Synergistic interactions of targeted therapy within signal transduction pathways

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A thesis is submitted in partial fulfilment of the requirements for the award of the degree of
DOCTOR OF PHILOSOPHY
of the
UNIVERSITY OF PORTSMOUTH

March 2012
Abstract

Introduction: Cancer of the same origin show considerable heterogeneity in sensitivity to chemotherapy both clinically and in vitro, and show rapid adaptation to chemotherapy based on gene expression. This study tested the hypothesis that anti-cancer drug exposure could render tumour-derived cells more susceptible to second agents, particularly those with specific molecular targets in survival pathways and with the knowledge of cellular pathways, determine new more effective molecularly designed regimens.

Materials and Methods: Single agent, combinational and sequential chemosensitivity of a series of cell lines and tumours was assessed using the ATP-based tumour chemosensitivity assay (ATP-TCA). Sensitivity data was correlated with gene expression, measured by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) in a TaqMan Array following extraction of mRNA from cell samples and standardisation to the housekeeping gene (PBGD). Mutation analysis kits utilising Amplification Refractory Mutation System (ARMS) and scorpion technology were used to establish the presence of activating mutations in EGFR, KRAS, BRAF and PI3K.

Results: Heterogeneity in cellular sensitivity to cytotoxic and targeted agents was observed. While gene expression was seen to show some correlation with sensitivity to signalling pathway combination targets, the complexity associated with cellular adaptation prevented the prediction of response to second agent sequential treatment.

Discussion: This study has identified potential novel combinations for use in ovarian cancer. This combination of EGFR and PI3K inhibitors has shown greater sensitivity in cell based assays compared with single agent activity and could become the focus of future clinical trials. Successful application of 384 well ATP-based chemosensitivity assays has shown to be a valuable tool for future cell based research. The quantity of viable tumour derived cellular material is diminishing; therefore, methods developed
here will continue to provide the means to complete these types of studies in the future.
Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>A, T, G and C</td>
<td>Adenine (A), Thymine (T), Guanine (G) and Cytosine (C) nucleotide bases</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ATP-binding cassette, sub-family B, member 1</td>
</tr>
<tr>
<td>ABCC1</td>
<td>ATP-binding cassette, sub-family C, member 1</td>
</tr>
<tr>
<td>ABCG2</td>
<td>ATP-binding cassette, sub-family C, member 2</td>
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<td>ABI</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha-Fetoprotein</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B (designation from AKR mouse strain)</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic Protease Activating Factor 1</td>
</tr>
<tr>
<td>ApppI</td>
<td>1-adenosin-5’-yl ester 3-(3-methylbut-3-enyl) ester triphosphoric acid</td>
</tr>
<tr>
<td>ARMS</td>
<td>Amplification Refractory Mutation System</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATP7A</td>
<td>ATPase, Copper Transporting, Alpha Polypeptide</td>
</tr>
<tr>
<td>ATP7B</td>
<td>ATPase, Copper Transporting, Beta Polypeptide</td>
</tr>
<tr>
<td>ATP-TCA</td>
<td>ATP- Tumour Chemosensitivity Assay</td>
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<tr>
<td>ATP-TSCA</td>
<td>ATP-Tumour Sequential Chemosensitivity Assay</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under Curve</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2 Associated Death Promoter</td>
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<tr>
<td>BAK</td>
<td>Bcl-2 Homologous Antagonist/Killer</td>
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<td>Bcl-2-Like Protein 2</td>
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<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
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<tr>
<td>BID</td>
<td>BH3 Interacting Domain Death Agonist</td>
</tr>
<tr>
<td>BIM</td>
<td>Bcl-2-like Protein 11 (apoptosis facilitator)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-raf Murine Sarcoma Viral Oncogene Homolog B1</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Ca</td>
<td>Carcinoma</td>
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<td>CA125</td>
<td>Cancer Antigen 125 or Carbohydrate Antigen 125</td>
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<td>c-Abl</td>
<td>V-abl Abelson Murine Leukemia Viral Oncogene Homolog 1</td>
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<td>Complete Assay Medium</td>
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<td>CDDP</td>
<td>Cis-diamminedichloroplatinum</td>
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<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinases</td>
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<td>Complementary DNA</td>
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<td>Cl</td>
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<td>CI90</td>
<td>Combination Index- calculated for 90% cell death</td>
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<td>CIN</td>
<td>Chromosomal Instability</td>
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<td>c-myc</td>
<td>V-myc Myelocytomatosis Viral Oncogene Homolog</td>
</tr>
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<td>CO2</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>Comet</td>
<td>Single Cell Gel Electrophoresis Assay</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle Threshold</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>DiSC</td>
<td>Differential Staining Cytotoxicity Assay</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
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<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
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<td>Deoxyribonucleic Acid</td>
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<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxribonucleotide Triphosphates</td>
</tr>
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<td>dUTP</td>
<td>Deoxyuridine Triphosphate Nucleotide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>EdU</td>
<td>5-Ethynyl-2’-deoxyuridine</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
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<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic Translation Initiation Factor 4E</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>ER</td>
<td>Oestrogen Receptor</td>
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<tr>
<td>ErbB</td>
<td>Epidermal Growth Factor Receptor (EGFR) Family</td>
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<td>ERCC1</td>
<td>Excision Repair Cross-Complementing 1)</td>
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<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
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<td>FCS</td>
<td>Foetal Calf Serum</td>
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<td>FISH</td>
<td>Fluorescence <em>in situ</em> Hybridisation</td>
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<td>FKHR</td>
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<td>FTI</td>
<td>Farnesyl Transferase Inhibitors</td>
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<tr>
<td>GGPP</td>
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<tr>
<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine Triphosphatase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HEPES</td>
<td>Hydroxyethyl Piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HER</td>
<td>Human Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
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<tr>
<td>HER3</td>
<td>Human Epidermal Growth Factor Receptor 3</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Hypoxia-Inducible Factor</td>
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<td>Hypoxia-Inducible Factor 1 α</td>
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<tr>
<td>HSP60</td>
<td>Heat Shock Protein 60</td>
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<td>Abbreviation</td>
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<td>IC90</td>
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<tr>
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<td>v-Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog</td>
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<td>Lactate Dehydrogenase</td>
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<td>LuLu</td>
<td>Luciferin-Luciferase</td>
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<td>MAP</td>
<td>Mitogen Activated Protein</td>
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<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein inase</td>
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<tr>
<td>Mcl-1</td>
<td>Induced Myeloid Leukemia Cell Differentiation Protein</td>
</tr>
<tr>
<td>MDM2</td>
<td>p53 E3 Ubiquitin Protein Ligase Homolog (mouse)</td>
</tr>
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<td>Multidrug Resistance</td>
</tr>
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<td>MDR1</td>
<td>Multidrug Resistance Protein 1</td>
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<td>MDT</td>
<td>Modular Dispense Technology</td>
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<td>MEK</td>
<td>Mitogen Activated Protein Kinase Kinase</td>
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<td>Modified Eagles Medium</td>
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<td>Hepatocyte Growth Factor Receptor</td>
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<td>Maximum Inhibitor</td>
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<td>microRNA 34a</td>
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<td>Mismatch Repair</td>
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<td>Matrix metalloproteinases (MMP’s)</td>
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<td>Media Only</td>
</tr>
<tr>
<td>MRP5</td>
<td>ATP-binding cassette, sub-family C (MRP), member 5</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite Instability</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
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<td>Mammalian Target of Rapamycin Complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian Target of Rapamycin Complex 2</td>
</tr>
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RLU
MTS  3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT  3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD+  Nicotinamide Adenine Dinucleotide
NADH  Reduced Nicotinamide Adenine Dinucleotide
NFκB  Nuclear Factor Kappa light chain enhancer of activated B cells
NSCLC  Non Small Cell Lung Cancer
NTC  Non-Template Control
p21  Cyclin Dependent Kinase Inhibitor 1
p27  Cyclin Dependent Kinase Inhibitor 1B
p38  Mitogen Activated Protein Kinase (including subunits 11-14)
p53  Tumour Protein 53
p70S6K  70 kDa Ribosomal protein S6 kinase
p73  Tumour Protein p73
P85α  Phosphatidylinositol 3-Kinase Regulatory Subunit Alpha
PBGD  Porphobilinogen Deaminase
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
PDGFR  Platelet Derived Growth Factor Receptor
PGK  3-Phosphoglyceric Phosphokinase
PI  Propidium Iodide
PI3K  Phosphatidylinositol-3 Kinase
PIK3CA  Phosphatidylinositol-3 Kinase, Catalytic, Alpha Polypeptide
pip3  Phosphatidylinositol (3,4,5)-Triphosphate
PKC  Protein Kinase C
PR  Progesterone Receptor
PSA  Prostate-Specific Antigen
PTEN  3'-Phosphatase with Tensin Homology
PUMAα  p53 Up-Regulated Modulator of Apoptosis
qRT-PCR  Real Time Polymerase Chain Reaction
Rad51  RAD51 Homolog
RAS  Small GTPase (Rat sarcoma)
Relative Light Units
RNA
Rnase
ROS
RTK
RT-PCR
S6K
S6K1
SCC
SIDR
STAT
T790M
Taq
TCER
TDC
TdT
Tg
TKI
TLDA
Topo1
TUNEL
UK
UNG
Activation
VEGF
WST
wt
XIAP
XTT
ZSTK474

Ribonucleic Acid
Ribonuclease
Reactive Oxygen Species
Receptor Tyrosine Kinase
Reverse transcription Polymerase Chain Reaction
S6 Kinase
S6 Kinase 1
Squamous Cell Carcinoma
Strathclyde Institute for Drug Research
Signal Transducer and Activator of transcription
Substitution of Threonine 790 with Methionine
Thermus aquaticus (DNA Polymerase)
Tumour Cell Extraction Reagent
Test Drug Concentration
Terminal Deoxynucleotidyl Transferase
Thyroglobulin
Tyrosine Kinase Inhibitors
TaqMan Low Density Array
Topoisomerase 1
Terminal Deoxynucleotidyl Transferase dUTP Nick End-Labelling
United Kingdom
Uracil-N-Glycosylase
Vascular Endothelial Growth Factor
8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (Water soluble Tetrazolium salt)
2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine
X-Linked Inhibitor of Apoptosis
2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine

XXI
Acknowledgements

The work in this thesis was performed in the Translational Oncology Research Centre at Queen Alexandra Hospital, Portsmouth, with Funding provided by Portsmouth Hospitals NHS Trust, CanTech Ltd, and the Rocky Appeal charitable funds.

I am grateful to Professor Cree for his continued guidance and support throughout this project and for all the opportunities that were provided through working in a translational research department.

I am indebted to many colleagues who supported me throughout this journey. To all the surgeons, physicians and nurses’ thank you for your assistance in obtaining consent from patients included in these studies. To Dr Louise Knight, thank you for your guidance and support in the beginning and for the collaboration and inclusion of my work on the mevalonate pathway for publication. To Louise Bolton thank you for your help in the analysis of data from mutation testing and finally to Penny Johnson, thank you for the use of your amazing histopathology skills, that while not included here, will help in the publication of work included in Chapter 6 -

I would also like to send my gratitude and sincerest thanks to all those patients that donated tissue essential for these studies and to the families of those whom are no longer with us.
Dedication

For my family

It is your support and patience that fuelled my desire to complete this thesis.

Thank you for not continually asking ‘so, when are you going to finish’.

For mum

You will be the only member of the family who will have read this thesis cover to cover. Although you will not have understood the majority of the work enclosed in these pages, you will pick most of the spelling mistakes. Thank you and sorry, as this will unlikely be the last thing I ask you to proof read.
Chapter 1 - Introduction
1.1 Hypothesis and Aims

We suggest that it is possible to drive neoplastic cells to adapt to low doses of anti-cancer treatment that will render them more susceptible to a second dose or course of anti-cancer treatment.

1. To determine whether anti-cancer drug exposure can render tumour-derived cells more susceptible to second agents, particularly those with specific molecular targets in survival pathways.

2. To use knowledge of cellular pathways to determine new more effective molecularly designed regimens.
1.2 Cancer Hallmarks and Tumour Biology

It has been over a decade since the seminal paper by Hanahan and Weinberg was published in the year 2000 outlining the hallmarks of cancer (1). They believed then; that a small number of underlying principles could explain the entirety of the cancer formation process. These six fundamental principles included; self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (1). Since its publication a vast amount of knowledge has been learned about why cells grow uncontrollably and the paper has recently been revised to include further hallmarks (2).

![Diagram of Cancer Hallmarks]

**Figure 1: Hallmarks of cancer (2)**

Unfortunately, the amount of knowledge learned is not necessarily proportional to its use in successful therapeutics. This comparison will not however remain unbalanced, advances in diagnostics, greater use of molecularly targeted treatments, individualised therapies and more effective maintenance treatments are beginning to appear. With the inclusion of four new hallmarks by Hanahan et al in 2011 (Figure 1) to include
deregulation of energy supplies, immune system evasion, genetic instability/mutation and inflammation (encompassing the effect cancer cells have on their surrounding environment), the ability to exploit treatments targeting combinations of these ten cancer hallmarks is now present and could lead to real benefits for patients (2).

Examples of how these 10 hallmarks of cancer are related to molecular mechanisms and cancer treatment are examined below.

1.2.1 Evading Growth Suppressors

Antigrowth signals have the ability to block proliferation and maintain quiescence and homeostasis in normal cells. For cancer cells to flourish they must evade these anti-proliferative signals. This can be achieved via cell cycle deregulation or entering irreversibly into postmitotic differentiated states.

The replication of normal cells is a highly regulated and accurate process. From the standpoint of chromosomes, there are four phases constituting the cell cycle. These comprise of DNA synthesis (S) and mitosis (M) which alternate with one another, separated by two "gap" phases (G2 and G1) required for preparation and growth. During the S phase DNA synthesis/replication occurs providing two sister chromatids (joined at the centromere) resulting in the existence of duplicated genetic material within the cell, ready to be divided into two daughter cells. The G2 phase follows successful completion of the S phase in which the cell prepares for mitosis. Both mitosis and cytokinesis define the M phase of the cell cycle. This stage can be further sub-divided to include: prophase; prometaphase; metaphase; anaphase; telophase and cytokinesis. The result is nuclear division and the production of two daughter cells that are genetically identical to each other. The G1 phase follows cytokinesis and is where cells increase in size, produce RNA and synthesise proteins. Cells may pause within this phase and become quiescent entering a temporary resting period (G0). These then require stimulation by growth factors before entering back into the cell cycle. Moving through these phases is mediated carefully with safeguards and checkpoints in place to ensure DNA is intact and that the cell is functioning normally. Deregulation of the cell cycle occurs when these checkpoints are disrupted which can lead to malignancy.
These cell cycle check points are mediated by cyclins and cyclin dependent kinases (CDKs) (3). The pattern of cyclin expression varies throughout the different phases defining the relative position of a cell within the cycle. The level of CDK’s within a cell is fairly stable; each must bind their appropriate cyclin in order to be activated. Once these complexes form, they have the ability to add phosphate groups to a variety of protein substrates, which in turn control processes in the cell cycle (4). Cell cycle inhibitors halt the progression of the cell through the cycle and are instrumental in prevention of tumour formation. Oncogenes such as c-myc also have the ability to activate or repress target genes involved in cell-cycle progression (5). C-myc has been shown to be amplified in various cancers and its deregulation linked to increases in cyclin A and cyclin E expression which are required for the transition of cells from G1 to S phase (6).

1.2.2 Sustaining Proliferative Signalling

Normal cells do not have the ability to actively proliferate without stimulation via growth signals. Cancers manage to circumvent this stimulatory dependence in many ways including; mutation of oncogenes, structural alteration of regulatory proteins such as AKT or Ras, deregulation of cellular receptors and autocrine growth factor stimulation.

AKT finds itself at the centre of a diverse signalling cascade involved in many pathways, one of which can result in continued proliferative signalling (Figure 2). This abnormal activation has being widely implicated in many cancers (7). Elevated AKT activation can result from a number of points within its signalling pathway. An example of enhanced AKT activation can be due to altered phosphatidylinosital-3 kinase (PI3K) activation resulting from; mutations (8) or amplification of growth factor receptors (such as ErbB family members) (9), RAS oncogenes (10), or PI3K itself. Overexpression and mutations of AKT itself can also contribute to the elevated expression in human cancers (11). Loss of function mutations as well as decreased expression of the 3'-phosphatase with tensin homology (PTEN) which shuts off PI3K signalling may also contribute to continual activation of AKT resulting in continued proliferative signalling (12, 13).
1.2.3 Resisting Cell Death

Proliferation is not the only means by which cancer cells can expand their numbers. Acquiring the ability to avoid programmed cell death (apoptosis) is also a major contributory factor. Avoidance of apoptosis can occur through several processes, of which loss of function or mutations involving tumour suppressor genes (e.g. p53) are frequently found in many tumours (14).

Apoptosis is controlled by an array of cell signals involved in an energy-dependent cascade of molecular events. These either originate intrinsically (mitochondrial pathway) or extrinsically (death receptor pathway) (15). In many cancers proapoptotic signalling components are shown to be downregulated or lost (Bax, Bak, Bid, Bim) while antiapoptotic components can be found to be upregulated (Bcl-2, Bcl-XL, Bcl-W) (14).
1.2.4 Enabling Replicative Immortality

Normal cells have a finite replicative potential, progressing through a definitive number of doublings before senescence. This replication limit is known as the Hayflick limit (16). For cells to develop into large tumours they at some point must breach this barrier and acquire unlimited replicative potential.

The ability of tumour cells to maintain telomeric DNA underlies their ability to possess unlimited replicative potential (17). Telomeres form a protective region of repetitive nucleotide sequences at each end of a chromosome. They are vulnerable to dysfunction due to their progressive shortening during each round of DNA replication. It has been shown that in advanced cancers, telomerase (the enzyme which adds DNA sequence repeats to the end of DNA strands) is reactivated serving to maintain telomere length and directly regulate cancer-promoting pathways (18).

1.2.5 Inducing Angiogenesis

Like normal cells tumour cells require the delivery of nutrients and oxygen and the ability to remove metabolic waste and carbon dioxide. These requirements are met by the development of a tumour neovasculature environment. The continued sprouting of these new vessels (angiogenesis) help sustain the expanding neoplastic growth of malignant tumours (19). These angiogenic regulators are often disrupted in tumour cells leading to possibilities of anti-angiogenic targeted therapy (20-24).

As an example of successful treatment targeting a hallmark of cancer bevacizumab (Avastin), an inhibitor of vascular endothelial growth factor (VEGF), was approved by the FDA in 2004 for the treatment of metastatic colorectal cancer. This monoclonal antibody binds to human VEGF (angiogenesis inducer) preventing it from binding to endothelial surface receptors lining blood vessel walls, thus preventing formation and growth of new blood vessels (25).

1.2.6 Activating Invasion and Metastasis

Tumour metastasis involves a succession of changes resulting in the development of distinct macroscopic tumours in another non-adjacent organ via transportation in the bloodstream or the lymphatic system. This is achieved when malignant cells break...
away from the primary tumour by the degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMP's). This family of zinc-dependent enzymes coordinate the breakdown of extracellular matrix and have been shown to be expressed by both tumour and stromal cells (26).

The loss of E-cadherin in many human carcinomas provides strong support for its role as a key suppressor of metastasis (27-29). E-cadherin is a key cell-cell adhesion molecule forming adherens junctions with adjacent epithelial cells, giving sheet like structures.

In ovarian cancer, E-cadherin expression in cells found floating in ascites and at metastatic sites is lower than in the primary tumour (30). Although this process is still not fully understood, cells with low E-cadherin expression have been found to be more invasive, while its absence predicts poor patient survival (31-36).

1.2.7 Deregulating Cellular Energetics
Cancer cells have been shown to have an altered glucose metabolism (37). These cells develop the ability to adjust their metabolism in order to support their need for rapid proliferation (38-40). Studies have shown that several oncogenic and tumour suppressors can directly activate hypoxia-inducible factor 1 (HIF-1) which directly controls the transcription of glycolysis enzymes required for cancer cell progression and metastasis (41, 42).

1.2.8 Avoiding Immune Destruction
Throughout the last few decades several hypotheses on cancer cell immune evasion have been suggested. The most recent concept is that of immunoediting (43-45). If cancer cells survive immune elimination they are able to enter an equilibrium phase. The immune system exerts a selective pressure on the unstable tumour cells leading to the acquisition of immunosuppressive characteristics (such as cytokine secretion and Fas-L expression constructive to lymphocyte death). This immune evasion helps promote tumour growth and metastasis (46).
1.2.9 Genome Instability and Mutation

Genomic instability is a characteristic of almost all human cancers. These consist mainly from, chromosomal instability (CIN), microsatellite instability (MSI) and karyotypic instability associated with loss of telomeric DNA (18, 47). Chromosomal instability is associated with poor prognosis in solid tumours (48). It can be characterised by abnormal chromosome structures and numbers as well as abnormal mitoses. Microsatellite instability is caused by defects in the normal DNA repair process leading to unchecked damaged DNA. The sections of DNA known as microsatellites consist of a sequence of repeating units which can lengthen or shorten and become unstable.

1.2.10 Tumour Promoting Inflammation

In healthy tissues, inflammation is associated with the innate immune system whose functions include wound healing, fighting infections and cellular/tissue repair. The inflammatory process can contribute to the tumour micro-environment by producing chemicals such as growth/survival factors and matrix modification enzymes. Inflammatory cells can also promote tumour formation by the release of mutagenic chemicals such as reactive oxygen species (ROS) (2).

With the mass of knowledge regarding cancer hallmarks and their effects on neoplastic development expands, this ensures the continued enrichment of understanding required for the continued development into effective treatments.

1.3 Cancer Treatment and Therapy

The treatment of cancer is variable and is dependent on numerous factors including the type, location and mass of the tumour as well as the stage of disease and general health of the patient. Designing successful treatment regimes relies on a multifaceted approach to directly remove or kill the cancer cells through surgery, radiation, chemotherapy, hormonal treatments, targeted therapy, antibodies or vaccines.

Our modern-day use of chemotherapy in cancer therapy has its origins on the battlefields of the First World War. However, it wasn’t until the mid 1940’s that agents such as methotrexate were becoming more commonly used to treat child hood
leukaemia (49, 50). Although success has been achieved with new molecular targeted treatments, many cancer regimens still rely on older cytotoxic based drugs such as cisplatin for their foundation.

1.3.1 Platinum Anti-tumour Compounds

Platinum-based antineoplastic drugs still serve as one of the most important anticancer drug families used clinically. These agents are able to bind to and cause crosslinking of DNA which can result in inhibition of DNA repair and/or DNA synthesis in cancer cells ultimately triggering apoptosis.

Defects in DNA repair mechanisms caused by mutations in BRCA1 and BRCA2 have been shown to render tumour cells more sensitive to inter-strand DNA crosslinking agents such as cisplatin and carboplatin (51, 52). This could suggest a potential marker of platinum sensitivity in BRCA1/2 deregulated tumours.

Platinum resistance continues to be a major hurdle in cancer chemotherapy. Cisplatin sensitivity is not only regulated by its uptake, efflux or interaction with its target DNA, signalling responses to cisplatin-induced DNA damage may play a major role in the cancer cells fate (53). Due to renal and neurotoxicities associated with cisplatin, intensive efforts were made to devise analogues with fewer of these toxicities. This work led to the development of carboplatin, which produces primarily haematopoietic toxicity and appears to have an anti-tumour effect similar to cisplatin, previously shown in vitro to be equimolar (Cree IA, personal communication).

Cisplatin enters the cells either passively by diffusion or mediated by a carrier such as copper. Cellular efflux is by the ATP-dependent transporters, ATP7A and ATP7B. Cisplatin is a complex of platinum which forms strong chemical bonds with cellular thiols, such as glutathione and metallothionein. On interaction with DNA, cisplatin stalls cell proliferation by inhibiting DNA synthesis, followed by the activation of DNA damage responses. The resulting cisplatin-DNA adducts are then repaired via the nucleotide excision repair system, inducing cell-cycle arrest (via p53 and c-Abl). Damage induced p53 activation leads to the induction of p21, GADD45, proapoptotic PUMAα, caspase-6, caspase-7, microRNAs (miR-34a) and promotes apoptosis by
binding and inhibiting the antiapoptotic Bcl-xL. Apoptosis may also occur via activation of the mismatch repair system, leading to the activation of JNK and p38 MAPK and stabilization of p73. The kinases (PKC, ERK, and AKT) are also involved in the regulation of cisplatin-induced cell death.

A number of mechanisms of cellular resistance to platinum compounds have been identified (54). These mechanisms include decreased uptake/increased efflux of the platinum compound, inactivation of the drug by cellular thiol compounds, enhanced repair of the platinum-related DNA damage, the absence of mismatch repair, and genotoxic induced stress activating multiple signal transduction pathways. MicroRNAs (miR-214) can also promote cisplatin resistance by down regulating PTEN and activating AKT (55).

It has been suggested that the mismatch repair system plays a vital role in the cytotoxicity of cisplatin as these proteins recognise the cisplatin cross-links. Mispairing caused by these cross-links triggers the mismatch repair (MMR) pathway. This produces a cycle of ineffective DNA repair. The repetitive cycles of MMR leads to growth arrest, accumulation of p53 and induction of apoptosis. However, in cells that are MMR deficient, the mispairs are not removed and apoptosis is not induced rendering the cells resistant to platinum as the cell continues to synthesise DNA containing mutations (56).

1.4 Drug Resistance Mechanisms

Despite a new era of molecular characterization of disease and the discovery of novel targets for drug development, cancer mortality rates have seen little change. The inevitable development of resistance to each new pharmaceutical entity continues to form an obstacle to successful drug treatment in cancer.

1.4.1 Drug Pumps

The overexpression of drug transporters has long been considered the predominant mechanism responsible for multidrug resistance (MDR), with increased expression of
members of the ATP binding cassette (ABC) transporter family playing a key role in this resistance.

Of the 48 ABC transporters there are at least 12 which function as drug efflux pumps. These allow efflux of an assortment of substrates including ions, sugars, amino acids, lipids, toxins and anticancer drugs. The most widely categorised of the ABC transporters are ABCB1 (also known as MDR1 or P-glycoprotein), ABCC1 (also known as MRP1) and ABCG2 (also known as BCRP) (57, 58).

MDR1 is an ATP-dependent efflux pump with broad specificity for anticancer drugs including vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes but not platinum compounds. These classes of transporters have long been associated with a decrease in anticancer drug accumulation in those cells exhibiting multidrug resistance. The inhibition of ABC transporter-mediated drug efflux has been a target for possible cellular resensitisation to chemotherapeutic agents (59).

It has been shown that many cells with an acquired resistance to cisplatin often exhibit reduced drug accumulation. It is generally believed that reduced cisplatin accumulation in platinum resistant cells is due to a decrease in uptake rather than increase efflux as seen in other forms of drug resistance (53).

1.4.2 Mutations

Signalling pathways downstream of EGFR include the Ras/MAPK, PI3K/AKT and STAT pathways (60). Constitutive activation of these pathways through mutation un couples downstream signalling from upstream receptor inhibition (61). Normally, binding of GTP to Ras is transient, and following activation RAS triggers a diversity of intracellular signalling before returning to its inactive state. Mutations in codons 12 or 13 of KRAS result in RAS mutants which are insensitive to RAS-GTPase activating proteins, while mutations in codon 61 inhibit the intrinsic GTPase activity of KRAS (62). The consequence of these mutations results in a failure to convert active RAS-bound GTP into its inactive form GDP. This allows constitutive activation of downstream signalling, independent of stimulation (63). KRAS mutations are found to be present in
approximately 40% of patients and have become biomarkers to predict for intrinsic resistance to EGFR-targeted agents (64-66).

Activating mutations in BRAF affect downstream signalling through MAPK and appear to occur in a mutually exclusive manner with KRAS mutations (67). Although occurring less frequently BRAF mutations also appear to have intrinsic resistance to EGFR inhibitors (68, 69). Mutations in BRAF (V600E) may give rise to the subset of wild-type KRAS (wtKRAS) patients who do not respond to EGFR-targeted agents (68, 70).

1.4.3 Development of New Signalling Pathways

Many of the targets downstream of receptor tyrosine kinases can be trans-activated via a multitude of signalling cascades. The inherent feedback mechanisms associated with specific targeted treatment can lead to intrinsic resistance of these inhibitors. Sirolimus (Rapamycin) has been shown to have therapeutic activity in a variety of tumours; however, feedback mechanisms through the AKT/PI3K or MEK/ERK pathways can result in considerably reduced effects (71-73).

Evidence suggests that despite tumour cells having adopted dominant growth factor pathways, they are not necessarily incapable of responding to other growth factors that may be present. The ability to switch to alternative growth signalling pathways during drug treatment represents an apparent means of limiting the actions of these highly targeted therapies. Therefore, although EGFR inhibition may efficiently reduce EGFR signalling, IGFs acting through the IGF-1R can readily reactivate downstream signalling molecules and thereby re-establish tumour cell growth (74).

In a percentage of gefitinib-relapsed patients affected by NSCLC, resistance occurs due to the amplification of the MET gene encoding for the receptor tyrosine kinase for hepatocyte growth factor (75, 76). The overexpression of MET and its interaction with other ErbB family members, mainly HER3, continues the downstream activation of signalling pathways in the presence of EGFR inhibition (77). It has been shown that in these instances inhibition of MET can restore sensitivity to EGFR inhibitors (75, 76).
1.5 Molecular Mechanisms for Sequential Therapy

1.5.1 Sequential Therapy

Sequential chemotherapy consists of the administration of non-cross resistant chemotherapy in a predetermined order for a defined number of cycles. Regimens are designed so that one anti-cancer agent is used before switching to an alternate anti-cancer agent. This can help eliminate cells which have developed resistance to the primary agent. Mechanisms involved in resistance to chemotherapy usually involve up-regulation of resistance mechanisms, or down-regulation of target genes. This adaptation has been shown to occur within hours of drug exposure (78).

A cross-over effect has been observed within some clinical trials, in which patients treated with one type of chemotherapy show sensitivity to an alternative regimen following failure of the treatment to which they were first allocated (79). In these instances the mechanisms involved could specify a basis for sequential administration.

1.5.2 Log Cell Kill

As far as cancer chemotherapy has come, its practice still adheres to the concept of logarithmic cell kill (80). This concept indicates that a constant fraction of tumour cells will be killed by per cycle of drug dosing, regardless of initial body burden (81, 82).

In single agent therapy the exponential growth in tumour cell number can be disrupted by one or more cycles of treatment, although the corresponding drop in the log number of cells is unlikely to be effective for long. The graphical response after each treatment shows an expected reduction of cell number to the right of which the cell number resumes at its previous growth rate (Figure 3).
1.5.3 Design of Sequential Regimens

The interval between cycles of chemotherapy has generally been the minimum time required to recover from drug toxicity (84). However, with increased observation of drug resistance mechanisms this time scale may not be appropriate. Where drug resistance is partially reversible or the mechanism by which resistance occurs is targetable a more molecular approach to sequential therapy may be more advantageous.

The idea of sequential chemotherapy is to maintain dose intensity and preclude any cumulative toxicity by increasing drug diversity by reducing drug cross-reactivity.

The late 1970’s- early 1980’s saw the impact of the design of new chemotherapy regimens using mathematical modelling. One such model ‘the worst drug rule’ states that if two non-cross-resistant drugs are used intermittently in treatment, then the drug with the weaker killing rate has to be applied first and/or for a longer period of time. This would ensure that the most effective drug would then kill the remaining fractions of the more resistant tumour cells (85).
Ciardiello et al believe that chemotherapy should be administered prior to targeted receptor inhibition (86). The reasoning behind this sequence comes from the situation in which the receptor inhibitor exerts its potential synergy with chemotherapy. The chemotherapy is inducing or has induced maximal cell damage; therefore the subsequent receptor blockade could interfere with the repair of this cell damage (86).

Based on Di Nicolantonio et al, and Mercer et al, a successful molecular based sequential regimen would include a primary chemotherapeutic agent followed in close succession by a specific inhibitor against a target upregulated in response to the primary treatment (78, 87). This would require identification of specific stress responses in a population of heterogenic cancer cells, and would have to consider dose and time of administration.

Recovery from dormancy status or recovery of proliferation provides an additional rationale for delay of additional courses of chemotherapy, particularly in slow growing solid tumours.

Synchronizing cells with drugs that inhibit DNA synthesis or arrest cells in mitosis can be exploited with agents that specifically target the cell cycle. This process however, may also synchronize non-neoplastic target cells providing little improvement in the overall therapeutic effect. This effect can be shown to be beneficial in hormone-dependent tumours. Hormones have been shown to manipulate metastatic breast cancer cells, where cells were arrested cytokinetically with tamoxifen and then pulse-stimulated into the cycle with oestrogen. Chemotherapy can then be delivered at the time of maximum synchronization allowing for a greater portion of cell kill (84).

1.6 Molecular Targeted Therapy and Individualised Treatments

It could be said that there are as many types of cancers as there are people. Everyone is genetically different and so no two cancers are exactly alike. Recent studies have shown that differences in some small molecule targeted drugs can be explained by the absence or inclusion of specific mutations in related genes. This has inevitably initiated the rationale for molecular diagnostic testing for clinical decisions regarding chemotherapy administration.
The first molecular target used to aid cancer therapy was the receptor for the female sex hormone oestrogen which is required by many breast cancers for growth. Drugs selected for this target include tamoxifen which is a potent oestrogen receptor (ER) antagonist and newer drugs such as the aromatase inhibitors (88, 89).

The expression of HER2 is associated with poor prognosis in many cancers. In those with breast cancer, treatment with the anti-HER2 antibody trastuzumab (herceptin) as a monotherapy or combined with regular chemotherapy may be beneficial. HER2, ER and progesterone receptors (PR) are routinely tested for expression via immunohistological staining, which helps aid chemotherapeutic regimen choice. HER2 expression is driven by gene amplification, which is assessed by fluorescence in situ hybridisation (FISH) (90).

Treatment with small molecule inhibitors of EGFR have shown to benefit non small cell lung cancer (NSCLC) patients that carry certain somatic mutations in their EGFR gene. There is also emerging evidence that mutations in KRAS may be predictive of a lack of sensitivity to either gefitinib or erlotinib in these lung tumours (91). It has been shown that mutations in EGFR, KRAS, BRAF and HER2 are frequently mutually exclusive in lung adenocarcinomas. This represents alternate pathways with which these tumours could continue to activate downstream pathways such as PI3K/AKT and MAPK (92).

Antibody therapies directed against EGFR (cetuximab) have been shown to be clinically effective in a subset of colorectal cancer patients with a wild-type KRAS gene. In the UK at present, patients with metastatic colorectal cancer are tested for KRAS mutations prior to administration of EGFR antibody therapy. The presence of mutations in BRAF and PIK3CA genes found in those tumours which do not respond to anti-EGFR treatment suggests the need for further genotyping for these genes among KRAS wild-type tumours when being considered anti-EGFR therapies (65, 68).

It is fast approaching a time where a single gene strategy for individualised treatment will become inadequate. The genetic roots of cancers are proving to be more complicated than current testing provides. Many tumours have a variety of mutations...
thought to be important to chemosensitivity, as well as mutations within the same signalling pathways being found to occur in many tumour types. With greater knowledge of the correlation of these mutations with sensitivity to chemotherapy new targeted agents will be developed with tailored individualised tests for their detection.

A number of studies have applied next-generation sequencing technologies to sequence cancer genomes to find mutations in other genes which may be targeted successfully (93, 94). Although effective, this type of approach (whole genome sequencing) is still cost prohibitive for current widespread diagnostic applications. A possible alternative to this costly methodology is exome sequencing. This method captures and sequences only in the coding exons (exomes) within the genome. With the popularity of this technique increasing, many cancer based exome studies are underway (95-99).

1.7 EGFR Pathway in Cancer Treatment

The epidermal growth factor receptor (EGFR) is a member of the erbB family of receptor tyrosine kinase proteins. This family also includes HER2/neu (erbB2), HER3 (erbB3), and HER4 (erbB4). These are type I transmembrane growth factor receptors that activate intracellular signalling pathways in response to extracellular signals. Phosphorylation of the tyrosine kinase domain followed by homo/hetero-dimerization between different receptors of the erbB family leads to protein activation; followed by activation of downstream signalling cascades (Figure 24, Page 84) ultimately resulting in cell growth, differentiation, metabolism, cell survival and angiogenesis.

Several members of the protein tyrosine kinase family have been shown to be activated in cancer cells and can often be associated with a poor prognosis. EGFR overexpression can lead to inappropriate activation of signalling cascades, which can lead to uncontrolled cell proliferation via pathways such as the RAS/RAF/MEK/MAPK pathway. In NSCLC an increased EGFR copy number can be associated with improved survival, suggesting that increased expression of mutant and/or wild-type EGFR could determine response to tyrosine kinase inhibitors (TKI’s) such as gefitinib (100, 101). Studies have shown that factors, such as high expression levels of EGFR and other ErbB
family members, might constitutively activate AKT and sensitize cells to EGFR inhibitors (102, 103).

Gefitinib (Iressa®, Astra Zeneca) is an orally active tyrosine kinase inhibitor (TKI) targeted to the ATP-binding domain of EGFR (HER1; erbB1). However, in the absence of mutation, the mechanism of drug sensitivity to gefitinib is not fully understood as activity does not always correlate with the level of EGFR expression by the tumour cell. Studies have shown there to be great heterogeneity in the degree of inhibition observed when tumours were tested against single agent gefitinib as well as in combinations with other agents (104).

Erlotinib (Tarceva®, Roche) specifically targets the epidermal growth factor receptor binding reversibly to its ATP binding site. Like gefitinib the mechanism of erlotinib sensitivity in the absence of mutation is not fully understood.

The discovery of the correlation between EGFR mutations and EGFR-targeting drugs in NSCLC has lead to a more evidence-based therapeutic strategy, where treatment is considered based on patients mutation status (105-110). Routine mutation analysis to determine drug sensitivity in these tumours prior to drug administration is becoming more common place for such drugs as erlotinib and gefitinib (111). This is beneficial not only financially as these drugs can be expensive, but also means patients will not be required to undergo ineffective or inappropriate treatment.

Correlation between EGFR mutations and clinical outcome with specific TKI’s is not found in all tumour types (112). A phase II study in ovarian cancer identified no mutations in 20 patients who responded to gefitinib therapy (stabilization or partial response) (113). It maybe that in some tumours other pathways such as the EGFR/ERK1/2 and AKT pathways have greater relevance for cell survival and proliferation (114, 115).

After variable time on treatment, cancer cells can become resistant to EGFR inhibitors. The main source of this resistance is the development of the mutation T790M (substitution of threonine 790 with methionine). Threonine 790 has been shown to be...
an important factor in determining inhibitor specificity in the ATP binding pocket as it located at the entrance to a hydrophobic pocket in the back of the ATP binding cleft. Therefore, the T790M mutation is thought to sterically block binding of TKIs such as gefitinib and erlotinib (116, 117).

Another mechanism of acquired resistance to EGFR inhibitors can be demonstrated by continued activation of downstream signalling pathways (including PI3K). Some studies have suggested that if cancer cells can find a way to effectively activate PI3K independent of EGFR activity, it will become resistant to EGFR TKIs (118, 119).

The PI3K/AKT signalling pathway is frequently deregulated in cancer (120, 121). Tyrosine kinase receptors (EGFR) bind PI3K, either directly or indirectly via adaptor molecules. These active kinases generate pip3 at the lipid membrane which in turn facilitates the phosphorylation of AKT. This allows AKT to transmit signals to many downstream substrates including mTOR, influencing a variety of key cellular functions such as cell growth, survival, proliferation and metabolism. The phosphatase PTEN dephosphorylates pip3 terminating PI3K signalling, acting as the pathways negative feedback loop. PTEN is a tumour suppressor and loss of PTEN function or mutation has been linked to more aggressive and resistant tumours (122, 123). The ability of PI3K to directly activate AKT has lead to its interest as a potential drug target (124, 125).

Progress has begun on the identification and development of novel PI3K inhibitors (61, 126). ZSTK474 (2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine) (LC Laboratories, Massachusetts USA) is an experimental PI3K inhibitor which has been shown to inhibit all four PI3K isoforms in an ATP-competitive manner and has been used in these studies (127).

HER3 is unique among the ErbB family due to its lack of tyrosine kinase activity. Due to its inactivity, it is not a direct target of kinase inhibitors. It does however have six tyrosine phosphorylation sites which effectively couple the protein to the PI3K/AKT pathway via P85α when it is activated by dimerization with other HER molecules. Binding sites for PI3K (P85α) are also found in EGFR and HER4 but not HER2. It has been shown that EGFR-targeting drugs preferentially inhibit AKT phosphorylation in a
dose-dependent manner. This suggests that greater sensitivity to EGFR targeting drugs would be seen in HER2/HER3 and EGFR/HER3 heterodimers (114). It could therefore be considered that over expression of HER3 could also contribute to resistance to EGFR and HER2 inhibitors.

1.8 Ras Pathway in Cancer Treatment

Given the vital role of the Ras-Raf-MEK-ERK signalling pathway in cell proliferation and survival it is unsurprising that alterations in this pathway are highly prevalent in human cancer.

Mutations in the KRAS gene have been shown to lead to a constitutively active ras protein which subsequently leads to increases in proliferation and malignant transformation (128). In colorectal cancer, treatment with the EGFR inhibitor cetuximab (Erbitux®) is not effective among patients with a mutated KRAS gene. KRAS mutation status in colorectal cancer is used as a clinical diagnostic tool to predict resistance to the anti-EGFR antibody, cetuximab (129-132). KRAS mutations have also been shown in gynaecological tumours. However, their significance to pathway signalling is a much debated question (133-136). Examples of this are found in two studies where mutations in KRAS and BRAF were shown to correlate with overexpression of activated ERK1/2 in ovarian serous tumours (135), which is in contrast to findings in endometrial cancer where ERK activation occurs in a KRAS and BRAF independent manner (137). This discrepancy may suggest that the effect of KRAS mutations may be organ specific.

Many effector pathways downstream of RAS are comprised of kinase cascades, which provide multiple pathway points for potential therapeutic intervention. Two such pathways, the RAF/MEK/ERK and PI3K pathways have shown promise for therapeutic targets (138-141).

Studies have documented the hyperactivation of MEK1/MEK2 and ERK1/ERK2 in solid tumour malignancies (142). This activation has been shown to directly influence RAS pathway dynamics (143). The blockade of ERK1/2 by small molecule MEK1/2 inhibitors has shown a variety of anti-proliferative effects in various tumour cell models. Despite
a strong rationale for the use of MEK1/2 inhibitors in the treatment of cancer their effectiveness has yet to prove evaluable (142). It is likely that MEK1/2 inhibitors will find their therapeutic value with their use in combinations with either other targeted or conventional cytotoxic agents. This has already been seen with the combination of ERK1/2 and PI3K inhibitors producing synergistic effects on tumour regression (144).

1.9 Mevalonate Pathway in Cancer Treatment

The mevalonate pathway performs several key functions within cells including synthesis of cholesterol and the non-sterol isoprenoids farnesyl and geranylgeranyl pyrophosphate (FPP, GGPP). These are important in prenylation of small GTPase proteins such as RAS. The process of prenylation involves farnesylation and geranylgeranylation from the mevalonate metabolite farnesyl pyrophosphate (FPP) as shown in Figure 4.

While farnesylation is usually required for the translocation of Ras to the cell membrane during its activation, NRAS and KRAS can be geranylgeranylated in the presence of farnesyl transferase inhibitors (FTIs). This gives rise to a possible explanation for the limited clinical efficacy of these agents (145-147). Ras signalling has shown to be involved in many cancers, as part of activated growth receptor signalling pathways (e.g. Raf/MEK/ERK) or activating mutations during carcinogenesis where mutations upstream and downstream of RAS can result in growth stimulatory effects (148). Therefore, inhibition of the mevalonate pathway could be used to treat cancers with multiple effects, including theoretical effects on the RAS pathway.

Drugs that disrupt the mevalonate pathway already exist. 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) inhibitors such as the statins can reduce the entry of mevalonate into the pathway by inhibiting its synthesis from 3-hydroxy-3-methylglutaryl coenzyme A (Figure 4). This blockade prevents the formation of downstream products, including cholesterol, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP).

Inhibitors of farnesyl pyrophosphate synthase (FPPS) are also widely available. The nitrogen containing bisphosphonates such as zoledronic acid form a class of drugs
which are widely used for treatment of osteoporosis and bone disorders including tumour associated bone disease (149). The enzymatic blockade of FPPS by zoledronic acid results in the depletion of intermediate isoprenoids (FPP, GGPP), many of which are needed for post-translational protein prenylation (150).

Figure 4: Mevalonate Pathway- N-bisophosphonates inhibit FPP-synthase, leading to accumulation of IPP, which generates ApppI from AMP. ApppI has been found to be toxic to cells (151), while statins inhibit HMG-CoA reductase.
In addition to the loss of protein prenylation brought on by FPPS inhibition, zoledronic acid has been shown to cause the accumulation of intra-cellular isopentenyl pyrophosphate (IPP). Subsequently this accumulation induces the biosynthesis of the pro-apoptotic ATP analog ApppI (1-adenosin-5’-yl ester 3-(3-methylbut-3-enyl) ester triphosphoric acid) which has been shown to interfere with mitochondrial function and induce apoptosis (151-155). Apoptosis brought about by ApppI is thought to occur through the blockade of mitochondrial ADP/ATP translocase (151).

**Prediction and Measurement of Response to Chemotherapy**

The need for rapid, reliable and accurate assessment of cell viability and proliferation is a prerequisite for all *in vitro* and *in vivo* studies employed to determine the effective drug response of solid tumours.

Cell viability can be defined as the number of ‘healthy’ cells in a sample irrespective of whether the cells are actively dividing or are quiescent. The simplest way to test viability after a toxic agent is administered is to count the remaining live cells. This can be achieved by direct visualisation for morphological changes or by observed changes in membrane permeability inferred from the exclusion, uptake or retention of certain dyes.

An alternative method to detect cell viability is to measure metabolic activity. Rather than relying on cell membrane integrity to determine viability these assays determine the ability of the cell to perform biochemical reactions resulting in detectable by-products. Methodologies for the detection of metabolic activities can include absorbance, fluorescence and luminescence.

Assays that determine cell viability are useful for determining chemotherapeutic response as they can also detect non-rapidly dividing cells such as many primary solid tumours for which proliferation rate is relatively low (156).

The measurement of cellular proliferation is defined by the number of cells that are actively dividing in a culture. Measuring cellular proliferation can be achieved via the use of clonogenic assays to determine the number of colonies produced from a single
cell in the presence of toxic agents. Proliferation can also be directly determined through the measurement of the incorporation of labelled precursors into DNA. This measurement of DNA synthesis is proportional to the amount of cell division occurring within the assay culture. Cell proliferation can also be measured indirectly by observing molecules that regulate the cell cycle or by quantifying their amounts via Western blots, ELISA, or immunohistochemistry.

1.9.1 Cell-Based Assays for the Detection of Cell Viability and Proliferation

The prediction of tumour sensitivity to anticancer agents has been widely explored, generating a variety of methodologies by which to measure response to treatment. There is no gold standard test used by all to determine the response to cellular toxicity and many techniques are still currently employed, many of which are briefly outlined below.

1.9.1.1 Spectrophotometric and Colorimetric Assays

The lactate dehydrogenase assay measures either the number of cells via total cytoplasmic LDH or membrane integrity by the amount of cytoplasmic LDH released into the medium. Lactate dehydrogenase (LDH) is an enzyme which catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+. These products supply energy to living cells (157). This assay is based on the reduction of NAD by LDH which is responsible for the stoichiometric conversion of a tetrazolium dye. This coloured compound can then be measured via a spectrophotometer.

Many dyes can also be used to test viability by selectively colouring dead tissues or cells. This is based on the principle that live cells possess intact cell membranes and so will exclude certain dyes (trypan blue, eosin, or propidium) whereas dead cells will not.

Trypan blue is a toluidine derivative. When this dye is mixed with a cell population, viability can be visually examined to determine whether cells have taken up or excluded the dye. A viable cell with an intact membrane will have a clear cytoplasm whereas a nonviable cell will have taken in the dye and will appear blue.
Detection of cell survival can be determined via dye inclusion methods. Neutral red (3-amino-m-dimethylamino-2-methylphenazine hydrochloride) is a weak cationic supravital dye that has the ability to penetrate cell membranes by non-ionic diffusion and accumulate intracellularly in lysosomes (158). Cell survival can be measured via the quantification of the number of viable cells following drug exposure (159).

The differential staining cytotoxicity (DiSC) assay can be used to test efficacy of drugs against tumour cells in vitro identifying optimal therapy. Tumour cells are placed in culture and treated with drugs of interest from between 4-6 days. Cells are then stained within the culture plate with fast green dye before removal to slides and counterstaining with haematoxylin and eosin (H&E). A live cell with an intact membrane precludes staining with the green dye. Therefore, drug sensitivity can be measured by calculating the ratio of live cells in the drug treated samples to the number of live cells in the untreated controls (160).

Long term effects of cytotoxic and cytostatic drugs can be evaluated via the use of clonogenic assays. These can measure the proliferative ability of single cells to grow and produce viable colonies over a period of weeks (161). After this time viable colonies can be fixed and stained before being counted on a stereomicroscope or via image analysis. By comparing the number of colonies in a drug exposed plate with drug free control plates drug sensitivity or resistance can be determined.

The MTT, MTS, XTT and WST are colorimetric assays based on the ability of living cells to convert tetrazolium salts into formazan crystals determining mitochondrial activity. The solubilised formazan reagent can then be measured spectrophotometrically. In most cell populations the total mitochondrial activity is related to the number of viable cells and so these assays can be used to measure the in vitro cytotoxic effects of drugs (162).

The principle of the sulforhodamine B assay is to measure the total biomass of a cellular sample via staining of the cellular proteins with the Sulforhodamine B. The measurement of corresponding drug-induced cytotoxicity and cell proliferation is detected by the ability of the protein dye sulforhodamine B to bind electrostatically
and pH dependently on the protein basic amino acid residues of trichloroacetic acid-fixed cells (163).

The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals (164). Following drug exposure a caspase specific peptide conjugated to a colour reporter molecule (p-nitroaniline) is added to the cell lysate. Cleavage of the peptide by the caspase releases the chromophore, which can be quantified spectrophotometrically. As caspase detection is not discriminatory of the cell type undergoing apoptosis, it is more useful alongside other cell death end point assays.

During the early events of apoptosis, oligo- and mononucleosomes are generated and released in the cytoplasm by the internucleosomal cleavage of chromatin (165). These cytoplasmic histone associated DNA fragments can be detected via enzyme linked immuno assay (ELISA). In this assay cytoplasmic nucleosomes can be immobilised in culture plates by sequential administration of antibodies. The first antibody is directed against species specific histones, followed by the addition of an antiDNA-antibody conjugated to an enzyme. After a final wash step to remove any unbound antibodies, an enzymatic substrate is added to produce a visible signal (colorometrically/photometrically). When compared with an internal standard the extent of apoptosis activity can be evaluated.

1.9.1.2 Fluorometric Assays

Glucose 6-phosphate dehydrogenase (G6PD) is a cytosolic enzyme and is part of the pentose phosphate pathway. As G6PD generates NADPH, it plays a crucial role in cellular antioxidant defence. G6PD has been shown to leak from cells when its plasma membrane integrity is compromised (166). Glucose 6-phosphate (cell death) is detected by the coupled-enzyme reaction G6PD to 6-phosphogluconate and the reduction of resazurin to a red-fluorescent resorufin (166). The resulting fluorescence signal produced by this reaction is proportional to the amount of G6PD released into the cell medium. This in turn correlates well with the number of dead cells found in the cellular sample.
The single cell gel electrophoresis (Comet) assay is used for the evaluation of DNA damage and repair. This assay involves the encapsulation of cells in low-melting-point agarose followed by cell lysis and electrophoresis of the remaining lysed cellular suspension. DNA damage is determined via fluorescent or silver staining and analysed either manually or automatically via imaging software. This process helps in the detection of single/ double-strand DNA breaks, DNA cross-links, base/ base-pair damages and apoptotic nuclei (167).

An early event within apoptosis is the externalization of phosphatidylserine (PS) to the cell surface membrane which serves as a signal by which cells destined for death are recognized by phagocytes (168). The annexin V assay uses labelled annexin V protein to bind to PS, identifying apoptotic and dead cells which can be counted using flow cytometry or fluorescence microscopy.

The detection of nuclear DNA fragmentation and thus apoptosis can also be achieved by using the terminal deoxynucleotidyl transferase dUTP Nick End-Labelling (TUNEL) assay. Cells undergoing apoptosis cleave their DNA with nucleases leaving free 3'-hydroxyl ends. The detection of this apoptotic event is achieved via transfer of a fluorescent-labelled deoxyuridine triphosphate nucleotide (dUTP) to the free 3'-hydroxyl ends using the enzyme terminal deoxynucleotidyl transferase (TdT). The labelled dUTP can then be detected by light/fluorescence microscopy of flow cytometry. Considerations need to be made regarding necrotic cells and those cells in the process of DNA repair or gene transcription as they can produce false positives (169).

When the intrinsic apoptotic pathway is triggered, cytochrome c is released from the mitochondria into cytosol where it is able to bind to Apaf-1. The resulting cytochrome c/Apaf-1 complex activates caspase-9 initiating downstream caspase signalling (170). Detection of cell death via the cytochrome c release assay is achieved by detecting cytochrome c translocation from mitochondria into the cytosol during cellular apoptosis. This can be done on living or fixed cells via fluorescence and electron microscopy.
1.9.1.3 Chemiluminescent Assays

The ATP endpoint assays are based on the fact that all living cells require ATP to function, whether this be in biosynthesis, cellular signalling or in the cellular structure of DNA. The comparison of ATP content from remaining drug exposed cells to those of drug free control gives a direct measurement of cellular death and drug potency. This assay uses bioluminescence to detect cellular ATP through the conversion of D-luciferin in the presence of ATP and O$_2$ by the enzyme luciferase to produce AMP, CO$_2$ and Light (171). This method is able to measure the ATP presence of single cells, with ATP concentration being linear up to $10^8$ cells making it highly sensitive (156).

This assay methodology (ATP tumour chemosensitivity assay (ATP-TCA)) has been used to assess tumour chemosensitivity in these studies. This method was chosen for its superior sensitivity, where the presence of as few as 5 viable cells per well following drug exposure can be detected. Fibroblasts, mesothelial cells and other stromal cells can proliferate in adherence-based culture systems adding ‘background noise’ to most assay types. This phenomenon is minimised in the ATP-TCA by the use of round-bottomed polypropylene plates, which inhibit cell adherence. The use of a serum-free medium also suppresses non-transformed cell proliferation. Unlike other assays the ATP-TCA does not require the incorporation of tetrazolium dyes which can have the potential for interference with certain drugs (172, 173). A disadvantage to this technique is the inability to distinguish between the effects of inhibited glucose metabolism/mitochondrial function resulting in decreased intracellular ATP concentrations and drug induced cell death. However, this disadvantage is offset by the fact that the ATP-TCA has also shown good correlation between ex vivo sensitivity and clinical response in some solid tumours (79, 171, 174-176).

Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) is an important enzyme in the glycolysis pathway, catalyzing the oxidative phosphorylation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. The release of GAPDH from dying cells is coupled to the enzyme 3-Phosphoglyceric Phosphokinase (PGK) to produce ATP. The ATP can then be detected via luciferin-luciferase bioluminescence methodology measuring cytotoxicity and proliferation (177). The generation of ATP is an ongoing
process; therefore time point and kinetics methods may also be measured via this methodology.

1.9.1.4  Radioactivity Assays

The thymidine incorporation assay introduces a radioactive nucleoside (3H-thymidine) to proliferating cells. This becomes incorporated into new strands of chromosomal DNA during mitotic cell division. The radioactivity of the DNA recovered from the cells is used in order to determine the extent of cell division that has occurred in response to the drug being tested.

This assay can be adapted to exclude the use of radioactivity by using 5-bromo-2'-deoxyuridine (BrdU). This BrdU assay detects the incorporation of this pyrimidine analog into cellular DNA during proliferation. By using a monoclonal antibody against BrdU with an enzyme or fluorochrome conjugated secondary antibody, proliferating cells can be detected.

This methodology has been developed further in the form of the Click-iT® EdU assay (Life Technologies, UK). Unlike the BrdU assay it does not use antibody based detection methods and therefore does not require DNA denaturation. This assay instead uses a copper catalyzed covalent reaction between an alkyne and an azide to determine EdU incorporated cell proliferation (178).

1.9.1.5  Real Time Cytotoxic Assays

Cytotoxic cell based assays usually have finite endpoints where the living/dead cell population is determined. These assays capture only a snapshot of the incidence of cell death and usually require the destruction of the cell to measure outcome. Novel assays are now being used to probe and quantify cytotoxicity in real time. These include assays that measure plasma membrane integrity markers such as propidium iodide (PI), SYTOX Green, SYTOX Red, and YO-PRO 1 (Invitrogen, UK) which can be observed via time lapsed microscopy or flow cytometry. Real time cytotoxicity assays have also been developed in microfluidic devices, which have the ability to characterise drug metabolites and cytotoxicity simultaneously (179).
1.9.2 Serum Tumour Markers

Serum tumour markers consist of substances that are secreted by tumours and are detectable at a level proportional to tumour burden. The use of serum markers to measure response to therapy is appealing due to its non-invasive nature and the ability to be repeated at every stage of treatment using immunoassays. A few examples of serum tumour markers are outlined below.

Carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) are glycoproteins found during foetal development. The abnormal presence of these proteins found in some cancers and their reduction following tumour resection or chemotherapy has lead to their use as markers for tumour response (180-184).

Cancer antigen 125 (CA125) has found a use in ovarian cancer for the detection of recurrence following treatment where it is able to identify a percentage of patients with asymptomatic recurrence. Evidence suggests that early chemotherapeutic treatment of recurrence based solely on CA125 increase does not prolong survival. There is still controversy as to the use of CA125 monitoring during follow-up (185-187).

The prostate-specific antigen (PSA) is used in the detection of prostate cancer. In men with healthy prostates serum PSA is present in small quantities. However, this level becomes increased in prostate related disease including prostate cancer. It has become a useful tumour marker for the early detection of prostate cancer (188).

Thyroglobulin (Tg) is a glycoprotein produced solely by thyroid follicular cells. The level of Tg is known to be increased in the majority of patients with either benign or malignant thyroid nodules (189). This serum tumour marker tested serially over time is used to detect residual disease post surgical and radiation treatment (190).

Tissue polypeptide-specific antigen (TPS) is a cytokeratin 18 associated tumour proliferation marker, with increased serum levels found at the time of rapid cell division (191). It has been shown to predict response to 5-FU-based chemotherapy in patients with advanced colorectal cancer (192).
Various tumour markers are available to clinicians to aid diagnosis or determine a patient’s response to surgery or chemotherapy; however, many are not tumour specific and their use still remains controversial (193).

1.9.3 Molecular Markers

There are an increasing number of methodologies used for the detection of molecular markers in the study of cancer. The use of molecular markers in predictive and prognostic oncology is on the rise, resulting in their use for identification of patients most likely to respond to specific therapies as well as those most likely to experience disease recurrence, or suffer toxicity.

Tests to detect these markers include assays for molecular or genetic analyses of tumour tissue. Detection of mutations in EGFR confers sensitivity to EGFR inhibitors in non small cell lung cancer (NSCLC) while mutations in KRAS confer resistance to anti-EGFR therapy in colorectal cancer (131, 132). The detection of CK19 or mammaglobin during intraoperative breast sentinel lymph node testing, infers the presence of metastases which requires further surgical treatment (194).

Methodologies used for the detection of such molecular markers are detailed below.

1.9.3.1 Polymerase Chain Reaction (PCR)

This technique is based on the ability to take a single copy of a piece of DNA and amplify it across several orders of magnitude producing millions of identical copies. The DNA sample is subjected to repetitive thermal cycling in which the DNA is first heated to allow separation into single strands (denature). The temperature is then lowered to allow primers to anneal to their complementary sequences. The temperature is then raised to allow DNA polymerase enzymes to attach to the priming site and extend new stands of DNA. These denature (94-96 °C), anneal (50-56 °C) and extension (72 °C) steps are repeated between 20-40 times resulting in a doubling of DNA product at each cycle.
The use of quantitative PCR methods allows estimation of the quantity of target DNA with a sample. This can be assessed after PCR amplification via gel electrophoresis or in real time using fluorescent probes.

As DNA polymerases require a DNA template for PCR, this technique would seem to be limited to the analysis of DNA samples. However, the process by which either RNA or mRNA can be reverse transcribed into complementary DNA (cDNA) (reverse transcription PCR (RT-PCR)) allows for the quantification of gene expression which can act as markers for possible response to chemotherapy (195). This response can be examined by measuring the abundance of the gene-specific transcript in pre and post drug exposure samples.

Real-time PCR (qRT-PCR) using TaqMan probes measures the accumulation of fluorescence with each PCR cycle. Fluorescence is measured from the probe containing a fluorescent reporter and a quencher. When the probe is intact the fluorescing reporter is in close proximity to the quencher allowing the transfer of light emitting energy from reporter to quencher. This results in the reporter’s suppressed signal. When the PCR enzyme extension phase reaches the annealed probe, the enzyme cleaves the probe. A fluorescent emission from the reporter increases as it moves further from the quencher.

Interpretation of qRT-PCR can be done in two way quantitatively and qualitatively. Qualitative measurement of PCR is used for detecting a specific DNA product. Its presence or absence can signify a marker for specific response to treatment (143, 196, 197).

Quantitative measurement not only detects the presence of a specified DNA product, but also deduces its quantity. Absolute quantification requires either a standard DNA sample of known quantity for standard curve comparison or for the quantity of DNA originally present in a PCR sample to be known. Relative quantification is based on stable internal reference genes (housekeeping genes) allowing determination of differences of target gene expression (198).
Scorpion primers contain a hairpin loop tail consisting of a specific probe sequence, a fluorophore and a quencher. The signal from fluorophore attached to the 5’ end of the probe is suppressed by the quencher attached to the 3’ end. The hairpin loop is attached to the 5’ end of a primer via a PCR blocker. Following the PCR extension phase the probe binds to its complementary strand, with this the hairpin loop opens so that the fluorophore is no longer quenched and a fluorescent signal is produced (199).

Scorpions can be adapted for use in mutation or allelic discrimination with an Amplification Refractory Mutation System (ARMS). The ARMS Scorpions are used to both amplify and discriminate between two alleles of a target oligonucleotide. These reactions consist of scorpions for each allele. The probe part of the scorpions are identical and it is the difference between the primer parts which are used to discriminate between the two alleles. This system is already used for the characterisation of mutations in EGFR and KRAS to determine the practical use of anti-EGFR based therapies (200, 201).

1.9.3.2 DNA Sequencing

DNA sequencing is used to map out the sequence of the nucleotides (A, T, G and C) that comprise a strand of DNA. Through sequencing identification of important genes can provide clues about the underlying mechanism of tumour cell biology. Mutations identified within these genes have lead to their development as molecular markers for the use in identification of effective and non-effective therapies (EGFR in non small cell lung cancers (NSCLC) and KRAS in colorectal cancers). Some of the methodologies by which these markers are identified are detailed below.

The chain-terminator (dideoxy sequencing/ Sanger) method of sequencing involves using a purified DNA polymerase enzyme and a primer specifically constructed so that its 3’ end is located next to the DNA sequence of interest to synthesize DNA chains of varying lengths. This is accomplished through the use of dideoxynucleotide triphosphates (ddNTPs) in the reaction mixture in addition to the normal nucleotides (NTP’s) found in DNA. These modified dideoxynucleotides lack the 3’ hydroxyl (OH) group required for the addition of further nucleotides. Following their integration into a DNA sequence they effectively terminate the chain. Termination via ddNTP occurs
periodically during DNA synthesis resulting in numerous DNA fragments of varying length. These can then be separated using gel or capillary tube electrophoresis (202, 203).

Pyrosequencing is based on the principle of sequencing by synthesis, which detects the release of pyrophosphate upon nucleotide incorporation and its reaction with another chemiluminescent enzyme. With hybridisation of the primer to the single-stranded PCR amplicon serving as a template, sequential addition of deoxribonucleotide triphosphates (dNTPs) can begin. Addition of dNTPs is performed sequentially and the light produced by each incorporation event is recorded (202).

Like pyrosequencing, the Ion Torrent sequencing method is based on sequencing by synthesis. This technology uses an ion sensor to detect hydrogen ions released during DNA polymerization. These sensors measure the change in voltage from the release of hydrogen ions when the matching nucleotide is added. These readings can then be translated into a DNA sequence.

Future technologies are fast approaching with the possible applications of real-time sequencing technologies, using single molecule real time sequencing or exonuclease nanopore sequencing methodologies (204, 205).

1.9.4 Microarrays

Array based technology offers the user the ability to evaluate large numbers of experiments simultaneously. Microarray technologies have been widely used to research cancer related genetic markers and changes in gene expression (206).

Some of the first array based technology such as the spotted microarrays rely on the transfer of probes onto solid surfaces or chips. The cDNA or oligonucleotide probes are flooded with a fluorescently labelled sample allowing for the comparison between the gene expression of pre and post chemotherapy samples and normal and neoplastic cells (207).
Real-time PCR arrays have shown some promise for the prediction of tumours to chemotherapy (208-210). The use of microfluidic cards to test for tumour gene expression profiles lends itself to practical clinical application. Its application reduces both lab based work time and the risk of human error associated with manual pipetting.

There is an extraordinary potential of microarray technology in the field of clinical oncology. Understanding the complexity of cancer has greatly increased the understanding of how heterogenic individual tumours can be. Soon diagnosis and prediction of response will rely on detection and quantification of multiple markers provided by array based technology.

### 1.9.4.1 Microsatellite Instability Testing

Microsatellites are short repeating sequences of DNA usually 2-6 base pairs in length. These repeated sequences are both common and normal; however cells that have mutations in DNA repair genes can accumulate errors in these regions resulting in these repeated sequences becoming longer or shorter. The manifestation of unusually long or short microsatellites within a person’s DNA is referred to as microsatellite instability (211).

Microsatellite instability (MSI) can be associated with defective DNA mismatch repair in various solid tumours including endometrial, ovarian, colorectal and gastric cancers (211-213). Although microsatellite instability status has shown to be a good prognostic factor for colorectal tumours, its predictive value for chemosensitivity remains controversial (214-217).

Analysis of MSI involves the comparison of the allelic profiles of both microsatellite markers generated by amplification of DNA and from matching normal and test samples. Positive MSI results in the presence of alleles in the test sample which are not found in the corresponding normal samples.
Chapter 2 - Materials and Methods
2.1 Introduction
This chapter describes the various methods used during the experimental aspect of this PhD.

2.2 ATP-TCA
The ATP-Based tumour chemosensitivity assay (ATP-TCA) was performed as described Andreotti et al., (1995). This assay is available in kit form from DCS Innovative Diagnostik Systeme (Hamburg, Germany) as the TCA-100 kit though an in house method was used here. This method is described below. Methods were carried out in accordance with the control of substances hazardous to health (COSHH) regulations and under standard laboratory safety regulations to Good Laboratory Practice standards using standard operating procedures.

2.2.1 Preparation of Assay Reagents
Preparation of assay reagents was performed under sterile conditions within a BioQ Microfuge Class II Hood. All ATP-TCA assays for both human specimens and cultured cells were carried out in Complete Assay Medium (CAM) (available from DCS Innovative Diagnostik Systeme, Hamburg, Germany). This medium is serum free and does not contain growth factors. To prevent infection CAM is supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co Ltd, Poole, Dorset, Cat No. P0781) and also 10 mM HEPES (Sigma H9136).

2.2.2 Transportation of Tumour Material
Ethics was approved for the use of excess tumour tissue not required for diagnosis ((07/MRE08/2) North West Research Ethics Committee).

Solid tumour material was transported in universals containing 10 ml Dulbecco’s Modified Eagles Medium (DMEM) (Sigma D6171) to which 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma P0781), 10 mg/ml gentamicin (Sigma, UK; G1272) and 10 mM HEPES (Sigma H0887) has been added. Tissue from potentially contaminated sources (such as those who come in contact with the body’s natural flora) were transported in the same medium, with 2.5 μg/ml amphotericin B (Fungizone, A2942;
Sigma) and 1 µg/ml metronidazole (Flagyl®; Rhône Poulenc Rorer Limited, Eastbourne, UK).

Ascites samples, as well as pleural fluids, were transported in 250 ml bottles, containing 25 ml DMEM (Sigma D5671) to which 100 IU/ml penicillin, 100 µg/ml streptomycin (Sigma P0781) and 10 mM HEPES (Sigma H9136) has been added. As a precaution to prevent blood clots forming while in transit 5000 IU heparin sodium (Monoparin®, CP Pharmaceuticals Ltd, Wales) was added.

Tumour samples that required overnight delivery were packed in a polystyrene box and kept chilled with an icepack. To prevent freezing, which would result in cell death and an inadequate cell yield for assay; the sample was separated from the icepack by paper towels. The sample along with its paper work, including patient history details and consent form was then sent via overnight delivery.

2.2.3 Initial Preparation of Tumour Samples

Upon receipt of solid tumour specimens, samples were placed into a 100 mm diameter x 15 mm deep sterile petri-dish. Excess fat and connective tissue was excised and discarded. The sample was then dissected into 0.5 – 2.0 mm³ pieces using sterile scalpels and transferred into universals containing a previously prepared enzyme solution. If the tumour sample was large, i.e. greater than 5 x 10 x 10 mm, then more than one container was set up with equal amounts of tumour material going into each digest. The dissected sample was gently digested overnight in the collagenase solution (Sigma, UK; C8051) reconstituted with Complete Assay Medium (CAM; DCS Innovative Diagnostik Systeme, Germany) and incubated at 37°C and 5% CO₂ (Table 1). The mixture was shaken or inverted at intervals to disrupt the specimen and encourage dissociation.

Table 1: Collagenase concentrations used to digest different tumour tissues

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>Collagenase Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soft Tissue Tumours</td>
<td>1.0</td>
</tr>
<tr>
<td>All other Tumours</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Following overnight digestion the sample was removed from the incubator, gently inverted forming a homogenous cell suspension, then replaced back into the incubator for 30 minutes.

Ascites samples already being single cell suspensions, do not require enzymatic dissociation. These samples were split equally and aliquoted into 50 ml universals under sterile conditions. The samples were centrifuged at 300 g for 10 minutes and the supernatant discarded.

The dissociated cells were washed in a medium consisting of DMEM supplemented with 1 M HEPES, 100 IU/ml penicillin, 10 mg/ml streptomycin and 10 mg/ml gentamicin. Media was added to the samples which were then centrifuged at 300g for 10 minutes. The supernatant was discarded and the process repeated. The supernatant was kept separate from the sample after each wash until the cells had been counted. After the second wash the cells were resuspended in 10 ml CAM (less if the specimen was small) and the universal was stood vertically to allow any undigested material to sediment out.

The cell viability and concentration were then assessed using the trypan blue exclusion method. If tumour cells were present, viable cells were separated from erythrocytes and debris using Ficoll-Hypaque density gradient separation.

2.2.4 Trypan Blue Exclusion

Cell number and viability were routinely assessed using a haemocytometer and the trypan blue exclusion method. Equal volumes of cell suspension and a 0.4% solution of trypan blue (Sigma T8154), normally 20 µl, were mixed in a polypropylene (Eppendorf) container and pipetted onto the haemocytometer. Dead or dying cells are unable to pump trypan blue out of the cytoplasm and so appear blue under the microscope, while viable cells remain clear (218).

As well as assessing the percentage of viable cells within a sample, this method also allowed the amount of non-tumour cells to be estimated. It was not uncommon to find non-tumour cells in samples; these included mainly red blood cells. These were
removed by Ficoll-Hypaque density gradient separation (see next section). The use of specialised media (CAM) and polypropylene culture plates ensured that lymphocytes and other non-malignant cells did not survive in culture and therefore did not interfere with the assay result (171).

2.2.5 Ficoll-Hypaque Density Gradient Separation

Ficoll-Hypaque density gradient separation (219) was performed following the manufacturer’s instructions. 10 ml of Ficoll-Hypaque (Histopaque; Sigma 1077-1) was pipetted into sterile polystyrene 30 ml universal containers. Equal volumes of the digested specimen was carefully layered on top of the Ficoll-Hypaque using a sterile Pasteur pipette and tilting the universal at a 45° angle taking care not to mix the two layers. The sample was then centrifuged at 400 g for 30 minutes at room temperature. After this period the blood cells were sedimented as a pellet, leaving the tumour-derived cells forming an interface between the Ficoll-Hypaque and CAM. The interface containing the purified tumour cell suspension was transferred into a separate sterile 30 ml universal using a sterile Pasteur pipette. The cells were then washed twice with 10 ml CAM by centrifugation at 300g for 10 minutes. Cell number and viability were then reassessed using the trypan blue exclusion method.

2.2.6 Preparation of Chemotherapeutic Agents

Standard cytotoxic drugs used in the assay were obtained as vials for injection from the pharmacy at Queen Alexandra Hospital in Portsmouth where available. All of the chemotherapeutic drugs or combinations were tested in triplicate at 6 dilutions, corresponding to 200%, 100%, 50%, 25%, 12.5% and 6.25% of the estimated Test Drug Concentration (TDC). The TDC is based on the peak plasma concentration and the protein binding for the individual drugs and has been adjusted to provide correlation with clinical response rates (171). In each 96-microwell culture plate four drugs or drug combinations can be tested. The remaining wells in the plate were used for maximum inhibitor (MI) and no drug media only (MO) controls. The plate design used is shown in Figure 5. All the chemotherapeutic drugs were prepared following manufacturer’s instructions and divided into aliquots which were then stored either at room temperature, 4°C, -20°C or -80°C (220). A list of all the drugs used including the stock and test drug concentrations can be found in Table 2.
An 800% test drug concentration (TDC) solution of each drug to be tested was prepared by diluting the stock solution into 2 ml, 5 ml or 10 ml of complete assay media (CAM). Table 2 shows the volume that needs to be added to 5 ml CAM to obtain an 800% TDC solution, as well as the TDC for all the drugs used in this study. Drug combinations were tested by adding the constituent drugs together at their 800% TDC at the beginning of the assay before serially diluting them.

**Table 2: Drug stock and test drug concentrations**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Test Drug Concentration μg/ml</th>
<th>Stock Concentration mg/ml</th>
<th>Test Drug Concentration μM</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetuximab</td>
<td>185</td>
<td>2</td>
<td>1.27</td>
<td>145781.6</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>300.1</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.4</td>
<td>5</td>
<td>3.26</td>
<td>429.9</td>
</tr>
<tr>
<td>Farnesol</td>
<td>2</td>
<td>5</td>
<td>10</td>
<td>222.37</td>
</tr>
<tr>
<td>Farnesyl Diphosphate</td>
<td>4</td>
<td>12</td>
<td>23</td>
<td>382.326</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>5.0</td>
<td>12.5</td>
<td>12</td>
<td>411.5</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>0.4</td>
<td>1</td>
<td>1</td>
<td>446.9</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>12.0</td>
<td>40</td>
<td>40</td>
<td>299.7</td>
</tr>
<tr>
<td>Geranylgeraniol</td>
<td>5</td>
<td>20</td>
<td>10</td>
<td>450.449</td>
</tr>
<tr>
<td>Mevalonate Low</td>
<td>0.7</td>
<td>4</td>
<td>5</td>
<td>148.16</td>
</tr>
<tr>
<td>Mevalonate Medium</td>
<td>30</td>
<td>4</td>
<td>200</td>
<td>148.16</td>
</tr>
<tr>
<td>Mevalonate High</td>
<td>228</td>
<td>4</td>
<td>1537</td>
<td>148.16</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>13.6</td>
<td>6</td>
<td>16</td>
<td>853.9</td>
</tr>
<tr>
<td>PD98059</td>
<td>13.4</td>
<td>5</td>
<td>50</td>
<td>267.28</td>
</tr>
<tr>
<td>Sirolimus</td>
<td>0.9</td>
<td>0.46</td>
<td>1</td>
<td>914.2</td>
</tr>
<tr>
<td>Treosulfan</td>
<td>20</td>
<td>50</td>
<td>72</td>
<td>278.3</td>
</tr>
<tr>
<td>Zoledronic acid</td>
<td>10</td>
<td>0.8</td>
<td>34</td>
<td>290.1</td>
</tr>
<tr>
<td>ZSTK474</td>
<td>0.45</td>
<td>2</td>
<td>1.08</td>
<td>417.4</td>
</tr>
</tbody>
</table>
2.2.7 Drug Panel for ATP- Assay’s

The panel of drugs used for each assay was chosen after researching relevant literature and consultation with Professor Cree. The concentrations of these agents were determined from phase 1 data, corresponding literature and discussions with Professor Cree.

2.2.8 Preparation of 96 Well Plates

ATP-TCAs were performed in round bottomed polypropylene plates (Corning Life Sciences, UK; 3790). 100 µl of CAM was pipetted into each well of rows B-H with a multi-channel pipette. The pre-prepared 800% TDC drug dilution (100 µl) was added to triplicate wells of row B. Using a multichannel pipette the drugs were serially diluted down the plate from rows B-G, while the excess 100 µl remaining after the serial diluting was discarded giving dilutions corresponding to 200-6.25% of the TDC. Two controls were included in each plate: a no drug control consisting of media only (MO) in row H corresponding to 100% viable cells and a maximum inhibitor (MI) control in row A which killed all cells present giving a zero ATP count. The MI solution was made up of 0.02% v/v Triton X-100 (Sigma, UK; T8787) in DMEM. The plate was now ready for addition of the tumour cells.

Cells obtained from enzymatic dissociation of solid tumour material or malignant effusions as described previously were adjusted to 200,000 or 100,000 cells/ml in CAM respectively. 100 µl of cell suspension was then added to each well of the 96-microwell plate giving final cell concentrations of 20,000 for solid tumour specimens and 10,000 for the ascites specimens. Plates were incubated in a 95% humidified, 37°C, 5% CO\textsubscript{2} incubator for 6 days, and checked periodically for overgrowth and infection. After the incubation period the ATP was extracted from the cells and measured.

Any cells that were not required for assays were cryopreserved in DMEM supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin, 10% foetal calf serum (FCS) (Labtech International, East Sussex, UK, cat. 4-101-500) and 10% DMSO (Sigma D5879) or the complete cryopreservation medium- recovery culture cell freezing media (Life Technologies, Paisley, UK, cat. 12648-010).
2.3 ATP Extraction and Measurement

2.3.1 ATP Extraction

A reagent for the extraction of ATP was prepared. HEPES buffer powder (101.7 mg/ml; Sigma H-4034), ammonium meta-vanadate (1.267 mg/ml; Sigma A-1183) and Triton X-100 (0.5%; Sigma T-8787) were dissolved in sterile water (B.P. Water for Irrigation, Pharmacy, Queen Alexandra Hospital), and the solution was titrated to pH=7.8 with 5.0 M sodium hydroxide. The solution was then left to stand overnight in a sterile safety cabinet uncovered until the colour of the liquid changed from yellow to clear. The solution was then filter-sterilised using a 0.22 µm filter and 20 ml syringe and aliquoted into 30 ml sterile universal containers, which were stored in the refrigerator. ATP was extracted from cells by the addition of 50 µl of ATP extraction reagent to each well of the 96-well plate. The cells were immediately mixed by gentle pipetting 4-6 times using a multichannel pipette. Pipette tips were discarded after each drug triplicate. Plates were incubated at room temperature for a minimum of 20 minutes and a maximum of one hour before the ATP was read.

2.3.2 Preparation of Reagents

The ATP in the wells was measured using a luciferin-luciferase counting reagent. D-luciferin (Cat. 800-LN) and recombinant luciferase (Cat. 700-LF) were purchased from R&D systems (Abingdon, UK) and a luciferin-luciferase counting reagent was prepared as described below.

Firstly, a 10x HEPES buffer was prepared by mixing 4.766 mg/ml HEPES powder (Sigma H-0887) and 1.22 mg/ml magnesium sulphate heptahydrate (Sigma M-9397). The solution was basified to pH=7.7 with sodium hydroxide and filter sterilised; D-luciferin was then dissolved in it (0.732 mg/ml buffer). Secondly, a 0.5 M Tris-succinate buffer containing 1% BSA was prepared by dissolving 0.226 mg/ml TRIZMA®-succinate (Sigma T-9632) and 25.9 µl/ml bovine albumin 35% solution (Sigma A-7409), and adjusted to pH=7.5 with sodium hydroxide. This buffer was used to reconstitute the recombinant luciferase (1.0 mg/ml); the solution was then incubated on ice for 1 hour to let the luciferase dissolve completely. The final reagent was then prepared combining 102.5 ml of 10x HEPES buffer containing D-Luciferin with 24.75 ml of recombinant luciferase
(1.0 mg/ml) in 0.5 M Tris-succinate buffer and 41.25 ml of 3% BSA. All incubations were performed in light-tight vials.

The solution was stored at -20°C in 2.0 ml aliquots in light-tight boxes. Before use each 2.0 ml aliquot was diluted with 18 ml of dilution buffer, which consisted of a 1 in 10 dilution of the HEPES buffer prepared to dissolve D-luciferin. ATP was measured using a Berthold Diagnostic Systems MPL1 luminometer (Berthold Diagnostic Systems, Pforzheim, Germany). All luminescence measurements were performed using the manufacturer’s instructions.

The Adenosine 5'-triphosphate standard disodium salt hydrate (Sigma, UK, cat. FLAAS-1VL) was reconstituted using sterile water to give a final concentration of 250 ng/ml. The solution was filter sterilised through a 0.2 µm filter and aliquoted into 40 µl aliquots, stored at –20°C and protected from light for a maximum time of 6 months.

2.3.3 ATP- Standard Curve

An ATP standard curve was performed as a quality control prior to reading any ATPTCA plates, to ensure that all reagents and equipment were functioning properly. For this procedure, 50 µl of dilution buffer was added to nine wells of a white 96 well microplate (Thermo Life Sciences, UK, cat. 7905). To prepare the ATP, 10 µl of the previously aliquoted 100 µg/ml stock solution was added to 4 ml of dilution buffer giving a 250 ng/ml ATP solution; 25 µl of this was added to the first well of the white plate containing dilution buffer. The solution was mixed using the pipette and then 25 µl was transferred to the 2nd well. The mixing was repeated through to the ninth well to give final ATP concentrations of 83.33; 27.76; 9.253; 3.084; 1.028; 0.342; 0.114; 0.038 and 0.012 ng/ml. 50 µl of luciferin-luciferase reagent, was added to each of the nine wells and the plate placed into a luminometer (MPL1, Berthold Diagnostic Systems, Germany). The light output, which is directly proportional to ATP concentration, was then measured. For the test ATP-TCA to be evaluable the average MO reading must reach that of the 1.028 ng/ml ATP value in the standard curve (Figure 6- ATP Standard curve).
2.3.4 Reading of ATP-TCA Plates

At the end of the incubation period (either 5/6 days), cells in the TCA plates were checked for the presence of infections (if any problems were observed the plate was discarded). On rare occasions, single wells were found to be infected and these were excluded from analysis. To read the ATP levels in the ATP-TCA plates following cell lysis (ATP extraction), 50 µl from each well of the 96-well culture plate was transferred into a fresh white 96-well plate (Corning, UK, cat. 3362), using fresh pipette tips for each drug triplicate. To each well, 50 µl of the previously prepared luciferin-luciferase counting reagent was added and the luminescence was read using the luminometer, following the manufacturer’s operating instructions.

2.4 Data Capture and Analysis

2.4.1 Data Capture and Calculation of Sensitivity

The data produced from each ATP-TCA plate was entered into an Excel (Microsoft®) spreadsheet that calculated the percentage tumour growth inhibition at each concentration, the IC$_{50}$, IC$_{90}$ (concentration of drug required to cause 50% and 90% inhibition) and the area under the concentration vs. inhibition graph (Index$_{AUC}$) for each drug. The percentage tumour growth inhibition at each drug concentration was
used to plot curves for each drug or combination. As the variation between the wells that were averaged to calculate the percentage tumour inhibition is small (typical coefficient of variance of less than 10%), error bars have not been included on most graphs as they are usually smaller than the markers on the graphs. The percentage tumour growth inhibition was calculated as follows:

\[
\% \text{ Inhibition} = \left[ \frac{1.0 - (\text{Test-MI}) \times 100}{(\text{MO-MI})} \right]
\]

Test = mean counts for test drug wells
MI = mean counts for maximum inhibitor wells
MO = mean counts for medium only wells

To make comparison between different tumours easier a sensitivity index (Index$_{SUM}$) for each drug in each tumour was calculated. This involved summing the percentage tumour growth inhibition and subtracting this figure from 600 (Index$_{SUM} = 600 - \text{Sum Inhibition 6.25.........200}$. It has been suggested that Index$_{SUM}$ values $>350$ confer comparable clinical resistance, values $<300$ confer comparable clinical sensitivity and values between 300 and 350 confer equivocal sensitivity (221, 222).

### 2.4.2 Calculation of Synergy

The effects of drug combinations compared with their single agent counterparts were analysed using the methods determined by Poch et al (223, 224) which compares the expected (additive) effect with the actual achieved effect at each drug concentration. This method is often better suited to the data produced by the ATP-TCA than other methods commonly used to evaluate combination as it is able to deal with drugs which produce a shallow dose response curve.

Combination indices (CI) calculated by the Chou and Talalay (225) methods were determined at 50% and 90% cell death. These were defined as follows:

\[
\text{CI}_{A+B} = \left[ \frac{(D_{A/A+B})/D_{A}}{D_{A}} \right] + \left[ \frac{(D_{B/A+B})/D_{B}}{D_{B}} \right] + \left[ \alpha(D_{A/A+B} \times D_{B/A+B})/D_{A}D_{B} \right]
\]
Where $CI_{A+B} = CI$ for a fixed effect ($F=50\%$ or $90\%$) for the combination of cytotoxic $A$ and cytotoxic $B$; $D_{A/A+B} = concentration$ of cytotoxic $A$ in the combination $A + B$ giving an effect $F$; $D_{B/A+B} = concentration$ of cytotoxic $B$ in the combination $A + B$ giving an effect $F$; $D_A = concentration$ of cytotoxic $A$ alone giving an effect $F$; $D_B = concentration$ of cytotoxic $B$ alone giving an effect $F$. $\alpha = parameter$ with value $0$ when $A$ and $B$ are mutually exclusive and $1$ when $A$ and $B$ are mutually non-exclusive. The combination index CI calculates synergism $<0.8$; additivity $>0.8$ and $<1.2$; antagonism $>1.2$ (226).

### 2.5 ATP – Sequential Assay (ATP-TSCA)

The ATP – sequential assay (ATP-TSCA) was developed in order to obtain sequential information regarding drug sensitivity or resistance in samples when one drug is given before another. This method is comparable to the standard ATP-TCA, but modified to determine the effects of sequential treatment. The method and development of this assay are described in Chapter 3.3: Methods and Results ATP – Sequential Assay (ATP-TSCA).

### 2.6 Sequential Molecular Biology Plate

This plate was produced alongside the standard ATP-TCA and ATP-TSCA plates for inclusion in the sequential TaqMan array studies. Plates were split into two halves, one half containing the media only control cells and the other the relevant drug (Drug used for pre-treatment in sequence assay). The plates were incubated for $24$ hours before cells were harvested and stored in RA1 2ME where at a later date RNA could be extracted and multiple targets tested in a TaqMan Low Density Array (TLDA).

### 2.7 RNA Extraction

#### 2.7.1 Nucleic Acid Isolation and Digestion

This process was completed using the Ambion® RecoverALL™ Nucleic acid Isolation kit using their total nucleic acid isolation protocol (Life Technologies, Paisley, UK, cat. AM1975).

Sample lysates were slowly thawed from $-20^\circ C$ to $7^\circ C$ in the fridge, and then allowed to come to room temperature. During this time, vials of nuclease free water were pre-heated to $95^\circ C$ in a heat block. Once thawed $100\%$ ethanol was added to each sample
and carefully mixed via pipetting. Samples were then added onto the pre-prepared filter spin columns and the lids closed. Samples were then centrifuged at 10,000g for 30-60 seconds passing the lysate through the filter. The flow through was then discarded and the filter cartridge re-inserted into the same collection tube. This process was repeated until all the lysate sample mixture had passed through the filter. Following filtration, the samples underwent two wash steps. Wash 1 consisted of adding 700 μl of wash 1 to the filter followed by centrifugation at 10,000g for 30 seconds. After discarding the flow through 500 μl of wash 2/3 was added to the filter and again passed through the filter. After the second wash step the flow through was discarded and the filter cartridge replaced back into the same collection tube, the assembly was then spun again to remove any residual fluid from the filter.

The following solutions were combined to make a DNase mix (Table 3). When multiple samples were being processed a master mix was made. Each sample had a 60 μl DNase mix added to the centre of the filter cartridge. The tubes were capped and then incubated at room temperature for 30 minutes. After incubation with the DNase mix the samples were washed as before once with 700 μl of wash 1 and then twice with 500 μl of wash 2/3. They were then spun a further time to remove residual fluid. The fluid free filter cartridges were then transferred to fresh collection tubes where the nucleic acid could be eluted. Nuclease free water (55 μl) pre-heated to 95˚C was applied to the centre of the filter and allowed to sit at room temperature for 1 minute with the cap closed. The nucleic acid was then eluted from the filter via centrifugation at maximum speed and collected in the collection tube. A second 55 μl of nuclease water was used to elute further nucleic acid into the same collection tube leaving a total close to 110 μl of collected eluate. Samples were then quantified and purity checked using 1.3 μl of each undiluted sample with the NanoDrop spectrophotometer.

Table 3: DNase and master mix concentrations.

<table>
<thead>
<tr>
<th></th>
<th>1 Sample Volume (μL)</th>
<th>10 Samples Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Buffer</td>
<td>6</td>
<td>60</td>
</tr>
<tr>
<td>DNase</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>50</td>
<td>500</td>
</tr>
</tbody>
</table>
2.7.2 Reverse Transcription

Isolated RNA was immediately converted into cDNA using ABI High Capacity cDNA conversion kits. Samples were diluted when necessary in nuclease free water (final volume 105 µl) to give a working concentration of approximately 100 ng/µl. Data from NanoDrop readings were inputted into an Excel workbook (Microsoft®) which performed the dilution calculations necessary to complete cDNA and RT Negative reaction mixes. These mixes were added and chilled in a cooling block until ready to load into the Thermal Cycler (Peqlab, UK). Thermal cycler conditions were set to the manufactures ‘manual method’ settings. After the run was completed samples were pulse microfuged at 10,000g for 30 seconds before cDNA quantity was checked via a NanoDrop spectrophotometer (Thermo, UK) ready for future PCR based experiments.

2.8 DNA Extraction

2.8.1 Nucleic acid Isolation and Digestion

This process was completed using the Ambion® RecoverALL™ Nucleic acid Isolation kit using their total nucleic acid isolation protocol.

Sample lysates were slowly thawed from -20°C to 7°C in the fridge; then allowed to come to room temperature. During this time vials of nuclease free water were pre-heated to 95°C in a heat block. Once thawed 100% ethanol was added to each sample and carefully mixed via pipetting. Samples were then added onto the pre-prepared filter spin columns and the lids closed. Spin columns were then centrifuged at 10,000 g for 30-60 seconds passing the lysate through the filter. The flow through was then discarded and the filter cartridge re-inserted into the same collection tube. This process was repeated until all the lysate sample mixture had passed through the filter. Following filtration the samples underwent two wash steps. Wash 1 consisted of adding 700 µl of wash 1 to the filter followed by centrifugation at 10,000 g for 30 seconds. After discarding the flow through 500 µl of wash 2/3 was added to the filter and again passed through the filter. After the second wash step the flow through was discarded and the filter cartridge replaced back into the same collection tube; the assembly was then spun again to remove any residual fluid from the filter.
The following solutions were combined to make an RNase mix (Table 4). When multiple samples were being processed a master mix was made. Each sample had a 60 μl RNase mix added to the centre of the filter cartridge. The tubes were capped and then incubated at room temperature for 30 minutes. After incubation with the RNase mix the samples were washed as before once with 700 μl of wash 1 and then twice with 500 μl of wash 2/3. They were then spun a further time to remove residual fluid. The fluid free filter cartridges were then transferred to fresh collection tubes where the nucleic acid could be eluted. Nuclease free water (55 μl) pre-heated to 95°C was applied to the centre of the filter and allowed to sit at room temperature for 1 minute with the cap closed. The nucleic acid was then eluted from the filter via centrifugation at maximum speed and collected in the collection tube. A second 55 μl of nuclease water was used to elute further nucleic acid into the same collection tube leaving a total close to 110 μl of collected eluate. Samples were then quantified and purity checked using 1.3 μl of each undiluted sample with the NanoDrop spectrophotometer.

### Table 4: RNase and master mix concentrations.

<table>
<thead>
<tr>
<th></th>
<th>1 Sample</th>
<th>10 Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase A</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>50</td>
<td>500</td>
</tr>
</tbody>
</table>

#### 2.9 Calculation of DNA and RNA with NanoDrop

The NanoDrop spectrophotometer was used to quantify gDNA, RNA and cDNA samples. Prior to sample reading the computer software needs to be opened and the correct sample type highlighted. Each measurement session always began with a blanking cycle. This assures the instrument is working well and that any buffer absorbance is not a concern. Before administration onto the measurement pedestal tubes were vortexed to ensure samples were mixed thoroughly. Once administered the sampling arm was then closed and the spectral measurement initiated. Between sample measurements both the upper and lower pedestals were wiped clean using a soft laboratory wipe, thus preventing sample carryover in successive measurements. The NanoDrop software uses Beer’s law to calculate sample concentration in ng/μl based on absorbance at 260 nm. The following absorption coefficients were used: (i) ds DNA: 1.0 absorbance units at 260 nm in a 1 cm cuvette = 50 μg/ml, (ii) RNA: 1.0
absorbance units at 260 nm in a 1 cm cuvette = 40 µg/ml. Purity is also measured and is calculated by the ratio of sample absorbance at 260 and 280 nm. Ratios of ~1.8 for DNA and ~2.0 for RNA are generally accepted as pure. If the ratio is noticeably lower in either case, it may indicate the presence of protein, phenol or other contaminants. Sample data is automatically stored in archive files and easily retrieved when needed.

2.10 Running the TaqMan Low Density Array (TLDA)

The TLDA is comprised of a 384-well micro fluidic card, designed to be used within an Applied Biosystems 7900HT Fast Real-Time PCR System (Figure 7). Hundreds of real-time PCR reactions can be performed simultaneously with minimal amounts of sample, allowing 1 to 8 samples to be run in parallel against 12 to 384 targets. Targets were chosen after researching relevant literature and looking for targets that were presumed relevant in cell proliferation and differentiation, drug pumps and detoxification, DNA damage and repair, apoptosis as well as others more specific to cell and drug type (Table 5). The panel also included four housekeeping genes used for controls.

TaqMan arrays were run according to manufacturer’s instructions. Each sample was made up with TaqMan ×2 Universal Master Mix and mixed with an equal volume of cDNA to give a final concentration of 300 ng/µl. All four samples were then pipetted into two consecutive ports (100 µl per port) of the 384 well cards. The loaded TaqMan array was then placed, port side upwards, into a balanced centrifuge (Sorval Legend) and spun at 380 g for 1 minute to fill the card. This was checked and the card spun again to remove any air bubbles. The card was then placed into a TaqMan array slide sealer, sealed, and the loading ports cut from the card before being loaded into an AB 7900HT thermal cycler. PCR was performed for 90 min with the following conditions: AmpErase UNG Activation for 2 min at 50°C; AmpliTaq Gold DNA Polymerase Activation for 10 min at 94.5°C; followed by 40 cycles each of Melt Anneal/Extend for 30 sec at 97°C and 1 min at 59.7°C. The 'Auto Threshold Cycle' function was performed at the end of the run and resulting cycle threshold (Ct) data from the array card was transferred to a Microsoft Excel spreadsheet. Controls were checked, and the data transferred to a Microsoft Access database before further analysis.
Ct values were standardised by reference to porphobilinogen deaminase (PBGD), the least variable housekeeping gene of the four present on the array. Standard methods of PCR analysis (delta Ct) were not used in analysis of this assay. Instead these studies used a logarithmic gene expression ratio (GER) calculated as $\ln(2^{\frac{Ct(test)}{2^{\frac{Ct(PBGD)}}}})$ and used for comparison with ATP-TCA data.

As these studies were performed over a prolonged period of time, during the course of experimentation standard control material obtained from pooled cDNA samples were run alongside non template controls (NTC) and RT negative samples testing card and sample Ct variability.

**Figure 7: TLDA microfluidic Card (adapted from TaqMan array microfluidic cards user guide)**
Table 5: Genes included on TaqMan array, classified by their major contribution to drug resistance mechanisms.

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>DNA repair</th>
<th>Proliferation</th>
<th>Pumps/Detox</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>ATM kinase</td>
<td>APC C-term</td>
<td>ATP7B</td>
</tr>
<tr>
<td>APAF1</td>
<td>BRCA1</td>
<td>APC N-term</td>
<td>BCRP</td>
</tr>
<tr>
<td>BAD</td>
<td>ERCC1</td>
<td>β TUBULIN III</td>
<td>CES1</td>
</tr>
<tr>
<td>BAX</td>
<td>ERCC2</td>
<td>COX2</td>
<td>CES2</td>
</tr>
<tr>
<td>BCL2</td>
<td>GTF2H2</td>
<td>EGFR</td>
<td>cN II</td>
</tr>
<tr>
<td>BCL-x(L)</td>
<td>MGMT</td>
<td>HER2</td>
<td>DPD</td>
</tr>
<tr>
<td>BID</td>
<td>MLH1</td>
<td>HER3</td>
<td>FPGS</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>MSH2</td>
<td>HER4</td>
<td>γH2AX</td>
</tr>
<tr>
<td>FAS</td>
<td>MSH6</td>
<td>HIF1A</td>
<td>GCLC</td>
</tr>
<tr>
<td>FASL</td>
<td>RAD51</td>
<td>KI67</td>
<td>GCLM</td>
</tr>
<tr>
<td>HSP60</td>
<td>TOPO I</td>
<td>P16</td>
<td>GSTT</td>
</tr>
<tr>
<td>HSP70</td>
<td>TOPO IIα</td>
<td>P21</td>
<td>hENT1</td>
</tr>
<tr>
<td>HSP90</td>
<td>TOPO IIβ</td>
<td>P27</td>
<td>hENT2</td>
</tr>
<tr>
<td>IAP2</td>
<td>XPA</td>
<td>PS3</td>
<td>MDR1</td>
</tr>
<tr>
<td>IGF1</td>
<td>XRCC1</td>
<td>TS</td>
<td>MRP1</td>
</tr>
<tr>
<td>IGF1R</td>
<td>XRCC5</td>
<td>VEGF</td>
<td>MRP2</td>
</tr>
<tr>
<td>IGF2</td>
<td>XRCC6</td>
<td></td>
<td>MRP3</td>
</tr>
<tr>
<td>IGF2R</td>
<td></td>
<td></td>
<td>MRP4</td>
</tr>
<tr>
<td>IGFBP1</td>
<td></td>
<td></td>
<td>MRP5</td>
</tr>
<tr>
<td>IGFBP2</td>
<td></td>
<td></td>
<td>MRP6</td>
</tr>
<tr>
<td>MCJ</td>
<td></td>
<td></td>
<td>MRP8</td>
</tr>
<tr>
<td>MCL1</td>
<td></td>
<td></td>
<td>MTII</td>
</tr>
<tr>
<td>mTOR</td>
<td></td>
<td></td>
<td>MVP</td>
</tr>
<tr>
<td>NFκB</td>
<td></td>
<td></td>
<td>OPRT</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>18S</td>
<td></td>
<td>RRMI</td>
</tr>
<tr>
<td>PTEN</td>
<td>HPRT</td>
<td></td>
<td>SOD1</td>
</tr>
<tr>
<td>STAT3</td>
<td>PBGD</td>
<td></td>
<td>TAP1</td>
</tr>
<tr>
<td>SURVIVIN</td>
<td>SDHA</td>
<td></td>
<td>TAP2</td>
</tr>
<tr>
<td>XIAP</td>
<td>TBP</td>
<td></td>
<td>TAP4</td>
</tr>
</tbody>
</table>
2.11 Analysing TaqMan Low Density Array Raw Data

TaqMan data was analysed using a logarithmic gene expression ratio. The cycle number is first converted to presumed copy number (i.e. $2^{-Ct}$) based on data having similar primer efficiencies. This allows the gene expression ratios to be obtained without skewing the data. It is then standardised against the best housekeeping gene for that data series (i.e. $2^{-Ct(\text{test})}/2^{-Ct(\text{PBGD})}$). Data is then normalised by natural log transformation of this value, (i.e. $\ln(2^{-Ct(\text{test})}/2^{-Ct(\text{PBGD})})$).

2.12 TaqMan Low Density Array Descriptive and Inferential Statistical Analysis

Descriptive statistics such as mean, median, range, variation and distribution were calculated via Microsoft Excel 2007 spreadsheets using relevant statistical function formulae.

Correlation was calculated using Pearson’s correlation coefficient ($r$). This is calculated as a measurement of the strength associated between two variables.

$$r = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sqrt{(\sum X^2 - \frac{(\sum X)^2}{N})(\sum Y^2 - \frac{(\sum Y)^2}{N})}}$$

The closer the scatter of points is to a straight line, the higher the strength of association between the variables. Data ranges from -1 to +1 with zero indicating no linear relationship. Positive correlated variables increase or decrease together, while negative correlated variables increase as the other decreases and vice versa. It should also be considered that just because two variables are related, it does not automatically mean that one variable directly causes the other.

Significance of correlation was calculated with GraphPad InStat3 (www.graphpad.com) using the two-tailed P value where normality and random sampling was assumed. The resulting P value answers the question that if the two variables really are not
correlated at all, what is the chance that randomly selected subjects would have a correlation coefficient as large as that observed in the analysed experiment.

2.13 Cell Culture

Chosen from an in-house panel (TORC-20), cell lines were selected for their close in vitro resemblance in terms of chemosensitivity in the ATP-TCA to human tumour derived cells harvested directly from patients (227). These were purchased from either ATCC (LGC Promochem, Middlesex, UK) Cancer Research UK or donated from Horizon Discovery (Cambridge, UK). Cell lines were supplied as frozen aliquots and subsequently grown under the direct methods from the companies obtained. All cell lines grew as adherent cultures.

2.13.1 Cell Lines

Cell lines in this study included cells originating from ovarian (JAMA2), breast (MCF10a), lung (NCI-H23) and oesophageal (OE19) tissue. Cell lines were expanded for 3 passages from the frozen aliquots received to produce a stock prior to experimentation and did not exceed 10 passages in total. This kept any long term culture changes from affecting cellular assay results.

2.13.2 Cell Culture Media

All cell lines were grown in standard growth medium specified by the corresponding company of purchase. These were supplemented with 10% foetal calf serum (FCS) (Labtech International, East Sussex, UK, cat. 4-101-500) or 5% horse serum (Invitrogen, Paisley, UK, cat. 26050-088), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma P0781). The cells were incubated at 37°C in a humidified 5% CO₂ incubator until they were sufficiently confluent. Prior to experimentation, Ficoll-Hypaque density gradient centrifugation was used to separate pure cell populations from debris or dead cells, followed by viability estimation and cell counting by Trypan blue exclusion as previously described in paragraph 2.2.4.

2.13.3 Passaging Cell Lines

When sufficiently confluent, adherent cell lines were trypsinised using a 1:10 dilution of a 10x trypsin-EDTA solution (5.0 g/L trypsin in 0.2% EDTA; obtained either from GIBCO BRL, Cat. 35400-027, or from Sigma, Cat T4174) flasks were washed with
phosphate buffered saline to remove traces of serum containing media before the addition of trypsin. Trypsinisation could be sped up by putting the flask containing the trypsinised cells into the incubator for no longer than 3 minutes. After cells were completely detached from the flask, the trypsin activity was inhibited by the addition of growth media containing foetal calf serum (FCS) and the cells pelleted by centrifugation at 300g for 10 minutes before being washed twice in growth media to remove any traces of trypsin. The cells were counted using the trypan blue exclusion method as described in paragraph 2.2.4. Cells were then ready for either experimentation or further culturing.

2.13.4 Seeding Cell Line Flasks and Cell Line Plates

Cells were seeded according to supplier’s guidelines while taking into account of doubling times. Cells were reconstituted from frozen pellets stored in a liquid nitrogen dewar and defrosted under cold running water. Once defrosted, vials were wiped with an alcohol soaked towel and placed into the class 2 safety hood. The cells were added drop wise to a 30 ml universal containing 10 ml of pre-warmed growth media. Cells were centrifuged at 300g for 10 minutes, supernatant discarded, and the cell pellet resuspended in fresh media. A further two washes were carried out before the pellet was finally resuspended in 10 ml of growth media checked for viability via trypan blue and transferred to a 25 cm$^2$ flask (Fisher Scientific, Loughborough, UK, cat. TKV-123-011R) and incubated in an incubator at 37ºC. After 48-96 hours, cells were generally confluent and could be passaged and prepared for subsequent experiments.

2.13.5 Storage of Cell Lines in Liquid Nitrogen

Early passage number cells were divided into aliquots of about $10^6$ cells per ml and stored in a liquid nitrogen dewar cylinder. Standard storage procedures used were that of the European Collection of Cell Cultures (ECACC, Centre for Applied Microbiology & Research, Salisbury, Wiltshire, England).

Cells passaged from culture were washed and adjusted to $3 \times 10^5$ cells per ml in growth media containing 10% dimethylsulphoxide (DMSO) (Sigma, UK, cat. D5879) and 50% foetal calf serum. The cells were then divided into aliquots of 1 ml in polypropylene cryovials (Corning, UK, cat. 430658 or 430659) and placed in a ‘Mr Frosty’ cryo-
container (Nalgene, US, cat. 5100-0001) and then immediately placed in a -80°C freezer. After 24 hours in a -80°C freezer the cryovials containing cells were transferred to a rack in a liquid nitrogen dewar.

2.14 Mutation Analysis using DxS TheraScreen Kits for EGFR, KRAS, BRAF and PI3K

The TheraScreen mutation analysis kits (DxS Ltd, Manchester, UK) utilizes two technologies: amplification refractory mutation system (ARMS) and Scorpions for detection of mutations in Real-Time PCR. The high sensitivity and specificity provided by the combined primer-probe leads to the detection of a low percentage of mutant DNA in a background of wild type genomic DNA. Each kit includes a control assay alongside its corresponding mutation assays.

2.14.1 ARMS

The amplification refractory mutation system (ARMS) discriminates between the mutation and wild-type DNA by selectively amplifying the mutated target sequence located at the 3’-end of the primer. In the presence of targetable mutant DNA the ARMS primer will hybridise and be extended by Taq DNA polymerase. This results in the generation of detectable PCR product.

2.14.2 Scorpions

The detection of the amplified mutated DNA is performed using Scorpions. The Scorpion primer is held in a hairpin loop conformation by complementary stem sequences of around six bases which flank a probe sequence specific for the target of interest (228). In this conformation the fluorescent reporter dye (attached to the 5’-end) is in close proximity with a quencher molecule and so no signal is produced. Following PCR, extension and the subsequent denaturation and annealing steps, the hairpin loop unfolds and if the correct product has been amplified the probe sequence binds to the specific target sequence on the newly synthesized strand. Since the fluorescent dye is no longer in close proximity to the quencher a fluorescent signal is generated (229).

All procedures were carried out in accordance with manufacturers’ guidelines and standard operating procedures in use in the Queen Alexandra molecular pathology
diagnostic laboratory. Copies of manufactures guidelines can be found in Chapter 8.3: electronic appendices EGFR/KRAS/BRAF/PI3K Therascreen.
Chapter 3 - Technical Development
3.1 Introduction

3.1.1 Adaptation

Cellular adaptation has been shown to occur within hours of drug exposure (78). Cells respond to the stress induced by chemotherapeutic agents and induce changes to either promote resistance or cellular death. Mechanisms involved in resistance to chemotherapy usually involve up-regulation of resistance mechanisms, or down-regulation of target genes. To study these changes array technology was considered.

3.1.2 TaqMan Low Density Arrays

The study of cancer genetics and signalling pathways is not novel, but its importance now ranges from how we identify tumours to how we treat them and is constantly adapting to advances in molecular techniques. Single genes or proteins are unlikely to be looked at in isolation, unless they happen to be the specific targets to the drugs studied. It is for this reason that array technology has begun to prosper allowing multi-gene signature studies. Instead of screening very large numbers of genes using hybridization arrays to generate signatures, the approach used here was hypothesis-driven. Using current knowledge of the pathways involved in drug resistance and sensitivity a set of candidate genes was generated (Table 5). These targets are specific to areas of known interest in cell proliferation and differentiation, drug pumps and detoxification, DNA damage and repair, apoptosis as well as others targets specific to cell and common drug types. Through analysing these multiple targets it was believed that a more complex profile of tumour sensitivity could be established. These array data along with in vitro cellular assay data were expected to provide a better understanding of how tumours adapt to chemotherapy in the initial stages; looking particularly at tumour sensitivity, cellular stress and ultimately resistance or cell death.

The Applied Biosystems (ABI) custom TaqMan arrays require minimal amounts of sample and allow for 4 samples to be run in parallel against 96 candidate genes and controls. Accuracy is insured as the arrays do not require liquid-handling robotics or complex pipetting to load samples, while the sealed wells decrease the risk of contamination. The custom assays with specific gene targets are delivered preloaded
and dried in microfluidic card formats and individually wrapped and barcoded to ensure no human errors in labelling.

Each individual well of the array houses an individual real time PCR reaction. Real time PCR is based on the detection of fluorescence produced by a reporter molecule while the PCR reaction is taking place (Figure 8). The amount of fluorescence increases as the reaction proceeds due to the accumulation of the PCR product with each cycle of amplification. This method has many advantages over other traditional methods, including greater precision and sensitivity, numerical results and no post PCR processing. TaqMan probes contain a reporter dye and a non-fluorescent quencher. While the probe is intact the close proximity of the quencher to the reporter dye results in fluorescence suppression (230). Fluorescence from the reporter is only released when the two dyes are physically separated via hybridization or nuclease activity. The measurement output is the threshold cycle (Ct). It is the intersection between the amplification curve and the threshold line and determines the relative amount of cDNA starting template found in each sample. The cycle threshold is determined as the cycle in which the first detectable significant increase in fluorescence is detected. After each PCR cycle the amount of DNA theoretically doubles, so samples that differ by a factor of 2 would be expected to be 1 cycle apart and samples that differ by a factor of 10 would be approximately 3.3 cycles apart.

Each TaqMan gene expression assay on the array is given an assay ID number (supplied by the manufacturer (electronic appendices 8.3: TaqMan Array Card Map)) consisting of a 2 letter prefix designating the species for which the assay was designed (Hs = Homo sapiens) as well as a 2 letter suffix designating the assay placement (Table 6). For example, the TaqMan gene expression assay for human PBGD is: Hs00609297_m1, indicating that this is a human multi-exonic gene assay that does not detect genomic DNA.
Table 6: TaqMan gene expression assay placement nomenclature: The alphabetical suffix of the assay ID designates assay probe placement and sensitivity.

<table>
<thead>
<tr>
<th>Assay Suffix</th>
<th>Assay Placement</th>
</tr>
</thead>
<tbody>
<tr>
<td>_m</td>
<td>An assay whose probe spans an exon-exon junction of the associated genes and will not detect genomic DNA.</td>
</tr>
<tr>
<td>_s</td>
<td>An assay whose primers and probes are designed within a single exon, such assays will detect genomic DNA.</td>
</tr>
<tr>
<td>_g</td>
<td>An assay whose probe spans an exon-exon junction, but the assay may detect genomic DNA if present in the sample.</td>
</tr>
</tbody>
</table>

The assay was designed to a transcript belonging to a gene family with high sequence homology. The assays are designed to give between 10 CTand 15 CT difference between the target gene and the gene with the closest sequence homology. An assay, therefore, will detect the target transcript with 1,000 to 30,000-fold greater discrimination (sensitivity) than the closest homologous gene.

3.1.2.1 Housekeeping Genes

Housekeeping genes serve as a common denominator to which target gene expression can be normalized. A suitable housekeeping gene should be adequately expressed in the tissue of interest, and show minimal variability in expression between samples, under experimental conditions. Therefore, housekeeping genes are chosen from genes
that are always expressed because they code for proteins that are constantly required by the cell and most likely present under any conditions.

The identification of stable housekeeping genes for real time RT-PCR is a critical requirement for both accurate and meaningful analysis of target gene expression. Fluctuations in housekeeping gene expression can result in misrepresentation of differences in target gene expression. Obtaining reliable measurements depends on the choice of control genes which is achieved by selecting relevant stable housekeeping genes (231, 232). Five housekeeping genes were chosen for the array (Table 5) allowing the most suitable gene for the sample data to be used for standardisation.

Control cDNA made from a cocktail of human tumour samples were used to test inter and intra assay variability (Electronic Appendices -8.3: Control cDNA repeats). The control cDNA was also used to test operator and climate variability (samples run at varied points throughout the day). These tests included three operators running assays over three days at varied times. This gave rise to a coefficient of variation value for five repeats over three days being less than 3% when Ct values were available.

3.1.2.2 Data Transformation, Standardisation and Normalization

Normalization refers to the division of data by a common variable in order to cancel out that variable’s effect on the data.

Data was analysed using a new method formulated with help from statisticians from Applied Biosystems (Foster City, San Francisco, US). The cycle number is first converted to presumed copy number (i.e. \(2^{-\Delta C_{t}}\)) assuming similar primer efficiencies. This allows the gene expression ratios to be taken without skewing the data. It is then standardised against the best housekeeping gene for that data series (i.e. \(2^{-\Delta C_{t}(\text{test})}/2^{-\Delta C_{t}(\text{PBGD})}\)). Data is then transformed by natural log transformation of this value, (i.e. Ln(\(2^{-\Delta C_{t}(\text{test})}/2^{-\Delta C_{t}(\text{PBGD})}\))) giving the data a normal distribution.

Due to the design of the arrays it was not possible to use an efficiency corrected method (relative standard curve method) when normalizing data. The array cards only
hold positions for 4 samples per card meaning only a 4 point standard curve could be carried out. In this instance the gene expression ratio (GER) method was more appropriate.

### 3.1.3 Sequential Therapy

When carrying out a standard ATP-TCA it is possible to test drug combinations as well as single agent activity. To test drugs in sequence a new assay needed to be developed. Knowing that the limitation to any chemosensitivity assay, molecular or cellular, was that it was only as good as the drugs that are available, much thought needed to be taken in choosing which drug sequences to study. Unfortunately, cytotoxic chemotherapy is not good enough to provide a cure for every patient. This almost certainly reflects the biology of the tumour and the adaptability of neoplastic cells to drug exposure. Therefore, overcoming this adaptation will inevitably be a challenge. By looking at the mechanisms involved in cellular adaptation, resistance and re-growth it may be feasible to see trends in the way tumour cells adapt to certain types of chemotherapy to which new scientifically guided sequential regimens can be developed to utilise this information.

### 3.1.4 High Throughput Drug Screening

As advances in cancer screening and earlier tumour detection continues, the amount of fresh tumour material available for research declines. If cellular assays are to continue they must be adapted to use fewer cells. Moving these 96 well assays from 96 to 384 well footprints would be the most logical step forward as long as both the variability and reproducibility continued to produce the same consistent results as the original 96 well assays. Manual pipetting with 384 well plates using relatively small volumes is time consuming and often imprecise due to human error. It would therefore be more beneficial to use an automated liquid handling system such as the Janus Modular Dispense Technology (MDT) with the addition of a 96 well head and serial dilution tool (Perkin Elmer, Seer Green, UK).

The development of anticancer agents commonly relies on screening potential drugs against cell lines. These however, show significant differences in their behaviour from tumour-derived cells in primary cell culture or xenografts. Nevertheless, cell lines
continue to be used because of their convenience and utility for high throughput screens. Cell line models do have their uses: for instance isogenic cell models (Horizon Ltd, Cambridge, UK) have been generated with a parental line and subsequent lines that include specific isogenic mutations. These cells would be useful for the development for specific molecularly targeted drugs where targets fall in mutated regions.

### 3.2 Methods and Results TaqMan Low Density Array (TLDA)

Many published studies on cellular adaptation and resistance to anti-cancer drugs have investigated the development of resistance using cell lines generated in the lab after prolonged and step-wise exposure to anti-cancer drugs. While this approach provides cells with acquired resistance, these cells do not represent the true nature of the *in vivo* situation, where patients are administered chemotherapy cyclically over a period of weeks. Results from this study have shown the limitations on reproducibility when using cell lines to study expression changes in stress responses (Figure 9).

![Figure 9: Loss of gene expression during cell culture](image)

**Figure 9: Loss of gene expression in the ovarian cell line JAMA2 during 6 weeks in cell culture showing an overall relative decrease from 1 to -1 equating to 4 times the cDNA product.**
A change in the gene expression of the ovarian cell line JAMA2 has shown to occur in as little as 6 weeks (passaging at once a week intervals). While this loss is seen throughout many of the genes tested the remaining housekeeping genes not used for standardisation remained relatively stable (Figure 10).

![Stability of remaining housekeeping genes when standardised to PBGD](image)

**Figure 10: Stability of remaining housekeeping genes in the ovarian cell line JAMA2 in culture over 6 weeks when standardised to PBGD.**

Duplicate flasks of cells from the same passage, harvested and exposed to 2.5 µM cisplatin simultaneously showed a difference in adaptation to some genes tested (Figure 11). This suggests that cell lines that were originally derived from single clones could utilise different cellular adaptation mechanisms when subjected to the same stress inducing factors. As signal transduction usually involves multiple steps there is opportunity for diverse pathway branching and cross-talk to occur.

Differences in gene expression from multiple cell line experimentation could also result from confluency at cell harvest. This could account for differences seen in proliferative genes where overcrowding can promote apoptosis or senescence (233).
expression may also alter in over confluent cells due to contact and nutrient inhibition (234, 235). Alteration in signalling pathways has also been observed with respect to culture media, where differences in biologically relevant pathways are seen between culture in DMEM and MEM (236).

Figure 11: Heterogeneity of JAMA2 cell line response to gene expression when cells were treated simultaneously

Increasingly some studies have demonstrated that over-subculturing cell lines change their properties over time (237-243). It is important to ensure reliable and reproducible results so where cell line models must be used it may be useful to avoid the use of cell lines that have been in culture too long. Primarily cells that are in culture are under stress from being in an ‘alien’ environment. The stress of culture creates pressure for cells to adapt and evolve giving rise to situations favourable to genotypic and phenotypic changes. As cells continue to grow and are subcultured these changes accumulate giving rise to a line of cells so far removed from their original patient origin that they no longer function like their in vivo counterparts (227).
It is for these reasons that where possible, tumour cells derived directly from patients were used for these studies, reducing the amount of manipulation and culturing prior to experimentation.

3.3 Methods and Results ATP – Sequential Assay (ATP-TSCA)

The translation of the ATP-TCA from the current format to include testing for sequential chemotherapy regimens is designed to give more information on the interaction between the tumour sample and/or the different drugs used. At the start of these studies, many methodologies were tried and tested. The initial experiments used ovarian cell lines (JAMA2) as opposed to tumour derived cells, given they were readily available in large cell quantities and being homogenous would hopefully provide greater reproducible results.

In addition to testing a number of molecularly targeted agents, it was the intention to test representatives of all major cytotoxic drug classes, both cycle-specific and non-cycle dependent type. Experiments were carried out with cells originating from tumours where drugs already had an established use (e.g. the use of lung cell lines in gefitinib treatment). Initial experiments carried out with the ovarian cell line JAMA2 were approached by looking back at ATP-TCA results obtained over the previous six years (79). It was observed that the most efficient combination following failure of first line platinum treatment was the combination treosulfan plus gemcitabine. As much data on this drug combination existed in the labs’ database, this was the first sequence used to test the efficiency of the new sequential assay.

Cells were grown to between 80-95% confluence in either 25 cm$^2$ or 75 cm$^2$ (Sigma, UK, cat: CLS3289, CLS3290) cell culture flasks. In these initial experiments two identical flasks were seeded. Once at confluence, culture media was discarded and the flasks washed with 2 ml PBS (Sigma, UK, cat: D8537). Either 15 ml CAM or 15 ml CAM supplemented with cisplatin was added to the culture flasks. These were incubated at 37°C and 5% CO$_2$ for 24 hours before being passaged and cells plated according to the standard ATP-TCA protocol (Chapter 2.13.3 Passaging Cell Lines, Chapter 2.2.8 Preparation of 96 Well Plates).
Pre-incubation times were initially taken from observations from Di Nicolantonio et al. (78) who saw changes in cellular response occurring within 24 hrs of drug exposure. The ATP-TCA assay is optimal over a 6 day period therefore, consideration for pre-incubation times followed by sufficient time for second drug exposure and subsequent cell death needed to be factored into the design. Standard (100% Test Drug Concentration (TDC)) concentrations of the initial drug in the sequence were not used for all drugs, as the idea of the sequential assay was to determine whether preliminary drug exposure could render tumour cells more susceptible to second agents. Thus pre-exposure with a concentration of cisplatin designed to induce a cellular response and not cell death was required. This was devised from results obtained from standard ATP-TCA results from 379 ovarian tumours (Figure 12). A concentration in which roughly between 10 and 20% (2.5 µM) tumour growth inhibition was achieved over 6 days was considered suitable to promote cellular adaptation within 24 hours at both ends of the sensitivity spectrum. This also took account for the overall increase in sensitivity associated with cell lines in long term culture (243).

![Sensitivity of Ovarian tumour Derived cells to Cisplatin (n=379)](image)

**Figure 12: Heterogeneity of ovarian tumour derived cells (n=379) to cisplatin (error bars indicate the most and least sensitive tumour while the blue series shows mean cisplatin sensitivity).**
Results from the sequential assay shown in Figure 13 showed increased resistance to gemcitabine and its combination with treosulfan when cells were pre-exposed to cisplatin. However, treosulfan showed an increased sensitivity at lower concentrations when pre-exposed to cisplatin for 24 hours.

![Graph showing sensitivity of the cell line JAMA2 to treosulfan and gemcitabine alone, in combination and with 24 hour pre-treatment with 2.5µM cisplatin pre-treatment.](image-url)

**Figure 13:** Sensitivity of the cell line JAMA2 to treosulfan and gemcitabine alone, in combination and with 24 hour pre-treatment with 2.5µM cisplatin. With cisplatin pre-treatment carried out in culture flasks

This experiment showed that a sequential assay was possible although it would need to be adapted further to be suitable for use with tumour-derived cells which would not be cultured in flasks. The pre-incubation step was therefore adjusted to take place in 96 well plates (Figure 14). These parameters were then tested in parallel with JAMA2 cells both pre-incubation in flasks and pre-incubation in 96 well plates before the addition of the second drugs (Figure 15). Pre-incubating the drugs in the plates prior to the addition of the second drug combination produced less erratic results, however the general cell sensitivity was greater. This could be due to the lack of stress which is
caused by passaging the cells with trypsin and removing them from the flask before plating in the sequential assay, or that the cell seeding density exposed to cisplatin could be more accurately defined.

To ensure that the activity shown in the 96 well pre-incubation assay was due to the sequence in which the drugs were administered and not synergy of the combination, a wash step was added. This eliminated traces of the preliminary drug from the media and ensured the effect seen was due to the adaptation brought on by the first drug followed by the subsequent effect of the addition of the second drug.

![Graph showing sensitivity of cell line JAMA2 to treosulfan and gemcitabine alone, in combination and with 24 hour pre-treatment with 2.5µM cisplatin.](image)

**Figure 14:** Sensitivity of the cell line JAMA2 to treosulfan and gemcitabine alone, in combination and with 24 hour pre-treatment with 2.5µM cisplatin. With cisplatin pre-treatment carried out in culture plates.

Time course experiments were carried out to ascertain if there was an optimum time at which to add different second agents where second agents were associated with
specific targets. These involved testing agents such as gefitinib and cetuximab 1-6 days post 24 hr cisplatin exposure (see adaptation chapter, page 89: Figure 30 - Figure 34). Results were then compared with molecular data obtained using TaqMan low density array's to determine the level of expression of apoptosis, proliferation and resistance mechanism associated genes during this time.

The lack of response of cells to second agent chemotherapy may be explained by a higher percentage of resting cells (G0-phase cells) and these being resistant to the subsequent chemotherapy (244, 245). To overcome this phenomenon, the capacity of cytokines to switch on cells into the division cycle and progress to the chemosensitive phases (S-, M-phases) was examined. Supplementation with epidermal growth factor (EGF) was used to try to promote tumour re-growth normally occurring in vivo, so that the subsequent active cells could be hit with effective sequential chemotherapeutic agents. Results for these experiments showed little or no difference between EGF

Figure 15: Sensitivity of the cell line JAMA2 to gemcitabine alone, in combination with treosulfan and with 24 hour pre-treatment with 2.5µM cisplatin. With comparison of cisplatin pre-treatment carried out in flasks and in culture plates.
treated, single agent or sequential treatments in the cell lines tested (Figure 16). A switch to lung cancer cell lines was due the fact that gefitinib had been licensed for use in NSCLC.

![Figure 16: Sensitivity of NCI-H23 lung cancer cells (which does not harbour an EGFR mutation) to single agent and sequential cisplatin and gefitinib with and without EGF supplement](image)

3.4 Methods and Results ATP – High Throughput Assay (ATP-THCA)

Using 384 well plates and the ATP-TCA based cell assay, high throughput screening of agents with unknown or possible anticancer properties using primary cell cultures was devised. Custom programs were designed and created for the Janus MDT (Perkin Elmer, Seer Green, UK) using its 96 tip head and serial dilution tool to increase accuracy and reproducibility.

Initial programs were designed to test the growth capabilities of different volume 384 well plates. These plates were chosen to mimic the ATP-TCA 96 well plates as closely as
possible. Therefore, plates were chosen with round bottoms in polypropylene ensuring cells would not adhere to the surface of the plates. This also ensured that when tumour derived cultures were used the plates would not support the growth of non-neoplastic cells.

Deep Well Plates (Greiner, Gloucestershire, UK, cat. 781271) incubated with 100 µl CAM were compared with shallow well plates (Corning, UK, cat. 3656) incubated with 40 µl CAM at 5000 cells per well (densities obtained from previous studies (246)). Results (Figure 17) indicated that cell survival was greater in the shallow plates (MO average 514 relative light units (RLU) in deep well versus 1497 RLU in Shallow Well). This is probably due to better gas exchange in the shallow plates which had a higher surface area to volume ratio. Shallow well plates were therefore used for all subsequent work.

![Figure 17: Comparison of relative light units for media only wells in deep and shallow well plates.](image)

The volume of tumour cell extraction reagent (TCER) used to lyse cells and the cell lysate to luciferin-luciferase (LuLu) volume ratio added to white plates following the incubation needed to be scaled down and optimised to ensure that this did not alter
the results. In the higher volume deep well plates the ATP concentration might have been diluted by the addition of a higher volume of the extraction reagent, giving rise to lower than expected ATP values.

Six plates were set up with varying volumes of TCER and varying ratios of tumour lysate to LuLu. These experiments were conducted with 2,500 cells per well of an ovarian ascites sample from which sufficient cells were available to assess the optimum volumes used in the white plates to measure ATP levels. These were compared over both a 5 and 6 day incubation period.

Index sum variation for the cytotoxic drugs tested was relatively low over all 4 plates read at the 5 day incubation period (coefficient of variation (CV) all below 20%), shown for cisplatin in Table 7. However, the higher RLU values seen with the 20 μl:20 μl cell lysate to LuLu ratio produced better results with greater MO values giving rise to better variance results. No great difference was seen with the different TCER volumes, however again the greater RLU value was seen in those with 5 μl volumes, suggesting that this is enough reagent to lyse the cells and not dilute the ATP content (Table 7).

**Table 7: Intra-assay variability of cisplatin in 384 Shallow plates with various TCER and Lulu volumes**

<table>
<thead>
<tr>
<th>Drug</th>
<th>TCER Volume (μl)</th>
<th>Lulu Volume (μl)</th>
<th>Index SUM plate triplicate 1</th>
<th>Index SUM plate triplicate 2</th>
<th>Index SUM CoV (%)</th>
<th>Mean MO (RLU)</th>
<th>MO CoV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>5</td>
<td>10</td>
<td>206</td>
<td>285</td>
<td>16%</td>
<td>819</td>
<td>44%</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10</td>
<td>10</td>
<td>248</td>
<td>310</td>
<td>11%</td>
<td>1395</td>
<td>21%</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>5</td>
<td>20</td>
<td>469</td>
<td>498</td>
<td>3%</td>
<td>1995</td>
<td>44%</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10</td>
<td>20</td>
<td>326</td>
<td>431</td>
<td>14%</td>
<td>1177</td>
<td>26%</td>
</tr>
</tbody>
</table>

Those plates read on the 6th day of incubation produced slightly erratic results with a media only CV of 88% (Table 8). These were located in the bottom edge of the plate showing a range of 276-2698 relative light units (RLU). It was observed that during this 6 day incubation period evaporation of media at the edges of the plates occurred. This left some wells with less than 40 μl total volume before ATP extraction. Due to this phenomenon any single well assays using the entire 384 well plate will need to be performed with a 5 day incubation period or exclude the outer wells from use.
Comparison of 384 well shallow plates with the original 96 well plates showed that the
downscaling of the ATP-TCA from 96 to 384 wells was successful (Figure 18-Figure 20).
This method as well as being useful for tumours with limited cell numbers would be
useful for screening large numbers of drugs.

Table 8: Raw data showing relative light units (RLU) from a 384 Well plate 6 Day
incubation of an ATP-THCA (Media only wells in bold at bottom of plate)

<table>
<thead>
<tr>
<th>Cisplatin</th>
<th>Gemcitabine</th>
<th>Cisplatin + Gemcitabine</th>
<th>Doxorubicin x3</th>
<th>Paclitaxel</th>
<th>Paclitaxel + Cisplatin</th>
<th>Treosulfan</th>
<th>Tresulfan + Gemcitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 0 0 0</td>
<td>3 0 0 2</td>
<td>0 0 3 0</td>
<td>2 3 0 0</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>108 194 214</td>
<td>0 2 4 4</td>
<td>3 3 0 3</td>
<td>3 0 3 0</td>
<td>6 0 2 0</td>
<td>0 0 0 1</td>
<td>154 82 117</td>
<td>15 9 2</td>
</tr>
<tr>
<td>239 394 330</td>
<td>0 1 2 3</td>
<td>0 0 0 0</td>
<td>0 0 1 0</td>
<td>74 29 41</td>
<td>1 64 12</td>
<td>227 113 258</td>
<td>7 2 0</td>
</tr>
<tr>
<td>370 465 434</td>
<td>22 55 80</td>
<td>17 2 16</td>
<td>3 6 2 0</td>
<td>182 147 168</td>
<td>249 168 121</td>
<td>442 411 274</td>
<td>5 7 0</td>
</tr>
<tr>
<td>365 399 417</td>
<td>0 1 2 3</td>
<td>0 0 1 0</td>
<td>0 0 1 0</td>
<td>103 74 196</td>
<td>109 145 66</td>
<td>565 259 562</td>
<td>0 32 0</td>
</tr>
<tr>
<td>510 539 563</td>
<td>12 12 17</td>
<td>26 12 77</td>
<td>90 339 155</td>
<td>446 915 750</td>
<td>476 409 333</td>
<td>855 644 751</td>
<td>86 63 33</td>
</tr>
<tr>
<td>531 561 586</td>
<td>104 120 229</td>
<td>350 732 550</td>
<td>300 337 464</td>
<td>593 406 229</td>
<td>587 454 731</td>
<td>39 36 47</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 18:** Comparison of cisplatin sensitivity in the ATP-TCA and ATP-THCA assays
Figure 19: Comparison of cisplatin sensitivity in the ATP-TCA and ATP-THCA assays

Figure 20: Comparison of paclitaxel sensitivity in the ATP-TCA and ATP-THCA assays
3.4.1 ATP-THCA for use in Screening Large Compound Libraries

A large library of 5605 plant extracts was obtained (Strathclyde Institute for Drug Research (SIDR), University of Strathclyde, Glasgow, UK). Each extract was screened in 3 recurrent ovarian tumours and inhibitory effects calculated by percentage inhibition. Cells were isolated from fresh tumour samples. These were incubated with 12.5 µg/ml drug compound for 5 days in 384 well polypropylene plates (352 compounds per plate) before being lysed and ATP levels measured by luciferin-luciferase assay. This assay was performed using the Janus MDT liquid handling robot with a 96 well tip head and serial dilution tool (Perkin Elmer, Seer Green, UK).

There were 113 positive hits for sensitivity (Electronic Appendices 8.3: SIDR Single well, high through put screen); these were classified where greater than 80% inhibition was achieved in all 3 tumour samples. These positive hits were then tested further for luciferase inhibition to rule out any compounds inhibiting the assay’s detection system.

Luciferase sensitivity plates were setup with 40 µl of the 12.5 µg/ml extract added to an 83.33 ng/ml ATP solution (10µl/well) in a 96 well white plate (Thermo Life Sciences, UK, cat. 7905). 50 µl of Luciferin-luciferase was then added before reading in a luminometer. From this screen 33 extracts were excluded due to their incompatibility with the luciferin-luciferase assay (Electronic Appendices 8.3: SIDR Luciferase assay).

The remaining 80 extracts were then tested again in a further 3 recurrent ovarian tumour samples; over 5 drug concentrations in triplicate (14 extracts per plate). Alongside these extracts 4 combinations of known cytotoxics were tested, examples of these can be seen in Figure 21 - Figure 23 (Full 80 extract data; Electronic Appendices 8.3: SIDR Dilution Screen). Plate layouts were designed so that blank wells were left at either side of the plate compensating for any evaporation (Technical development Chapter: 3.4). Extracts that had previous activity but whose activity had now diminished, were now excluded (n=33). This was probably due to extract instability caused by multiple freeze thaw actions associated with prior experimentation. While this did not necessarily exclude the extracts for their sensitivity any extract to be taken
further for drug development would need to be stable past a few freeze thaw actions required for testing, as well as possible future drug manufacturing and dispatch.

Figure 21: Sensitivity of 3 recurrent ovarian tumours to treosulfan + gemcitabine in the 384 well high throughput screen

Figure 22: Example of sensitivity of 3 recurrent ovarian tumours to a plant extract in the 384 well high throughput screen (example 1)
3.5 Discussion

The continued development of pre-existing assays to incorporate sequential and small volume high throughput methodologies has been successful. The combined data with that of the TaqMan array technology will inevitably provide a more detailed picture of tumour cell response and adaptation to therapy.

TaqMan array data has already been shown to be well suited to investigate the presence of resistance mechanisms in ovarian, lung and melanoma cancers alongside cellular chemosensitivity testing and clinical results (208-210). It also has many benefits for future use as it requires only small fragments of tumour tissue which can be obtained from archived formalin fixed paraffin embedded specimens as well as fresh tissue. This can allow for large retrospective studies from pre-existing clinical drug trials in which patient outcome is already known.

The adaptation of the standard ATP based tumour chemosensitivity assay to include testing of sequential regimes will be evaluable for study presented in this thesis. Its
application in studying how cells adapt to changes in chemotherapy will also improve understanding of drug scheduling (continued in Chapter 4 - Adaptation and Chapter 7 - Targeting the Mevalonate Pathway). However, the potential use of results generated from low dose drug exposure in this type of assay in predicing any clinical significance is not clear, as these agents would not be used in patients at these concentrations. Heterogeneity of dose escalation response to platinum agents is evident and so this type of assay may therefore be more suited to targeted agents or drugs with cytostatic responses, where cells could be pre-exposed to drugs in similar concentrations to those seen in vivo.

The development of 384 well cell assay applications allowed for development of a novel screening approach for large libraries of potential anti-cancer compounds. This design enables large numbers of agents to be tested against tumour-derived cells where previously the cell numbers required for such studies could not be achieved without the use of cell lines. Collaborative studies with SIdR continue, with further development to discover active fractions of the positive compound hits found here. Further use of this 384 well platform is continued in Chapter 5 - Cisplatin and RAS Effector Inhibition, where this assay was used to study drug combinations at multiple concentrations in a checkerboard style pattern.
Chapter 4 - Adaptation
4.1 Cellular Adaptation to Chemotherapy

Oncologists have long been studying how tumours adapt to first line chemotherapy by observing how effectively patients respond to subsequent treatment. This has helped shape the way standard regimes for particular tumours have developed. Cancer cells can start adapting to anti-cancer agents within 24 hours (78); so the potential to exploit common adaptation results early on could lead to a more defined, molecular driven target based regime design. This could give rise to possibilities for greater enhanced sensitivity for second-line, sequential or maintenance therapies.

Figure 24: Downstream receptor tyrosine kinase signalling (e.g. EGFR) including relevant targets found on the TaqMan gene expression array and important for cell survival, proliferation, protein synthesis and angiogenesis.
Cisplatin kills cancer cells by damaging DNA and inhibiting DNA synthesis and is one of the most widely used drugs for first line cancer treatment, particularly for lung and ovarian cancers. How cells respond to cisplatin-induced DNA damage determines cisplatin sensitivity (53). For these reasons cisplatin is the most logical drug to look at with regards to adaptation and sequential therapy strategies.

EGFR and its downstream signalling pathways have been identified as being associated with cisplatin sensitivity. Cisplatin has also been shown to stimulate internalisation of EGFR and activate downstream signals involving p38, PI3K and AKT (247, 248). Intrinsic intracellular protein-tyrosine kinase activity brought about by EGFR leads to activation of multiple downstream signal transduction pathways shown in the simplified diagram in Figure 24. In addition, EGFR inhibitors have been useful for patients who failed cisplatin-based therapy, but less effective when administered in combination. Poor combination effects are thought to result from gefitinib interfering with cisplatin entry into cells (104, 247, 249, 250).

Effects of cisplatin on the EGFR pathway remain unclear, but the potential role for sequential treatment makes it an important starting point to examine (251).

4.2 Results

4.2.1 Single Agent Sensitivity

Tumour derived cells (n=19) from 8 melanomas, 6 lungs, 3 ovarian and 2 other solid tumours were tested for their sensitivity to gefitinib. Cellular sensitivity showed some heterogeneity in its response to gefitinib. However, resistance (Index\textsubscript{SUM} >350) was seen in 89% (17 of 19) of tumours tested. All tumours and cell lines tested for sensitivity to cetuximab showed resistance with an Index\textsubscript{SUM} >400.

4.2.2 Sequential Assay

Using the sequential assay (Chapter 3.1) tumour derived cells were tested for their sensitivity to gefitinib alone compared with sensitivity to gefitinib following 24 hr cisplatin pre-exposure (2.5 µM). Single agent gefitinib showed a trend for resistance with Index\textsubscript{SUM} results ranging between 315 and 646. Sequential results with 2.5 µM cisplatin pre-exposure showed little change in sensitivity (Figure 25). The exception to
this was four tumours of differing origin, a cisplatin resistant recurrent ovarian tumour, a cutaneous melanoma, a pancreatic tumour and a squamous cell lung carcinoma (donated prior to routine mutation analysis and therefore EGFR mutation status for this tumour is unknown). These tumours showed greater gefitinib activity when pre-treated with cisplatin and examples of their sensitivity can be seen in Figure 26 - Figure 29. However, cellular inhibition in these tumours did not exceed 60%, with only the ovarian tumour producing a sensitivity index of less than 300 (IndexSUM =269) considered sensitive.

![The effect of Gefitinib alone and sequentially with low dose Cisplatin on Tumour derived cells](image)

**Figure 25:** Effect on 19 individual tumours from differing origin with gefitinib alone and sequentially with low dose cisplatin (Pre-exposure for 24Hrs), showing an increase in sensitivity (reduction in IndexSUM) of some tumours when pre exposed to cisplatin (2.5 µM).
Figure 26: Sensitivity of a recurrent ovarian tumour to gefitinib alone, in combination and in sequence with low dose cisplatin 2.5 µM (Pre-exposure 24Hrs)

Figure 27: Sensitivity of a melanoma tumour to gefitinib alone, in combination and in sequence with low dose cisplatin 2.5 µM (Pre-exposure 24Hrs)
Figure 28: Sensitivity of a pancreatic tumour to gefitinib alone, in combination and in sequence with low dose cisplatin 2.5 µM (Pre-exposure 24Hrs)

Figure 29: Sensitivity of a squamous cell lung carcinoma to gefitinib alone, in combination and in sequence with low dose cisplatin 2.5 µM (Pre-exposure 24Hrs)
4.2.3 Sequential Assay and Cellular Recovery

Gefitinib sensitivity could be enhanced in both cell lines and tumour derived cells where previously no increase in sensitivity was observed. This was achieved when a recovery period was introduced between primary cisplatin exposure (2.5 µM) and the serial dilution of gefitinib (Figure 30 - Figure 32). This phenomenon could also be repeated with another EGFR inhibitor, cetuximab (Erbitux) (Figure 33, Figure 34). Although sensitivity was shown to be increased in these samples, this increase was still not enough to put the index$_{SUM}$ marker of sensitivity below 300, considered to reflect clinical sensitivity (175).

Figure 30: Sensitivity of the lung cancer cell line NCI-H23 to gefitinib alone and sequentially with low dose cisplatin with time delays

Figure 30: Sensitivity of the lung cancer cell line NCI-H23 to gefitinib given over a delayed time course following 24 Hrs cisplatin treatment
Figure 31: Sensitivity of the oesophageal cancer cell line OE19 to gefitinib alone and sequentially with low dose cisplatin with time delays.

Figure 32: Sensitivity of an ovarian tumour to gefitinib alone and sequentially with low dose cisplatin with time delays.

Figure 31: Sensitivity of the oesophageal cancer cell line OE19 to gefitinib given over a delayed time course following 24 Hrs cisplatin treatment.

Figure 32: Sensitivity of an ovarian tumour to gefitinib given over a delayed time course following 24 Hrs cisplatin treatment.
Figure 33: Sensitivity of the oesophageal cancer cell line OE19 to cetuximab given over a delayed time course following 24 Hrs cisplatin treatment.

Figure 34: Sensitivity of a lung adenocarcinoma to cetuximab given over a delayed time course following 24 Hrs cisplatin treatment.
4.2.4 Downstream RTK Gene Expression targets and sensitivity to gefitinib

Material for gene expression analysis was available in 47% of samples (9 of 19). This sample size was unfortunately too small to make any statistical claims; however, gene expression of targets downstream and including growth receptors showed some heterogeneity in expression levels. The gefitinib sensitive ovarian tumour (Figure 26) showed relatively elevated expression of EGFR, HER2 and HER3 receptors and lower expression of receptors IGFR1/2, survival targets Bcl-2 and p53 and proliferation targets p21 and p27 when compared with more resistant tumours (Figure 35).

![Figure 35: Waterfall plots of 9 tumours (Labelled O-ovarian, L-lung and S-skin melanoma) showing increasing resistance (bar chart) to cisplatin and gefitinib alone and in sequence with relation to their pre-treatment relative gene expression (standardised to PBGD) of growth factor receptors and downstream targets (highlighted green and red for the greatest and least expression)](image-url)
4.2.5 Sequential Gene Expression and Mutation analysis

Material for sequential gene expression analysis (9 of 19) showed a trend for tumours to exhibit changes in expression within classes of genes involved in apoptosis and growth as well as inflammation, resistance and drug transport, showing the expected changes in gene expression for these tumours in response to cisplatin exposure (Table 9).

Table 9: Changes in gene expression (> 1Ct) of tumours exposed to cisplatin (2.5 µM) compared with drug sensitivity (Index_{SUM}) of cisplatin and gefitinib alone and sequentially (full data Chapter 8.3: Electronic Appendices- Sequential GER)

<table>
<thead>
<tr>
<th>Tumour Number Corresponding to Figure 35</th>
<th>Tumour Type</th>
<th>Drug Sensitivity (Index_{SUM})</th>
<th>Change in Gene Expression greater than one Ct when exposed to 24Hr Cisplatin (2.5µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ovary Ca</td>
<td>344</td>
<td>IGF1, PTEN, BCL2, Fas-L, MDR1, MRP8</td>
</tr>
<tr>
<td>7</td>
<td>Ovary Ca</td>
<td>426</td>
<td>COX2, MTII, PTEN, Fas</td>
</tr>
<tr>
<td>9</td>
<td>Ovary Ca</td>
<td>419</td>
<td>DPD, HER4, IGFBP1, K67, MDR1</td>
</tr>
<tr>
<td>8</td>
<td>Lung Ca</td>
<td>546</td>
<td>MRP4, TAP4</td>
</tr>
<tr>
<td>1</td>
<td>Lung Ca</td>
<td>543</td>
<td>PTEN, MRP8</td>
</tr>
<tr>
<td>2</td>
<td>Lung Ca</td>
<td>402</td>
<td>HER4, TAP4</td>
</tr>
<tr>
<td>4</td>
<td>Skin Melanoma</td>
<td>358</td>
<td>COX2, HER4, MDR1</td>
</tr>
<tr>
<td>5</td>
<td>Skin Melanoma</td>
<td>378</td>
<td>MRP6, MRP8, MTII</td>
</tr>
<tr>
<td>8</td>
<td>Skin Melanoma</td>
<td>426</td>
<td>CES1, EGFR, IGFI, MBR4</td>
</tr>
</tbody>
</table>

There was some variation in the genes activated or suppressed between individual tumours and no clear distinction between tumours that were sequentially sensitive and tumours that were resistant.

Gene expression data for the recurrent ovarian tumour which responded to sequential treatment with cisplatin and gefitinib (Figure 26), showed changes greater than 1 Ct (double/half the cDNA material) value in Cox2, MTII, PTEN and FAS (Table 9). Gene expression for this tumour following cisplatin exposure and tumour recovery showed a marked increase in PTEN expression (Table 10). Enough material was available in this instance for further testing for activating mutations thought to be important in gefitinib sensitivity (Table 13, Page 119). Results showed it to be negative for EGFR, KRAS, BRAF and PI3K mutations suggesting that its sensitivity was due to its gene expression profile rather than any of the mutations tested.
Table 10: Changes in gene expression of the sequential cisplatin and gefitinib sensitive recurrent ovarian carcinoma following drug exposure and recovery (comparisons made with media only/ drug free control starting point, cell exposure to cisplatin (24 hours at 2.5 µM) and cells exposed to cisplatin washed and left to recover with measurements made at 24hr increments. Full data Chapter 8.3: Electronic Appendices- Sequential GER)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Drug Free Control</th>
<th>24 Hr Cisplatin</th>
<th>24 Hr Cisplatin 1 Day Recovery</th>
<th>24 Hr Cisplatin 2 Day Recovery</th>
<th>24 Hr Cisplatin 3 Day Recovery</th>
<th>Pathway Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>1.29</td>
<td>1.53</td>
<td>1.02</td>
<td>1.06</td>
<td>0.89</td>
<td>Growth receptors</td>
</tr>
<tr>
<td>HER2</td>
<td>1.30</td>
<td>1.30</td>
<td>1.16</td>
<td>1.07</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>HER3</td>
<td>0.83</td>
<td>1.16</td>
<td>0.82</td>
<td>1.02</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>HER4</td>
<td>-3.69</td>
<td>-3.96</td>
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<td>-3.47</td>
<td></td>
</tr>
<tr>
<td>IGF1R</td>
<td>-0.23</td>
<td>-0.11</td>
<td>-0.08</td>
<td>0.17</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>IGF2R</td>
<td>1.54</td>
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4.3 Discussion

4.3.1 Gefitinib Sensitivity

Gefitinib resistance was seen in 89% (17 of 19) of tumours tested which is in accordance with previous studies and clinical data (104). Sensitivity to gefitinib seen in
NSCLC clinical samples has shown to be increased with the presence of activating mutations within the EGFR gene (115, 252, 253). The phenomenon in which EGFR mutations suggest possible sensitivity to anti-EGFR targeted therapy may not be reflected in all tumour types. EGFR mutations are found less frequently in ovarian (250) and colorectal (254, 255) cancers but still show variable sensitivity to anti-EGFR based therapy (251, 256-258).

The increased gefitinib sensitivity following short low dose cisplatin exposure was independent of tumour type occurring in some tumours but not others. This suggests mechanistic similarities to either cisplatin exposure or gefitinib sensitivity within these tumours.

Gefitinib has shown little clinical effect outside NSCLC EGFR mutated cancers as a single agent or in combinational or sequential settings (104). This intrinsic resistance may in some part be due to the continued cellular pathway activation via alternate or compensatory signalling pathways (IGFR and MET) (61, 75, 76, 259-261). Alternative strategies to target multiple aspects to this pathway may prove more fruitful. As by combining inhibitors of EGFR with alternate or continued signalling pathway targets, mechanisms of resistance may be circumvented.

**4.3.2 Cetuximab Sensitivity**

Sequential results for cetuximab following cisplatin exposure in oesophageal and lung cancer cells (cell line and primary tumour cells respectively) showed an increase in sensitivity when anti-EGFR antibody therapy is delayed between 1 and 3 days. These data also showed similar effects with the tyrosine kinase (EGFR) inhibitor gefitinib.

Response to delayed schedules has also indirectly been shown within the phase II trials with cisplatin based chemotherapy and tyrosine kinase inhibitors (TKI’s) in lung cancer patients (262, 263). The sequence employed by Zwitter et al had cisplatin given on day 2 and the TKI erlotinib given 3 days later interspersed with gemcitabine given on days 1 and 4. Positive results were seen for this dosing schedule but it is not clear whether these results were in part due to activating mutations with EGFR.
Cetuximab has been shown to sensitisise breast cancer cells to cisplatin. Activity is thought to be achieved by cetuximab suppressing cellular DNA repair capacity, thus prolonging the presence of cisplatin induced DNA adducts or by increasing apoptosis and arresting the surviving cancer cells in the G(2)/M phases of the cell cycle (264, 265).

### 4.3.3 Gene Expression

Sufficient material for gene expression analysis was only available in around half of the tumour samples and so no statistical comparison could be effectively performed. However, molecular data showed greatest changes in expression from cisplatin exposure within genes associated with drug transport, drug resistance, apoptosis, proliferation and growth. Adaptation within these genes look to be as heterogenic as the phenotypic response they induce.

Ability of the EGFR inhibitor gefitinib to inhibit functions of transporter proteins (e.g. BCRP and MDR1) by binding at their ATP binding sites may be found to be useful in MDR cancer cells (266). However, the relevance here to cisplatin is minimal as this agent does not seem to be pumped via these proteins (267).

While cisplatin treatment has been shown to activate the EGFR pathway through phosphorylation of AKT and ERK this has been shown to occur in the absence of ERCC1 (268, 269). Gene expression data for those tumours with sufficient material showed ERCC1 to be actively expressed in all tumours tested but expression did not change extensively in response to cisplatin exposure (Electronic Appendices 8.3: Sequential GER).

Cisplatin recovery experiments with the recurrent ovarian tumour sensitive to cisplatin and gefitinib sequential therapy continued to show minimal changes in ERCC1 gene expression. A similar lack of expression change was seen with AKT and ErBb family members, where expression peaked slightly within the first 24 hours before slowly declining. However PTEN showed a 10 fold increase in magnitude post cisplatin exposure.
Cisplatin resistance can involve deregulation of the AKT/PTEN pathway separate from EGFR (270). Future work could look to intervene with RTK/AKT/PI3K signalling inhibitors to reverse resistant phenotypes with standard therapy or as targeted combinations, such as those used in Chapter 6 - targeting EGFR and PI3K pathways.

4.3.4 Conclusions and Future Prospects

Toxicity effects of cisplatin can be severe for many patients. In such cases cisplatin dose regimens may need to be reduced or changed even if tumour sensitivity is seen. The ability to use a low dose cytotoxic to sensitize tumour cells to a second more tolerable anti-cancer agent is extremely desirable. Results for the sequential administration of cisplatin and EGFR inhibitors gefitinib and cetuximab are shown to be inconsistent and full explanations for sensitivity are, for the most part, still elusive.

These data show the need for an array based approach to cellular adaptation and signalling studies, as cells have shown to utilize a variety of mechanisms to avoid drug induced stress and death. It was optimistic to think that these mechanisms could be so simply exploited to predict individual responses and be utilisable in effective sequential treatments. Other studies have compounding evidence for a multifaceted approach to drug resistance involving adaptation of mechanisms that are both drug- and tissue-dependent (271).

The practical development of the sequential cellular and gene expression assay designs provided are evaluable tools which demonstrate the level of complexity of interconnecting pathways that work in harmony to either drive the cell into death or adapt for survival. It is however, unclear if the use of low dose cisplatin concentrations would reflect clinical outcomes where much higher doses are more commonly used.

One way to move forward from this approach would be to use these techniques to look more closely at targets in and around known pathways that are utilised by neoplastic cells with the options for future pathway specific arrays.
5.1 Introduction

Platinum compounds are DNA damaging agents, with cisplatin being one of the most widely used anti-cancer drugs. Cisplatin has long been augmented with other compounds to increase its efficacy. The intention here is to look at known molecular mechanisms to find novel approaches to cisplatin combinations. Signalling pathways have been shown to regulate cisplatin sensitivity. Therefore an ideal starting point would be combinations with specific agents targeting signalling pathways that contribute to cisplatin activity or resistance. This has already been shown with the VEGF inhibitor SU5416 which sensitizes ovarian cancer cells to cisplatin via suppression of nucleotide excision repair activity (272) or with the limited activity of the sequential administration of cisplatin and EGFR inhibitors gefitinib and cetuximab (Chapter 4 - ).

Genotoxic stress like those produced by cisplatin can induce multiple signalling pathways. Of those the MAP kinase pathways including ERK are structurally related and run in parallel, with end points resulting in regulation of cell proliferation, differentiation and survival (273). Their involvements with receptors such as EGFR, HER2, IGFR and PDGFR along with RAS and RAF oncoprotein participation make it an ideal target for therapeutic intervention alongside cisplatin.

Induction of ERK1/2 activity in response to cisplatin has been documented and is suggestive that ERK1/2 activation could provide cells with partial protection against the cytotoxic effects of cisplatin (274). Activation of ERK, induced by cisplatin exposure precedes p53-mediated DNA damage responses. ERK has the ability to directly phosphorylate p53 causing up-regulation of p21, GADD45, and MDM2 (53) thus, ERK activation may lead to cell cycle arrest allowing time for the repair of cisplatin-induced DNA damage via p53. Inhibition of ERK may therefore increase tumour cell sensitivity to cisplatin.

A link between IGF1R signalling through the PI3K pathway and cisplatin resistance has been shown with ovarian cancer cell lines (275). Moreover the inactivation of downstream targets of the AKT/PI3K pathway has also been shown to sensitize ovarian cells to cisplatin (276, 277).
Treatment of other cisplatin resistant cells lines has shown a decrease of BCL2/BCLxL expression when treated with an mTOR inhibitor. Decreased levels of these anti-apoptotic proteins can contribute to increased apoptosis within these cells (278). These resistant cells may also use alternative mechanisms such as the activation of the PI3/AKT survival pathway to oppose the insult from cisplatin. It is therefore logical to explore targets within these pathways to both enhance cisplatin sensitivity as well as reverse its resistance.

Since many pathways downstream of RAS are controlled by multiple signalling cascades direct inhibition of RAS would not be sufficient to block them (62). Due to the overlapping of RAS signalling, increased signalling through one RAS effector pathway may occur at the expense of another.

It is therefore logical to look at combinations based on downstream RAS effector pathways such as MEK/ERK, PI3K or mTOR as viable alternatives to direct RAS inhibition with the possibility of combining these with the standard cytotoxic cisplatin.

5.2 Results

Tumour material from 4 ovarian adenocarcinomas (1 chemo-naive and 3 with previous platinum/paclitaxel based treatment) was obtained and sensitivity tested against cisplatin alone and in combination with inhibitors of cisplatin related signalling pathways. These include active inhibitors of MEK1/2 (PD98059, LC Laboratories USA), PI3K (ZSTK474, LC Laboratories USA) and mTOR (sirolimus (rapamycin), LC Laboratories USA). Where possible these data have been compared with real-time PCR gene expression profiles and relevant mutational analysis.

5.2.1 Single Agents

Data shows some sensitivity to cells exposed to single agent sirolimus and ZSTK474 but not cisplatin and PD98059 over a range of concentrations thought to be clinically achievable. Taking an Index$_{SUM}$ < 300 as a threshold for sensitivity, all tumour-derived cell samples showed sensitivity to ZSTK474 while 50 % (2 of 4) of samples showed sensitivity when exposed to sirolimus. All samples showed resistance to PD98059 with
index£sum ranging from 372-457. Cisplatin resistance was also seen in all ovarian samples tested (all Index£sum >400) regardless of their prior exposure to cisplatin in vivo (1 chemo-naive, 3 post platinum + paclitaxel treatment) (Figure 42).

5.2.2 Cisplatin Combinations

Using the adapted 384 well ATP-HTCA assay, combinations of cisplatin with cisplatin related signalling targets could be analysed with dose escalating concentrations in a checkerboard style pattern; allowing analysis of dilutions of drug with static concentrations of another (8.3 Electronic Appendices: Checkerboard Design).

Even though the MEK kinase inhibitor PD98059 showed minimal single agent activity not exceeding 50 % cellular inhibition (Index£sum range 372-457) at low cisplatin concentrations, PD98059 could help dramatically increase the sensitivity in these ovarian tumours(Figure 36,Figure 37).

![Figure 36: Sensitivity of ovarian tumour derived cells to cisplatin alone and over differing concentrations of PD980959 (n=4)](image)

Figure 36: Sensitivity of ovarian tumour derived cells to cisplatin alone and with stepwise concentrations of the MEK inhibitor PD98059
This increase in sensitivity can be represented by a decrease in the mean $\text{Index}_{\text{SUM}}$ from a resistant 410 (356-450) to a sensitive 195 (148-237). Results showed that by increasing the concentration of PD98059 this could augment cisplatin sensitivity independently of cisplatin dose escalation.

Figure 37: Sensitivity of ovarian tumour derived cells to the MEK inhibitor PD98059 alone and with stepwise concentrations of Cisplatin

Ovarian cells showed a stepwise increase in sensitivity with the increase of static concentrations of the PI3K inhibitor ZSTK474 when combined with cisplatin (Figure 38). The combination of cisplatin and 2.16 µM ZSTK474 exceeded 90% tumour cell inhibition giving rise to a mean $\text{Index}_{\text{SUM}}$ of 89 (35-131). This combinational effect was less pronounced when looking at static concentrations of cisplatin. Activity of ZSTK474 was shown to be greater with the addition of cisplatin at between 1.3-2.5 µM (Figure 39).

Addition of static doses of sirolimus (between 1.3-2 µM) to cisplatin concentrations increased ovarian tumour cell inhibition (Figure 40). The activity of this combination was shown to be effective mainly due to the activity of sirolimus. However, at higher sirolimus concentrations cisplatin could increase mean tumour cell inhibition from 61% to 87% (Figure 41).
Figure 38: Sensitivity of ovarian tumour derived cells to cisplatin alone and with stepwise concentrations of the PI3K inhibitor ZSTK474

Figure 39: Sensitivity of ovarian tumour derived cells to the PI3K inhibitor ZSTK474 alone and with stepwise concentrations of cisplatin
Figure 40: Sensitivity of ovarian tumour derived cells to cisplatin alone and with stepwise concentrations of the mTOR inhibitor sirolimus

Figure 41: Sensitivity of ovarian tumour derived cells to the mTOR inhibitor sirolimus alone and with stepwise concentrations of cisplatin
5.2.3 Gene Expression and Mutation Status

Tumours were tested for mutations in EGFR, KRAS, BRAF, and PI3K (Table 13). Results showed them to be negative for all mutations tested suggesting that combinational sensitivity was due to the gene expression profile and not the mutations tested.

Expression of multiple markers of DNA repair was observed in 3/4 tumour samples, with the sample taken from the chem-naive patient (tumour 2) showing relatively increased levels (Figure 42).

![Figure 42: Sensitivity of ovarian tumour derived cells to cisplatin, PI3K inhibitor ZSTK474, mTOR inhibitor sirolimus and MEK inhibitor PD98059 alone and in combination (both with relative dose escalation), in relation to relative gene expression ratio data of signal transduction pathway targets (highlights show positive (green) and negative (pink) expression in relation to PBGD).](image-url)
5.3 Discussion

5.3.1 Single Agents

Resistance to cisplatin was seen in all tumours tested regardless of prior treatment before tumour sample retrieval. This could result from a multitude of factors as tumours showed heterogeneous expression of efflux pump genes as well as increased expression of multiple targets of signalling pathways and apoptosis related genes. Cisplatin resistance has shown to be related to AKT/mTOR survival pathway, with resistant cells expressing a higher level of activated AKT (279).

ZSTK474 was the most effective single agent tested with all tumours showing sensitivity with an Index_{sum}<300. PI3K has been shown to be a promising target for ovarian tumours, whether this is due to its possible disruption of AKT or other pathway targets. It is important to consider that targeting downstream targets of AKT rather than AKT directly is more beneficial, as AKT also mediates certain biologically important cellular processes such as glucose metabolism which could give rise to more toxic side effects for patients (280).

The mTOR inhibitor sirolimus showed some sensitivity in these cisplatin resistant cells. Sensitivity of mTOR inhibitors including sirolimus can be limited in some tumours due to the presence of negative feedback loops, where AKT and ERK become phosphorylated through mTORC1 and mTORC2 (281). Sirolimus has shown to only partially block translation by efficiently inhibiting S6 kinase 1 (S6K1) but not eIF4E. Due to the inhibition of S6K1-dependent feedback loops rapamycin also indirectly upregulates PI3K activity promoting cell survival (282).

5.3.2 Combinations

Resistance was seen in all tumours to the MEK inhibitor PD98059. Its combination with cisplatin showed little increase in activity suggesting there was little evidence for a role of the RAS/RAF/MEK/ERK pathway in these tumours. However, at low cisplatin concentrations PD98059 was shown to increase sensitivity more noticeably.
This study showed an increase in sensitivity to sirolimus and cisplatin combinations when compared with either single agent in ovarian tumours. Inhibition of mTOR has been shown to restore activity of cisplatin in lung cancer (278, 283). Sirolimus inhibition has been shown to activate AKT/ERK via feedback mechanisms. This suggests a role of the MEK/ERK pathway in enhancing cisplatin induced apoptosis (284, 285), with prolonged ERK activation promoting cell death (284).

This study has shown that augmenting cisplatin with the PI3K inhibitor ZSTK474 may be an effective strategy for ovarian cancer. It has already been shown that inhibition of PI3K activity decreases the survival of the cells exposed to Cisplatin (248, 286). As AKT activation has been shown to occur in many cisplatin resistant tumours, the addition of PI3K inhibitors to first line cisplatin treatment may result in a more prolonged response with the option of reducing cisplatin concentrations (286).

### 5.3.3 Checkerboard Dose Escalation

Using the checkerboard design to evaluate dose escalation studies with these combinations proved invaluable as simple combination analysis would not have shown the extent of sensitivity seen with these agents at different static dose escalations.

Combining molecular targeted agents with cisplatin in some instances has shown that it may be possible to use lower doses of cisplatin (than those currently employed clinically) to achieve similar or greater cellular inhibition than those seen with single agent cisplatin. Although PD98059 never reached clinical evaluation, combinations of this type of inhibitor along with cisplatin could be beneficial. Combinations could give rise to a reduction in side effects such as nephrotoxicity associated with cisplatin toxicity without reducing the cytotoxic activity within patients who are unable to tolerate higher dose cisplatin regimens.

### 5.3.4 Gene Expression

Previous studies comparing TaqMan array and ATP-TCA drug sensitivity in ovarian cancer had shown correlation of 10 genes with cisplatin sensitivity (Rad51, IGF1R, p53, Topo I, MRP5, survivin, HSP60, BCRP, Mcl-1 and Ki67 in order of greatest contribution) (209). With such small numbers here statistical correlation was not performed.
However, expression of genes could be observed within known mechanisms of chemoresistance, membrane drug pumps and DNA repair (see Electronic Appendices: 8.3; GER RAS effector Inhibitors, for full data).

Cisplatin resistance was seen in all tumours tested however; those with greatest resistance showed to have reduced levels of over 5 DNA-repair mechanisms. A trend in sirolimus sensitivity could be seen with EGFR, HER2 and HER3 expression suggesting the potential rationale for combinations of this mTOR inhibitor with a RTK inhibitor.

Greatest resistance was seen for the MEK inhibitor PD98059 however, there were no target genes directed for the RAS arm of RTK pathway to associate this with. Greater sensitivity was seen with ZSTK474 and sirolimus single agents and combination with cisplatin. Gene expression data for theses tumours showed greater expression of multiple targets within the PI3K/AKT/mTOR pathway. This may suggest that the PI3K/AKT/mTOR pathway is more prominent than the RAS/RAF/MEK/ERK pathway in ovarian tumours.

5.3.5 Conclusion

Although platinum agents are still one of the most widely used cytotoxic agents in ovarian cancer, resistance and toxicity are still major problems. While this study included small numbers of tumour samples, evidence of greater sensitivity could be seen with combinations of cisplatin and inhibitors of targets downstream of RAS. Evidence for the reduced cisplatin concentration used in these combinations may provide a rationale for patients who develop severe platinum based toxicity.
Chapter 6 - Targeting EGFR and PI3K Pathways
6.1 Introduction

EGFR is found to be expressed in ovarian cancer (287). Anti-EGFR antibodies and small molecule inhibitors have been used in ovarian cancer patients with little or no success in terms of clinical response (250, 288). This is consistent with previous data on TKIs activity in ovarian cancer primary cell cultures where only occasional evidence of gefitinib activity was observed and not at a level likely to produce clinical response (104). While data from non-small cell lung cancer (NSCLC) would suggest that this may be due to a lack of activating mutations, it has also been suggested that an anti-EGFR strategy may also fail due to the presence of alternate activated pathways (287, 289). Inhibition of a solitary signal transduction pathway is often inefficient due to activation of alternative signalling cascades or receptor switching (261, 290-292). The PI3K/AKT/mTOR pathway is of importance in ovarian cancer (293) and is activated by a number of known molecular defects, particularly PIK3CA mutation or amplification in up to 30% and PTEN loss in up to 40% of patients (294, 295). In addition, the insulin like growth factor (IGF) pathway, as well as other human epidermal growth factor receptor (HER) pathways are present and thought to be active in ovarian cancer (296, 297).

Given the observation of limited activity of gefitinib and the potential for combination with inhibitors of the PI3K/AKT/mTOR pathway, it was decided that the best course of action would be to examine the possibility that EGFR targeted agents would be more effective against ovarian cancer in combination with other inhibitors specific to the RTK/AKT/PI3K/mTOR pathway (Figure 43). Di Nicolantonio and colleagues developed a panel of isogenic human cell lines by employing homologous recombination (by knock-in) to characterize the response of the specific mTOR inhibitor everolimus to those cells containing specific mutations (298). Using paired cell lines (isogenic and parental); drug sensitivity versus resistance was accurately assessed, with any phenotypic changes being a direct result of the introduced mutations. The DNA-modifications made to these commercially available cell lines are made within the endogenous gene so as to closely recapitulate the genetic events leading to the desired disease of study.
Figure 43: Growth factor receptor tyrosine kinase pathway signalling including points at which gefitinib, erlotinib, ZSTK474 and sirolimus interact

This comparative technique was also employed here. Isogenic cell lines for ovarian cancer were not available, so instead the nearest alternative were chosen. These were the human non-tumorigenic immortalized breast epithelial cells MCF10a containing known activating mutations in EGFR, BRAF, AKT and PI3K along with the parental line which is wild type for these mutations. These were tested for sensitivity against EGFR/AKT/PI3K/mTOR pathway inhibitors gefitinib (Iressa), erlotinib (Tarceva), sirolimus (Rapamycin) and ZSTK474, a pan-PI3K inhibitor (299). These combinations were then tested against primary cell cultures from human ovarian tumours to determine whether this strategy might have potential for clinical application.
6.2 Results

In both isogenic cell lines and tumour-derived cells, the ATP-TCA was used to determine the effect of EGFR inhibitors (gefitinib and erlotinib) alone and in combination with inhibitors of the AKT/PI3K/mTOR pathway. In the primary cell cultures these data have been compared with RT-PCR gene expression profiles and relevant mutation analysis to determine mechanisms of sensitivity and resistance.

6.2.1 Effect of Single Agents on Isogenic MCF10a Cell Lines

The parental MCF10a cell line showed greater resistance to gefitinib than those with mutations in EGFR, PI3K, BRAF or AKT (Figure 44). Greatest sensitivity was seen within the PI3K mutated cells where Index$_{SUM}$ values decreased from a relatively resistant 423 to considerably more active 120 and 64 for the H1407R and E545K mutations of PI3KCA respectively. MCF10a cells were more sensitive to erlotinib (Index$_{SUM}$ = 188) than gefitinib (Index$_{SUM}$ = 423). The reasons for this are unclear. Changes in sensitivity caused by the mutations were less pronounced in response to erlotinib exposure. The PIK3CA mutation E545K still became the most sensitive phenotype, but the effect of an AKT mutation produced a slightly more resistant phenotype (Index$_{SUM}$ = 227) when compared with the parental line (Index$_{SUM}$ = 188). However, both showed sufficient cellular inhibition to be classed as active agents in this setting with an Index$_{SUM} < 300$ (104).

All mutated MCF10a cell lines showed sensitivity to the PI3K inhibitor ZSTK474 (Index$_{SUM} < 300$) with EGFR and AKT mutations having no effect on the activity of this agent. Results with these mutations were similar to the parental line. PI3K mutations conferred greater sensitivity to ZSTK474 compared with the parental line, while cells containing the BRAF mutation V600E showed a slightly more resistant phenotype.

Sirolimus was the least active of the four inhibitors tested (Figure 44). Only MCF10a cells harbouring EGFR and PIK3CA (E545K) mutations showed sensitivity with Index$_{SUM}$ 272 and 254 respectively which was an increase compared with the parental line Index$_{SUM}$ 333. Cells containing the BRAF mutation (V600E) again showed the most resistant phenotype with an Index$_{SUM}$ 533.
6.2.2 Effect of Combinations on Isogenic MCF10a Cell Lines

Combination of the EGFR inhibitors with either ZSTK474 or sirolimus resulted in greatly increased cellular inhibition (Figure 44), with sensitivity Index$_{SUM}$ values in all MCF10a cell lines falling below 200. The effects of mutations on sensitivity were still observable with cells containing BRAF mutations, though these showed less sensitivity to combinations when compared with the parental line. MCF10a cells containing the PI3K mutation H1047R showed little sensitivity to single agent sirolimus. In combination with EGFR inhibitors these cells were still seen to be more resistant than their parental counterparts and more resistant than MCF10a cells containing the PIK3CA E545K mutation. Interestingly, MCF10a cells containing PI3K mutations (E545K and H1047R) showed greater sensitivity to EGFR inhibitor gefitinib than the cells containing the EGFR mutation (ΔE746-A750).

Activity at higher concentrations in all combinations was mainly due to synergy (100% with EGFR and PI3K inhibitors 83% (5 of 6) for EGFR and mTOR inhibitors). This is reflected by the combination indices calculated by the Chou and Talalay method on the median dose-response curve (Table 11) (225). Antagonism was seen more predominately for drug combinations at lower concentrations where only 17% (1 of 6) of cells showed any synergy.

Table 11: Effect of combining EGFR inhibitors with PI3K and mTOR inhibitors on MCF10a cell lines using Chou and Talalay combination index calculated for 50% cell death (CI50) and for 90% cell death (CI90), where synergy is <0.8, additivity is between 0.8 and 1.2 and antagonism is >1.2

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Figure 44: Sensitivity of gefitinib, erlotinib, ZSTK474 and sirolimus alone and in combination on the parental MCF10a cell line compared to isogenic clones with EGFR, BRAF, AKT and PI3K mutations Compared with it’s Wt
6.2.3 Effects of Single Agents on Tumour Derived Cells

Heterogeneity between ovarian tumours was observed for all four targeted agents. The majority of ovarian tumours tested were found to be resistant to EGFR inhibitors with only 2/10 for gefitinib and 1/10 for erlotinib respectively (1 in 10), showing strong single agent activity with an \( \text{Index}_{\text{SUM}}<300 \) (Figure 45). Greater sensitivity was seen with the PI3K inhibitor ZSTK474 with 4/10 tumours showing single agent sensitivity (Figure 45). Inhibitors of mTOR (sirolimus) have been shown to elicit a predominately cytostatic response (300) and so not surprising only 1/10 tumours showed any sensitivity in this type of assay where cell death is the end point (Figure 45).

![Figure 45: Mean sensitivity of ovarian tumour cells to the EGFR, mTOR and PI3K inhibitors alone and in combination in Ovarian tumours (n=10)](image)

6.2.4 Effect of Combinations on Tumour Derived Cells

In the ovarian tumour samples the combinations of EGFR inhibitors with PI3K and sirolimus inhibitors showed greater sensitivity compared with their corresponding single agent activity (Figure 45).
Some of the activity observed with gefitinib and ZSTK474 was due to both synergism and additivity at lower concentrations (50% and 20% respectively), while 50% of tumours also showed additivity at higher doses. This is reflected by the combination indices calculated by the Chou and Talalay method on the median dose-response curve (Table 12) (225). Antagonism was seen more predominately for this combination at higher concentrations where 50% of samples had >1.2 combination index for 90% inhibition.

Table 12: Effect of combining EGFR inhibitors with PI3K and mTOR inhibitors for ovarian tumours using Chou and Talalay combination index calculated for 50% cell death (CI50) and for 90% cell death (CI90), where synergy is <0.8, additivity is between 0.8 and 1.2 and antagonism is >1.2

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</tbody>
</table>

The combination of erlotinib and ZSTK474 showed similar effects to that of the gefitinib and ZSTK474 combination (example of individual tumour sensitivity (Figure 46). A pronounced increase in sensitivity could be seen with co-administration of these agents. Results showed synergy at lower concentrations while antagonism was seen at 90% inhibition, where 70% of samples had >1.2 combination index (Table 12).

The combination of sirolimus and EGFR inhibitors though not as effective as the EGFR and PI3K combinations showed some increase in sensitivity to its combination compared to individual single agent activity (example of individual tumour sensitivity Figure 47). Although some synergy was observed the sensitivity increase seen with this combination was predominantly due to an additive effect (Table 12). An inverse relationship could be seen in the single agents activity of both EGFR inhibitors and sirolimus, whereby sensitivity to one referred resistance to the other (Figure 48 and Figure 49).
Figure 46: Example of sensitivity of an ovarian tumour to EGFR and PI3K inhibitors alone and in combination

Figure 47: Example of sensitivity of an ovarian tumour to EGFR and mTOR inhibitors alone and in combination
6.2.5 Mutation Status of MCF10a and Ovarian Tumours

MCF10a cell lines included mutations in EGFR (exon 19 deletion E746-A750), BRAF (c.1799T>A - V600E), PI3K (c.3140A>G - H1047R and c.1633G>A - E545K) and AKT (c.49G>A - E17K). All cell line mutations were confirmed where ARMS kits for the relevant mutation were available (Qiagen Ltd, Manchester). Confirmation of mutations
were obtained for all but AKT in this way. The parental MCF10a cell line was also screened against all available mutation tests to confirm mutation exclusion.

Tumours were tested for common mutations with EGFR, KRAS, BRAF and PI3K genes (Table 13). All tumours tested negative for the common mutations of EGFR, PI3K and BRAF. A single serous adenocarcinoma was shown to have a KRAS c.35G>T (G12V) mutation. The G12V mutation results in an amino acid substitution at position 12 in KRAS, from a glycine (G) to a valine (V). This tumour showed decreased sensitivity to all EGFR, PI3K and mTOR inhibitors tested alone and in combination, with Index\textsubscript{SUM} values indicative of resistance (504 gefitinib, 455 erlotinib, 347 ZSTK474 and 396 sirolimus. Combination Index\textsubscript{SUM} values for gefitinib + ZSTK474 303, Erlotinib + ZSTK474 303, gefitinib + sirolimus 401 and erlotinib + sirolimus 336). Interestingly gefitinib showed greater single agent resistance than erlotinib.

Table 13: Activating mutations tested within EGFR, KRAS, PI3K and BRAF genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Deletions in exon 19</td>
<td>Detects the presence of any of 19 deletions but does not distinguish between them</td>
</tr>
<tr>
<td></td>
<td>T790M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L858R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L861Q</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G719X</td>
<td>Detects the presence of G719S, G719A or G719C but does not distinguish between them</td>
</tr>
<tr>
<td></td>
<td>S768I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insertions in exon 20</td>
<td>Detects the presence of any 3 insertions but does not distinguish between them</td>
</tr>
<tr>
<td>KRAS</td>
<td>Gly12Ala</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly12Asp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly12Cys</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly12 Ser</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly12Val</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly13 Asp</td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td>V600E/K</td>
<td>Detects the presence of V600E or V600K but does not distinguish between them</td>
</tr>
<tr>
<td>PI3K</td>
<td>H1047R</td>
<td>Detects the presence of E545D or E545K but does not distinguish between them</td>
</tr>
<tr>
<td></td>
<td>E542K</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E545D/K</td>
<td></td>
</tr>
</tbody>
</table>
6.2.6 Gene Expression and Sensitivity

Relative gene expression data for ovarian tumours showed varying expression levels of multiple growth factor receptors including EGFR, HER2, HER3, IGF1R and IGF2R (Figure 50 and Figure 51) many of which showed some linear dependence with EGFR, PI3K and mTOR inhibitor sensitivity (p<0.05) (Table 14). EGFR inhibitors showed contrasting correlating gene expression data compared with PI3K and mTOR inhibitors where positive correlation of target genes seen in EGFR inhibitors was seen to be negatively correlated in ZSTK474 and sirolimus (Table 14).

Table 14: Correlation of relative gene expression with drug sensitivity using paired Pearson’s correlation.

<table>
<thead>
<tr>
<th>Drug</th>
<th>gene</th>
<th>Pearson’s r</th>
<th>Pearson’s r²</th>
<th>Linear correlation p &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefitinib</td>
<td>EGFR</td>
<td>-0.68</td>
<td>0.46</td>
<td>0.0447</td>
</tr>
<tr>
<td></td>
<td>HER2</td>
<td>-0.67</td>
<td>0.45</td>
<td>0.0479</td>
</tr>
<tr>
<td></td>
<td>IGF1</td>
<td>0.72</td>
<td>0.52</td>
<td>0.0289</td>
</tr>
<tr>
<td></td>
<td>IGF2R</td>
<td>0.71</td>
<td>0.50</td>
<td>0.0327</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>Bcl2</td>
<td>0.74</td>
<td>0.54</td>
<td>0.0236</td>
</tr>
<tr>
<td></td>
<td>HER3</td>
<td>-0.73</td>
<td>0.54</td>
<td>0.0248</td>
</tr>
<tr>
<td></td>
<td>IGF1</td>
<td>0.83</td>
<td>0.69</td>
<td>0.0054</td>
</tr>
<tr>
<td></td>
<td>IGF2R</td>
<td>0.74</td>
<td>0.55</td>
<td>0.0224</td>
</tr>
<tr>
<td></td>
<td>MRP1</td>
<td>0.68</td>
<td>0.46</td>
<td>0.0431</td>
</tr>
<tr>
<td></td>
<td>TAP4</td>
<td>0.83</td>
<td>0.69</td>
<td>0.0104</td>
</tr>
<tr>
<td>ZSTK474</td>
<td>EGFR</td>
<td>0.69</td>
<td>0.48</td>
<td>0.0383</td>
</tr>
<tr>
<td></td>
<td>HER3</td>
<td>0.90</td>
<td>0.82</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td>TAP4</td>
<td>-0.73</td>
<td>0.53</td>
<td>0.0397</td>
</tr>
<tr>
<td>Sirolimus</td>
<td>HER3</td>
<td>0.81</td>
<td>0.66</td>
<td>0.0080</td>
</tr>
<tr>
<td></td>
<td>IGF2R</td>
<td>-0.74</td>
<td>0.55</td>
<td>0.0217</td>
</tr>
<tr>
<td></td>
<td>MRP1</td>
<td>-0.74</td>
<td>0.55</td>
<td>0.0219</td>
</tr>
<tr>
<td></td>
<td>TAP4</td>
<td>-0.71</td>
<td>0.50</td>
<td>0.0486</td>
</tr>
</tbody>
</table>
Figure 50: Waterfall plots reflecting sensitivity of ovarian tumours to RTK pathway inhibitors and their relation to relevant gene expression targets (highlighted cells indicate relative gene expression where target Ct value is more than double (green)/half (pink) that of PBGD).
Figure 51: Waterfall plots reflecting sensitivity of ovarian tumours to combinations of RTK pathway inhibitors and their relation to relevant gene expression targets (highlighted cells indicate relative gene expression where target Ct value is more than double (green)/half (pink) that of PBGD).
6.3 Discussion

Combinations of EGFR and PI3K inhibition show synergistic activity against ovarian cancer, despite the absence of EGFR mutations examined in this tumour type. There is heterogeneity of activity of the different drugs and combinations with different mutations in the MCF10a cell line. Similar heterogeneity is seen in clinical trials with single agents in ovarian cancers and it will be important to define the pathways to future stratify patients for optimal treatment schedules. The gene expression data indicate that this is feasible but suggests that determinants of resistance may not be limited to the target pathways, but also involve both the apoptotic potential of the cell and classical drug resistance mechanisms relating to drug efflux pumps.

In MCF10a cell lines, PI3K mutations lead to higher activity of EGFR inhibitors and PI3K inhibitors, while AKT mutation E17K leads to excessive growth with the mTOR inhibitor sirolimus. Limitations of sirolimus activity in the E17K mutated line could be due to the amalgamation of multiple factors. These could include the continued PI3K and AKT activation via feedback loops caused by mTOR inhibition with sirolimus, as well as activation of AKT1 by means of pathological localisation to the plasma membrane stimulating downstream signalling caused by the E17K mutation (301-303).

Mutations in PI3K (H1047R) have been shown to enhance HER2-mediated transformation by amplifying the ligand-induced signaling output of the ErbB family of RTK’s (304). It could be assumed that because PI3K mutations drive ErbB related receptor addiction in these cells it would make them more susceptible to RTK inhibition (like those seen here with gefitinib).

EGFR/PI3K and EGFR/mTOR combinations were all more active than their single agents, especially when PI3K mutations were present. However, it was found that E545K cells were more sensitive than H1047R suggesting that not all PI3K mutations will result in the same activity in combination.

The number of ovarian tumours studied was too small to determine whether this is an issue in practice as PIK3CA mutations are rare in ovarian cancer. The reality that not all
activating mutations within a given gene are comparable may also be important in other tumour types and recapitulates data from Di Nicolantonio et al suggesting that not all KRAS mutations in colorectal cancer are equally effective in conferring resistance to anti-EGFR antibodies (298).

This study identified varied inhibition of ovarian tumours when exposed to EGFR, PI3K and mTOR inhibitors. The greatest single agent activity was observed with the PI3K inhibitor ZSTK474 confirming the importance of PI3K signalling within these tumours. Evidence for the deregulation of PI3K/AKT signalling in ovarian cancer includes gain of function mutations, amplifications of PI3K and AKT genes as well as allelic imbalance and mutations in PTEN (305).

All drug combinations showed greater activity than their single agent counterparts, with ZSTK474 and EGFR inhibitor combinations showing greatest activity. Antagonism with these combinations was seen at higher concentrations suggesting that there may be a biologically optimum dose beyond which activity is lost. The strategy to hit multiple aspects to signalling pathways thought to be key to ovarian cancer control of growth and survival proved to be more effective than single agent administration in all of the tumours studied. With inhibition of EGFR via tyrosine kinase inhibitors, continued expression of HER2, HER3, IGFR and c-MET goes unchecked. Subsequent heterodimerisation of these unchecked receptors has been shown to increase downstream signalling via the PI3K-AKT pathway resulting in resistance to tyrosine kinase inhibitors gefitinib and erlotinib (306). Therefore, the addition of further agents targeting such PI3K compensatory pathways or multi-targeted HER inhibitors (e.g. lapatinib) might result in greater effects. Such a strategy is feasible but, would of course require phase I trials with careful monitoring of toxicity.

Expression of genes involved in growth factor receptors and substrates, apoptosis and drug transport were seen to correlate well with the activity of single agent EGFR inhibitors in ovarian tumours. In these agents increased levels of EGFR, HER2 or HER3 conferred sensitivity to these inhibitors, while increased IGF2R expression inferred greater resistance (Figure 50). The inverse to this relationship could be seen with ZSTK474 and sirolimus sensitivity. The inverse relationship between the effects of gene
expression for these receptors may give rise to some of the antagonism seen with the combination of these inhibitors and is explicable in terms of the pathway (Figure 43).

In the presence of the EGFR inhibitor gefitinib, PI3K signalling can be maintained by an activated IGF1R pathway (74, 119). Therefore it could be suggested that for effective single agent anti-EGFR therapy to be effective in EGFR wild type ovarian tumours it would require cells to have active EGFR dominant ErbB signalling in the absence of IGFR signalling pathways. In anti-EGFR resistant tumours showing this profile, further alternate signalling mechanisms may be employed including continued signalling via MET by driving ErbB3 dependent activation of PI3K.

Cellular Inhibition with PI3K inhibitor ZSTK474 was shown to be more effective in tumours with low receptor tyrosine kinase (RTK) gene expression. It has been shown that inhibition of PI3K can result in feedback upregulation of expression and phosphorylation of multiple RTK’s in breast cancer (307). The identification of these feedback mechanisms emphasise the need for a multiple approach to treatment, by combining RTK and PI3K inhibitors as shown here some of this effect may be muted.

This study shows the possible importance of two drug transporters in the sensitivity of erlotinib, ZSTK474 and sirolimus in ovarian cancer. Both gefitinib and erlotinib previously have shown direct inhibition of drug efflux function of MDR1/ABCB1 and BCRP/ABCG2. Their expression has been found to be elevated in cells with acquired gefitinib resistance (252, 308). The expression of ATP Binding Cassette (ABC) transporters MRP1/ABCC1 and TAP4/ABCB4 had a negative effect on drug sensitivity to the EGFR inhibitor erlotinib, whereby drug resistance increased along with the level of gene expression. However, the reverse was true for the sensitivity to ZSTK474 and sirolimus where an increase in transporter expression meant an increase in sensitivity.

Some limitations were imposed on this study by our use of cells from ascites. This limited our ability to perform immunohistochemistry as most cells obtained were used in the primary cell culture experiments and no corresponding histological material was obtained. However, primary tumour material is still probably more relevant than cell lines from long term culture with hyperactivated growth pathways. Unfortunately, the
sample size for this study meant that there were very few tumours with activating mutations within relevant targets. Breast epithelial isogenic cell line MCF10a data showed very similar results to ovarian tumours in relation to drug sensitivity and showed the importance for molecular characterisation of tumours, where possible including gene expression as well as mutation analysis.

In conclusion, this study shows very encouraging activity of a combination of EGFR and PI3K inhibitors: combined HER inhibitors such as lapatinib may also be interesting though they were not tested here. Further work should include PTEN and AKT mutation analysis as well as PIK3CA amplification (possible via fluorescent in situ hybridisation). Possible Phase I/II clinical trials with these agents should include pharmacodynamic endpoints and molecular characterisation to identify patients most likely to benefit from this strategy.
Chapter 7 - Targeting the Mevalonate Pathway
7.1 Introduction

The mevalonate pathway provides cells with isoprenoids which are fundamental for cell growth and survival. Inhibition of targets involved in this pathway has shown promising results for the treatment of some cancers (149, 309). Two such agents fluvastatin and zoledronic acid have shown activity at points distant to each other along this pathway and have shown to be directly effective in tumour cell inhibition (Figure 4, page 23) (147, 310). The rationale behind using these two inhibitors within a single pathway stems from the idea that you could amplify the single pathway blockade limiting cross reactivity by targeting points at distant sites.

Statins such as fluvastatin are potent competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, and are widely used to treat patients with high cholesterol and coronary heart disease (311). Although still controversial, statin use in patients diagnosed with ovarian cancer has shown to be associated with improved survival (312, 313).

The newer N-bisphosphonates such as zoledronic acid (Novartis) are inhibitors of farnesyl pyrophosphate synthase (FPPs), reducing the amount of both FPP and GGPP available for Ras prenylation (310, 314). In addition to the loss of prenylated proteins, zoledronic acid causes intracellular accumulation of isopentenyl pyrophosphate (IPP) which consequently induces the biosynthesis of the pro-apoptotic ATP analog AppPi (1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester triphosphoric acid) (150, 152, 153). AppPi has the subsequent ability to directly induce apoptosis through the blockade of mitochondrial adenine nucleotide translocase (152).

Studies have shown direct activity of the N-bisphosphonate zoledronic acid in an ATP-based tumour chemosensitivity assay (ATP-TCA) against breast and ovarian tumours (147, 310). Studies here show the continuation of the ATP-TCA mevalonate studies carried out by Knight et al. examining effects of fluvastatin sequentially and in combination with zoledronic acid against primary ovarian cancer cells in vitro.
7.2 Results

A total of 11 ovarian tumours were tested for sensitivity to zoledronic acid and fluvastatin, alone and in combination. Alongside these, 8 ovarian tumours were tested for sequential administration of these agents. Zoledronic acid (hydrated sodium salt) was obtained from Novartis (Basel, Switzerland) and fluvastatin (344095-25) was obtained from VWR International (Leicestershire, UK). Both drugs were diluted in complete assay media (CAM) to concentrations previously thought to be clinically achievable (Zoledronic acid 2.2 – 69 μM and fluvastatin 0.7 – 24 μM). Combinations of zoledronic acid and fluvastatin were tested by simultaneous addition as per the method outlined in the methods chapter (2.2 ATP-TCA).

7.2.1 Effects of Single Agents

There was considerable heterogeneity between individual tumours in their response to zoledronic acid and fluvastatin (Figure 52- Figure 54). Data shows sensitivity to cells exposed to both single agents at clinically achievable concentrations. Taking an Index<sub>SUM</sub> < 300 as a threshold for sensitivity, all tumour-derived cell samples showed sensitivity to zoledronic acid while 82% (9 of 11) of samples showed sensitivity when exposed to fluvastatin.

7.2.2 Effects of Combination

Sensitivity was seen in all ovarian tumours to the zoledronic acid and fluvastatin combination with less heterogeneity at higher concentrations compared with their single agent counterparts (Figure 54). This combinational sensitivity shows greater activity compared with either single agent (Figure 55). Using combinational indices (225) data shows synergistic effects for zoledronic acid and fluvastatin combinations in 82% (9 of 11) of samples (Table 15) where values were < 0.80. The median CI50 and CI90 values were 0.67 (0.131-1.29) and 0.37 (0.11-1.85) respectively both showing synergy. Only 1 sample showed any additivity and antagonism for CI50 and CI90.
Figure 52: Heterogeneity of ovarian tumour derived cells to zoledronic acid

Figure 53: Heterogeneity of ovarian tumour derived cells to fluvastatin
Figure 54: Heterogeneity of ovarian tumour derived cells to the combination of zoledronic acid and fluvastatin

Figure 55: Chemosensitivity of tumour derived cells to zoledronic acid and fluvastatin alone and in combination.
Table 15: combinational indices showing the synergistic effects of zoledronic acid and fluvastatin on the number of ovarian samples (225)

<table>
<thead>
<tr>
<th>Effect</th>
<th>$C_{90}$</th>
<th>$C_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synergy ($&lt;0.80$)</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Additivity ($&gt;0.80$ AND $&lt;1.20$)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Antagonism ($&gt;1.20$)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

7.2.3 Sequential Effects

Sequential drug experiments show that ovarian tumour cells exposed to fluvastatin exhibit little or no differences in their sensitivity to fluvastatin regardless of whether they have been pre-exposed to zoledronic acid (Figure 56). Greater changes in zoledronic acid sensitivity were observed when fluvastatin was given as a 24 hour pre-treatment, 71% (5 of 7) of samples showed an increase in zoledronic acid resistance when pre-exposed to fluvastatin compared with 29% (2 of 7) which showed an increase in zoledronic acid sensitivity (Figure 57).

Figure 56: Effect of sequential zoledronic acid and fluvastatin on ovarian tumour derived cells
Substrate replacement experiments were conducted with a single tumour derived ovarian cancer from which large numbers of cells were obtained. Substrates included farnesyl diphosphate (FPP) (Echelon Biosciences Inc, US, cat: I-0150), mevalonate (Sigma, UK cat: M4667), farnesol (Sigma, UK, cat: F203), and Geranylgeraniol (GGOH) (Sigma, UK cat: G3278) these were tested via simultaneous addition or pre-incubation using a static concentration.

No evidence of toxicity was seen when cells were exposed to farnesol, GGOH, mevalonate and FPP alone (Figure 58). Replacement and pre-incubation experiments were performed in various combinations with zoledronic acid and fluvastatin showing that in certain incidences the pathway blockade could be reversed (Figure 59- Figure 61).
FPP addition only weakly reversed the effects of fluvastatin; the combination of zoledronic acid and fluvastatin and combination of zoledronic acid, fluvastatin and mevalonate (5 µM) (Figure 59).

Addition of farnesol and GGOH to zoledronic acid and zoledronic acid and fluvastatin showed no effects sequentially or in combination (Figure 60 and Figure 59).

A reversal of fluvastatin activity was shown in the presence of increasing mevalonate concentration from an IndexSUM of 185 for single agent fluvastatin to 499 with 1537µM of mevalonate. Mevalonate had little effect of downstream inhibition of the zoledronic acid pathway alone or in combination with fluvastatin (Figure 62).

Figure 58: Sensitivity of an ovarian tumour to mevalonate pathway substrates as well as zoledronic acid and fluvastatin alone and in combination
Figure 59: Sensitivity of an ovarian tumour sample to zoledronic acid alone and in combination with fluvastatin and mevalonate with FPP substrate replacement.

Figure 60: Sensitivity of an ovarian tumour to zoledronic acid alone and in combination with fluvastatin and GGOH substrate replacement (→ indicating sequential administration + indication combination administration).
Comparison of Ovarian tumour sensitivity of Zoledronic Acid alone and in combination with Fluvastatin when the substrate Farnesol is reinstated

Figure 61: Sensitivity of an ovarian tumour sample to zoledronic acid alone and in combination with fluvastatin and with farnesol substrate replacement (-> indicating sequential administration + indication combination administration)
Figure 62: Sensitivity of an ovarian tumour to fluvastatin alone and in combination with zoledronic acid with mevalonate substrate replacement over 3 concentrations (µM low, µM medium and µM high)
7.3 Discussion

It has been suggested that the underlying mechanism for the activity of zoledronic acid and fluvastatin involves Ras prenylation (147). Their use in combination has shown to produce enhanced effects compared to single agent activity in ovarian cancer (Figure 55).

Observations of a marked reduction in sensitivity seen in some tumours when zoledronic acid follows fluvastatin sequentially are in contrast to what would be expected. When given before the bisphosphonate statins should, theoretically, be able to significantly reduce the entry of mevalonate into the pathway, allowing zoledronic acid to further block any remnants of downstream substrates resulting in increased efficacy of the combination. Sequential data suggests that in some samples zoledronic acid is far less effective when given after fluvastatin. Short exposure to fluvastatin may be enough to alter pathway dynamics rendering zoledronic acid less effective or, this effect may be a result of alternative mechanisms in play, such as an altered function of Ras specific to these samples.

Suggestions have been made for a metabolite of ATP known as ApppI (Figure 4, Page 23) to be responsible for some of the toxic effects of N-bisphosphonates (151, 152, 154, 155). Sequential treatment in which zoledronic acid is given prior to fluvastatin shows no real increase in sensitivity compared with single agent fluvastatin. The main synergistic qualities from zoledronic acid in this combination may therefore come from the accumulation of ApppI over time where exposure exceeds 24 hours.

The pharmacologic effects of fluvastatin are greatly reversed by treatment with mevalonate, suggesting that the downstream inhibition of geranylgeranylation and farnesylation of cellular proteins plays a critical role in its anticancer effect. This effect is less substantial when zoledronic acid is added to this combination. Zoledronic acid activity was not comparably reversed by FPP, while no effect was seen with GGOH or the farnesylated protein farnesol, suggestive of an effect more consistent with the alternative ApppI mechanism.
The direct anti-cancer synergistic results obtained from the combination of zoledronic acid and fluvastatin in ovarian tumour derived cells puts forth a rationale that it may be useful for treatment of ovarian cancer.
Chapter 8 - Discussion and Future Work
The purpose of this study was the development and adaptation of techniques to investigate neoplastic molecular pathways and how they react when exposed to standard and targeted chemotherapy. With the growing difficulty to obtain viable fresh tissue in the quantities required for these studies it was necessary to look closer at the tumour types which would generate sufficient cellular material. This came mainly in the form of ascitic fluid from ovarian cancer patients. Ascites is the accumulation of fluid in the peritoneal cavity and in ovarian cancer this can be rich in cellular material. Many patients with ovarian ascites will undergo palliative paracentesis, the contents of which are routinely discarded. Obtaining this material has been valuable to these studies.

Ovarian cancer is the second most common gynaecological cancer in the UK. Its high mortality is in part due to the difficulty of making an early diagnosis as ovarian cancer often develops without well-defined symptoms. This leads to a large majority of patients presenting with advanced cancer (stage III) at diagnosis (315). First line treatment for this disease is usually aggressive and can include a combination of surgery, chemotherapy or radiotherapy. Relapse can often occur within 6 months with residual tumour becoming highly resistant.

There are many mechanisms by which cells resist drugs. Often exposure to one drug results in the acquisition of resistance to a broad range of chemically unrelated compounds (multidrug resistance). These studies have further confirmed that this adaptation process can begin to occur in a matter of hours, with up/down regulation of target genes involved in proliferation, DNA repair, apoptosis, cellular detoxification and drug pumps (78).

8.1.1 Targeting Cells through Adaptation

The idea of molecular chess and specific predictive oncology using designer sequential therapy is unlikely to come to fruition at present. It may be very difficult to predict the precise cellular adaptation paths a specific tumour will take when given that a single clonal tumour cell line taken from the same passage on the same day and exposed to the same drugs can react in similar but non-identical ways (JAMA2 experiments Chapter-3.1.1). Tumours can follow similar cellular adaptation profiles but often react
to drugs in dissimilar ways. It may be possible to try using small biopsies soon after treatment and even within the first 24 hours to see in which direction the tumour is adapting so as to tailor treatment, but predicting the outcome of the adaptation model can currently only be guess work which negates the rational for precise informed predictive sequential regimens. It may be useful to look at gene amplification or copy number alongside gene expression profiles, but the algorithms required to predict sensitivity from these data will be complex. Nevertheless, it may be possible to use drugs in combination to target connecting pathways and prediction of these may be feasible.

It should be remembered that not all resistance is acquired through adaptation. Consideration must also be given to the role of somatic selection. It is known that within the vast population of cells making up the tumour mass not all tumours contain identical mutations. This however, can become a problem when certain mutations have a survival advantage over neighbouring cells, allowing them to proliferate into an expanding clonal cluster of drug resistant cells. This situation can arise following therapeutic intervention where a selective pressure is placed on sensitive cells allowing resistant clones to continue to grow and invade (316, 317).

Even though studies looking at single genes showed both similarities and significant differences related to sensitivity to chemotherapeutic agents in some cases the bigger picture can be easily overlooked. Those results that were excluded as outliers or seemed to contradict all other results may have found other means by which to act. Looking at single genes may suggest how these tumours will react to a given drug exposure, but as shown in these cellular adaptation profiles, profiling multiple genes can give more information as to the behaviour of the individual tumour. If we are to continue to improve therapies and regimens, strategies that are more individualised will be required. It has long been known that tumours of the same type are extremely heterogenic and through studying these cellular and gene expression profiles we can see their adaptation to chemotherapy is no exception. Repeated tests alongside treatment to show how the tumour is adapting and advancing can only help improve understanding of individual responses allowing a more direct and specific regime to be employed.
8.1.2 Multiple Molecular target Approach to Treatment

Evidence presented here shows the importance of the tyrosine kinase receptors including EGFR, HER2/3 and IGFR1/2 as well as the importance of their signalling pathways through both RAS and PI3K/AKT in solid tumours. The cross-signalling involved with these pathways suggests a rationale for the deregulation of these signalling cascades via multiple modes of action. It has been shown that, highly selective or specific blocking of single sites involved in signaling pathways has been associated with limited or sporadic responses (318). Improved understanding of the complexity of signal transduction processes and their roles in cancer suggest a strategy for simultaneous inhibition of several key pathway positions to help optimize overall therapeutic benefit associated with preventing alternative mechanisms associated with bypassing pathway inhibition.

8.1.3 Targeting Cellular Signalling through RAS

Statins may influence ovarian cancer biology through disruption of Ras signalling by inhibiting the enzyme HMG-CoA reductase, halting the mevalonate pathway and thus RAS protein prenylation which is required for signalling. Active RAS signalling has been shown to occur in high-grade ovarian cancers, suggesting that the RAS pathway may be important in the maintenance rather than the establishment of tumour growth and metastasis (311, 319). By combining fluvastatin with another mevalonate pathway inhibitor zoledronic acid, sensitivity was increased showing a synergistic relationship with these agents in the ovarian tumours tested in this study. While this combination may in fact work by promoting accumulation of the cytotoxic by product ApppI, disruption of protein prenylation and farnesylation through the inhibition of the mevalonate pathway remains an attractive strategy. The zoledronic acid fluvastatin combination may still prove to be a useful combination for future applications in patients with ovarian cancer, and clinical trials are probably warranted as there are few maintenance options for patients with recurrent ovarian cancer.

Platinum compounds such as cisplatin are still one of the most widely used classes of drugs for the treatment of ovarian cancer. The efficacy of these agents has been successfully augmented with many drugs such as paclitaxel to provide combination therapy which is used routinely as standard treatment.
Although extensive DNA damage from cisplatin treatment can induce cell death by apoptosis, several signalling pathways, including AKT and MAPKs, are known to regulate cisplatin induced apoptosis (53). Cisplatin combinations with MEK, PI3K and mTOR inhibitors in this study showed some increase in sensitivity compared with single agent activity. However, more interesting was that the activity of these targeted drug combinations could still be achieved when reduced concentrations of cisplatin were introduced.

By combining platinum with molecular targeted therapy it may be possible to lower the dosage of cisplatin currently employed without losing sensitivity, which could help alleviate its side effects, which include nephrotoxicity and neurotoxicity.

8.1.4 Targeting Cellular Signalling through EGFR

Growth factor receptors are overexpressed and/or dysregulated in many ovarian tumours, with EGFR overexpression occurring regularly (320-324). Despite its overexpression, anti-EGFR treatments have had little success in ovarian cancer. In other cancers, EGFR-activating mutations are associated with enhanced sensitivity to EGFR inhibitors. However, these mutations are rare in ovarian cancer and are therefore not suitable predictive markers for this disease.

In general, tyrosine kinase inhibitors (TKIs) have shown variable activity in solid tumours despite initial evidence for the key importance of the pathways they disrupt. The discovery that responsive subsets of these patients had activating mutations in relevant receptors initiated increased interest for these mutations as markers for sensitivity. In particular, lung cancers that harbour the EGFR kinase domain mutations show greater response to small molecule EGFR inhibitors such as gefitinib and erlotinib. Unfortunately, the activity seen with these inhibitors is limited as cancers invariably develop resistance mechanisms against these drugs (325). The most common mechanism of acquired resistance to the EGFR inhibitors gefitinib and erlotinib in lung cancer is the development of a secondary EGFR mutation, T790M which increases the affinity of the EGFR for ATP, thereby reducing the efficacy of these drugs (117).
Other EGFR inhibitor resistance mechanisms include the amplification of the MET oncogene. When MET is overexpressed it activates HER3 independently of EGFR (or HER2), and is able to maintain downstream signalling through both PI3K and ERK in the presence of gefitinib.

There is increasing evidence to support the importance of the involvement of the upregulation of HER3 gene expression with its ability to increase the signalling potential of other HER family receptors (326, 327).

This study has shown the importance of the interplay between HER family receptors and IGF1/2R. Sensitivity to EGFR inhibitors gefitinib and erlotinib is seen when increased expression of EGFR, HER2 and HER3 receptors combine with low levels of IGF1R and IGF2R receptors (Chapter 6 - . It may be interesting to investigate if the inverse relationship seen here with tyrosine kinase inhibitors occurs in other tumour types. These receptors may also prove useful as potential molecular markers for sensitivity to EGFR inhibitors in patients expressing this profile. The inhibition of IGF1R has shown to enhance response to HER targeted drug treatment in many solid tumours (328-331), so it is no wonder that many inhibitors of IGF1R are currently in phase I/II trials (332).

Resistance to EGFR inhibitors by maintenance of PI3K signalling can also occur through activation of the IGF1R signalling pathway (261). The importance of the PI3K pathway as well as its re-activation associated with acquired resistance to TKIs in EGFR addicted cancers strengthens the support for use of PI3K inhibitors in multiple targeted treatment strategies. Results here show the potential of incorporating PI3K pathway inhibitors with EGFR target therapies for an effective strategy to overcome acquired resistance as well as the transactivation associated with these signalling pathways. Early safety and tolerability studies are already under way for solid tumours with the combination of PI3K inhibitors (XL765 and XL147, Exelixis, San Francisco, CA) and the EGFR inhibitor erlotinib (333, 334).
8.1.5 Targeted Treatment the Present and Future Strategies

We are firmly in the midst of the molecularly targeted era of personalised oncology. Cancer patients can start to expect treatment regimes based more dominantly on their individual tumour’s signalling and molecular type rather than the empirical approach oncologists have previously seen to be successful in the past. This will inevitably require patients to have adequate amounts of diagnostic tissue for multiple histological and molecular techniques. With increased efficacy of early detection and tumour screening, in many tumour types the supply of viable diagnostic material is rapidly becoming inadequate. Novel methods, such as isolation of circulating DNA from blood will become vital for future diagnostic strategies (335-340). It will eventually require significant thought to future planning with regards to diagnostic services including close collaboration between all arms of cellular pathology, blood science, molecular pathology and cytogenetics (341).

8.1.6 Future Work

Collaborative work with the University of Strathclyde will continue based on the use of the automated 384 well based assay (ATP-THCA) methods, with the possibility of evaluating individual fractions of the compounds which were found sensitive. The checkerboard adaptation to this method is also currently in use for other drug combination evaluations for use in collaborative industry based projects.

Studies identifying a possible benefit for combinational EGFR and PI3K targeted treatment is ongoing with future grant proposals being submitted by Prof. Cree based on work completed here. The aim is to extend these observations into possible clinical trials, all while monitoring the molecular mechanisms involved in patient response.
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Appendices
8.2 Publications

Full text copies of publications can be found in the electronic appendices.

First author publications:


Other publications relevant to this thesis:


8.3 Electronic Appendices

1. Therascreen Instruction Manuals EGFR, KRAS, PI3K and BRAF
2. TaqMan Array Card Map
3. Control cDNA Repeats (Intra/Inter assay variability, operator and time controls)
4. SIDR Single Well High Throughput Screen
5. SIDR Luciferase Assay
6. SIDR Dilution Screen
7. Sequential GER
8. Checkerboard Design
9. GER RAS Effector Inhibitors
10. Full Text Publications