The Role of Heat Shock Protein 90 Inhibition in the Treatment of Pancreatic Cancer

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine

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April 2008

ABSTRACT

The Role of HSP90 Inhibition in the treatment of Pancreatic Cancer

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Pancreatic ductal adenocarcinoma (Pancreatic Cancer) is a disease characterised by resistance to conventional chemotherapeutics. The HSP90 chaperone protein is an important regulator of several cellular pathways involved in the generation of the neoplastic phenotype. Inhibition of HSP90 function using geldanamycin is known to potentiate the effect of chemotherapeutics. In this work geldanamycin is shown to potentiate the effects of 5-Fluoro Uracil in the pancreatic cancer cell line Suit2. This potentiation is dependant on the scheduling of treatment and also the environment of the pancreatic cell line. In the context of serum-starvation there is a reversal of the potentiation. The apoptotic protein APAF-1 may mediate the effects of HSP90 inhibition. Serum starvation can induce nuclear localisation of HSP90 within 24 hours and is associated with modulation of the pro-apoptotic protein APAF-1. Treatment with geldanamycin also modulates APAF-1. APAF-1 is a key mediator of the apoptotic pathway. HSP90 and APAF-1 represent stress pathway proteins. In a yeast model system the ribosomal protein Rsa4p/YCR072c could represent an evolutionary homolog to APAF-1, linking ribosomal function with aging and apoptosis

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ABBREVIATIONS AND ACRONYMS

°C	Centigrade
17AAG	17 aminoallyl geldanamycin
17DMG	17 demethyl geldanamycin
5FU	5 Fluoro-Uracil
AAMP	Angio Associated Migratory Protein
AD	Activation Domain
ADP	Adenosine Diphosphate
AHA	Activator of HSP90 ATPase
AIF	Apoptosis Inhibition Factor
Akt	Protein Kinase B
ALT	Alternate Lengthening of Telomeres
APAF-1	Apoptotic Protease Activating Factor-1
APC	Anaphase Promoting Complex
APES	3-aminopropyltriethoxysilane
Asp	Aspartate
ATM	Ataxia-Telangectasia Mutant
ATP	Adenosine Triphosphate
ATPase	Adensosine Triphosphatase
Bak	Bcl2 homologous Antagonist/Killer
BASS2	Bax Antagonists Selected in Saccharomyces2
BASS3	Bax Antagonists Selected in Saccharomyces3
BASS4	Bax Antagonists Selected in Saccharomyces4
Bax	Bcl2 Associated X-protein
Bcl-2	B cell CLL/Lymphoma 2
Bcl-$_{XL}$	Bcl2 like
BD	Binding Domain
Bid	BH_3 Interacting Domain death agonist
BLAST	Basic Local Alignment Search Tools
BM	Basement Membrane
BMK1	Big MAP Kinase 1
BMP	Bone Morphogeneic Protein
BSA	Bovine Serum Albumin
CAK	Cycle Activating Kinase
CARD	Caspase Activation and Recruitment Domain
Cdc	Cell Division Control
cdc37	Cell Division Control 37
cdk	Cyclin Dependent Kinase
Cdk2	Cyclin Dependent Kinase3
Cdk4	Cyclin Dependent Kinase4
Cdk6	Cyclin Dependent Kinase6
CED3	Caenorhabditis elegans Death 3
CED4	Caenorhabditis elegans Death 4

CED9	Caenorhabditis elegans Death 9
CHFR	Checkpoint with Forkhead and Ring Finger
CHIP	Carboxy terminus of Hsc70 Interacting Protein
Chk1	Checkpoint Kinase 1
Chk2	Checkpoint Kinase 2
CIP	Calf Intestinal Phosphatase
CO_2	Carbon Dioxide
CPP32	Caspase 3
CT	Carboxy Terminal Construct of APAF-1
CycB	Cyclin B
CycD	Cyclin D
CycE	Cyclin E
CycH	Cyclin H
DAG	Diacyl Glycerol
dATP	Deoxy Adenosine Triphosphate
DBA	Diamond Blackfan Anaemia
DED	Death Effector Domain
DIABLO	Direct IAP binding Protein with Low pI
DKC1	Dyskeratosis Congenita 1
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-Protein Kinase
DNAse	DNA phosphatase
dTMP	Deoxythymidine Monophosphate
dTTP	Deoxythymidine Triphosphate
dUMP	Deoxyuridine Monophosphate
ECL	Enhanced Chemoluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
Erb-B2	Epidermal Growth Factor recebtor B2
ERC	Extra chromosomal rDNA Circle
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescence Activated Cell Sorter
FADD	Fas Adaptor Death Domain
Fas-L	Fas ligand
FCS	Foetal Calf Serum
FdUMP	5 Fluordeoxyuridine Monophosphate
FdUrd	5 Fuoro depxyuridine
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FKBP51	FK Binding Protein 51
FKBP52	FK Binding Protein52
FL	Full Length
FUTP	5-Fluoro Uridine Triphosphate
G1/S	Gap Phase 1/DNA synthesis cell cycle interface
G2/M	Gap Phase 2/Mitosis cell cycle interface
GA	Geldanamycin

GAL4	Galactose gene
GDEPT	Gene Directed Enzyme Pro Drug Therapy
GH	Glycine Histidine diPeptide
Glu	Glutamate
G-protein	GTPase protein
GR	Glucocorticoid Receptor
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HAT	Histone Acetylase
HBSS	Hanks Balanced Salt Solution
HDAC	Histone Deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HER2	Human Epidermal Growth Factor receptor-2
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
HIF	Hypoxia Inducible Factor
his	Histidine
HOP	HSP70/HSP90 Organizing Protein
HRP	Horse Radish Peroxidase
hrs	Hours
HSE	Heat Shock Element
HSF	Heat Shock Factor
HSP40	Heat Shock Protein 40
HSP70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90
hTERT	Human Telomerase Reverse Transcriptase
IAP	Inhibitor of Apoptosis Protein
IC50	50% Inhibition of Growth Concentration
IHC	Immunohistochemistry
IMS	Inter Membrane Space
INK4A	Inhibitor of cyclin dependent Kinase 4A
IP_3	Inositol Triphosphate
kan	Kanamycin
kDa	Kilo Daltons
LAP	Latency Associated protein
LB	Luria Bertani
LD50	50% Lethality Concentration
leu	Leucine
LTBP	Latent TGFβ Binding Protein
M	Mitosis
MAP2K	Mitogen Activated Protein Kinase Kinase
MAP3K	Mitogen Activated Protein Kinase Kinase Kinase
MAPK	Mitogen Activated Protein Kinase
MDM2	Murine Double Minute 2
MEK	MAP Kinase Kinase
met	Methionine
mins	Minutes
μM	Micro Molar
MMP	Matrix Metalloproteinase

MOM	Mitochondrial Outer membrane
M-phase	Mitosis Phase
MoMLV-RT	Moloney Murine Leukaemia Virus Reverse Transcriptase
MPK	Mitogen Protein Kinase
mRNA	Messenger RNA
MTT	d-[4,5-dimethylthiazol-2-yl]-2,5,-diphenyltretrazolium bromide
N	NH_3 Terminal
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NB-ARC	Nucleotide Binding R Gene CED4 homology domain
NCI	National Cancer Institute
NH_3	Amino group
NOD	Nucleotide Binding Oligomerization Domain
NP	Non-deterministic Polynomial
NT	Amino terminal construct of APAF-1
P1/P3	Position1/4 amino acid residue
P13	Phosphoinositide-3 kinase
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDAC	Pancreatic Ductal Adenocarcinoma
PIP_2	Phosphatidyl-inositol 4,5 biphosphate
PKC	Protein Kinase C
Plk	Polo Like Kinase
pMol	Pico Moles
PMSF	Phenylmethylsulfonylfluoride
pRb	Retinoblastoma Protein
PRIMA	p53 Reactivation and Induction of Massive Apoptosis
PTP	Permeability Transition Pore
Puma	p53 Upregulated Mediator of Apoptosis
RAF-1	Ras Associated Factor
rDNA	Ribosomal DNA
RIP	Receptor Interacting Protein
RNAse	Ribonucleic Acid Phosphatase
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RpS3	Ribosomal Protein S3
rRNA	Ribosomal RNA
RT	Reverse Transcriptase
RTK	Receptor Tyrosine Kinase
SAPK	Stress Associated Protein Kinase
SD	Synthetic Dropout
SDS	Sodium Dodecyl Sulfate
sec	Seconds
SHR	Steroid Hormone Receptor
Sir2	Silent Information Regulator-2
SLIP	Stuart Linn Immunoprecipitation Buffer

Smac	Second Mitochondrial Activator of Caspases
SMAD	Small body size Mothers Against Decapentaplegic
SODD	Silencer Of Death Domain
SS	Serum Starvation
TAE	Tris Acetic acid EDTA
TBS	Tris Buffered Saline
TEMED	N'N'N'N'-tetramethylethylenediamine
TGFβ	Transforming Growth Factor β
TIMP	Tissue Inhibitors of Matrix mettaloproteinases
TNF	Tumour Necrosis Factor
TNF-R	Tumour Necrosis Factor-Receptor
TPA	Phorbol 12- myristate 13- acetate
TPR	Tetratricopeptide Repeat
Tris	Tris(hydroxymethyl)aminomethane
TS	Thymidylate Synthase
TSG	Tumour Suppressor Gene
Tween	Polyethylene Sorbitan Monolaurate
ura	Uracil
VDAC	Voltage Dependent Anion Channel
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau
W	Watts
WD	Tryptophan-Aspartic Acid diPeptide
WD40	Tryptophan-Aspartic Acid diPeptide Repeat Motif
Y2H	Yeast Two Hybrid Screen
YCA-1	Yeast Caspase 1
YEPD	Yeast Extract Peptone Dextrose
yPCD	Yeast Programmed Cell death

AMINO ACID CODES

AMINO ACID	CODE
Alanine	A
Arginine	R
Asparagine	N
Aspartic Acid	D
Cysteine	C
Glutamine	Q
Glutamic Acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

GENETIC CODE

A/a Adenosine
T/t Thymine
C/c Cytosine
G/g Guanine

SECTION 1: INTRODUCTION

Caveat Lector:

In this introduction cancer will be discussed as a disease whose aetiology lies in the disruption of cellular pathways of growth and replication and the enhancement of survival and stress pathways. The chaperone protein HSP90 is increasingly recognised as a significant contributor to the maintenance of these pathways. The structure and function of HSP90 will be described before an in-depth analysis of the molecular hallmarks of cancer with particular relevance to pancreatic cancer. Following on from this I will explore the concept of modelling cancer in yeast before looking at one particular stress pathway - apoptosis- in a yeast model. The clinical context for the experimental work described is pancreatic cancer, and I will begin with this.

1.1 PANCREATIC CANCER THE CLINICAL PROBLEM

1.1.1 The Pancreas

The first mention of the word *pancreas* ("all flesh"), is seen in Aristotle's *Histoire Animalium* [1]. However, it is debateable the relevance of this term to what we now consider the pancreas proper. Galen gives a description of the pancreas in his *On the usefulness of the parts of the body* [2].Galen ascribes a functional role to the pancreas of supporting the abdominal vasculature, and Andreas Vesalius accepted both name and role of pancreas in his masterwork *De Humanis Corporis Fabrica* [3]. Galen's interpretation of pancreatic function was

repeated over the next 1500 years, in some ways holding back scientific investigation into the pancreas.

1.1.2 Historical Context Of Cancer Of The Pancreas

The first reported description of macroscopic cancer of the pancreas was given by Giovanni Morgagni, Professor of pathological anatomy in Padua, in 1761.In 1858 DaCosta, a physician from Philadelphia, described cancer of the pancreas in conjunction with microscopic evidence of adenocarcinoma. The Nineteenth century began to see pancreatic cancer recognised as a clinical entity [4]. The American surgeon Nicholas Senn presented *"The Surgery of the Pancreas as based on Clinical Researches"*, in particular he gives a succinct description of pancreatic cancer "- carcinoma of the pancreas, probably not so rare as it was claimed, usually started in the head of the organ, that jaundice and occasionally duodenal obstruction resulted". Senn then alluded to the difficulty in providing a surgical cure because "… the disease as a rule develops primarily in the head of the organ, a location which, in itself, precludes the propriety of an operation" [5]. However, within 12 years resection of the head of the pancreas had been achieved in Italy by Alessandro Codivilla. Codivilla performed a block resection of the pancreatic head and part of the duodenum. Unfortunately the patient died at 24 days with post-mortem findings of generalised metastases [6]. The German surgeon Walter Kausch performed the first successful resection of the pancreatic head in 1909 in a two-stage procedure [7]. The patient died 9 months later of acute cholecystitis with no recurrence noted at autopsy, although it was noted that the primary tumour was an ampullary cancer.

In 1935 the American surgeon Allen Oldfather Whipple carried out a pancreato-duodenectomy for ampullary cancer, the patient survived 28 months and died of liver metastases. Whipple was responsible for popularising pancreato-duodenectomy and standardising the technique.[8] Alexander Brunschwig performed the first resection for "true" pancreatic cancer in 1937, but the patient died within 4 months [9]. As more of this type of surgery was carried out it became clear that results for cancer of the pancreatic head were much worse than those operations performed for cancers of the ampulla, duodenum and lower bile duct. This was reflected in pronouncements such as that made by the Cattel and Pyrtrek of the Lahey Clinic "It must be admitted that no patient with carcinoma of the head of the pancreas has yet been cured or survived 5 years or more. In the light of our present experience, we must modify the operative procedure or abandon radical surgery for carcinoma of the head of the pancreas" [10]. This surgical credo reached its nadir in the 1970's when George Crile claimed that patients with an adenocarcinoma of the head of the pancreas will live longer and more comfortably if no attempt is made to biopsy or remove the cancer [11]. At this time operative mortality for pancreatoduodenectomy commonly exceeded 20% with redoubtable morbidity- leaving surgical success as judged by operative survival with no appreciation of the complications that were regarded as inevitable for this procedure [11, 12].

1.1.3 Pancreatic Cancer: The Modern Era

The renaissance of pancreatic cancer treatment started in the early 1980s when reports entered the literature showing that pancreatic resection could be achieved with acceptable mortality and morbidity, but also with significant survival benefit [13, 14]. Today pancreatic resection can be routinely performed in high-volume specialised centres with significantly reduced mortality [15].

In a reflection of Cattel and Pyrteks comments, a modification of the original Kausch-Whipple procedure, has been introduced; the pylorus preserving pancreatoduodenectomy appears to have similar survival benefit, is less radical in resection and has potential benefits.

Running in parallel with this change in clinical practice has been the expansion of basic science research into the molecular events that underlie pancreatic cancer, providing potential targets for novel therapeutic agents.

1.1.4 Pancreatic Cancer Today

Pancreatic ductal adenocarcinoma (PDAC) is the most common and deadly form of pancreatic cancer. PDAC is the 4th to 5th leading cause of cancer-related death in the Western world and is characterized by an incidence:mortality ratio approaching unity. Worldwide it is predicted that in the year 2000 there will have been 217000 new cases of pancreatic cancer and 213000 deaths [16]. The only modality consistently shown to exert a positive influence on survival is surgery which today is safer than ever when practised in centres of suitable quality and experience [17].

Adjuvant therapy for pancreatic cancer can improve 5-year survival and quality of life and chemotherapy regimens appear to be most beneficial [18], although at present the best modality and level of benefit are not clearly defined. The benefits of radiotherapy in the adjuvant setting are disputed and combination chemo-radiotherapy appears to give no benefit over chemotherapy[19].

Optimism for treatment of pancreatic cancer must be tempered by the overall dismal 5-year survival rate. Surgery offers the best chance of cure, but most patients present with advanced disease unamenable to resection. Of those that undergo surgery median survival is up to 20 months with an overall 5-year survival rate of 17-24%, with virtually all patients dead at 7 years.

Accordingly new treatment paradigms are required. Conventional chemotherapy relies on indiscriminate targeting of all replicating cells. Basic science research into pancreatic cancer aims to pinpoint the underlying molecular abnormalities thus allowing potential therapeutic targets to be identified. It is hoped that such an approach will allow the introduction of more effective treatments for pancreatic cancer.

1.2 PROTEIN FOLDING AND HEAT SHOCK PROTEINS

1.2.1 The Protein Folding Problem

The fundamental paradigm of molecular biology maintains the one-way transfer of information from DNA through linear mRNA to three-dimensional proteins. The information contained within linear mRNA is converted into a chain of

amino-acids that finally forms a three-dimensional protein. Obviously functional integrity of proteins is dependant upon their correct three-dimensional conformation. *A priori* there must be a relationship between amino acid sequence and final tertiary conformation of protein [20], however the precise details of this relationship have resisted interpretation; this is the protein-folding problem. Indeed, the protein folding problem has been investigated by mathematicians and is considered to be NP-complete- a class of problems unlikely to be solved by conventional computational algorithms.

Protein folding *in vivo* is an energy dependent process in which the final outcome, functional protein, is of critical importance to the cell. Newly synthesised polypeptide chains leave the ribosome in a vectorial manner with the NH_3 terminus produced first. Evidence suggests that protein domain folding can only occur once the domain is fully translated, however the hydrophobic residues synthesised as the chain elongates readily interact within the water rich intra-cellular milieu. This promotes the formation of collapsed "folding-intermediates" that may be refractory to final conformational requirements, or predispose to aggregation. Furthermore nascent polypeptide chains formed on adjacent ribosomes can interact, leading to further aggregation [21], although presumably this may be beneficial in the production of dimerically active proteins.

Cellular proteins known as chaperones have evolved to assist in the folding of linear proteins that emerge from the ribosome or the refolding of denatured proteins in response to proteotoxic cellular stress. Molecular chaperones direct proteins towards their final functional conformation- hence the name- and

consist of several classes of structurally distinct families. Of particular relevance are the heat shock family proteins.

1.2.2 Heat Shock Proteins And The Heat Shock Response- an historical overview

Heat shock proteins (HSPs) were originally identified in the fruit fly *Drosophila melanogaster*. *Drosophila* salivary gland cells contain 4 polytene chromosomes; these are chromosomes that contain DNA that has replicated enormously during development. During development banding patterns can be seen on light microscopy reflecting increased gene expression. In the 1960s it was noted that increasing the ambient temperature of isolated salivary gland cells led to a different pattern of banding, and by the 1970s it was clear that this new banding pattern was due to specific transcription genes coding for *heat shock proteins* (HSPs). Further work established that this heat shock response was ubiquitous in eukaryotes and was a specific example of a more general cellular stress response [22].

The cellular stress response is highly conserved amongst organisms and is probably one of the most ancient of gene-protein-gene circuits. It is initiated by a number of cellular stressors and the induction of HSP production is controlled at the transcriptional level. In unstressed cells Heat Shock Factors (HSFs) are present in cytosol and nucleus as inactive monomers bound to the protein HSP70. Cellular stress results in aggregation of monomeric HSF into trimeric HSF. Trimeric HSF are competent DNA binding proteins and bind to specific Heat Shock Elements (HSEs) that are present within the HSP promoters, with resultant production of HSPs. It is believed that the increased HSP70 production

will lead to conversion of trimeric HSF to inactive monomeric HSF, closing the gene-protein-gene circuit and bring the cell back to its original state. Of particular relevance to protein folding are the HSP70 and HSP90 family of chaperone proteins.

1.2.3 The HSP70 Super-chaperone family

HSP70s are ubiquitously expressed and conserved in all prokaryotes and eukaryotes. Prototypical HSP70 is a 70kDa molecule consisting of two functional domains: Carboxy-terminal substrate binding domain [23], NH_3-terminal ATP binding domain [24].

The substrate-binding domain recognises linear polypeptide sequences rich in hydrophobic residues. Such sequences would ordinarily be expected to be hidden deep within correctly folded proteins- accordingly their exposure could imply misfolding and permit recognition by HSP70 [25-27]. Indeed analysis suggests on average a potential HSP70 binding site occurs every 40 residues, implying that HSP70 could bind to nearly all proteins. The binding and release of proteins recognised by HSP70 (client proteins) is dependent on the moderation of intrinsic substrate affinity by ATP binding and hydrolysis cycles. ATP binds at the 44kDa NH_3 terminus and ATP bound HSP70 has rapid substrate binding and release kinetics, ADP bound HSP70 has slower substrate binding and release kinetics [28, 29]. The functional paradigm for HSP70 is derived from work on its bacterial homolgue DnaK.

1.2.4 The HSP70 Cycle

HSP70 has the ability to bind to nearly all unfolded proteins and provides an iterative folding program enabling proteins to reach their correct functional configuration [30]. Upon completion of this program proteins are released from HSP70 and enter the cellular pool of active proteins with final destination dependent on multiple factors.

HSP70 can interact with other chaperone families, client proteins can be transferred to a chaperonin to reach its final native state [28]. HSP70 can also interact with dimeric HSP90 to shuttle a certain subset of client proteins down an alternative-folding pathway [31] (Figure 1).

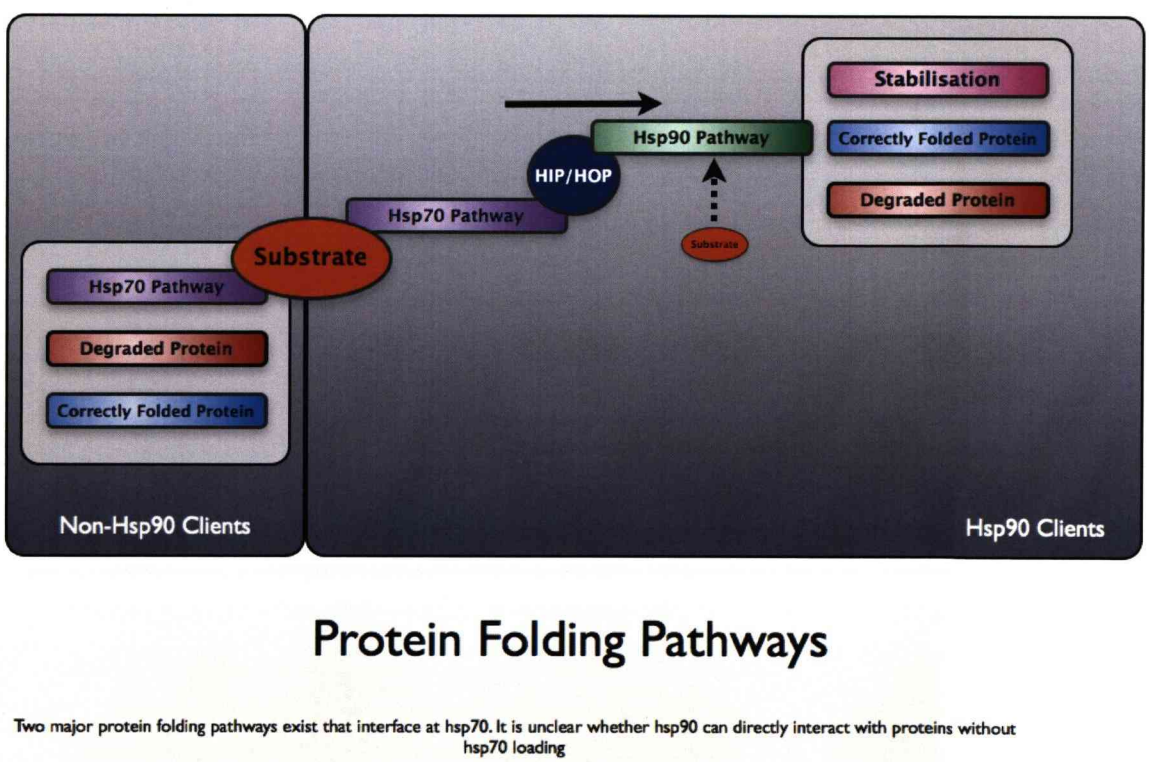


Figure 1:HSP90 AND HSP70 FOLDING PATHWAYS
HSP70 can fold proteins directly or direct a subset of proteins along a HSP90 dependent pathway

HSP90 utilises a different protein-folding strategy to HSP70 and also appears to have distinct biological properties of importance.

1.3 HEAT SHOCK PROTEIN 90

1.3.1 A note on terminology

HSP90 substrate proteins that undergo conformational change are known as *clients*. Proteins that can modulate the folding program of HSP90 are known as *co-chaperones*, some of which are essential to HSP90 function.

Heat Shock Protein 90 (HSP90) is a highly conserved, ubiquitously expressed, essential protein. It was originally identified as being upregulated in conditions of heat-shock, but has been better characterised in its role in the cellular stress-response. HSP90 acts in concert with the HSP70 super-chaperone system, but also would appear to have functions outwith this system. HSP90 does not appear to play a major role in chaperoning nascent polypeptide chains at the ribosome [32], but seems to be the principal chaperone involved in maturation and/or activation of signal transduction proteins, principally steroid hormone receptors and signalling kinases (reviewed in Young *et al.*[33]). The mechanism of client maturation is described below.

1.3.2 HSP90 structure and function

HSP90 exists in the eukaryotic cytosol as two isoforms (HSP90α and HSP90β, the major and minor form respectively). There also exist homologs in other species. All share structural similarities implying a degree of mechanistic similarity

HSP90 is a 732 amino-acid protein with three functional domains (Figure 2):

i. Carboxy-terminal dimerisation domain

ii. Middle domain implicated in client protein binding

iii. Amino-terminal ATP binding domain

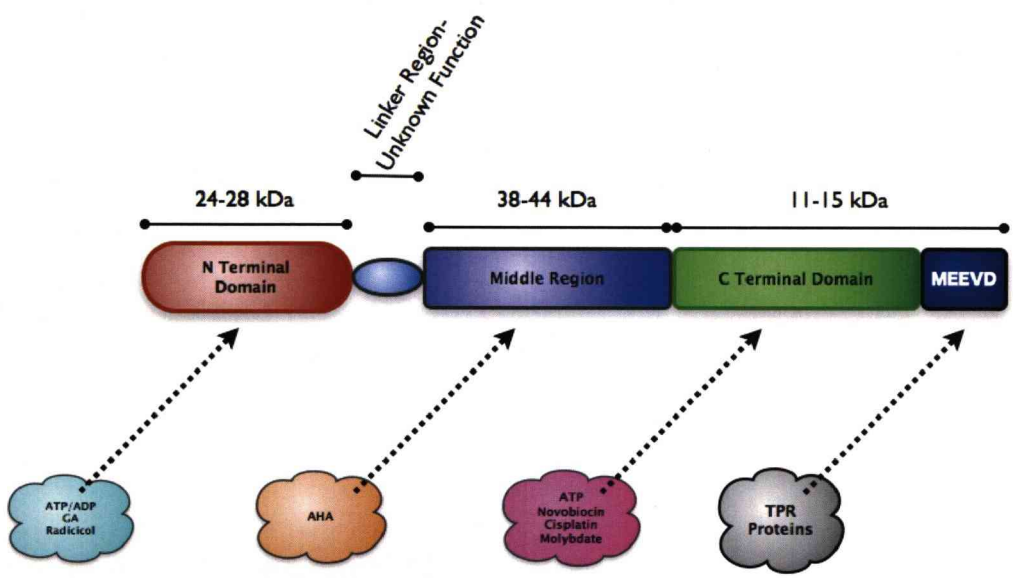


Figure 2: HSP90 DOMAINS AND BINDING SITES
HSP90 contains 3 functional domains that interact with defined proteins

HSP90 acts as a constitutive homodimer with the C-terminal 190 residues forming the main dimerisation domain [34]. The N-terminal 236 residues contain a nucleotide binding "pocket" structurally related to a superfamily of homodimeric ATPases including DNA gyrase and topoisomerase II [35]. The middle domain (residues 272-629) is believed to be essential for substrate binding. In addition client interaction is regulated by numerous co-chaperones that compete for

binding to HSP90 (e.g. Aha1 competes with Hop and cdc37 [36]).

1.3.3 The HSP90 Cycle

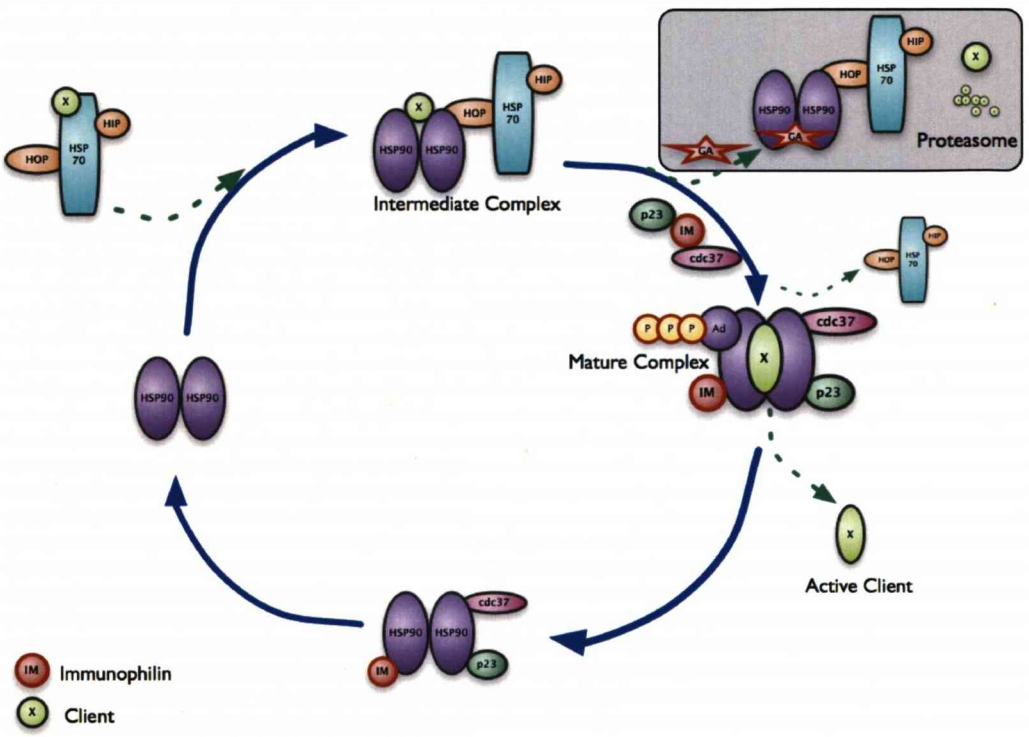


Figure 3: THE HSP90 CYCLE
HSP90 acts through an ATP-dependant iterative cycle to fold protein clients. In addition the HSP90 cycle can be augmented by co-chaperones.

Although in cell-free systems HSP90 can independently bind and cause folding of proteins in the cell it functions in combination with other chaperones and co-chaperones.

The current model for HSP90 function (Figure 3) is derived from seminal work describing the maturation of the glucocorticoid receptor (GR) in rabbit reticulocyte lysate [37]. This receptor-HSP90 heterocomplex pathway consists of a 5 protein minimal system. The system requires HSPs 40,70 and 90 in addition to Hop and p23. The pathway is driven by ATP hydrolysis cycles and also requires potassium and molybdate ions. Although this prototypical system was described

for steroid hormone receptor maturation, the 5 protein minimal system also allows assembly of RAF-1 with HSP90 and reconstitution of reverse transcriptase [38].

There appear to be two ATP dependant phases in the maturation of the GR. Phase 1 involves presentation of the GR to the HSP90 system by HSP70. HSP70-HSP40 bind the GR and in the presence of ATP form a "primed complex" that will be presented to HSP90 forming an initial multimeric complex. In this way HSP90 would bind HSP70 processed clients that have achieved a near-active or active conformation. This could provide a pool of readily available products that could be activated by HSP90 in response to appropriate stimuli. In phase 2 there is ATP dependent conformational maturation of the GR into a steroid binding competent state.

1.3.4 The initial complex in the HSP90 cycle consists of HSP40-HSP70-Hop-HSP90.

The bridging protein HSP70/HSP90 organising protein (Hop) is characterised by the presence of three TPR (tetratricopeptide repeat domains). Simplistically, the TPR1 domain contributes to HSP70 binding and the TPR2a domain contributes to HSP90 binding (with some influence from the TPR2b domain, although in reality TPR2a/2b domains may also affect HSP70 binding [39]). Essentially, Hop allows simultaneous binding of HSP70 and HSP90 and ensures that HSP70 clients are in close proximity to HSP90 [40, 41]. Hop provides the spatial context for client dissociation from HSP70 towards HSP90.

Steroid binding incompetent glucocorticoid receptor (GR) is rendered competent by addition to the minimal system describe above. The GR alters its conformation from having a closed hydrophobic cleft to one where the cleft is exposed to allow interaction with steroid hormones.

The initial complex interacts with GR and there is a change in HSP90 conformation with associated activation of the GR.

Hop binding to HSP90 inhibits nucleotide binding. Nucleotide free HSP90 exists as an extended conformation in which the N-termini are not dimerised. This open state is favourable towards client (GR) binding. HSP70 can lift the Hop-mediated inhibition of nucleotide binding once it binds to Hop-HSP90 and also permit p23 binding- essential for formation of the mature HSP90-client complex.

Nucleotide binding induces a significant change in the topology of the HSP90 complex. Upon ATP binding there is dimerisation of the N-termini with subsequent interaction between them and the middle region of HSP90. This "closed state" of the HSP90 complex resembles a molecular clamp that stabilises the Client-HSP90 complex. The close association of the N-termini also facilitates the ATPase activity of the NH_3 terminus allowing ATP hydrolysis to occur. This conformation also allows binding of p23 [42, 43]. The ATP bound state of HSP90 is considered a mature complex. This mature complex is characterised by the binding of p23, which can only recognise the ATP-HSP90 state. p23 allows the ATP hydrolysis dependant dissociation of the HSP90-substrate complex. The ATP binding site of HSP90 is of particular interest as it is the site of action of the benzoquinoid HSP90 inhibitors such as geldanamycin (GA).

The precise mechanism of Hop release/p23 binding is unclear and there almost certainly are a number of intermediate complexes throughout this phase of the cycle. HSP70 appears to be released from the complex along with Hop. Hop release will "free up" a TPR binding site on HSP90. This TPR site can be utilised by TPR containing proteins such as immunophilins e.g. FKBP52. These TPR proteins enter late in the HSP90 cycle once Hop has been disassembled. They allow a whole class of proteins to interact with the HSP90 machinery and associated pathways. It is suggested that the immunophilins link steroid receptors with receptor-trafficking pathways.

Active GR-steroid complexes move swiftly into the nucleus along the cytoskeleton, but this can re abrogated by inhibition of HSP90 with the drug geldanamycin [44, 45]. Final cleavage of the γ-phosphate of ATP is associated with GR release [46].

The HSP90 cycle as described above is simplified. Progression of client proteins through the cycle is affected by co-chaperones. The cycle can be accelerated by the co-chaperone Aha1 (Activators of the HSP90 ATPase) [47]. The rate-limiting step of the cycle is the N-terminal dimerisation and Aha1 is believed to act here. Aha1 could stabilise the dimerisation or perhaps enhance interaction of the NH_3-terminus with middle domain residues essential for ATP hydrolysis.

Interestingly Panetrou *et al* showed that p23 could inhibit ATP hydrolysis in the presence of Aha1. It may be that Aha1 and p23 cooperate to coordinate progression of the HSP90 cycle, although it is noted that work has also shown that p23 itself can enhance client release [46].

Further regulation of the HSP90 cycle could occur through the differing binding characteristics of phosphorylated HSP90. Hyperphosphorylated HSP90 is not immunopreciptated with target proteins in contrast to unphosphorylated HSP90. Mutant truncated forms of the viral protein σ1 can bind to HSP90 but are refractory to folding. In these cases binding to HSP90 induces hyperphosphorylation suggesting that substrate binding is the initiator of phosphorylation, presumably related to stages I/II of the cycle. It could be that this HSP90-kinase (as yet unknown) adds another level of control to the cycle [48]. This chaperone complex has far greater specificity than HSP90 alone due to targeting proteins such as cdc37. Client interaction is initially indirect through bridging proteins known as targeting proteins. HSP90 targeting proteins include the immunophilins FKBP51 and FKBP52 which bind to TPR domains previously occupied by Hop and the kinase-specific targeting protein cdc37 which binds to a different but overlapping region [49, 50].

1.3.5 Co-chaperones and HSP90

The HSP90 complex is regulated by the presence of regulatory co-chaperones. The best-studied class of co-chaperones are known as TPR proteins. TPR proteins typically contain three 34 amino-acid helix-turn-helix tetratricopeptide repeats (TPRs). These domains are linked to further active domains (e.g. peptidylproloyl isomerase in FKBP52) but can also be linked to further TPR domains to allow interaction between two proteins (e.g. HSP70 and HSP90 bridged by Hop). As alluded to above TPR proteins allow the HSP90 pathway

to interact with other pathways, recently the TPR protein CHIP was shown to link HSP90 with the ubiquitination pathway of proteasomal degradation [51].

Hop

Hop recognises the carboxy terminal MEEVD motif of HSP90 through one TPR domain and also recognises the GPTIEEVD domain of HSP70 through a separate TPR domain. Hop provides the spatial context for HSP90-HSP70 pathway interaction but also appears to inhibit the HSP90-ATPase by preventing nucleotide binding to HSP90. Hence, substrate loading via HSP70 is initially to nucleotide free HSP90.

p23

Nucleotide binding to HSP90 allows conformational change of HSP90 with dimerisation and formation of the HSP90-client clamp. The TPR protein p23 binds specifically to the ATP bound conformation of HSP90.

cdc37

The targeting protein cdc37 has been investigated in its role to target protein kinase to HSP90. Recruitment of cdc37 to HSP90 is initially through Hop. Cdc37 will bind both HSP90 and its target kinase (e.g. cdk4). Cdc37 inhibits the HSP90 ATPase [52], keeping HSP90 in a state amenable to client binding. The details by which kinase folding and activation occur are not known.

1.3.6 Role of HSP90

HSP90 acts in the maturation and activation of proteins (e.g. steroid hormone receptor, see above) but HSP90 can also sequester proteins such as HSF1 and act to provide a pool of rapidly released active protein in response to appropriate stimuli (e.g. p53 see later) (Figures 4-7).

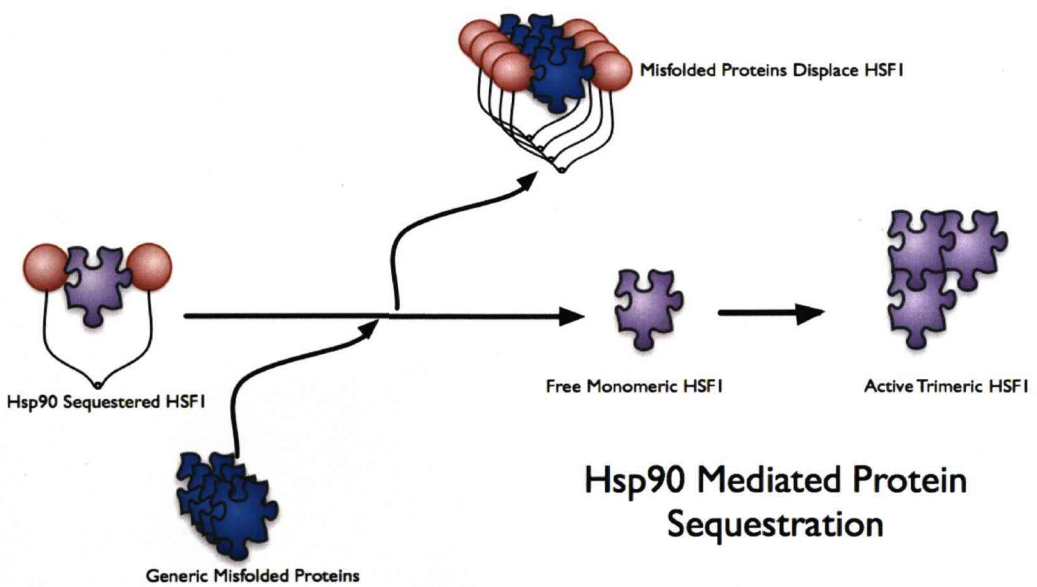


Figure 4: HSP90 MEDIATED PROTEIN SEQUESTRATION
In addition to its roles in protein folding/maturation/activation, HSP90 can sequester proteins until release is induced by appropriate stimuli (e.g. Heat Shock Factor-1, HSF1).

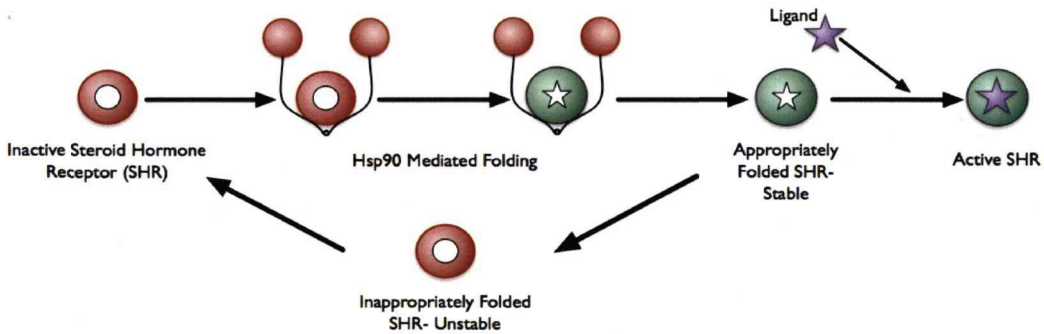


Figure 5: HSP90 MEDIATED PROTEIN STABILISATION
HSP90 can stabilise the steroid hormone receptor (SHR) in a ligand-binding competent form.

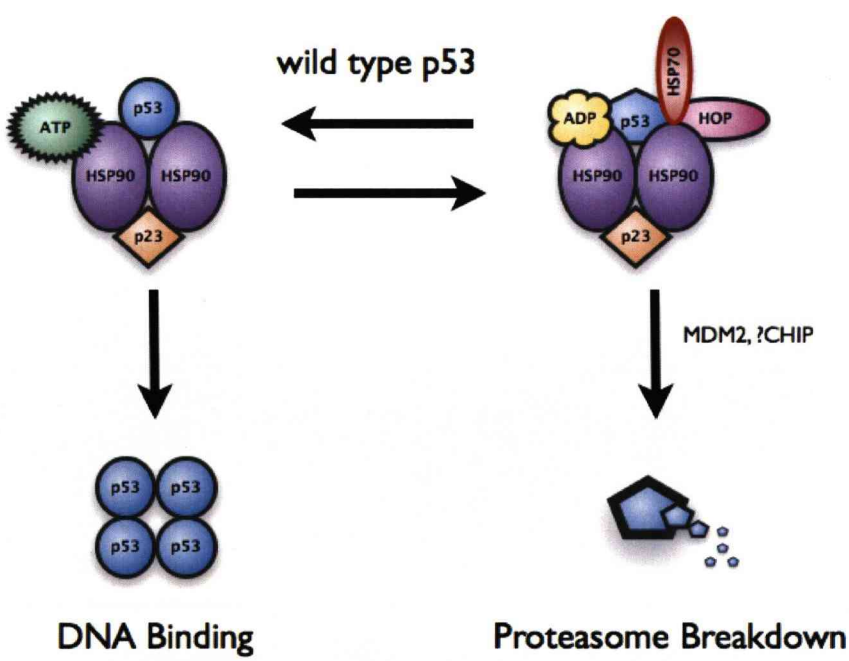


Figure 6: HSP90 CHAPERONING OF WILD TYPE p53
HSP90 can influence wild type p53 activity. HSP90 can promote both DNA binding of p53 and also p53 breakdown via the MDM2/proteasome pathway.

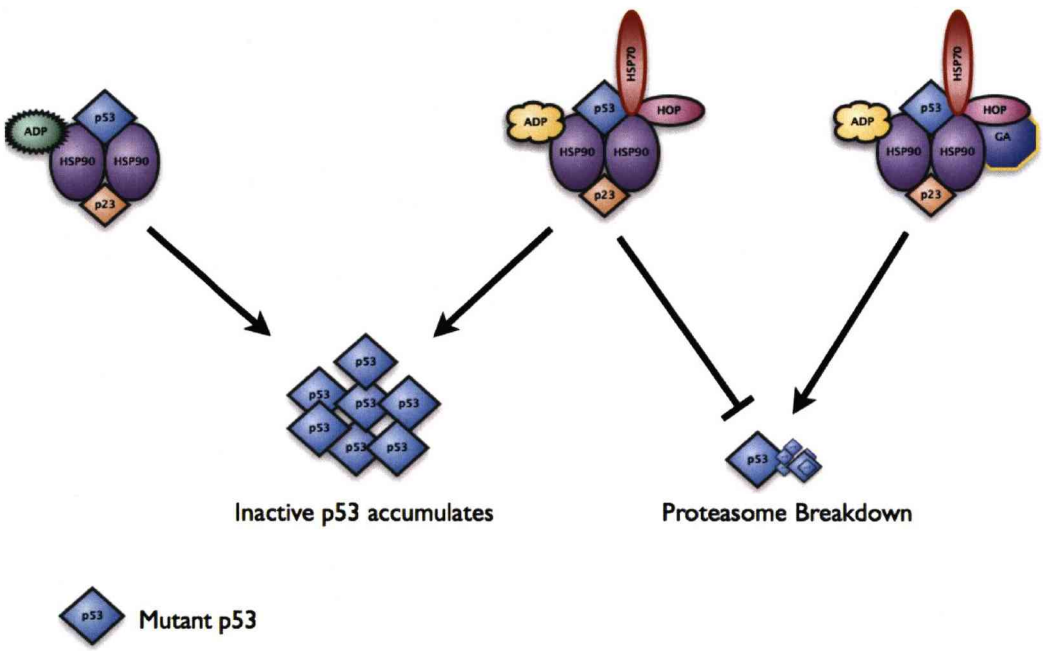


Figure 7: HSP90 CHAPERONING OF MUTANT p53
HSP90 also chaperones mutant p53. HSP90 can inhibit proteasomal degredation of mutant p53. This can be inhibited by geldanamycin (GA).

1.3.7 Cellular Clients of HSP90

The Picard laboratory has an online resource of known HSP90 clients (http://www.**picard**.ch/downloads/HSP90interactors.pdf). A significant number of HSP90 clients are signalling proteins some of which have been implicated in carcinogenesis by mutation and/or over-expression (Figure 8).

Hallmark Trait	Relevant Hsp90 Clients
Mitogen Independence	ErbB2, Raf-1, Akt
Insensitivity to Anti-Growth Signals	Plk, Wee1, Myt1
Evasion of Apoptosis	Apaf, RIP
Limitless Replicative Potential	hTERT
Sustained Angiogenesis	HIF
Invasion and Metastasis	MMP

G1/S Hsp90 Regulated Proteins	G2/M Hsp90 Regulated Proteins
cdk2	cdk1
cdk4	Myt-1
cdk6	Wee-1
cyclinE	survivin

Figure 8: HSP90 AND THE HALLMARKS OF CANCER
HSP90 clients have been assoiated with the phenotypical hallmarks of cancer and cell-cycle regulation.

1.3.8 Inhibition of HSP90

Specific inhibition of HSP90 can be achieved pharmacologically. The best-studied HSP90 inhibitors are members of certain antibiotic classes. Non-antibiotic inhibitors of HSP90 are less well described. HSP90 inhibition can lead to release of protein previously bound.

Geldanamycin and its derivatives

Specific inhibition of all heat-shock proteins has not been achieved pharmacologically, however there exists a class of antibiotics that appear to specifically inhibit HSP90. The benzoquinone ansamycin antibiotics were originally identified as potential cancer therapeutics in a screening program

undertaken by the National Institute of Health. However, at this time their mode of action was unclear. In 1992 Whitesell reported the benzoquinoids possessed tumouricidal activity that was not due to inhibition of signalling molecules such as *v-src* [53]. Further work revealed that that there was specific binding to and inhibition of HSP90 [54]. The archetypal benzoquinoid antibiotic is geldanamycin (GA).

GA shows nanomolar affinity for the nucleotide-binding domain of HSP90-much more than ATP or ADP. GA binding directs HSP90 into a conformation that facilitates client protein degradation, essentially reducing the half-life of HSP90 client proteins, and in addition GA may also induce client release [55]. Assessment of GA in human tumour models revealed it to have significant hepatotoxicity [56] but further work showing that manipulation of the 17 position of GA could be achieved without compromising HSP90 binding allowed derivatives of geldanamycin to be constructed. 17-allylaminogeldanamycin (17AAG) is metabolized to 17-aminodethoxygeldanamycin by cytochrome P450 and has tumouricidal activity. 17AAG also appears to be better tolerated than GA *in vivo* [57, 58]. Further manipulation of the GA skeleton has seen the compound 17-demethylgeldanamycin (17 DMG) introduced as a novel GA derivative with better solubility characteristics [59]. The function of HSP90 is inextricably linked to its ability to bind nucleotides (ATP and ADP). Client proteins associate with HSP90 in its ADP bound state (through HOP/HSP70) forming the intermediate complex (Figure 9).

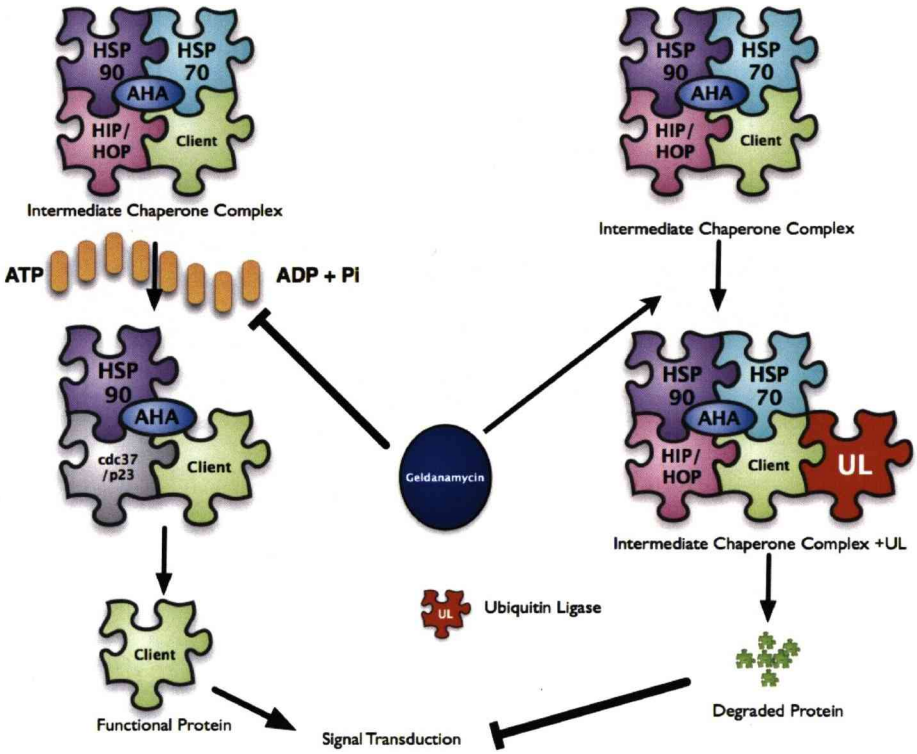


Figure 9: GELDANAMYCIN AND THE INTERMEDIATE AND MATURE COMPLEXES

The mature complex maintains stability of HSP90 clients. However, HSP90 inhibitors such as geldanamycin prevent the formation of the mature complex and permit the activation of a degradation pathway mediated through Ubiquitin ligase binding.

When ATP binds to HSP90 there is dissociation of HOP/HSP70 and alternative co-chaperones enter the complex (e.g. cdc37,p23) forming a more stable mature complex. The mature complex maintains stability of HSP90 clients and the integrity of signalling pathways. Nucleotide mimics like GA disrupt ATP binding to HSP90. GA will prevent formation of the mature complex and degradation of the HSP90 complex may take place through CHIP and the proteasome.

Radicicol

The macrolide antibiotic radicicol has been shown to inhibit HSP90. Radicicol binds to the N-terminal domain of HSP90 but with a higher affinity than GA related drugs. Radicicol is structurally unrelated to GA family members [60].

Novobiocin

Novobiocin is a member of the coumarin family of antibiotics that inhibit bacterial DNA synthesis through binding to DNA gyrase. It has been shown that Novobiocin inhibits HSP90. Unexpectedly Novobiocin does not seem to bind at the N-terminal ATP site that shares structural similarity to DNA gyrase. Instead Novobiocin appears to bind to an occult ATP binding site at the C-terminus. Novobiocin binding to HSP90 is of low affinity and high concentrations are required to achieve client destabilisation. Interestingly work suggests that Novobiocin alters HSP90 conformation to one in which client release is preferred [61]. Furthermore Novobiocin binding to HSP90 destabilises GA-HSP90 binding [62].

Cisplatin

The platinum compound *cis*-diamine-dichloro-platinum is a widely used chemotherapeutic agent. Conventionally cisplatin is believed to exert its tumoricidal effect through the formation of DNA adducts that interfere with DNA transcription. Cisplatin is also known to interact with RNA, cytoskeletal

proteins and phospholipids and it is reported that cisplatin binds to the carboxy terminus of HSP90 in a region overlapping the Novobiocin site and interferes with nucleotide binding [62].

Purine-Based Inhibitors

Purine based molecules have been engineered to target the N-terminal ATP binding site and act in a similar way to GA and its derivatives. The prototypical molecule designed was PU3 [63]. A more potent derivative PU24F-Cl has been described [64].

Taxol

Both HSP70 and HSP90 have been found to be targets of Taxol [65].

Hypericin

Hypericin has been shown to enhance ubiquitinylation of HSP90 thereby abrogating HSP90 dependent functions with subsequent proteasomal degradation of client proteins [66]. The precise mechanism by which Hypericin targets HSP90 for ubiquitinylation is unknown.

1.4 THE MOLECULAR BASIS OF CANCER

Cancer is characterised by the clonal expansion of somatic cells that have undergone deregulation of proliferative and developmental control. Cancer usually arises as a result of initial stochastic mutations in genes responsible for metazoan growth control. The "multiple-hit" hypothesis promotes the view that

several genetic abnormalities combine to give the neoplastic phenotype. These "Hallmarks of Cancer" have been described by Hanahan and Weinberg [67](Figure 10) and can be applied to any cancer.

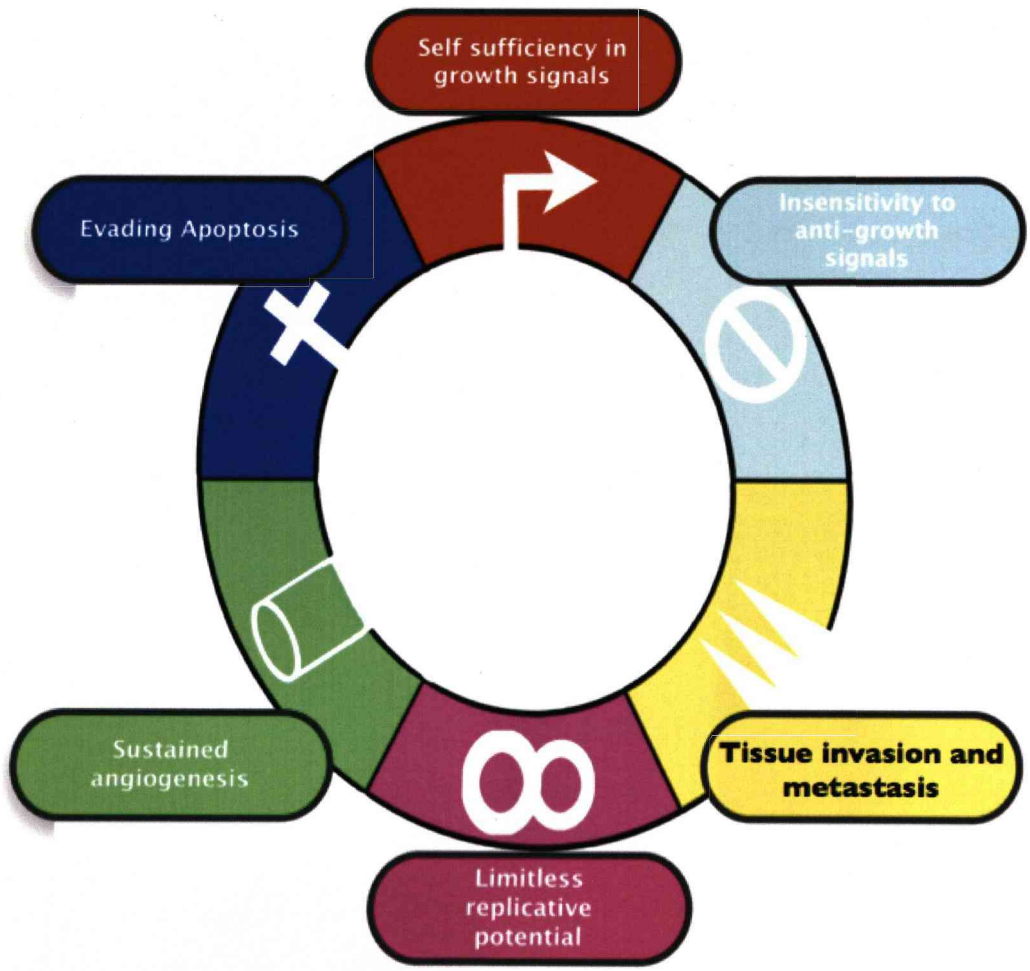


Figure 10: THE HALLMARKS OF CANCER
Six phenotypical features or hallmarks of cancer have been described.

Cellular pathways are responsible for the maintenance of cellular function and integrity. By a similar token maintenance of cellular pathways is therefore essential for normal cellular function and integrity.

The most critical pathways with respect to carcinogenesis are those of cellular proliferation and cellular survival/death. This coupling of proliferative signals

with growth-inhibitory mechanisms normally allows close control to be exercised over the accumulation of new cells.

Stress and survival pathways act to ensure that cellular integrity can be preserved in response to hostile stimuli that would normally cause cell death. These are primitive pathways that would be part of cellular responses early in evolutionary history. As cells have evolved the molecules responsible for stress and survival have become integrated with novel pathways such as programmed cell death. In this way we can see why stress pathways have members that effect many different aspects of cellular proliferation and accordingly present an attractive target for therapeutic intervention. Although tempting to think of these pathways as distinct entities it is clear that molecular crosstalk ensures that the response of the cell to any stimulus is the net output of all pathways activated at that particular time.

The chaperone protein HSP90 is central to the cellular stress and survival pathways; from this stems a pivotal role for HSP90 in the pathogenesis of cancer.

1.4.1 The Cell Cycle

The cell-cycle and its regulation is central to our understanding of cancer.

The cell cycle is considered as 4 components: M-phase, G1, S-phase and G2. A cell that is not on a trajectory ending in cell division is considered to be quiescent (G0 phase). The cell-cycle contains *checkpoints* that must be passed to allow cell-cycle progression. The checkpoints allow halt the cell-cycle and initiate consequent pathways of growth arrest, DNA repair or apoptosis. The

best-studied checkpoints are at the G1-S and G2-M interfaces. Other checkpoints such as the CHFR checkpoint and the M phase spindle checkpoint and mitotic exit pathways have been described.

Upon mitogenic stimulation the cell enters the cell-cycle in the first gap phase (G1). G1 ends at the essential proliferative checkpoint at the G1-S interface. After the G1-S checkpoint has been passed the cell undergoes DNA synthesis and chromosome replication (S-phase). Following S-phase a further gap (G2) phase is entered. A further essential proliferative checkpoint is described at the G2-M interface. If G2-M is negotiated then the cell undergoes mitosis (M-phase). Mitosis is a complex process by which replicated chromosomes segregate with eventual cytokinesis and physical cell-division. Following successful cytokinesis each daughter cell can re-enter G1 for further division or become quiescent and exit the cell-cycle into G0. By definition terminally differentiated cells are in G0 phase.

The G1-S Checkpoint

Transduction of mitogenic signals will stimulate the cell to move from G1 into S phase. As alluded to earlier, progression into S phase is a tightly regulated event. The principal proteins controlling entry into S phase are the cyclins and a group of cyclin dependent kinases (cdks). Cyclins were originally described in sea urchin ova, but have been shown to be ubiquitous proteins [68].

Cyclin D (cycD) has a low basal expression but early in G1 it is upregulated and forms a complex with Cdk4. The CycD-cdk4 complex is competent to bind and phosphorylate the retinoblastoma protein (pRb).

Hypophosphorylated pRb has a characteristic pocket domain that can bind the E2F transcription factor family of proteins. The pRb-E2F complex binds DNA at E2F binding sites and pRb transrepresses DNA transcription at these sites. Mitogenic signalling and upregulation-activation of CycD-cdk4 allows phosphorylation of pRb. This initial cycle of pRb phosphorylation reduces transrepression and permits restricted expression of a number of proteins including cyclin E, allowing a further cycle of pRb phosphorylation by cdk2-cyclin E. Hyper-phosphorylated pRb is incompetent at E2F binding and pRb transrepression is eliminated [69]. The E2F family of transcription factors (E2F1-5) upregulate a host of genes involved in S phase progression when not bound by pRb.

The formation of the CycD-cdk4 complex requires the presence of cyclin dependent kinase inhibitors (CKIs) $p27^{kip1}$ and $p21^{cip1}$. Although originally described as general inhibitors of cdks, it now appears that they have a dual role and have greater inhibitory effects on cyclin E than cyclin D complexes, which ironically require these CKIs to form[70].

The INK4 (inhibitor of cdk4) group of CKIs specifically inhibit cycD-cdk4 activity. It appears they bind to cdk4 before it can interact with cyclin D. Cdk4 has been investigated extensively as a target of HSP90, specifically for binding of HSP90 to cdk4 being determined by the targeting protein cdc37 [71, 72]. Cdc37 protects cdk4 from inhibition by p16 and acts as an oncogene overall by reducing "contact inhibition" (the phenomena by which cells grown in culture reach a monolayer and undergo no further growth). Expression of cdk4 in yeast is toxic, but co-expression with p16 or cdc37 will overcome this [73].

INK4 proteins bind to cdk4 across a region contiguous with the cdc37-binding domain. This leads to competitive inhibition of cdk4 binding. INK4 proteins also compete with Cip/Kip CKIs for cdk4 binding. This means that there will be inhibition of any cdk4 not bound to HSP90 and also releasing Kip/Cip allowing sequestration in the nucleus where they can inhibit CycE-cdk2, again inhibiting progress through G1 [74].

p53 and the G1/S checkpoint

The tumour suppressor gene p53 has attracted enormous study due its position at the nexus of cellular mechanisms that prevent neoplastic transformation. p53 inactivation has profound effects on both cell-cycle arrest and apoptosis. Indeed to many oncologists p53 is truly the guardian of the genome.

Normally p53 has a very short half-life and exists at low levels through the action of MDM2. MDM2 is a nuclear protein that acts as an ubiquitin ligase for nuclear p53, targeting it for proteasomal degradation. In response to genotoxic stimuli p53 is stabilised by a variety of mechanisms and becomes refractory to MDM2 mediated degradation. Escape from the MDM2 feedback pathway does not appear to be potentiated by an increase in p53 production [75].

The effects of p53 are mediated through its role as a transcription factor. Numerous p53 target genes have been identified and most have important roles in cell-cycle control and apoptosis. An important p53 target is the p21 gene that encodes the p21 cyclin dependent kinase inhibitor that inhibits cell-cycle progression. In common with many proteins p21 also has other effects, notably

p21 can bind PCNA (proliferating cell nuclear antigen) thus inhibiting DNA polymerase. In this way DNA replication is restrained, allowing cell-cycle progression to be blocked and DNA repair programs to be initiated. As well as inducing cell-cycle arrest p53 also upregulates pro-apoptotic proteins such as Bax and APAF-1 [76].

The G2/M Checkpoint

Following successful chromosome replication in S-phase the cell enters a second gap phase G2. The G2/M checkpoint controls the initiation of mitosis. Progression through G2 is controlled by cyclin dependent kinase cdc2 (also known as cdk1) and cyclin B (cycB). Dephosphorylated cdc2 is inactive but initial monophosphorylation allows kinase activity in the presence of cyclinB, phosphorylating substrates promoting mitosis (e.g. nuclear laminin breakdown, kinesin related motor protein Eg5). Further phosphorylation of cdc2 renders it inactive. Activation of cdc2 can be initiated by CAK (complex of cdk7, cyclinH and Mat1) which will phosphorylate cdc2 at threonine 161. Wee1 and Myt1 can hyper-phosphorylate and inactivate cdc2 (at tyrosine 15 and tyrosine 15/threonine 14 respectively). The cdc25 family of proteins (cdc25 A-D) can act as phosphatases at these sites reactivating cdc2 [77]. To allow completion of mitosis the cdc2/cycB complex is degraded. The anaphase promoting complex (APC) interacts with a motif known as the "destruction box" present on cycA and cycB and target them for ubiquitination. The APC is inhibited in the absence

of tension at the centromeres; therefore cycB degradation will begin after the last of the chromosomes are aligned on the metaphase plate (spindle checkpoint). Activation of the G2/M checkpoint involves inhibition of the cycB/cdc2 complex. ATM is activated, as is the DNA activated-protein kinase (DNA-PK). There is subsequent p53 stabilisation and activation with upregulation of p21. In addition cellular PI3 kinases (of which one is DNA-PK) activate the checkpoint kinases Chk1 and Chk2, these inhibit cdc25C and hence cycB/cdc2. The precise details by which Chk1/2 inhibit cdc25 proteins is unknown, but phosphorylation of the carboxy terminus of cdc25 by Chk1 can inhibit activity directly and NH_3 phosphorylation allows binding of a 14-33 protein targeting cdc25B for destruction via the proteasome [78].

1.5 THE MOLECULAR BIOLOGY OF PANCREATIC CANCER

Hanahan and Weinberg have defined the neoplastic phenotype as the net contribution of seven defined although overlapping features. We will discuss the general molecular biology and then changes found in pancreatic cancer with respect to each of these features. We will also introduce areas where HSP90 contributes to these features before summarising the role of HSP90 in the development of the neoplastic phenotype.

The current model of pancreatic carcinogenesis centres on a quartet of genetic changes (in K-ras, p16, p53 and SMAD4- see later, reviewed in Magee *et al*[79]). Much work has focused on interactions between pancreatic cancer and its environment (tissue invasion and metastasis), cell-cycle defects (mitogen

independence etc.) and angiogenesis. Of particular relevance to the work presented here is the role of apoptosis and accordingly this will be discussed at length.

1.5.1 Mitogen Independence

Neoplasia means "new growth" and cellular proliferation is the clearest feature of most cancers. Normally there is tight regulation imposed upon cell division and proliferation. The framework for organised and co-ordinated cell division and *a priori* cell proliferation is the cell-cycle.

Mitogenic stimulation is of paramount importance in entering the cell cycle. In cancer cells proliferation is independent of mitogenic stimulation. This disruption of a normal signalling pathway with consequent inappropriate cell cycling is a defining feature of the cancer phenotype.

Normally the cell responds to a legion of extra-cellular growth factors and other stimuli and a coordinated response to such mitogenic stimuli is essential. A number of mitogen pathways have been described and we will discuss the MAPK pathway, the PKC pathway and also their relationship with receptor tyrosine kinases (RTKs).

MAPK pathway

MAPK (Mitogen Activated Protein Kinase) pathway is responsible for the transduction of growth signals to the nucleus and involved in many cellular processes including cell-cycle progression. The MAPK pathway is a

hierarchical system consisting of three protein kinase levels. This scaffolding of a multi-component system provides for regulation and control. The primary level is the MAPK kinase kinase (MAP3K) that integrates the initial signalling to the MAPK pathway. MAP3K will activate the second level of MAPK kinases (MAP2K), which in turn activate MAPK. The tertiary MAPK provide the signalling link to downstream effector pathways.

Four distinct subgroups within the MAPK family have been described:

1. Extracellular signal related kinases (Ras-MAPK pathway)
2. c-jun N terminal or stress activated protein kinases (JNK/SAPK)
3. ERK/big MAP kinase 1 (BMK1)
4. p38 protein kinases

Intracellular MAPK activation can be stimulated by ligand binding to cell-surface growth factor receptors (e.g. EGFR- Epidermal Growth Factor Receptor). There are a number of pathways that allow ligand-receptor-MAPK signal transduction. Of particular relevance in cancer, and particular pancreatic cancer is the Ras-MAPK pathway [79] (Figure 11).

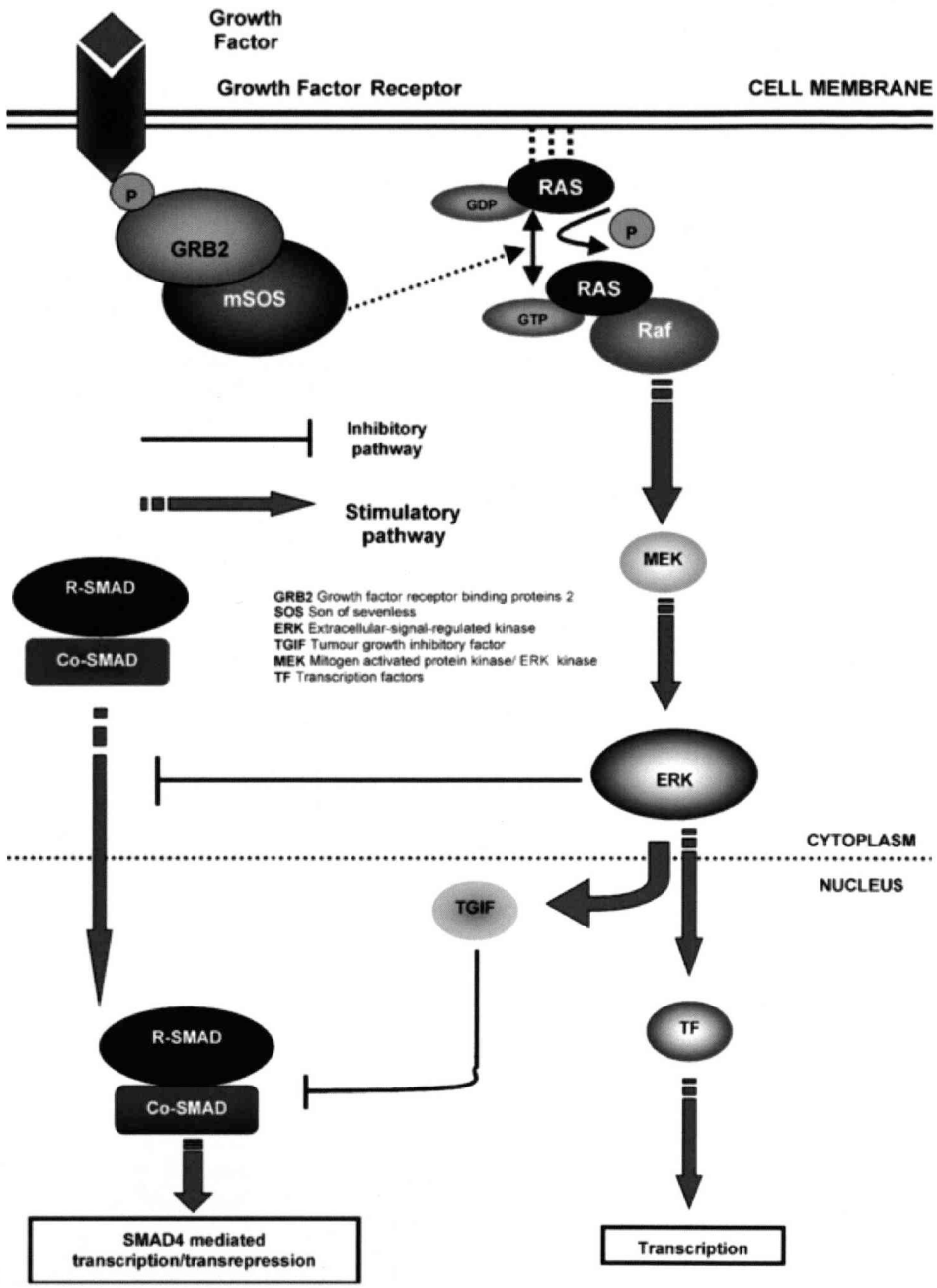


Figure 11: MITOGEN ACTIVATED PROTEIN KINASE (MAPK) PATHWAY

The MAPK pathway is an important signal transduction pathway for cellular growth/division and of great significance in carcinogenesis.

Mitogen activated protein kinase pathways are highly conserved in eukaryotes from yeast to humans [80]. The best-characterised MAPKs are the extra-cellular signal regulated kinases (ERKs). These proteins act as transcription factors when they have been phosphorylated by a dual action tyrosine/threonine protein

kinase. This level of the pathway is also well conserved, and is called the mitogen activated protein kinase kinase (MAP2K). The MAP2K in the Ras pathway is MEK. In turn MAP2Ks are activated through phosphorylation by a specific serine/threonine dual kinase, called the mitogen activated protein kinase kinase kinase (MAP3K) [81]. The most well characterised specific MAPK pathway in humans is that stimulated by the Ras cell surface GTPase.

K-Ras is a member of the RAS signal transduction protein family and is encoded by the *K-RAS2* gene situated on the short arm of chromosome 12 (12p12). *K-RAS2* is the cellular counterpart of a Kirsten rat sarcoma virus gene that encodes a 21-kilo Dalton membrane bound protein with intrinsic GTPase activity.

In response to diverse mitogenic stimuli, wild-type (wt) K-Ras binds to GTP and in this state activates a number of downstream effector cascades (e.g. the RAF/MAPK pathway). This is achieved in part by promoting effector molecule translocation to the cell membrane although additional steps are required for full activation of effector interaction, for example phosphorylation of RAF. After signalling, GTP is hydrolysed to GDP in essence turning off wt K-Ras signalling. The commonly occurring mutations of *K-RAS* alter the ability of the K-Ras protein to hydrolyse GTP. Thus the K-Ras protein becomes constitutively active, persistently stimulating its downstream targets and behaves as though under constant mitogenic stimulus.

GTP bound RAS activates the serine/threonine kinase RAF that lies upstream of MPK. The MAPK pathway proteins RAF and MEK have been identified as HSP90 clients [82-84] and HSP90 appears important in maintaining the integrity of MAPK signalling.

PKC-MAPK axis

The protein kinase-c (PKC) family are a set of disparate serine-threonine kinases. PKC family members have homologous domains but have marked differences in location (tissue-specific and sub-cellular), substrate specificity and co-factor requirements. PKCs have critical roles in intracellular signalling pathways, notably the MAPK pathway.

PKC isoenzymes are conventionally classified on the basis of structure and co-factor requirements. Classic c-PKCs ($\alpha,\beta1,\beta2,\gamma$) require calcium and diacylglycerol (DAG) as co-factors whereas novel n-PKCs ($\delta,\varepsilon,\eta,o$) only require DAG and are calcium independent. Atypical, a-PKCs, (ζ,λ,μ,ι) do not require calcium or DAG for activation.

PKC activation has been best studied for the c-PKCs. Ligand binding to cell surface receptors induces phospholipase Cγ which catalyses the hydrolysis of phosphatidyl-inositol 4,5 biphosphate (PIP_2) to generate the second messengers DAG and inositol 1,4,5 triphosphate (IP_3). IP_3 mobilises calcium from intracellular stores and DAG increases the affinity of PKC for calcium, thereby providing the appropriate context for c-PKC activation to occur. Phorbol esters such as phorbol 12- myristate 13- acetate (TPA) can substitute for DAG and activate c-PKC in the absence of calcium, although calcium lowers the threshold of TPA concentration for this to occur. Phorbol esters are metabolically inert, thus persistently stimulating c-PKC leading to depletion of intracellular stores, because PKCs are cleaved after activation. Interestingly PKC isoenzymes vary in their response to phorbol esters. There are differences between *in vivo* and *in vitro* responses, and also between responses in different cell lines.

Activation of PKC pathways with TPA in a pancreatic cancer cell line has been shown to induce apoptotic proteins [85].

Receptor Tyrosine Kinases

The best-studied tyrosine kinase is that of the receptor tyrosine kinase (RTK) ErbB2 (HER2/neu). ErbB2 is a transmembrane RTK that transduces mitogenic and survival signals from extra-cellular growth factors [86]. ErbB2 can work alone as a homodimer or as a heterodimeric complex with other Erb family members. ErbB2 is an oncoprotein and has been shown to interact with the chaperone HSP90 [87].RTKs link extracellular stimuli (e.g. mitogens) with intracellular pathways such as MAPK.

It is of importance to note that other important RTK pathways implicated in oncogenesis have been shown to be modulated by the chaperone protein HSP90.

Mitogen independence and Pancreatic Cancer

K-RAS

The commonest genetic alteration in pancreatic cancer is mutation of the *K-RAS* proto-oncogene, occurring in 75-100% of cases depending on the reported series [88]. The frequency of *K-RAS* mutation is the highest of any somatic cancer and is a target for therapeutic intervention [18]. K-RAS gain of function is believed to be an early event in pancreatic cancer [79] and appears to precede other mutations. Interestingly *K-RAS* mutation can also be seen in benign pancreatic conditions such as chronic pancreatitis.

P16(INK4a)

In pancreatic cancer the tumour suppressor gene $P16^{INK4A}$ is reported to be functionally lost in around 85% of cases. Microdissection of preneoplastic lesions has demonstrated that $P16^{INK4A}$ loss occurs later than K-ras mutation in the development of pancreatic cancer [79].

RTK and Pancreatic Cancer

ErbB2 is overexpressed in a number of cancers including pancreatic cancer [89]. and its expression appears to be associated with improved differentiation [90].

Oncogene addiction and tumour suppressor gene hypersensitivity

The overexpression of numerous oncogenes (e.g. *K-RAS*) can give rise to a state where by the cancer cell becomes dependant on this persistent abnormal signalling for survival. Work with myc transformed cells showed that cessation of abnormal myc stimulation led to widespread apoptosis, differentiation and proliferative arrest [91]. Earlier work in pancreatic cell lines showed that antisense *K-RAS* induced growth inhibition in mutant *K-RAS* subtypes but not those with wild-type *K-RAS* [92]. Similar findings in breast cancer using anti-sense Her2/neu have been described [93].

Experimental work has shown that reintroduction of wild type tumour suppressor genes (TSG) into cancer cells with previous respective TSG loss induces growth arrest or apoptosis. The dramatic effects of TSG reintroduction at first glance appear at odds to effects expected with repair of a single step in carcinogenesis [94]. This TSG hypersensitivity is a reflection of the multiple roles these proteins play- often being the nexus of numerous cellular pathways. In

order to survive the cancer cell must maintain basic functions such as cellular integrity, energy utilisation, cellular growth and division in an operable state albeit deranged to a significant degree. TSG mutation of pro-apoptotic proteins could result in cell survival being wholly dependent on this small number of mutation (in contrast to the vast genomic instability that forms the context of most cancers). Some authorities believe that targeting of such TSGs alone (e.g. Bcl2 family members) maybe sufficient as an effective therapy [95]. However *in-vivo* cancer is likely to have many deranged pathways favouring cell survival, including anti-apoptotic pathways, proliferation pathways, immune evasion pathways etc. It could be argued that a better approach to cancer therapy would be to target proteins that contribute to multiple pathways deranged in cancer. Such a target is HSP90.

1.5.2 Insensitivity to Anti-Growth Signals

The requirement for growth stimulatory factors is coupled to a number of growth restraining mechanisms that gate the entry to the cell-cycle. Normally there appears to be an inverse relationship between proliferative capability and cellular differentiation. This inverse coupling restricts proliferation to a smaller population of stem-cells. Such restriction enables tighter control over the entry of individual cells into the cell cycle.

A drawback of this is that cells would need to be terminally differentiated before proliferation would cease. Such cells in a permanent post-mitotic state would be refractory to further physiological mitogenic stimuli. As an alternative, anti-growth signal can induce the cell to leave the cell-cycle and enter the quiescent G0 phase, thus leaving the possibility for mitogen stimulated re-entry in the cell-

cycle. Anti-growth signals are gated to the cell-cycle. The best-studied mechanisms relate to the G1-S checkpoint and appear to act when proliferation is sensed to be inappropriate. As mentioned above pRb is central to the G1 checkpoint and it appears pRb forms the crux of many anti-growth signalling pathways. One of the best-studied anti-growth signalling pathways is the TGF-β axis.

TGF-β Axis

Normally, Transforming Growth Factor-β (TGF-β) signalling results in growth arrest. Members of the TGF-β super-family include TGF-βs, bone morphogenetic proteins (BMPs) and activins. The family have a wide range of biological functions including cell proliferation and apoptosis (Figure 12).

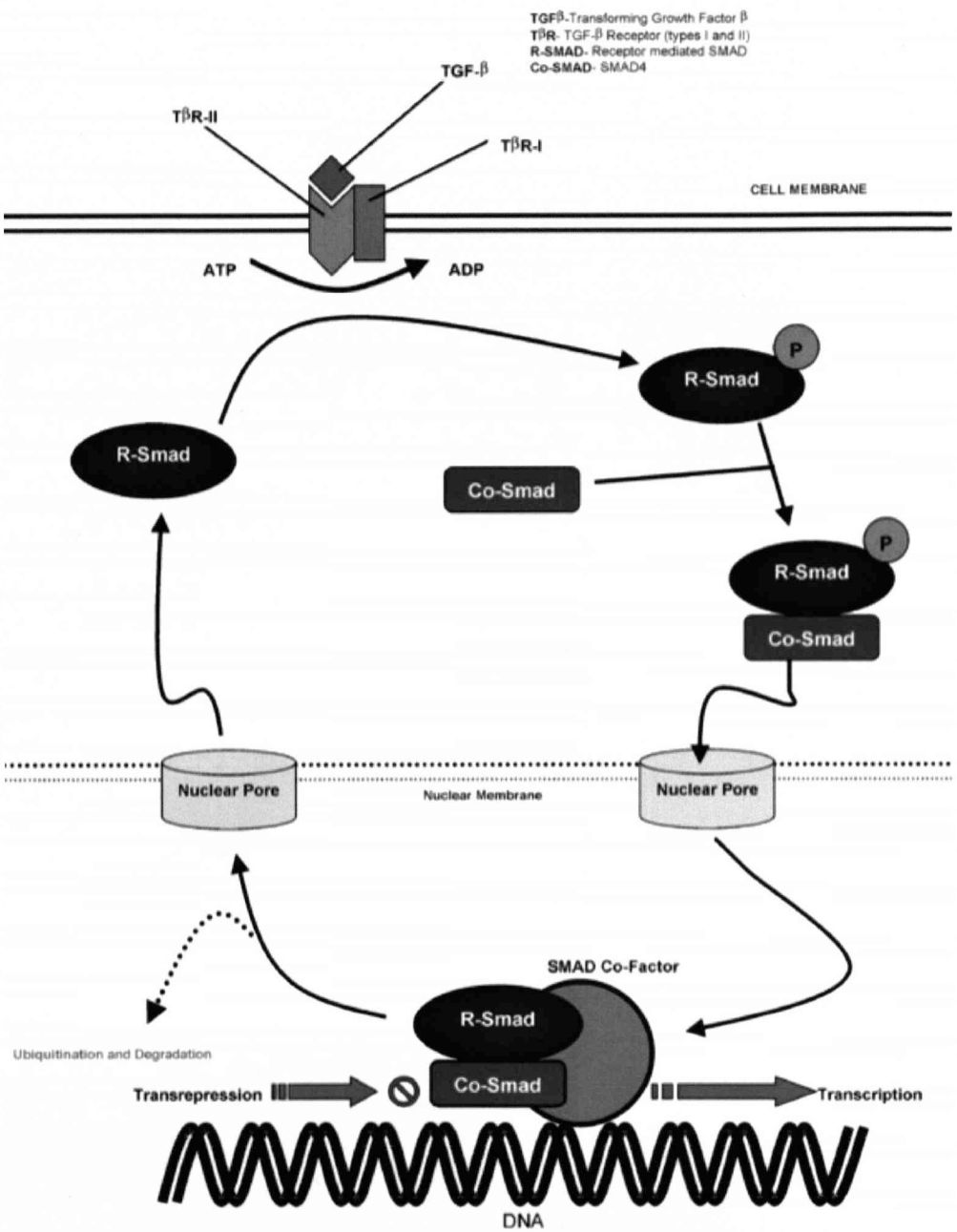


Figure 12: TGF-β AND SMAD PATHWAYS

TGF-β and SMAD pathways are important pathways in the development of pancreatic cancer.

Normally TGF-β exists in the extracellular space as a complex with the latency-associated protein (LAP) and the latent TGF-β binding protein (LTBP). Once released from this complex TGF-β binds to a heterodimeric transmembrane receptor consisting of 2 components- TβR-I and TβR-II. Binding of TGF-β to

TβR-II forms a complex that binds to TβR-I. This enables phosphorylation and activation of TβR-I by TβR-II. SMAD proteins are responsible for the transduction of signals induced by TGF-β binding to its membrane bound receptor. TβR-I phosphorylates the cytoplasmic proteins Smad2 and Smad3, which are termed receptor regulated or R-Smads. These R-Smads associate with Smad4 (common mediator Smad or Co-Smad). The co-Smad/R-Smad heterocomplex translocates to the nucleus where it can co-operate with a host of cofactors leading to the up- or down-regulation of transcriptional activity. This transcriptional regulation appears to be mediated by chromatin remodelling by histone acetylases (HATs) and histone deacetylases (HDACs).

Evidence has shown that TGF-β signalling through Smad4 upregulates *P21*$^{CIP1/WAF1}$ [96] thereby arresting the cell in G1, and that TGF-β mediated growth inhibition is mediated through pRb-E2F transrepression [97]. There are also inhibitory SMAD proteins (I-Smads, Smad6 and Smad7), which abrogate Smad pathway signalling.

Insensitivity to anti-growth signals in pancreatic cancer

All three mammalian isoforms of TGF-β are seen in pancreatic cancer [98].

Inhibitory SMAD proteins have been shown to be elevated in pancreatic cancer samples when compared to normal pancreatic tissue and such over-expression may confer TGF-β resistance and enhanced tumorigenicity [99, 100].

SMAD4 (DPC4) was originally identified through chromosomal mapping of an area with a propensity for deletions in pancreatic and biliary cancer, 18q21 [101]. It was the fourth marker found to be deleted in pancreatic cancer hence, *DPC4*, but

is now termed *SMAD4* as it represents a member of the evolutionary conserved *SMAD* family. The term SMAD is derived from the founding members of this protein family: the *Drosophila melanogaster* MAD (mothers against decapentaplegic) protein and the *Caenorhabditis elegans* protein SMA (small body size). Smad4 is the only co-SMAD identified in mammals [102]; Smad4 loss represents a feature strongly associated with PDAC

p53

The p53 tumour suppressor gene is located on the short arm of chromosome 17 (17p13). More than 50% of human cancers contain mutations of p53 [103]. When activated in response to genotoxic stress, p53 forms a tetramer that binds to DNA and upregulates transcription of genes responsible for cell-cycle arrest and/or apoptosis. Around 90% of p53 mutations occur in exons encoding the DNA binding domain (exons 5,6,7 and 8). More than 50% of PDAC harbour a p53 mutation of which 78% are point mutations, and the remainder are either small intragenic deletions or insertions [104].

Mutated p53 protein has a much longer half-life than wild-type p53 protein. The short half-life of wild-type p53 protein is dependent on a feedback loop with the oncoprotein MDM2 that is itself upregulated by wild-type p53 and MDM2 induces destruction of p53 via ubiquitination. Mutant p53 protein cannot bind DNA to upregulate MDM2 and therefore mutant p53 accumulates in the cell. Accumulation of p53 protein is used as a marker for the presence of mutant p53, although in conditions of stress wild type p53 protein can transiently accumulate in the cell. Specificity can be improved by the use of mutation specific antibodies.

The cellular level of p53 is low due to degradation by the ubiquitin-proteasome pathway. Transient interactions of the HSP90 machinery with wild type p53 can contribute to this pathway through CHIP [105] but also allow a pool of p53 to exist in an activation-competent state. This pool of activateable p53 can be rapidly released in response to genotoxic stress and allow p53 dependent programs of cell-cycle arrest, DNA repair or apoptosis to take place (Figures 6). Mutant p53 is characterised by a DNA-binding incompetent conformation. In contrast to the transient interactions seen with wild type p53, the chaperone machinery could have prolonged interactions with mutant p53. Such prolonged interactions with the chaperone machinery may result in a failure of targeting for degradation, contributing to the accumulation of cellular mutant p53. Inhibition of HSP90 may interrupt the aberrant interactions with mutant p53 and allow escape from the HSP90 cycle by re-establishing ubiquitination [106] (see Figure 7). Recently a breast cancer model has shown that mutant p53 could be reactivated through the actions of a therapeutic molecule termed PRIMA. The mechanism of action of PRIMA is unknown but a proteomic screen identified HSP90 as a possible mediator [107].

1.5.3 Evasion of Apoptosis

Both mitogen independence and insensitivity to anti-growth signalling, previously discussed, are undoubtedly pivotal in driving unregulated proliferation. Proliferative signals can also be other growth-inhibitory mechanisms, for example the shedding of epithelia in the gastrointestinal tract. Probably, the most important cellular growth-inhibitory process in metazoans is programmed cell death; of which the best studied is apoptosis.

The growth of any cancer is not only determined by the inherent replicative state of the tumour (i.e. cell-cycle kinetics) but also by the rate of cell-loss (i.e. cell-death).

Apoptotic cell death is a program of carefully orchestrated events induced by a number of physiological and pathological stimuli. It is characterized by cell membrane disruption, breakdown of cytoskeletal proteins, cytoplasmic loss and organised chromosomal breakdown with eventual nuclear fragmentation. This all takes place in the absence of an inflammatory response.

Apoptosis was first described in the nematode *Caenorhabitis elegans* and has been shown to be conserved in all metazoans. The apoptotic program has two major components:

1. Sensor and Signalling pathways
2. Effector pathways

Sensor pathways monitor the homeostatic state of the cell and its environs. They integrate signals that induce apoptosis and in turn activate the effector mechanisms. The effectors of apoptosis are the cysteine aspartate proteases (caspases); a family of inactive zymogens that are proteolytically activated following apoptotic stimuli. Although much control and regulation can occur at the sensor level, once the effector mechanisms have been activated there is rapid progression towards cell death, typically within 30-120 minutes.

The apoptotic program in the nematode consists of four proteins, the *C elegans* death proteins (CED) –3,-4,-9 and egl-1 (although egl-1 is not essential). There are human homologs of all the CEDs, but in addition humans and higher metazoans have evolved apoptotic proteins not present in the nematode.

Initiation of apoptosis occurs through an extrinsic and an intrinsic pathway, both of which meet at the apoptotic crux- the mitochondrion.

The extrinsic pathway

The cell membrane is the interface between the intracellular and extracellular environments. Changes in the extracellular milieu can be recognised by membrane bound receptors and transduced into mitogenic or apoptotic signals. Of particular relevance to apoptosis are the membrane bound death receptors such as the TNF-receptor family and CD95/Fas/Apo1. The CD95/Fas/Apo1 mechanism is the best described and contributes to immunological cell-death. Activation of the Fas pathway requires receptor interaction with Fas-ligand (FasL) at the cell surface. Normally FasL is expressed by T-cells, and accordingly this method of apoptosis is a feature of T-cell based immunity. It is of note that many auto-immune diseases show inappropriate expression of both Fas and FasL. Fas/FasL induces apoptosis through activation of caspase-8. Following ligand binding there is aggregation of receptors to form membrane bound signalling complexes. FADD (Fas adaptor death domain protein) can bind Fas and also has a death effector domain (DED) that recruits procaspase-8 to the complex with consequent high local concentration of zymogen. It is believed that this allows auto-activation through an "induced-proximity" model. This model proposes that the intrinsic protease activity of pro-caspase-8 is enhanced in these conditions, forming active caspase-8. Caspase-8 then acts upon effector caspases such as caspase-3.

Both haematological cancers (Burkitt's lymphoma, Hodgkins's lymphoma, chronic lymphocytic leukaemia) and solid cancers (colonic cancer, oesophageal cancer) show altered Fas activity.

Despite co-expressing Fas and Fas-L, pancreatic cancers display resistance to Fas-L mediated apoptosis and this may be mediated by Fas associated phosphatases [108]. More recently it has been shown that the resistance to Fas mediated apoptosis could be dependant on activation of the JNK/p38/MAPK pathway [109]. Ligand binding to the TNF receptor normally induces apoptosis, yet pancreatic cancers are resistant to TNF-α induced apoptosis [110]. Ozawa *et al.* have shown that there is increased expression of "silencer of death domains" (SODD/BAG4) mRNA in pancreatic cancer in comparison to other tumour types (liver, oesophagus, colon and stomach) [111]. SODD's block death signalling pathways downstream of TNF-R1 but not Fas [112], and are believed to act by preventing self association of receptor death domains, therefore inhibiting inappropriate receptor signalling.

The intrinsic pathway and the mitochondrion

Central to the intrinsic pathway of apoptosis is the mitochondrion. The mitochondrion is described as the powerhouse of the cell, and in many respects is the powerhouse of apoptosis. The mitochondrion is an intracellular organelle characterised by a double membrane structure. The inter membrane space (IMS) of the mitochondrion contains numerous pro-apoptotic factors (e.g. cytochrome c, Smac/DIABLO, AIF) sequestering them from the cytosol and the nucleus. The release of these pro-apoptotic factors stimulates the apoptotic program in a number of ways: Smac/DIABLO and Htra2/Omi will inactivate the inhibitors of

apoptosis (IAPs), again contributing to activation of the caspase cascade, mitochondrial AIF (apoptosis inducing factor) and endonuclease G can enter the nucleus and contribute to DNA degredation. The best understood of these factors is cytochrome c. Normally cytochrome c is part of the oxidative phosphorylation pathway, however it is also one of the components of the apoptosome- a multimeric complex responsible for caspase-9 activation in the cytosol.

The intrinsic pathway of apoptosis requires permeabilisation of the mitochondrial membrane to allow pro-apoptotic factors such as cytochrome c access to the cytosol or nucleus. The exact mechanism of membrane permeation is not clear but it is essential that permeation be tightly regulated to ensure premature or inappropriate release of pro-apoptotic factors is minimised. The Bcl-2 super family of proteins plays a major role in regulating mitochondrial membrane stability and regulation of apoptosis [113].

The Bcl-2 Super Family

In the nematode, CED-9 is an anti-apoptotic protein that binds to and sequesters the death effector protein CED-4. CED-9 homologs form the Bcl-2 superfamily. These proteins have structural similarity and have three sub-families. Bcl-2 is an oncogene which in follicular lymphoma is linked to an immunoglobulin locus by the chromosome translocation t(14:18). Bcl-2 acts by inhibiting cell death not by directly promoting proliferation. Bcl-2 family members share a number of domains (Bcl Homology, BH). Group I proteins such as Bcl-2 and Bcl-x_L have 4 domains. They also have a carboxy terminus hydrophobic transmembrane domain, localising them to intracellular membranes such as the mitochondria. Group I proteins are anti-apoptotic; in contrast group II proteins such as Bax and

Bak contain apoptotic activity. Group II family members do not possess the BH4 domain. Group III proteins are a heterogeneous collection that shares the small BH3 motif (~12-16 residues) such as Bid.

Pro-apoptotic members such as Bak and Bax can be activated following interaction with Bid, also binding of other BH3-only proteins such as Noxa, Puma, Bad and Bim to anti-apoptotic members such as Bcl-2 and Bcl-x_L results in activation of Bax and Bak. Thus BH3-only proteins mediate the balancing act between pro- and anti-apoptotic signals.

Mitochondrial Membrane Permeabilisation

Bcl-2 family proteins disrupt mitochondrial membrane integrity in an, as yet, unknown fashion. A number of hypotheses have been proposed.

A. *Bcl-2 proteins form channels*

   The structural similarity between Bcl-X_L and the pore forming diptheria toxin [114] suggests that Bcl-2 members may form pores in the mitochondrial membrane to allow contents to pass. Although supported by *in vitro* data it is not clear whether such pores would allow passage of proteins[115].

B. *Bcl-2 form channels as part of multimeric complex*

It is known that Bcl-2 members can bind to and regulate the mitochondrial voltage dependent anion channel (VDAC) [116]. It may be that this could allow egress of proteins. The same caveat as A applies.

C. *Bcl-2 members rupture the mitochondrial membrane*

Alteration of mitochondrial homeostasis in response to apoptotic stimuli could lead to membrane breakdown and release of cytochrome c. This would obviate the need for a specific channel. The VDAC is part of the permeability transition pore (PTP). PTP opening leads to rapid loss of membrane potential and swelling of the mitochondrion, with associated cytochrome c loss.

D. *Bcl-2 members alter membrane dynamics*

Recent work demonstrated that Bax and Bid can permeabilise a model liposome system *without* the need for additional proteins such as VDAC [117]. It may be that Bax induces formation of lipidic pores that allow escape of mitochondrial proteins such as cytochrome c and DIABLO. Recent work has suggested that Bid could contribute to VDAC closure. It was suggested that this could alter mitochondrial membrane dynamics leading to mitochondrial dysfunction [118].

No matter which of these hypotheses holds true the end result is passage of cytochrome c and other "death factors" such as DIABLO into the cytosol.

In addition to their role in release of apoptotic factors from the mitochondrion the BH4 domain of Bcl-2 and Bcl-XL can bind to the C terminal part of APAF-1

(to the CED-4 like part and the WD-40 domain), preventing association of APAF-1 and Caspase-9 [119]. This process seems to be conserved from nematodes to humans since in *C. elegans* CED-9 binds to CED-4, preventing it from binding and activating CED-3. However other work suggests that this APAF-1 and Bcl-2 family members do not interact *in vivo* [120].

Caspases and apoptosis

Cytochrome C release is the initial step in the assembly of a multimeric complex known as the apoptosome. The apoptosome is responsible for activation of caspase-9 and the caspase cascade that results in the morphological features of apoptosis.

Caspases, as alluded to earlier, are a group of proteases that recognise a 4 residue motif (X-Glu-X-Asp) and cleave after the carboxyl terminal Aspartate (P1), although some redundancy with Glutamate has been reported. The P3 residue is almost invariably Glutamate. The caspases share structural similarity and can be divided into two groups; initiators and effectors. All caspases are produced as zymogens and undergo proteolytic processing before becoming active. The activation of an effector caspase such as caspase-3 is performed by initiator caspases such as caspase-9. Note that initiator caspases have intrinsic autoactivity.

Invariably caspases contain one of two protein-protein interaction domains: A death effector domain (DED) or caspase recruitment domain (CARD). These motifs allow interaction with other adaptor proteins mediating a spatial environment conducive to activation and cleavage to occur.

The caspases cleave a diverse number of target proteins. Substrate processing leads to cellular damage that manifests itself as the morphological hallmarks of apoptosis (see above).

APAF-1, caspase-9 and the apoptosome

Cytochrome C released from the mitochondrion binds to the adaptor protein APAF-1 and in the presence of dATP or ATP assembles in to the 1.4mDa multimeric complex called the apoptosome [121, 122]. The apoptosome recruits pro-caspase-9 through CARD-CARD interaction with APAF-1 and facilitates auto-activation of pro-caspase-9. It is interesting to note that processed caspase-9 is only marginally active in the absence of the apoptosome; in the presence of the apoptosome caspase-9 activity increases 1000 fold [123]. Caspase-9 activation and cleavage results in activation of the caspase cascade. Pro-caspase-9 can activate apoptosis without proteolytic processing [124].

The primary component of the apoptosome is the adaptor protein APAF-1 that we will describe in some detail here.

APAF-1

The *a*poptotic *p*rotease *a*ctivating *f*actor-1 (APAF-1) was first described in 1997 as a human homologue of the nematode *Caenorhabditis elegans* protein CED-4 [125]. CED-4 is a central protein in the nematode apoptotic programme and similarly APAF-1 is central to the apoptotic programme in mammals. This is reflected in the phenotype of APAF-1 knockout mice, which have several abnormalities reflecting a lack of developmental cell-death (i.e. apoptosis) resulting in embryonic death, although small proportion survive [126]. Before

describing the functional role of APAF-1 I will discuss the structure of this protein.

APAF-1 Structure

APAF-1 is encoded by a mRNA transcript of 7042 bases, within this is a 3.7 kilobase coding sequence that is translated into an 1194 amino acid protein with a molecular weight of 130 kilodaltons. The 5' untranslated region contains an internal ribosome entry site (IRES), that can be recognized by certain ribonucleotide binding proteins (e.g. upstream of N-Ras: unr). The importance of this with regard to APAF-1 function has yet to be determined [127].

APAF-1 has three domains:

i. N terminal *ca*spase *r*ecruitment *d*omain (CARD): Residues 1-85

ii. Nucleotide binding oligomerisation domain (NOD)/R-gene/CED4 homology domain homology domain (NB-ARC): Residues 86-412

iii. WD40 repeat domain: Residues 602-1194

APAF-1's CARD has homology to the nematode protein CED-3, and the NB-ARC shares significant homology to CED-4, upon which the identification of APAF-1 was made. The NB-ARC domain is shared by CED-4, APAF-1 and plant pathogen resistance genes known as R-genes [128], this latter group being a family of ATPases. APAF-1 is believed to be a member of a group of adaptor proteins that contain an N-terminal CARD, a central nucleotide binding oligomerisation domain (NOD) and a C-terminal sensing domain for intracellular ligands [129]. APAF-1 also contains 12 WD40 repeat motifs. WD40

motifs are common motifs in proteins involved in intracellular signalling; the best studied being the Gβ subunit of heterotrimeric G proteins.

APAF-1 is known to have a number of splice variants [130]:

1. APAF-1S

This is the original sequence identified and has the structure outlined above.

2. APAF-1L

This isoform has an 11 residue insert after residue 98 within the NBARC domain

3. APAF-1XL

This is the same as APAF-1L but has an extra 43 residue WD40 repeat insert between the fifth and sixth WD40 repeats.

4. APAF-1LC

This is the APAF-1S isoform with the extra WD40 repeat as above.

There is some debate in the literature about the biological action of each of these isoforms and it is likely that more isoforms may exist.

Disruption of APAF-1 dependent pathways by APAF-1 loss can contribute to carcinogenesis. DNA methylation of the APAF-1 promoter is a feature of malignant melanoma [131].

Caspase-9 activation requires the assembly of the multimeric holoenzyme apoptosome. The apoptosome is proposed to consist of a heptameric complex with the APAF-1 centred at the hub and their WD40 repeats acting as extended spokes [132]. The apoptosome binds seven monomers of inactive procaspase-9. Thus procaspase-9 is locally concentrated. This provides a scaffold for

recruitment of further procaspase-9 monomers to allow efficient pro-caspase-9 dimerisation and the formation of an active site to drive caspase-9 activation [133].

Downstream of caspase-9, the effector caspases act. Caspase-3 is recruited into the apoptosome and cleaved by caspase-9. There is some cross-talk at this level between intrinsic and extrinsic pathways of apoptosis in that caspase-3 is also cleaved by Fas mediated apoptosis through caspase-8. Further caspase recruitment and cleavage magnifies the apoptotic signal

Until recently it was believed that the apoptosome was essential for activation of the caspase cascade. However in the haematopoetic system lack of APAF-1 or caspase-9 seems not to affect cell number, indeed over expression of Bcl-2 produces an increase in lymphocyte number. It would appear that the apoptosome is not essential for programmed cell death. Furthermore, apoptosis was still seen and could be blocked by caspase inhibition.

There is massive attrition of APAF-1 -/- embryos, but some survive and become healthy adults [126], although infertile. In addition post-mitotic neurones lacking APAF-1 die normally, in contrast to other APAF-1 or caspase-9 deficient cell lines [134].

Taken together these results point to a role of the apoptosome in caspase cascade amplification, or alternatively there may be apoptotic redundancy involving perhaps APAF-1 isoforms.

The caspase cascade is revealing itself as a plastic system. The linearity of caspase activation has more cross-talk than previously thought. Caspase-2 can be activated upstream of mitochondrial disruption and apoptosome activation [135].

Further regulation of the apoptotic programme can be achieved by HSP90. APAF-1 is an HSP90 client protein. HSP90 is a negatively regulator of APAF-1 activation by cytochrome c. Thus HSP90 pathways can abrogate apoptosis- enhancing the neoplastic phenotype.

In pancreatic cancer allelic loss at 12q is seen in around 60% of cases- this is the same locus as APAF-1 (12q23) [136].

1.5.4 Sustained Angiogenesis

New blood vessel formation is essential for tumour growth as the tumour will expand pushing cells away from existing vasculature, depriving the tumour of oxygen, nutrients and access to serum mitogens. The tumour also has an increased energy demand. Vascularistion has two components, vasculogenesis and angiogenesis. Vasculogenesis refers to the assembling of vessels in the embryo from endothelial precursors and angiogenesis refers to the sprouting of these primitive vessels. In cancers the neo-vascularisation occurs predominantly by sprouting or intussusception of pre-existing vessels. The vasculature seen in cancer is noted for both its chaotic organisation and irregular pattern. Consequently, oxygen and nutrient delivery will be sub-optimal resulting in a net hypoxic state for the tumour. At distances greater than 200μm (the diffusion limit of oxygen) from vessels there will be profound hypoxia and acidosis. These conditions exert an immense selection pressure for the development of pro-angiogenic clones. This is achieved by the expression of hypoxia inducible transcription factors (HIFs) that can upregulate angiogenic factors such as vascular endothelial growth factor (VEGF) [137]. VEGF is a soluble growth factor that binds to membrane bound receptor tyrosine kinases (RTKs) expressed by

endothelial cells. VEGF is a 38-46 kDa dimeric glycoprotein that has chemotactic as well as mitogenic properties. In pancreatic cancer the predominant isoforms seen are VEGF-A and VEGF-C[138].

In addition to pro-angiogenic signals (eg. VEGF and angiogenin) there are also anti-angiogenic signals. Such signals include thrombospondin-1 and β-interferon. The angiogenic status of any cancer is the net result of the balance between pro- and anti-angiogenic factors.

Normoxia is associated with low levels of angiogenic factors. This is a result of the relative lability of these molecules in association with low baseline production. However hypoxia increases angiogenic factor activity through enhanced stability and also increased protein production.

HIF-1α is degraded via an E3 ubiquitin ligase that contains a substrate recognition factor called VHL (von Hippel-Lindau). Hypoxia impairs VHL function, with consequent accumulation of HIF-1α.

HIF-1α is a client protein of HSP90 and HSP90 inhibition can reduce HIF transcriptional activity [139, 140] and HIF dependant VEGF production. It was proposed that HSP90 was essential for mediating the formation of a DNA-binding competent form of HIF, or recruiting additional co-factors. It would appear that HSP90 inhibition also promoted VHL mediated HIF degradation by the proteasome.

In a leukaemia model VEGF elevated levels of Bcl2 and inhibited APAF-1, promoting cell-survival, through VEGF-stimulated HSP90 association. HSP90 inhibition reverses this, blocking VEGF mediated survival effects by preventing accumulation of Bcl2 and inhibiting APAF [141].

HIF-1α appears to be able to upregulate the Met gene that encodes the Met receptor tyrosine kinase. Combination with the met ligand promotes tumour invasion [142]. Met is also an HSP90 client protein and protein level can be reduced by geldanamycin, also resulting in increased apoptosis [143].

1.5.5 Limitless Replicative Potential

Cancer cells have an unlimited ability to replicate. The proliferation of normal cells in culture appears to be limited to 60-70 cell doublings, after which the ability to divide is lost (called the Hayflick Limit). In human fibroblasts the loss of p53 or pRb can circumvent this limit but a second point of growth arrest is reached. This second point is termed "crisis". From a crisis population there can emerge a small number of cells that have acquired the ability to divide and proliferate with no further limitation. These cells have undergone "immortalisation". The cellular mechanisms that are circumvented to achieve this state seem to involve the ends of chromosomes known as "telomeres".

Human telomeres contain multiple repeats of the hexanucleotide sequence "GGGTTA". They normally extend for thousands of bases. With each cell division there is shortening of the chromosome ends (and hence the telomeres) due to the inability of DNA polymerases to replicate chromosomal 3 prime DNA (resulting in a 3' overhang which would normally be blunt ended by exonucleases). As the telomere shortens a point is reached, called the "Hayflick

limit", where the cell will normally arrest as a result of a p53 dependent signal. Should this point be overcome for any reason (such as the loss of p53) a point known as M2 or "crisis" is reached whereby the cell can no longer pass the DNA break checkpoint in G2 and the cells will undergo apoptosis in a p53-independent manner. If cells manage to survive this there will be fusion of DNA ends causing a "breakage fusion bridge" cycle, resulting in extreme DNA damage, which would nearly always prove fatal, but in the case of the successful cancer cell may provide the stimulus for advantageous mutations which produce more aggressive tumours [144]. Cancer cells must replace lost telomere hexanucleotide sequences. This is achieved in two ways: Firstly through upregulation of *telomerase*, which is seen in 80-90% of cancer cells [145], and less commonly through alternative lengthening of telomeres (ALT) [146].

Telomerase is a holoenzyme of telomere reverse transcriptase (TERT) and an RNA component that acts as a template for telomere DNA synthesis.

Introduction of TERT into mammalian cells can extend life span and maintain telomere length.

Regulation of telomerase appears to be a complex and recent work has shown that AkT is essential for TERT activation [147]. Furthermore a complex of Akt and HSP90 is needed for assembly of the telomerase complex [148], a process that can be blocked by HSP90 inhibition with geldanamycin. More recent work has shown that the HSP90-Akt-TERT complex can be modulated by the HSP90 inhibitor Novobiocin to increase apoptosis [149].

Suehara *et al.* showed that the telomerase activity in 20 cases of PDAC was significantly higher compared to normal tissue, and greater than that seen in

pancreatic adenomas and chronic pancreatitic tissue [150]. During the proposed progression from normal tissue to carcinoma via pancreatic intraepithelial neoplasias (PanINs) telomere length decreases suggesting that telomerase activation is a relatively late event[151].

1.5.6 Tissue invasion and metastasis

At some point in the neoplastic progression there will be local invasion and subsequent metastasis. As the tumour grows there will an inevitable reduction in nutrient supply and available space to grow into. Angiogenesis will bring an increased nutrient supply, but the problem of "living space" remains. In order to metastasise successfully three barriers must be broached:

i. Normal-cancer cell interface and the surrounding extracellular matrix (ECM)
ii. Basement membrane (BM)
iii. Vascular/lymphatic endothelium.

There is a great deal of similarity in composition between the ECM and BM [15]. Under normal conditions the ECM is a semi-solid structure, but in response to appropriate stimuli the structure becomes more fluid. Not surprisingly this fluidity of the protein rich ECM depends on the action of proteolytic enzymes. Matrix metalloproteinases (MMPs) are a family of zinc ion containing proteolytic enzymes that are involved in physiological remodelling of the ECM and are inhibited by specific tissue inhibitors of MMPs (TIMPs). PDAC exhibits an excess of MMP formation when compared to normal pancreatic tissue [152, 153], and there exists an imbalance between MMP and TIMP production in PDAC [154]. It is thought that this imbalance leads to matrix degradation and tumour

invasion. The stromal cells can be co-opted by malignant cells to produce matrix-degrading proteases [155]. This interaction can modify tumour cell and stromal cell behaviour through signalling pathways associated with cell-matrix adherence molecules (integrins). Expression of the integrin α and β subunits will vary dependant on the local environment. Thus integrin binding for "normal" ECM may be altered resulting in preferential binding to degraded ECM components, thereby endowing cancer cells with trophic abilities facilitating migration and invasion [156, 157].

Associated with local tissue invasion in PDAC is the presence of desmoplasia. It is now believed that desmoplasia is induced by PDAC. Human pancreatic cell lines transplanted into nude mice invoke a fibrotic response similar to desmoplasia [158]. Given that no human fibroblasts were transplanted, the fibrosis could only have derived from murine fibroblasts. This was confirmed by the absence of staining for human connective tissue components in these areas of fibrosis. There was further support for a tumour derived desmoplastic response in that supernatants derived from pancreatic cell lines gave a time-dependant growth advantage to human fibroblasts [159]. Lohr *et al.* have shown the pivotal role of TGF-β in the induction of the desmoplastic response [160]. TGF-β can stimulate the collagenase matrix metalloproteinase MMP-2 [161]. Activation of MMP-2 in pancreatic cancer was found to be strongly associated with the desmoplastic response in pancreatic cancer and to be indicative of invasive potential of pancreatic cancer cell lines [162]. Interestingly, extracellular HSP90 has been found to be associated with MMP2 activation and tissue invasion [163]. Other factors that may mediate desmoplasia include cytokine growth factors such as fibroblast growth factor (FGF).

A group of proteases known as the cathepsins play a role in tissue invasion. Cathepsins are cysteine proteases and cathepsin B and cathepsin L degrade the extracellular matrix. Studies have shown the levels of these two enzymes to be elevated in PDAC and high levels are associated with shorter post-resection survival [164]. Another group of proteins involved in tissue invasion and metastasis are the cadhereins. These proteins are expressed in a variety of tissues and are intimately involved in cell-cell adhesion and play a role in morphogenesis of epithelial tissues. The predominant member of this family is E-cadherin that is found mainly on the lateral wall of epithelial cells and exists as a dimer in areas of intercellular contact known as the adherens junction. Loss of E-cadherin causes reduced cell-cell adhesion increased cell motility- features that are absolute requirements for tissue invasion and metastasis. Reduced expression of E-cadherin has been reported using immunohistochemistry in areas of diffusely growing pancreatic cancer whereas areas of compact tumour showed only a minimal decrease in E-cadherin [165]. A later study using in-situ hybridisation of mRNA on resected pancreatic cancer specimens showed that a high MMP-9:E-cadherin ratio (greater than 3.0) at the periphery of tumours was a significant negative prognostic factor [166].

1.6 HSP90 AND CANCER

HSP90 has defined roles in the maintenance of cellular homeostasis. As a molecular chaperone it acts to ensure the correct folding of proteins. Entwined with this function HSP90 appears to be central in dictating the fate of its client proteins, in particular permitting proteasomal degradation or active protein release depending on specific circumstances.

In normal cells HSP90 can be upregulated by cellular stresses such as hypoxia and nutrient deprivation [167, 168]. Certainly cancer cells exist in a stressful extracellular environment (relative hypoxia, nutrient deprivation, non-efficient metabolic pathways) as well as a stressful intracellular milieu (oncoprotein overexpression, non-efficient metabolic pathways). This imparts selective pressure that would support wild-type HSP90 overexpression (to date HSP90 mutations have not been described in malignancy). In cancer cells HSP90 is expressed at 2-10 times normal levels [169], a highly significant amount given that HSP90 accounts for around 2% of total cellular protein in normal cells. This degree of overexpression is indicative of the importance of HSP90 in the cancer cell.

Analysis of the multiple cellular targets of HSP90 shows disproportional representation of proteins involved in signal transduction pathways. The maintenance of such pathways is central to cellular survival, more so in the disrupted homeostatic environment of cancer. Accordingly there exists a strong argument for focussing on HSP90 as a therapeutic cancer target because HSP90 inhibition could result in simultaneous effects on multiple oncogenic pathways. Such a strategy may result in therapeutic activity against a wide variety of

tumour types or allow synergy in combination with standard chemotherapeutic drugs.

The discussion above introduced the hallmarks of cancer and briefly illustrated the contribution of HSP90. I will next describe further contributions of HSP90 in the pathways that are central to carcinogenesis.

1.6.1 HSP90 associated pathways

Tyrosine Kinases

In addition to the ErbB2 pathway (see previously) HSP90 modulates signalling through a number of other RTKs. HSP90 binds to src and sequestrates it in an inactive form as well as playing a role in its membrane recruitment [87]. The protein Wee1 is a TK that signals to cdk1 (cdc2) in the nucleus thereby permitting a G2/M arrest. HSP90 is implicated in Wee1 assembly [170]. Met is a RTK that is stimulated by HGF/SF (hepatocyte growth factor/scatter factor). Aberrant Met activation signalling is crucial in the development of tumour progression and metastasis [171]. HSP90 has been implicated in Met function and HSP90 inhibition reduces Met levels[172].

Serine-Threonine Kinases

In addition to the MAPK pathway (see previously) HSP90 modulates signalling through other serine-threonine kinases. Akt (also known as protein kinase B) is a signalling target of phosphoinositol-3-kinase. Akt plays a significant role in cell-growth regulation. Akt was first described in insulin pathways but has been shown to phosphorylate several proteins associated with cell-death pathways [173].

Cell-cycle regulators such as cdk4/cdk6 are serine-threonine kinases and again are HSP90 client proteins [71].

Steroid Hormone Trafficking

The regulation of glucocorticoid and progesterone receptors is HSP90 dependent and implicated in oncogenesis of prostate cancer [174].

Transcription Factors

HSP90 plays a significant role in HIF-1 signalling (see previously). In addition to the mechanisms described above HSP90 is implicated in the fate of mutant forms of p53 by inhibiting MDM2 mediated degradation [175].

Apoptotic Factors

HSP90 is implicated in Akt regulation, which in turn regulates cell-death pathways. In addition HSP90 also interacts with other cell-death proteins such as RIP [176]. HSP90 can negatively regulate APAF-1 and also maintain APAF-1 levels [177].

HSP90 has been shown to play a pivotal role in evolutionary progression. It appears that HSP90 can buffer mutant proteins formed by random mutation in the genome. The negative effects of these mutant proteins are dampened by HSP90, with these proteins being functionally sequestrated. When a threshold level of stress or selective pressure is reached these mutations are uncovered[178]. More detail has been added with findings suggesting that HSP90 can interact

with chromatin and hence epigenetically influence gene expression [179]. Given the accelerated rate of mutation and evolutionary progression/regression seen in cancer, HSP90 may act in a similar manner in cancer as in evolution.

1.6.2 Inhibition of HSP90 and Cancer

A major problem that exists for conventional (and many experimental) chemotherapeutics is the concept of tumour specificity. The ideal chemotherapeutic should only target the neoplastic cell. Conventional chemotherapeutics target cycling cells, undoubtedly many cancer cells are cycling (and at any given time many cancer cells will not be dividing) but there are also numerous non-neoplastic cell types that are actively cycling. The toxic effects of the chemotherapeutics manifest themselves as hair loss (hair follicle cells), sterility (germ cells), diarrhoea (gastrointestinal epithelial cells) and mucositis (oral epithelial cells). There do exist strategies to improve the specificity of drug delivery (e.g. gene directed enzyme pro-drug therapy, GDEPT) but these are experimental.

Recent work has shone light on observations with the GA family of HSP90 inhibitors. Ansamycins such as GA appear to be selectively retained in tumour masses *in vivo* despite HSP90 being abundant in both normal and neoplastic cells, in addition the cytotoxic potency of 17AAG exceeds the apparent binding affinity for recombinant HSP90 by two orders of magnitude. The reason for this is that tumour cells contain a high affinity activated form of HSP90 with high ATPase activity, conversely HSP90 present in normal cells is an inactive, uncomplexed form of low-affinity [180] (Figure 13).

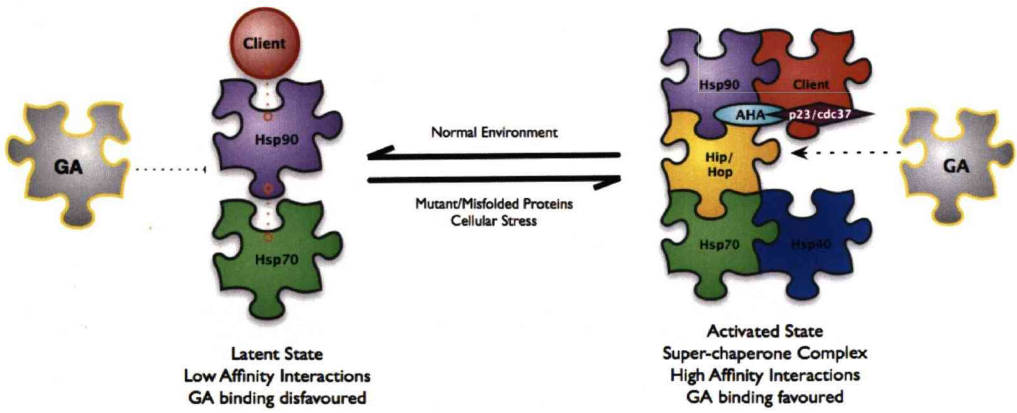


Figure 13: GELDANAMYCIN BINDS PREFERENTIALLY TO THE ACTIVATED HSP90 COMPLEX

Pharmacological inhibition of HSP90 switches the function of HSP90 from one of protein stabilisation and activation to one of protein breakdown. Evidence suggests that protein breakdown can occur through recruitment to the complex of the E3 ubiquitin ligase CHIP with subsequent proteasomal degradation of client proteins [51], but this does not rule out more passive protein degradation as an additional mechanism.

1.6.3 HSP90 inhibition and potentiation of chemotherapeutics

The dose (D)-response(R) relationship of most chemotherapeutics can be expressed as $R \alpha kD$ where k is dependant on the kinetics of the drug in question. Unfortunately as D increases so do adverse responses, essentially limiting the dose that can be given to patients. Strategies employed by clinicians to improve side-effect profiles include combination treatment with two or more differing compounds (with differing side effect profiles) in the hope that an equal clinical response can be seen with lowered doses of the agents. HSP90

antagonists have been used in both experimental and clinical settings as sole agents or in combination with established chemotherapeutics.

The HSP90 inhibitor 17-allylamino demethoxygeldanamycin (17AAG) is completing phase I/II clinical trials as a single agent cancer therapeutic [181]. There are numerous reports of synergy with chemotherapeutics in combination therapy with 17AAG or more commonly by its progenitor drug GA. This apparent generality of potentiation suggests that HSP90 has a global effect accentuating the action of other chemotoxic agents. This may be distinct from its mode of action as a single agent. It has been proposed that HSP90 inhibition lowers the "apoptotic threshold" when used in combination with other chemotherapeutics, perhaps by reducing the level of apoptosis inhibitors [182] or increasing the activity of pro-apoptotic proteins such as APAF-1 [177]. Interestingly the scheduling of HSP90 inhibition appears important. When combined with paclitaxel 17AAG must be given *after* paclitaxel to enhance apoptosis [183]. In contrast to combination use, as a single agent, HSP90 inhibitors probably exert most of their anti-tumour activity through depletion of proteins essential for cell division, such as Cdk4 and Raf1 [181].There have been concerns that HSP90 inhibition may prevent the HSP90 mediated buffering of mutations allowing expression of a more aggressive phenotype, in addition HSP90 inhibition can stimulate the heat-shock response through heat shock factor 1 activity. The heat shock response is cytoprotective and could lead to a state of "drug tolerance" developing in the cancer cell. Such a response may require alteration in the schedule of HSP90 inhibitor administration (see above). Much published work on HSP90 inhibitors with varying chemotherapeutics has shown at least additive effects or potentiation.

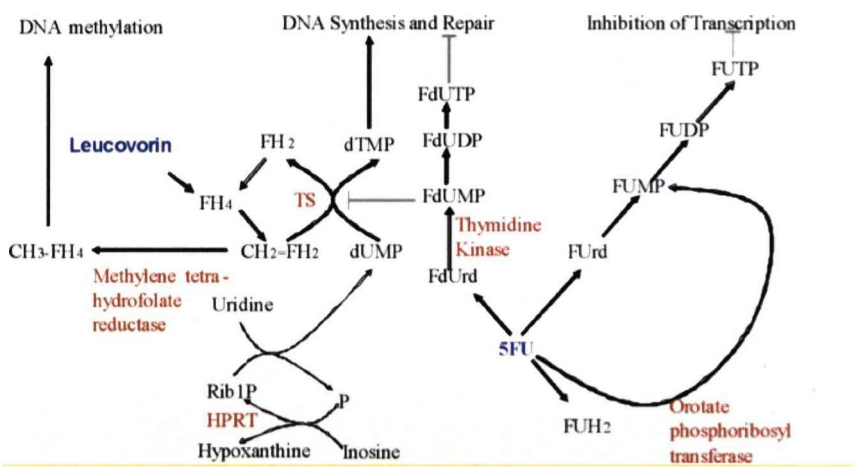
Therapeutic	Mode of Action	Model System	Endpoint	Reference
Paclitaxel	Microtubule inhibitor	Breast cell line (SKBr3/BT474)	Enhanced apoptosis	[183]
Doxorubicin	Inhibit DNA synthesis	Breast cell line (BT549/SkBr)	Enhanced apoptosis	[183]
PKC-412	FLT-3 kinase inhibitor	Leukaemia cell line (MV4-11)	Enhanced cell death	[184]
UCN-01	PKC inhibitor	Leukaemia cell line (U937)	Enhanced cytotoxicity	[185]
LBH589	Histone deacetylase inhibitor	Leukaemia cell line (MV4-11)	Enhanced apoptosis	[186]
Imatinib	Tyrosine Kinase inhibitor	CML cells	Enhances apotosis	[187]
Herceptin-GA conjugate	HER2 antagonist	Mice tumour xenograft	Increased survival	[188]

To date there are no published data on the combination of geldanamycin family members with the conventional chemotherapeutic 5 Fluoro-Uracil (5FU). 5FU is the most widely used drug in resectable and advanced pancreatic cancer. This thesis is concerned with the possible potentiation of 5FU by geldanamycin and it is appropriate to discuss 5FU is some detail.

1.6.4 5-Fluoro-Uracil

5-Fluoro Uracil (5FU) is the most studied chemotherapeutic in pancreatic cancer. 5FU belongs to the fluoropyrimidine family of chemotherapeutic agents. 5FU exerts its main effect by inhibiting the enzyme thymidylate synthase (TS). TS catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). dTMP is subsequently converted to

the triphosphate form (dTTP) for DNA synthesis. 5FU enters the cell via the action of nucleoside transporters found in the cell membrane. In Figure 14 the dUMP/dTMP pathways is illustrated. 5FU is rendered active by metabolism to 5-fluoro deoxyuridine (FdUrd) by the enzyme thymidine phosphorylase, FdUrd is then phosphorylated by thymidine kinase to FdUMP which inhibits TS. This results in reduction of production of dTTP essential for DNA synthesis and repair [189]. 5FU can also disrupt DNA synthesis via its conversion to 5-fluoro uridine triphosphate (FUTP). This occurs via an alternative metabolic pathway to that of TS inhibition. FUTP is incorporated into DNA and induces DNA damage and transcription arrest.



TS Thymidylate Synthase
dUMP (deoxyuridine monophosphate)
dTMP (deoxythymidine monosphate)
dTTP (deoxythymidine triphophate)
FH4 Tetrahydrofolate
FH2, Dihydrofolate

CH2=FH4 Methylene tetrahydrofolate
CH3-FH4 Methyl tetrahydrofolatete
dUMP Deoxyuridine Monophosphate
TMP Thymidine monophosphate
FdUMP 5-Fluorodeoxyuridine monophosphate
FUMP 5-Fluorouridine-monophosphate
FdUrd 5- Fluoro deoxyuridine

Figure 14: 5FU METABOLIC PATHWAYS

Responders to 5FU treatment would be expected to express TS and two studies have correlated TS expression with improved patient survival [190, 191]. However,

although 5FU is widely used in pancreatic cancer, overall response rates are low and limited by toxicity (reviewed in Magee *et al*[192]).

1.7 YEAST AS A MODEL ORGANISM FOR INVESTIGATING APOPTOSIS AND CANCER

The two yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been used as models of multiple cellular pathways. *S. cerevisiae* is also known as brewers yeast and divides by budding in contrast to the fission yeast *Ss. Pombe*. Although the use of yeast in the study of cellular pathways has a long heritage, the investigation of apoptosis in a yeast model is a recent introduction.

The eukaryote *S cerevisiae* is the best-studied model of mammalian cellular pathways. Its utility comes from its well-understood genetics and ease of manipulation. SC has many pathways and proteins homologous to those seen in mammalian cells.

1.7.1 Yeast and apoptosis

Unicellular organisms such as yeast must maintain their internal cellular environment in response to fluctuations in the external milieu (temperature variation, nutrient deprivation, viral infection etc). Such fluctuations can be rapid and prolonged, thus stressing the cells ability to respond and hence survive. It is now clear that yeast possess a repository of genomic programs that can be

activated in response to environmental stress. Of particular relevance to this thesis is the sporulation response to nutrient starvation and the concept of yeast "apoptosis". I will introduce the concept of a program for cell death in yeast first before giving an overview of the sporulation program of budding yeast.

From the metazoan standpoint individual cells form the substrate of programmed cell death and this is determined by the need for organism survival or tissue remodelling. For unicellular organisms cellular death is death of the organism. However, particularly in yeast and bacteria there is the concept of the "herd". The herd is the repository of the genome, albeit replicated in near-identical copies throughout each unicellular, isogenic member. Herd stresses and threats such as viral pathogens and nutrient insufficiency can be responded to by loss of affected members, thus allowing survival in the remaining population, preserving and propagating the genome.

Yeast can undergo cell-death with morphological features of apoptosis

Identification of an apoptotic feature in yeast came from work in which *CDC48* mutants were shown to undergo cell-death with morphological features suggestive of apoptosis (exposure of phosphatidyl serine, cell fragmentation and chromatin margination), although a DNA ladder was not seen[193].

Yeast show an apoptotic response to mammalian apoptotic proteins

Further progress occurred serendipitously when several groups reported that heterologous expression of pro-apoptotic proteins in yeast led to cell death [194, 195]. Pro-apoptotic proteins were being used as bait in yeast two-hybrid screens

with unexpected lethality. Notably human Bax was found to be lethal in yeast. It was demonstrated that apoptotic features such as phosphatidyl serine (PS) exposure, membrane blebbing, nuclear condensation and DNA cleavage accompanied the lethal Bax phenotype - features of metazoan apoptosis [196]. Intriguingly, this lethal phenotype is rescued when Bax is co-expressed with the anti-apoptotic proteins Bcl2 or Bcl-xl [197]and also Bax expressing yeast show release of cytochrome c into the cytoplasm [198]. In addition the Bax related protein Bak can induce an apoptotic phenotype in the fission yeast *Ss. pombe* that is suppressed by Bcl-xl [199].

The mitochondrion is implicated in Yeast Programmed Cell Death

The mitochondrion is the seat of metazoan apoptosis, driving the initial caspase activation and subsequent apoptotic cascade. In yeast data suggest a role for the mitochondrion in yeast programmed cell death (yPCD).

A mitochondrial channel has been described that is Bax-dependent yet is absent from yeast that over-express Bcl-2 [200]. The diameter of the pore is believed to be compatible with cytochrome c passage. It is tempting to assume that cytochrome c release plays a similar role in yPCD to mammalian apoptosis. However data supporting this hypothesis are unclear. Certainly Bax expression releases cytochrome c from the mitochondria but work has also shown that it may not be essential for Bax mediated cell death [201]. Petite yeast lack functional mitochondria and Bax expression induces growth inhibition rather than death, suggesting that competent mitochondria are required for Bax mediated yPCD [194]. This was supported by Zha *et al* who showed that the BH3 dimerisation domain

of Bax responsible for mitochondrial targeting was essential for killing mammalian and yeast cells [202].

Yeast cell stress can induce morphological changes of apoptosis

Heterologous expression of human pro-apoptotic proteins is not the only trigger of cellular changes of apoptosis. The cell stressors acetic acid, H_2O_2, and NaCl induce cell death with markers of apoptosis[203-205]. Although these are pathological stressors, physiological stimuli of yeast programmed cell death have been described. Serevin and Hyman demonstrated that a mating-type pheromone signal could induce yeast cell-death that was accompanied by DNA fragmentation. This could be abrogated by cytochrome c disruption indicating the role of the mitochondrion [206]. This coupling of yeast cell death with a physiological signal suggests a cellular program in response to environmental cues.

Yeast contain caspases

Madeo *et al* described the existence of a caspase-related protein in yeast. Yeast caspase 1 (YCA1) is induced by cellular stresses H_2O_2, acetic acid and aging. YCA1 is processed in a similar way to caspases and is proteolytic for caspase substrates [195]. Overexpression of YCA1 enhances yeast cell death and gene disruption reduces cell death. Encouraging, as these data are, more work is awaited to clarify the nature of YCA1 substrates and true *in vivo* role (reviewed by Jin and Reed[207]).

The Bax phenotype can be used as a screening tool for anti-apoptotic agents

Using the lethal Bax-expressing yeast phenotype as a substrate for identifying potential human apoptosis inhibitors resulted in the identification of 2 novel human genes.

The genes BASS1 and BASS2 (Bax antagonists identified in *Saccharomyces*) not only inhibited Bax mediated yeast death but also prevented apoptosis in mammalian cell-lines [208]. The yeast based screen pulled out 5 proteins (SON2, MAS70, BASS2/3/4). Of these MAS70 and BASS2 are known to interact with HSP90. MAS70 is also known as Tom70.

Tom70 is an import receptor on the mitochondrial outer membrane (MOM). HSP90 and HSP70 present mitochondrial pre-proteins to Tom70. After presentation there is transfer of pre-protein to the Tom70 core domain. It appears that this step is followed by Tom70 homodimerisation forming a high molecular weight complex. It has been suggested that ATPase cycling by HSP90/70 may provide the energy to allow transfer of pre-protein to the import channel [209].

For Tom70 it is striking how the assembly of a multimeric complex at the mitochondrion is associated with HSP90 function. The assembly of the multimeric apoptosome assembly is essential for apoptosis and HSP90 can inhibit APAF-1 oligomerisation [177]. Curiously in yeast HSP70 appears to be required for Tom70 function although this could be simply because of the relative paucity of HSP90 in the yeast cytoplasm.

The release of cytochrome c following expression of Bax could imply the presence of an APAF-1 like protein in yeast associated with cell death.

To date an APAF-1 analogue has not been described in yeast. However in this thesis I report that a BLAST (Basic Local Alignment Search Tools) search strategy using the APAF-1 sequence has shown that the yeast protein YCR072c has significant homology to APAF-1.

1.7.2 YCR072c and APAF-1

A BLAST search of the APAF-1 sequence shows significant homology to YCR072c (17.90% identity, E-value 4.35E-25, http://www.signalling-gateway.org/molecule/query?afcsid=A00302&type=homologs&adv=latest). The identity rises to 28.3% with 58% amino acid conservation in the WD40 region. The E-value indicates that this similarity is highly unlikely to have occurred by chance alone. The yeast protein YCR072c (GenBank reference GI:1907211) is a 515 amino acid protein with a predicted molecular weight of 57 kilodaltons. The homology between APAF-1 and YCR072c is in the C terminal domains containing the WD40 repeats. We will briefly describe the WD40 motif and its functional consequences.

1.7.3 WD40 Motifs

The WD40 motif consists of a 44-60 residue sequence that typically contains the glycine-histidine (GH) dipeptide 11-24 residues from its amino terminus and the tryptophan-aspartic acid (WD) dipeptide at the C-terminus. It must be realised though that there is much variability in these motifs and that neither the GH or WD dipeptide is absolutely conserved. The WD40 motif imparts a structural

constraint on tertiary protein structure, leading to three-dimensional topology that resembles a propeller shape [210].

The WD40 repeats give rise to a surface that allows interaction with other proteins. Three types of interaction have been described. Firstly the WD40 surface facilitates binding of multiple proteins and permits transient interactions among those proteins. The best-studied proteins for this interaction are the G-beta proteins [211] and yeast Hat2 [212]. Secondly, the WD40 surface can provide interactions between subunits of a protein complex, for example 7 of the subunits of the snoRNP U3 particle are WD40 proteins [213]. Thirdly the WD40 can act as a surface to allow conformation integrity within a protein- see below. The APAF-1 WD40 surface serves two functions. Normally the CARD is bound by 2 WD40 repeats. Cytochrome c binding to the WD surface displaces the CARD allowing assembly of the multimeric apoptosome to take place.

1.7.4 YCR072c is a lethal haploid strain

YCR072c has been "knocked out" by single allele disruption with a Kanamycin resistance cassette. Haploid yeast bearing this disrupted allele alone are non-viable (YCR072c-/0). Diploid yeast with only one copy of the disruptant are viable (YCR072c+/-).

One technique of yeast genetics allows us to manipulate yeast into undergoing a process of sporulation. When under stress yeast will undergo sporulation in which the diploid yeast undergo meiosis. In this manner the diploid yeast cell becomes 4 haploid daughter cells all enclosed in a tough outer wall- a structure known as a tetrad. Using a dissecting microscope it is possible to separate each of the 4 spores and analyse their growth individually. YCR072c+/- yeast treated

this way will allow us to investigate whether APAF-1 is functionally related to YCR072c.

1.7.5 The Sporulation Program of Budding Yeast

Unicellular *Saccharomyces cerevisiae* predominantly undergoes asexual reproduction through the budding program. However under certain circumstances a gametogenetic program can be initiated to allow sexual reproduction. Gametogenesis involves two stages- halving of the chromosomal complement and then packaging of these haploid products into gametes. In *Saccharomyces cerevisiae* the gametogenetic program is termed the Sporulation Program.

Sporulation is a complex developmental program coupling DNA replicatory and segregatory events with cyto-architectural changes in response to specific stimuli.

In yeast an environment that is nitrogen depleted as well as having a nonfermentable carbon source initiates the sporulation program. Recently DNA microarray analysis has provided a description of the genes involved in the sporulation program [214].

The production of haploid gametes from diploid parents is achieved through meiosis. The meiotic process has three crucial differences from mitosis:

i. The production of chiasmata by recombination
ii. The protection of centromere-proximal sister chromatid cohesion
iii. The mono-orientation of sister kinetochores during meiosis I

The sporulation program has distinct morphological stages (Figure 15).

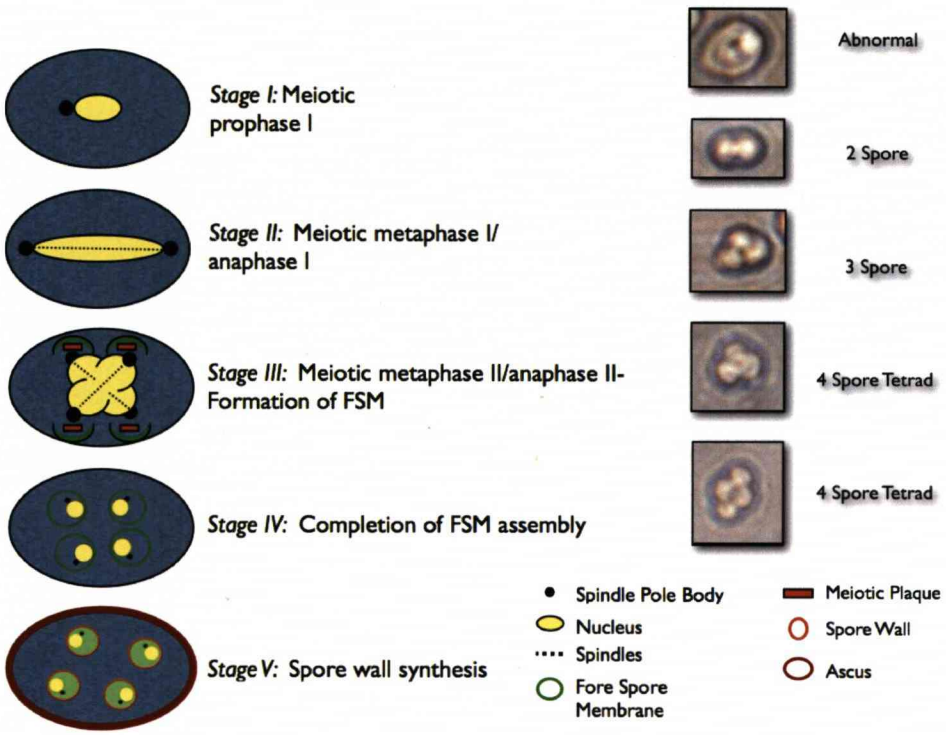


Figure 15: YEAST SPORULATION
Yeast sporulation involves the formation of 4 haploid spores from one diploid yeast cell.

The final result is the production of 4 haploid ascospores enclosed within a tough protective wall known as the ascus. The ascus can be dissolved using zymolyase. The spores can then be individually manipulated on to drop out media to investigate expression of markers genes tagged with auxotrophic markers. This is termed Tetrad dissection and requires the use of a specialised dissecting microscope.

1.8 AIMS

To investigate the role of HSP90 inhibition in modulating the response of pancreatic cancer to apoptotic stimuli.

1.9 OBJECTIVES

1. Test if HSP90 inhibition sensitises to drugs
2. Examine the effect of the order of drug treatment on combination response
3. Examine the effect of serum deprivation on combination response
4. Examine modulation of HSP90 during treatments
5. Investigate the mechanism of HSP90 interaction with the apoptotic program

SECTION 2: MATERIALS AND METHODS

2.1 MAMMALIAN CELL CULTURE

2.1.1 Cell Lines

Mutation	SUIT2	PANC1
K-ras	12 Asp	mutant
p16	Homozygous deletion	Homozygous deletion
p53	273 His	mutant
Smad-4	Wild type	Wild type

2.1.2 Tissue Culture Vessels

Most tissue culture was carried out in Corning 75cm^2 canted neck, 0.2µm filtered, vented cap tissue culture flasks (Cat. No:3376) (T75). Immunofluorescence was carried out in Nalge Nunc Lab Tek II 8 chamber glass slides (Cat. No:154534). 96 well cell culture cluster plates were also acquired from Corning (Cat. No:3596). Liquid nitrogen resistant tubes for freezing of cell stocks were purchased from Nalge Nunc (Cat. No: 363401).

2.1.3 Media and Reagents

All cell cultures were established in GibcoBRL RPMI 1640 liquid medium with L-Glutamine (RPMI) (Cat No: 21875 – 091). Foetal calf serum (FBS) used was GibcoBRL E. C. Approved (Cat. No: 10106 – 169). When trypsin was used for removing adherent cells from tissue culture flasks GibcoBRL 1X Liquid Trypsin (Cat No: 25050 – 030) was used. Penicillin and streptomycin were added to all media and were purchased from Sigma (Cat. No: P-4333). Hanks basic salt solution (HBSS) was used to wash cells, it was also purchased from Gibco-BRL (Cat. No: 14170-088). Propidium iodide for FACS was from Sigma (Cat. No. P4170).

2.1.4 Chemotherapeutics

All drugs were used at the concentrations stated in the text. GA was a gift from Prof. Len Neckers (National Cancer Institute, Rockville, USA). TPA (phorbol 12-myristate 13- acetate (PMA, TPA) (Sigma, Cat. No: P-8139). 5FU (Sigma, F6256).

2.1.5 Passaging of Cells

All cell lines were grown in T75 culture flasks, from frozen stocks in 12mLs of RPMI with 10% FCS and 1% penicillin/streptomycin (complete RPMI). The cells were incubated at 37°C in a humid atmosphere of 5% CO_2 in a Heraeus D-6450 incubator. Once 80% confluent, they were split into either further culture flasks or frozen.

2.1.6 Trypsinisation of Cells

Before many procedures, cells must be removed from the culture flask to which they are adhered. Cells were first washed in two changes of pre-warmed HBSS then 1.5mL of trypsin was added to the flask. The flask was tilted to wash all cells in trypsin and then excess trypsin was aspirated from the culture dish. Cells were incubated at 37°C in a humid atmosphere of air and 5% CO_2 until they dissociated from the flask.

Once the cells were completely dissociated from the flask, at least 5mLs of FBS containing medium (i.e. freeze media, or complete RPMI) was added to the cells to inhibit the trypsin.

2.1.7 Freezing of Cells

A confluent T75 flask of cells was trypsinised, as above, and when cells were no longer adherent, freeze medium was used to inhibit the action of trypsin. Freeze medium consists of 90% FCS with 10% dimethyl sulphoxide (DMSO) (Sigma, Cat. No: D-5879). This mixture is filter sterilised through a 0.2µm MiniSart (Sartorius, Cat No: 16534) filter before use. One T75 produces enough cells to make 5mls of frozen stocks. Therefore, 5mls of freeze media were added to the T75, which was then shaken gently to suspend the cells in media. 1mL aliquots of the cell suspension were then placed into 1.8mL liquid nitrogen storage tubes, which were flash frozen into liquid nitrogen.

To thaw cells and restart a culture, the cells were removed from liquid nitrogen and allowed to thaw rapidly in a waterbath at 37°C before being placed into a T75 containing 12mLs of pre warmed media immediately after the last crystals disappeared. Four to eight hours after inoculating the culture, the cells were adherent to the tissue culture flask and were washed in 12mLs pre-warmed HBSS. The medium was then replaced before continuing to passage as normal.

2.1.8 96-well Plate MTT Analysis

Cells were grown and trypsinised in 4ml as described above. To this suspension 1 ml of FCS was added and spun down in a centrifuge, again as described above. The supernatant was aspirated and the pellet resuspended in 10ml of HBSS.

100µL of cell suspension was mixed with 100µL of 0.2% Trypan Blue solution (Sigma, T-6146). After thorough mixing an aliquot was transferred to a haemocytometer. The number of live (non-blue) cells per square were counted and as the average of 4 squares. This was multiplied by 2 x 10^4 to give total cells/mL. From this figure a solution of 1000 cells/200µL (media with FCS, serum free or containing

drugs) was made up. 1000 cells were plated onto each well using an 8-channel pipette. Cells were grown in serum or serum-free conditions as dictated by the text. Changes in media or drugs was performed by careful aspiration of media and gentle wash in HBSS before the media was replaced and the cells treated as according to the text. Following treatment the cells were incubated with 0.5mg/ml of MTT (d-[4,5-dimethylthiazol-2-yl]-2,5,-diphenyltretrazolium bromide) (Sigma, M2003).. This is metabolised to an insoluble formazan dye by mitochondrial enzymes associated with metabolic activity. The cells were incubated with MTT for 3 hours at 37°C. After discarding the MTT solution, the resultant formazan crystals were solubilised in DMSO and the optical density at 570nm was determined using an Anthos 2001 plate reader (Anthos Labtec Instruments, Salzburg, Austria). The data were processed using "Stingray" software (DAZDAQ Ltd., Ringmer, East Sussex, UK). Predicted additive effect was produced by combining mean reductions for the two drugs. Errors for this value were produced by subtracting the standard error from the means for each treatment. For time courses duplicate plates were used so as to allow different plates to be assayed for each time point.

Statistical analysis was performed using the Mann-Whitney U test.

2.1.9 CaspAce Assay

The CaspAce assay (Promega, Madison, WI, USA) was used to determine capase 1 and CPP32 (caspase 3) activity according to the manufacturers protocol.

2.1.10 FACS Analysis

Cells were grown in T25 tissue culture flasks and treated according to the text. Cells were harvested and suspended in 1ml of FCS followed by 1ml of medium prior to fixing with ice-cold 70% ethanol for 30 minutes. Fixed samples were centrifuged (5 minutes at 300g) and cell pellets were resuspended in 100µl of PBS containing RNase (1mg/ml) and incubated fro 5 minutes at 4°C. The volume was increased to 1ml with 1% bovine serum albumin and propidium iodide (50µg/ml) and the suspensions incubated at 4°C for 30 minutes. The stained cells were analysed by FACScan (Becton-Dickinson) and subsequently by WinMDI v2.8 software (Scripps Institute).

2.2 MICROBIOLOGICAL WORK

2.2.1 Media

All of the following are sterilised by autoclaving unless otherwise stated.

Luria Bertoni (LB) Media:
1% Bactotryptone (Difco, Cat No: 211705)
0.5% Bacto-Yeast Extract (Difco, Cat No:212750)
1% NaCl (Sigma, Cat No. 57653)

Luria Bertoni (LB) Agar
1% Bactotryptone (Difco, Cat No: 211705)
0.5% Bacto-Yeast Extract (Difco, Cat No:212750)
1% NaCl (Sigma, Cat No. 57653)
2% Agar (Difco, Cat No: 214010)

Luria Bertoni (LB) Amp Agar

1% Bactotryptone (Difco, Cat No: 211705)
0.5% Bacto-Yeast Extract (Difco, Cat No:212750)
1% NaCl (Sigma, Cat No. 57653)
2% Agar (Difco, Cat No: 214010)
50mg/ml Ampicillin (Sigma, Cat No: A-0166)

STET Medium

50mM Tris-HCl, pH 8.0 (Calbiochem, Cat No: 648310)
50mM EDTA, pH 8.0 (Sigma, Cat No: E5134)
0.5% v/v Triton X-100 (Sigma, Cat No: X-100)
8% w/v sucrose (Supelco, Cat No: 47289)

YEPD Media

1% Bacto-Yeast Extract (Difco, Cat No: 212750)

2% Peptone (Difco, Cat No: 211677)

This was autoclaved and allowed to cool then glucose was added (BDH, Cat No: 101176K) to a final concentration of 2%

YEPD AGAR

1% Bacto-Yeast Extract (Difco, Cat No: 212750)

2% Peptone (Difco, Cat No: 211677)

This was autoclaved and allowed to cool then glucose was added (BDH, Cat No: 101176K) to a final concentration of 2%

2% Agar (Difco, Cat No: 214010)

10x YNB (Yeast Nitrogen Base)

8.75g YNB (Sigma, Cat No: Y-0626)

25g Ammonium Sulphate (Sigma, Cat No: A5132)

Make up to 500mls with H_2O

This was filter sterilised

Yeast Lysis Buffer

1% (w/v) SDS (BDH, Cat No: 444464T)
100mM NaCl (Sigma, Cat No: 57653
10mM Tris (BDH, Cat No: 443864E
1mM EDTA (Sigma, Cat No: E5134)

Pre Sporulation Media

0.8g Bacto Yeast Extract (Difco, Cat No. 212720)
0.3g Bacto Peptone (Difco, Cat. No. 211677)
10g Glucose (Sigma, Cat. No. 47829)
in 100ml sterilised water

Sporulation Media

10g Potassium Acetate (Sigma, Cat. No. 236497)
10g Potassium Acetate (Sigma, Cat. No. 236497)
Required amino acids at 25% less than SD media
in 1000ml sterilised water

Synthetic Dropout (SD) Media

2% Agar (Difco, Cat No: 214010)
2% Glucose (Sigma, Cat. No. 47829)
This was made up to 400mls with H_2O, then autoclaved and allowed to cool. Then the following were added:
50ml 10xYNB
Adenine (Sigma, Cat No: A-9126) to 50mg/l
Histidine (Sigma, Cat No: H-6125) to 20mg/l
Leucine (Sigma, Cat No: L5652) to 30mg/l
Lysine (Sigma, Cat No: L-5626) to 30mg/l
Tryptophan (Sigma, Cat No: T-0254) to 20mg/l
Tryptophan (Sigma, Cat No: T-0254) to 20mg/l
Amino acids were omitted as appropriate for each dropout medium

Yeast RNA Extraction Buffer 1

0.5M NaCl (Sigma, Cat 57653)
200mM Tris-HCl (pH 7.5) (BDH, Cat 443864E)
10mM EDTA (Sigma, Cat E5134)
This was treated overnight with 1% DEPC and then sterilized by autoclaving

Yeast RNA Extraction Buffer 2
50mM NaOAc (pH 5.3), (Sigma, Cat No: S-1429) 10mM EDTA (Sigma, Cat No: E-5134) 1% SDS (Sigma, Cat No: L3771) This was treated overnight with 1% DEPC and then sterilized by autoclaving

50x TAE Buffer
242g Tris (Calbiochem, Cat No: 648310) 57.1ml Glacial Acetic Acid (Sigma, Cat No: A-6283) 18.6g EDTA (Sigma, Cat No: E-5134) Make up to 1000ml with deionised water

2.2.2 Strain Genotypes

XL1-Blue E.coli:

*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$

F'[*proAB*$^+$ *lacI*^{q} *lacZ*DM15 Tn*10*(*tet*^{r})]

YCR072c+/-

BY4743 [Mat a/a; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YCR072c::kanMX4/YCR072c]

2.2.3 Microbiological Culture

Bacterial Culture.

Bacterial strains were grown in LB media containing 50mg/ml ampicillin at 37^{O}C (unless otherwise stated) with shaking at 280RPM in 50ml Falcon tubes (Alpha, Cat No: LW1120) or sterile conical flasks using an Innova 4300 shaking incubator.

Yeast Culture

Yeast strains were grown in YEPD media or SD media (unless otherwise stated) at 30^{O}C with shaking at 250RPM in sterile conical flasks using a Gallenkamp shaking incubator.

Sporulation

Yeast were grown up on fresh agar plates and inoculated into a 250ml sterile conical flask containing pre-sporulation media. Yeast were pre-sporulated to reach log-phase growth at 30°C in a Gallenkamp shaking incubator. A 1 ml aliquot was inoculated into a sterile 4 litre conical flask containing 250ml of sporulation media and incubated at 30°C for 3-5 days in a Gallenkamp shaking incubator. Sporulation analysis was undertaken using an aliquot pipetted onto glass microscope slides and examined under light microscopy.

2.3 PROTEIN WORK

2.3.1 Solutions and Reagents

Phosphate Buffered Saline pH 7.5

Reagent	**Amount**
Na_2HPO_4 (Sigma, S-0876)	62.5mMol
NaH_2PO_4 (Sigma, S-0751)	15.5mMol
NaCl (Sigma, S-9888)	75mMol

Tris Buffered Saline pH 7.6-

Reagent	Amount
Tris(hydroxymethyl)methylamine (BDH 103156X)	30.3g
NaCl (Sigma S9888)	40.85g
HCl	19ml
Deionised water	Make up to 1000ml

Acid Alcohol

Reagent	Amount
Ethanol (Sigma E7148)	700ml
Concentrated HCl	10ml
Deionised water	Make up to 1000ml

Scot's Tap Water

Reagent	Amount
$NaHCO_3$ (Sigma S6297)	3.5g
$MgSO_4$ (Sigma M7506)	20g
Tap Water	1L

SLIP (Stuart Linn IP buffer)

Reagent	Amount
HEPES pH7.5 (Sigma, H-3784)	50mM
Glycerol (Sigam, G-7893)	10%
Triton X100 (Sigma, X-100)	0.1%
NaCl (Sigma S-7653)	150mM
Protease Inhibitors (Roche, 1836153)	1 tablet per 10ml
Phenylmethylsulfonyl Fluoride PMSF (Sigma, P-7626)	1mM

Nuclear/Cytoplasmic Extraction Buffers

Buffer's A, B and C

Reagent	**Buffer A**	**Buffer B**	**Buffer C**
NaCl (Sigma S9888)	50mM	50mM	350mM
HEPES pH 8.0	10mM	10mM	10mM
Sucrose (Sigma S1888)	0.5M	-	-
Glycerol (Sigma G8773)	-	25%	25%
EDTA (Sigma E5134)	1mM	1mM	1mM
Spermidine (Sigma S9389)	0.5mM	0.5mM	0.5mM
Spermine (Sigma S2876)	0.15mM	0.15mM	0.15mM
Triton X100(Sigma T8532)	0.2%	-	-
Protease Inhibitors (Roche, 1836153)	1 tablet per 10ml	1 tablet per 10ml	1 tablet per 10ml
PMSF (Sigma P7626)	1mM	1mM	1mM

Sample Loading Buffer

Reagent	**Amount**
Glycerol (Sigma G8773)	10%
Tris-Cl pH 6.8 (Sigma T87602)	60mM
SDS (Sigma L3771)	0.2%
Bromophenol Blue (Sigma 32768)	0.001%
β-mercaptoethanol (Sigma, M-6250)	2mMol

This was the final 1X concentration for the protein samples, a concentrated 4X solution was used to allow addition of samples

Electrophoresis Running Buffer

Reagent	Amount
Tris Base (Sigma T87602)	25mM
Glycine (Calbiochem, 3570)	250mM
SDS (Sigma, L-3771)	0.1%

Electrophoresis Transfer Buffer

Reagent	Amount
Tris Base (Sigma T87602)	25mM
Glycine (Sigma G8898)	200mM
Methanol (Sigma M1775)	20%

SDS-Polyacrylamide Stacking Gel (10ml aliquot)

Reagent	Amount
Distilled Water	7.225ml
40% Acrylamide Bis 19:1 (Biorad,1610144)	1.275ml
Tris 1M, pH 6.8	1.25ml
10% SDS (Sigma L3771)	0.1ml
10% w/v Ammonium persulfate APS, (Sigma, A-1433)	0.1ml
N'N'N'N'-tetramethylethylenediamine TEMED (Sigma, T-8133)	10µL

TEMED is the polymerising agent and is added *last*, before the gel is poured.

10% SDS-Polyacrylamide Running Gel (10ml aliquot)

Reagent	Amount
Distilled Water	4.8ml
40% Acrylamide Bis 19:1 (Biorad,1610144)	2.5ml
Tris 1.5M, pH 8.8	2.5ml
10% SDS (Sigma L3771)	0.1ml
10% w/v Ammonium persulfate APS, (Sigma, A-1433)	0.1ml
N'N'N'N'-tetramethylethylenediamine TEMED (Sigma, T-8133)	8μL

TEMED is the polymerising agent and is added *last*, before the gel is poured.

2.3.2 Antibodies

Primary Antibodies

Antibody	Host	Supplier	Cat No.
HSP90	Rat	StressGen	SPA840
APAF-1	Rabbit	Prosci	PSC2015
Actin	Mouse	Santa-Cruz	Sc8432
Caspase-9	Mouse	StressGen	AAM139
Cdc37	Mouse	Transduction Laboratories	C50820

Secondary Antibodies

Antigen	Signal	Supplier	Cat No.
Mouse	HorseRadish Peroxidase HRP	Santa-Cruz	Sc2004
Rabbit	HRP	Santa-Cruz	Sc2005
Mouse	FITC	Sigma	F2883
Rabbit	Texas Red	Vector	TI1000

2.3.3 Protein Extraction and Quantification

Whole Cell Protein Extraction

For Western Blot analyses, proteins were extracted from mammalian cells. Cells were first trypsinised and separated from the culture vessel. Once this was completed, the cell suspension was transferred to a 15mL centrifuge tube (Alpha, Cat. No: LW3078) and was spun to form a pellet at 1,200 RPM for 10 minutes in a Beckman TJ-6 centrifuge. The medium was carefully aspirated through a Pasteur pipette and enough Stuart Lynn Immunoprecipitation Buffer (SLIP) was added to resuspend the pellet of cells (50-100μL).

SLIP without PMSF and protein inhibitors can be made up in solution and stored at 4°C indefinitely. The protease inhibitors are added fresh and the solution can be stored at -80°C. PMSF is added fresh from new stock of 100X each time.

This cell suspension was then transferred to a 1.5mL microcentrifuge tube (Fisher, Cat. No: TUL-800-120 B), which was incubated on ice for 10 – 15 minutes until the suspension began to clear. At this point it was spun at 15,000 RPM at 4°C for 10 minutes in a Sorvall Super T21 centrifuge. When this was complete, the clear supernatant was removed from the pellet. The lysate contains total cell protein and can be quantified and standardised before storing in a solution of 1X SDS at –20°C.

Nuclear and Cytoplasmic Protein Extractions

Cells were harvested as described above and then spun at 6500rpm in a micro-centrifuge for 3 minutes at 4°C. The supernatant was removed. The pellet was resuspended in 500μL of Buffer A and left to lyse over 10 minutes at 4°C. Following this the suspension was spun at 6500rpm for 3 minutes at 4°C. The supernatant

containing the cytoplasmic fraction was then stored immediately at -80°C before protein quantification. The pellet was resuspended in 500µL of Buffer B and spun at 6500rpm for 3 minutes at 4°C. The supernatant was then discarded. The pellet was resuspended in 20µL of Buffer C and left for 30 minutes at 4°C. This suspension was spun for 3 minutes at 6500rpm and the supernatant containing the nuclear fraction was stored at -80°C before protein quantification.

Protein Quantification

For protein quantification the Bradford protein assay has been used. A proprietary assay solution concentrate (Bio-Rad, Cat. No: 500 - 0006) was used, diluted 1 part assay solution to 4 parts distilled water. 1ml of this solution was added to 5 eppendorfs.

A 1:2 serial dilution from a stock 50mg/ml bovine serum albumin (BSA) (Sigma, A-2153) was undertaken and 2µL added to the 1ml Biorad assay. The protein concentrations of 50, 25, 12.5, 6.25, 3.125µg/ml were assayed by spectrophotometry at 620nm and a standard curve obtained. Following this protein samples were analysed in a similar way and protein concentration estimated. To avoid the denaturation of the protein during storage, the protein solutions were mixed at this point with 4X protein sample loading buffer, so that the eventual concentration of SDS was 1X.

2.3.4 Polyacrylamide gel electrophoresis and Western Blotting

Polyacrylamide gel electrophoresis requires two separate gels: a resolving gel for the separation of proteins and a stacking gel for the alignment of proteins before their

entry into the resolving gel. Bio-Rad mini-gel apparatus was used for all protein electrophoresis and transfer.

Resolving gels of 10% were used. Volumes that were required are shown in the Table. The gel was made in a 50mL centrifuge tube, which was vortexed to mix the gel before being poured. A small sample of gel was retained to demonstrate when it has solidified.

After pouring the separating gel it was covered with water,this ensures an even surface to the gel with no bubbles at the surface. Bubbles below the gels' surface were avoided by ensuring that the glass plates were clean and grease free before use by cleaning with absolute ethanol.

The stacking gel was prepared once the resolving gel had set. The water was poured and blotted off the surface of the gel with Whattmann paper. The stacking gel was then made in a 50mL centrifuge tube and poured onto the surface of the resolving gel. A plastic comb was put into place at the top of the stacking gel. As the gel sets it shrinks, therefore to ensure that full wells were achieved extra stacking gel was poured onto the top of the gel until it had set thoroughly. Once the stacking gel was set, the comb was removed and the position of the wells was marked in marker pen on the gel apparatus.

Protein loading and elctrophoresis

Once both resolving and stacking gels had been poured and allowed to set, the gels were assembled in an electrophoresis tank and electrophoresis buffer was poured on. Any bubbles in the electrophoresis buffer below the gel apparatus were removed with a large gauge needle and syringe before proteins were loaded. A narrow gauge needle and syringe were used to wash any unpolymerised acrylamide out of the wells

in the gel. The maximum volume that can be loaded per well is 24μL. Boilates of protein sample (sample denatured at 94°C, for 5 minutes) were readied.

When proteins were loaded, electrophoresis buffer was topped up and a potential difference of 200V was applied. A protein marker ladder (SeeBlue2, Invitrogen LC5625) was run in addition to allow protein sizes to be calculated.

Coomassie brilliant bluestaining of polyacrylamide gels

To visualise the total protein loaded on a polyacrylamide gel, the gel can be stained using a 0.15% w/v solution of Coomassie brilliant blue (BDH, Cat. No: 44329) in 10%v/v glacial acetic acid (Sigma, Cat. No: A-6283), 30%v/vMethanol in distilled water.

Gels were rocked at room temperature with this solution for 2-4 hours once, and then the solution was poured off and retained for future use. This stains the entire gel, and to visualise the protein the same solution, without Coomassie brilliant blue, was incubated with the gel. To better visualise the proteins, an incubation time of 2-4 hours at room temperature, or overnight at 4°C was used.

After destaining, the gel was dried to preserve it. The two pitfalls of gel drying are cracking and shrinkage or distortion of the gel. To avoid these, the gels were first soaked in glycerol for ten minutes and then rehydrated in distilled water until they had regained their initial size. After this, the gels were laid onto a piece of cling film and then two pieces of Whattman paper were placed on top of them.

This sandwich was then placed on a Heto-Dry GD-1 gel drying apparatus, with the cling film uppermost. The flexible cover was then placed across the gel dryer and the heater and vacuum were applied for 2 hours. The gel dryer worked at a temperature of 80°C, had to be switched off a few minutes before the vacuum.

Protein Transfer

Protein transfer from polyacrylamide gel to Hybond-P PVDF membrane (Amersham Pharmacia Biotech, Cat. No: RPN303F) was carried out using a 20% methanol buffer as described above. This buffer was chilled before use.

Sections of PVDF membrane of sufficient size to cover the entire gel were cut and marked before being dipped in 100% methanol to wet. The wetted membrane was then transferred to soak in distilled water for ten minutes, before being soaked in the chilled transfer buffer for five minutes. At the same time, 2 pieces of sponge and 3 sheets of Whattman filter paper per gel were also soaked in transfer buffer for 20 minutes. After soaking, a transfer sandwich was made, the black side of the transfer cassette had a piece of sponge and two sheets of Whattmann paper placed upon them before first the gel and then the membrane was laid on top. The sandwich was completed with a further piece of Whattman paper and a sponge.

The completed cassette was placed into an electrophoretic transfer electrode and cassette holder, submerged in an electrophoresis tank with transfer buffer. When complete, a potential difference of 100V was applied for one hour.

ImmunoBlotting

ImmunoBlotting was carried out using the membrane as produced above. First the membrane was washed once for ten minutes in washing buffer (Phosphate Buffered Saline with 0.1% v/v polyoxyethylenesorbitan- Tween20 Sigma, P-1379). It was then incubated in blocking buffer for one hour at room temperature on a rocker, rocking at 30RPM.

After blocking, the membrane was washed well in washing buffer, with three quick washes, followed by three ten-minute washes, each on a rocker at 80 RPM. Each time the maximum volume of washing buffer possible was used.

During these washes the primary antibody solution was made up. The antibody was diluted in 15 mLs of blocking buffer (5% w/v non fat milk powder (Biorad, 1706404 in washing buffer)), which was then applied to the membrane immediately after the final wash. The primary antibody was incubated for two hours at room temperature, with rocking at 30RPM.

After primary antibody incubation, the antibody was removed from the membrane and transferred to a 50mL centrifuge tube to be stored at –80°C the membrane was then washed with first three quick washes and then three ten minute washes each rocking at 80 RPM.

Secondary antibody incubation was then carried out, again with the antibody diluted in blocking buffer. This incubation was carried out at room temperature for one hour. It was followed by washes of the membrane, however the third long wash was followed by a 10 minute wash in plain PBS to remove traces of Tween 20.

To visualise the Blot, Amersham enhanced chemiluminesence (ECL) kit (Amershm Life Sciences, Cat. No: RPN 2100) was used along with Kodak Biomax Light film.

3mLs of each ECL kit solution were mixed in a 15mL Falcon tube in the dark. This mixture was then dropped onto the protein-containing surface of the membrane. The membrane was then incubated for 1 minute before the ECL solutions were removed by Blotting onto Whattman paper. The membrane was then wrapped in cling film and placed into a light proof cassette. A piece of film was placed over the membrane and exposed for two minutes. This film was then placed in developer (Kodak, Cat. No:190 1859 for both developer and fixer) for two minutes, and fixer for a further

two minutes, before being washed in tap water. On the basis of this film, further exposures were made to produce the clearest Blot.

2.3.5 Immunohistochemistry

The antibodies were used with the Dako Envision anti-rabbit secondary antibody and antiAPAF-1 antibody described above. The Envision polymer consists of several secondary antibody residues conjugated to a dextran backbone molecule, which has many horseradish peroxidase molecules attached to it. This produces a system that is not susceptible to endogenous biotin, and which is both highly sensitive and simple to use.

Samples

Paraffin wax embedded samples of tissue from pancreatic resections obtained from the Department of Pathology at the University of Liverpool, were used as samples.

Preparation of Slides

3-aminopropyltriethoxysilane (APES) Coating of slides

APES (Sigma, Cat. No: A-3648) was used to coat microscope slides, acting to keep microtome sections adhered to slides. Metal staining racks were filled with new, clean, glass slides, ensuring that no grease or dirt touched the surface of the glass slides. Once several racks were full, they were immersed, one rack at a time, for 30 seconds, in acetone. When the slides were removed from the acetone they were rinsed again in fresh acetone for 30 seconds. After this the slides were immersed in a 2% APES / Acetone solution for 2 minutes. The slides were then rinsed in distilled water for 2 minutes and dried overnight taking care not to allow dust to fall onto the

damp slides. Once dry, coated slides can be stored in a dust free area for up to six months.

Microtome sectioning

Again, sections were placed on APES coated slides. Chilled paraffin embedded blocks were cut into 5μm thick sections using a standard microtome.

Immunohistochemical staining (IHC)

Firstly sections were prepared by removing the paraffin wax with two changes of xylene. These washes were carried out for three and a half minutes each. Next, the sections were transferred from the water insoluble xylene media to ethanol, they were then rehydrated in dilutions of alcohol, beginning in two changes of absolute alcohol and moving through 90% and 70% dilutions, each for 30 seconds.

At this point the sections had their endogenous peroxidases blocked by incubating the sections with 1% hydrogen peroxide in methanol for 12 minutes. To remove the alcohol, the sections were then washed for 30 seconds in running tap water, before being placed into 10mM EDTA at pH 8.0. In this solution, the sections were heated in a microwave oven on full power (850W) for 10 minutes to retrieve epitopes that were otherwise unavailable to the antibody. Both HSP90 antibodies used required this microwave pre-treatment.

When antigen retrieval was complete, the sections were again washed in running tap water, before being transferred to Shandon Sequenza coverplates (Cat. No: 7211013) and placed into Shandon Sequenza staining racks. The sections were then washed twice with 3mLs of TBS with 5 minutes between washes. After this, primary antibodies were applied to the sections in 5% BSA / TBS solution. 100μL of antibody solution was added to each section.

Primary antibody incubations were optimised for 40 minutes at room temperature, using the dilution of antibody stated above. At the end of this incubation, two washes of TBS were completed and each was left to incubate for five minutes. After these washes, 100μL of secondary antibody was added the antibody solution was supplied pre diluted. The Envision Polymer antibodies were incubated for exactly 30 minutes, before being washed off with two further TBS washes for five minutes each.

The final part of the immunostaining was the placement of a chromagen. To prepare the DAB solution, one drop of chromagen was added to 1mL of the supplied chromagen buffer. This was added to the slide in a volume of 100μL and left to incubate for 10 minutes. After this, the excess chromagen was washed off, first in 3mL of distilled water and then, once removed from the Sequenza staining rack, in running tap water for 2 minutes.

After excess chromagen had been washed from the slides they were counterstained with haematoxylin (Surgipath Cat. No:16102). The slides were dipped into a bath of haematoxylin four or five times for approximately 1 second each time. After this, the excess was first washed off in running tap water for 30 seconds and then more was removed in acid alcohol. After four or five dips in acid alcohol, the haematoxylin was turned blue using Scott's Tap Water, which required an incubation of approximately 20 seconds.

For visualisation and long-term storage of the slides, they were mounted using DPX mountant (BDH, Cat. No: 0038062) and a borosilicate glass coverslip. To achieve this, the sections were dehydrated, once again using graded alcohols, and then they were transferred to xylene. Washes in the various alcohols were for 30 seconds each, whilst transfer to xylene was achieved through two washes in clean xylene, each of which lasted for 2 minutes. The sections were then mounted using the inversion

technique with DPX mountant and 24mm x 40mm coverslips (Shandon Cat. No: 67761319).

2.3.6 Immunofluoresence

Sample Preparation

Samples for immunofluorescence were prepared upon LabTekII 8 chamber slides. $3X10^6$ cells were seeded onto each chamber in a total volume of cell suspension of 400μL. The cells were allowed to grow overnight in a humidified incubator at 37°C in 5% CO_2, before any further treatments were used.

Fixation of samples for immunofluoresence.

Samples were fixed using 4% paraformaldehyde (BDH, Cat. No: 29447) in PBS, with a pH of 7.4. 1g of paraformaldehyde was weighed in a fume hood and dissolved in 25mLs of PBS. To allow dissolution, a few drops of 1M NaOH (BDH, Cat. No: B168450) were added to the paraformaldehyde and the solution was gently heated. Once the paraformaldehyde was completely dissolved, the pH of the solution was adjusted to 7.4 using 1M and 100mM HCl (and NaOH if necessary). This solution was not stored, but made up as it was required.

After aspiration of the media from the chamber slides, and washing of the cells with 400μL of HBSS, 400μL of paraformaldehyde was added to each chamber. This was left to incubate and fix the cells for ten minutes before being aspirated and washed off with PBS.

Immunofluorescence

To make the cells permeable to antibodies, the cells were subjected to three ten minute washes in 0.2% Triton X-100 made up in PBS. After three washes, the cells

were incubated for ten minutes with 5% BSA in 0.2% Triton X-100 in PBS. This acted to block any non-specific protein binding capacity that the cells may have had, allowing the antibody to interact only with its correct epitope.

Following the blocking wash, the primary antibody incubations were carried out. When more than one primary antibody was to be used, they were applied as a mixture together, all antibody solutions for immunofluoresence were diluted in 5% BSA in 0.2% Triton X-100 in PBS. A volume of 50μL of antibody solution is sufficient to cover the base of a single chamber. Primary antibody incubations were carried out at room temperature for one hour.

On completion of the primary antibody incubation samples were washed three times, for ten minutes each time, with 0.2% Triton X-100 in PBS, in preparation for the secondary antibody incubation. Fluorophore labelled secondary antibodies are sensitive to light, therefore these were stored in the dark and incubations were also carried out in the dark. Once again, a volume of 50μL was required to cover the base of the chamber, the secondary antibody was incubated, again at room temperature, but for only 20 minutes, with the chamber slide wrapped in aluminium foil.

Once the secondary antibody has completed its incubation, the chambers were washed using 0.2% Triton X-100 PBS three times for ten minutes each, and finally a wash of plain PBS to remove detergent residues. After these washes, the chambers were removed from the slide, using the device supplied with the LabTek slides, and are mounted. A Dako proprietary mounting medium specifically designed for fluorescence microscopy was used (Cat. No: S3023). The mountant was applied to a glass coverslip in a thin strip along the centre and the slide was inverted onto this, ensuring that the mountant ran to all edges of the slide. To attach the coverslip firmly to the slide, colourless nail polish was applied around the edge of the coverslip.

Visualisation of the fluorescence was carried out using a Nikon Eclipse E600 fluorescence microscope with mercury light source and filters for 515-555nm to visualise FITC staining and 600-600nm to visualise Texas Red staining. Confocal microscopy was also carried out, using a Carl Zeiss laser scanning microscope (LSM) 510.

2.4 PREPARATION OF CONSTRUCTS

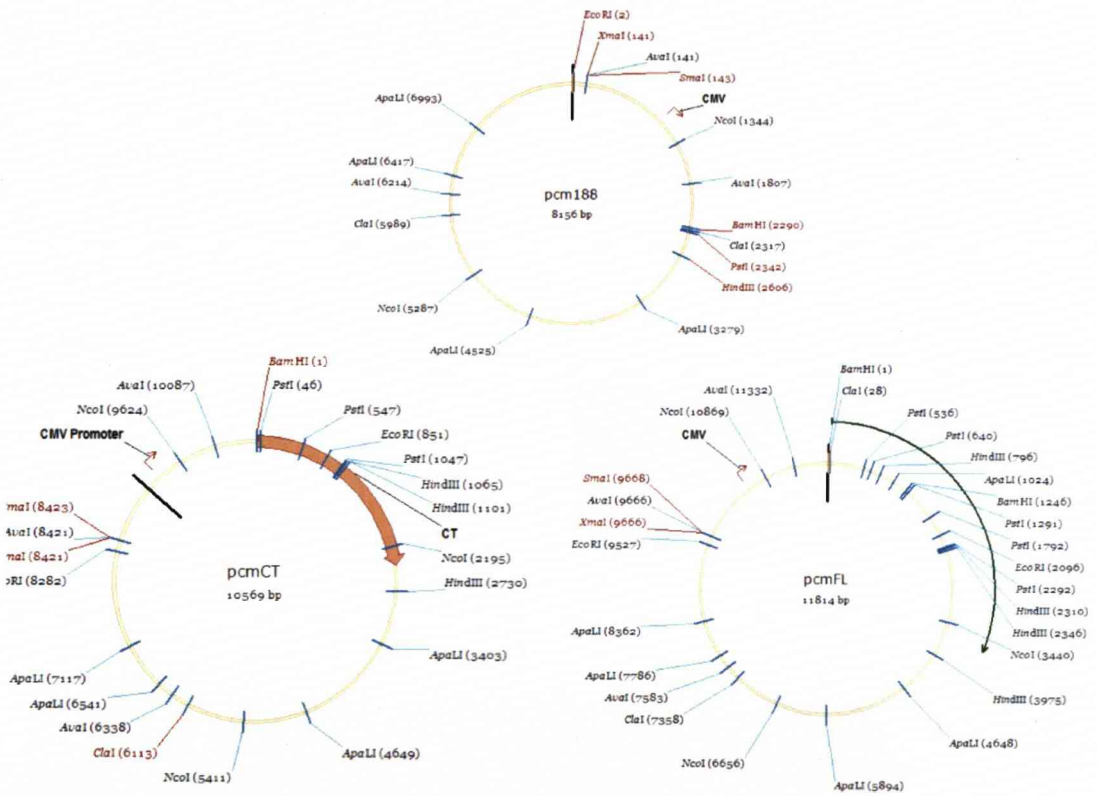


Figure 16: YEAST PLASMID MAPS
Three yeast plasmids were used. The vector pCM188 was used to subclone C-terminal APAF-1 (CT) and full-length APAF-1 (FL).

2.4.1 Polymerase Chain Reaction (PCR)

PCR was carried out using Techne Progene PCR machines. Primers were designed using Primer Designer Computer Software (Scientific and Educational Software). All primers were purchased from Gibco Life Technologies, Paisley, Scotland. Primers were used at a concentration of 20pmoles/50µl reaction and

dNTPs (Roche Biochemicals, Cat No: 1969064) were used at a concentration of 0.2mM.

2.4.2 PCR amplification of C-terminal APAF-1 (CT)

Using pCM188-FL as a template a Carboxy-terminal construct was amplified using the following primers:

FWD: 5'-ggatccaggatggtcagagaatag

REV: 3'-ggatcctgcaatactgcacaac

The conditions were 95°C for 10 minutes, followed by 30 cycles of [95°C for 30 secs, 52°C for 30 secs, 72°C for 2 minutes] followed by 72°C for 10 minutes followed by 4°C overnight.

2.4.3 PCR amplification of N-terminal APAF-1 (NT)

Using pCM188-FL as a template an amino-terminal construct was amplified using the following primers:

FWD: 5'-gaattcagacacgcaaacacaaata

REV: 3'-agcggatcctacataacacctttgtaggc

The conditions were 95°C for 10 minutes, followed by 30 cycles of [95°C for 30 secs, 62°C for 30 secs, 72°C for 2 minutes] followed by 72°C for 10 minutes followed by 4°C overnight.

2.4.4 Cloning into pMOS-Blue

PCR products were subcloned into pMOS-Blue using a kit supplied by Amersham (Cat No. RPN 5110) according to the manufacturers protocol.

2.4.5 Restriction Digests

DNA was digested with the following enzymes at 37^{O}C in the buffers supplied by the manufacturers. *Bam*HI (Krammel Biotech Cat No. 012704, Promega Cat No. R6021), *Eco*RI (Krammel Biotech Cat No. 010106, Promega Cat No. R601A), *XmaIII* (Fermentas Life Sciences), *MluI* (Fementas Life Sciences), *Pst*I (Promega Cat No. R6111), *Pvu*I (Krammel Biotech Cat No. 013711).

2.4.6 Dephosphorylation of DNA

DNA was dephosphorylated using CIP (Calf Intestinal Alkaline Phosphatase) (Promega Cat No: M1821) according to the manufacturers' protocol.

2.4.7 Ligation of DNA

DNA was ligated into target vectors using T4 DNA Ligase (New England Biolabs Cat No: 202S or Promega, Cat No: M1801) in the buffer supplied by the manufacturer. DNA was typically ligated into vectors using a molar ratio of 1:1 or 3:1 insert : vector.

2.4.8 Transformation of MOS-Blue competent *E coli*

MOS-Blue competent E.coli (Amersham) were transformed according to the heat-shock protocol supplied by Amersham with the cells.

2.4.9 Cloning of APAF-1 and CT into the yeast expression vector pCM188

pcDNA3.1-APAF-1 was a gift of Dr X. Wang, Howard Hughes Institute, Texas, USA. pCM188 was purchased from Euroscarf. pCM188 contains a *URA3* marker allowing growth in Uracil deficient media.

To manufacture pCM188-FL APAF-1, pcDNA3.1 APAF-1 was cut using *XmaIII*. The APAF-1 insert was gene purified from the agarose gel. The vector pCM188 was cut with *XmaIII* and ligated with the full-length APAF-1 insert. From the clones produced, DNA was isolated and an orientation digest performed with *EcoR1*. The pCM188-FL clone was transformed into uracil deficient plates to assess for success.

To manufacture pCM188-CT primers were designed to amplify the C-terminal of APAF-1 (amino acids 615-1149). The primers were also designed to introduce a 5'-terminal *BamH1* site and a 3' *MluI* site. The CT DNA insert was amplified from pcDNA3.1-APAF-1 and gel-purified. pMOS Blue was digested with *BamH1* and *MluI* and the CT insert was ligated into this. The pMOS Blue-CT construct was digested with *BamH1* and *MluI* and the insert was gel purified and ligated into pCM188 which had been digested with *BamH1* and *MluI*.

All constructs underwent DNA sequencing to ensure correct sequences were present.

2.4.10 Cloning of NT and FL-APAF-1 into pAS2.1

pGAD424 was cut with *BamHI* and then dephosphorylated using CIP. pcDNA-APAF was cut using *BamHI*. This gave an insert that would insert in frame into

pGAD424. The insert was gel-purified and ligated into pGAD424. Orientation digests with *EcoR1* were performed to ensure correct orientation. This gave pGAD424-FL.

2.4.11 Yeast Transformation by electroporation

Yeast to be transformed by electroportation were grown to mid-log phase in YEPD media and harvested by spinning at 4500 RPM in a Sorvall SL250-T rotor that had been pre-cooled to $4^{O}C$. Yeast cells were washed 3 times in ice-cold water and once in ice-cold 50ml 1.2M Sorbitol. Yeast cells were then resuspended in 500ml 1.2M Sorbitol and placed on ice. 60ml yeast were then placed in a sterile electroporation cuvette (BioRad 0.4 cm gap Cat No. 165-2088) and DNA added. Yeast were transformed using a Biorad Gene Pulsar II electroporator at 250V, 25mF and 200Ẉwith time constants generated typically being between 4.5 and 5.0ms. Following electroporation, 1ml ice cold 1.2M Sorbitol was added to the transformed yeast and cells transferred to a 1.5ml eppendorf tube. Cells were then pelleted by spinning at 4500 RPM for 5 minutes in a microcentrifuge (Labnet, Cat No. C-0160) and spread onto SD- Agar plates. Yeast were incubated at $30^{O}C$ for between 48 and 72 hours to allow growth of transformants to occur.

2.4.12 Yeast transformation by Lithium Acetate for the Yeast Two Hybrid Screen

Cells were inoculated into 2ml YEPD medium and grown overnight at 30°C to OD_{600}=3-5. Cultures were diluted into 50ml 30°C fresh YEPD and regrown at 30°C

for 5 hours in shaking incubator to a final optical density of 0.8-1.2. Cells were harvested by spinning in a centrifuge at 5000 rpm for 5 minutes. The cells were washed with 10ml of sterile water and then with 10ml of TE/LiAc buffer (0.1M Lithium acetate, 10mM Tris-HCl (pH8), 1mM EDTA). The cell pellet was resuspended in 0.5ml TE/LiAc buffer.

100µL of cell suspension was added to a sterile Eppendorf tibe containing 1µg plasmid DNA and 50µg of sonicated and denatured calf thymus carrier DNA. To this mixture 280µL of 50%PEG4000 was added. The solution was mixed thoroughly. This solution was incubated at 30°C without agitation for 45 minutes. 43µL of DMSO was added and the solution was inverted several times to mix.

The solution was heated to 42°C for 5 minutes and then spun down in a centrifuge at 5000 rpm for 5 seconds. The pellet was washed in 1ml of sterile water. 0.5ml of YEPD was added to the solution and incubated in a shaking incubator for 1 hour at 30°C.

100µL aliquots of the transformed cells were plated on the test medium as described in the results.

2.4.13 Yeast Episomal Plasmid Isolation

Yeast were grown overnight in 2ml YPAD at 30^{O}C in a shaking incubator at 280 RPM. The yeast were then harvested by spinning at 4000 RPM in a centrifuge for 5 minutes. Yeast were resuspended in Yeast Lysis buffer and 300mg of Acid Washed Glass beads (Sigma, Cat No. G-8772) were added. An equal volume of Phenol-Chloroform (Sigma, Cat No. P-2069) was then also added on ice and yeast were vortexed 3 times for 1 minute with rests on ice in between. Each tube was then spun at 13000 RPM in a centrifuge for 5 minutes and the top layer was removed and placed in a fresh tube. An equal volume of Chloroform (Sigma, Cat No. C-0549)

was then added and the 2 layers mixed to form an emulsion. The layers were separated by spinning in a microcentrifuge as before. The top layer was removed and placed in 2.5 volumes of 100% Ethanol. This was incubated at room temperature for 30 minutes and then at –80^{O}C for a further 30 minutes. DNA was then harvested by spinning at 17500 RPM in a Sorvall SL-250T rotor for 30 minutes at 4^{O}C. The supernatant was then removed and the pellet washed in 250ml 70% ethanol. The DNA was collected by spinning at 17500 RPM in a Sorvall rotor for 10 minutes at 4^{O}C. The ethanol was then removed, the DNA pellet air-dried and resuspended in 20ml sterile H_2O.

2.4.14 DNA Sequencing

DNA was sequenced using an ABI377 sequencer using the BigDye terminator kit supplied by Perkin-Elmer. This was carried out by Miss Alison Mason or Mr Gerry Lepts according to the manufacturers protocol.

2.4.15 RNA Isolation from Yeast

RNA was isolated from yeast using an acid phenol extraction protocol. Briefly, yeast were grown to mid-log phase in liquid SD- culture and harvested by spinning at 4500 RPM for 5 minutes at room temperature in a Sorvall SL-250T rotor. The media was removed and cells washed in 1ml ice-cold RNase-free water. Cells were resuspended in 400mls of TES Buffer or 400mls AE Buffer and transferred to an RNase-free microfuge tube. An equal volume of ice cold acid-phenol (Sigma Cat No. P-4682) was added and the two layers formed were mixed by vortexing briefly. The homogenous solution was incubated at 65^{O}C for 40 minutes with brief vortexing every 10 minutes. Tubes were then placed on ice for 5 minutes and the layers separated by spinning at 13000 RPM in a Sorvall SL250-T rotor for 5 minutes at

$4^{O}C$. The aqueous layer was removed and added to an equal volume of acid-phenol. These were mixed by brief vortexing and separated by spinning in a centrifuge as before. The aqueous layer was removed and added to an equal volume of chloroform (Sigma Cat No. C-2432). The aqueous layer was extracted as before. The aqueous layer was then added to 1ml Isopropanol (Sigma Cat No. I-9516) and placed on ice for 5 minutes. Precipitated RNA was pelleted by spinning at 13000 RPM for 10 minutes at $4^{O}C$ in a Sorvall rotor and the isopropanol was removed. The pellet was washed in 1ml Ethanol (Sigma Cat No. E-7023) and the ethanol removed. The pellet was air-dried and RNA resuspended in 50ml RNase-free water.

2.4.16 RNA Analysis

The amount of RNA isolated was quantified by spectrometry using the Optical Density at 260nm wavelength (OD-260) of the isolated RNA and the purity was determined by the OD-260/OD-280 ratio. Quality of isolated RNA was analysed on a 1% agarose gel. If the 28S and 18S rRNA bands were visible on the gel (for example see Figure 38a), then the isolated RNA was stored at $-20^{O}C$. If the rRNA bands were not visible then the isolated RNA was discarded.

2.4.17 Making cDNA

Isolated RNA was first treated with DNAseI to remove any genomic DNA present in the sample. To the RNA was added an equal volume of Yellow Core Buffer (Promega, Cat No. Z317A), 5ml $MnCl_2$ and 10U RQ1 DNAse (Promega, Cat. No: M6101). This was incubated at $37^{O}C$ for 30 minutes. RNA was then extracted by addition of 0.1 volumes 3M NaAc (pH 5.3) and an equal volume of Phenol-Chloroform. This was then vortexed to form an emulsion and the 2 layers were then separated by spinning at 14000 RPM for 5 minutes at $4^{O}C$. The top layer was

removed, added to an equal volume of 100% Ethanol and placed at -80^{O}C for 30 minutes. The precipitated RNA was then pelleted by spinning in a centrifuge at 14000 RPM for 15 minutes at 4^{O}C. The supernatant was removed, the pellet air-dried and then resuspended in 10ml MilliQ water.

To make cDNA from the purified RNA, a Moloney Murine Leukemia Virus Reverse Transcriptase (MoMLV-RT) was used (Gibco Life Technologies, Paisley, Scotland, Cat No. 28025-013) using the following protocol. 5ml RNA was added to 3ml RNase-free water, 100ng Oligo (dT) Primer (Gibco Life Technologies, Cat No. 18418-012) and 1ml of 10x RNA Buffer. This was incubated for 2 minutes at 90^{O}C and immediately cooled on ice. This was then spun in a microcentrifuge briefly to collect any condensate. To this was then added 4ml 5x First Strand Reaction Buffer (Gibco Life Technologies, supplied with MoMLV-RT), 1ml 10mM dNTP Mix (Gibco Life Technologies, Cat No. 18427-013) 3ml RNase-free water and 2ml MoMLV-RT (400U). This was then incubated at 37^{O}C for 90 minutes and then the MoMLV-RT was inactivated by incubating the reaction at 65^{O}C for 15 minutes. As a negative control, all the above steps were carried out on the other 5ml of purified RNA, *to which no* MoMLV-RT had been added. cDNA was then stored at –20^{O}C prior to further use.

SECTION 3: RESULTS

REMINDER OF OBJECTIVES

1. Test if HSP90 inhibition sensitises to drugs
2. Examine the effect of the order of drug treatment on combination response
3. Examine the effect of serum deprivation on combination response
4. Examine modulation of HSP90 during treatments
5. Investigate the mechanism of HSP90 interaction with the apoptotic program

3.1 CELL-LINE EXPERIMENTS

The aim of this thesis is to examine the role of inhibiting a stress related protein (HSP90) in modulating the response of pancreatic cancer to apoptotic stimuli. Tissue culture is the most convenient way to study efficacy of new drugs and the National Cancer Institute (NCI), recognizes that cell lines are the most appropriate way of preclinical testing of potential chemotherapeutic agents [215]. However it must be appreciated that cells growing in a plate will never behave (or have the same levels of sensitivity) as tumour cells in situ. Accordingly, it is recognised that the different nature of stress in cell culture and in situ means that cell culture is not ideal for this study, however, alternatives (such as directly using animal models) are impractical, hence, the recommendation by the NCI. This work must be appreciated within the context of recognised limitations of tissue culture.

The tissue culture work presented here is based on the pancreatic cell line Suit2. Suit2 is known to demonstrate chemoresistance to 5FU [216] and therefore was an ideal cell line to investigate whether HSP90 inhibition could potentiate 5FU.

3.1.1 The response of Suit2 to 5FU and GA precludes isobolar analysis

The conventional *in vitro* assessment of combination treatments is centred on the creation of an isobologram [217]. The isobologram is a graphical means of assessing whether synergy between two treatments is present.

The conventional approach to investigating synergy/additivity between compounds is to construct an isobologram. An example of an isobologram is given in figure 17.

The x and y axes of the isobologram are the concentrations of the drugs investigated.

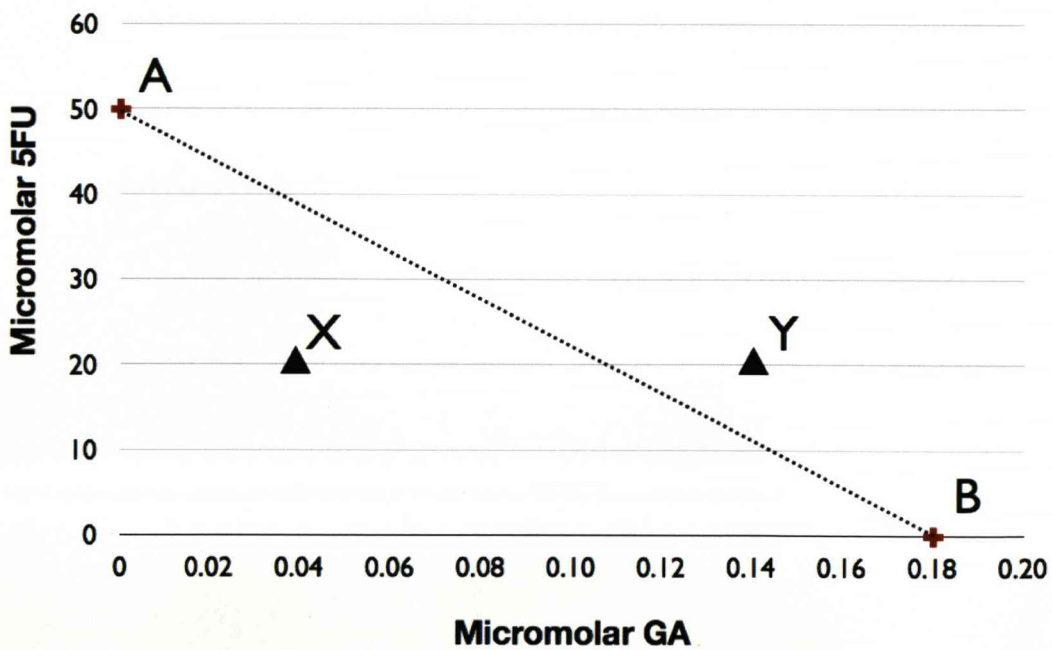


Figure 17: EXAMPLE ISOBOLOGRAM FOR 50μM 5FU AND 0.18μM GA GIVING ZERO GROWTH RATE OVER 72 HOURS FOR SUIT2

Point A is the cytostatic concentration of 5FU, point B is the cytostatic concentration of GA. The line AB is the *line of additvity* (see text). Zero growth rate at a concentration of 20micM 5FU with 0.04micM GA (point X) would demonstrate synergy between 5FU and GA. Zero growth rate at a concentration of 20micM 5FU and 0.14micM GA would demonstrate an additive effect of 5FU and GA.

Point A represents the effective concentration of 5FU (e.g. 50micM) and point B represents the effective concentration of GA (e.g 0.18micM). The *line of additivity* connects points A and B. Combination of drugs at concentrations below this line that give similar effects to single doses are considered to be acting synergistically, whereas combinations of drugs at concentrations above this line are considered to be additive in their effects. For example, if no cell growth was seen at a combination of 20micM 5FU and 0.04micM GA (point X) that would be considered synergistic, but no cell growth seen at a combination of 20micM 5FU and 0.14micM GA would be considered additive.

The isobologram depends on the use of rigorous endpoints for example 50% lethality (LD50). Work has shown that GA's principal activity is cytostatic rather than cytotoxic [218]. In order to test for enhancement of GA activity measurement of cell growth rather than cell death (LD50) is therefore more appropriate. 5FU is cytotoxic, cytotoxicity can also be measured by assaying cellular vitality over time. I therefore set out to create an isobologram for the conventional chemotherapeutic 5FU and the HSP90 inhibitor geldanamycin (GA).

Initial experiments looked at the growth of cells in 5FU and GA (Figures 18 and 19). This showed that both 50μM 5FU and 0.18μM GA gave no significant increase in vitality over a 72 hour period. However, as seen in Figure 20 the overall absence of increase in vitality with 5FU treatment was the result of an initial increase followed by a compensating decrease (a flattened, inverted "U"); in contrast GA simply caused an absence of increase (probably as a result of cytostasis). The endpoints are therefore incompatable and open to misinterpretation.

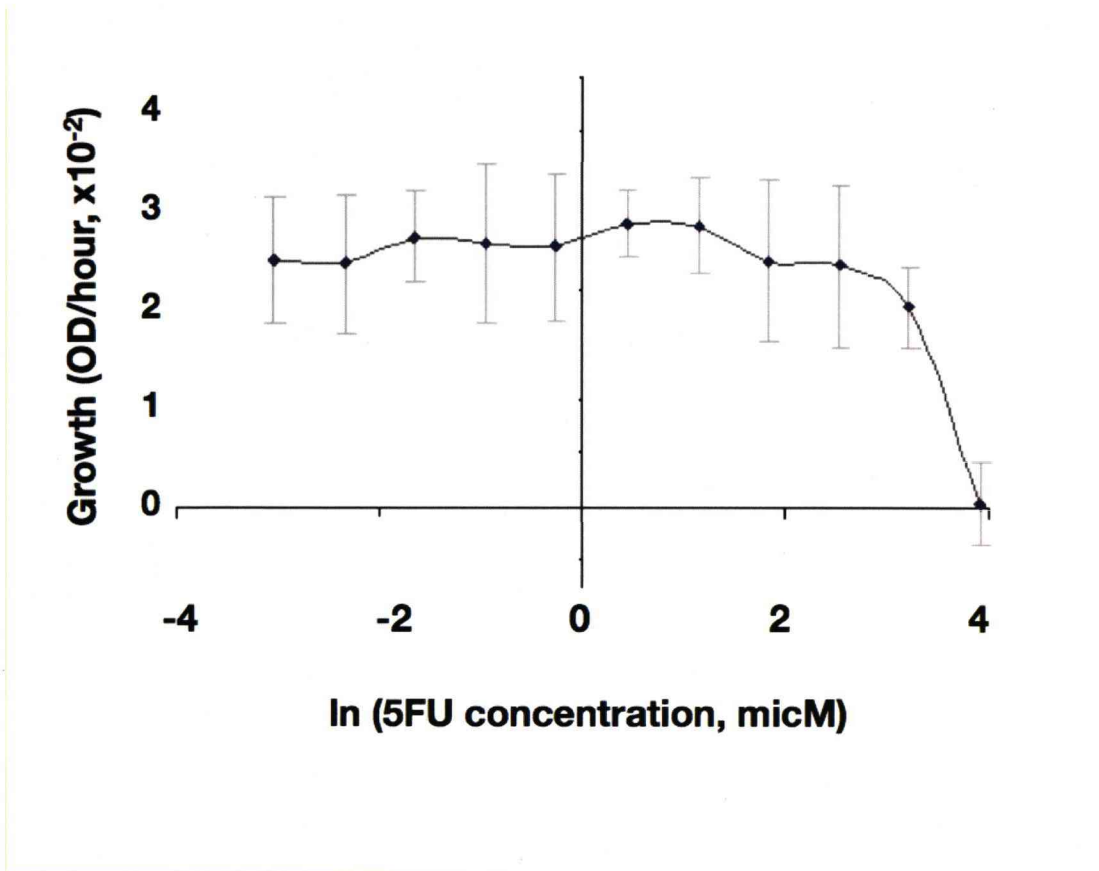


Figure 18: SUIT2 GROWTH IN 5FU

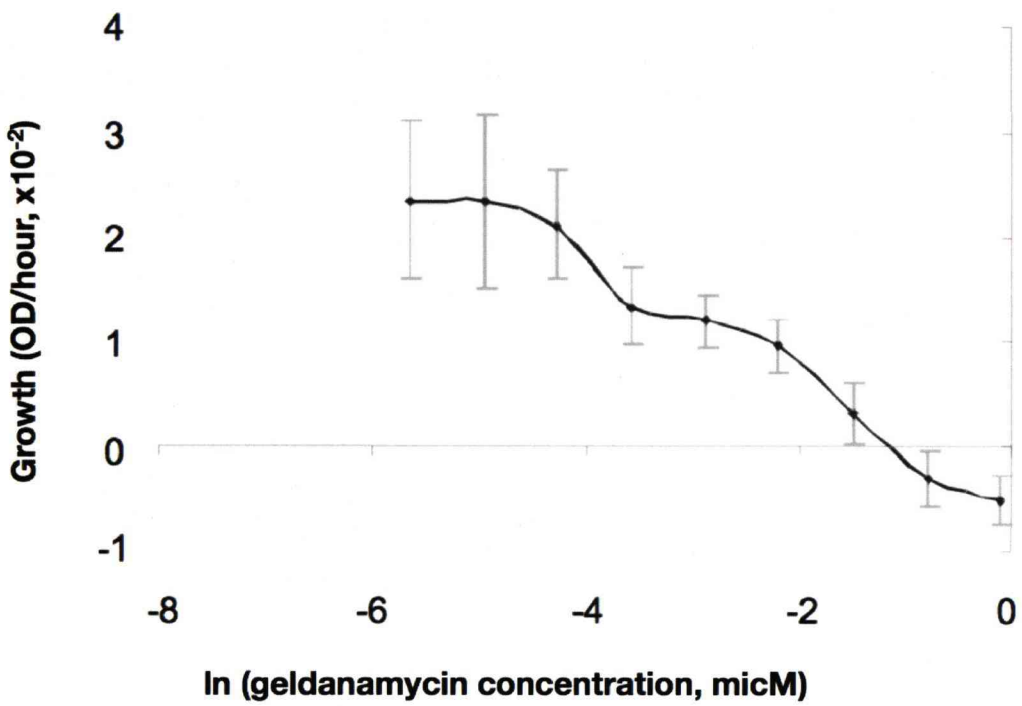


Figure 19: SUIT2 GROWTH IN GELDANAMYCIN

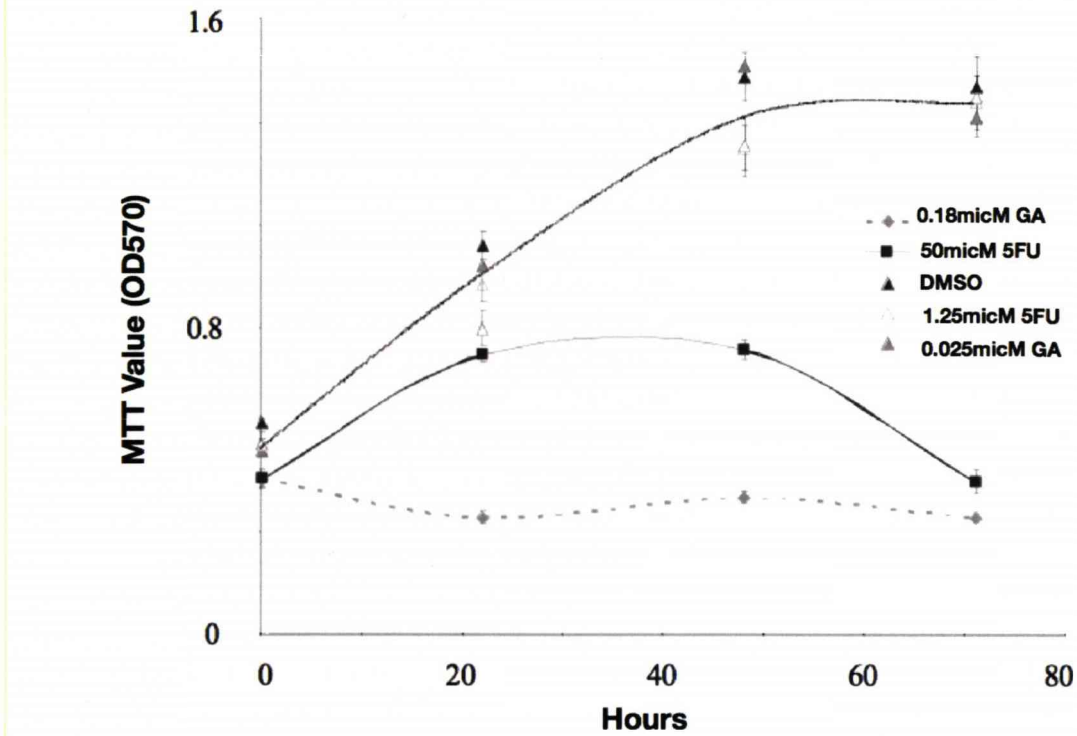


Figure 20: SUIT2 GROWTH CURVES OVER 72 HOURS IN VARYING CONDITIONS

SUIT2 cells were plated and grown in 5FU (50micM, 1.25micM) and GA (0.18micM, 0.025micM). DMSO is the solvent only negative control.

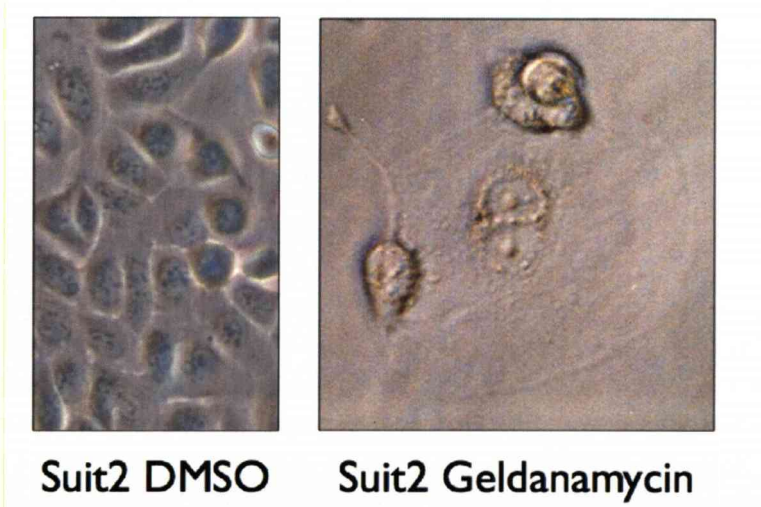


Figure 21: SUIT2 CYTOLOGY IN GELDANAMYCIN

Geldanamycin induces distinct changes in SUIT2 morphology, note the markedly flattened cytoplasm displayed.

For example, consider an experiment where we initially see responses to 5FU as described previously (Figure 22A). Let us use a lower concentration of 5FU that will

not cause enough cell death over a 72 hour period to give an overall absence of growth; (note that the 5FU allows an initial phase of growth), the endpoint of no growth is only reached when the cell death matches this growth (Figure 22C, note that zero cell growth occurs at t_2). In the same experiment a concentration of GA is used that allows growth, albeit at a lower level (Figure 22B) the endpoint of no growth is clearly not reached with this GA concentration. Let us assume a protective effect of GA (the opposite of synergy), GA reduces the cell death gradient of the 5FU – but the GA will cause a reduced level of cells after the initial period of growth in the presence of 5FU than would be the case if 5FU was on its own, for the purposes of argument this could even be assumed to be an increase on the level that would be seen with GA alone (again the opposite of synergy). It is quite possible that in this experiment the end point of no growth is reached, because the downward curve although less steep starts from a lower point. In an isobologram this will appear as synergy – when in fact the biological effect was the opposite (Figure 22D).

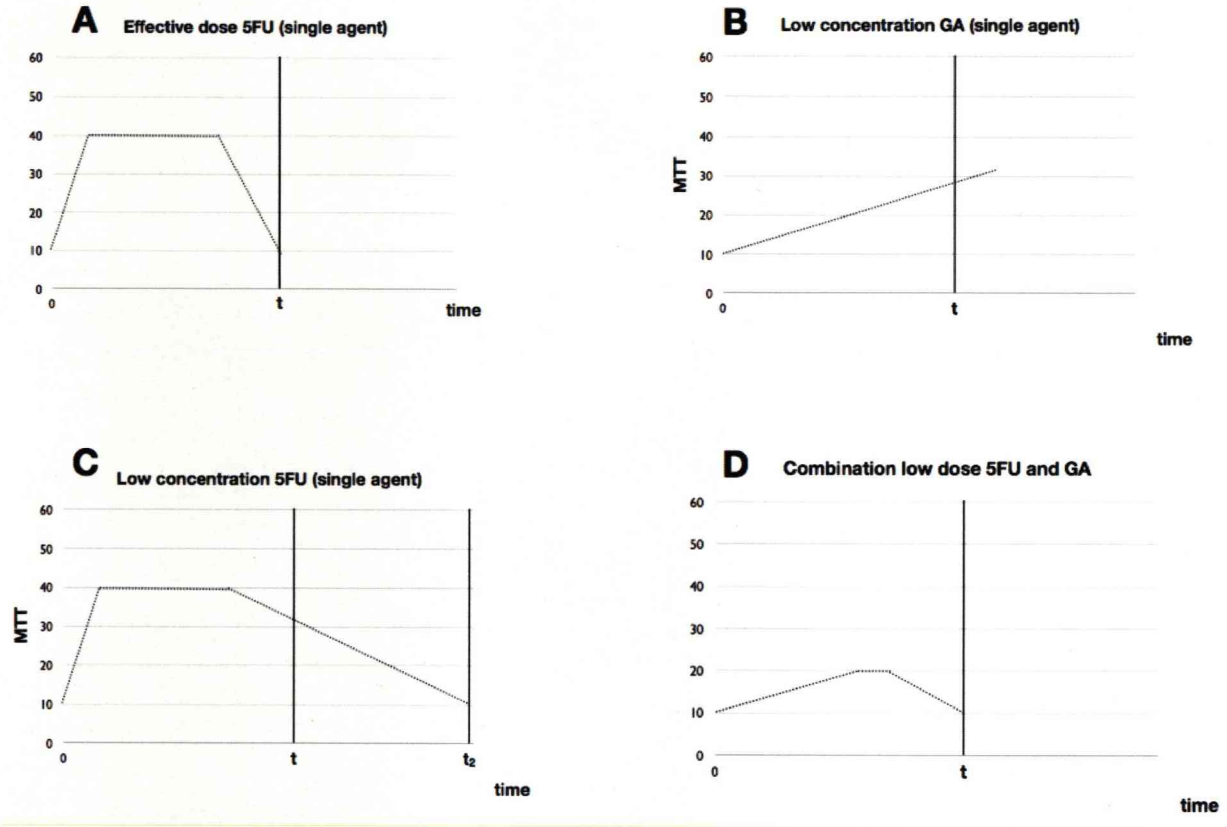


Figure 22: DIFFERING GROWTH CURVES CAN LEAD TO MISLEADING RESULTS

A: Growth of cells in effective dose of 5FU (e.g.50micM). Note at time t there is overall zero growth

B. Using a low concentration of GA can permit some cell growth, clearly zero growth rate at time t is never reached

C: Using a lower concentration of 5FU will flatten the cell death phase (lower gradient) and will reach overall zero growth at a later time t_2 (so the endpoint is not reached).

D: Combination of lower concentration GA and 5FU, note that the gradient of cell death is less steep than 5FU alone (there is no synergy). However there is a net zero growth at time t because the effect of 5FU is starting at a lower point. This would imply synergy but would be misleading.

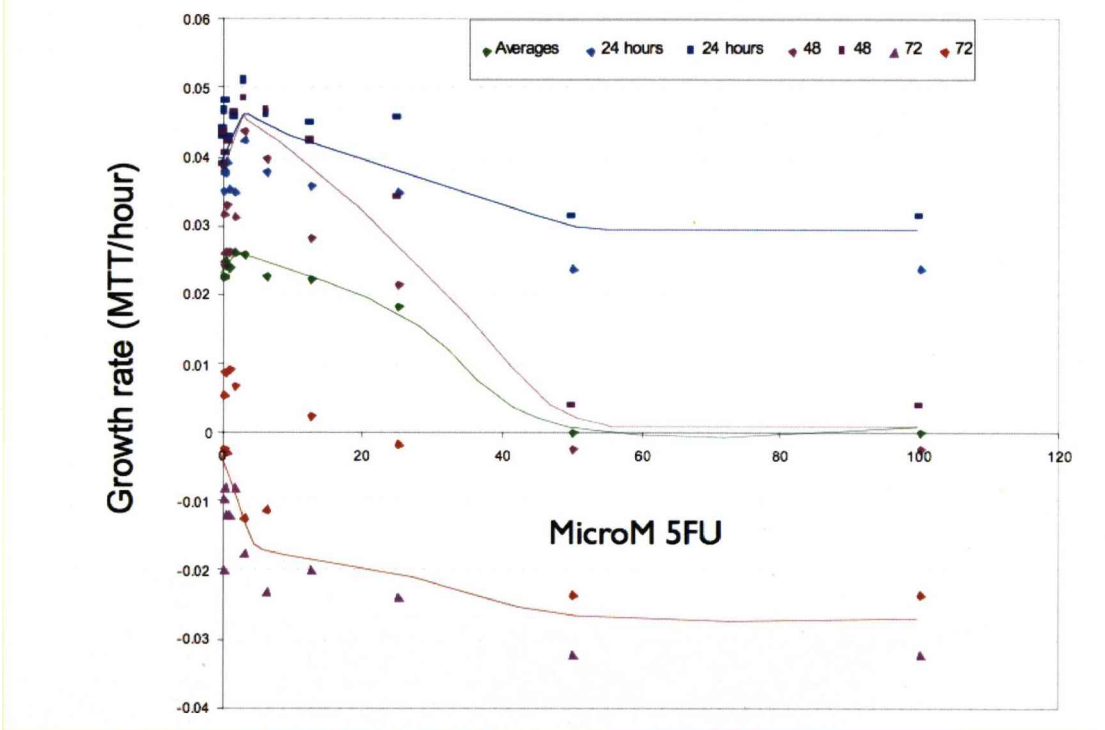


Figure 23: SUIT2 GROWTH IN 5FU

Growth rate versus concentration of 5FU assayed at 24, 48 and 72 hours. Note how at 24 hours there is relatively little variation in growth rate across a wide range of concentrations of 5FU. This will reduce the likelihood of erroneous results introduced by dilution errors.

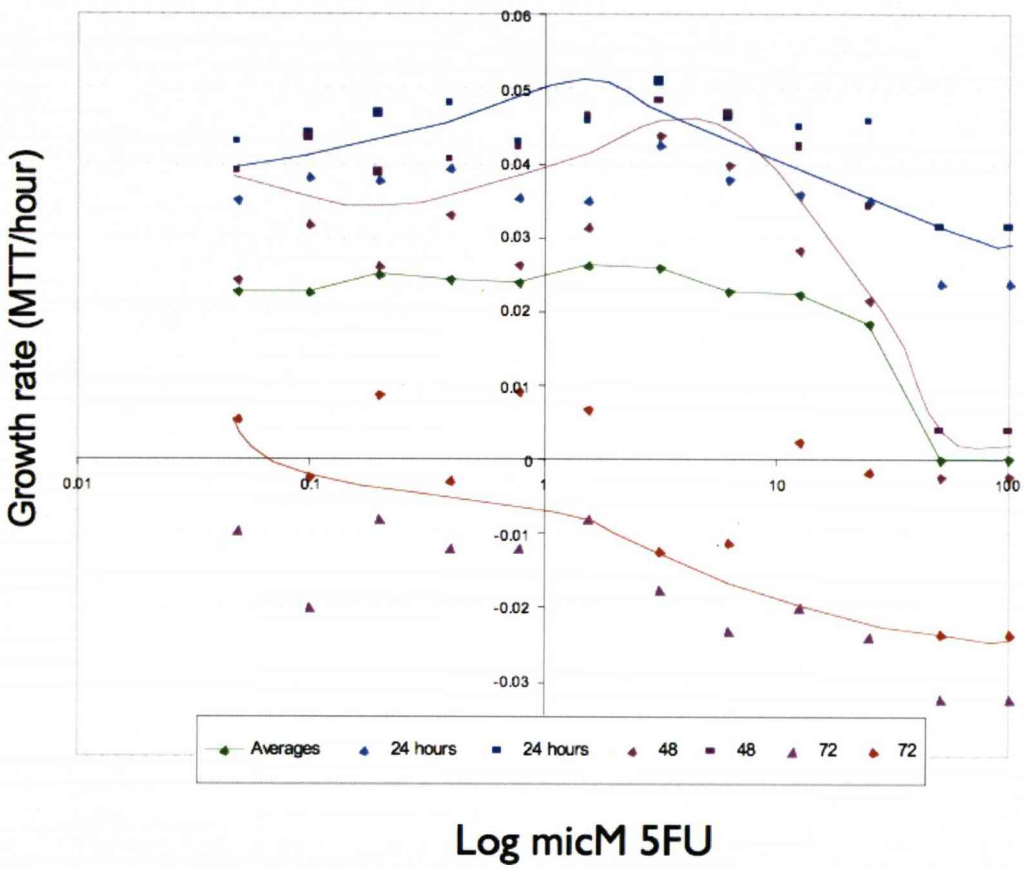


Figure 24: SUIT2 GROWTH IN 5FU (LOGARITHMIC SCALE)
As figure 23 but using a logarithmic scale.

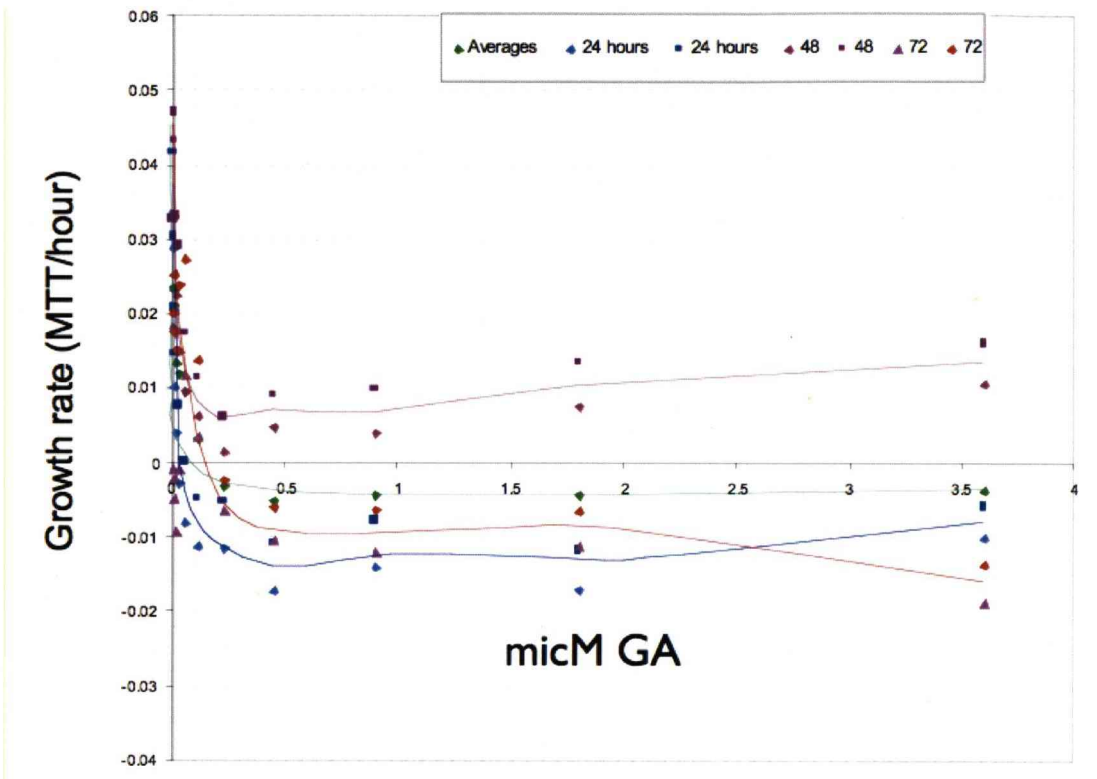


Figure 25: SUIT2 GROWTH IN GELDANAMYCIN
Growth rate versus concentration of GA assayed at 24, 48 and 72 hours.

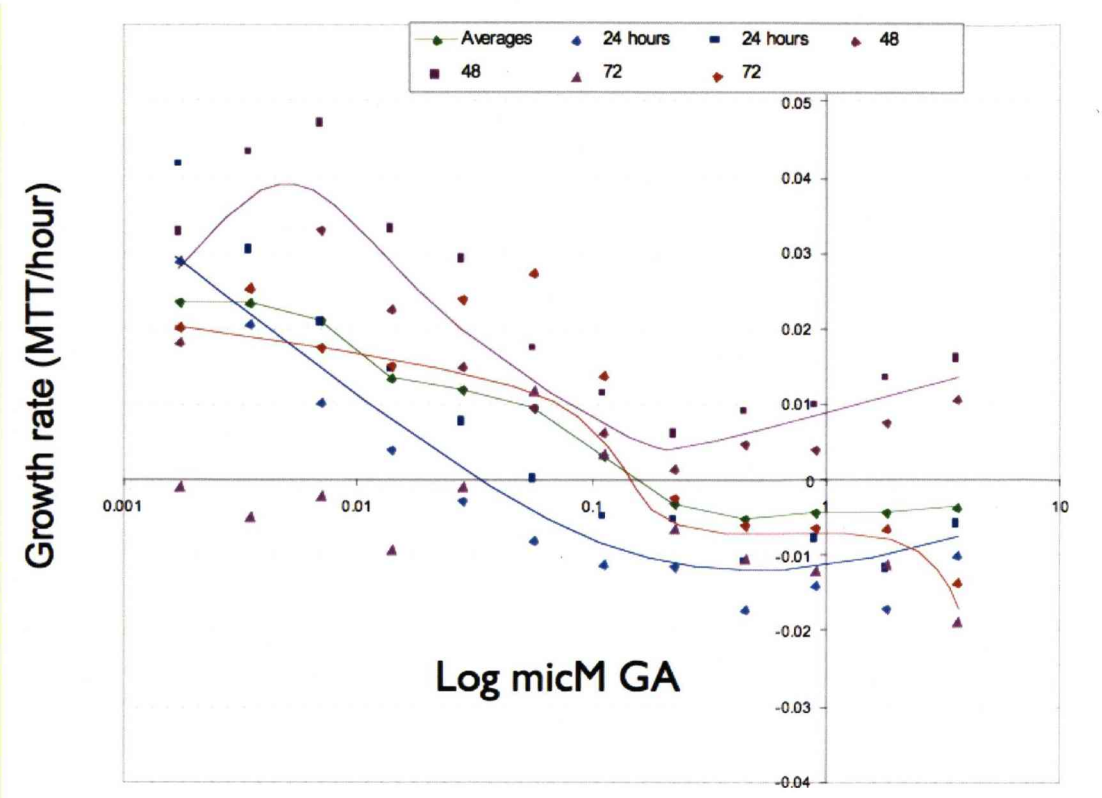


Figure 26: SUIT2 GROWTH IN GELDANAMYCIN (LOGARITHMIC SCALE)
As figure 25, but logarithmic scale.

Taken together, these results indicated that isobolar analysis of 5FU and GA in the cell line SUIT2 would be difficult to perform and open to misinterpretation. Accordingly we sought another metric of synergy. We hypothesised that comparison of single or combination drug treatment would give differing results when compared to solvent control and to each other. It seemed reasonable to use a 1/10 dilution of the

concentrations of 5FU and GA that we had investigated previously. Therefore we used a concentration of 5micM 5FU and 0.018micM GA (18nM GA).

A time period of 24 hours was taken for subsequent single point analysis as growth rate over 24 hours was significantly lower with 50 μM 5FU than at lower concentrations, but the impact of increasing 5FU below this critical point was negligible (Figures 23 & 24). In contrast at 48/72 hours lower concentrations of 5FU also had an impact on cell growth in a graduated fashion, which would potentially accentuated any dilution errors with 5FU in subsequent experiments (Figures 23 & 24). With GA there is an impact on growth rate with each graduation of concentration (at 24, 48 and 72hours) (Figures 25 and 26).

As seen in figure 20, 18nM GA had little effect on cell growth and vitality, however there was distinct cytological evidence of an effect seen on light microscopy. SUIT2 cells treated with 0.18micM GA show distinct flattening of the cytoplasm. An extreme example is shown in figure 21. In addition we found that combination of 18nM GA with 5micM 5FU gave significant results of particular biological relevance (see next section).

3.1.2 Combination treatment with GA and 5FU is at least additive and may be synergistic

5FU is the best-studied chemotherapeutic agent in pancreatic cancer but unfortunately response rates are low and toxicity a major problem in clinical practice. 5FU is an S phase specific, fluorinated pyrimidine that is metabolised intracellularly

to its active form fluorodeoxyuridine monophosphate (FdUMP) via the de novo pyrimidine pathway [219]. FdUMP acts as an inhibitor of thymidylate synthase, thereby reducing the production of thymidine that is essential for DNA synthesis. In addition to this the 5FU metabolites can be incorporated into DNA enhancing cytotoxicity (5fluordeoxyuracil triphosphate) and into RNA inhibiting protein translation (5 fluorouracil triphosphate).

We treated the pancreatic cancer cell line Suit2 with the HSP90 inhibitor geldanamycin (GA) at sublethal levels as previously described in combination with sublethal levels of 5-FU (5μM). Vitality was then measured with MTT at time zero and after 24 hours. GA caused a small decrease in vitality in comparison to treatment with the solvent DMSO alone. 5FU caused a greater decrease and the combination of 5FU and GA caused the greatest reduction in vitality ($p<0.001$ versus DMSO control), although the results are suggestive of a synergistic effect, the effect of the two drugs was not significantly greater than a predicted additive effect (Figure 27).

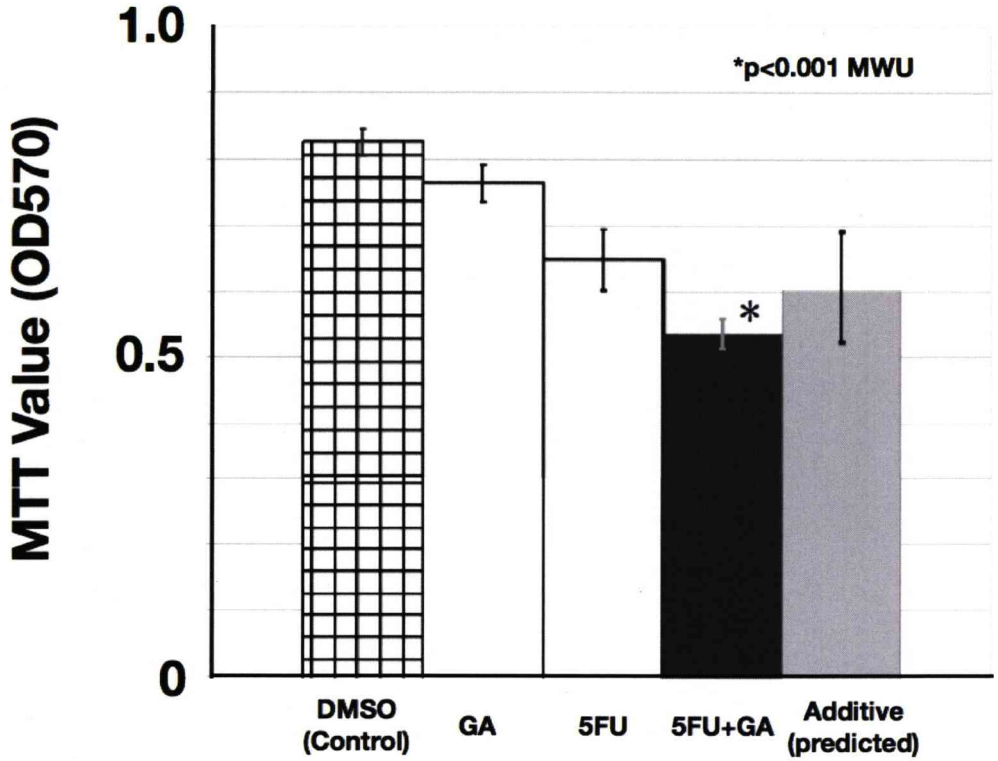


Figure 27: COMBINATION TREATMENT WITH 5FU AND GELDANAMYCIN IS AT BEST ADDITIVE

Following 24 hours of acclimatization in 96-well plates, SUIT2 cells were treated with 18nM geldanamycin (GA), 5μM 5FU or a combination of the two drugs. At 48 hours vitality was assessed.

3.1.3 Combination treatment with GA and 5FU is synergistic following sensitization with GA

The additive effect of combination treatment was encouraging and we sought to determine whether manipulation of our experimental protocol could demonstrate sensitisation of 5FU by GA. We investigated whether the order of treatment with 5FU or GA could affect our previous findings.

If cells were pre-treated with GA for 24 hours before treatment with 5FU the cells were resistant and MTT values after 24 hours of 5FU treatment were indistinguishable from values following 24 hours of treatment with solvent (Figure 28). Similarly the cells pre-treated with GA were resistant to subsequent treatment with GA or 5FU, but treatment of these cells with a combination of GA and 5FU caused a significant reduction in vitality (compared to control).

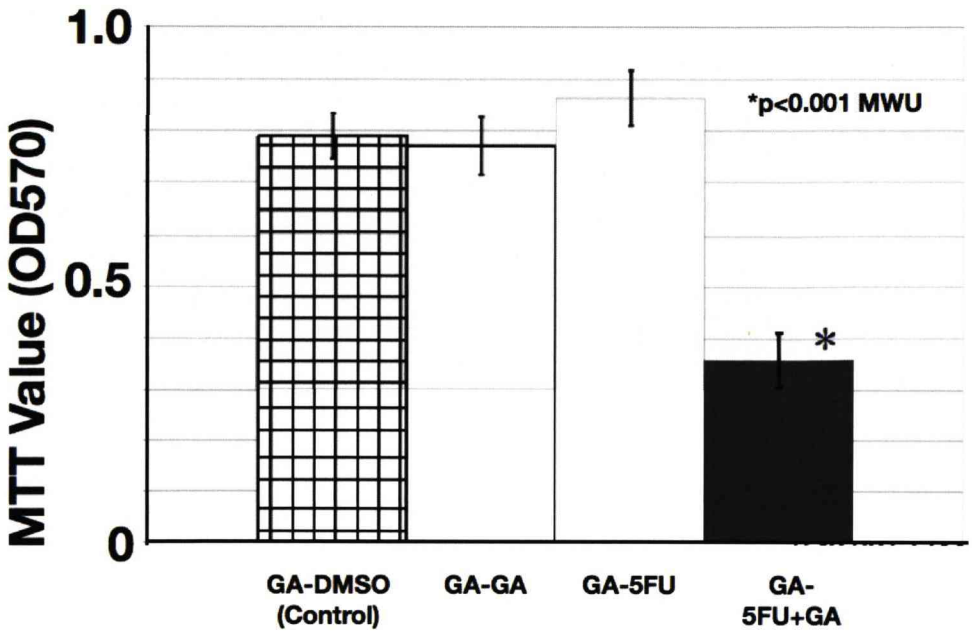


Figure 28: PRE-TREATMENT WITH GELDANAMYCIN POTENTIATES COMBINATION THERAPY

SUIT2 cells were established as in figure 27 but in the presence of 18nM geldanamycin (pre-treatment) and then treated with DMSO, further geldanamycin (GA), 5FU or a combination of 5FU and GA. The period of geldanamycin pre-treatment potentiates combination therapy.

The observation that pre-treatment with GA impairs subsequent 5FU treatment is consistent with a model whereby GA causes a level of cell-cycle arrest, reducing the number of cells in S-phase- 5FU is most actively toxic during S-phase- and hence reducing 5FU toxicity. Figure 29 demonstrates the reduction in S phase population.

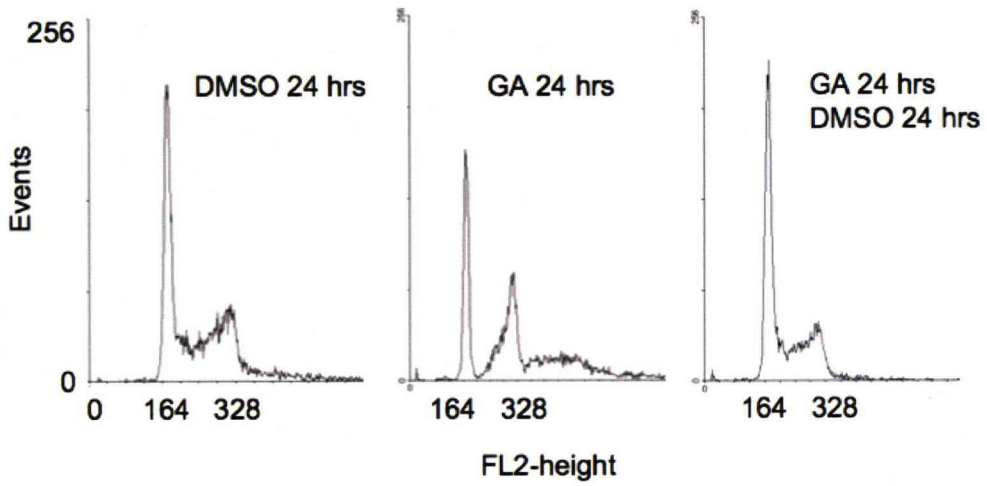


Figure 29: FACS ANALYSIS OF TREATED CELLS
FACS analysis of cells following 24 hours of treatment with 0.18nM geldanamycin (GA) and 24 hours after the restoration of normal culture conditions following GA treatment

However, removal of GA allows the cell-cycle to return to a normal profile (the situation in which GA is removed and treatment with 5FU started) and we would expect a reduction in toxicity of the GA/5FU combination, this was not the case. Combination of GA/5FU following pre-treatment with GA was synergistic. This will be considered in the discussion section.

Reversing the schedule and pre-treating with 5FU showed only a marginal increase in the effect of combination GA/5FU and would be consistent with a purely additive effect (Figure 30).

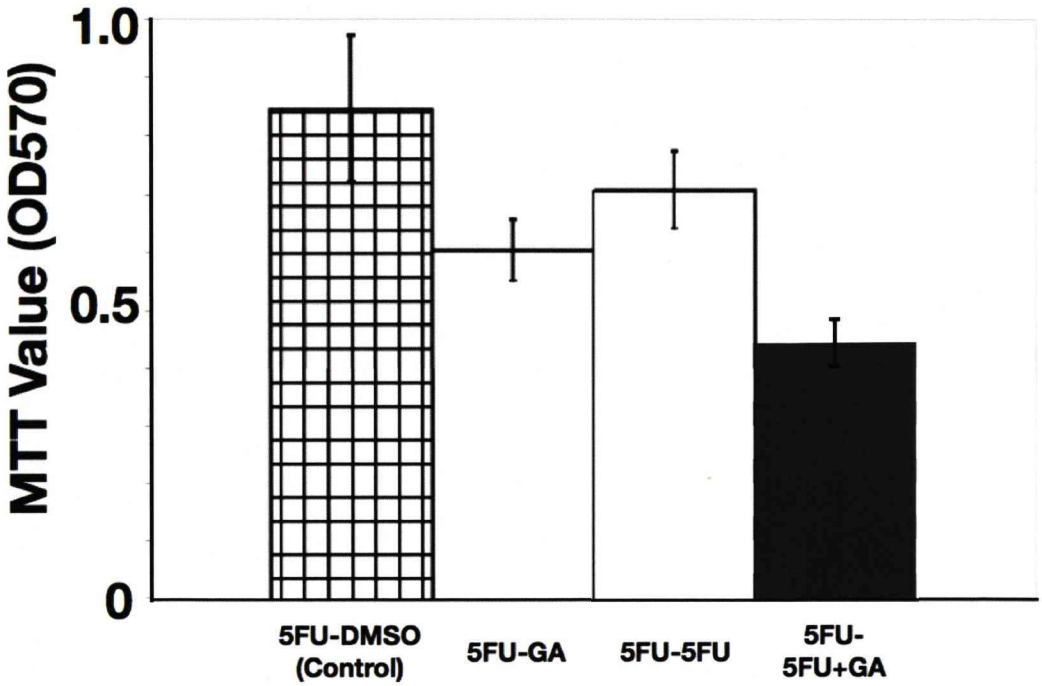


Figure 30: EFFECTS OF PRE-TREATMENT WITH 5FU
SUIT2 cells were established as in figure 28 but in this series the pre-treatment was with 5μM 5FU and then cells were treated with 18nM GA, further 5μM 5FU or a combination of 5FU and GA.

3.1.4 Effect of serum starvation on drug treatment

The next objective was to see how, on the simplest level possible, factors typical of the *in-situ* tumour that influence cellular stress responses would influence cellular response to cytotoxic agents. Pancreatic cancer is a relatively avascular cancer characterised by a dense fibrous desmoplastic stroma. This has implications as regard the effectiveness of the microvascular supply of the tumour itself. An avascular microenvironment would be characterised by relative hypoxia, and nutrient shortage. Also as the tumour grows and possibly metastasises the exposure to mitogens will decrease, by definition the cancer cells will be mitogen independent, meaning they will not be dependent on specific mitogens to divide, but this does not mean that they are unaffected by the presence of mitogens. Whilst sophisticated models for hypoxia and nutrient limitation are complex (both to operate and to interpret) [220] mitogen

deprivation is relatively easy to reproduce simply by culturing cells in the absence of foetal calf serum.

Cells were grown in serum containing media for 24 hours and then this media was replaced with serum-free media and then the cells were treated with GA, 5FU or GA/5FU for 48 hours (Figure 31 transition to serum free).

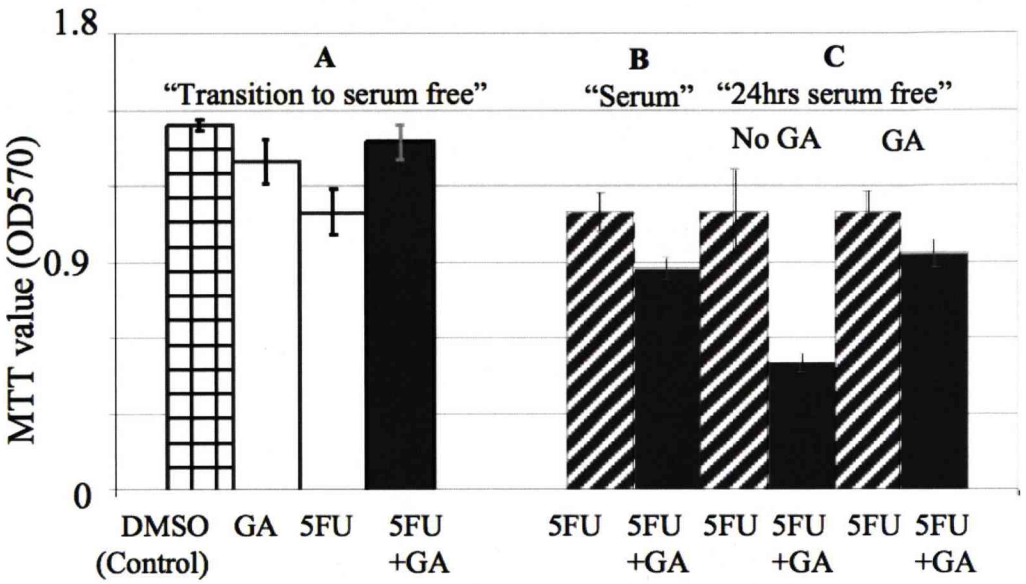


Figure 31: TRANSITION TO SERUM-FREE CONDITIONS REVERSES POTENTIATION

A: Following 24 hours of acclimatization with serum SUIT2 cells were treated with 18nM GA, 5μM 5FU or a combination of the two drugs in serum-free conditions.
B: A further representation of the data from figure 26 for comparison.
C: Cells were acclimatized in serum-free conditions and remained in serum-free conditions following drug therapy.

Both GA and 5FU caused a significant reduction in vitality ($p<0.001$ MWU) but in strong contrast to the results seen with serum containing media combination GA/5FU did not reduce vitality. Combination treatment allowed significantly increased vitality than 5FU alone ($p<0.001$ MWU) and greater vitality than GA alone but this did not reach significance ($p=0.38$ MWU). MTT values obtained in different growth conditions cannot be compared directly, but relative changes in MTT value in two different experiments can be compared if the change is opposite and statistically significant in both cases.

I next sought to establish whether this reversal of synergy with combination GA/5FU was because of the absence of serum or because of the transition to serum-free conditions. I altered the experimental protocol and maintained cells in serum-free media for 24 hours before addition of drugs in serum-containing media. For comparison purposes Figure 31 shows the effect of combination treatment of GA/5FU versus 5FU alone. After a period of 24hrs serum starvation, the effect of cytotoxic agents was never tested in the presence of serum.

Cells that have been grown in serum-free conditions and then treated in serum containing conditions with GA/5FU show lower vitality than those treated with 5FU alone. This is true even when cells are pre-treated with GA during the 24 hours of serum-starvation (Figure 31).

3.1.5 Combination treatment of Suit2 cells with TPA and GA

HSP90 inhibition can sensitise cells to 5FU. This could represent an interaction between the pathways affected by HSP90 and those affected by 5FU. Alternatively, this could be a more general effect whereby HSP90 protects the cells from damage caused by toxins. To investigate this I used TPA. TPA is toxic to pancreatic cell lines but by a different pathway to 5FU (see introduction). It is well documented that 5FU exerts its cellular effect through apoptosis but it was not known whether this was true of TPA in Suit2.

As discussed in the introduction, Caspase-9 cleavage is an early step in the intrinsic pathway of apoptotic programmed cell death ("apoptosis"). Caspase-9 cleavage requires the formation of a multimeric protein complex termed the apoptosome. Central to apoptosome formation is the pro-apoptotic adaptor protein APAF-1.

Western blot analysis of the floating, treated cells shows cleavage of capsase-9 only in the presence of serum-starvation and TPA treatment (Figure 32). This is highly suggestive of activation of the apoptotic programme.

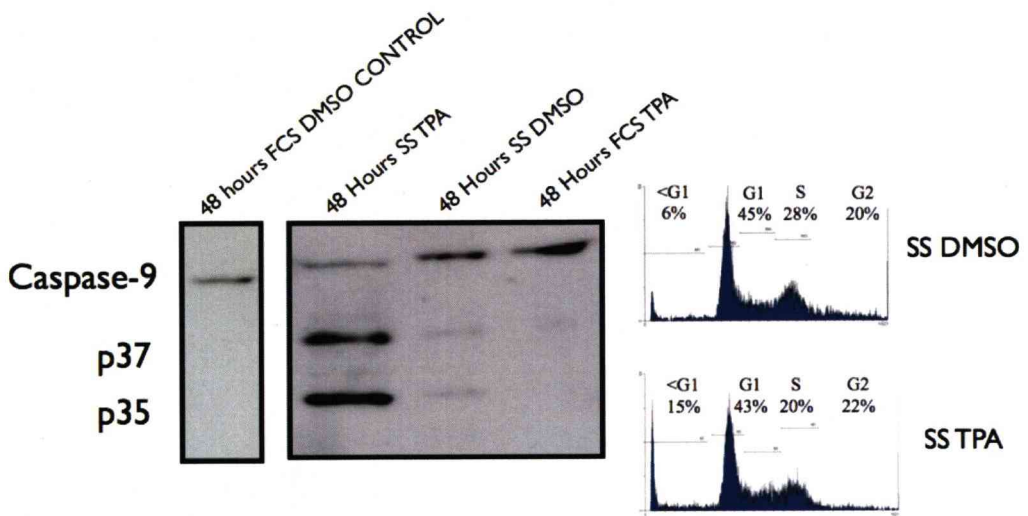


Figure 32: TPA INDUCES CASPASE-9 CLEAVAGE AND INCREASES SUB-G1 POPULATION

Left hand side: Western blot probed for Caspase-9 with whole cell lysates of SUIT2 following 0.16μM TPA treatment in the presence (FCS) or absence of serum (SS).Caspase-9 cleavage is seen with TPA treatment in the absence of serum.

Right hand side: FACS analysis of SUIT2 cells treated with TPA or DMSO in the absence of serum. There is an increase in the sub-G1 population with TPA treatment.

A Caspase-3 activity assay shows activation of Caspase-3 in response to TPA within 24 hours. A similar assay was carried out for Caspase-1 but this showed no increase in caspase activity (Figure 33). This suggests that the activity of TPA is through the intrinsic apoptotic pathway rather than the extrinsic pathway.

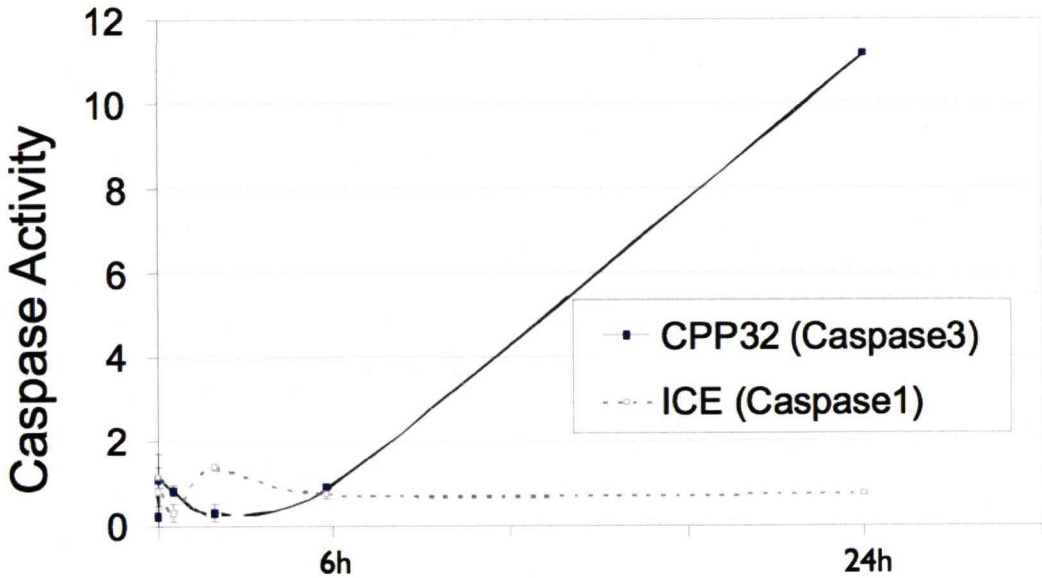


Figure 33: TPA INDUCES CASPASE-3 ACTIVITY BUT NOT CASPASE-1
Caspase assay demonstrating caspase-3 activity but not caspase-1 activity with TPA treatment of SUIT2 cells.

Further analysis of DNA content of the treated cells using FACS analysis showed an increase in the sub-G1 population of serum-starved, treated cells (Figure 32)- consistent with apoptotic cell-death.

I repeated the experimental protocol using serum-containing media and serum-free media in a similar way to the GA/5FU series. GA was used at 18nμM and TPA at 0.16μM. Treatment after transition to serum-free conditions shows that TPA reduces vitality to a similar degree as GA, however combination of the two drugs has no effect suggesting the drugs counteract each other. In contrast treatment in the presence of serum with TPA and GA has more effect than TPA alone. This is consistent with the results seen with 5FU (Figure 34).

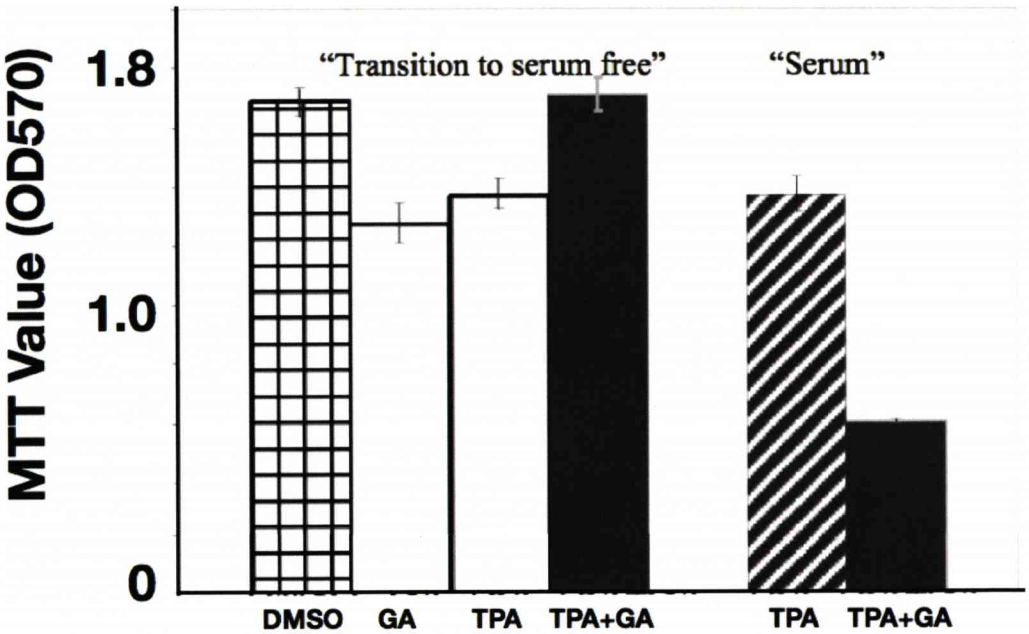


Figure 34: TRANSITION TO SERUM-FREE CONDITIONS REVERSES POTENTIATION WITH TPA

Following 24 hours of acclimatization with serum, cells were treated with 18nM geldanamycin (GA), TPA or a combination of the two drugs in serum free conditions ("Transition to serum free"). In "Serum" drugs were applied in serum containing media.

3.1.6 Serum starvation induces nuclear translocation of HSP90 reaching a maximum at 16-24 hours

A recent patent application [221] showed that nuclear export inhibitors could potentiate the action of 17AAG. It was noted that potentiation was lost if nuclear export inhibition was applied after HSP90 inhibition. Stress has been shown to induce nuclear accumulation of HSP90 [222] and I next sought to see if the transition to serum-free conditions was associated with nuclear accumulation of HSP90 Under normal

growth conditions HSP90 exhibits cytoplasmic localisation on immunofluorescence (IF), however, serum withdrawal is associated with profound translocation of HSP90 from cytoplasm to nucleus (Figure 35). I next sought to confirm this shift using western blot analysis of sub-cellular fractions (Figure 35). Here actin is used as a control for the cytosolic compartment, the coomassie blue stain allows comparison of total protein content. Translocation is seen to begin at around 8 hours (12%) and is maximal between 16 and 24 hours (87-92%) (Figure 36).

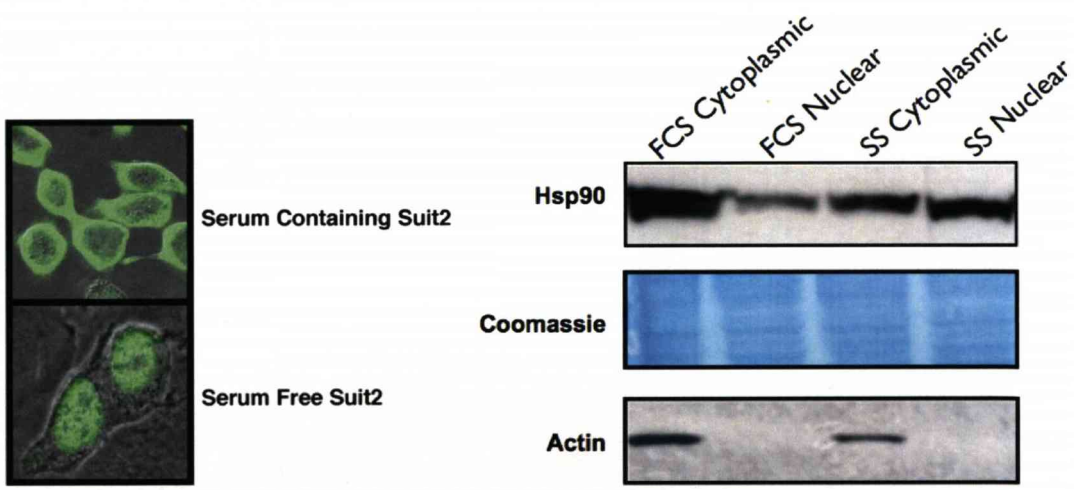


Figure 35: SERUM STARVATION INDUCES NUCLEAR TRANSLOCATION OF HSP90

On the left are micrographs of SUIT2 cells immunstained for Hsp90 (green) in serum containing and serum-free conditions. On the right is a Western blot of subcellular fractions of SUIT2 showing an increase in nuclear Hsp90 in serum-free (SS) conditions. Actin is shown as a cytoplasmic protein control, the Coomassie Blue stain shows overall protein levels.

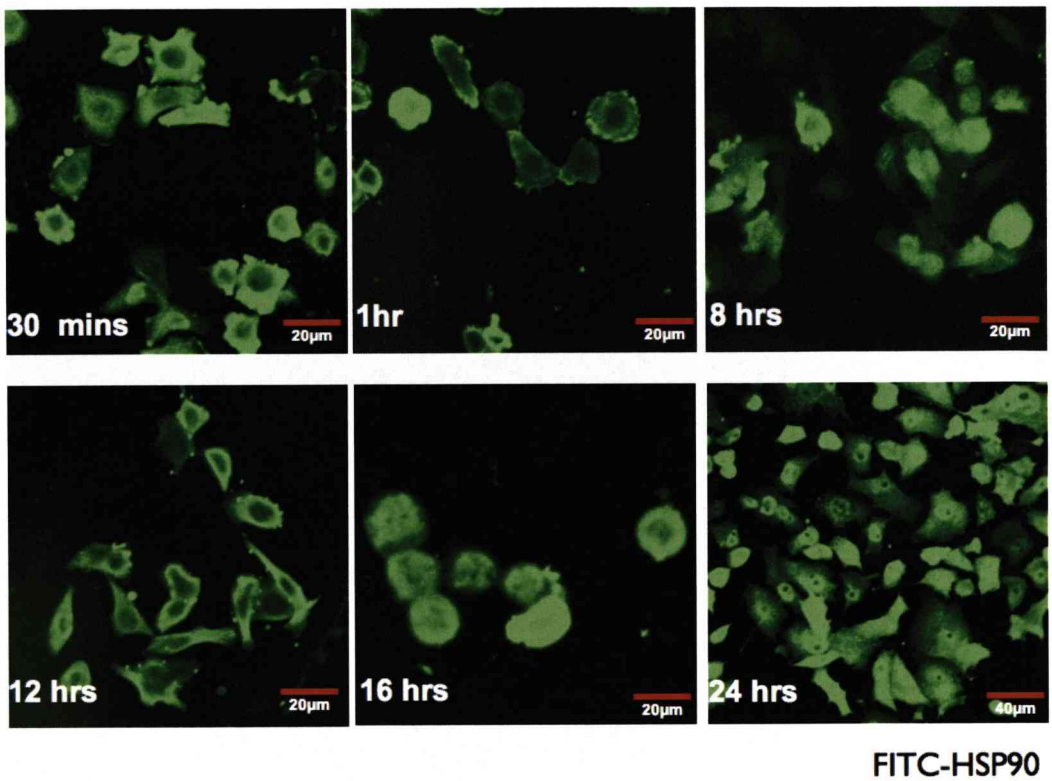


Figure 36: NUCLEAR TRANSLOCATION OF HSP90 IS MAXIMAL AT 16-24 HOURS

Micrographs of SUIT2 cells immunostained for HSP90 (green) showing increasing nuclear localisation of HSP90 with increasing time in serum free media, maximal at 16-24 hours.

3.1.7 HSP90 entry to the nucleus is preceded by cdc37

HSP90 acts in concert with a number of targeting proteins, the best studied of which is cdc37. Cdc37 plays a significant role in targeting protein kinases to HSP90. In serum starvation the shift of HSP90 to the nucleus could alter the spectrum of bound HSP90 clients. I next sought see if cdc37 showed evidence of alteration under conditions of serum-starvation. Western blot analysis indicates a shift of cdc37 to the nuclear fraction that precedes the transportation of HSP90 (Figure 37). In this Figure note that at 8 hours nuclear cdc37 is increased and only later at 16 hours does the nuclear fraction of HSP90 increase. In particular the ratio of nuclear HSP90 to cdc37 changes markedly. The Coomassie Blue gels demonstrate equivalent protein loading of the lysates.

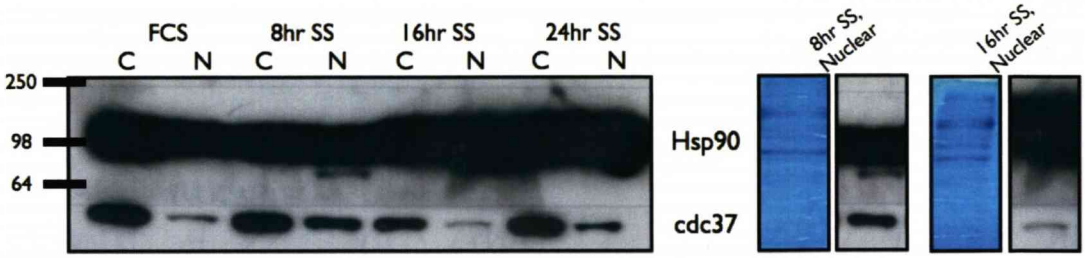


Figure 37: ENTRY OF HSP90 TO THE NUCLEUS IS PRECEEDED BY CDC37
Immunoblot of SUIT2 nuclear and cytoplasmic lysates probed for HSP90 and cdc37 in serum containing media (FCS) and at timepoints in the presence of serum-free media (SS). The Coomassie Blue stain shows total protein content. Note how the increase in cdc37 nuclear staining precedes that of HSP90 from 8 to 16 hours.

3.1.8 APAF-1 is modulated by serum-starvation and geldanamycin treatment

I established that serum-starvation had a profound effect on HSP90 localisation and sensitivity to TPA. I also established that TPA sensitivity could be achieved using the specific HSP90 inhibitor geldanamycin (GA). I next sought to determine the cellular substrate for this phenomenon. The HSP90 client protein APAF-1 is central to the apoptotic program through the formation of the apotosome and its subsequent cleavage of Caspase-9. I next investigated the role of APAF-1.

Western blot analysis showed that APAF-1 appears to exist in two forms in cultured Suit2 cells grown in serum containing media; a full length form (FL) and a truncated C-terminal form (CT) migrating with an Mr of approximately 64 kDa. Upon withdrawal of serum the C-terminal form is lost (Figure 38). IF data shows that initially APAF-1 and HSP90 colocalise, but on serum deprivation the shift of HSP90 to the nucleus reduces this (Figure 39). Further analysis with an amino-terminal antibody did not demonstrate a signal, implying the carboxy-terminal APAF-1 does not contain the CARD domain. Time course experiments show this occurs by at least 4 hours and is unaffected by TPA treatment. In contrast TPA treatment in serum containing media has no effect on this profile.

I next looked at the effect of geldanamycin on APAF-1 expression in cells grown in serum containing media. In the presence of geldanamycin there appears to be stabilisation of the CT form of APAF-1 (Figure 38).

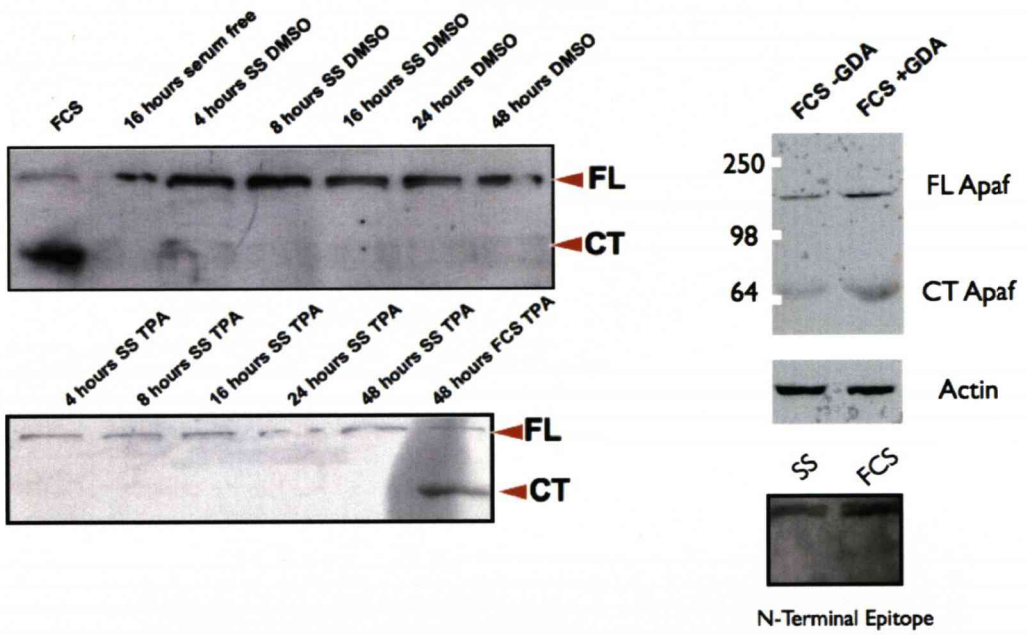


Figure 38: APAF-1 IS MODULATED BY SERUM-STARVATION AND GELDANAMYCIN

Serum withdrawl (SS) is associated with loss of the C-terminal isoform of APAF-1. Geldanamycin appears to stabilise the CT isoform of APAF-1.

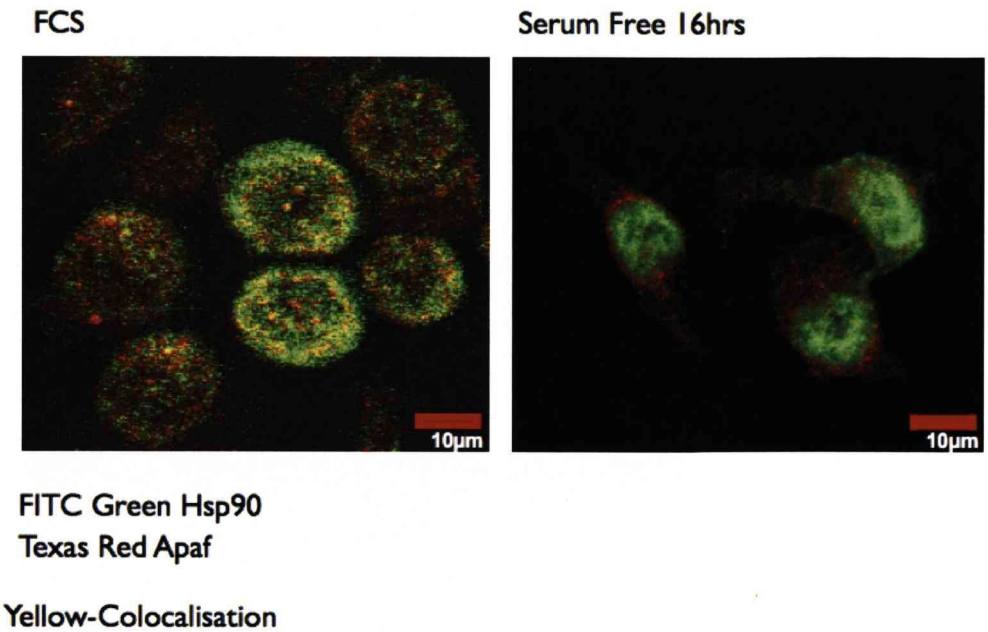


Figure 39: SERUM-STARVATION IS ASSOCIATED WITH HSP90 DISSOCIATION FROM APAF-1
Colocalisation (yellow) of HSP90 (green) and APAF-1 (red) in the presence of serum is lost on serum withdrawal and translocation of HSP90 into the nucleus.

3.1.9 HSP90 and APAF-1 are expressed *in-vivo* in Pancreatic Cancer

The above results led to an examination of whether HSP90 and APAF-1 were expressed *in vivo* pancreatic cancer. Loss of APAF-1 has been described in malignant melanoma and shown to contribute to the malignant phenotype [131]. There existed the possibility that APAF-1 may also be lost in pancreatic cancer because pancreatic cancer is associated with the familial melanoma syndrome [79]. Accordingly the presence of APAF-1 in our cell line data may not represent the true expression of APAF-1 *in vivo*. Immunohistochemistry of pancreatic cancer samples shows expression of both HSP90 and APAF-1 (Figures 40 & 41).

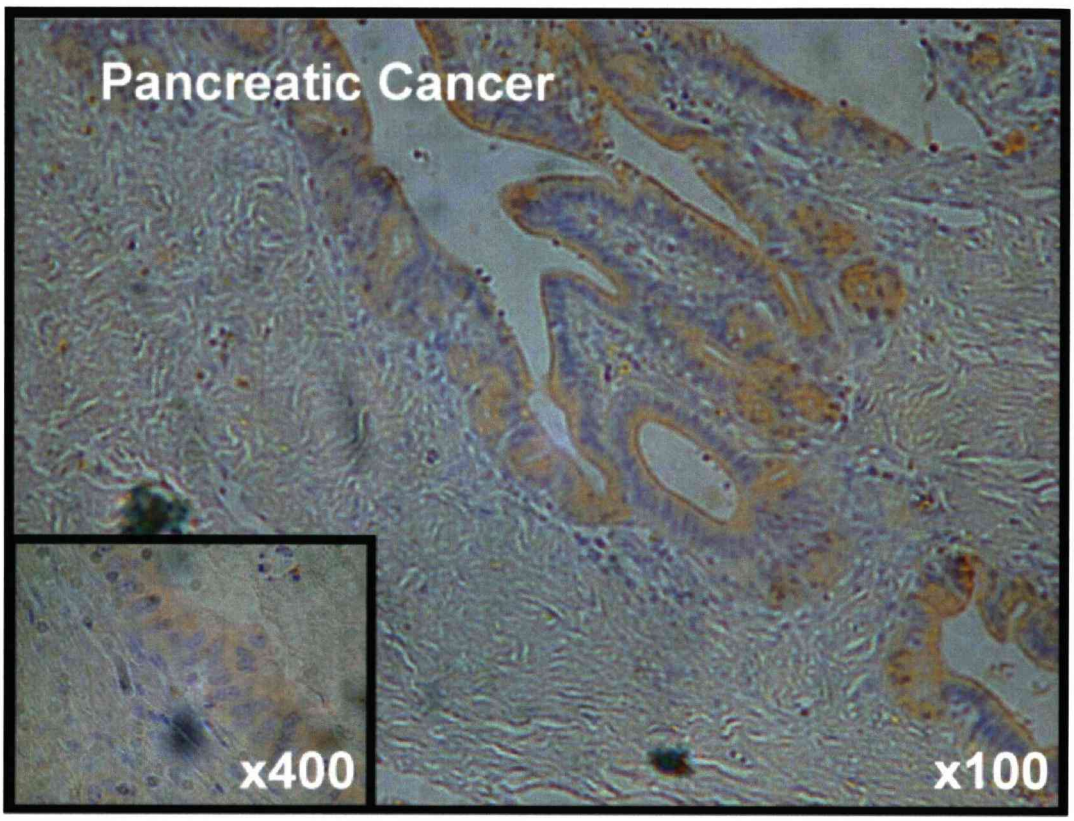


Figure 40: APAF-1 IS EXPRESSED IN-VIVO IN PANCREATIC CANCER
Immunohistochemistry of APAF-1 (brown) in human pancreatic cancer showing cytoplasmic location.

APAF-1 is located in the cytoplasm but HSP90 shows marked nuclear staining (Figure 41) (M Burkitt, University of Liverpool Department of Surgery). Recent work in the department of surgery has shown that nuclear HSP90 staining is related to tumour size, suggesting that cell-death in pancreatic cancer may abrogated by nuclear localisation of HSP90.

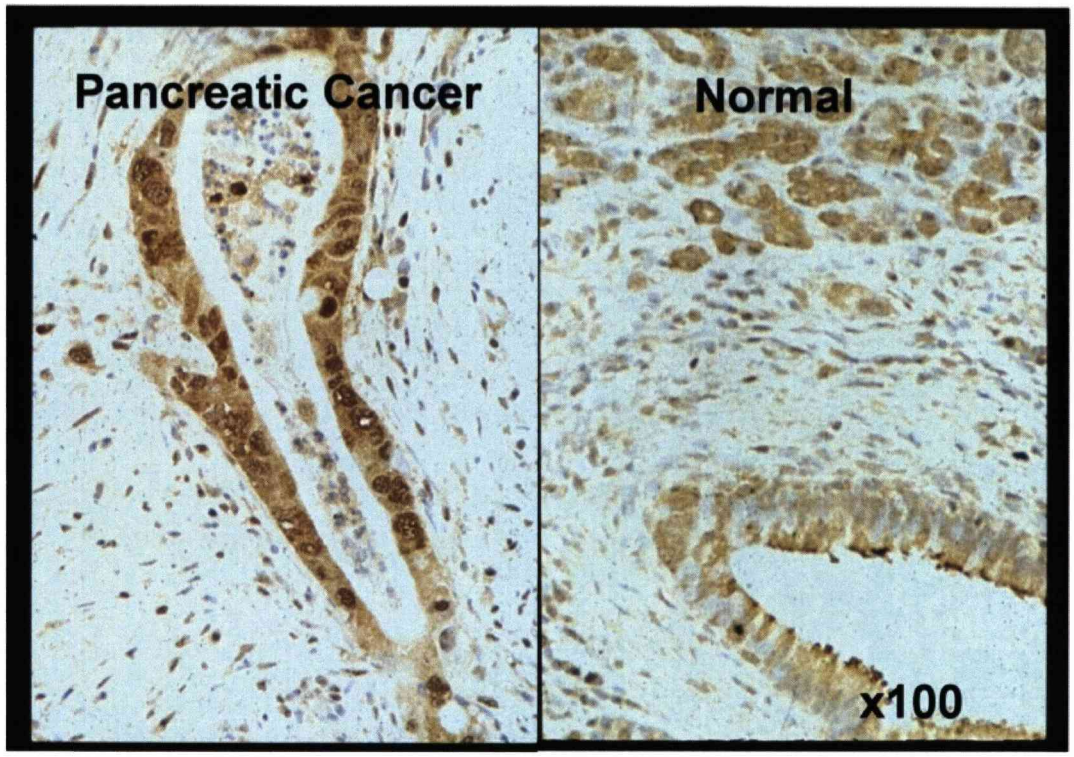


Figure 41: NUCLEAR HSP90 IS A FEATURE OF IN-VIVO PANCREATIC CANCER
Immunohistochemistry of HSP90 in human pancreatic cancer and normal pancreas.

3.2 YEAST EXPERIMENTS

Inhibition of HSP90 can potentiate chemotherapeutics, however there are many documented clinical problems with the currently available HSP90 inhibitors (e.g. hepatotoxixity with GA derivatives, solubility in DMSO)[223]. There is a need to identify new HSP90 inhibitors that are more suitable for clinical use. Unfortunately, there are no readily available methods to screen candidate compounds for the ability to inhibit HSP90, indeed, identification of HSP90 antagonists up until this point has been serendipitous. Geldanamycin was identified due to its cytotoxic effects and not because it was suspected to interact with HSP90. Derivatives of GA have been investigated empirically[224], and although these modifications have been discussed in terms of predicted interaction with HSP90 these discussions have dealt exclusively with improvement of existing interactions[225] and not with exploitation of new interactions.

The previous work showed that the HSP90 target protein APAF1 as well as being a *bona-fide* client also contributed to the chemotherapeutic action of HSP90 . We therefore decided to investigate whether it was possible to create a yeast-based screening tool for HSP90 inhibitors that would utilise the APAF1-HSP90 interaction. If this were successful it would be possible to screen compounds not only for their ability to inhibit HSP90 (potentially not contributing to a therapeutic effect) but also their ability to alter APAF1 pathways (with definite chemotherapeutic effects).

Most current work investigating HSP90 inhibitors is focussed on pharmacological manipulation of the prototypical compounds geldanamycin and radicicol or purine scaffold compounds. Manipulation of these compounds will not identify new compounds that act outside the known mechanisms of the prototype compounds and such purely biochemical approaches are labour intensive and slow.

We wanted to develop a screening system that would allow us to investigate drugs that interfere with the HSP90 -APAF1 interaction; we did not wish this screen to be limited to interaction with the N-terminal ATPase site of HSP90 (a target already extensively studied by other groups). A yeast model seemed to us ideal as this would maintain many of the HSP90 co-chaperones, but would be considerably simpler than a cell line based assay. A cell line assay is essential to determine how compounds could behave in a clinical setting, the outputs (modulation of cell growth or viability) directly relate to the desired function of test compounds, regardless of the mechanism. However, if the question is identification of candidate compounds with a specific mechanism, the sensitivity of cell lines becomes a confounding factor. Yeast are easier to culture than cell lines, they have co-chaperones essential for HSP90 function and, due to simplicity, are less prone to side affects of test compounds. The

following experiments were all aimed at the eventual development of such a screening system.

We proposed investigating yeast-based systems, initially the Yeast Two-Hybrid (Y2H) system. The Y2H approach has the potential advantage of high throughput screening and giving specific information of particular protein-protein interactions. In a Y2H system the binding domain and activation domain linked proteins would be known based on confirmed HSP90 -protein interaction that could be disrupted by confirmed HSP90 inhibitors such as geldanamycin derivatives. Such a system could then be used to assay HSP90 inhibitory candidates. The HSP90 -protein partners could be HSP90 and any suitable known HSP90 binding partner e.g. Cdc37.
We proposed that an interaction between APAF1 and HSP90 could be an effective output for a yeast two-hybrid screening tool. In contrast to Cdc37, APAF1 is involved in apoptotic cell death and, as we were interested in apoptotic cell death, was therefore relevant for investigation of novel chemotherapeutics.
A Y2H screening tool would be based on the interaction between APAF1 and HSP90. In the absence of HSP90 inhibition the GAL4 gene would be constitutively active, however in the presence of a HSP90 inhibitor there would be disruption of the APAF1-HSP90 interaction with consequent reduction in GAL4 activity. This would provide an output signal for HSP90 inhibitory activity.

3.2.1 The conventional Yeast Two-Hybrid system cannot demonstrate an interaction between APAF1 and HSP90

As part of the work investigating the utility of the yeast two-hybrid system for assaying novel HSP90 inhibitors we proposed APAF1 as the HSP90 interacting protein.

The Yeast Two-Hybrid (Y2H) system is based on the finding that most yeast transcription factors contain multiple functional domains. Of particular relevance are the DNA-binding domain (BD) and the activation domain (AD). Brent and Ptashne showed that fusion of the GAL4 AD to the LexA BD was sufficient for expression to occur [226]. Initial work with the Y2H concept demonstrated that fusion of GAL4-BD with SNF1 and GAL4-AD with SNF4 allowed the activation of the *his3* and *β-galactosidase* reporter genes under the control of the Gal1 promotor in a yeast strain lacking the natural Gal1 activator (Gal4), as the SNF1 and SNF4 proteins bind together and so bring BD and AD together. This system has been extensively used to identify genes for proteins, or regions of proteins, that when cloned in frame with the AD and BD will allow transcriptional activation of reporter genes. This is widely taken as evidence that the cloned proteins bind to each other, although on occasions such binding may be indirect.

The yeast strain used carry mutations in genes required for amino-acid biosynthesis. In this set of experiments the yeast cannot grow in a medium that does not contain leucine, tryptyophan and histidine. The introduced plasmids containing the AD and BD fused proteins carry genes coding for proteins that can synthesise leucine and tryptophan and therefore act as markers of successful transformation with these plasmids. The HIS3 gene can only be activated by interaction of the AD and BD proteins and growth in media deficient in leucine, tryptophan and histidine confirms protein interaction. In a conventional Y2H screen a known "bait" protein is fused to the BD and used to screen for candidate "prey"-AD proteins. For our purposes both

bait and prey are known (HSP90 and APAF1) and such terms are meaningless. We will refer to HSP90-AD and APAF1-BD. In the initial set of experiments we used HSP90-AD and as a positive control we used Cdc37-BD.

I initially set up the experimental protocol using an N-terminal APAF1 (residues 1-621) construct. Our initial plan was to quality control the Y2H system with a known positive control (Cdc37 interacting with C-terminal HSP90), and then if the system worked perform more experiments. N-terminal APAF1 was chosen because it contains the CARD domain and was specific to APAF1, in contrast to the C-terminal WD40 regions. An N-terminal fusion of APAF1 was chosen because of the relative straightforward nature of the cloning process (see Materials and Methods). Briefly, N-terminal APAF1was cloned in frame with the DNA binding domain of the yeast vector pAS2.1. The construct was verified by DNA sequencing and used in a standard Y2H screen with pGAD424-HSP90 constructs.

There was no interaction demonstrated between full length or N terminal HSP90 with N-terminal APAF1 (Figures 42 and 43)

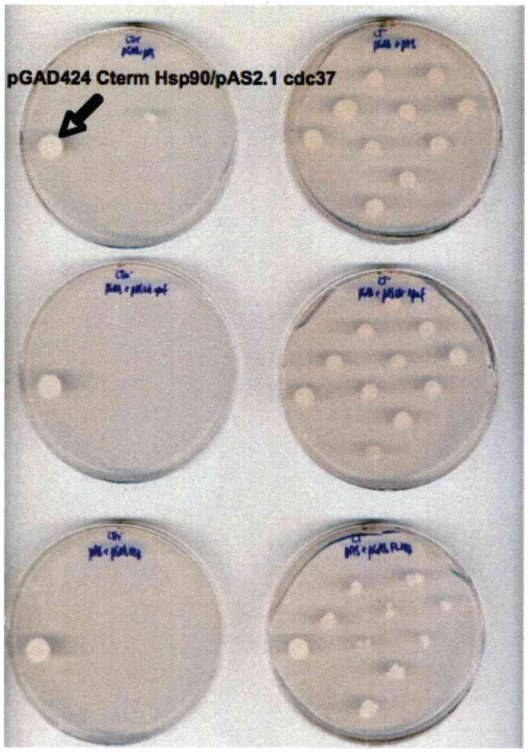


Figure 42: YEAST TWO HYBRID SCREEN CONTROLS
The positive control used is pGAD424 CtermHSP90/pAS2.2cdc37. These plates show no false positive interaction between the constructs used.

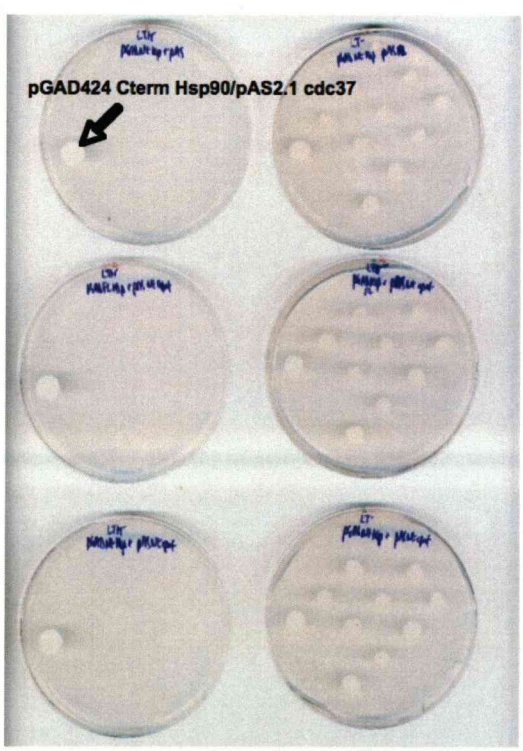


Figure 43: YEAST TWO HYBRID SCREEN INTERACTION
It was not possible to demonstrate interactions between HSP90 and APAF-1

The conventional cloning approach with full length HSP90 is to fuse it at its N terminal domain to a transcriptional activation domain. This did not demonstrate interactions with Cdk4, Raf1, APAF1 or any other target protein (our own observations and personal communication Professor Peter Piper, University of Sheffield). It became clear at a later date that only the pGAD424-C-terminal HSP90 construct can be used in a yeast two-hybrid screen.

It was decided that the Y2H system with APAF1was not robust enough to offer a means to assay potential HSP90 inhibitors. Therefore a second approach was investigated that would also utilise the HSP90 target protein APAF1. This utilised a novel structural similarity between APAF1 and the yeast protein YCR072c.

3.2.2 The essential yeast protein YCR072c shares homology with APAF1

The brewers yeast *Sacchromyces cerevisiae* contains a protein known as YCR072c (GenBank reference GI:1907211). This is a 515 amino acid protein with a molecular weight (predicted) of 57 kilodaltons. YCR072c is a WD40 repeat containing protein. It contains 8 WD40 motifs in its C-terminus (residues 141-514). YCR072c has no known function in yeast, but it does share significant homology to human APAF1 (Figure 44)

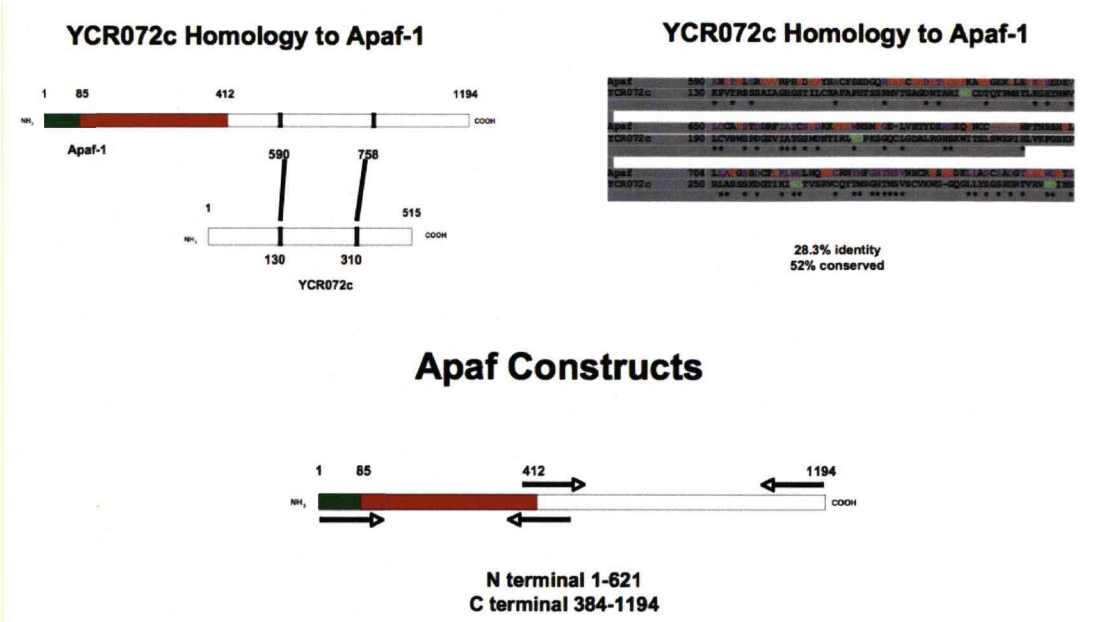


Figure 44: APAF-1 HOMOLGY TO YCR072C AND APAF CONSTRUCTS
APAF-1 shares homology with the yeast protein YCR072c. C-terminal and N-terminal APAF-1 constructs were then used in yeast experiments.

The homologous regions are within the WD40 repeat regions of each protein. YCR072c has also been reported to be show homology to Angio Associated Migratory Protein (AAMP) [227] but has recently been identified as Rsa4p- a protein essential for rRNA processing [228].

The yeast strain YCR072c+/- has a single allele gene disruption of YCR072c achieved via introduction of a Kanamycin resistance gene. Haploid yeast spores without YCR072c (YCR072c-) are non-viable. Diploid yeast (YCR072c+/-) with a single disrupted allele, however, are viable.

We hypothesised that introduction of APAF1 into haploid YCR072c could rescue the lethal phenotype and therefore confirm APAF1 as a functional homologue of YCR072c. This would give us a functional assay based on APAF1. The output of this assay (i.e. rescue of the lethal phenotype) would then allow us to investigate whether this could be related to HSP90 function (use of geldanamycin and other

HSP90 inhibitors). It would also allow us to investigate apoptotic pathways in yeast (for example, can phenotype rescue with APAF1 be inhibited by other heterologous proteins). Finally, the presence of an APAF1-like protein in yeast was intriguing given the recent data published supporting an intrinsic program of cell-death in yeast [195] and this assay could allow us to investigate this further.

Proposed Output of HSP90 -APAF1 Sporulation based Screening Tool

Following sporulation the YCR072c+/- diploid cell forms 4 haploid spores. These would be dissected out and grown on appropriate media. Due to the lethal YCR072c knockout two of the four spores would be non viable and not grow. Transformation of YCR072c+/- with APAF1 should allow rescue of this lethal phenotype, i.e. APAF1 substitutes for the knocked out YCR072c allele. In this case all four spores would be viable and grow. This phenotype rescue forms the basis of the proposed screening tool. Next we would introduce known HSP90 inhibitors that can disrupt the HSP90 -APAF1 interaction (such as geldanamycin), this would lead to abrogation of the APAF1 mediated viability in YCR072c- haploid spores (shown diagrammatically in figure 45). Following from this it will be possible to screen other potential HSP90 inhibitors using this system and compare their effects to the baseline effect mediated by geldanamycin.

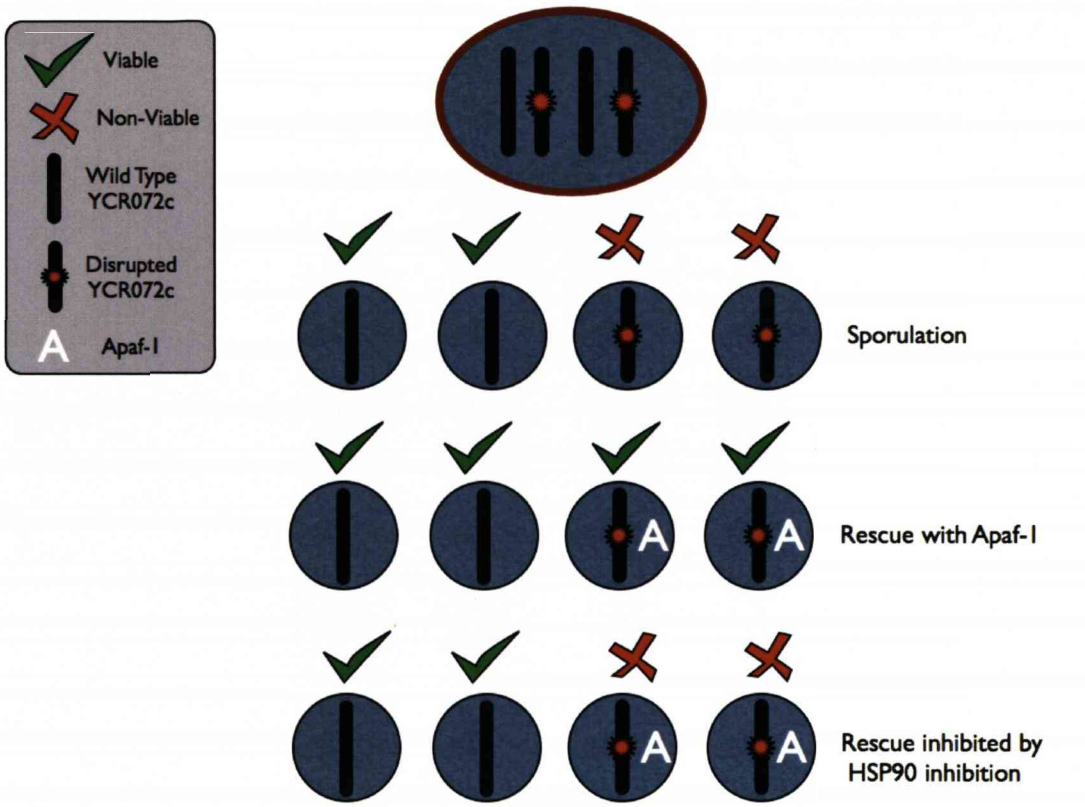


Figure 45: PROPOSED OUTPUT FROM THE PROPOSED SPORULATION BASED SCREEN

Sporulation of YCR02c+/- yeast will only give 2 viable spores. With APAF-1 there will be rescue of the YCR072c- spores, i.e. all 4 spores are viable. This rescue is inhibited by the presence of HSP90 inhibitors.

3.2.3 Construction of pCM188-Apaf Plasmids

All YCR072c strains were verified using auxotrophic growth plates.

Full length APAF1 and a C-terminal construct containing the WD40 repeat region of APAF1 were cloned into the yeast expression vector pCM188 (see Materials and Methods). Care was taken to ensure that a good Kozak sequence was present in the construct to allow maximal expression of the protein. Initial Western blot analysis did not conclusively demonstrate protein expression because of native yeast proteins "washing out" the signal. Reverse transcriptase-PCR analysis of the transformed yeast showed the presence of construct mRNA indicating their competency and suggesting protein was being expressed (Figures 46 and 47). In Figure 46 the characteristic pattern of RNA species is displayed showing that good quality RNA was extracted. From this cDNA was made (see materials and methods) and PCR

performed using primers designed for the N terminal (FL construct) and C-terminal (CT construct). The results demonstrate that the constructs were competent to produce transcripts when transformed into YCR072c. However, the identification of an actual phenotype would be needed before any conclusions could be drawn.

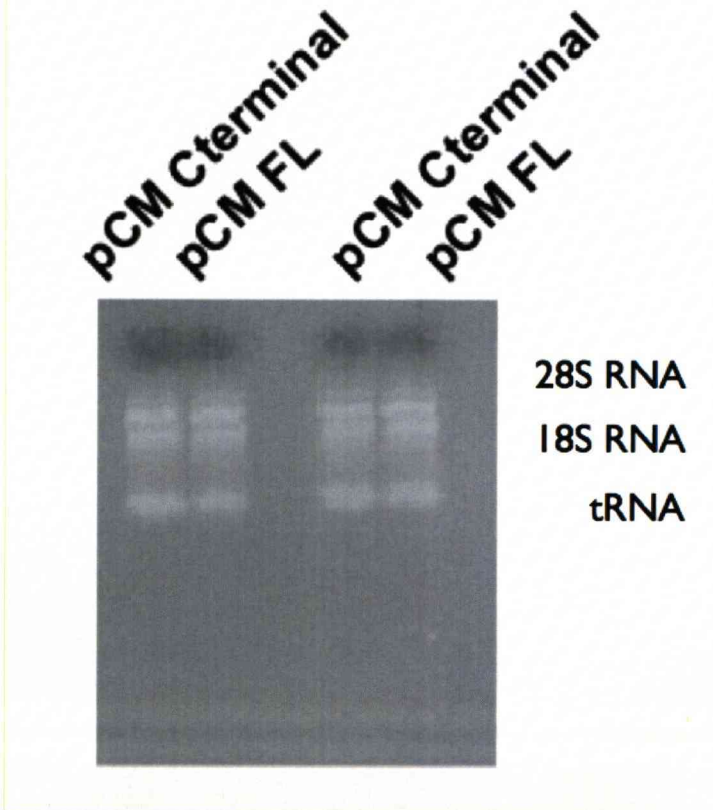


Figure 46: mRNA EXTRACTION
Good quality mRNA was extracted from the transformed yeast cells to allow RT-PCR analysis

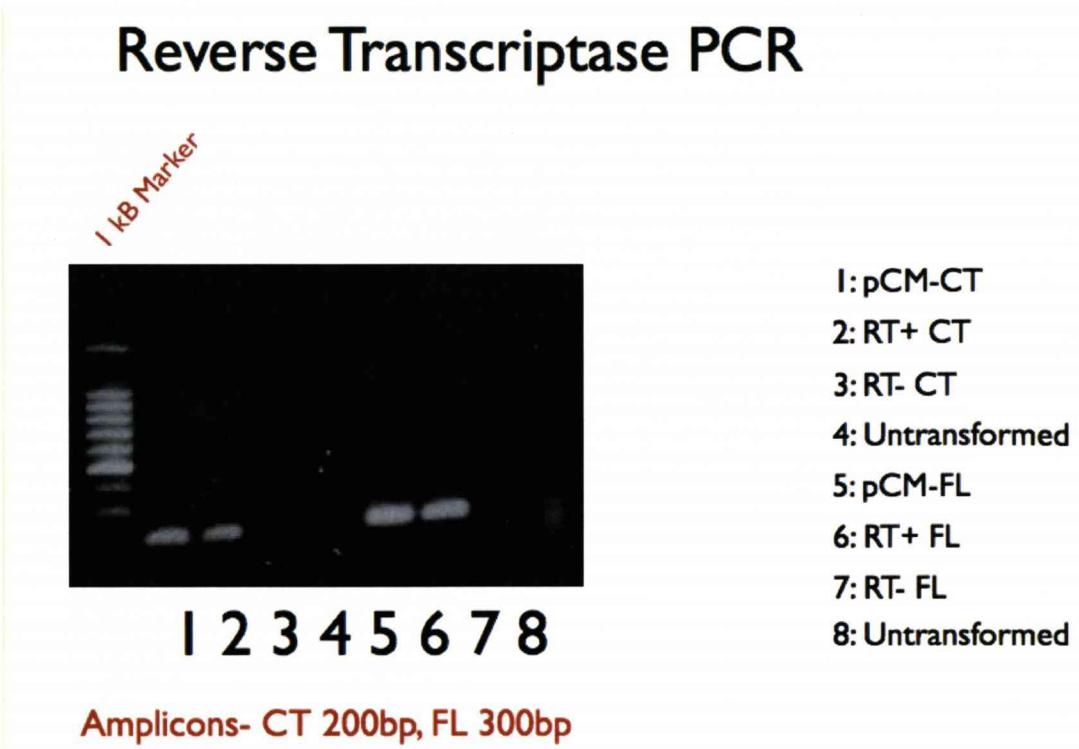


Figure 47: RT-PCR ANALYSIC OF YCR072C AND CONSTRUCTS
The APAF-1 constructs were being expressed the yeast cells

3.2.4 Sporulation Analysis of APAF1 in YCR072c

The ideal means of assessing rescue of haploid YCR072c by APAF1 is through tetrad analysis. This technique involves two stages- yeast sporulation and tetrad dissection.

Sporulation

When yeast are subjected to environmental stress (prototypically carbon and nitrogen deprivation) they undergo a survival process termed sporulation. Sporulation is essentially yeast meiosis and as such involves chromosome reduction but also has a stage of spore morphogenesis. In meiosis there is chromosomal replication and then recombination of homologous chromosomes. There next follows two consecutive nuclear divisions with separation of homologous chromosomes (meiosis I) and then

sister chromatids (meiosis II). The four haploid daughter cells are termed spores and are enclosed in a protective wall called the ascus (Figure 48). With YCR072c+/- there would be two spores containing YCR072c and 2 spores containing the Kanamycin disrupted, inactive gene. These latter 2 will be non-viable. If APAF1 is a true homolog of YCR072c there should be rescue of this lethal phenotype.

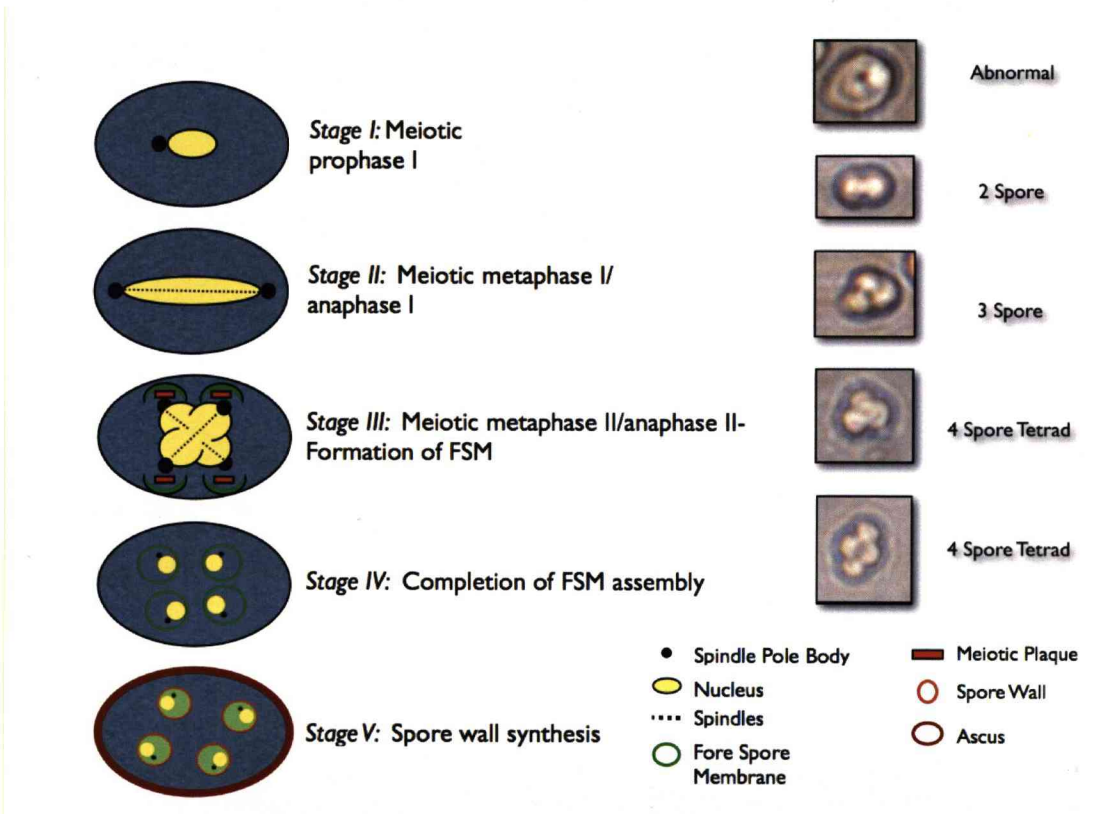


Figure 48: YEAST SPORULATION

Tetrad Dissection

The ascus-4 spore complex is termed a tetrad. Individual spores can be dissected out following dissolution of the ascus. Using a dissecting microscope it is possible to place the individual spores on an agar plate, and thus investigate their growth responses to varying environmental stimuli (e.g. auxotrophic requirements).We looked to see if APAF1 containing haploid YCR072c spores would be viable, thus confirming our hypothesis.

Sporulation of YCR072c

Sporulation of budding yeast is best produced by initial growth in high glucose media (pre-sporulation media) and removal of a log phase aliquot into sporulation media. Using this method a sporulation frequency of over 90% can be achieved. However, utilising this method, the YCR072c strain produced very poor rates of sporulation. The immediate consequence of this was very few tetrads available for analysis. In order to see if this was due to a technical shortcoming the yeast strain BY4743 (the pure wild type strain on which the YCR072c+/- knockout is derived) and the non-related yeast strain CG1945 were also sporulated in identical conditions. Using CG1945 a sporulation frequency of 83% was achieved (figure 49). The BY4743 strain sporulated at a frequency of 24% (this is a strain that sporulates at low frequencies [229, 230]) . Despite alteration of the sporulation protocol it was not possible to increase sporulation frequency to a degree whereby sporulation analysis by dissection would be efficient.

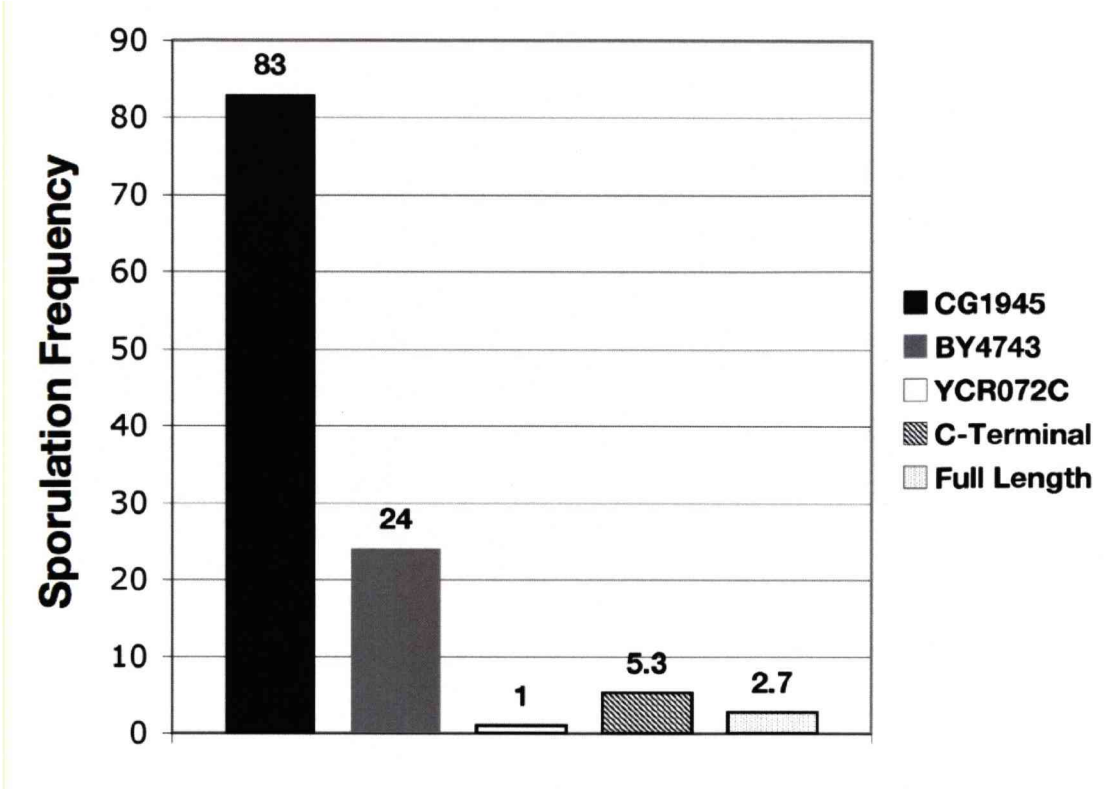


Figure 49: SPORULATION FREQUENCY OF YEAST STRAINS

The poor sporulation frequency of the YCR072+/- strain was puzzling, why should a single heterozygous knockout albeit in an essential gene have such a phenotypical consequence? The poor sporulation frequency observed with the YCR072c+/- strain could be a direct result of the YCR072c gene knockout. Analysis of genetic databases showed that expression of YCR072c is specifically repressed during sporulation [214] indicating a role in the sporulation program and hence could be contributing to the poor sporulation frequency observed. Based on this observation we hypothesied that our APAF1 constructs could influence the sporulation frequencies we had seen.

We looked at the following criteria when evaluating sporulation (see Figure 48):

i. Yeast cell of normal appearance

ii. Abnormal/Enlarged

iii. 2 spore formation

iv. 3 spore formation

v. 4 spore formation

Sporulation Analysis

YCR072C was transformed with either empty vector (pCM188), a carboxy terminal APAF1 construct (CT), or full-length APAF1 (FL). Yeast were then subjected to a standard sporulation protocol and sporulation frequencies analysed (see Materials and Methods).

We noted that in all cases the APAF1 constructs increased the ability of the yeast to undergo enter and complete sporulation (abnormal cells and tetrads, $p<0.001$ Chi Squared, Figure 50). In particular the C-terminal gave consistently better results than the Full length APAF1 construct (possibly due to its greater homology to YCR072C).

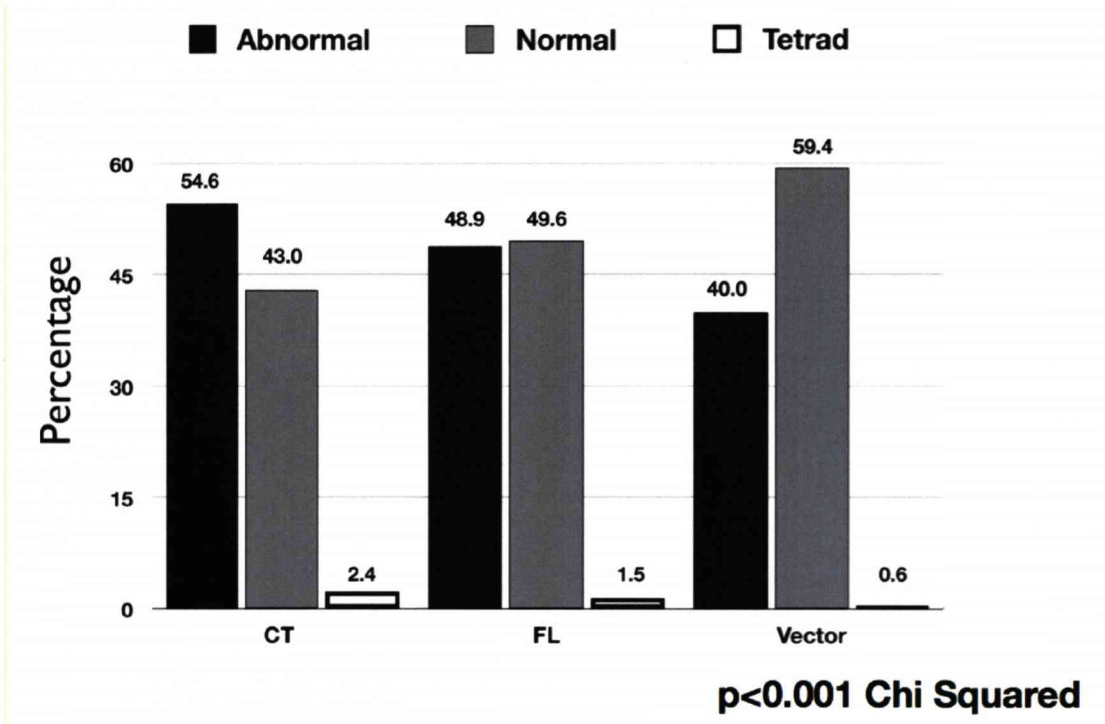


Figure 50: HISTOGRAM SHOWING PERCENTAGE SPORULATION (AND STAGE) FOR EACH CONSTRUCT

Sporulation frequency was assayed in YCR072c using vector, C-terminal APAF1 (CT) and Full-Length APAF1. Successful sporulation was indicated by the presence of tetrads. Attempted sporulation was indicated by the presence of abnormal cells. Normal cells were considered not to have undergone sporulation.

SECTION 4: DISCUSSION

4.1 COMBINATION THERAPY IN CANCER

Pancreatic cancer remains largely resistant to conventional therapies [192], although early-stage pancreatic cancer treated with surgery and adjuvant 5FU based chemotherapy has significant survival benefit [19]. To improve survival further, combination therapy with established agents has been suggested and is being actively investigated [192].

Combination therapy for cancer has been based on four precepts:

1 Combination of agents that have non-overlapping toxic profiles will allow an increase in overall active agent dose without increasing overall toxicity

2 Combination therapy will help circumvent the development of resistance to the component agents

3 Combination therapy can possibly improve toxicity in heterogenous tumour populations with differing resistance/sensitivity profiles

4 Combination therapy may be synergistic

With regard to HSP90 inhibitors there have been numerous reports of synergy when combined with other chemotherapeutics (see Introduction). However the presence of synergy *in-vitro* may not be translated into *in-vivo* synergy or clinical synergy (*vis-à-vis* murine models to human trials). This thesis investigates the role of HSP90 inhibition in pancreatic cancer.

The commonest method of assessing synergy is the isobologram method (reviewed by Tallarida[217]). As explained in the results section, the construction of an isobologram is

dependent on the definition of identical endpoints for the comparator drugs. Pancreatic ductal adenocarcinoma is characterised by resistance to chemotherapeutics (reviewed in [192]) and as we hypothesised that HSP90 inhibition could alter this resistance, we chose to use a pancreatic cell-line noted for its chemoresitance [216]. It was not possible to induce lethality in the Suit2 cell-line at physiologically relevant concentrations. This precludes the use of LD50 as an endpoint for isobolar analysis. I attempted to define surrogate endpoints but felt this would compromise use of the isobologram.

The published data shows that cellular metabolic activity will respond rapidly to potentiation of cytotoxics, while low levels of HSP90 inhibitors alone will principally act to reduce cell division [218]. Accordingly we used the MTT assay to assess metabolic level, rather than absolute survival/growth. In this way rapid changes in the metabolic health (vitality) of the cell could be identified.

The number of agents that have been found to be synergistic with HSP90 is increasing (see Introduction). These agents have differing modes of action, yet show enhancement by HSP90 inhibition. In the experiments performed we demonstrated that HSP90 inhibition could potentiate both 5FU and TPA, but that this was altered by serum-starvation. In the serum-deprived context there was mutual inhibition rather than synergy. The experimental results indicate that synergy is dependent on the tumour environment and may even be reversed under certain conditions. The consistency of these observations with two differing agents that have differing modes of action suggest that HSP90 inhibition is influencing the cells non-specific response to a toxic stimulus.

We will discuss the effect of serum-starvation first.

4.2 HSP90 INHIBITION CAN HAVE BOTH PRO AND ANTI-APOPTOTIC EFFECTS

HSP90 antagonists can display pleiotropic antagonism, i.e. both pro and anti-apoptotic features. In the model in Figure 51 the action of HSP90 is shown (Serum Present side). Nascent folding of proteins initially involves the HSP70 super-family. A specific group of proteins requires HSP90 for maturation. These proteins are targeted to HSP90 by co-chaperones such as cdc37, following "priming" by folding HSP70. ATP binding to HSP90 activates the "clamp" mechanism of HSP90 and the client is held in place. A pool of free client will be maintained by release from HSP90 following ATP hydrolysis. HSP90 inhibition acts by preventing the formation of the mature client-HSP90 complex. Hence this pool of free client protein is reduced. GA can shift the client-HSP90 multimer towards CHIP mediated proteasomal destruction [51].

HSP90 has also been shown to be capable of binding activated clients, bringing about inactivation of transcriptional regulatory complexes [231] and activated steroid receptors [232]. This is described in the right hand side of the serum present model. HSP90 is an essential step in the maturation of nascent client protein to the point where it can be activated, but it is also inhibitory as shown on the diagram and by the role of targeted protein degradation. This inhibitory effect was described by Pandey *et al.*[177] in the case of the pro-apoptotic protein APAF-1. This model explains how HSP90 can have pro- and anti-apoptotic effects.

4.3 GA IS PROTECTIVE AGAINST 5FU DURING TRANSITION TO SERUM FREE CONDITIONS

Serum-starvation was investigated because it was felt to be the simplest way to model the relatively avascular environment of pancreatic cancer. Withdrawal of serum has multiple effects, the reason it causes the synergy between GA and 5FU (or TPA) to be reduced is not clear. The profound shift in HSP90 from the cytosol to the nucleus may contribute to this phenomenon. In the patent application by Johnson *et al.* they demonstrate that use of a nuclear export inhibitor can reduce synergy in breast and colon cell lines [221]. The prevention of nuclear export would lead to a situation whereby HSP90 that shuttles between nucleus and cytosol accumulates in the nucleus. The initial results with serum containing media show that under normal tissue culture conditions the pro-apoptotic effects of GA subsume its anti-apoptotic effects. However, the serum-free data suggests that during serum-starvation the anti-apoptotic effects (i.e. survival) can be amplified.

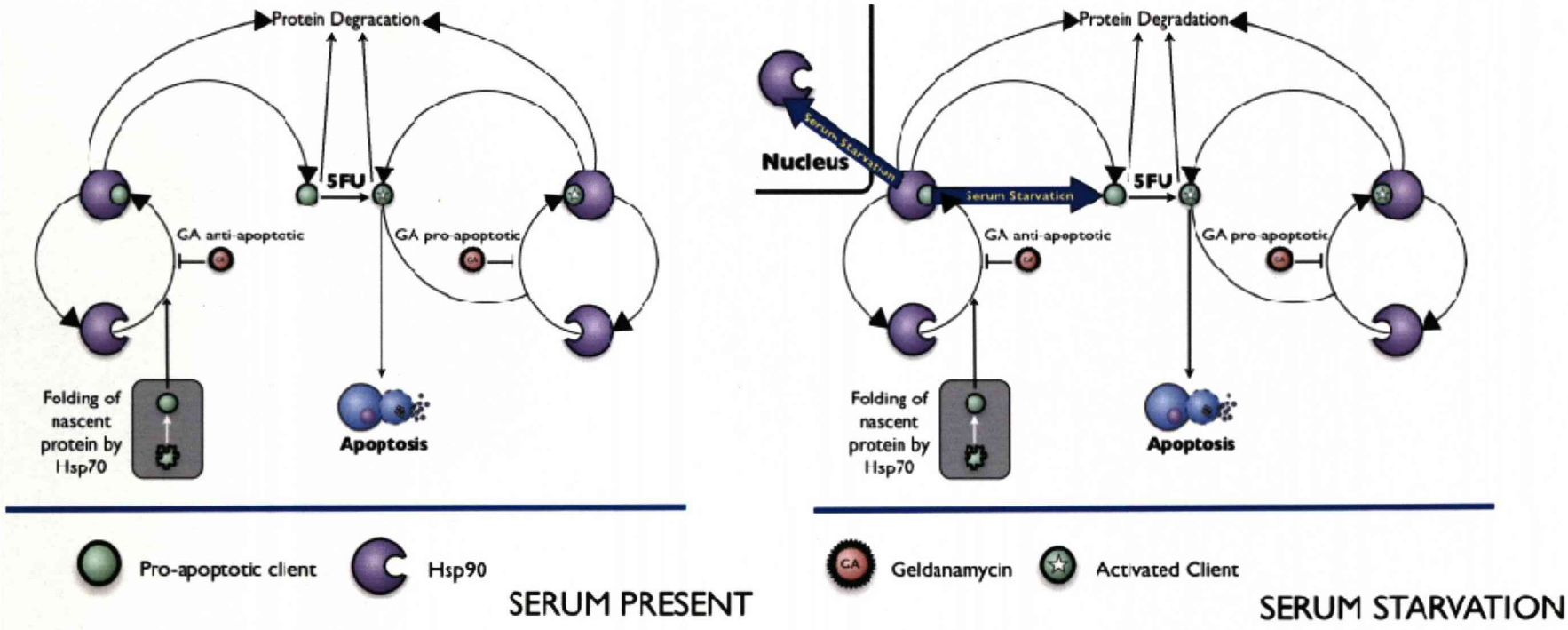


Figure 51: MODEL OF ACTIVITY OF HSP90 AND ITS ANTAGONISTS

During serum-starvation there is a rapid reduction of cytoplasmic HSP90 (certainly maximal between 16 and 24 hours). The shift of HSP90 to the nucleus will result in the release of previously bound client, in the context of a pro-apoptotic client this is consistent with the known sensitisation of many cell-lines to apoptosis during serum-starvation [233]. In this scenario GA would continue to reduce the processing of client by HSP90. Before serum-starvation most processed client would be maintained inactive or targeted for destruction, so the anti-apoptotic effect of GA would be mitigated. During nuclear localisation of HSP90 most client-HSP90 binding will be transient (the broader arrow leading from bound client to the activation ready pool in the model (Serum Starvation side).

The anti-apoptotic effect on the left side of the diagram will be significant, as HSP90 binding at this stage remains essential for eventual protein activation. In contrast the pro-apoptotic effect of GA, on the right side of the serum-starvation model, is reduced, as GA will be blocking what would in any case be a transient and non-essential interaction. After a period of serum-starvation a steady state will be resumed (the broad arrows will only apply during transition to serum-free conditions), hence the protective effect of GA is only seen during the transition from serum containing to serum-free media.

4.4 POTENTIATION OF 5FU BY GA FOLLOWING GA PRE-TREATMENT

The results also demonstrate that pre-treatment with GA with subsequent combination treatment using 5FU is potentiated. The effect of pre-treatment with GA can also be explained on the basis of the known mechanism of HSP90 action. The pool of apoptotic client available for activation will decline in the presence of GA.

With 5FU treatment, activation of this reduced pool may be insufficient to induce apoptosis. Binding of HSP90 to the activated client will reduce the apoptotic pressure, so the same pool of activated client in the presence of GA may be inadequate to induce cell death. 5FU treatment would therefore be predicted to be less effective after GA treatment unless the GA treatment was continued.

4.5 HSP90 INHIBITION CAN BE CYTOPROTECTIVE

Although most investigation of HSP90 inhibitors has shown at least additive effects when combined with chemotherapeutics, HSP90 inhibitors can sometimes protect against cell death. HSP90 antagonists can contribute to cell survival through the activation of the cytoprotective heat shock response. In addition, the scheduling of GA administration (whether GA is administered before or after conventional chemotherapeutics) can lead to reduced effectiveness of combination chemotherapy and, finally, HSP90 inhibition has been reported to antagonise the conventional chemotherapeutic cisplatin. We will discuss the general cytoprotective effect that can be seen with HSP90 inhibition first.

HSP90 plays a significant role in the generation of the heat shock response. Normally HSP90 will sequester the HSF (heat shock factor) in the low affinity intermediate state (see Introduction). In response to proteotoxic stress the HSP90 pool preferentially binds to the greatly increased numbers of misfolded cellular proteins. This displaces HSP90 from the HSFs. The unbound HSFs translocate to the nucleus where they become transcriptionally active after trimerisation. All HSFs bind to consensus heat shock elements (HSEs) within the promotor regions of heat shock proteins. Although four HSFs have been described (HSF1-4), HSF-1 is the predominant factor inducing the heat shock response. HSF-1 is an absolute

requirement for the drug-induced heat shock response [234]. HSFs will upregulate multiple heat shock protein families with resultant cytoprotection. HSP90 itself is upregulated and is believed to complete a feedback cycle whereby it will be able to sequester cytoplasmic HSF and return the cell to the status quo.

Geldanamycin will disrupt HSP90-HSF binding and release sequestered HSF, allowing it to become transcriptionally active. In untransformed cells this will stimulate a heat shock response that may well ameliorate the cytotoxic effects of GA [234]. There have been reports that failure of a cell to activate the heat shock response renders it more sensitive to HSP90 inhibitor mediated toxicity [235, 236]. However, the heat shock response seen with HSP90 inhibition is not of the same magnitude as that seen with metabolic poisons such as heavy metals. This may be because the pleiotropic effects of HSP90 inhibition may affect kinases that phosphorylate HSF to aid trimerisation and potentiate their transcriptional activity [237, 238]. Finally, the cancer cell is a stressed cell and the heat shock response may be maximal and HSP90 inhibition will be unlikely to contribute further.

The effectiveness of the combination of GA with Paclitaxel was found to be dependent on the schedule of GA administration [183]. If GA was given before Paclitaxel then there was a reduction of apoptosis. This was thought to be because GA shifted the cells towards an Rb dependent G1 arrest and paclitaxel was a G2/M active agent. In our data with 5FU, pre-treatment with GA left the cells resistant to 5FU (5FU is most active in S phase, i.e. S phase will not be entered after GA arrests the cells in G1), this would be consistent with the Munster findings. However, combination of 5FU and GA following pre-treatment with GA would therefore be predicted to be less effective, this was not the case. In these experiments the single drugs (GA or 5FU) had no effect but the combination caused a significant reduction in vitality. This was considered to be a form of synergy. The FACS data show that

GA treated cells show a reduction in the S phase population and an apparent increase in G2 and post-G2 cells. Removal of GA allows complete recovery of the cell-cycle profile within 24 hours. As continuous GA treatment gives the greatest reduction in S-phase and 5FU in combination with continued GA resulted in the greatest reduction in vitality, the protective effect of GA is unlikely to be as a result of cell-cycle arrest.

HSP90 inhibition has been reported to be anatagonistic in a colon cancer cell model[239]. Here both GA and 17AAG antagonised the action of Cisplatin in a cell-cycle independent manner. The authors of this report ascribed the negative effect of HSP90 inhibition to p53 and differential caspase activation. However, although the possible role of Cisplatin as an HSP90 inhibitor was briefly discussed, this was not expanded upon and there remains the possibility that HSP90 inhibitory effects of Cisplatin may have contributed to the results noted.

4.6 APAF-1 SPECIES CAN BE MODULATED BY SERUM-STARVATION AND GA

The apoptotic protein APAF-1 is a recognised client of HSP90 [177]. In the report by Pandey *et al.*[177] they describe full-length APAF-1 to be negatively regulated by HSP90. In our set of experiments we noted that APAF-1 was present in human pancreatic cancer tissue and was a relevant subject for investigation in the pancreatic cell line Suit2.

APAF-1 was present as a full-length form (FL) and as a smaller Mr form that reacted to the C-terminal antibody we used. In serum containing cell culture both species are present, but on serum withdrawal there is marked and rapid loss of the CT species. The sequence of both FL and CT APAF-1 species is unknown although the data showing caspase-9 cleavage is consistent with a functional APAF-1/Apoptosome

complex. There are reports of isoforms of APAF-1 that are characterised by the extra WD40 regions [130] and also data showing that APAF-1 is proteolytically processed into a predominantly WD40 containing protein. The CT species here is probably the breakdown product of APAF-1 reported as being produced in response to an apoptotic stimulus [240].

The behaviour of the isoforms in response to serum and serum-starvation can be explained with reference to the models above. In the presence of serum and the absence of any apoptotic stimulus we expect to see a pool of activateable APAF-1 held by HSP90 ready for release. The observation of the truncated form of APAF-1 suggests that there is ongoing processing of APAF-1. In the context of apoptotic activation by cytochrome C release this would allow release of the active Caspase-9, but in the absence of an apoptotic signal this probably just represents turnover of the protein. Blocking HSP90 with GA increases the concentration of the fragment, suggesting that this turnover of APAF-1 can occur in the absence of HSP90 binding, potentially when the APAF-1 protein fails to bind HSP90 after translation. In the case of serum starvation the movement of HSP90 into the nucleus would not prevent binding of HSP90 to APAF-1, but, I would suggest, would make this binding transient, releasing full length APAF-1. HSP90 would normally regulate the processing of APAF-1, presumably giving the cleavage product, so premature release will reduce the concentration of this product. The immunofluorescence data in Figure 39 shows initial colocalisation of HSP90 with APAF-1. This is markedly reduced on serum-starvation. The cellular content of HSP90 is much greater than that of APAF-1; accordingly much of the APAF-1 signal will be subsumed by the HSP90 signal and the yellow of co-localisation more subtle. Note that there are areas in the cells where there is APAF-1 not colocalising with APAF-1, this would represent APAF-1 that can be processed independently of HSP90.

The results also indicate that the modulation of APAF-1 species is achieved rapidly (certainly by four hours as displayed by the Western data in Figure 38). Such rapid alteration in protein levels and activity cannot be regulated at the transcriptional level and requires input at the protein level.

4.7 SERUM-STARVATION IS ASSOCIATED WITH NUCLEAR TRANSLOCATION OF HSP90

Serum-starvation was found to modulate the response of the cell line to combination HSP90-inhibitor treatment. The most notable finding was that serum-starvation induced the translocation of HSP90 from the cytosol to the nucleus. This was maximal at 16-24 hours. The immunofluorescence data shows convincing evidence of a compartment shift for HSP90. The nuclear HSP90 appears to be excluded from the nucleoli. Although no stain for chromatin was used, three-dimensional analysis of the HSP90 staining is conclusive for its nuclear location. HSP90 is not considered to have an orthodox nuclear localisation signal (NLS) but does possess a carboxy-terminal cytoplasmic localisation signal [241]. Putative functions of nuclear HSP90 include telomere maintenance (through hTERT [149]), centromere function [242] and interestingly, ribosome biogenesis [243]. Although the IF data is strong on the qualitative aspects of HSP90 (i.e. location) it is a poor assay of quantity (i.e. amount). The Western blot data confirms the increase in the nuclear fraction of HSP90. The Western blot also shows HSP90 to be present in the nuclear fraction even in the presence of serum. This is not seen in the IF images. A small proportion of HSP90 is known to be present in the nucleus and it is likely that the Western data is picking this up and that the IF was not sensitive enough to show nuclear HSP90 in the serum containing state. It is also possible that the nuclear fractions in the Westerns may not have been "pure" and some cytoplasmic HSP90 could have been

present, although this is unlikely given the absence of Actin in the nuclear fraction controls.

In the presence of serum HSP90 would be predicted to bind cytoplasmic clients (e.g. APAF-1, Raf-1) and following nuclear translocation HSP90 binding may be preferential to nuclear proteins such as telomerase and cdk4. The shift of cdc37 towards the nucleus from the cytoplasm may reflect an increase in targeting of HSP90 to nuclear clients.

The staining of HSP90 in human pancreatic cancer tissue shows marked nuclear localisation. This staining is heterogeneous [244] and may represent differing areas of response of HSP90 to stimuli that promote nuclear localisation. The work withSuit2 suggests these stimuli may include reduced extra-cellular mitogen signalling. The work presented here does not demonstrate that nuclear localisation of HSP90 *in vitro* or *in vivo* can increase or decrease the sensitivity of tumour cells to combination treatment with HSP90 antagonists and other chemotherapeutics. However, if the localisation of HSP90 in tumours is dynamic, perhaps influenced by levels of vascularisation, then the potentiating effect of HSP90 in stable regions of the tumour may be compensated by reduced activity in cells that are in transition. This may promote the development of small populations of cells that will never regress unless the HSP90 inhibitor is removed allowing partial regression of tumours followed by recurrence. This should be considered when analysing the results from clinical trials of therapy with HSP90 inhibitors in combination with other agents.

4.8 EXTENDING THESE STUDIES TO OTHER CELL LINES

The cell-line results presented earlier are all based on the pancreatic cell-line SUIT2. SUIT2 was chosen because its origins (derived from a pancreatic cancer liver metastasis) were felt to represent the commonest clinical scenario of advanced

pancreatic cancer. As discussed in the introduction, at this stage of neoplastic progression there has been selection pressures for an aggressive phenotype with characteristic survival attributes.

The characteristics of any compound considered for use in pancreatic cancer therefore must include activity against metastatic cells. Accordingly, the cell-line SUIT2 was deemed suitable for investigation with geldanamycin because it is derived from a pancreatic cancer liver metastasis. This is clinically important because it is metastatic pancreatic cancer that causes patient death. In addition SUIT2 is noted for its chemoresistant properties, again, this is particularly apposite because of the relatively poor response of patients to conventional chemotherapeutics.

The use of cell-lines derived from primary pancreatic cancer tissue (e.g. PANC-1) could give misleading results i.e not be applicable to cells that have developed the capability to metastasise. The evolutionary progression of cancer cells shows not only gain of metastatic characteristics but a loss of other features- a process termed de-differentiation. In many ways the metastatic cell is built purely for survival and shows increasing resistance to drugs. The cell at this point may have very little in common with the tumour it is derived from, and accordingly may respond completely differently to treatment.

Limited resources meant that only confirmatory experiments were performed on other pancreatic cell lines and full analysis was only possible on SUIT2 (again to re-emphasise, the chemoresistance of SUIT2 was felt to be more typical of metastatic pancreatic cancer, possibly because of SUIT2 derivation from a pancreatic cancer liver metastasis). However, the basic observations of nuclear translocation upon

serum withdrawal, presence of APAF were confirmed in other cell lines (PANC1) (Figures 52 & 53).

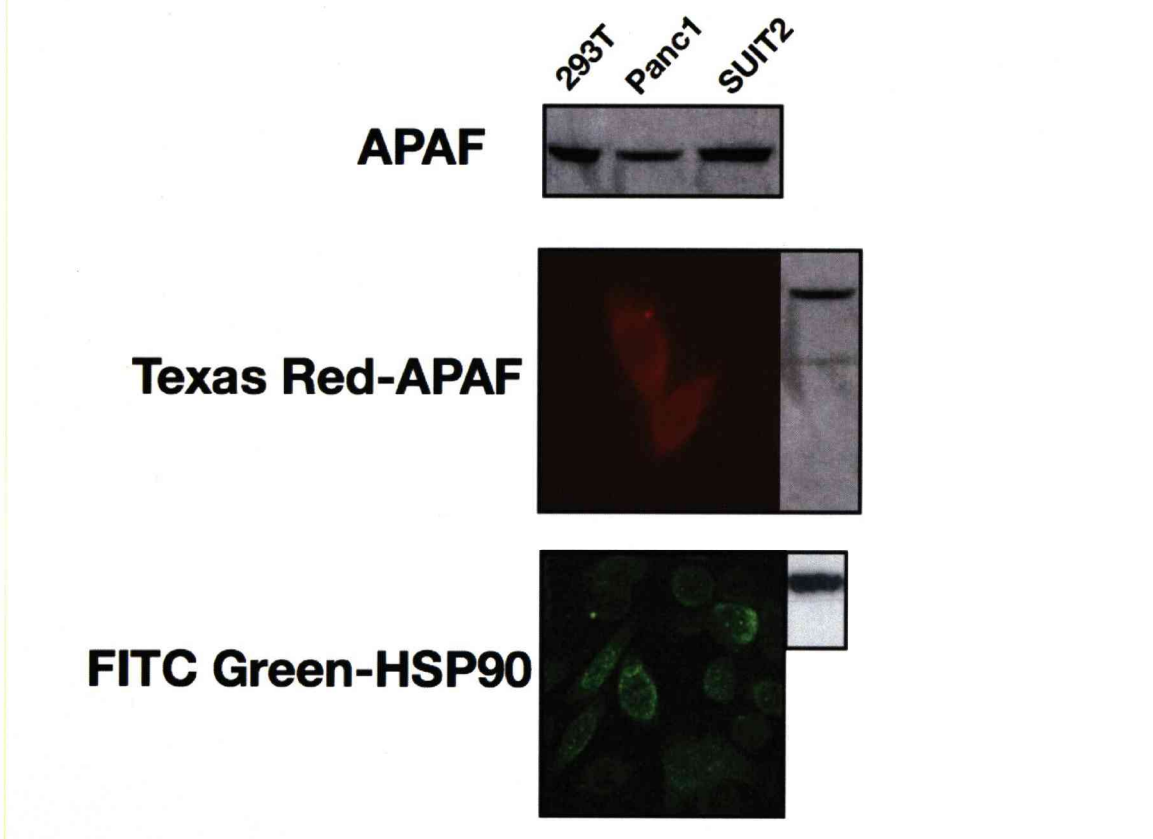


Figure 52: HSP90 AND APAF-1 ARE PRESENT IN THE CELL-LINE PANC1
Western blot showing APAF-1 in both PANC1 and SUIT2 (293T is the positive control cell-line). Immunofluorescence showing APAF-1 in PANC1. Immunofluorescence showing normal cytoplasmic location of HSP90 in PANC1 cells under serum-containing conditions.

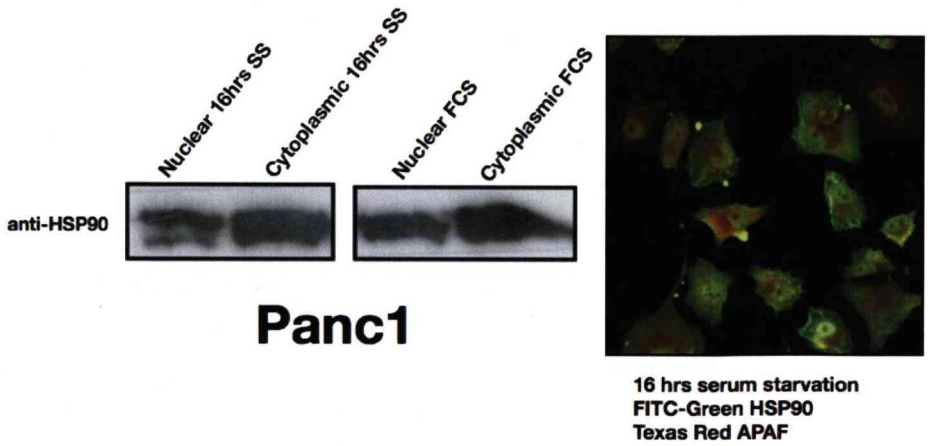


Figure 53: SERUM STARVATION OF PANC-1 CELLS RESULTS IN NUCLEAR TRANSLOCATION OF HSP90

It would be premature to extrapolate the results seen with the clonally derived SUIT2 cell line into that of heterogenous *in vivo* cancer. However, in terms of a model for the mechanism of action of combination use of Geldamaycin and chemotherapeutics a single cell line is acceptable. Indeed a number of groups have used single cell-line experiments (using SUIT2) to model the effects of pancreatic cancer therapeutics [245, 246].

Further work based on this model can be undertaken using a bank of cell-lines (both pancreatic and non-pancreatic) to assess their response to GA with or without serum-starvation. Such experiments can easily accommodate combination treatment with chemotherapeutics. The model described posits that APAF-1 could be a mediator of geldanamycin's effects. In order to elucidate whether APAF-1 is the actual mediator experiments using APAF-1 -/- cell-lines, or targeted repression of APAF-1 expression (for example using RNA inhibition) would be required. Such experiments have not been performed and are part of future work that can be carried out.

It is clear that the use of a chemoresistant cell-line such as SUIT2 with its limited apoptotic response to chemotherapeutics means that the output of experiments is small. Ideally, cell-lines with greater response to apoptotic stimuli such as 5FU/TPA would allow greater resolution of the effects associated with serum-starvation and HSP90 inhibition. This represents a strong argument for the investigation of other cell-lines with a more favourable response to chemotherapeutics, although for the reasons stated above, these may not be pancreatic cell-lines.

Finally, it is recognised that in the experimental protocols used the endpoints chosen may affect the apparent activities seen, particularly when the growthrates appear to change through the time course of the experiment.

4.9 RELEVANCE FOR CLINICAL USE

Geldanamycin was the first HSP90 antagonist developed. Unfortunately it is an unsuitable drug for human use because of its hepatotoxic side-effects. However GA is suitable for *in vitro* studies because of its prototypical mode of action and as such its derivatives can have similar effects [239]. The recent introduction of GA derivatives such as 17AAG with better hepatotoxicity profiles has allowed phase I/II clinical trials to begin [181]. Alongside their clinical use, 17AAG and 17DMG have become more widely available to allow *in vitro* work to be performed.

The work presented here adds to our knowledge of HSP90 inhibition by adding caveats to the belief that HSP90 antagonism can potentiate chemotherapeutics. We have shown that in pancreatic cancer there may be environmental factors that could reverse any potentiation seen.

Although the work here represents the effects of GA (i.e. blocking the nucleotide binding site of HSP90) this may not translate to similar effects with HSP90

antagonists with differing modes of action. Further work needs to be done with agents such as Novobiocin, because differences in effect may contribute to our knowledge of HSP90.

4.10 LINKING APAF-1 TO YEAST

The cell-line work described above demonstrates that inhibition of HSP90 can modulate the response to pro-apoptotic signals. We looked at whether APAF-1 could be a mediator of the effects of HSP90 inhibition. The data showed that APAF-1 could be modulated by both HSP90 inhibition and serum-starvation.
We initially looked at whether the APAF-1 and HSP90 interaction could form the basis of a screening tool for novel HSP90 inhibitors. The Yeast two-hybrid system was investigated but unfortunately this did not prove effective. We next looked at whether a functional assay based on the effect of APAF-1 in a yeast model could form the basis of a screening tool. These results led us consider a link between APAF-1 and the yeast protein YCR072c.

As described in the introduction the heat shock response is an archaic, evolutionary conserved program. Metazoans have retained the basic elements of this survival response and integrated them into more advanced stress response programs. Such a program is programmed cell death, of which apoptosis is of crucial importance in cancer. The mitochondrion is the powerhouse of apoptosis but the mitochondria also plays an important role in another stress response- that of ribosome biogenesis and metabolic regulation. Recent work has suggested that YCR072c contributes to ribosome biogenesis and this has implications for the experimental results derived here.

4.11 RIBOSOME BIOGENESIS, STRESS AND CANCER

The ribosome is the seat of all protein synthesis and the production of mature ribosomes is a highly coordinated, multistep program that largely takes place in the nucleolus (reviewed by Ruggero[247]). The ribosome itself is a heterogeneous multimeric machine constructed from 4 rRNA species and 80 ribosomal proteins. Our understanding of eukaryotic ribosome biogenesis is largely derived from studies on the budding yeast *Saccharomyces cerevisiae*. There are three ribosome RNA transcripts formed in the nucleolus (18S, 5.8S and 25S) and these are transcribed as a large primary transcript known as pre-rRNA (45S). A separate 5S rRNA species is independently transcribed. The pre-rRNA transcripts undergo a series of maturation steps and finally produce 18S, 5.8S and 25S rRNA. During rRNA processing the rRNA species must associate with ribosomal proteins within the nucleolus before forming the characteristic small (S,40S) and large (L, 60S) ribosomal subunits which are assembled and transported to the cytoplasm to initiate protein synthesis. The assembly of the rRNA with ribosomal proteins (r-proteins) involves a number of r-subunit assembly factors, which do not form part of the final ribosome machine. The rRNA is derived from an rDNA (ribosomal RNA) template. Synthesis of rRNA from the rDNA template appears to be gated to the cell-cycle- it is maximal in S and G2 phases, repressed in mitosis and increased in G1 [248-250]. This variation in rRNA synthesis is dependent on the activity of PolI and the transcription factor UBF (upstream binding factor). UBF is phosphorylated in response to mitogen stimuli (e.g. cdk4-cycD [251]) and cell-cycle progression. The phosphorylated form of UBF affects its ability to bind DNA and mediate protein-protein interactions required for the assembly of the complex required for transcription [252, 253].

In yeast there are approximately 900 genes that show altered expression during environmental stress [254, 255]. One set of genes that were found to be transiently

repressed were those that encode proteins in ribosome structure and biogenesis. It has been estimated that exponentially dividing yeast produce approximately 40 new ribosomes per second[250]. This ensures that ribosome biogenesis is a significant consumer of cellular energy resources, which can be as high as 50% of cellular energy [250]. Accordingly repression of ribosome biosynthesis under adverse environmental conditions may liberate energy for other cellular processes. In addition the reduction in translation could also prevent the synthesis of proteins that could interfere with the stress response. Recently the stress responsive yeast protein Yar1 has been reported tointeract with RpS3 (a component of the 40S subunit) and Ltv1 (a component of the 43S preribosomal particle), thus linking stress responses with ribosome biogenesis [256].

In yeast the molecular response to glucose deprivation (as in sporulation) involves a shift from fermentation to oxidative phosphorylation. This shift to oxidative phosphorylation in the context of caloric restriction is associated with an increased lifespan [257]. It is not clear precisely how this affects cellular senescence but the protein SIR2 is required. Sir2 (Silent information regulator 2) acts as a NAD-dependant histone deacetylase to suppress recombination and turns off transcription at multiple genomic loci [258, 259]. In yeast there is a finite number of divisions that a mother cell can undergo. This limited replicative capacity present within mother cells can be used as a metric of yeast senescence. With multiple rounds of cell division there is increased rDNA recombination and a shortening of lifespan, however an extra Sir2 copy can increase rDNA stability and extend lifespan[260]. Sir2 may act to couple cellular energy utilisation to longevity and Sir2 levels may be increased by the altered NAD/NADP ratio seen in oxidative phosphorylation, or in addition, through reduction in the levels of the Sir2 inhibitor Nicotinamide [258]. Sir2 is related to the mammalian Sirt protein family that contains 7 proteins (Sirt1-7) [260]. Although

yeast Sir2 appears only to interact with chromatin, the Sirt proteins are known to interact with other proteins such as p53 and forkhead transcription factors [258].

In our yeast experiments using the heterozygote YCR072c+/- we noted that induction of sporulation was inefficient and of low frequency. Despite numerous modifications to the experimental protocol it was not possible to achieve adequate sporulation. DNA microarray work to explore the temporal program of gene expression during sporulation has revealed that the expression of YCR072c is altered significantly [214]. YCR072c is an essential gene that plays a role in sporulation and the poor sporulation rates seen in this strain probably reflects YCR072c loss.

Our data shows that introduction of an APAF-1 construct can improve sporulation frequency and increase passage through meiosis. This is intriguing. The results with the C terminal construct consisting entirely of the WD40 repeat region appears to be more effective than the full length APAF construct containing the N-terminal CARD domain.

Initially the suspicion was that this is a general effect mediated by the similarity in the WD40 domains of APAF-1 and YCR072c, however recent work has suggested a deeper connection.

YCR072c has recently been identified as the nucleolar protein Rsa4p [228]. Rsa4p is required in ribosome biogenesis, specifically in the processing of rRNA and trafficking of the 60S ribosomal subunits. Rsa4p is considered to be the 20th WD40 protein described as having roles in ribosome biogenesis. Given the context of only 55 yeast WD40 proteins [210] there seems to be a disproportionate over-representation of WD40 proteins in ribosome biogenesis. The WD40 motif is thought to provide a scaffold for further protein binding and interaction. The APAF-1 constructs did not rescue completely the YCR072c sporulation phenotype. This may be because

ribosome biogenesis requires expression of ribosomal proteins to be coordinated both spatially and temporally. This is not possible in a plasmid construct driven by a constitutive promoter.

There is a growing corpus of data that supports a primitive program of cell death within yeast (see Introduction) and there has been a report implicating the yeast WD40 protein Mdv1/Net1 along with Dnm1 and Fis1 in yeast programmed cell death. These data suggest a deeper link between WD40 proteins and stress than might be at first thought.

4.12 AGING, STRESS AND APOPTOSIS

The conceptual link between metazoan programmed cell death and the unicellular yPCD is difficult. Evolutionary theory would predict that yPCD would be a negative trait, however potential benefits to the colony would exist in the context of viral infection, nutrient deprivation etc. However, by definition there must be a *program* of controlled steps that lead to cell death with minimal fallout for the colony as a whole. In yeast the sensescence program represents a controlled process by which individual yeast die, albeit over approximately 30 generations. The yeast aging program links genomic instability with cell death. In *S cerevisiae* there is asymmetric cell division giving a larger mother cell and a smaller daughter cell. With each round of division the mother cell displays a specific phenotype characterised by circular rDNA species. The circular rDNA species are produced by homologous recombination of chromosomal rDNA. The rDNA circles are extra-chromosomal and have the ability to replicate. The extrachromosomal rDNA circles (ERC) segregate asymmetrically during cell division, almost exclusively ending up in the mother cell. Coincidental with ERC number increase there is fragmentation of the nucleolus [261].

Finally there is induction of a senescent program and cell death. This response can be ameliorated by SIR2.

It is possible that constituents of the yeast ageing-program may contribute to yPCD. Certainly, SIR2 contributes to yeast senescence and influences the production of ribosomal DNA circles and YCR072c/Rsa4p are involved in ribosome biogenesis and the sporulation program. Increasing longevity (i.e. preventing senescence) in yeast can be achieved through the sporulation program. In higher organisms metabolic stress responses can increase longevity (see Sir2 above). I suggest that the ribosomal proteins could provide a link between stress and cell death in unicellular organisms. These proteins may have contributed to the evolution of apoptotic proteins such as APAF-1.

4.13 THE RIBOSOME AND CANCER

Recent work has demonstrated a direct link between ribosomal proteins and cancer. Mutations in ribosomal proteins have been described in conditions with an increased risk of cancer. The *DKC1* gene is mutated in dyskeratosis congenita. Dyskeratosis congenita is characterised by premature aging and an increased susceptibility to squamous cell carcinomas. *DKC1* encodes dyskerin, a a pseudouridine synthase that mediates post-transcriptional modification of rRNA [262]. In Diamond-Blackfan anaemia (DBA) there is an abnormal small ribosomal S19 protein [263]. DBA is associated with an increased incidence of haematological malignancies.

4.14 THE APAF-1 WD40 DOMAINS MAY BE RELATED TO YCR072C

The nematode protein CED-4 is the primitive form of mammalian APAF-1 [125]. CED-4 does not contain WD40 regions, yet the WD40 motif provides the scaffold for apotosome formation and function [132]. The origin of mammalian APAF-1's WD40 region remains unknown. However, the work presented here suggests that APAF-1's WD40 region can contribute to the function of the yeast protein YCR072c. From the above discussion it is clear that the regulation of ribosome biogenesis can be considered part of ancient stress responses, but also that it can contribute to cancer. It may be that YCR072c is an evolutionary ancestor of the WD40 region of APAF-1.

SUMMARY

The work described here shows that the HSP90 antagonist geldanamycin can potentiate chemotherapeutics in a pancreatic cell-line. In addition the work demonstrates that during transition to serum-free conditions this potentiation can be reversed to such an extent that there is mutual inhibition. These effects are seen with two different agents- suggesting that HSP90 inhibition influences the cells non-specific response to a toxic stimuli possibly through APAF-1. The results presented here suggest that any benefit from co-treatment with HSP90 inhibitors may be exquisitely dependent upon the micro-environment of pancreatic cancer *in-vivo*. The identification of APAF-1 as a mediator of the potentiation seen with HSP90 inhibitors formed the basis for a yeast based screening system for novel HSP90 inhibitors. The work presented here shows that a sporulation based screening tool for HSP90 inhibitors based on the phenotype of YCR072c transformed with APAF-1

could be possible. Finally, as a result of this work we note a potential yeast homolog of APAF-1, Rsa4p.

FUTURE WORK

CELL LINE WORK

Response of cells to HSP90 inhibition and chemotherapeutics

The majority of the work shown has been performed in on cell-line (although preliminary work was repeated in PANC-1). Future work should involve a bank of cell-lines, including non-pancreatic cells. It is anticipated that the use of non-pancreatic cell-line may allow easier induction of apoptosis to allow easier exploration of the apoptotic programme induced by HSP90 inhibition.

The SUIT2 system presented should be explored *in-vivo.* A nude mice model using SUIT2 xenografts would be the next step. As well as looking tumour response to single and combined geldanamycin/chemotherapeutic, microscopic examination of the xenografts would be undertake to see whether there is differential cell death depending on distance from nutrient blood vessels (as a surrogate marker of serum-withdrawl)

HSP90 translocation to the nucleus

The profound shift of HSP90 to the nucleus requires further investigation. It is not known whether HSP90 is functionally sequestered within the nucleus or acts upon nuclear targets. Future work should address this question. We propose investigating whether blocking nuclear import will abrogate the effects of HSP90 inhibition.

YEAST WORK

The sporulation phenotype seen with APAF-1 transformants needs to be assessed in the presence of HSP90 inhibition. We hypothesise that the improvement in sporulation frequency will be abolished by treatment with geldanamycin- this would form the basis of a potential screening tool for novel HSP90 inhibitors.

SECTION 5: PUBLICATIONS ARISING FROM THIS WORK

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