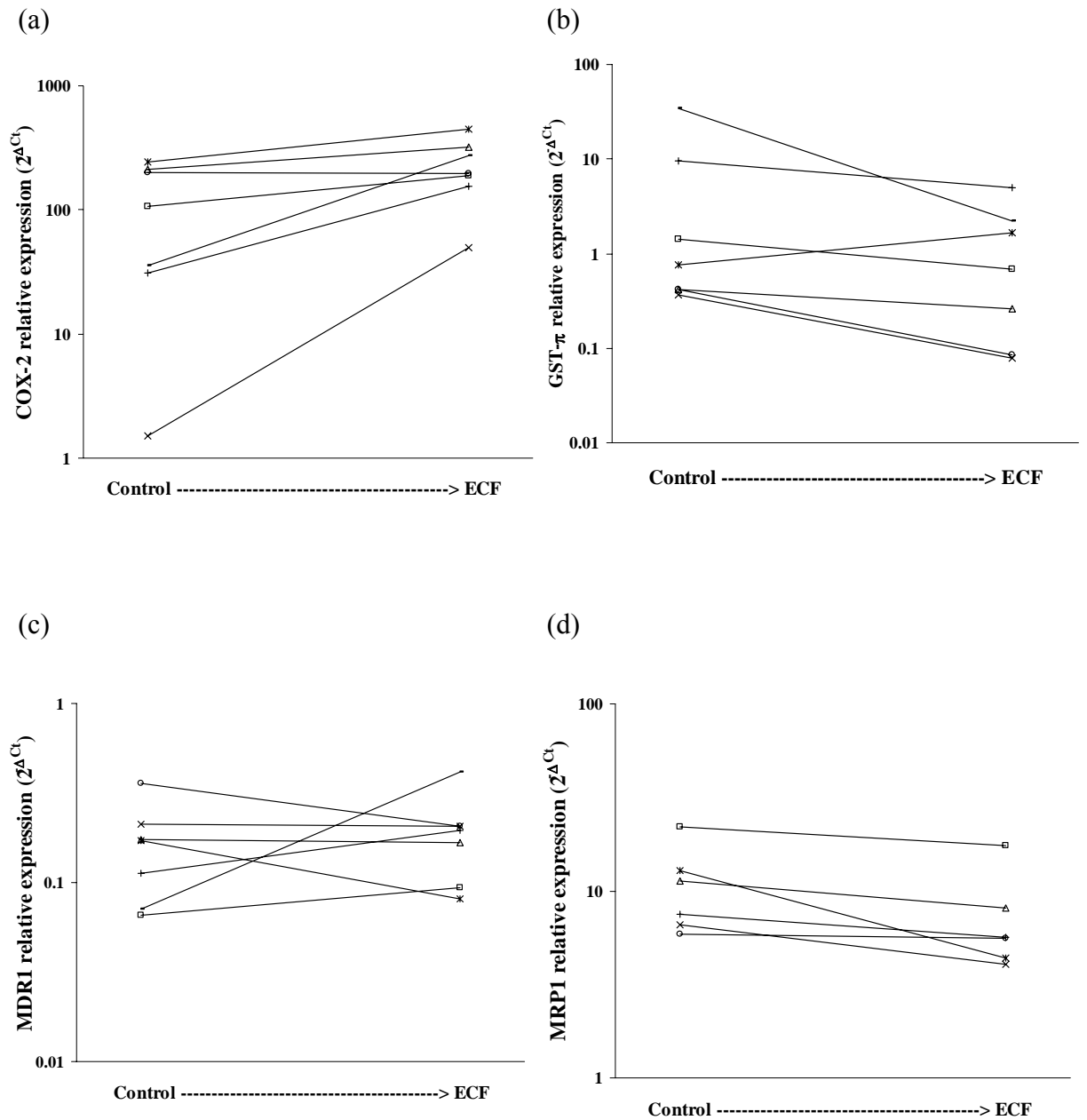


Figure 11.5 Changes in COX-2 (a), GST- π (b), MDR1 (c) and MRP1 (d) gene expression after ECF exposure in the ATP-TCA in tumour derived cells from 7 oesophageal cancer patients.

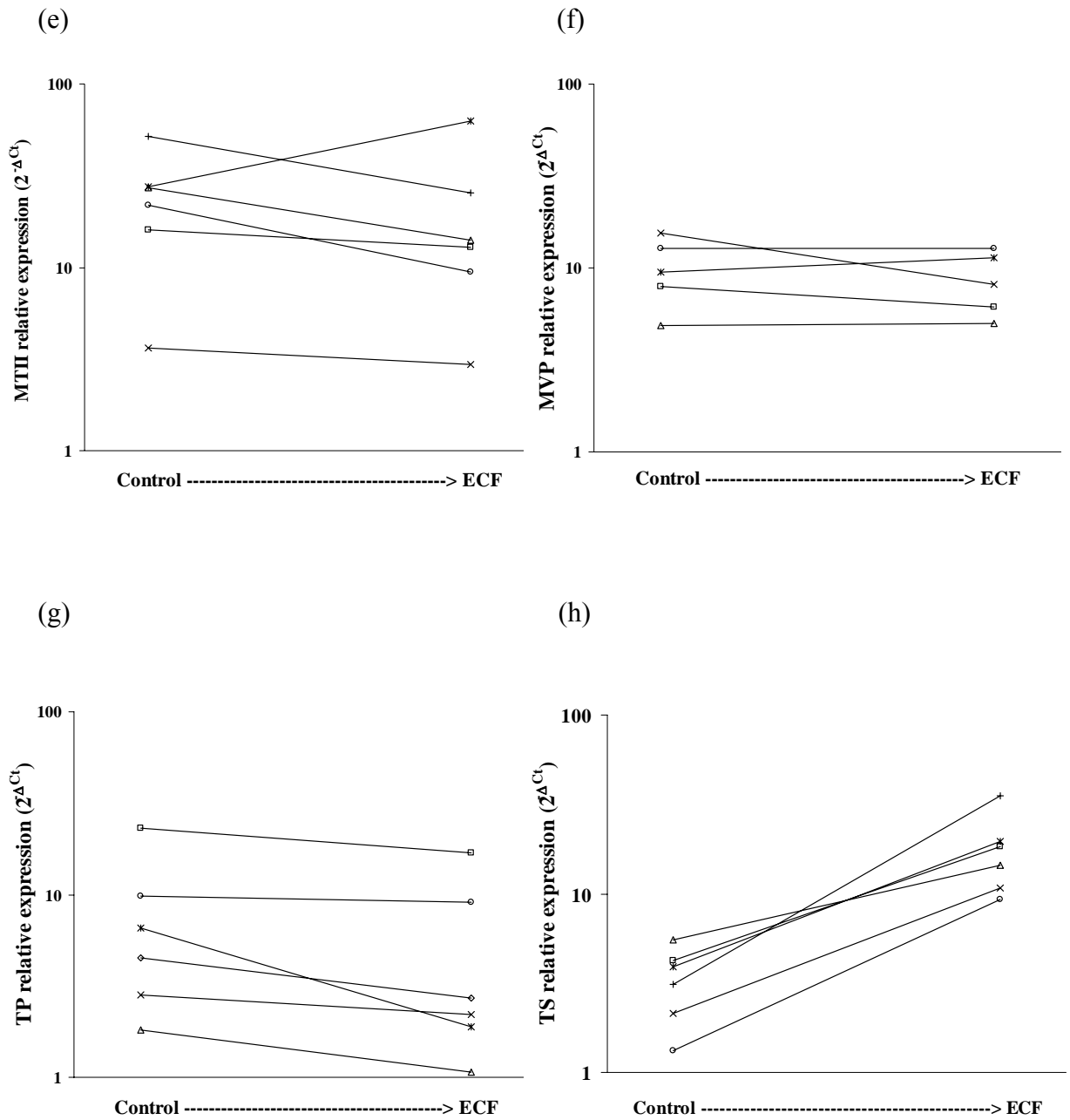


Figure 11.5 (continued). Changes in MTII (e), MVP (f), TP (g) and TS (h) gene expression after ECF exposure in the ATP-TCA in tumour derived cells from 6 oesophageal cancer patients.

11.3.4 Gene expression changes induced by *in vivo* ECF chemotherapy

Twelve oesophageal adenocarcinoma biopsies were collected from 6 patients before and after treatment with 2 courses of ECF. The median values of expression for each target gene are shown in table 11.4. Due to the limited number of samples, no statistically significant differences were found. However, a trend of variation in certain genes was noted (figure 11.6 a-h). For example ECF chemotherapy induced up-regulation of the mRNA levels of COX-2 and TS in 5/6 samples.

Among the drug transporters, there was an increase of the mRNA levels of MDR1 in 5/6 paired samples following ECF chemotherapy (figure 11.6 c). We also noted a trend towards increased expression of MRP1 and MVP (table 11.4). Notably, three tumours showed concomitant up-regulation of MVP and MRP1 (figure 11.4 d,f). Furthermore, the increased expression of MRP1 paralleled that of GST- π in 2 samples (figure 11.4 b), which is consistent with the mechanism of detoxification of MRP1 involving transport of glutathione-conjugated molecules.

It should be noted that these results were all obtained from tumour samples that included normal cells present in the tumour as well as neoplastic cells, and could be affected by stress responses in normal cells as well as neoplastic cells. We therefore examined the immunohistochemical expression of those genes that were found to produce the same effect in at least 5/6 samples, namely COX-2 and MDR1, to try to confirm these data from changes in protein levels.

11.3.5 Chemotherapy-induced changes in protein levels in biopsy material

Immunohistochemistry (IHC) was performed on all 12 oesophageal biopsies for which molecular biology data had been obtained and paraffin embedded material was available. However one sample was considered by a consultant histopathologist too small to be assessed. Overall, we were able to confirm an increased expression of the MDR-1 encoded protein, P-gp, in 2 tumour samples obtained from patients who had been exposed to ECF chemotherapy (figure 11.7a). In one case P-gp positivity was already present in the pre-chemotherapy sample, and was found also in the post-chemotherapy specimen. The remaining two cases were P-gp negative at diagnosis and remained such after treatment. COX-2 positivity was found in all samples but one post-chemotherapy

specimen (figure 11.7b); in this instance the decrease in protein level was paralleled by a decrease in the mRNA levels. Overall, this data showed that there was little correlation between the mRNA and the degree of expression of protein levels determined by IHC. Confounding factors may be the presence of non-cancerous cells as mentioned above, but also the nature of IHC, which is at best a semiquantitative technique.

Table 11.4 Relative expression of mRNA levels ($2^{-\Delta C_t}$) in tumour samples after *in vivo* treatment with ECF. The p values have been calculated using non parametric statistics (Wilcoxon matched pairs test for paired samples with a Bonferroni's correction; statistical significance was taken at $p < 0.006$).

Target gene	Paired oesophageal samples (n=12)		p
	Pre-chemo	Post-chemo	
COX-2	30.2	88.8	0.219
GST- π	1.61	1.85	0.844
MDR1	0.114	0.426	0.0313
MRP1	4.58	10.8	0.438
MT II	12.5	24.1	0.844
MVP	6.39	9.11	0.562
TP	3.56	3.01	>0.999
TS	1.27	2.27	0.438

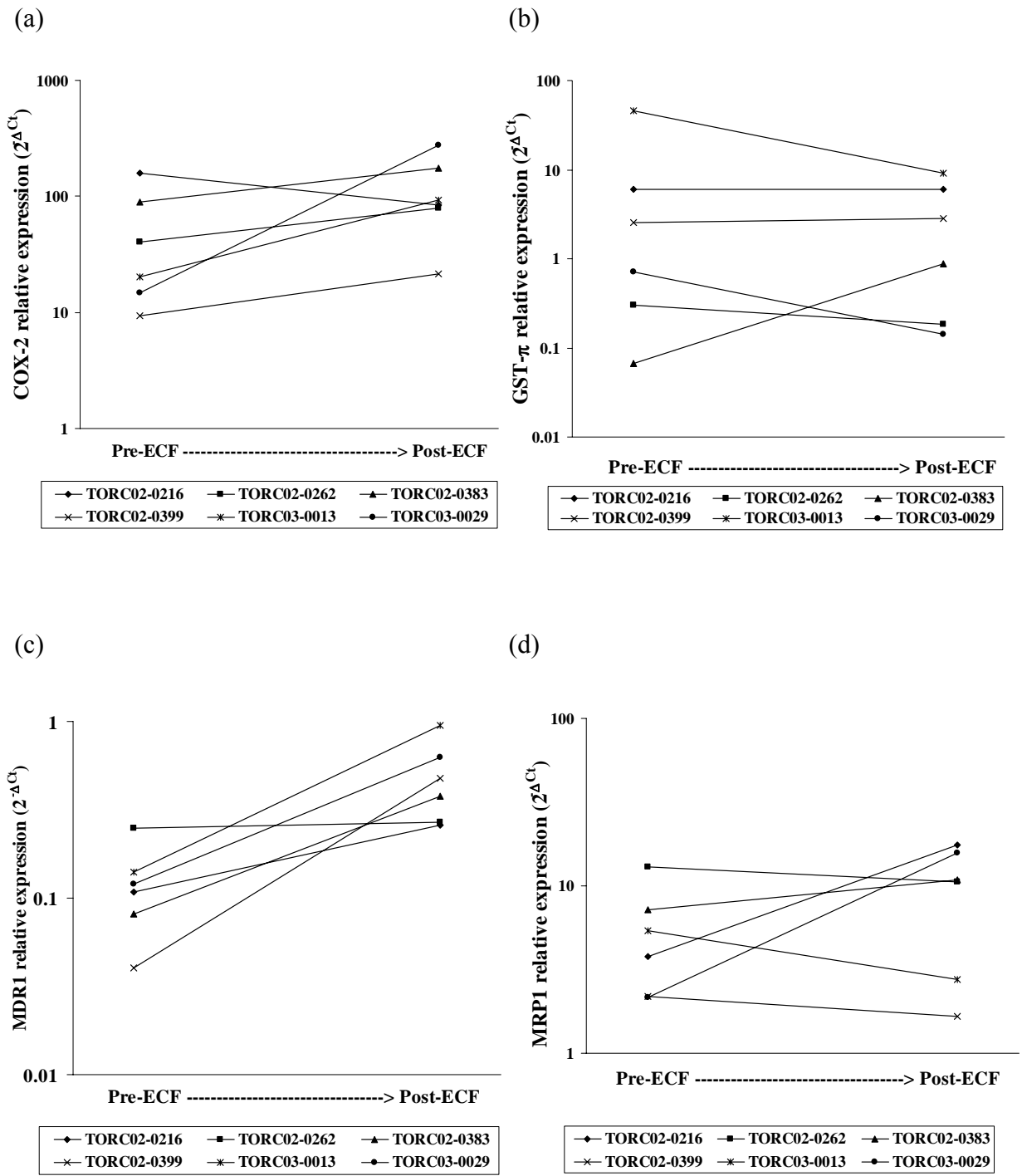


Figure 11.6 Changes in COX-2 (a), GST- π (b), MDR1 (c) and MRP1 (d) gene expression in paired oesophageal biopsies obtained before and after in vivo treatment with ECF.

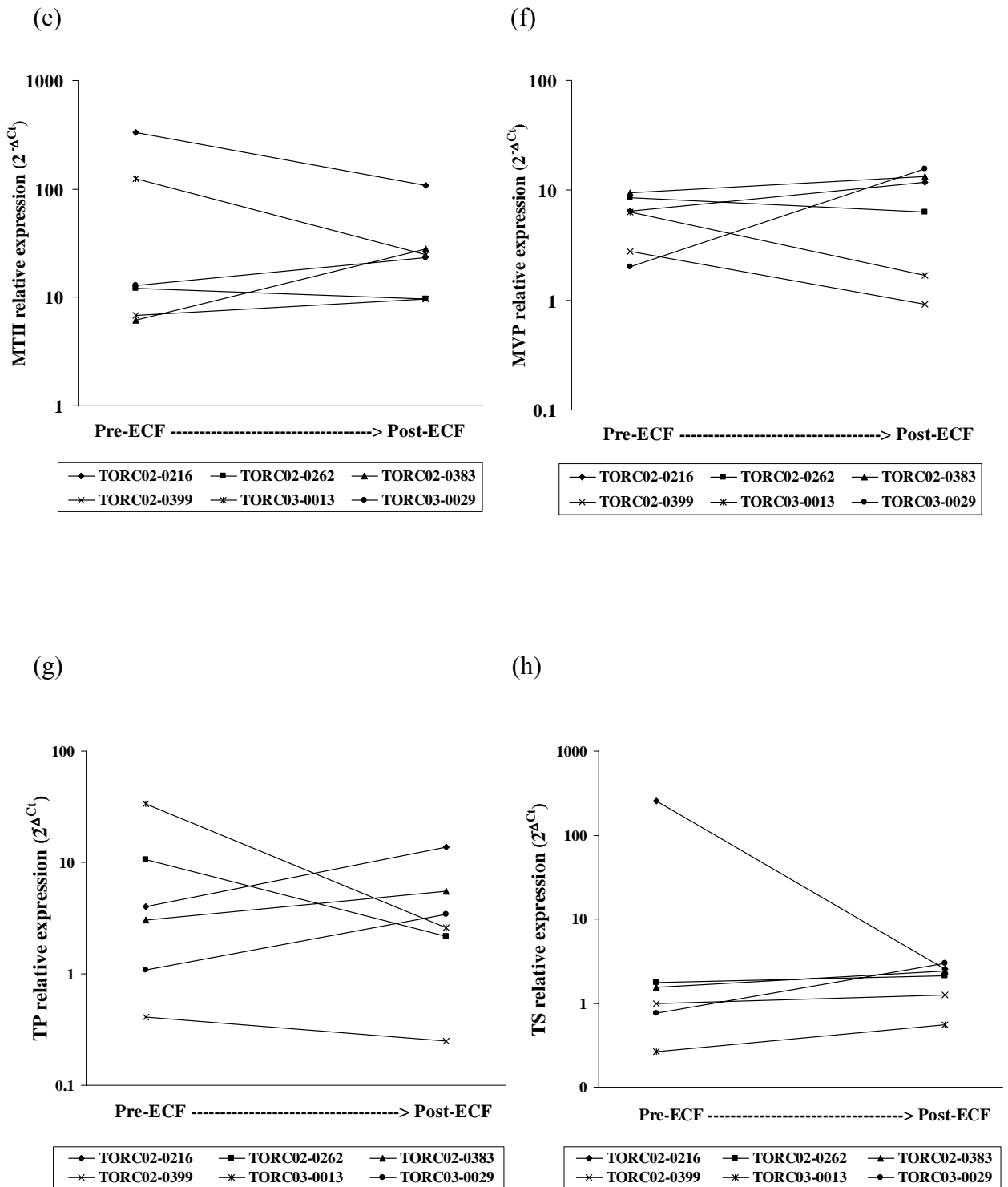
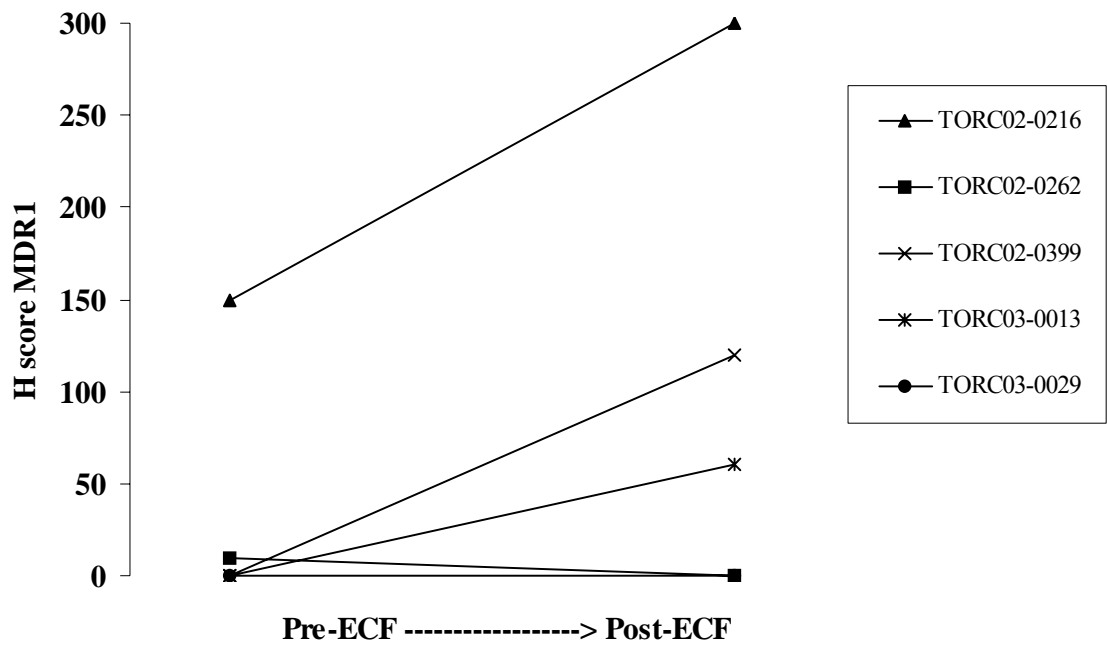


Figure 11.6 (continued). Changes in MTII (e), MVP (f), TP (g) and TS (h) gene expression in paired oesophageal biopsies obtained before and after in vivo treatment with ECF.

(a)

MDR1 IHC expression in paired oesophageal biopsies



(b)

COX-2 IHC expression in paired oesophageal biopsies

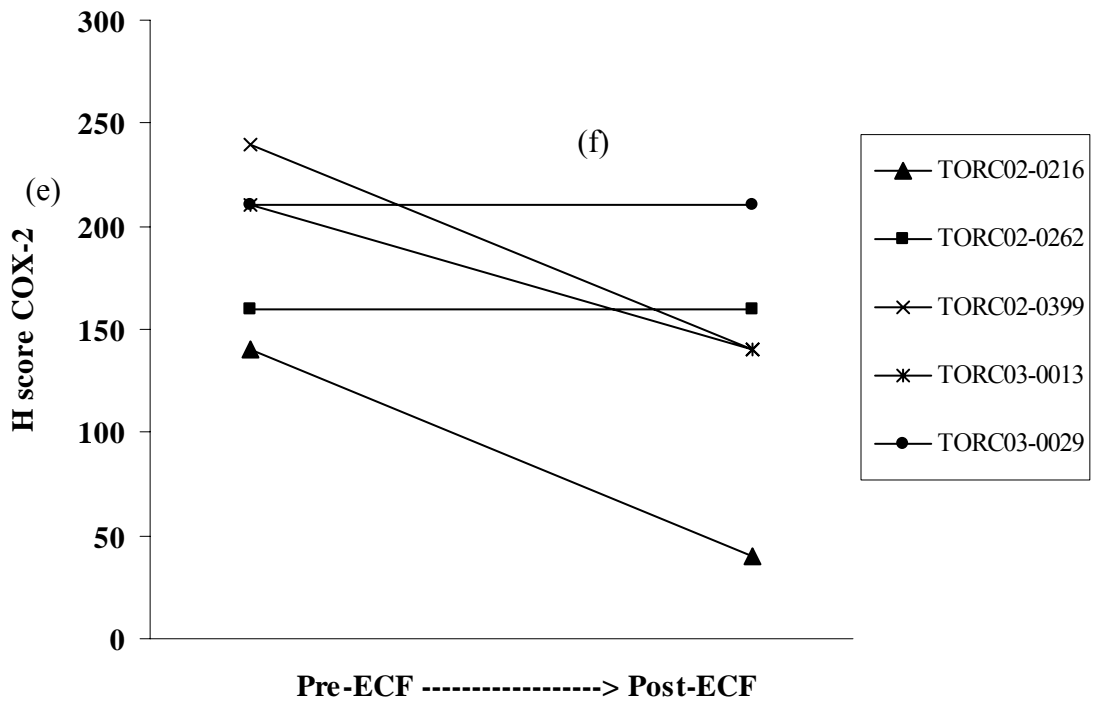


Figure 11.7 Changes in MDR1 (a) and COX-2 (b) protein levels determined by IHC in paired oesophageal biopsies obtained before and after *in vivo* treatment with ECF.

11.4 Discussion

The results shown in this chapter confirmed those previously obtained in chapter 9, and suggest that there is rapid adaptation to chemotherapy in tumour-derived cells. This adaptation is mediated by down- or up-regulation of genes that are sometimes correlated to the mechanism of action of the individual chemotherapeutic agent, but are often implicated in general survival or resistance pathways. The data presented in this chapter suggest that short-term cell culture methods with exposure to chemotherapy of patients' cells could provide a suitable model for studying resistance mechanisms, and ultimately predicting response in the clinic.

A large proportion of the published studies on resistance to chemotherapy have investigated the development of resistance using cell lines generated in the laboratory after prolonged and step-wise exposure to anti-cancer drugs. These *in vitro* models are not necessarily representative of the *in vivo* situation, when patients are usually administered one cycle of chemotherapy every 3-4 weeks. There are few studies in clinical samples. The approach presented in this thesis allows exposing the tumour cells to single drugs under carefully controlled conditions, even if this would be an inappropriate drug for that particular patient. In this cytotoxicity and molecular markers of resistance can be studied concomitantly in the same experiment. I have begun to explore less obvious forms of resistance using this technology, and show here that in some tumours, loss of MLH1 contributes to resistance to camptothecins, as previously shown in cell lines (Fedier *et al.*, 2001). In addition, our results are consistent with previous studies that found low levels of EGFR to correlate with topotecan resistance in cervical cancer cell lines (Ling *et al.*, 2001), and could provide an explanation for the synergy demonstrated by the combination of topotecan and gefitinib, an EGFR tyrosine kinase inhibitor (Ciardiello *et al.*, 2000).

One intriguing finding from our work is the marked up-regulation of COX-2 mRNA levels after 5-FU exposure in three different tumour types, while treatment with irinotecan tended to decrease the expression of this enzyme in primary colon cells. The only comparable data are from cell line studies. Recently, Yamaguchi *et al.* (2003) showed that SN-38 was able to inhibit phorbol ester (PMA)-mediated induction of COX-2 in human gastrointestinal cancer cell lines. Although the authors did not report a

direct effect of camptothecin derivatives on constitutive COX-2 levels, they suggested a link between TOPO I and COX-2. On the other hand, other studies suggest that the up-regulation of COX-2 by 5-FU may be part of a general anti-apoptotic response; Sun *et al.* (2002) have demonstrated that forced COX-2 expression attenuated apoptosis induction by 5-FU predominantly through inhibition of the cytochrome c-dependent apoptotic pathway; Grosch *et al.* (2001) have also shown that the selective COX-2 inhibitor celecoxib is able to induce cell cycle arrest and apoptosis in cultured colon cancer cells. It must be noted, however, that these studies all used cell lines, and that both in the case of Sun *et al.*, and Yamaguchi *et al.*, the authors stimulated or forced the expression of COX-2. Interestingly another study showed that in colorectal cell lines pretreated with 5-FU, re-growth was slowed by administration of Interferon- γ combined with indomethacin and phenylbutyrate (Huang *et al.*, 2000). As yet, I am uncertain whether the marked change in COX-2 expression following exposure to 5-FU represents part of a re-growth phenomenon, or whether it simply gives the cells a survival advantage (and may therefore render them resistant to 5-FU chemotherapy). Further studies are needed on this point, and it would be valuable to build measurements of COX-2 into clinical trials involving 5-FU. Our data also raise the possibility that combination of COX-2 inhibitors with chemotherapy might be useful and provide a molecular rationale for the observed efficacy of combinations of topoisomerase I inhibitors with 5-FU in gastro-intestinal cancers.

The potential for positive selection: molecular chess

It is common to show a cross-over with clinical trials of treatments with differing mechanisms of action, in which patients with treated with one type of chemotherapy show sensitivity to the alternative regimen following failure of the one to which they were allocated. The recognition that selection of a molecular phenotype by exposure to some anti-cancer agents leads to the expression of molecular targets for other drugs raises the possibility that it might be possible to enhance sensitivity to second-line or maintenance therapy by careful selection of patients for first-line therapy. It might therefore be possible to use tumour derived cells to guide such therapy to overcome the inherent heterogeneity of such changes between tumour cells which may underlie the variable results obtained from sequential chemotherapy to date.

Chapter 12 - General Discussion and Future Perspectives

12.1 General discussion

The work presented in this thesis has shown that considerable heterogeneity of chemosensitivity exists between different tumours corroborating some previous findings (Andreotti *et al.*, 1995; Myatt *et al.*, 1997; Cree *et al.*, 1999). Even a highly chemosensitive tumour such as retinoblastoma occasionally shows reduced sensitivity to anticancer agents (chapter 5). Drug resistance can be intrinsic or acquired following treatment with chemotherapy. Often exposure to one drug results in the acquisition of a resistance to a broad range of chemically unrelated compounds, a phenomenon that is called classical 'multidrug resistance' or MDR. Therefore cross-resistance patterns can be identified in the data obtained from chemosensitivity testing of fresh tumour cells even for unrelated drugs.

The data presented in this thesis has shown that drug resistance rapidly develops in tumour-derived cells exposed to cytotoxics *ex vivo* in short-term culture (chapters 9 and 11). The high reproducibility and accuracy of qRT-PCR -coupled with the use of multiple reference genes and rigorous data analysis (chapter 3) - allows the detection of even very small changes in gene expression that could be missed with less sensitive techniques such as cDNA microarray or traditional PCR with gel-based analysis. Using qRT-PCR, we were able to show up-regulation of MDR1 mRNA in a number of samples that had been exposed to anthracyclines in the ATP-TCA (chapter 9). In addition, all samples tested with doxorubicin showed decreased levels of TOPO II α expression after drug exposure. While some changes in gene expression can be predicted on the basis of the mode of action of the drug in question, as in the case of TOPO II α and doxorubicin, others appear not to be so strictly related to the drug-specific cytotoxicity. During the course of this thesis, evidence has been presented that 5-FU, an antimetabolite, consistently induces COX-2 expression (a gene which has recently been shown implicated in tumour cell growth) in tumour derived cells from breast and gastrointestinal carcinomas (chapter 11). Importantly, a similar increase of COX-2 expression induced by 5-FU based chemotherapy was also found in the cells of twelve oesophageal cancer patients when we compared the gene expression profile of pre- and post-chemotherapy biopsies. This change was not confirmed at the protein level by immunohistochemistry, which is at its best a semiquantitative technique, although a high proportion of oesophageal cancer biopsies showed COX-2 positivity.

The relevance of these findings in the clinical setting has not been fully investigated yet, but the pilot study on the oesophageal tumour-derived cells suggest that cancer cells can adapt *in vivo* to chemotherapy by rapidly altering their molecular phenotype. Indeed, some studies have shown that the expression of MDR1/P-gp is up-regulated within hours of anti-cancer drug treatment *in vivo* in patient samples (Abolhoda *et al.*, 1999; Hu *et al.*, 1999; Stein *et al.*, 2002; Tada *et al.*, 2002), although this effect was not observed in all patients. This may be an important factor when specific inhibitors of P-gp are used in clinical trials. So far clinical studies that have employed such inhibitors have been disappointing. This is probably due to the limited specificity and selectivity for P-gp of the inhibitors used in the early studies (causing unacceptable toxicities), but also to the lack of activity of these compounds when administered to an unselected cancer patient population, disregarding the expression of P-gp. It is notable that in some recent trials of P-gp inhibitors a benefit was only achieved in those patients with P-gp positive tumours (Baer *et al.*, 2002; Solary *et al.*, 2003).

In the course of this thesis we were able to test the *ex vivo* activity of a third-generation P-gp inhibitor, tariquidar, in the ATP-TCA (chapter 10). This inhibitor showed some reversal of resistance to doxorubicin, paclitaxel and vinorelbine in a small proportion of the samples tested. Notably, we failed to correlate the activity observed in the TCA with the degree of P-gp immunostaining, as not all P-gp positive tumours benefited from tariquidar. Other mechanisms, including some other MDR transporters, can also mediate resistance to doxorubicin, paclitaxel and vinorelbine. The clinical significance of these other transporters is less well established, but there was little correlation between the expression of P-gp and the *ex vivo* activity shown in the ATP-TCA by some MDR sensitive drugs, suggesting that these other mechanisms may be important. However, both doxorubicin (chapter 9) and the experimental agent XR5944 (chapter 7) failed to produce >95% inhibition in most P-gp positive tumours (particularly GI tumours, which often express multiple transporters).

In the case of XR5944, this finding might influence the choice of the clinical conditions chosen for trials of this new drug. However, while this compound has now been recognised as a MDR substrate by our and other studies (Mistry *et al.*, 2001), our attempt to generate a drug resistant leukaemia sub-line produced only a modest 10- to 15-fold increase of the IC₅₀ values of XR5944. Moreover, we found a stronger

correlation between P-gp positivity and IC₅₀ values rather than with the IC₉₀s of XR5944, suggesting that higher concentrations of this compound can probably overcome MDR-mediated resistance. It should be noted that the concentration of XR5944 required to produce >95% inhibition was often in the 100-200 nanomolar range, showing the exceptional potency of this cytotoxic when compared with many currently available drugs (chapter 7).

Current anticancer drug screening is carried out using a panel of cell lines grown as monolayers in serum-rich medium. While this method has undeniable advantages (cost, rapidity, reproducibility), there are some drawbacks. As shown in chapter 3 the chemosensitivity of cell lines differ substantially from that of tumour-derived cells. Primary cultures can be up to 10-fold more chemoresistant than established cell lines. This is true for virtually all cytotoxics (Andreotti *et al.*, 1994, chapter 4), including the experimental agents XR5000, XR11576 and XR5944 that were employed in this study. Possible explanations for this phenomenon include the homogeneity of cell lines versus the heterogeneity observed in tumour-derived cells; the highly-proliferative status of cell lines compared with the relatively quiescent cell population usually found in solid tumours; and the fact that cell lines are usually grown as single cells, while the dissociation of solid tumours often yields small clumps of cells. The work presented in chapter 3 has also shown that altering cell culture conditions can partly affect chemosensitivity. Cell lines grown in low-serum media or even in serum-free appear more resistant than cells cultured in standard or high-serum concentrations. This is especially true for drugs that kill cells in the S-phase of the cellular cycle, further suggesting that chemosensitivity is somewhat also determined by the highly proliferative nature of cell lines.

The studies described in chapters 6, 7 and 10 have shown the usefulness of tumour chemosensitivity assays with primary cells for new drug development. For example, XR5000 was shown to be active *ex vivo* only at concentrations that exceeded 5 µM, which represents about twice the plasma levels achievable in patients (Caponigro *et al.*, 2002; Dittrich *et al.*, 2003a). Therefore the ATP-TCA was able to predict the lack of activity of this compound well before the results of the phase II clinical trials were available (Neale *et al.*, 2000; Caponigro *et al.*, 2002; Dittrich *et al.*, 2003 a & b). The results in chapter 6 demonstrated that XR11576, a compound originated from the same

medicinal chemistry programme as XR5000, is about 20-fold more potent than XR5000, while its isomer, XR11612, is only 10-fold more potent. XR11576 was found to be active in a great variety of solid tumours, including gastrointestinal cancers that are usually refractory to chemotherapy. In keeping with previous studies in cell lines, the activity of XR11576 was not particularly affected by MDR mechanisms or topoisomerase levels, consistently with the proposed role of dual topoisomerase inhibitor of this compound. XR11576 has also been tested in combination with some standard cytotoxics, and some cases of synergy were identified with vinorelbine and doxorubicin.

Chemosensitivity tests are not only a tool to assess new drugs, but they have the potential to identify new regimens that might be clinically useful. In the past the ATP-TCA has been used to develop two combinations, mitoxantrone plus paclitaxel, and treosulfan plus gemcitabine. Both have been successfully evaluated in second-line ovarian cancer patients. In this thesis the ATP-TCA, in two series of ovarian tumour samples, has identified at least additive activity between vinorelbine and doxorubicin tested at concentrations which approximate those achievable with liposomal preparations (chapter 8). This combination is currently being assessed in the clinic: the early results in advanced breast and ovarian cancer are encouraging.

In conclusion, it is clear that the mechanisms which affect the chemosensitivity of individual patients to chemotherapy are complex. However, this thesis shows that application of a combination of molecular methods such as qRT-PCR, immunohistochemistry, and cellular assays such as the ATP-TCA can determine subsets of patients who might benefit from particular drugs, and that such methods can also assist the development of new drugs and combinations.

12.2 Future work

There are several directions in which the work presented here could be continued.

First of all, the work presented in chapter 4 is currently being extended by Dr Augusta Fernando at the Translational Oncology Research Centre in Portsmouth using other cell lines derived from tumour types other than skin melanoma. If successful this would lead to the generation of a panel of human tumour cell lines that - grown under selected conditions - would mimic the chemosensitivity profile of primary cultures. This panel could then be used to aid in the development of new anti-cancer agents, thereby overcoming the need for fresh tumour-derived cells.

The pilot study on the chemosensitivity of retinoblastoma described in chapter 5 formed the basis of a successful grant application awarded jointly to St Bartholomew's hospital in London and to Prof Ian Cree in Portsmouth to extend the initial findings. This study is attempting to correlate retinoblastoma chemosensitivity to the expression of drug resistance proteins such as MDR1/P-gp and MRP1 in the hope that inhibitors of these molecules could help some patients.

The mRNA data shown in chapters 9 and 11 awaits confirmation at the protein level with more sensitive techniques than immunohistochemistry. It is envisaged that ELISA-based assays could be useful for this purpose.

The studies in gene expression changes induced by chemotherapeutic agents are currently being continued by Ms Louise Knight and Mr Sanjay Sharma at the Translational Oncology Research centre in Portsmouth. We aim to extend these observations to other cytotoxic drugs, and to different molecular mechanisms.

Finally the "Molecular Chess" hypothesis that giving one drug to a patient would alter the patient's response to another unrelated drug at relapse needs to be proven in the clinical setting with clear evidence in accompanying translational studies that the expected changes have occurred in the patients treated.

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APPENDIX

Formulas and macros used to analyse the ATP-TCA data presented in this thesis. The equations employed to calculate IndexSUM, IndexAUC, IC50 and IC90 are listed below. The percentage of inhibition at each drug concentration tested (Inh%) was calculated according to the equation shown in Chapter 2.2.19.

$$\text{IndexAUC} = 50 * (\text{Inh}_{200} + \text{Inh}_{100}) + 25 * (\text{Inh}_{100} + \text{Inh}_{50}) + 12.5 * (\text{Inh}_{50} + \text{Inh}_{25}) + 6.25 * (\text{Inh}_{25} + \text{Inh}_{12.5}) + 3.125 * (\text{Inh}_{12.5} + \text{Inh}_{6.25})$$

$$\text{IndexSUM} = 600 - (\text{Inh}_{6.25} + \text{Inh}_{12.5} + \text{Inh}_{25} + \text{Inh}_{50} + \text{Inh}_{100} + \text{Inh}_{200})$$

IC50 = $((50-y_4)/(y_2-y_4)*(y_1-y_3))+y_3$; where the values y_1, y_2, y_3 and y_4 are calculated using the following macros:

If $Inh_{6.25} < 49.99$ and $Inh_{12.5} > 50$ and $Inh_{25} > 50$ is true, then $x_1 = 6.25$; if false, $x_1 = 0$

If $Inh_{6.25} < 49.99$ and $Inh_{12.5} < 49.99$ and $Inh_{25} > 50$ and $Inh_{50} > 50$ is true, then $x_1 = 12.5$; if false, $x_1 = 0$

If $Inh_{12.5} < 49.99$ and $Inh_{25} < 49.99$ and $Inh_{50} > 50$ and $Inh_{100} > 50$ is true, then $x_1 = 25$; if false, $x_1 = 0$

If $Inh_{25} < 49.99$ and $Inh_{50} < 49.99$ and $Inh_{100} > 50$ and $Inh_{200} > 50$ is true, then $x_1 = 50$; if false, $x_1 = 0$

If $Inh_{50} < 49.99$ and $Inh_{100} < 49.99$ and $Inh_{200} > 50$ is true, then $x_1 = 100$; if false, $x_1 = 0$

If $Inh_{100} < 49.99$ and $Inh_{200} > 50$ is true, then $x_1 = 200$; if false, $x_1 = 0$

$y_1 = \Sigma(x_1)$

If $Inh_{6.25} < 49.99$ and $Inh_{12.5} > 50$ and $Inh_{25} > 50$ is true, then $x_2 = Inh_{6.25}$; if false, $x_2 = 0$

If $Inh_{6.25} < 49.99$ and $Inh_{12.5} < 49.99$ and $Inh_{25} > 50$ and $Inh_{50} > 50$ is true, then $x_2 = Inh_{12.5}$; if false, $x_2 = 0$

If $Inh_{12.5} < 49.99$ and $Inh_{25} < 49.99$ and $Inh_{50} > 50$ and $Inh_{100} > 50$ is true, then $x_2 = Inh_{25}$; if false, $x_2 = 0$

If $Inh_{25} < 49.99$ and $Inh_{50} < 49.99$ and $Inh_{100} > 50$ and $Inh_{200} > 50$ is true, then $x_2 = Inh_{50}$; if false, $x_2 = 0$

If $Inh_{50} < 49.99$ and $Inh_{100} < 49.99$ and $Inh_{200} > 50$ is true, then $x_2 = Inh_{100}$; if false, $x_2 = 0$

If $Inh_{100} < 49.99$ and $Inh_{200} > 50$ is true, then $x_2 = Inh_{200}$; if false, $x_2 = 0$

$y_2 = \Sigma(x_2)$

If $Inh_{6.25} > 50$ and $Inh_{12.5} > 50$ is true, then $x_3 = 6.25$; if false, $x_3 = 0$

If $Inh_{6.25} < 50$ and $Inh_{12.5} > 50$ and $Inh_{25} > 50$ is true, then $x_3 = 12.5$; if false, $x_3 = 0$

If $Inh_{6.25} < 50$ and $Inh_{12.5} < 50$ and $Inh_{25} > 50$ and $Inh_{50} > 50$ is true, then $x_3 = 25$; if false, $x_3 = 0$

If $Inh_{6.25} < 50$ and $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} > 50$ and $Inh_{100} > 50$ is true, then $x_3 = 50$; if false, $x_3 = 0$

If $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} < 50$ and $Inh_{100} > 50$ and $Inh_{200} > 50$ is true, then $x_3 = 100$; if false, $x_3 = 0$

If $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} < 50$ and $Inh_{100} < 50$ and $Inh_{200} > 50$ is true, then $x_3 = 200$; if false, $x_3 = 0$

$y_3 = \Sigma(x_3)$

If $Inh_{6.25} > 50$ and $Inh_{12.5} > 50$ is true, then $x_4 = Inh_{6.25}$; if false, $x_4 = 0$

If $Inh_{6.25} < 50$ and $Inh_{12.5} > 50$ and $Inh_{25} > 50$ is true, then $x_4 = Inh_{12.5}$; if false, $x_4 = 0$

If $Inh_{6.25} < 50$ and $Inh_{12.5} < 50$ and $Inh_{25} > 50$ and $Inh_{50} > 50$ is true, then $x_4 = Inh_{25}$; if false, $x_4 = 0$

If $Inh_{6.25} < 50$ and $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} > 50$ and $Inh_{100} > 50$ is true, then $x_4 = Inh_{50}$; if false, $x_4 = 0$

If $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} < 50$ and $Inh_{100} > 50$ and $Inh_{200} > 50$ is true, then $x_4 = Inh_{100}$; if false, $x_4 = 0$

If $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} < 50$ and $Inh_{100} < 50$ and $Inh_{200} > 50$ is true, then $x_4 = Inh_{200}$; if false, $x_4 = 0$

$y_4 = \Sigma(x_4)$

IC90 = $((90-y_4)/(y_2-y_4)*(y_1-y_3))+y_3$; where the values y_1, y_2, y_3 and y_4 are calculated using the following macros:

If $Inh_{6.25} < 89.99$ and $Inh_{12.5} > 90$ and $Inh_{25} > 90$ is true, then $x_1 = 6.25$; if false, $x_1 = 0$

If $Inh_{6.25} < 89.99$ and $Inh_{12.5} < 89.99$ and $Inh_{25} > 90$ and $Inh_{50} > 90$ is true, then $x_1 = 12.5$; if false, $x_1 = 0$

If $Inh_{12.5} < 89.99$ and $Inh_{25} < 89.99$ and $Inh_{50} > 90$ and $Inh_{100} > 90$ is true, then $x_1 = 25$; if false, $x_1 = 0$

If $Inh_{25} < 89.99$ and $Inh_{50} < 89.99$ and $Inh_{100} > 90$ and $Inh_{200} > 50$ is true, then $x_1 = 50$; if false, $x_1 = 0$

If $Inh_{50} < 89.99$ and $Inh_{100} < 89.99$ and $Inh_{200} > 90$ is true, then $x_1 = 100$; if false, $x_1 = 0$

If $Inh_{100} < 89.99$ and $Inh_{200} > 90$ is true, then $x_1 = 200$; if false, $x_1 = 0$

$y_1 = \Sigma(x_1)$

If $Inh_{6.25} < 89.99$ and $Inh_{12.5} > 90$ and $Inh_{25} > 90$ is true, then $x_2 = Inh_{6.25}$; if false, $x_2 = 0$

If $Inh_{6.25} < 89.99$ and $Inh_{12.5} < 89.99$ and $Inh_{25} > 90$ and $Inh_{50} > 90$ is true, then $x_2 = Inh_{12.5}$; if false, $x_2 = 0$

If $Inh_{12.5} < 89.99$ and $Inh_{25} < 89.99$ and $Inh_{50} > 90$ and $Inh_{100} > 90$ is true, then $x_2 = Inh_{25}$; if false, $x_2 = 0$

If $Inh_{25} < 89.99$ and $Inh_{50} < 89.99$ and $Inh_{100} > 90$ and $Inh_{200} > 90$ is true, then $x_2 = Inh_{50}$; if false, $x_2 = 0$

If $Inh_{50} < 89.99$ and $Inh_{100} < 89.99$ and $Inh_{200} > 90$ is true, then $x_2 = Inh_{100}$; if false, $x_2 = 0$

If $Inh_{100} < 89.99$ and $Inh_{200} > 90$ is true, then $x_2 = Inh_{200}$; if false, $x_2 = 0$

$y_2 = \Sigma(x_2)$

If $Inh_{6.25} > 90$ and $Inh_{12.5} > 90$ is true, then $x_3 = 6.25$; if false, $x_3 = 0$

If $Inh_{6.25} < 90$ and $Inh_{12.5} > 90$ and $Inh_{25} > 90$ is true, then $x_3 = 12.5$; if false, $x_3 = 0$

If $Inh_{6.25} < 90$ and $Inh_{12.5} < 90$ and $Inh_{25} > 90$ and $Inh_{50} > 90$ is true, then $x_3 = 25$; if false, $x_3 = 0$

If $Inh_{6.25} < 90$ and $Inh_{12.5} < 90$ and $Inh_{25} < 90$ and $Inh_{50} > 90$ and $Inh_{100} > 90$ is true, then $x_3 = 50$; if false, $x_3 = 0$

If $Inh_{12.5} < 90$ and $Inh_{25} < 90$ and $Inh_{50} < 90$ and $Inh_{100} > 90$ and $Inh_{200} > 90$ is true, then $x_3 = 100$; if false, $x_3 = 0$

If $Inh_{12.5} < 90$ and $Inh_{25} < 90$ and $Inh_{50} < 90$ and $Inh_{100} < 90$ and $Inh_{200} > 90$ is true, then $x_3 = 200$; if false, $x_3 = 0$

$y_3 = \Sigma(x_3)$

If $Inh_{6.25} > 90$ and $Inh_{12.5} > 90$ is true, then $x_4 = Inh_{6.25}$; if false, $x_4 = 0$

If $Inh_{6.25} < 90$ and $Inh_{12.5} > 90$ and $Inh_{25} > 90$ is true, then $x_4 = Inh_{12.5}$; if false, $x_4 = 0$

If $Inh_{6.25} < 90$ and $Inh_{12.5} < 90$ and $Inh_{25} > 90$ and $Inh_{50} > 90$ is true, then $x_4 = Inh_{25}$; if false, $x_4 = 0$

If $Inh_{6.25} < 90$ and $Inh_{12.5} < 90$ and $Inh_{25} < 90$ and $Inh_{50} > 90$ and $Inh_{100} > 90$ is true, then $x_4 = Inh_{50}$; if false, $x_4 = 0$

If $Inh_{12.5} < 90$ and $Inh_{25} < 90$ and $Inh_{50} < 90$ and $Inh_{100} > 90$ and $Inh_{200} > 90$ is true, then $x_4 = Inh_{100}$; if false, $x_4 = 0$

If $Inh_{12.5} < 90$ and $Inh_{25} < 90$ and $Inh_{50} < 90$ and $Inh_{100} < 90$ and $Inh_{200} > 90$ is true, then $x_4 = Inh_{200}$; if false, $x_4 = 0$

$y_4 = \Sigma(x_4)$

Publications

List of publications

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