Drug metabolism and chemotherapy resistance

A thesis submitted for the degree of Ph D

by

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abstract

Development of drug resistance is a major limitation of chemotherapeutic treatment of cancer Resistance arises as a consequence of the genomic instability of tumour cells, and usually relies on the perversion of mechanisms used by normal cells to protect themselves against environmental toxic agents One of these mechanisms is the increased expression of xenobiotic metabolising enzymes, in an attempt to reduce drug activation or enhance its detoxification Cytochromes P450 are a family of enzymes responsible for biotransformation of both xenobiotic and endogenous compounds These enzymes can metabolise a number of chemotherapeutic drugs and thus they have the potential to influence the sensitivity of tumour cells to anticancer agents

Incubation of Adriamycin and Vincristine with recombinant cytochrome P450 3A4 (CYP3A4) was shown to decrease their toxic effects on A549 cells Epithelial lung BEAS-2B cells transfected with CYP3A4 cDNA showed a modest increase in resistance to Adriamycin, which was reversed when simultaneously treated with the CYP3A inhibitor 17α-ethynyl oestradiol (17 AEE) Treatment of HL60 cells with the CYP1B1 inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) did significantly increase mRNA expression and ethoxyresorufin-O-deethylase (EROD) activity of this enzyme, but failed to induce changes in the toxicity profiles of 5-fluorouracil, Cisplatin, Taxol or Taxotere Pulse selection of MCF-7 cells with Taxotere increased Taxotere resistance and greatly enhanced expression of CYP1B1 mRNA and EROD activity Knock-down of CYP1B1 expression by siRNA resulted in decreased cell survival, but did not substantially enhance Taxotere toxicity The role of P450 NADPH reductase was also investigated, and it was found that MDA 231 cells transfected with this enzyme were more sensitive to Mitomycin C, Adriamycin and 5-fluorouracil-induced toxicity This appeared to be due to increased oxidative stress as evidenced by lower NADPH levels and increased ROS production in MDA R4 cells Expression of a set of key enzymes involved in glutathione turnover was also found to be altered in a panel of drug-selected cell lines In order to study the mechanisms involved in the development of resistance, two metabolically competent cell lines were pulse-selected with Taxol, Taxotere and Cisplatin These cells showed increased resistance to the drugs they were pulsed with and also displayed cross-resistance to taxanes and Vincristine Expression of CYP3A4 and CYP3A5 was increased in pulse-selected cells as compared to their parental counterparts, the same was found of MDR1 To analyse the role of these proteins in drug resistance, combination assays were performed with

cytotoxic drugs in the presence of a CYP3A inhibitor, an MDR1 inhibitor or both Simultaneous treatment with 17 AEE did not appreciably affect Taxol toxicity in any of the cell lines tested, however, combination of Taxotere with the MDR1 inhibitor GF120918 dramatically enhanced the toxicity of the anticancer drug The study of xenobiotic metabolism enzymes in tumours could result in the discovery of novel and attractive targets for adjuvant therapy that can maximise the effect of chemotherapeutic agents and circumvent some types of resistance

Abbreviations

17 AEE	17α-ethynyl oestradiol
5FU	5-fluorouracil
Adr	Adriamycin
AhR	Aryl hydrocarbon receptor
ANF	α-naphtoflavone
ATCC	American Tissue Culture Collection
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CisPt	Cisplatin
Cpt	Carboplatin
CYP	Cytochrome P450
Da	Daltons
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate (N= A, C, T, G or U)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetracetic acid
EROD	Ethoxyresorufin-O-deethylase
FCS	Fetal Calf Serum
GCSH	γ-glutamylcysteine synthetase, heavy subunit
GCSL	γ-glutamylcysteine synthetase, light subunit
GGTP	γ-glutamyl transpeptidase
GSH	Glutathione
GSSG	Oxidised glutathione
GST	Glutathione transferase
IC50	Inhibitory Concentration 50%
IgG	Immunoglobulin
IMS	Industrial Methylated Spirits
kDa	Kilo Daltons
MDR	Multiple Drug Resistance
MRP	Multidrug Resistance-associated Protein
MEM	Minimum Essential Medium
MMLV-RT	Moloney Murine Leukemia Virus-Reverse Transcriptase

mRNA	Messenger RNA
NADPH	β-Nicotinamide adenine dinucleotide 2'-phosphate reduced
NSAID	Nonsteroidal anti-inflammatory drug
OD	Optical Density
Oligos	Oligonucleotides
P450	Cytochrome P450
P450R	Cytochrome P450 NADPH reductase
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase Chain Reaction
P-gp	P-glycoprotein
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNasin	Ribonuclease Inhibitor
ROS	Reactive oxygen species
rpm	Revolution(s) Per Minute
RT-PCR	Reverse Transcriptase-PCR
SDS	Sodium Dodecyl Sulphate
siRNA	Small interfering RNA
TBE	Tris-boric acid-EDTA buffer
TBS	Tris Buffered Saline
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TE	Tris-EDTA
TEMED	N, N, N', N'-Tetramethyl-Ethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
Txol	Taxol
Txt	Taxotere
UHP	Ultra high purity water
v/v	volume/volume
w/v	weight per volume

Table of contents

Section 1.0

Introduction

1 1 Cancer chemotherapy

Cancer treatment has seen much progress in the past years Certain types of cancer are now almost completely curable thanks to developments in therapy over the last 10 years, however, other types remain difficult to treat Surgery can be an effective cure for certain types of cancer, but it is not always a possible therapeutic option some tumours are inoperable, as are non-solid tumours such as leukaemia and advanced (metastatic) cancers In these cases, chemotherapy and radiotherapy are the alternatives, and they have been used quite successfully

Development of drug resistance is the major limitation of chemotherapeutic treatment of cancer Cancer cells can use a number of different mechanisms to attenuate or altogether overcome the cytotoxic effects of anticancer agents This usually comes as a consequence of the genomic instability of these cells replication is accelerated and usual controls of DNA fidelity are defective or no longer functional Mutations, amplifications and/or deletions occur, many of which are lethal, but a subpopulation of cells in the tumour can be rendered resistant by these alterations, sometimes to more than one chemotherapeutic agent Resistance usually relies on the perversion of mechanisms used by normal cells to protect themselves against environmental toxic agents

Resistance to one or more drugs is referred to as intrinsic if the drug elicits no initial response, or as acquired if the tumour is initially responsive to therapy, but later turns refractory to treatment (Clynes *et al*, 1998) A number of mechanisms mediate resistance in cancer cells, the best known are

- Alteration of cellular target
- Alteration of apoptosis pathways
- Enhanced DNA repair
- Drug efflux
- Kinetic resistance
- Enhanced drug inactivation or decreased drug activation

Other mechanisms do not involve alterations of isolated cells but rather of the tumour as a whole, such as low vascularisation, which prevents chemotherapeutic drugs from reaching the cells inside the tumour, and hypoxia, which reduces the cytotoxic potential of free radical-generating drugs or radiation

1 1 1 Drugs used in cancer treatment

Platinum compounds

The best known members of this class of drugs are Cisplatin (also known as cis-diamminedichloroplatinum) and Carboplatin (diammine [1,1-cyclobutanedicarboxylato(2-)-0, 0']-platinum), discovered over 30 years ago and still widely used in cancer chemotherapy Both compounds are platinum (II) complexes with two ammonia groups located in the *cis-* position, while in one of the newer members of this class, Oxaliplatin, platinum is complexed by a 1,2-diamino cyclohexane and an oxalate ligand

For many years, the exact mechanism of action of platinum compounds remained unclear, it is now accepted that these drugs exert diverse biological effects via a number of different mechanisms Upon entrance to the cell, one or both platinum bonds are hydrolysed, and this hydrolysed species is the active metabolite responsible for the toxic effects (Lau *et al*, 2005) The main effect is probably the DNA damage caused by platinum adducts, evidence suggests that platinum chelates nitrogen and oxygen atoms present in single guanine molecules, while others claim that the binding of adjacent guanine bases by the metal is also responsible for the damage (Kelman *et al*, 1979) The resulting intra- and inter-strand crosslinking interferes with normal transcription and replication of DNA, and if the damage is not repaired, the cell will undergo apoptosis This type of damage can be reverted by the nucleotide excision repair pathway, but this activity has been shown to be low (Zamble *et al*, 1996) Other mechanisms by which platinum compounds could be toxic to cells include binding and subsequent disruption of phospholipids and phosphatidylserine in the cell membrane, and of RNA and sulphur-containing molecules in the cytoplasm (Fuertesa *et al*, 2003)

Cisplatin has been successfully used to treat epithelial malignancies such as ovary, bladder and testicular cancer, and also in prostate, cervix, head and neck tumours (Wernyj *et al*, 2004) Platinum complexes are very toxic to normal cells, and their side effects include peripheral neurotoxicity (which is the dose-limiting side effect), renal tubular damage, nausea and vomiting, ototoxicity and mild haematological toxicity (McKeage *et al*, 1995)

Resistance to platinum compounds, much like their biological effects, involves a number of different mechanisms (Wernyj *et al*, 2004) The compounds can be metabolically inactivated by conjugation with sulphur-containing molecules, mainly glutathione (GSH) and metallothionein Alternatively, the drug or some of its

metabolites can be pumped out of the cells by different transporters, including the multidrug resistance protein 2 (MRP2) and the P-type adenosine triphosphatase ATP7B copper transporter Enhanced DNA repair by enzyme overexpression has also been reported to confer resistance to platinum compounds Contradictory reports exist regarding the role of p53 in Cisplatin resistance, with most of the evidence pointing to wild-type p53 acting as a pro-resistance factor Other survival-related proteins with altered expression in platinum-resistant cells are XIAP, Bcl-2, AKT, Bcl-xl, Fas-L and NF-κB

Camptothecins

Camptothecins are synthetic analogues of the plant alkaloid Camptothecin, which is extracted from the bark, wood and fruit of the Asian tree *Camptotheca acuminata* (Pizzolato *et al*, 2003), the most commonly used are Topotecan (9-[(dimethylamino)methyl]-10-hydroxy camptothecin) and Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy camptothecin) They were developed in recent years during the search for a replacement of Camptothecin, both compounds retain its anticancer properties but are much less toxic to normal cells

The DNA replication enzyme Topoisomerase I has been found to be the target of camptothecins, explaining the S-phase specific toxicity of these drugs (Hsiang *et al*, 1988, Li *et al*, 1972) They stabilise the cleavable complex formed between DNA and Topoisomerase I during the process of separation and relaxation of the DNA strands for replication Complexes formed in this way generate single strand breaks that can be repaired and do not result in cell death, however, when one of these complexes meets a replication fork along the DNA strand, irreversible double strand breaks are generated which are impossible to repair, thereby inducing cell cycle arrest and apoptosis

Irinotecan is a water-soluble analogue of Camptothecin that is widely used in combination with 5-fluorouracil for the treatment of advanced colorectal cancer It is also commonly used in combination with Cisplatin against a number of gastrointestinal malignancies, non-small and small-cell lung cancer, mesothelioma, ovarian and head and neck cancer Once in the organism, carboxylesterases present in the liver and gastrointestinal tract cleave its dipiperidino side-chain, generating the active metabolite SN-38 This metabolite can be as much as 1000-fold more potent than Irinotecan, however, Irinotecan is not considered to be a prodrug, since some of the anticancer effects it exhibits cannot be attributed to SN-38 (Takeda *et al*, 1992) SN-38 is inactivated by glucuronide conjugation, carried out mainly by the uridine

diphosphate glucuronosyltransferase 1A1 (UGT1A1) isozyme The main side effects observed after treatment with Irinotecan are myelosuppression and diarrhoea
Decreased intracellular drug concentrations, slower progression through the cell cycle and decreased total activity of Topoisomerase I have all been implicated in resistance against Irinotecan (Kanzawa *et al*, 1990) Other reported mechanisms include decreased conversion of Irinotecan into SN-38 (Niimi *et al*, 1992), and activation of the NF-κB (Cusack *et al*, 2000) and the PI3K/Akt pathways (Koizumi *et al*, 2005)

Anthracyclines

The anthracyclines are antibiotic compounds produced by bacteria of the Streptomyces species, they are weak bases of complex structure, with an amino sugar attached to the anthraquinone planar nucleus (Nielsen *et al*, 1996) Adriamycin (also known as Doxorubicin) is a semisynthetic compound derived from an anthracycline originally isolated from *Streptomyces peucetius var caesius*
Like platinum compounds, anthracycline toxicity is mediated by a number of different mechanisms, the most important of which has yet to be elucidated It has been suggested that the primary effect is the stabilisation of the cleavable complex between Topoisomerase II and DNA strands, resulting in double strand breaks However, this is not the only mechanism of anthracycline-induced DNA damage because of its planar structure, it is believed that these drugs can intercalate directly between DNA bases, altering nucleic acid structure and function It can also bind covalently to DNA forming adducts, adding to the damage In addition, bioreductive activation of these drugs generates free radicals, which are extremely toxic to cells Because of their hydrophobicity, it has also been suggested that anthracyclines bind directly to cell membranes and induce lipid peroxidation
In general, anthracyclines have been successfully used in the treatment of leukaemias, such as acute myeloid leukaemia (Monneret, 2001), and in the case of Adriamycin, also in solid tumours such as breast, bile duct, endometrium, oesophagus, liver, osteosarcoma, soft tissue sarcoma and non-Hodgkin's lymphoma (Gewirtz, 1999) The main side effect of Adriamycin is cardiotoxicity, while nausea, vomiting, alopecia and secondary acute myeloid leukaemia have also been reported
Resistance to Adriamycin and its sister drug, Daunorubicin, can arise as a result of reduced intracellular drug concentration by active efflux by both multidrug resistance P-glycoprotein (MDR1) and multidrug resistance protein 1 (MRP1), increased drug inactivation (particularly by GSH and GSH transferases), decreased availability of

intracellular targets either by reduced expression or mutation, and reduced susceptibility to apoptosis (Den Boer *et al*, 1998)

Mitoxantrone

Mitoxantrone is a synthetic anthracenedione initially developed as a Adriamycin analogue, which explains their similar structure (Fox, 2004) It is the only compound of its kind to be approved for clinical use (Faulds *et al*, 1991) The mechanism of action is also similar to that of the anthracyclines its main effects appear to be intercalation into DNA and inhibition of Topoisomerase II (Hande *et al*, 1998), it has also been shown to exert immunosuppressive and anti-inflammatory effects However, it is not as efficient as the anthracyclines in the generation of free radicals, overall lower toxicity, including cardiotoxicity, as compared to Adriamycin has also been demonstrated

Mitoxantrone has been used in the treatment of metastatic breast cancer, acute and chronic leukemias and non-Hodgkin's lymphoma, although it does have some activity against Hodgkin's lymphoma, myeloma, melanoma, bladder, prostate, liver, head and neck and non-small cell lung cancer (Poirier, 1986) Some cross-resistance between Mitoxantrone and the anthracyclines exists, although Mitoxantrone can be active against some anthracycline- refractory tumours In some cases, it can altogether replace Adriamycin in sensitive patients, because of its lower toxicity Myelosuppresion, nausea, vomiting and cardiotoxicity are the main side effects

Epipodophyllotoxins

This family is comprised of drugs originally isolated from the mandrake plant (*Podophyllum peltatum)* and their analogues, Etoposide (also known as VP-16) and Teniposide are the best known members The anticancer properties of the natural extracts were discovered as early as the 19th century (Hande *et al*, 1998), it was their toxicity that prevented them from being used in cancer treatment and started the search for analogues with similar activity and better toxicity profiles Etoposide and Teniposide were synthesised in the 1960s

Initially, it was thought that these toxins exerted their anticancer effects by inhibiting microtubule assembly, however, they did so at concentrations much higher than those achieved in tumoral tissue In 1984, it was finally discovered that the target of epipodophyllotoxins was Topoisomerase II (Ross *et al*, 1984, Glisson *et al*, 1984) These drugs stabilise the interaction between the enzyme and DNA, thereby inducing

single and double strand breaks, the ultimate consequence of this is cell cycle arrest and induction of apoptosis The effect is more pronounced when cells are dividing rapidly, hence the selectivity against tumour cells

Etoposide is used in the treatment of acute myeloid leukaemia, Hodgkin's and non-Hodgkin's lymphoma, small cell and non-small cell lung cancer, breast, gastric and ovarian cancer Its main toxic effect is myelosuppression Resistance to Etoposide and other epipodophyllotoxins arises as a consequence of drug efflux by MDR1 and/or MRP1, alterations in Topoisomerase II that affect drug binding and kinetic resistance (i e , slow growing tumours) (Hande *et al* , 1998)

5-fluorouracil

5-fluorouracil (5-FU) is a pyrimidine analogue belonging to the family of the 5-fluoro pyrimidines It was firstly synthesised after observations that rat tumours incorporated more uracil than normal tissue (Rutman *et al* , 1964) The difference between 5-FU and native uracil lies in the presence of a fluorine atom instead of hydrogen at the carbon-5 position of the pyrimidine ring

Due to its virtually identical structure to uracil, 5-FU participates in reactions that lead to thymidine synthesis, reactions it actually needs to exert its toxic effects, the drug itself is not toxic, but its metabolites are Upon entrance into the cell, 5-FU can be converted into two different metabolites 5-fluorouridine (FUrd) and 5-fluoro-2'-deoxyuridine (FdUrd) FdUrd can then be used as a substrate by thymidilate synthase, a key enzyme in pyrimidine synthesis FdUrd forms a stable, covalent complex with the enzyme, inhibiting it Inhibition of thymidylate synthase is one of the main mechanisms of 5-FU-mediated cytotoxicity, impairment of pyrimidine synthesis results in inhibition of DNA synthesis and repair, increased concentration of thymidylate synthase substrates (i e , uridine triphosphate or UTP) and their incorporation into DNA, causing further damage All these events ultimately lead to programmed cell death (Grem *et al* , 2000)

On the other hand, FUrd undergoes phosphorylation to form 5-fluorouridine triphosphate (FUTP), which can be incorporated into RNA, and this effect correlates with cytotoxicity (Glazer *et al* , 1982) Numerous consequences arise from this, including alterations in RNA secondary structure, impaired conversion of nuclear RNA into ribosomal RNA, altered splicing and inhibition of mRNA polyadenylation, which decreases its stability These effects of course impair the ability of the cell to synthesise proteins Also, FUTP residues present in RNA can be transferred to cytoplasmic enzymes, resulting in their inhibition (Grem *et al* , 2000) A study by Jin *et*

al (1996) suggests that 5-FU could inhibit nitric oxide production without any detectable effect on nitric oxide synthase, it appears that this could be translated into growth inhibition

The clinical use of 5-FU is widespread now, alone or in combination The main indication is still colorectal cancer, but also pancreatic, gastrointestinal, breast and head and neck tumours (Longley *et al*, 2003) Several mechanisms can confer tumours increased resistance against 5-FU, among them are increased synthesis of thymidylate synthase (Peters *et al*, 2002), decreased activation of 5-FU by thymidine phosphorylase and increased catalysis of 5-FU active metabolites by dihydropyrimidine dihydrogenase (Banerjee *et al*, 2002)

Vinca alkaloids

Vincristine and Vinblastine were isolated over 40 years ago from the leaves of *Catharanthus roseus*, also known as *Vinca rosea*, synthetic analogues Vindesine, Vinorelbine and Vinflunine have been developed more recently

All members of this family can bind tubulin with high affinity After entering the cell, Vincristine binds to the growing end of microtubules, alterating their dynamics, and thus interfering with mitosis Vinblastine also affects microtubule kinetics by binding to tubulin, yet it does so at a different site Moreover, it appears to induce a conformational change, this change could increase microtubule stability, ultimately blocking mitosis (Jordan *et al*, 2004) At higher concentrations, they can also induce microtubule aggregation and even the formation of crystals

In spite of their very similar structures, Vincristine and Vinblastine have different spectra of activity, with Vincristine being succesfully used against childhood leukaemia and Vinblastine in the treatment of solid tumours (Hacker *et al*, 1991) They also differ in their toxicity, with leukopenia being the dose-limiting effect for Vinblastine and peripheral neuropathy for Vincristine All Vinca alkaloids are widely used as single agents in the treatment of rapidly growing tumours such as childhood and adult leukaemias and lymphomas, as well as in some solid tumours Cancer cells can acquire resistance against Vincristine by increasing drug efflux through MDR1 and MRP1 proteins, alterating the drug target through mutations in α and ß-tubulin, overexpressing certain isotypes of tubulin that are less sensitive to alkaloid binding and changing expression levels of microtubule regulatory proteins In addition, it seems that resistance can also be mediated by other tubulin isotypes (γ, δ and ε-

tubulin), which are associated to the centrosome, although it is not yet clear in which way this happens (Jordan *et al*, 2004)

Taxanes

Paclitaxel (also known as Taxol) is a natural plant product initially isolated from the bark of the pacific yew tree (*Taxus brevifolia*), because of its successful use in the treatment of several cancers, a semisynthetic analogue, Docetaxel (Taxotere) was developed shortly afterwards, derived from a similar compound found in the european yew tree which has quite similar, albeit not identical, properties

In a similar way than that of Vincristine, the taxanes exert their toxic effect by binding to ß-tubulin, alterating microtubule dynamics, however, while Vinca alkaloids have a tendency to destabilise these structures, taxanes appear to stimulate and stabilise microtubule polymerization (Schiff *et al*, 1979) it seems that their ability to cause this effect is related to the conformational change they induce in tubulin upon binding, this conformational change increases tubulin affinity for itself (Nogales *et al*, 2001)

Taxol has been successfully used in the treatment of breast, ovary, non-small cell lung cancer and Kaposi's sarcoma, while Taxotere is currently being used in brain, prostate and lung tumours Their main side effects, similar to the Vinca alkaloids, are myelosuppression and neurotoxicity It also shares a number of resistance mechanisms with the Vinca compounds, i e , efflux out of the cell by MDR1 (but not MRP1), molecular alterations in tubulin that lead to decreased drug binding, expression of endogenous microtubule-depolymerising agents and changes in microtubule dynamics (Jordan *et al*, 2004), alongside more general mechanisms such as decreased susceptibility to apoptosis, changes in lipid composition and increased secretion of interleukin-6 (Yusuf *et al*, 2003)

Mitomycin C

Mitomycin C is a natural product isolated from *Streptomyces lavendulae* Its chemical structure contains an aziridine functional group, consisting of a heterocycle with an amino group and two methylene groups

This drug undergoes activation by a number of intracellular enzymes including P450 NADPH reductase (P450R), DT-diaphorase and NADPH cytochrome c reductase (Cummings *et al*, 1998) In this reaction, the quinone group is reduced to a semiquinone by the addition of one electron, or to a hydroquinone by the addition of two When oxygen is present, the semiquinone will enter a redox cycle and produce

reactive oxygen species (ROS) that, although harmful to the cell, do not appear to significantly contribute to cytotoxicity In the absence of oxygen, the semiquinone rearranges to form the hydroquinone, a more stable structure, and this compound, together with downstream metabolites, generates mono- and bis-DNA adducts The mechanism of activation for Mitomycin C implies that this drug is more toxic to oxygen-deprived, hypoxic cells, which are usually found in solid tumours This is the molecular basis for the selective toxicity towards tumour cells displayed by Mitomycin C

Mitomycin C has shown a wide antitumoral spectrum and is active against breast, head and neck, cervical, prostate, bladder, pancreatic, gastric and non-small cell lung cancer (Verweij *et al*, 1990, Bradner, 2001) The main toxic side effects observed with its use are thrombocytopenia and leucocytopenia, although uremic-haemolytic syndrome, renal and cardiopulmonary toxicity have also been reported

Mitomycin C is an MDR1 substrate, so overexpression of this efflux pump renders cells resistant to the drug Decreased activity of bioactivating enzymes has also been reported as a resistance mechanism in cultured tumour cells (Singh *et al*, 1996), as well as the aberrant expression of DNA damage response proteins (Johnson *et al*, 1997)

1.2 Multidrug resistance in cancer

A well-known phenomenon observed in cancer cell lines is that, when cultured in the presence of constant or increasing concentrations of a toxic agent, such as a chemotherapeutic drug, cells develop resistance to this toxin Since most anticancer agents are mutagens, it is very likely that prolonged culture in the presence of the drug would induce mutations in the cells, some of these will have no effect on cell survival, some may even be lethal, but certain mutations can help cells adapt to their toxic environment and become resistant to the effects of the drug Another explanation for the development of resistance in chemotherapy-exposed cell lines is that treatment with anticancer agents can actually select an intrinsically resistant subpopulation within the cells Tumours are known to be heterogeneous, and it is not unreasonable to suppose that different phenotypes expressed by cell subpopulations will often display differential sensitivity to chemotherapy drugs

When evaluating the toxicity of different drugs on these resistant cell lines, it is often found that cells display resistance to the drug they were selected with and also to a number of others, whether or not structurally and/or mechanistically related This phenomenon gave rise to the expression Multi-drug resistance (MDR) The importance of this observation was shown when a similar behaviour was observed in tumours indeed, cancers that were treated with a particular drug often became resistant to it after a certain period of time Resistance was later shown to extend also to a number of anticancer agents that had not been used for the treatment

Although several different mechanisms are involved in the development of resistance, MDR is more often than not associated with certain proteins known as ATP binding cassette (ABC) transporters Members of this superfamily of proteins can act as efflux pumps and actively extrude a number of different compounds, including anticancer agents, from the inside of the cell, thereby reducing their toxic effects As their name suggests, efflux is active and depends on ATP hydrolysis, which provides the energy required for the transport process As the transport is active, these proteins can pump chemical compounds against a concentration gradient

All of these proteins are located in the plasma membrane albeit in different regions of polarized cells, with only a small fraction found in the Golgi apparatus (Schinkel *et al*, 2003) They can be divided in four classes according to their morphological structure

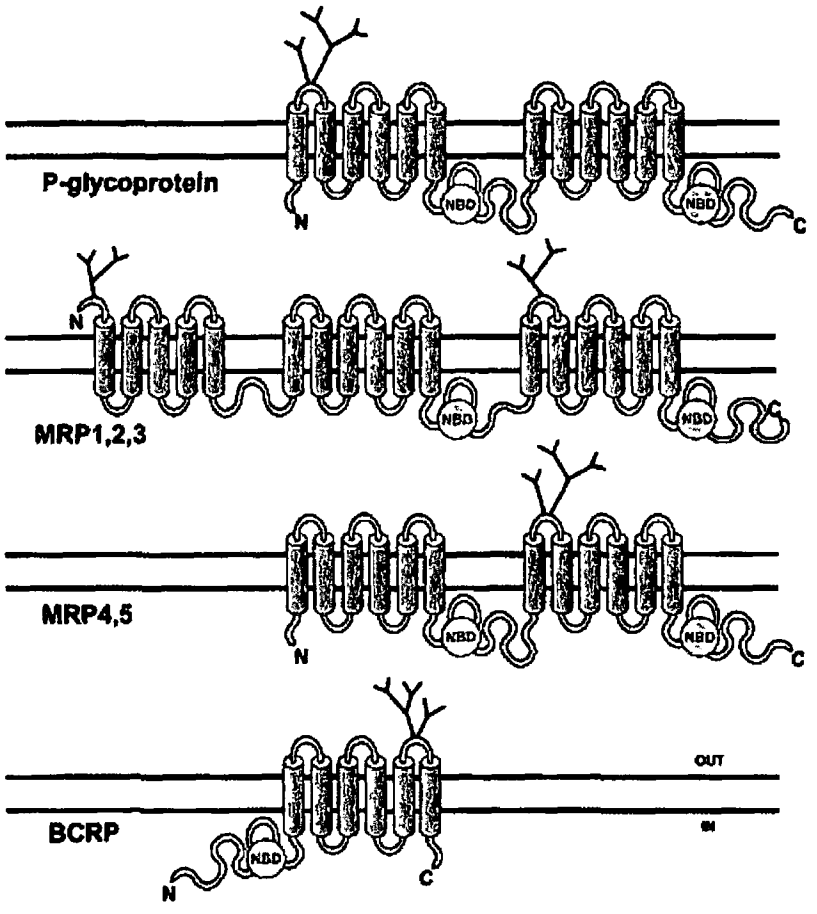


Figure 1 2 1 Comparative structures of ABC transporters superfamily members (From Schinkel *et al*, 2003), where NBD stands for nucleotide binding domain Branches represent sites of N-linked glycosilation

Overall structures of different ABC transporters are quite similar (Fig 1 2 1), displaying a variable number of the six transmembrane segment core connected by intra and extracellular loops, and containing one or more nucleotide binding domains (NBDs) The most obvious differences are in MRP 1,2 and 3, which have an additional domain, complete with five transmembrane segments (but lacking an extra NBD), and in BCRP, which is formed by a single domain Areas of heavy N-glycosylation can be found in all four classes, although they do not appear to be necessary for efflux (Schinkel *et al*, 1993) However, it is very likely that N-glycosylation acts as a signal for routing the protein to the plasma membrane, stabilising it

1 2 1 Multidrug resistance protein (MDR1)

This protein has received several alternative names, such as P-glycoprotein (Pgp), PGY1 and GP170 (Schinkel *et al*, 2003) It is a 170 kDa protein with heavy N-glycosylation in the extracellular loop closer to the N-terminal The structure is formed by six transmembrane segments connected by a short polypeptidic section which features phosphorylation sites for a variety of protein kinases and one of the two ATP binding sites, the other being located at the C-terminal

Substrates of MDR1 are extraordinarily diverse in structure, with only a few common factors amongst them they are relatively small organic compounds (the highest molecular weight that is actually transported is around 2kDa) which are amphipatic and usually uncharged or weakly basic, although some acidic compounds (i e, methotrexate, phenytoin) are also substrates of MDR1, albeit poor ones There is evidence that compounds are transported by binding to the intracellular side of MDR1 and then being flipped to the outside of the cell (Higgins *et al*, 1992), the fact that virtually all substrates are amphipatic supports the notion that MDR1 is a flippase It should be noted that the great majority of these substrates are hydrophobic and, as such, have the ability to diffuse passively through cell membranes, the presence of a pump makes it possible for them to be transported against a concentration gradient

MDR1 is usually located in secretory surfaces (Bosch *et al*, 1996), and this protein can be readily detected in high levels in liver (particularly in biliar canaliculi), proximal tubules of the kidney, intestinal and colonic epithelium, pancreatic ducts, bronchial mucosa, prostatic epithelia, ovarian follicles and pregnant uterine epithelium, as well as in the luminal face of endothelial cells that form the blood-brain barrier and also the blood-testis and blood-nerve barrier (Bosch *et al*, 1996, Schinkel *et al*, 2003) In all of these tissues, the physiological function of MDR1 appears to be that of protecting sensitive cells or organs from potential damage from toxins by excretion of these into the bloodstream, urine, faeces or other secretions

A number of chemotherapeutic drugs are MDR1 substrates, including anthracyclines (e g, Adriamycin), taxanes (Paclitaxel), Vinca alkaloids (Vincristine, Vinblastine), epipodophillotoxins (Etoposide) and other drugs, such as Topotecan and Actinomycin D This points to a potential involvement of MDR1 in chemotherapy resistance Indeed, transfection or transduction of MDR1 cDNA is sufficient to confer multidrug resistance, as shown by various studies (de Graaf *et al*, 1996, Mahon *et al*, 2003, Findling-Kagan *et al*, 2005) Overexpression of MDR1 is a common finding in cell lines cultured in the presence of chemotherapeutic drugs, inhibition of the high expression levels by antisense oligonucleotides or ribozyme transfection usually

restores sensitivity to cytotoxic drugs (Gao *et al*, 1998, Pan *et al*, 2001, Wang *et al*, 2003) A number of tumours have been shown to express high levels of this protein and this overexpression was directly correlated with poor prognosis (Ling *et al*, 1997) Expression of MDR1 in breast cancer was also found to correlate with tumour staging (Leonessa *et al*, 2003)

Implication of MDR1 activity in drug resistance opened the door to the development of a number of inhibitors, in the hope that coadministration with these compounds would improve the efficacy of chemotherapy The first compounds to show inhibitory activity against MDR1 were already known drugs, such as the calcium channel blocker Verapamil and the immunosuppressive drug Cyclosporin A These drugs were quickly replaced by second generation inhibitors, which were specifically designed for this purpose, these compounds display higher affinity for MDR1 and reduced pharmacodynamic effects A third generation of MDR1 inhibitors is now available, with even higher efficiency, examples of third generation MDR1 inhibitors are LY335979 and GF120918 (Hyafil *et al*, 1993, Dantzig *et al*, 1996)

1 2 2 The Multidrug Resistance Protein (MRP) family

The multidrug resistance protein (MRP) family comprises so far 9 members denominated MRP1 to MRP9 They can be divided according to their structure in two types MRP4, MRP5, MRP8 and MRP9 have a similar structure to MDR1 (i e , two six segment transmembrane domains and two NBDs), while MRP1, MRP2, MRP3, MRP6 and MRP7 possess an extra five segment transmembrane domain

Expression of MRP1, MRP4 and MRP5 can be found in several different types of tissue, whereas MRP2, MRP3 and MRP6 appear to be restricted to kidney, liver and gastrointestinal tract (Borst *et al*, 1999) These pumps can also be divided in two types according to their position on the cell membrane while MRP2 is located on the apical side of the plasma membrane, in a similar way to MDR1, MRP1, MRP3 and MRP5 are preferentially expressed on the basolateral side MRP1 is not exclusively found on the plasma membrane, with a fraction expressed in intracellular vesicles (Flens *et al*, 1996)

The physiological function of MRPs appears to be very similar to that of MDR1 in protecting vital areas from the toxic effects of circulating compounds, this has been demonstrated for MRP1 by experiments performed with MRP1 knock-out mice However, a protective role of MRP2-6 against xenobiotics has not yet been confirmed The fact that some of these proteins are expressed on the basolateral side of the cell

membrane does not interfere with their protective function, since toxins can be excreted out of the epithelium by pumping them into the circulation, this is true of the testicular tubes and the choroid plexus (Borst *et al*, 1999) Substrates of MRPs are organic anions that, unlike MDR1 substrates, do not diffuse passively through cell membranes Examples of physiological substrates of MRPs are leukotriene C4 (LTC4) and organic acids present in bile and liver cells

It has been demonstrated that MRP1 transports GSH, glucuronate and sulphate conjugated drugs, which would suggest that the pump has a binding site for these conjugating compounds However, several drugs transported by MRP1 are not conjugated *in vivo* This apparent contradiction has been explained by a number of studies, which have concluded that efficient export of substrates by MRP1 requires GSH This has been demonstrated by treatment of MRP1 overexpressing cells with GSH depleting agents, resulting in decreased resistance Moreover, increased expression of MRP1 in cells diminishes GSH levels, indicating that there is a basal export of GSH *out of* the cell even *in* the absence of drugs It is now believed that compounds pumped out of the cell by MRP1 are co-transported with GSH (Borst *et al*, 1999)

Several anticancer drugs have been reported as MRP1 substrates these include Vincristine, Vinblastine, Etoposide, Adriamycin, Cisplatin, Mitoxantrone and Methotrexate (Schinkel *et al*, 2003) Indeed, knock-out mice for MRP1 display increased sensitivity to the toxic effects of Etoposide (Wijnholds *et al*, 1997) Breast tumours positive for MRP1 expression were shown to have a lower response to chemotherapy than MRP1 negative tumours (Nooter *et al*, 1997b) Furthermore, two separate studies found a negative correlation between MRP1 protein expression and relapse-free survival (Nooter *et al*, 1997a, Filipits *et al*, 1999)

A number of inhibitors or MRP1 are now in experimental use, most of them well known drugs such as Sulfinpyrazone and Probenecid Indeed, Sulindac has been shown to considerably increase the cytotoxicity of anthracyclines, Vincristine and Etoposide when administered in combination, this effect has been attributed to the inhibitory effect of the anti-inflammatory agent on MRP1 (Duffy *et al*, 1998)

MRP2 transport is, like that of MRP1, dependent of GSH levels, as shown by experiments with GSH-depleting agents (Cui *et al*, 1999) Expression of MRP2 in lung cancer cells has been reported to correlate with resistance to Adriamycin and Cisplatin (Kool *et al*, 1997) Further confirmation of the role of MRP2 in drug resistance was obtained by transfecting HepG2 hepatoma cells with an MRP2 antisense construct, transfection resulted in sensitisation of cells to Cisplatin, Irinotecan and its derivative SN-38, Vincristine and Adriamycin (Koike *et al*, 1997) In

another study, MDCK cells transfected with MRP2 developed 5 to 10-fold resistance to Etoposide, Vincristine, Adriamycin and Cisplatin

MRP3 expression was shown to correlate strongly with resistance to Adriamycin and less substantially, albeit significantly, with resistance to Vincristine, Etoposide and Cisplatin (Young *et al*, 1999) Transfection of MRP3 can render cells resistant to Etoposide, Teniposide and Methotrexate, and also to Vincristine, albeit at lower levels (Kool *et al*, 1999, Zeng *et al*, 1999) This type of transport appears not to be dependent on GSH levels, since cotreatment with GSH-depleting agents did not result in decreased resistance

Few studies exist on the relationship between MRP4 expression and anticancer drug resistance, overexpression of this protein was reported to confer resistance to the nucleotide analogues 6-Mercaptopurine and Thioguanine (Chen *et al*, 2001) Similarly, HEK293 cells transfected with MRP5 displayed weak resistance to the same agents (Wijnholds *et al*, 2000)

MRP6, MRP7, MRP8 and MRP9 have been discovered very recently and their role in tissue protection and drug resistance has not yet been established

1.3 Drug metabolism

The body responds to noxious stimuli from the environment by activating a defense mechanism, the nature of this mechanism depends on the size of the agents that cause the stimulus Large foreign substances such as proteins, viruses and bacteria are dealt with by the immune system, while small chemical compounds can be enzymatically inactivated

The enzymatic detoxification system is for small molecules what the immune system is for bacteria and viruses it protects the body from environmental hazards The target molecules are extraordinarily variable in size and physicochemical characteristics therefore, the system must be very flexible, and allow for all sorts of substrates to be processed It is believed that families of enzymes involved in detoxification evolved from a single gene by amplification and mutation in response to environmental pressure, this explains why there are several enzyme isoforms The existence of different enzymes with similar activity leads to high metabolic efficiency as a result of overlapping activities a single substrate is metabolised by several enzymes and a single enzyme is able to biotransform a number of different compounds

Low specificity and overlapping of substrates are usually cited as advantages of the metabolic system, however, when different drugs are administered to a patient, competition for certain reactions can arise, with unexpected results in drug pharmacokinetics and toxicity It is therefore essential to check the metabolic pathways of all different drugs given to a patient at the same time, in order to avoid metabolic interactions and potential toxicity

An increase in water solubility will detoxify most hydrophobic compounds, hydrophilic compounds have reduced penetration into cells, while they are very soluble in urine and sweat, for example, this makes such compounds easier to excrete Hydrophobic substances can diffuse easily across cell membranes and accumulate inside the cell, where they can remain indefinitely, interfering in the complex homeostasis of the cell

The detoxification process is achieved in two phases In Phase I reactions, a small functional group is either added to or unmasked in the molecule, which usually results in changes in activity Phase II reactions involve the attachment (conjugation) of a large group to a reactive functional group present in the drug metabolites formed in this way can then be excreted in the urine, sweat or faeces Phase I reactions are believe to be preparative, that is, to make a certain chemical a better substrate for Phase II, which is the main detoxification step

Sometimes these reactions result in the formation of more active compounds, and this can lead to harmful (i e , if the toxicity of the metabolite is greater than that of the parental compound) or beneficial effects (for example, activation of a prodrug) It is mostly Phase I reactions that can activate a chemical, while Phase II metabolism will almost always result in detoxification

The liver is the major site of detoxification in the body, and is also where most enzymes related to drug metabolism are expressed However, since various types of tissue are exposed to foreign substances, expression of these enzymes can be found throughout the body as means of protection, especially in lung, kidney and gastrointestinal tract

Phase I	Oxidation	Small increase in hydrophilicity
	Reduction	
	Hydrolysis	
	Hydration	
	Dethioacetylation	
	Isomerisation	
Phase II	Glucuronidation/glucosidation	Large increase in hydrophilicity
	Sulfation	
	Methylation	
	Acetylation	
	Amino acid conjugation	
	Glutathione conjugation	
	Fatty acid conjugation	
	Condensation	

Table 1 3 1 Classification of metabolic reactions as Phase I or II (adapted from Gibson *et al)*

1 3 1 Phase I metabolism

As previously mentioned, these reactions prepare chemicals by adding or unmasking a polar group A xenobiotic can be modified in a number of different ways, as shown by the variety of Phase I reactions that exist These reactions can be carried out by cytochromes P450, by far, the most important Phase I *effectors*, or by other enzymes

1 3 1 1 Cytochromes P450

Cytochromes P450 are a family of enzymes implicated in the biotransformation of both xenobiotics and endogenous compounds Their primary functions are the synthesis of steroids and bile acids and the detoxification of several substances, such as drugs and environmental agents Mammalian P450s are membrane-bound They can be located in the endoplasmic reticulum of the cell or in mitochondria

Over 500 cytochromes have been described Even though the drug metabolising system of animals is similar to that of humans, there are several differences regarding isoforms and substrate specificity Mammals can express different sets of P450 isoforms and each one of these can be identical or completely different to the human counterpart For example, CYP2E1 is expressed in human, rat and rabbit liver, but while CYP3A6 is the only 3A member detected in rabbit tissues, humans can express 3A4, 3A5 and 3A7 isoforms Also, differences can be found regarding which isoform metabolises a particular substrate

Structure

All P450s possess a heme moiety as a prosthetic group (Fig 1 3 1) The heme group can bind carbon monoxide very tightly, and it is known that carbon monoxide-exposed microsomes will show a very strong absorption band at 450 nm in a difference spectrum, hence the name P450, where P stands for pigment

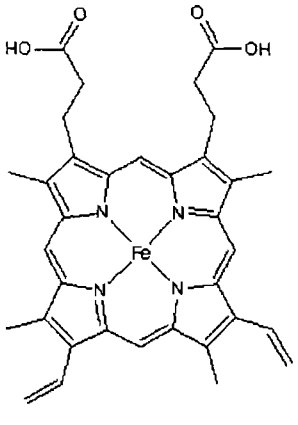


Figure 1 3 1 Heme group (from www newark rutgers edu)

P450s can be grouped into three classes according to the redox partner they use (Graham-Lorence *et al*, 1996), class I P450s require two redox cofactors, an iron-sulphur protein (ferredoxin) and a FAD-containing NAD(P)H-ferredoxin reductase for their catalytic activity, class II P450s need a FAD/FMN-containing NADPH-P450 reductase, and class III do not require a separate protein or proteins for reduction Examples of class I P450s are bacterial enzymes such as CYP101 (also known as P450cam) and CYP108, and mammalian enzyme CYP11A, which is involved in steroid synthesis Drug metabolising P450s such as the members of the CYP1, CYP2 and CYP3 family are all class II enzymes, as are steroidogenic microsomal P450s like CYP17 and CYP19 Finally, class III P450s include thromboxane synthase CYP5 and allene oxide synthase CYP74

The typical structure of cytochromes P450 can be seen in a simplified scheme in Fig 1 3 2 P450s are composed of two domains one has a predominant α-helix structure and comprises about 70% of the protein, while the other is formed mainly by β-sheet The α-helical domain comprises helices B' to K, helix L and also sheets β3 to β5, the β-sheet domain contains the sheets β1 and 2, and helices A, B and K' There is also a region in the protein termed the meander, which is a 14 or 15-residue section at the end of the K helix

The conserved residues among the P450 superfamily are only three, and consist of the cysteine located in the heme binding region, which helps coordinating the iron present in the heme group and is surrounded by a highly conserved sequence, and glutamine and arginine residues located in the K helix, facing the meander Highly conserved residues that are not present in all members of the P450 superfamily can also be found, including a threonine residue facing the active site and an acidic residue (usually glutamine or aspartate) located very close to it, which appear to be involved in molecular oxygen binding and bond-splitting (Raag *et al*, 1991)

Key structural features of P450s can be found when comparing the three-dimensional structures of enzymes belonging to different classes (Graham-Lorence *et al*, 1996) These features compose what is known as the core structure of the cytochrome, and are namely a four α-helix bundle, formed by three parallel helices (I, L and D) and an antiparallel one (E), helices J and K, sheets β1 and β2, the cysteine-containing heme binding loop and the meander The α-helical domain is topped by six helices, while another three α-helices and two β-sheets can be found at the bottom of this structure The fact that these features are so highly conserved among different family members suggests that they play important roles in protein folding and heme binding As expected in these proteins, the regions displaying the highest diversity and the lowest

conservation are those involved in substrate binding and recognition, and also in the binding of the different redox partners. In these cases, the core structure remains unchanged, but the helices and sheets adopt different lengths and positions in order to accommodate diverse compounds and cofactors.

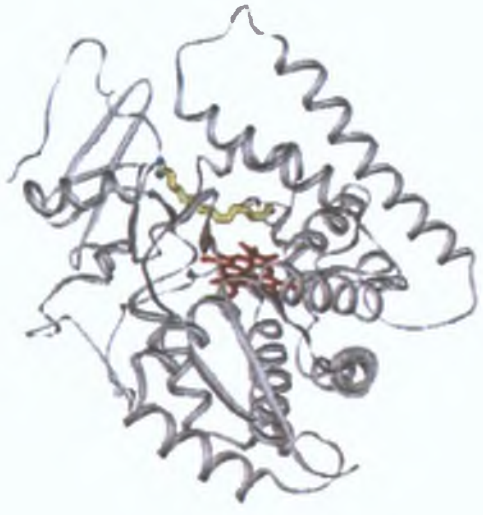


Figure 1.3.2: Typical structure of cytochromes P450 (from www.its.caltech.edu).

Catalytic cycle

Cytochromes P450 are the most important enzymes carrying out Phase I reactions. The families involved in drug metabolism are CYP1, CYP2 and CYP3. Cytochromes P450 catalyse a number of different reactions, such as hydroxylation, epoxidation, dealkylation and deamination, of which the most significant is probably hydroxylation. P450s are monooxygenases because they incorporate a single oxygen atom to the substrate. To do this, they must first break the bond between the two atoms of molecular oxygen: they achieve this by reducing the iron atom present in the heme group with two electrons donated by an accessory protein. Two electron-donor proteins usually work as P450 cofactors: the NADPH-cytochrome P450 reductase (P450R), present in the endoplasmic reticulum, and the Ferredoxin/Ferredoxin reductase complex located in mitochondria. The presence of these proteins is essential for P450 activity, as is also the presence of a lipid component, believed to influence substrate binding, electron transfer, conformational change and anchorage of the cytochrome (Nisimoto *et al.*, 1983). Cytochrome b5 is also considered as a P450 cofactor; even though it does not appear to be involved in all P450 reactions, it

can modulate catalysis by a number of different mechanisms (Schenkman *et al*, 2003) This battery of components works together as a system, known as the mixed function oxidase (MFO) system, of which cytochrome P450 is the terminal oxidase The P450 catalytic cycle is a very complex one, performed in several steps (Fig 1 3 3) Firstly, the substrate is bound to the iron atom located on the heme, which is in the ferric form (Fe^{3+}) This atom is coordinated by six different ligands, the catalytic activation of oxygen will only occur at a very precise location, specifically at the site of the sixth ligand, which is exchangeable (White *et al*, 1980) A first reduction is then carried out on the ferric atom with an electron donated by P450R, which obtains it from the reduction of NADPH In the adrenal gland mitochondria, this reaction is carried out in a slightly different way, with an extra protein, Adrenodoxin, transporting the electron from the NADPH-adrenodoxin reductase to the P450

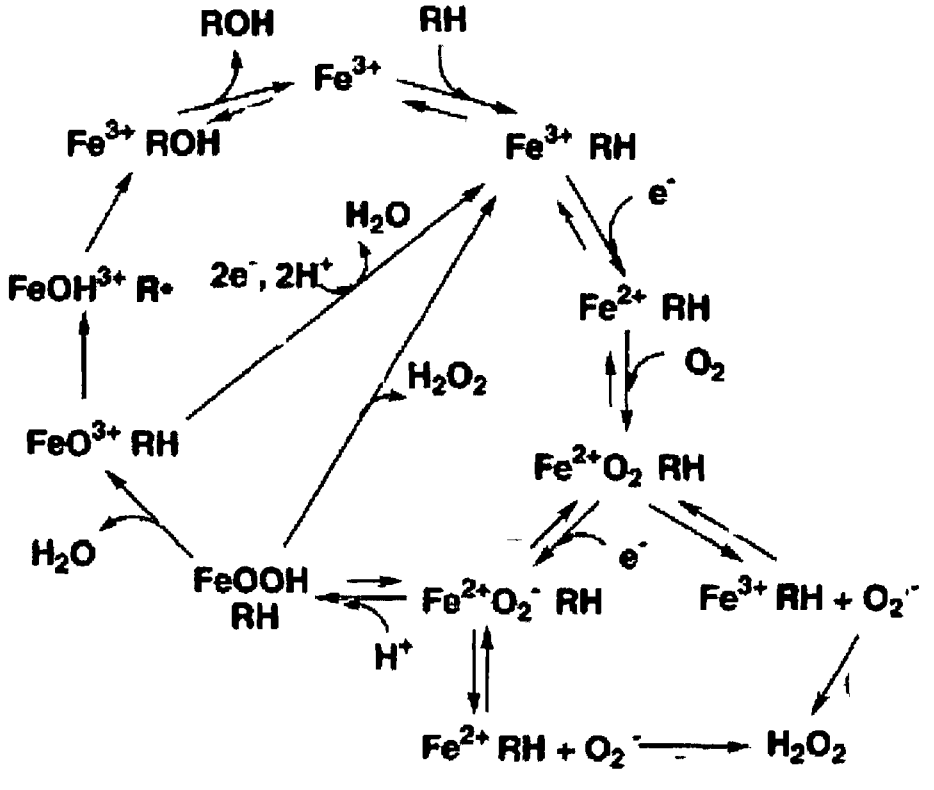


Figure 1 3 3 P450 Oxidation cycle (from Yun *et al*, 2000)

After the iron atom has been reduced to its ferrous form, it is coupled with an oxygen molecule, generating an unstable oxy-ferrous complex A redox reaction between the ferrous atom and the oxygen molecule ensues, where the ferrous atom returns to the

ferric state, while still bound to the substrate A second electron is incorporated, although the donating protein has yet not been identified, some suggest it is transported from the NADH-cytochrome b5 reductase to cytochrome b5 and then to P450s, while others claim it is donated by P450R There is a subsequent electron and oxygen rearrangement, by which an oxygen atom is incorporated to the substrate After the final step, the hydroxylated substrate is released into the cellular space, together with a water molecule

Certain substrates can induce uncoupling of the P450 cycle under specific conditions, resulting in the electrons being incorporated to the oxygen molecule, ultimately generating hydrogen peroxide This phenomenon is also known as the peroxide shunt (Coon *et al* , 1992)

Reactions

The following oxidation reactions are carried out by cytochromes P450

- Aliphatic oxidation
- Aromatic hydroxylation
- Epoxidation
- *N*-, *O*-, *S*- dealkylation
- Deamination
- *N*-hydroxylation
- Sulphoxidation
- Desulphuration
- Oxidative dehalogenation

Even though the principal reactions carried out by P450s are oxidative ones, they can also carry out reductive metabolism, particularly under low oxygen tensions In this case, electrons will be donated directly to the substrate with the participation of NADPH-cytochrome P450 reductase These reactions can result in chemical activation rather than detoxification, as is the case with organic compounds like carbon tetrachloride and halothane This is similar to what happens when the intestinal microflora reduces already detoxified compounds, reactivating them and restoring their toxicity

Contribution of partner enzymes to drug metabolism

P450R is a flavin-containing monooxygenase that, contrary to cytochromes P450, does not posses a heme group (Ziegler *et al.*, 1971). Its major function is to act as an electron bridge between NADPH and electron acceptors, using its cofactors, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), as electron acceptors and donors. The catalytic cycle is shown in Fig. 1.3.4.

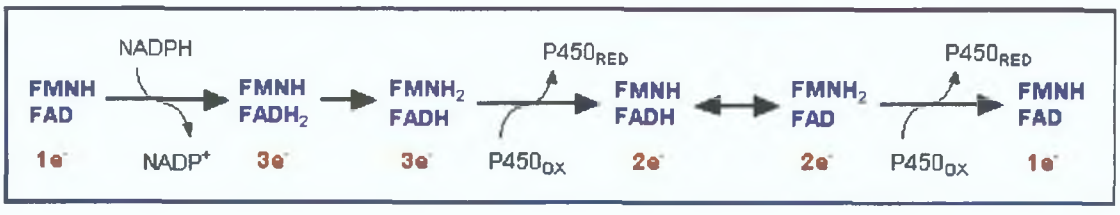


Figure 1.3.4: Sequential transfer of 2 electrons from NADPH to cytochromes P450 by P450R, using the FAD and FMN cofactors (from www.uky.edu/Pharmacy/ps/porter/CPR.htm).

The main acceptors of P450R are cytochromes P450, although other microsomal proteins such as cytochrome b5, heme oxygenase and fatty acid elongase might also be reduced by P450R. Cytochrome c, ferricyanide and certain lipids may also act as electron acceptors for P450R (Sevanian *et al.*, 1990; Backes, 1993; Shen *et al.*, 1993).

P450R is located in the endoplasmic reticulum of the cell and its expression is abundant in the liver, although it is widely expressed in the body, present in all different types of tissue studied. Expression of P450R appears to be regulated by the pithuitary-thyroid axis, as thyroid hormone is needed to sustain P450R expression (Waxman *et al.*, 1989). This means that expression of P450R is independently regulated from that of cytochromes P450, although some inducers are common to both types of enzyme. It is also important to notice that the *p450r* gene encodes a single protein that is able to interact with all different isoforms of cytochromes P450 (Shen *et al.*, 1993). In most tissues, P450R is expressed at much lower levels than cytochromes P450, and so constitutes a limiting factor for the reactions carried out by these enzymes.

P450R can carry out reductive metabolism of chemicals independently of P450s. Quinone anticancer agents such as Adriamycin and Mitomycin C are known to be

activated in this way to form an unstable intermediary that ultimately reverts to the quinone form, generating free radicals in the process The free radicals produced in this reaction appear to contribute to the toxic effects of these drugs, but the observed cytotoxicity is mainly due to the unstable intermediary, which acts as an alkylating agent, binding DNA and generating harmful adducts

Cytochrome b5 is a cylindrical membrane protein consisting of 6 helices and 5 β strands (Lu *et al*, 1995) There are 15 different forms of cytochrome b5, but the sequence is very well preserved across species (Schenkman *et al*, 2003) Its main function is to act as an intermediary in the transference of electrons between cytochromes P450 and reductases Cytochrome b5 can increase P450 activity in three different ways by transferring two electrons directly to P450 enzymes independently of P450R, by transferring a second electron to the oxyferrous form of P450s either from P450R or from cytochrome b5 reductase, and by allosterically stimulating P450 function without electron transfer (Porter, 2002)
Cytochrome b5 can also carry out reductive drug metabolism using NADH as a cofactor Chemical groups that can be reduced in this way are halogenated hydrocarbons, epoxides, heterocyclic azo (N=N) and nitro (N=O) compounds P450s and NADPH-cytochrome P450 reductase are also capable of catalysing azo- and nitro-reduction, involving substrates such as Chloramphenicol

1 3 1 2 Other Phase I enzymes

Oxidative enzymes

Enzymes other than P450s or the other components of the MFO system can carry out oxidative metabolism These enzymes are also located in the main organs responsible for detoxification, i e , kidney, lung and especially liver

- Alcohol dehydrogenase although the MFO system carries out some ethanol metabolism, particularly after induction, this enzyme is the major detoxification route under normal circumstances It uses NAD^+ as a cofactor to oxidise alcohols to aldehydes, a reaction that is reversible Other substrates are acetaldehyde and lipid peroxidation products Another role of

this enzyme, which is not related to drug metabolism, is in the synthesis of retinol (Ashmarin *et al* , 2000)

- Aldehyde dehydrogenase aldehydes generated in a previous step by alcohol dehydrogenase can be further oxidised to carboxylic acid by aldehyde dehydrogenase type 2, which is located in mitochondria and has low substrate affinity (Yokoyama *et al* , 2003) The other isoform is referred to as formaldehyde dehydrogenase and is not as relevant in drug metabolism The same oxidative reactions can also be carried out by aldehyde oxidase and xanthine oxidase

- Xanthine oxidase this enzyme is involved in the metabolism of a broad range of substrates, although its main role is in the oxidation of all xanthine-containing drugs, such as caffeine, and also purine analogues It catalyses the oxidation of hypoxanthine to xanthine and the oxidation of xanthine to uric acid This enzyme is expressed in milk-related tissues, although its role in milk production remains unclear (Martin *et al* , 2004)

- Aromatases these enzymes generate benzoic acid from cyclohexane-containing chemicals They require that the substrate is first derivatised with coenzyme A, and use molecular oxygen and FAD as cofactors

- Alkylhydrazine oxidase rearrangement and decomposition of alkyl hydrazines is of importance because these compounds are very toxic and can induce severe liver damage (Rumyantseva *et al* , 1991) Several commonly used drugs are hydrazine derivatives, including Isoniazide, Phenelzine and Hydralazine Their activation usually yields toxic and carcinogenic compounds

- Prostaglandin synthetase-mediated oxidation the synthesis of prostaglandins from arachidonic acid involves a two-step oxidation performed by the enzyme prostaglandin synthetase Co-oxidation of chemicals may occur as a side effect of these reactions, although this pathway may not be relevant in the detoxification of the drug Other peroxidases such as Myeloperoxidase and Lactoperoxidase also couple the oxidation of a substrate to the reduction of H_2O_2 and lipid hydroperoxidase

- Amine oxidases these enzymes can be divided into three groups, the flavin-containing monooxygenases (FMOs), the monoamino oxidases (MAOs) and the diamine oxidases This last group reacts mainly with endogenous substrates, and its role in xenobiotic metabolism is not relevant

  Similarly, the MAOs metabolise endogenous amines such as adrenaline and other catecholamines, and also other compounds like tyramine, which is incorporated with the diet, but not amphetamines, which are MFO substrates Only two isoforms exist, MAO A and MAO B, which differ in their substrate specificity, both use FAD as a cofactor Amines are transformed into aldehydes with the addition of an oxygen atom, which is not taken from molecular oxygen, but from water (Benedetti *et al* , 2001)

  The most important group is that of the FMOs, which metabolise compounds that contain nucleophillic nitrogen, sulphur, selenium or phosphorus (Hines *et al* , 1993) Five variants of this enzyme exist in humans, each with a particular location within the body, though most of the enzymatic activity is found in the liver All FMOs reside in the endoplasmic reticulum and require NADPH and molecular oxygen for catalysis Uniquely, these enzymes possess oxygen complexed to their active site in the absence of substrates, which means they are permanently activated Oxidation reactions can then be carried out with little or no extra energy, which explains why FMOs metabolise such a broad range of compounds

Hydrolases

Hydrolysis reactions involve the decomposition of a particular substance by water, facilitated by the presence of a hydrolytic enzyme It can also occur non-enzymatically, especially if nucleophillic groups surround the reactive site of the chemical When the cleavage is enzymatic, it is often stereoselective Esters, amides, hydrazides and carbamates are all good substrates for hydrolysis

- Esterases these enzymes can belong to two types, specific esterases that hydrolyse particular groups of compounds and non-specific esterases with broad substrate specificity The former are expressed in the liver, while the acetylcholinesterases, pseudocholinesterases and other esterases present in plasma comprise the latter

- Amidases amidases present in the liver microsomal fraction are responsible for the metabolism of most amide drugs However, the specificity of the plasma esterases is so low that they can actually hydrolyse amides as well Hydrolysis of amides is usually slower than that of esters

- Hydrazide and carbamate hydrolases these carry out the inactivation of less common functional groups in drugs such as the hydrazide group in Isoniazide

Hydration related enzymes

Hydration is similar to hydrolysis in that it involves inactivation of chemical compounds via reaction with water However, in the case of hydration the chemical is not dissociated by water, rather, the water molecule is added to the chemical Hydration is of particular importance in the detoxification of epoxides, which is mediated by epoxide hydrolases

- Epoxide hydrolases epoxides are very unstable chemical species generated by the chemical or enzymatic addition of oxygen to a double carbon bond These compounds are extremely reactive, and have been implicated in mutagenesis and carcinogenesis, their detoxification is then of maximal importance for the body

  Five forms of this enzyme exist in mammalian tissues, namely microsomal cholesterol 5,6-oxide hydrolase, hepoxilin A3, hydrolase, leukotriene A4 hydrolase, soluble hydrolase and microsomal expoxide hydrolase (Fretland *et al*, 2000) Their localisation is either microsomal or cytosolic, and their endogenous substrates are epoxides derived from steroids, cholesterol and arachidonic acid

These are the main Phase I metabolic reactions, other reactions of less importance also take place and are listed in Table 1 3 1

Other Phase I metabolic reactions
Ring cyclisation
N-carboxylation
Dimerisation
Transamidation
Isomerisation
Decarboxylation
Dethioacetylation
Disulfide reduction
Sulfoxide reduction

Table 1 3 1 Additional Phase I reactions

1 3 2 Phase II metabolism

Phase II reactions are often denominated as biosynthetic, since a new molecule is formed when drugs are combined with endogenous substrates such as glucuronic acid, sulphate, glutathione, amino acids and acetate, among others These groups cannot be used as such by the transferases involved in these reactions, in general, an activated form of the conjugating group is needed

1 3 2 1 Glutathione conjugation

Conjugation with glutathione (GSH) has been reported as one of the major Phase II reactions in mammalians (Kaplowitz *et al*, 1985) GSH is the most abundant non-protein molecule in the cell, it is formed by the amino acids Glycine, Cysteine and Glutamic acid Its synthesis and turnover are mentioned in detail in Section 1 5 2
Several endogenous and exogenous electrophiles can be conjugated with GSH This reaction is catalysed by the GSH transferases (GSTs), which have broad substrate specificity and low affinity These properties ensure that toxic compounds of various origins and structures will be detoxified, but it also means that a number of metabolic enzymes can be activated in response to a certain compound, some of which will not be relevant for its detoxification

In humans, there are six classes of GSTs, named alpha (α), mu (μ), pi (π), theta (τ), zeta (ξ) and omega (ω), each class division can contain one or more isoforms Expression levels of the different isoforms have been found to be tissue-specific (Salinas *et al* , 1999) GSTs are predominantly found in liver, although they are also present in other cell types and tissues, such as kidney, erythrocytes and intestinal cells

Two different GSH conjugation pathways exist one of them involves displacement of the halogen, sulphate, sulphonate, phospho or nitro group by GSH, while in the other case the conjugating group is added to an activated double bond or a strained ring system GSH conjugates can be directly excreted in urine or bile, but they usually undergo further transformation in the form of glutamate and glycine removal by γ-glutamyl transpeptidase, and subsequent *N*-acetylation of the cysteine conjugate This can be followed by cleavage of the C-S bond by the C-S lyase present in intestinal microflora, *S*-methylation and oxidation

Examples of chemical groups susceptible of GSH conjugation are epoxides, haloalkanes, nitroalkanes, alkenes, and aromatic halo- and nitro- compounds A wide range of xenobiotics can be conjugated with GSH, including endogenous substrates, such as oestrogens and cholesterol-5,6-oxide, and the carcinogen acrolein, pesticides such as DTT and the drugs Cisplatin and Acetaminophen Some compounds, such as bilirubin and heme, are transported but not metabolised by these enzymes, which earned them the name "ligandins" (Litwack *et al* , 1971) This is of importance in the matter of chemotherapy resistance it has been widely reported that GSTs can protect several cell types from anticancer agents, yet no conjugated drug can be found in these cells It has been suggested that the enzyme binds the drug and then sequesters it away from its target (Meyer *et al* , 1992)

1 3 2 2 Conjugation with sugars

Glucuronidation

Conjugation with the activated, energy rich form of α-D-glucuronic acid – uridine diphosphate (UDP)-glucuronic acid - is the major route of sugar conjugation and the most important Phase II transformation pathway It is carried out by UDP-glucuronosyltransferases (UGTs) these enzymes transfer UDP-glucuronic acid to a wide range of substrates, both endogenous, such as steroids, bile acids, bilirubin,

hormones and dietary compounds, and foreign-like drugs, environmental toxins and carcinogens Transfer of glucuronic acid generates a β-D-glucopyranosiduronic acid, also known as a glucuronide derivative (Tukey *et al* , 2000) The bond between the functional group and the glucuronide is sensitive to the enzymatic action of β-glucuronidase present in the intestinal microflora, which means that already detoxified compounds excreted in the bile or faeces can be reactivated and reabsorbed, this phenomenon is known as enterohepatic circulation

β-D-glucuronides can be attached to several different functional groups, including hydroxyl (alcohols or phenols), carboxyl, sulphuryl, carbonyl, and amino groups *O*-glucuronides are formed from phenols, alcohols and carboxylic acids, the first two generate glucuronide ethers, while acids form glucuronide esters They are often secreted into the bile, which makes it possible for them to be reactivated *N*-glucuronides are the result of conjugation with primary, secondary or tertiary amines, amides and sulphonamides Thiol groups present in drugs such as Antabuse are substrates of *S*-glucuronidation Direct conjugation of carbon can also occur

Although conjugation with glucuronides usually inactivates the chemical and facilitates its excretion, a few examples of activation by glucuronidation are known, the most studied being morphine-6-glucuronide, which is a more potent analgesic than morphine itself

50 different UGTs have been characterised in vertebrates, which have been grouped in the UGT1 and UGT2 families, isoforms are included in a family when they share 55% sequence homology with the established members UGTs grouped in subfamilies share 60% sequence homology

There are 16 human UGTs identified up to date (Table 1 3 2), nine of them are grouped in the UGT1A subfamily, while seven of them belong to the UGT2 family UGT1A9 is probably the most important isoform for drug metabolism

Human UGTs	
UGT1 family	UGT2 family
UGT1A1	UGT2A1
UGT1A3	UGT2B1
UGT1A4	UGT2B7
UGT1A5	UGT2B10
UGT1A6	UGT2B11
UGT1A7	UGT2B15
UGT1A8	UGT2B17
UGT1A9	
UGT1A10	

Table 1 3 2 Human UDP glucuronyl transferases

UGT activity is mainly found in the liver microsomal fraction, although other sites of glucuronidation have been described, including intestine, kidney and colon This is probably a consequence of the widespread distribution of UDP-glucuronic acid, which is an intermediary of glycogen synthesis and, as such, is present in all different tissues of the body

Conjugation with other sugars

Conjugation reactions carried out with other sugars have great importance in plant and insect metabolism, but their relevance to mammalian biotransformation of foreign compounds is limited Glucose, xylose and ribose are used as substrates and generate the corresponding *N*- or *O*-riboside or xyloside, reactions are analogous to glucuronidation, and the activated, UDP-bound form of the sugar is used as sugar source

1 3 2 3 Conjugation with sulphates

The transference of a sulphuryl (SO_3) group to a suitable functional group present in a drug is a Phase II reaction performed by sulphotransferases They require the activated form of sulphate for transference, which is 3'-phosphoadenosine-5'-

phosphosulphate (PAPS) PAPS is generated in the cytosol in two steps from ATP and sulphate by the sequential action of ATP sulphurylase and APS-kinase
The catalytically active enzyme is a homodimer, and the reaction is carried out in a sequential mechanism involving a ternary complex between the enzyme, the substrate and PAPS After a chemical rearrangement, the substrate is released with a sulphuryl group attached to it, while the phosphoadenosine monophosphate (PAP) can be rephosphorylated and take part in other reactions
Sulphotransferases can be divided into two groups cytosolic or membrane-bound Cytosolic transferases metabolise small compounds, such as hormones and drugs, while membrane-bound enzymes are responsible for the biotransformation of carbohydrates and proteins (Chapman *et al*, 2004) Human sulphotransferases are listed in Table 1 3 3

Human sulphotransferases
SULT1A1
SULT1A2
SULT1A3
SULT1B1
SULT1C1
SULT1C2
SULT1E1
SULT2A1
SULT2B1a
SULT2B1b

Table 1 3 3 Human sulphotransferases

Functional groups susceptible to sulphuryl conjugation are mainly phenols, although alcohols, amines and thiols have also been reported Paracetamol is the best known example of a drug metabolised by sulphurylation, while endogenous substrates include hormones, bioamines and steroids A number of drugs can be either glucuronidated or sulphated, and the two pathways will then compete for the substrate Glucuronidation is generally considered as a low affinity, high capacity

system, while the opposite happens with sulphurylation, competition will then depend on the availability of PAPS and the drug concentration

1 3 2 4 Methylation

This reaction involves the transference of a methyl group to an *N*, *O*, *C* or *S* moiety by methyltransferases This is only a minor pathway in drug metabolism, and it can often result in an increase in the drug hydrophobicity The co-factor *S*-adenosyl methionine (SAM) is required to provide the methyl radical SAM is a high energy intermediate and is synthesised from L-methionine and ATP by the L-methionine adenosyltransferase Several forms of methyltransferases exist, which are listed in Table 1 3 4

Human methyltransferases
Phenol O-methyltransferase
Catechol O-methyltransferase
Imidazole N-methyltransferase
S-methyltransferase
N-methyltransferase
Hydroxyindole O-methyltransferase

Table 1 3 4 The human methyltransferases

Most methyltransferases have very restricted substrate specifities, except for the non-specific *N*-methyltransferase expressed in lung and the *S*-methyltransferase, which metabolise several known drugs

1 3 2 5 Acetylation

Arylamine *N*-acetyl transferase (NAT) is best known as the enzyme responsible for Isoniazide metabolism, although other arylamines, including carcinogens, are substrates of this reaction Two forms of this enzyme exist in humans, known as

NAT1 and NAT2 (Sim *et al*, 2000) and both utilise Acetyl-CoA as a cofactor in the transference of an acetyl group onto an aromatic amine, hydrazine or hydroxylamine moiety NAT1 is expressed in almost all types of tissues in the body and is responsible for p-aminosalicylate and p-aminobenzoic acid metabolism, it is considered that this enzyme plays an important role in the metabolism of endogenous compounds, although not many substrates have been identified In contrast, NAT2 is expressed in liver and intestinal epithelium and catalyses the acetylation of Isoniazide, Dapsone, certain sulphonamides and carcinogenic arylamines NAT2 is a polymorphic enzyme, and the acetylation rates depend on the alleles expressed, this is translated into differential toxicity of Isoniazide between individuals (Upton *et al*, 2001)
Even though this is the major route for the metabolistm of aromatic amines, acetylation can also result in reduced water solubility of their metabolites, which can increase their toxicity

1 3 2 6 Conjugation with amino acids

Some drugs, such as carboxylic acids, can react with acetate to form CoA derivatives such as Acetyl CoA These derivatives can then be conjugated to amino groups, like those present in amino acids Amino acid conjugation can then be a reverse form of *N*-acetylation, in which the substrate is activated before being attached to a functional group present in an endogenous molecule
A different pathway of amino acid conjugation also exists, in which an aromatic amine or hydroxylamine moiety can be conjugated to the carboxyl group of an amino acid, this reaction requires prior activation of the amine by ATP
Both reactions are carried out by amino acid acyl transferases Glycine, glutamine, ornithine, arginine, proline, serine and taurine are the most common amino acids participating in both types of reactions Amino acid conjugates such as serine or proline *N*-esters of hydroxylamines can be unstable and degrade into active electrophiles, which can be carcinogenic

1 3 2 7 Conjugation with lipids

Fatty acids like palmitic and stearic acid can be attached to some Phase I metabolites, such as those derived from cannabinoids Carboxylic acid groups present in drugs can also be conjugated to form fatty acid or cholesteryl esters

1 3 2 8 Condensation

Some amines and aldehydes appear to react spontaneously to form larger molecules The best-studied case is that of Dopamine, which condenses with its own metabolite to form an active alkaloid Condensation could also be of enzymatical origin, although very little is known about these reactions

1 3 3 Phase III metabolism

Phase III metabolism would involve the further transformation of Phase II metabolites *N*-acetylcysteine conjugates derived from GSH conjugates that underwent further metabolism are sometimes considered Phase III products Others believe that enzymatic reactions performed by the intestinal microflora belong in a separate class, referred to as Phase III metabolism In any case, only Phase I and Phase II are firmly established as essential steps in drug metabolism

1.4 The cytochrome P450 superfamily

The cytochrome P450 superfamily is composed of 57 human CYP genes and 29 pseudogenes (a pseudogene being a defective gene that does not produce a functional protein) They have been grouped in families and subfamilies according to the following criteria two cytochromes belong to the same family if they share at least 40% of sequence homology, and to the same subfamily if they share at least 50% of sequence homology In total, 18 families and 43 subfamilies have been identified The different human families, their members and their functions are listed in Table 1 4 1

Family/ Subfamily	Members	Function
CYP1	2 subfamilies, 3 genes, 1 pseudogene	Drug metabolism, arachidonic acid and eicosanoid metabolism
CYP2	13 subfamilies, 16 genes, 16 pseudogenes	Drug metabolism, arachidonic acid and eicosanoid metabolism
CYP3	1 subfamily, 4 genes, 2 pseudogenes	Drug metabolism, arachidonic acid and eicosanoid metabolism
CYP4	5 subfamilies, 12 genes, 10 pseudogenes	Arachidonic acid, eicosanoid and fatty acid metabolism
CYP5	1 subfamily, 1 gene	Thromboxane A2 synthesis
CYP 7A	1 subfamily member	Bile acid biosynthesis/ 7-α hydroxylation of steroid nucleus
CYP 7B	1 subfamily member	Brain-specific form of 7-α hydroxylation
CYP 8A	1 subfamily member	Prostacyclin synthesis
CYP 8B	1 subfamily member	Bile acid biosynthesis
CYP11	2 subfamilies, 3 genes	Steroid biosynthesis
CYP17	1 subfamily, 1 gene	Steroid biosynthesis (17-α hydroxylase, 17/20-lyase)
CYP19	1 subfamily, 1 gene	Steroid biosynthesis (Aromatase)
CYP20	1 subfamily, 1 gene	Unknown
CYP21	1 subfamily, 1 gene, 1 pseudogene	Steroid biosynthesis (21-hydroxylase)
CYP24	1 subfamily, 1 gene	Vitamin D degradation (24-hydroxylase)
CYP26A	1 subfamily member	Retinoic acid hydroxylation
CYP26B	1 subfamily member	Probably retinoic acid hydroxylation
CYP26C	1 subfamily member	Probably retinoic acid hydroxylation
CYP27A	1 subfamily member	Bile acid biosynthesis
CYP27B	1 subfamily member	Vitamin D3 1-α hydroxylation
CYP27C	1 subfamily member	Unknown
CYP39	1 subfamily member	24-hydroxycholesterol 7α-hydroxylase
CYP46	1 subfamily member	Cholesterol 24-hydroxylation
CYP51	1 subfamily, 1 gene, 3 pseudogenes	Cholesterol biosynthesis (Lanosterol 14-α demethylase)

Table 1.4.1: The human P450 families (adapted from Nebert *et al.*, 2002).

1 4 1 Enzyme induction

A prominent characteristic of the P450 system is that many compounds are able to induce the expression of the enzymes responsible for their metabolism Induction is then unspecific, meaning that a single substrate can induce several enzymes at a time In some cases, these enzymes need not be cytochromes P450 some CYP3A4 inducers also increase expression of MDR1, an efflux pump (Schuetz *et al* , 1996)
Certain cytochromes are involved in steroid hormone synthesis this includes the sex hormones Expression of these cytochromes is therefore regulated in a sex- and age-specific manner (Jarukamjorn *et al* , 2001) However, P450s involved mainly in drug metabolism such as CYP1A2, CYP2B6 and CYP3A4 also show sex-specific regulation (Van Schaik, 2005), the reason for this regulation remains to be found
There are several mechanisms of induction, of which the aryl hydrocarbon receptor (AhR) pathway for the CYP1 family and the so-called orphan nuclear receptor pathway for CYP2, CYP3 and CYP4 families are probably the best studied
The AhR is a cytosolic protein that can bind ligands with high affinity, usually polycyclic aromatic hydrocarbons (PAHs) When the receptor-ligand complex is formed, it translocates to the nucleus with the aid of the AhR nuclear translocator protein (ARNT), and can subsequently bind to xenobiotic responsive elements in DNA But the AhR is involved in more important functions a study by Dohr *et al* (1997) showed coordinated regulation of mRNA expression of the AhR and cell cycle genes, suggesting that this receptor might be implicated in the regulation of cell proliferation Another report demonstrated that the AhR binds the retinoblastoma protein, known to induce cell cycle arrest (Puga *et al* , 2000), and together they have a synergic inhibitory effect on cell proliferation The retinoblastoma protein has recently been recognised as an AhR coactivator (Hankinson, 2005)
The orphan nuclear receptor superfamily is composed of constitutive androstane receptor (CAR), pregnane X receptor (PXR), steroid and xenobiotic receptor (SXR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR) and farnesoid X receptor (FXR), and they mediate the induction of CYP2, CYP3, CYP4 and CYP7A (Blumberg *et al* , 1998, Kliewer *et al* , 1999) The mechanism is common to all of them upon binding their specific ligand, these orphan receptors heterodimerise with the retinoid acid receptor (RXR) and then bind to specific sequences in DNA, activating the transcription of the respective family members Of these, the most relevant ones for drug metabolism are CAR, PXR and SXR
CAR can activate transcription of target genes even in the absence of any exogenously added ligand, and this basal activity is blocked by testosterone

metabolites This suggests that CAR-activated transcription is turned off upon hormone binding Expression of CAR is readily detected in the liver and the receptor is believed to regulate expression of CYP2B, which is a steroid hydroxylase, thus raising the possibility of CAR being involved in steroid homeostasis CAR has also been shown to activate expression of CYP3A genes, indicating that even though this receptor is mainly a CYP2B regulator, it can also affect the transcription of different P450 families (Xie *et al*, 2000)

Endogenous ligands of PXR are C21 steroids, known as pregnanes, such as progestagens, estrogens and corticosteroids It is not known whether PXR has a specific ligand for which it has high affinity or if its ability to bind several different steroidal ligands renders it as a general steroid sensor PXR is expressed in liver and intestine, tissues in which CYP3A expression can be induced, where it binds several different xenobiotics that modulate CYP3A expression Activation of PXR results in induction of CYP3A transcription and steroid (or drug) metabolism

SXR is closely related to PXR, showing a high degree of sequence homology (Blumberg *et al*, 1998) It is expressed mainly in the liver and also in lung, kidney and intestine (Miki *et al*, 2005), it has also been detected in breast carcinoma, but not normal tissue (Miki *et al*, 2006) Steroids such as corticosterone, oestradiol, dihydrotestosterone and pregnenolone are endogenous ligands of this receptor, while 1,25-dihydroxy vitamin D3 is not, traditional inducers of CYP3A4 such as dexamethasone, rifampicin and nifedipine also activate this receptor This receptor mainly modulates expression of CYP3A genes although, similar to CAR, can also induce the expression of CYP2B subfamily members (Xie *et al*, 2000) The finding that SXR can also activate the expression of MDR1 (Synold *et al*, 2001) shows the intimate connections that lie between the genes involved in cellular defence against xenobiotics

The intracellular vitamin D receptor (VDR) has been recently found to play a role in P450 induction Vitamin D is an active modulator of cell growth, proliferation and calcium homeostasis, and cytochromes P450 are responsible for its activation and catabolism It is not surprising then that vitamin D would induce its own metabolism by increasing CYP3A4 and also CYP2B6 and CYP2C9 expression via activation of VDR and heterodimerisation with RXR (Pascussi *et al*, 2003)

Down-regulation of CYP450 expression can also occur, as it has been reported that several proinflammatory cytokines, namely IL-1, IL-4, IL-6, TNFα and IFNγ, can suppress cytochrome expression in hepatocytes (Abdel-Razzak *et al*, 1993) This could be a major issue for cancer treatment, since many patients with advanced disease have high circulating concentrations of proinflammatory cytokines (Slaviero *et*

al , 2003), expression of these mediators can be stimulated by or released directly from the tumour Down-regulation of P450s occurs through several mechanisms, including decreased transcriptional activation or enhanced degradation by heme oxygenase and not only does it affect the overall P450 expression, but also its susceptibility of induction A recent study by Zhou *et al* (2006) shows that SXR and the key regulator of inflammation NF-κB reciprocally inhibit each other's activation, providing a molecular explanation for the observed decrease in P450 levels Several theories have been put forward to explain this phenomenon, but the reasons behind P450 down-regulation by cytokines remain the subject of speculation (Renton *et al* , 2001)

A recent report also shows that activation of Fas receptor suppresses CYP3A4 mRNA and protein expression (Chun *et al* , 2003), loss of detoxification mechanisms could make cells more sensitive to apoptotic stimuli

1 4 2 Enzyme inhibition

Inhibition of P450s can be competitive or non-competitive Competitive inhibition usually takes place when two (or more) drugs that are substrates of the same isoform are administered together and inhibit each other's metabolism Non-competitive inhibition can occur through different mechanisms, such as interference with an enzyme cofactor, suicide inhibition (i e , inactivation of the enzyme by the inhibitor), etc

Several commonly used drugs can inhibit P450s, this is of great importance in pharmacology, since co-administration of a P450 inhibitor, such as Ketoconazole, will impair the metabolism of other drugs, resulting in longer half lives and increased toxicity Much as with redundancy and overlapping phenomena observed with metabolism, several different drugs can inhibit one isoform

1 4 3 The P450 families

1 4 3 1 The CYP1 family

CYP1A

This subfamily is composed of two members, CYP1A1 and CYP1A2, involved in the metabolism of polycyclic hydrocarbons, aromatic and heterocyclic amines, among other compounds

CYP1A1 metabolises mainly polycyclic hydrocarbons Its expression is very low in liver and it is believed to be present almost exclusively in extra-hepatic tissue, and this at extremely low levels (Nebert *et al*, 2004) A recent study has contradicted these findings by demonstrating CYP1A1 protein expression and activity in 8 liver samples (Stiborova *et al*, 2005) Induction of enzymatic activity, usually referred to as aryl hydrocarbon hydroxylase (AHH) activity, occurs in response to treatment with polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methyl cholanthrene These xenobiotics are well known carcinogens and can be found in cigarette smoke and smog (Hankinson, 2005) PAHs can activate the transcription of CYP1A1 mRNA and therefore induce their own metabolism

There is evidence of cross talk between the AhR and the estrogen receptor (ER) pathways steroids are able to induce CYP1A1 transcription via an ER-mediated process This is probably an Ah-dependent pathway, as expression of ERα is believed to enhance CYP1A1 expression in breast cancer cells, rather than induce it *per se* (Spink *et al*, 1998) It has also been reported that testosterone and TCDD pathways negatively regulate each other's activity in the prostate cancer cell line LNCaP (Jana *et al*, 2000)

CYP1A2 is involved in the metabolism of aryl amines and amides, and also of aromatic amines, caffeine, oestradiol and certain drugs, such as Phenacetin, Clozapine and Propanolol It is also responsible for the activation of the antiandrogenic drug Flutamide, which is widely used in the treatment of prostate cancer Although a precise function for this enzyme in physiological or developmental pathways has not been described yet, CYP1A2 is highly conserved among mammals, and knock-out of the gene appears to be lethal in mice (Pineau *et al*, 1995) Knock-out mice for CYP1A2 die very early in their development from respiratory distress and display signs of lung immaturity, including expression of abnormal surfactant protein in the lung

CYP1A2 expression is constitutive in liver, where it accounts for about 15% of the total P450 content (Van Schaik, 2005), and negligible in extra-hepatic tissue Consistent with this, activity is readily detected in the liver in the absence of any inducers, while PAHs can significantly increase CYP1A2 activity levels in gastrointestinal tract, nasal epithelium and brain, and also in the liver (Nebert *et al*, 2004) Induction of this gene is also regulated by the AhR, although several differences exist between CYP1A1 and CYP1A2 regarding susceptibility of induction Both enzymes present overlapping activities, but their specific reactions are carried out faster than the shared ones They are also induced by the same compounds, with the exception of Isosafrole, which is a specific inducer of CYP1A2 but not of CYP1A1 (Boulenc *et al*, 1992)

CYP 1B

CYP1B1 is the only member of this subfamily, sharing a 40% sequence homology with CYP1A1 and CYP1A2 It can hydroxylate 17-β-estradiol in C4 and is involved in testosterone biotransformation It is also known to metabolise xenobiotics such as ethoxyresorufin, theophylline and caffeine

CYP1B1 mRNA is constitutively present in mammal steroidogenic tissues, such as rat granulosa cells (Dasmahapatra *et al*, 2002) Its expression can be induced by peptide hormones, cAMP and ligands of the AhR, in a similar way to that of CYP1A1 induction As with CYP1A1, there also seems to be cross talk between the ERα and the CYP1B1 expression pathways (Spink *et al*, 1998) There seems to be a tissue-specific regulation of the expression, since treatment with TCDD induces 1B1 mRNA in MCF-7 cells but not in HepG2 cells (Spink *et al*, 1994) Expression of CYP1B1 can be inhibited by treating cells with 2,4,3',5'-tetramethoxystilbene (TMS), which shows a 50-fold selectivity for CYP1B1 over CYP1A1, and 500-fold selectivity over CYP1A2 (Chun *et al*, 2001) Other non-selective inhibitors of CYP1B1 activity are α-naphtoflavone and Ketoconazole More recently, regulation of CYP1B1 expression by micro RNAs (miRNAs) has been described (Tsuchiya *et al*, 2006), it was discovered that transfecting MCF-7 cells with an antisense for the miRNA miR-27b, which has a matching sequence to that of the 3' untranslated region of CYP1B1, increased CYP1B1 expression and activity Furthermore, expression of miR-27b and CYP1B1 were found to be inversely correlated in breast tissue samples

A number of studies have failed to detect presence or activity of functional CYP1B1 protein in normal (non-tumoural) tissue (Murray *et al*, 1997, McFadyen *et al*, 1999) Murray *et al* (1997) have performed immunohistochemistry using a CYP1B1-specific

antibody in a number of clinical samples of cancerous and normal adjacent tissue from a wide range of tissues, namely bladder, breast, colon, oesophagus, kidney, lung, ovary, skin, stomach, uterus, connective tissue, lymph node, brain and testis CYP1B1 was detected in 122 tumour samples out of 127, and it was present in all different types of cancer However, the enzyme could not be detected in any of the 130 samples of normal tissue, not even in liver These results lead researchers to believe that CYP1B1 is a tumour-specific isoform, however, expression of CYP1B1 in normal tissue has been reported, albeit in much lower levels than in tumour tissue (Muskhelishvili *et al*, 2001, Gibson *et al*, 2003) Differential expression of cytochromes P450 in normal and tumoral tissue is further discussed in Section 1 4 5 Defects in the *cyp1b1* gene have been linked to primary congenital glaucoma It has been speculated that this enzyme is required to eliminate a signalling molecule failure to do so could lead to high concentrations of this molecule that would in turn lead to glaucoma The affected molecule may be a steroid (Stoilov *et al*, 1997)

1 4 3 2 The CYP2 family

The human CYP2A subfamily has three genes, CYP2A6, CYP2A7 and CYP2A13, and a pseudogene, CYP2A18P Of these, CYP2A6 is the most important isoform for drug metabolism and is expressed mainly in liver, with only very low levels detected in extrahepatic tissues (nasal mucosa, skin), and its substrates include coumarin, nicotine and the carcinogen aflatoxin B1, among other agents, this isoenzyme is also responsible for the activation of the 5-FU-prodrug Tegafur CYP2A6 is also responsible for the endogenous metabolism of retinoic acid, arachidonic acid and progesterone (Du *et al*, 2004)

The human CYP2B isoforms are CYP2B6, CYP2B7 and CYP2B8 CYP2B6 is expressed in liver, while CYP2B7 is found in lung, especially in Clara cells, and CYP2B8 in liver and intestine There is also a pseudogene, CYP2B7P, which contains a premature stop codon in its sequence Induction of expression is via the CAR orphan nuclear receptor, which can be activated by Phenobarbital Expression in humans is also regulated by growth and thyroid hormones Substrates metabolised by CYP2B6 include nicotine, coumarin, retinoic acid, 17ß-estradiol, estrone and testosterone

CYP2C8, CYP2C9, CYP2C18 and CYP2C19 comprise the CYP2C subfamily, which is one of the most important for drug metabolism, together, these four enzymes metabolise more than half of all frequently prescribed drugs There are also 8

pseudogenes, but they are all believed to be non-functional, except for CYP2C9-de1b. The reason for the multiplicity of members in this subfamily could be their tendency to recombination, conversion and transplicing, which even results in some isoforms being a hybrid of two different sequences (Finta *et al.*, 2000).

All members of this subfamily are constitutively expressed in liver, while CYP2C8 and CYP2C9 are also expressed in the intestinal tract. A recent immunohistochemical study using an antibody that reacts with CYP2C8, CYP2C9, CYP2C18 and CYP2C19 detected positive staining in samples of epithelium of the small and large intestine, gastric epithelium, bile duct, nasal mucosa, salivary glands, tracheobronchial glands, prostate, uterine cervix, kidney and adrenal cortex (Yokose *et al.*, 1999). The members of this subfamily are involved in retinal, arachidonic and linoleic acid and steroid metabolism, e.g. testosterone, oestradiol and progesterone, but they also contribute to the biotransformation of several drugs, such as Warfarin and Tolbutamide.

CYP2D6 is the only member of the CYP2D subfamily expressed in humans. Two other cytochrome genes are located upstream of *cyp2d6, cyp2d7* and *cyp2d8,* but the first is defective and is not expressed, while the second is a pseudogene. CYP2D6 is not detectable in all human livers, and this is probably because mutations that inactivate the gene are not lethal and therefore not subjected to selection pressure. This isoform is responsible for the metabolism of the noradrenaline- blocking drug Debrisoquine, along with several other drugs used in psychiatric treatment. Like the other CYP2 subfamilies, it is also involved in the biotransformation of retinal and steroids, along with tryptamine and its derivatives.

Only one member of the CYP2E subfamily is present in humans, although three transcripts have been detected in tissue samples (Botto *et al.*, 1994); it has been concluded that they all originate from the same gene. CYP2E1 is constitutively expressed in the liver, and its expression can be induced by a number of xenobiotics including ethanol, acetone and Pyrazole. Induction with these chemicals does not increase mRNA levels, so the net increase in protein expression is believed to be a combination of enhanced protein stabilization and decreased turnover. The most important role for this isoform is in the metabolism of ethanol, although it is also involved in the transformation of several compounds, such as the carcinogen N-nitrosodimethylamine, oestradiol, fatty acids, prostaglandins, ketone bodies and acetone. Even though in basal conditions the alcohol dehydrogenase and aldehyde dehydrogenase enzymes carry out most of the ethanol metabolism, CYP2E1 metabolism is rapidly and effectively induced by ethanol, and so takes over after significant alcohol ingestion.

Two *CYP2F* and two *CYP2G* loci have been found in humans, but only CYP2F1 appears to be functional, and is expressed mainly in lung epithelium It appears to have a protective function against pneumotoxins such as tobacco-derived nitrosamines (Du *et al*, 2004) Two pseudogenes have also been identified as members of the CYP2T subfamily, *cyp2t2p* and *cyp2t3p*, none of these genes appears to be functional

One CYP2J protein has been detected in humans, expressed in lung, skin and gastrointestinal tract, where it appears to be involved in first-pass intestinal metabolism (Zeldin *et al*, 1997) The endogenous substrates identified for CYP2J2 are fatty acids, such as arachidonic acid and linoleic acid, and testosterone Recently, CYP2J2 has also been identified as a vitamin d 25-hydroxylase (Aiba *et al*, 2006) The biologically active eicosanoids generated from arachidonic acid metabolism by CYP2J2 appear to be involved in a number of physiological functions such as vasoprotective effects, protection against oxidative stress, endothelial cell growth and angiogenesis (Yang *et al*, 2001, Wang *et al* ,2005, Spiecker *et al*, 2006)

A novel cytochrome P450, CYP2S1, has been detected in human lung and skin, while its mRNA is also present in trachea, stomach, small intestine and spleen (Karlgren *et al*, 2003) An interesting feature of this isoform is that its expression appears to be induced by polycyclic aromatic hydrocarbons and dioxin, much as the CYP1 family Furthermore, napthtalene has been identified as a CYP2S1 substrate, supporting the notion of this isoform's involvement in the metabolism of complex organic compounds Retinoic acid has also been identified as an endogenous substrate

CYP2R1, CYP2U1 and CYP2W1 have been recently identified in humans (Karlgren *et al*, 2003) CYP2R1 is the best studied of the three, and appears to be a vitamin D 25-hydroxylase (Cheng *et al*, 2003) High levels of its mRNA have been detected in pancreas, liver and kidney CYP2U1 expression was detected in thymus, heart and brain (Helvig *et al*, 2003), and its endogenous substrates are long chain fatty acids Little is known about CYP2W1, mRNA expression could not be found in any of the human tissues tested, although a transcript was detected in HepG2 hepatoma cells, and also in other transformed tissues, suggesting an association with cancer (Karlgren *et al*, 2003)

1.4.3.3 The CYP3 family

The CYP3A is the most abundantly expressed P450 subfamily in metabolically active tissues, fundamentally liver but also small intestine and renal epithelium. The metabolic reactions carried out by its members include *C*- and *N*-dealkylation, *C*-hydroxylation, dehalogenation, dehydration and nitroreduction. Adding another xenobiotic can stimulate the catalytic activity for a certain substrate: this phenomenon is known as positive cooperativity, and it is a unique feature of the CYP3A enzymes.

The spectrum of substrates metabolised by this subfamily is incredibly broad, including drugs, steroids and carcinogens. Substrate specificity is similar to that of MDR1 but overlap is not complete.

This subfamily comprises the CYP3A4, CYP3A5, CYP3A7 and CYP3A43 isoforms. The major isoform is CYP3A4, probably the most important of all P450 cytochromes, responsible for the metabolism of roughly 50% of all known drugs and also of carcinogens and endogenous steroids: it presents the broadest catalytic selectivity of any P450. It is expressed mainly in the liver, where it accounts for 30% of total protein, and also in the small intestine, its expression then decaying along the remainder gastrointestinal tract. It is also present in renal tissue, although only in 30% of tested human samples.

CYP3A5 is the predominant isoform in kidney and colon, but its expression in the liver is polymorphic, found in only 10-30% of human livers. The total amount of CYP3A5 hepatic protein, when present, is usually about 25% of that of CYP3A4. Recently, it was shown that the lack of CYP3A5 expression is due to the presence of a genetic polymorphism in this enzyme (Van Schaik, 2005); it is believed that about 80% of the Caucasian population are homozygous for this CYP3A5 form with defective expression.

CYP3A7 is the major isoform found in foetal liver and it can also be detected, albeit in low levels, in the adult organ. It is also expressed in adult endometrium and placenta. In the majority of individuals, CYP3A7 expression is repressed immediately after birth, but a polymorphism which results in the expression of CYP3A7 being under the control of the CYP3A4 promoter has been described (Van Schaik, 2005); this would result in CYP3A7 expression in the adult.

The previously described CYP3A3 isoform was for some time considered as a sequencing artefact; however, it was recently cloned and is now considered a *bona fide* member of the CYP3A subfamily (Gellner *et al.*, 2001). Its expression has been demonstrated in adult liver, kidney, pancreas and prostate, albeit at extremely low levels. The contribution of CYP3A43 to drug metabolism is thought to be negligible.

The selectivity of the different isoforms is quite poor, with overlapping substrate specificity, however, some differences in the catalytic activity exist For example, although Taxotere is metabolised by both CYP3A4 and CYP3A5, metabolism of Taxol appears to be carried out exclusively by CYP3A4 There is also considerable inter-individual variability in both the enzymatic activity and the susceptibility of induction Genetic polymorphisms of CYP3A isoforms also exist, leading to major inter-patient differences in the pharmacokinetics of commonly used drugs

Induction of these isoforms is usually through transcriptional activation, although increase of protein levels by stabilization might occur There is also a broad spectrum of inducers, some of which are substrates of these enzymes, such as anticonvulsant agents, barbiturates, pesticides, macrolide antibiotics, etc The induction process is mediated by the orphan nuclear receptors PXR and SXR and appears to be tissue-specific for example, expression of CYP3A4 is inducible in liver but not in colon

The CYP3A enzymes can be inhibited by a large number of compounds, the only common features amongst them being lipophillicity and relatively large molecular size Inhibition can be competitive or non-competitive, and the active compounds include macrolide antibiotics, antidepressants, glucocorticoids, antifungals, estrogens and Vinca alkaloids, among others

1.4.4 P450 activity and cancer

Several anti-cancer drugs are metabolised by the P450 system (Table 1.4.2), usually generating less or non-toxic derivatives, although some activation occurs. This is particularly important for oxazaphosphorines such as Cyclophosphamide or Ifosfamide, which require P450-mediated activation to exert their cytotoxic effect (De Raat *et al.*, 1977), and also for drugs like Tegafur, which is metabolised to 5-fluorouracil by the P450s (Ikeda *et al.*, 2000).

CYP450	Substrates
CYP1A2	Flutamide (activation), Erlotinib, Procarbazine, Tamoxifen, VP-16
CYP2A6	Tegafur (activation), Cyclophosphamide, Ifosfamide
CYP2B6	Cyclophosphamide (activation), Procarbazine, Ifosfamide (activation), Tamoxifen
CYP2C8	Taxol, Cyclophosphamide, Ifosfamide
CYP2C9	Cyclophosphamide, Ifosfamide, Tamoxifen, Tegafur (activation)
CYP2C19	Thalidomide, Cyclophosphamide, Ifosfamide, Tamoxifen
CYP2D6	Tamoxifen (activation), Adriamycin, Gefitinib/Iressa
CYP2E1	VP-16, Tamoxifen
CYP3A4/5	Cyclophosphamide, ((in)activation), Ifosfamide ((in)activation), Taxotere, Adriamycin, VP-16, Flutamide, Gefitinib/Iressa, Imatinib/Gleevec, Erlotinib, Irinotecan, Taxol, Teniposide, Tamoxifen, Vinca alkaloids, Busulfan

Table 1.4.2: Anticancer agents and P450 metabolism (adapted from Van Schaik, 2005).

Measurable P450 activity is readily detected in cell lines, P450 activity was studied in the NCI human tumour cell line panel (Yu *et al*, 2001) All cell lines exhibited significant P450 and P450R activity A negative correlation was found between general P450 activity and chemosensitivity to 10 standard anticancer agents, thereby suggesting that the cytochromes were contributing to cell resistance, probably by inactivating the drugs

CYP1A

The role of CYP1A1 in lung and colon cancer has been recently established The enzyme, expressed in lung and also along the gastrointestinal tract, is induced by the presence of polycyclic hydrocarbons present in cigarette smoke and charred foods, and also in polluted air Induction leads to enhanced activation of these compounds, and the activated metabolites are known to be carcinogenic (Nebert *et al*, 2004) Involvement of CYP1A1 in carcinogenesis was further confirmed after the discovery of a genetic polymorphism in the *cyp1a1* gene that results in three different phenotypes, A, B and C Alleles A and B are far less inducible than allele C, and the homozygous individuals for allele C were found to be at increased risk of developing cancer (Goto *et al*, 1996)

Other types of polymorphisms also exist, the most important of them probably being a point mutation in exon 7 of the *cyp1a1* gene The mutation results in and isoleucine to valine substitution and renders a far more active enzyme this can in turn lead to enhanced activation of environmental xenobiotics and a higher risk of developing cancer (Taioli *et al*, 1995) However, many anticancer drugs require activation by the target cell, and the mutation could result in a more efficient biotransformation of the drug and consequent enhanced effect Thus, the mutation in exon 7 could be a marker of anti-cancer drug sensitivity for certain tumours (Peters *et al*, 1997)

The role of CYP1A1 in other types of cancer remains somewhat obscure Expression of CYP1A enzymes was detected in 39% of breast tumour tissue samples, only in areas of invasive carcinoma (Murray *et al*, 1993), suggesting a relationship between enzyme expression and invasive phenotype Breast tumour 7-ethoxyresorufin O-deethylase (EROD) activity, mainly catalysed by CYP1A1, was not significantly different from that of normal tissue Moreover, no correlation was found between tumour EROD activity and stage or grade of malignancy (Iscan *et al*, 1999) On the other hand, expression of CYP1A was detected in 68% of urinary bladder tumour samples, and it was correlated with tumour grade (Murray *et al*, 1995)

A recent report by Leung *et al.* (2005) might explain some of the differences observed. The authors detected a novel spliced variant of CYP1A1, named CYP1A1v, which presents an 84bp deletion. This variant showed similar enzymatic activity when compared with CYP1A1; however, expression of CYP1A1v was not restricted to the endoplasmic reticulum, like that of CYP1A1, but rather it was detected in the nucleus and in mitochondria of ovarian cancer cells. Apparently, the deletion results in the creation of a translocation signal that alters intracellular distribution of CYP1A1.

Involvement of CYP1A1 in the development of resistance to chemotherapy also remains undefined. Expression of CYP1A1 and CYP1B1 enzymes was found to be high in anti-estrogen resistant human breast cancer cell lines; no gene amplification had occurred, so this was regarded as a regulatory alteration (Brockdorff *et al.*, 2000). However, expression of CYP1A1 was decreased in an Adriamycin-resistant variant of the MCF-7 breast cancer cell line as compared to the parental cells. This was also a regulatory phenomenon, as no mutation was detected in the gene and transfection with CYP1A1 cDNA did not result in an increase of enzymatic activity. No mRNA was detected following induction with TCDD, not that of CYP1A1 or other PAH-induced enzymes, which suggests an alteration in the regulation of several genes. MCF-7 cells with low levels of resistance to Adriamycin still displayed low AHH activity, implying that the loss of CYP1A1 expression is not necessary for the development of resistance. Metothrexate-resistant MCF-7 cells had also lost AHH activity, which made the authors suggest that this alteration occurs in response to general cytotoxic stress, rather than to specific Adriamycin effects (Ivy *et al.*, 1988).

CYP1B

Because of its ability to activate environmental procarcinogens, CYP1B1 has been proposed to play a role in carcinogenesis (Li *et al.*, 2000). However, if the functional protein is not expressed in normal tissue, this might not be an important factor.

The significance of CYP1B1 overexpression in cancer is not yet fully understood, but the fact that it can metabolise a number of relevant anti-cancer drugs suggests that it might be important in the development of resistance to chemotherapy. Rochat *et al.* (2001) have found that CYP1B1 is able to metabolise Flutamide, an antiandrogen used in the treatment of prostate cancer, with high efficiency, inactivating it. Also, McFadyen *et al.* (2001) found a significant decrease in the sensitivity of CYP1B1-transfected cells to Taxotere compared to the parental cell line. Cytotoxicity was restored by pretreating the cells with the CYP1 inhibitor α-naphtoflavone.

In a recent study, Tsuchiya *et al* (2006) studied the expression of miR-27b, an miRNA that has been shown to decrease CYP1B1 expression, in tissue samples from breast cancer and normal adjacent tissue A great proportion of patients showed decreased expression of miR-27b in cancerous tissue, accompanied by CYP1B1 overexpression, analysis of normal adjacent tissue did not show such a decrease, suggesting that downregulation of this miRNA was a specific tumoral event

CYP2

Since the members of the CYP2A subfamily are able to metabolically activate environmental procarcinogens, a role in tumour development has been proposed for them Given that nicotine and some nitrosamines commonly found in tobacco are good substrates of the CYP2A6 isoform, a recent study tried to find an association between sequence polymorphisms in the *cyp2a6* gene, smoking habits and lung cancer development Data suggest that a mutation in *cyp2a6* that results in a less active enzyme could lower the risk of developing smoking-induced lung cancer (Raunio *et al*, 2001) On the other hand, reduced activity of CYP2A6 could mean decreased activation of anticancer drugs like Tegafur, indeed, such an occurrence was reported for a patient with a reduced activity enzyme (Daigo *et al*, 2002)
Distribution of CYP2A6 was studied in human hepatocellular carcinoma samples 42% of them were positive for CYP2A6 protein Staining was very heterogeneous, and a survival analysis showed a tendency towards a more favourable prognosis in patients with CYP2A6-expressing tumours compared to those without detectable enzyme expression (Raunio *et al*, 1998) Expression of CYP2A enzymes has also been detected in a human glioma cell line (Vasquez *et al*, 1998), although its importance in tumour development or outcome was not studied
CYP2B6 is also associated with carcinogenesis induction of its expression was correlated with tumour development in rat liver (Diwan *et al*, 2001) and thyroid (Diwan *et al*, 1996) Also, selective inhibitors of CYP2B6 were able to block carcinogenic side effects of Tamoxifen (Stiborova *et al*, 2002) No correlation has been found to date between CYP2B6 expression and development of resistance to chemotherapy, although higher levels of CYP2B6 expression found in females as compared to males correlate with increased Ifosfamide activation (Schmidt *et al*, 2001) which might have therapeutic implications
The CYP2C subfamily is able to activate procarcinogens such as aflatoxin B1 and benzo[a]pyrene, which raises the possibility of a correlation between enzyme activity

and carcinogenesis However, no association has been found so far Sequence polymorphisms in *cyp2c9* and *cyp2c18* genes have been detected, and even though a correlation was found between polymorphic alleles and differences in the catalytic activation of cyclophosphamide (Chang *et al*, 1997), the influence of these mutations in chemotherapy outcome is still unknown

Nearly 5-10% of the Caucasian population carry a polymorphism in the *cyp2d6* gene that results in a low-activity enzyme this condition is denominated Debrisoquine polymorphism, because Debrisoquine metabolism is compromised in these individuals Several studies have linked this polymorphism with increased risk of developing leukaemia and bladder malignant melanoma (Smith *et al*, 1991, Wolf, 1991) This polymorphism might also have a role in chemotherapy outcome poor Debrisoquine metabolisers have lower levels of circulating Endoxifen, the active metabolite of Tamoxifen, which might result in lower therapy efficacy (Jin *et al*, 2005)

It has been reported that CYP2E1 can activate a large number of toxins and carcinogens, such as N-nitrosodimethylamine and benzene, and it has been suggested that polymorphisms in the *cyp2e1* sequence can be linked to increased incidence of lung, gastric or oesophageal cancer (Kato *et al*, 1992) Indeed, this enzyme appears to be dramatically induced by cigarette smoke (Villard *et al*, 1998) The same study also reports that pretreatment of mice with a CYP2E1 inhibitor before exposing them to tobacco smoke reduced the formation of DNA single strand breaks, suggesting that this isoform might be involved in lung DNA damage in smokers On the other hand, a recent report indicates that a polymorphism in *cyp2e1* may confer some protective effect against gastric cancer (Gonzalez *et al*, 2002) More recent reports also show that different allelic forms of CYP2E1 are associated with both increased and decreased risk of cancer (Cai *et al*, 2005, Iizasa *et al*, 2005, Li *et al*, 2005), and often were not independent predictory factors, but they were associated with lifestyle issues, such as smoking status Thus, the role of this enzyme in carcinogenesis remains undefined

It has recently been proposed that CYP2J2 has a role in the pathogenesis of cancer Indeed, transfection of this enzyme into cancer cells *in vitro* increased proliferation and protected cells from apoptosis induced by TNFα (Jiang *et al*, 2005) The same effects were observed after the exogenous addition of epoxyeicosatrienoic acids (EETs), which are products of CYP2J2 metabolism These derivatives of arachidonic acid activated the MAP kinase pathway, as well as the PI3-kinase/Akt pathway, and also increased the level of phosphorylation of the EGF receptor Moreover, these EETs have potent angiogenic effects, as shown by their ability to promote proliferation

and migration of endothelial cells, as well as capillary tubule formation (Wang *et al*, 2005)

Expression of CYP2R1, CYP2S1, CYP2U1 and CYP2W1 has been found to be increased in tumour as compared to normal tissue (Downie et al 2005, Kumarakulasingham et al 2005, Saarikoski et al 2005, Aung et al 2006, Karlgren et al 2006), moreover, CYP2W1 has emerged as a tumour-specific P450 These findings are revised in detail in Section 1 4 5 2

CYP3A

Association of sequence polymorphisms with increased risk of tumorigenesis and/or poor chemotherapy outcome has recently shown the importance of CYP3A4 in cancer development However, the precise role of this enzyme in carcinogenesis and/or cancer treatment is difficult to establish, since reports are sometimes contradictory Epipodophyllotoxins are substrates of the CYP3A4 isoform, individuals carrying a CYP3A4-V genotype (a sequence polymorphism in the 5' region of the *cyp3a4* gene) have a decreased risk of developing leukaemia after treatment with these drugs (Felix *et al*, 1998) On the other hand, the same polymorphism was associated with higher TNM (Tumour-Node-Metastasis) stage in prostate cancer patients (Rebbeck *et al*, 1998)

The fact that CYP3A4 is involved in the metabolism of several anti-cancer drugs raises the possibility of its expression influencing chemotherapy outcome Taxotere has been found to induce CYP3A4 expression both in hepatocytes and in peripheral mononuclear cells (Nallani *et al*, 2001, Fujitaka *et al*, 2001), i e induce its own metabolism This could lead to reduced drug availability

Two studies suggest a prominent role for CYP3A4 in influencing cell sensitivity to anticancer drugs In the first one, Chinese hamster ovary cell lines were transfected with a CYP3A4 expression plasmid, which rendered them able to metabolise Vinblastine with a similar or higher efficiency than that of human liver microsomes 50 and 35% of transfected cells remained viable after being exposed to 100 nM of Vincristine or Vinblastine, respectively, whereas over 90% of the parental cells were killed in these conditions (Yao *et al*, 2000) According to the second study, pretreatment of Caco-2 cells with antisense phosphorodiamiate morpholino oligomers (PMO) targeted to inhibit CYP3A4 expression resulted in an inhibition of Cyclophosphamide cytotoxicity and an enhancement of Taxol toxicity (Arora *et al*, 2002)

P450R

P450R is an integral part of cytochromes P450-mediated reactions, as well as an independently acting enzyme able to metabolise several commonly used drugs Many of the substrates of P450R are involved in cancer treatment, such as Mitomycin C This drug is activated into a much more toxic form mainly by P450R (Bligh *et al*, 1990) and indeed, a correlation has been shown between P450R expression and Mitomycin C activity in bladder cancer (Gan *et al*, 2001) Other anticancer agents like Tirapazamine show a requirement for P450R activity in order to exert their toxic effects (Patterson *et al*, 1995, Patterson *et al*, 1997) The role of P450R in Adriamycin-induced toxicity remains controversial In this case, P450R plays a dual role, decreasing Adriamycin toxicity by reducing the anthracycline ring and causing the loss of the sugar moiety attached to it, but it also transforms the drug into the semiquinone form, producing an active DNA-alkylating agent and also generating toxic free radicals in the process

However, it should be noted that, as is the case with cytochromes P450, P450R can also activate toxic chemical compounds that are harmful to the cell and may even induce transformation Pro-carcinogenic compounds such as Nitrofurazone and dinitropyrenes present in diesel engine emissions are activated by P450R into DNA-damaging compounds, resulting in the promotion and/or progression of carcinogenesis (Hiraku *et al*, 2004, Murata *et al*, 2004) Moreover, DNA damage and genomic instability were reported in cells overexpressing P450R in the absence of any substrate or toxic agent, suggesting that the very presence of this enzyme might result in transformation even in the absence of environmental toxins (Heine *et al*, 2006)

1 4 5 P450 expression in normal and cancer tissue

Detoxifying enzymes are usually found in the liver, though not exclusively many different tissues express cytochromes P450, especially those in close contact with environmental agents These include the lungs and the gastrointestinal tract, but also the renal epithelium Transformed cells do keep some of their normal characteristics, and cytochrome expression can be readily detected in several different types of cancerous cells (Murray *et al* , 1993, Murray *et al* , 1995, Murray *et al* , 1997)

Several studies have been conducted to detect P450 mRNA and protein in normal and tumoral tissue, and also to establish whether the expression patterns are changed during malignant transformation Some of these studies report overexpression of one or more P450s, while others indicate that the chronic inflammation that accompanies most solid tumours down-regulates P450 expression Even though there are a considerable number of articles on the subject, there is not yet enough information about cytochrome expression in normal and tumoral tissue, and thus it is difficult to make conclusions about their relevance

1 4 5 1 Techniques used for the study of CYP expression

Several different techniques can be used for detection of P450 expression amongst them are biochemical assays, such as activity measurement, reverse transcription-polymerase chain reaction (RT-PCR), Western blotting and immunohistochemistry (Murray *et al* , 1995)

Of these, biochemical assays such as activity measurement are the only ones that can demonstrate enzyme functionality, but they require a fairly large size of sample, preserved in excellent conditions Western blotting offers the advantage of easy identification of tissue proteins by combining antibody detection with molecular weight determination, but also requires significant amounts of sample RT-PCR is a good method for screening of numerous samples as it can provide extremely specific information, since there is no antibody cross-reaction that can interfere, however, mRNA expression does not always correlate with protein expression, for it does not take post-transcriptional regulatory events into account

The main drawback of these methods is that heterogeneous samples, such as those obtained from biopsies, are difficult to analyse These techniques cannot show the protein or mRNA of interest in a particular cell type, and for tumours there is always the risk of contamination with normal adjacent tissue Immunohistochemistry is the

method of choice when dealing with small samples (such as needle biopsies), from tissues with low content of the protein of interest, or in which the target is in small proportion It also allows precise location to a particular cell type and even to certain subcellular structures The main disadvantages of this technique are the possibility of cross-reaction with other proteins and the inability of some antibodies to work in paraffin-embedded tissue samples, the commonest preservation method

1 4 5 2 P450 Expression in normal and cancer tissue

Enzyme-specific expression studies

Some expression studies have focused on particular cytochrome isoforms and studied their expression in hepatic and various extra-hepatic tissues A study of CYP1B1 expression was carried out on a large cohort of paired samples (cancer and normal tissue) of bladder, breast, colon, oesophagus, kidney, lung, ovary, skin, stomach, uterus, connective tissue, lymph node, brain and testis (Murray *et al*, 1997) No CYP1B1 protein was detected in normal tissue, while expression of this enzyme was very common in all types of tumours, with positive staining in all samples, or all but one, of each particular type These results prompted the authors to suggest that CYP1B1 could be a tumour-specific isoform Recent studies, however, contradict this theory indeed, CYP1B1 appears to be overexpressed by tumours, but its presence is detectable in normal tissue by *in situ* hybridisation and immunohistochemistry (Gibson *et al*, 2003, Muskhelishvili *et al*, 2001) These contradictory results could be explained by the use of different antibodies and/or primers, and by post-transcriptional and/or translational modifications of CYP1B1

Another study of CYP2C and CYP3A expression in several neoplastic and non-neoplastic tissues was carried out by immunohistochemistry (Yokose *et al*, 1999) They found that hepatocytes, epithelium of the small and large intestine, bile duct, nasal mucosa, kidney and adrenal cortex all expressed enzymes of both subfamilies Epithelium of salivary glands, oesophagus, stomach, small intestine, colon, rectum, nasopharynx mucosa, prostate and uterus, plus serous cells of the respiratory tract, hepatocytes, myeloid cells, neutrophils and basophils stained positively for CYP2C, while epithelium of stomach, small intestine, colon, rectum, pancreas and gallbladder, plus hepatocytes, chief cells of the parathyroid, proximal kidney tubules, plasma cells and the corpus luteum of the ovary all reacted with the CYP3A antibody The presence of CYP3A was detected in carcinomas of six different organs, including

colon and liver carrcinoma, while CYP2C was present in 7 types of carcinomas of different tissue origin, indicating that P450s are present in both normal and tumoral tissue

Expression of novel members of the CYP2 family has been evaluated in recent studies Little is known about CYP2S1, a recently discovered P450 (Rivera *et al*, 2002, Rylander *et al*, 2001) In spite of being classified as a member of the CYP2 family based on DNA sequence homology, it is induced by dioxin and able to metabolise aromatic hydrocarbons, which makes it more similar to members of the CYP1 family Expression of CYP2S1 was studied by both immunohistochemistry and *in situ* hybridisation, which showed comparable expression patterns, in a variety of human tissues (Saarikoski *et al*, 2005) Staining of this particular enzyme was strongest in skin, tissues associated with the respiratory tract (nasal cavity, bronchus and bronchioli) and areas of the gastrointestinal tract, namely stomach, duodenum, colon and exocrine pancreas Moderate staining was found in uterine cervix, oesophagus, small bowel, rectum, bladder and adrenal glands Testis, prostate, ovary, breast, liver, kidney, spleen, lymph nodes and thyroid all displayed weak staining for CYP2S1, while the enzyme was undetectable in endometrium, myometrium and heart tissue Overall, CYP2S1 appeared to be mainly restricted to epithelial cell types and also in organs of epithelial origin, such as exocrine glands Staining was also performed in tumour tissue, revealing strong expression of CYP2S1 in ovarian tumours and in squamous cell carcinoma of the uterine cervix, while expression in lung carcinoma was variable

CYP2J2 is also a novel member of the CYP2 family, involved in the synthesis of eicosanoids, derivatives of arachidonic acid involved in tissue homeostasis and carcinogenesis (Wu *et al*, 1996) Its expression appears to be widespread and has been detected at the mRNA and/or protein level in lung, liver, kidney, ileum, jejunum, colon, stomach, pancreas, pituitary gland and especially in the heart, where it appears to regulate vascular homeostasis (Capdevila *et al*, 2000, Fleming, 2001, Wu *et al*, 1996) A recent study (Jiang *et al*, 2005) showed that CYP2J2 and its products promote cell proliferation and inhibit apoptosis, which prompted the authors to analyse the expression of this enzyme in human tumour samples CYP2J2 expression was increased in tumour compared to adjacent normal tissues in all of those samples where the enzyme expression was detectable, both at mRNA and protein level Elevated expression was observed in all types of tumour tested, including oesophageal carcinoma and adenocarcinoma, pulmonary carcinoma and adenocarcinoma, colon adenocarcinoma, breast, stomach and liver carcinoma However, samples of hyperproliferative non-tumoral tissues displayed no detectable

CYP2J2 expression CYP2J2 therefore appears to be involved in the development of cancer at a late stage

A recent study has suggested that expression of CYP2W1 is strictly tumour-specific (Karlgren *et al*, 2006), in contrast with other P450 isoforms that appear to be overexpressed in cancer samples but are also expressed in normal tissue This P450 has been only recently discovered (Karlgren *et al*, 2005) and yet had been shown to be overexpressed in gastric cancer samples as compared to normal tissue by serial analysis of gene expression (SAGE) and quantitative RT-PCR (Aung *et al*, 2006) Indeed, CYP2W1 mRNA expression was detected in 54% of all human tumours examined, with highest prevalence in adrenal gland and especially in colon tumours (Karlgren *et al*, 2006) Expression of CYP2W1 in colon tumours was also detected by Western blotting This enzyme was also present in HepG2 hepatoma cells at mRNA and protein levels, but not in other cell lines, absence of expression was later found to be due to hypermethylation of the CYP2W1 promoter No immunoreactivity was detected in normal tissue samples at mRNA or protein levels, not even in liver microsomes, supporting the fact that this isoform is tumour-specific

A recent study analysed the expression of CYP2C19 mRNA and protein in a panel of tumour and normal adjacent normal tissue from liver, colon, stomach, breast, oesophagus, lung, uterus, brain, pancreas, ovary and kidney (Wu *et al*, 2006) CYP2C19 mRNA was only found in liver and hepatic carcinoma samples, with significantly higher levels present in tumour as compared to normal samples Western blotting confirmed that the same expression profile was observed at protein levels

Brain

Expression of P450R, CYP1A1 and CYP1B1, among others, was demonstrated in the central nervous system in early reports (Rieder *et al*, 1998, Strobel *et al*, 1995, Yun *et al*, 1998) In a small study comprising 10 brain tumour samples, expression of CYP2C9 was detected in all samples, while CYP3A5 was detected in only 3 out of 10 (Knupfer *et al*, 1999) No immunoreactivity was shown for CYP3A4 All samples also expressed IL-1 and IL-6 receptors, suggesting that the lack of expression of certain P450s might be due to downregulation by these cytokines

Breast

In an early report, expression of CYP1A and CYP3A in primary breast cancer was studied with antibodies that could not distinguish between different P450 isoforms (Murray *et al.*, 1993). It was found that 39 and 22% of tumours expressed CYP1A and CYP3A protein, respectively. In both cases, staining was only found in areas of invasive carcinoma, which poses an interesting question as to whether P450s could be involved in invasion and/or metastasis.

Another report analysed mRNA expression of CYP1, 2 and 3 families by RT-PCR in human breast tissue and tumours (Huang *et al.*, 1996). In this case, CYP1A1 mRNA was found in roughly half of all normal samples and tumours, but the most interesting finding of this experiment was that higher levels of the enzyme were found in normal tissue adjacent to the tumour. This suggests that tumours may actually be able to stimulate expression of P450s by normal tissue. These results were confirmed by a later study (El-Rayes *et al.*, 2003), which showed that CYP1A1, CYP2E1 and CYP3A4 expression was significantly lower in malignant tissue as compared to normal adjacent tissue samples, as detected by Western blotting. Expression of CYP2B6 was also evaluated in the same samples and appeared to be higher in oestrogen receptor positive tumours than in negative ones.

Expression of CYP1B1 mRNA in normal breast and tumour tissue was present in all but one sample; CYP2C was present in every sample, while CYP2D6 was present in almost all normal and tumoral tissues (Huang *et al.*, 1996). Members of the CYP3A family CYP3A4 and CYP3A5 were found more often in normal samples than in tumours, but their expression was not coincidental, suggesting differential regulation. In another report, the expression profile of all major CYP forms was analysed in breast tumour and surrounding normal tissue (Iscan *et al.*, 2001). The levels of CYP1A1 mRNA were very low in normal and tumour samples, while CYP1B1, CYP2B6, CYP2C, CYP2D6 and CYP2E1 were readily expressed in both types of tissue. CYP2A6, CYP2A7, CYP2A13, CYP2F1, CYP3A4, CYP3A5 and CYP3A7 could not be detected in normal or tumoral tissue.

These results are in contradiction with those of a later study (Kapucuoglu *et al.*, 2003a), which assessed CYP3A4 expression in 25 paired set of breast tumour and surrounding tumour-free tissue by immunohistochemistry. The authors showed that all of the breast tumours evaluated and 68% of normal samples were positive for CYP3A4, with the mean score values for tumour staining being significantly higher than those of non-tumour tissue. This discrepancy might be explained by the existence of CYP3A4 variants. Indeed, in another study CYP3A4 RT-PCR revealed

two variants of enzyme mRNA a 380-bp present in 4 out of 5 tumour samples, and a 260-bp associated with normal tissue (Miller *et al*, 2003) This suggests that differential transcriptional and/or post-transcriptional regulation of the enzyme in tumoral tissue might affect expression results

Expression of P450s may also vary with different ethnic groups, as shown by a recent study (Oyama *et al*, 2005) the authors did not found detectable CYP3A in any of the 34 breast cancer specimens belonging to Japanese patients, contrary to the approximately 20% present in samples from Caucasian women This study also showed very low CYP2A6 prevalence (only present in one sample), while CYP1B1 was present in 82% of samples, especially in those from earlier stages of disease The authors failed to find a significant correlation between p53 and P450 expression in their breast tumour samples

The physiological importance of CYP3A4 expression was highlighted when it was shown that expression levels of CYP3A4 mRNA in breast cancer tumour could be inversely correlated with response to Taxotere, measured as reduction of 50% or more of the original tumour size (Miyoshi *et al*, 2002) This study suggested that tumoral CYP3A4 mRNA levels could be used as a predictor of therapeutic outcome Supporting these results, measurable P450 activity in breast cancer tissue was demonstrated by Ifosfamide turnover in tumour microsomes (Schmidt *et al*, 2004) CYP3A4 and CYP2C9 were detected in all analysed breast tumour samples by western blotting, while CYP2B6 was undetectable Enzymatic assays showed that Ifosfamide was metabolised by tumour microsomes, thus suggesting that intratumoral P450 activity should be considered as a potential factor that can influence therapeutic efficiency Furthermore, activity of P450R, necessary for P450-mediated metabolism, was increased in breast tumour tissues as compared to normal adjacent tissues in a recent study performed in 50 patients (Kumaraguruparan *et al*, 2006) The highest activity levels were detected in lower stage tumours and in those tumours present in premenopausal women, suggesting that upregulation of this enzyme is related to hormonal status and that it is an early event in tumour development

Another study focused on the co-expression of CYP2C family members and IL-6 (Knupfer *et al*, 2004) CYP2C8 and CYP2C9 mRNAs were detected in all the analysed breast cancer samples, while none of the tumours expressed CYP2C18 or CYP2C19 The CYP2C9 protein was detected in 9 out of 10 tumours, albeit weakly The low levels of P450 expressed were explained by the concomitant presence of IL-6 and its receptor in all of the samples

CYP1B1 expression has been associated with increased risk of carcinogenesis after it was found to catalyse conversion of 17ß-estradiol to 4-hydroxy estradiol, which is a

genotoxic compound The levels of this metabolite are elevated in breast and uterine cancer, suggesting enhancement of CYP1B1 activity in these tissues, maybe as a result of the carcinogenic process (Liehr *et al*, 1995) It is known that signalling of CYP1A1 and CYP1B1 expression involves cross talk with the oestrogen receptor and depends on the receptor status of the cell line

Immunohistochemical detection of CYP2E1 was performed on 25 paired samples of infiltrating ductal carcinoma and surrounding normal tissue (Kapucuoglu *et al*, 2003b), revealing the presence of the enzyme in all tumours and in all but one of the normal tissue samples However, expression of CYP2E1 in tumours was found to be significantly higher than in non-tumour tissue

Low expression of CYP1A1, CYP1A2, CYP1B1 and CYP3A4/5 was also found using immunohistochemistry in a large study comprising primary breast carcinoma samples from 393 patients (Haas *et al*, 2006) However, expression of CYP2E1 was detected in 44% of samples Correlation between P450 expression and disease features was also evaluated CYP3A4/5 expression was found to be associated with positive nodal status, while a positive correlation was established between CYP1B1 expression and poor tumour differentiation

Colon and Small intestine

Identification of rifampicin-inducible CYP3A in biopsies from small bowel mucosa was reported in an early study (Kolars *et al*, 1992), the same authors reported presence of CYP3A4 and CYP3A5 at both protein and mRNA levels in a later study (Kolars *et al*, 1994) CYP3A4 was reported to be the major isoform present in small intestine as early as in 1987 (Watkins *et al*, 1987) This finding was later confirmed by the characterisation of the P450 enzymatic profile of human enterocytes (Zhang *et al*, 1999) Although CYP1A1, CYP1B1, CYP2C, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 mRNAs were present in all samples, only CYP2C and CYP3A4 protein could be detected by Western blotting CYP3A4 was found to be the major P450 form expressed in small intestine

CYP3A5 was later identified as the predominant CYP3A isoform expressed in colonic tissue and cell lines (Gervot *et al*, 1996), while CYP3A7 was weakly detected in only some of the samples CYP3A4 was not found in any of the cell lines tested nor in colonic tissue samples Expression of CYP3A5 and CYP3A7 was not induced by rifampicin or dexamethasone, while hepatic CYP3A induction was observed after treatment with these agents, indicating differential regulation of CYP3A family

members A recent report showed measurable CYP3A activity in human normal colorectal epithelium and cancer, albeit with high interindividual variability (Martinez *et al*, 2002) The tumoral enzyme characteristics, such as affinity and turnover, were similar to those of normal CYP3A enzyme found in colonic or hepatic tissue

Presence of CYP1A1 in human small bowel was analysed by measuring 7-ethoxyresorufin O-deethylase (EROD) activity in a bank of microsomal preparations (Paine *et al*, 1999) Only 6 out of 18 samples displayed measurable EROD activity, and immunoreactive CYP1A1 protein was detected by Western blot only in those samples with the highest metabolic rates, CYP3A4 activity and protein were readily detected in all samples The great interndividual variability of CYP1A1 expression could not be attributed to smoking habits or concomitantly administrated medication, however, information about patient dietary habits was not available to the researchers

Expression of CYP1B1 was found to be significantly higher in 61 human colorectal adenocarcinomas as compared to 14 normal human large bowel samples by immunohistochemistry performed with two different antibodies (Gibson *et al*, 2003) Correlation could not be established between CYP1B1 expression, tumour stage or degree or lymph node invasion Presence of CYP1B1 in normal tissue was demonstrated, albeit at low levels Although the enzyme was mainly detected in colon epithelia, blood vessels also showed immunoreactivity Interestingly, CYP1B1 was expressed at higher levels in tumour as compared to normal vasculature

Proteomics constitutes a novel approach for the simultaneous analysis of multiple proteins in a sample Advantages of this technique include unequivocal identification of protein spots by liquid chromatography and mass spectrometry Identification of several different P450s in colorectal tumours and their corresponding liver metastases was performed using proteomic techniques (Nisar *et al*, 2004), revealing the presence of fourteen different P450s from the subfamilies CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, CYP3A, CYP4A, CYP4F, CYP8B and CYP27A in 3 out of 6 samples (the other three samples were apparently too small to be suitable for this study) An interesting finding was that the same enzyme pattern was detected in the liver metastasis and the surrounding, tumour-free liver tissue, indicating that the tumour environment is able to regulate intratumoral expression of different P450s

Recently, expression of a number of different P450s was studied in 264 primary colorectal cancers, 91 lymph node metastases and 10 normal colorectal samples by immunohistochemistry using a panel of P450-specific antibodies (Kumarakulasingham *et al*, 2005) The aim of this study was to detect an enzyme that might act as a prognostic marker It was found that all of the P450s studied were expressed in both normal and colorectal cancers, with the exception of CYP2F1, CYP3A7 and CYP4Z1,

which were absent in normal tissue. These results contradict previous findings, but discrepancies could be attributed to the use of different antibodies. Staining for CYP1B1, CYP2S1, CYP2U1, CYP3A5 and CYP51 was significantly stronger in colorectal cancer as compared to normal samples; this was a novel finding for CYP2U1. Similarly, all P450s were detected in lymph node metastasis, with CYP2S1 and 3A4 being the most commonly upregulated P450s. Expression of CYP51 and CYP2S1 was found to be associated with poor prognosis, with CYP51 emerging as an independent prognostic marker. Interestingly, for most P450s there was no correlation between expression in the primary tumours and their corresponding metastases, once again indicating that the tumour environment significantly affects expression of these enzymes.

Expression of P450s at the mRNA level does not always correlate with findings in protein expression. Indeed, expression of CYP2C, CYP2E1, CYP3A4 and CYP3A5 was studied in colon mucosa of normal and adenomatous tissue, revealing that only CYP3A5 mRNA appeared to be upregulated in adenoma as compared to normal adjacent tissue (Bergheim *et al.*, 2005). However, no differences were found at the protein level in the same samples. Interestingly, protein levels of CYP2C8, CYP3A4 and CYP3A5, but not 2E1, were much lower in normal tissue adjacent to tumour than in normal mucosa from disease-free patients, suggesting that the presence of the tumour might influence expression of these three P450s.

Kidney

Expression of CYP3A4 and CYP3A5 was demonstrated in human kidney by immunohistochemistry and Western blotting (Schuetz *et al.*, 1992). Later, CYP3A5 was shown to be the most prominent CYP3A subfamily member present in this tissue (Haehner *et al.*, 1996).

Renal tumours are known to respond poorly to chemotherapy, for they display intrinsic resistance to anticancer agents. Several studies were carried out in order to find alterations in P450 expression that could explain this increased resistance. Two reports compared P450 expression in paired samples of renal cancer and corresponding normal tissue in primary tumours of different histological types and staging. Immunohistochemical analysis demonstrated CYP3A presence in the cytoplasm of all tumour cells, with no differences in staining patterns amongst the various histological types (Murray *et al.*, 1999). CYP3A was also detected in normal renal tissue, in proximal tubular epithelial cells and collecting duct epithelium. Western

blot analysis confirmed these findings RT-PCR showed expression of CYP3A5 and CYP3A7 mRNA in all samples, normal and tumoral, without noticeable difference between malignant and non-malignant CYP3A4, however, was found in 90% and 65% of normal and tumour samples, respectively Where normal samples did not express CYP3A4, mRNA of this isoform was detected in the paired tumour samples The findings of these authors show that renal tumours have a much higher frequency of CYP3A expression than many other types of cancer

A similar analysis was carried out for CYP1A2 and CYP1B1 (Cheung *et al*, 1999) While CYP1B1, AhR and ARNT mRNA were found in all samples with no apparent difference between normal and tumoral, CYP1A2 expression was only present in 19% of tumours and 22% of normal tissues But the interesting finding of this study was the differential expression of CYP1A2 when CYP1A2 was present in the normal sample, it was not present in the paired tumour, and vice versa This suggests a complex regulatory phenomenon Expression of AhR and ARNT does not appear to play a role in this type of regulation, since their expression levels do not accompany the changes observed in CYP1A2 levels

However, when EROD activity was measured in 11 samples of renal cell carcinoma paired with corresponding normal adjacent tissue, together with a further 15 tumour samples, it was found that 70% of tumour samples displayed *measurable* EROD activity (McFadyen *et al*, 2004) Activity was inhibited by the CYP1B1 inhibitor alpha-naphtoflavone, indicating that this isoform was responsible for the increase No such activity was detected in any of the normal samples This study also detected measurable P450R activity in all normal and tumour samples

Liver

Liver is of course the main P450-expressing tissue, since it is the major detoxificating organ in the body But malignant transformation appears to deeply affect the liver's ability to metabolise drugs, since it was shown that primary and secondary hepatic tumours have decreased expression of several P450s and also of other Phase I and II enzymes (Philip *et al*, 1994) However, a recent report showed increased expression of multiple P450s hepatocellular carcinoma samples, as compared to normal adjacent liver tissue (Furukawa *et al*, 2004) Expression of the same P450s in peripheral blood leukocytes did not correlate at all, except for CYP4B1, indicating that this particular enzyme might be used as a marker of induction of P450 expression in the liver

Two recent reports have highlighted the importance of CYP2E1 expression as a prognostic biomarker Firstly, a human liver arrayed library was hybridised with probes generated from the mRNA isolated from hepatocellular carcinoma and normal adjacent tissues (Man *et al*, 2004) this method revealed that CYP2E1 was only expressed in normal liver, but not in cancerous tissue and only weakly in cirrhosis samples It appears that CYP2E1 expression decreases with the progression of malignant disease Another study showed low levels of CYP2E1 at both protein and mRNA levels in hepatocellular carcinoma samples as compared to normal adjacent tissue (Ho *et al*, 2004) Low expression of CYP2E1 correlated with aggressive tumour phenotype (poor differentiation, absence of capsule and younger age of patients), and was an independent prognostic factor for disease-free survival Again, expression of CYP2E1 was found to decrease with progression of malignant disease

A recent screen in the human genome found that 2 5% of individuals showed gains between 7q21 1 and 7q22 1, the chromosomal location of CYP3A4 (Lamba *et al*, 2006), a search for amplification of CYP3A4 was then performed in normal human livers, primary and secondary human tumours, human hepatic cell lines and immortalised cell lines CYP3A4 copy number was found to be normal in all samples, except for one cell line, primary human hepatocellular carcinomal cell line TONG/HCC This cell line showed an approximately 10-fold increase in CYP3A4 mRNA expression and also in catalytic activity Increase in the number of genomic copies, although it does occur, does not appear to be a common mechanism of CYP3A4 upregulation

Lung

Normal lung tissue is known to express CYP3A5 and CYP3A7, amongst other enzymes involved in xenobiotic metabolism (Kivisto *et al*, 1995) This is not unexpected, since this tissue is exposed to environmental toxins and therefore needs the metabolic machinery to protect itself from damaging agents However, not all P450s are present in pulmonary cells, as the same authors could not detect expression of CYP2D6 in normal or tumoral lung samples (Kivisto *et al*, 1996) Meanwhile, expression of CYP3A was detected in both normal and malignant tissue in this small study comprising 32 patients

Another study focused in the analysis of CYP1B1 expression by RT-PCR and immunoblotting in human tumour and non-tumour tissue (Spivack *et al*, 2001) CYP1B1 protein was detected in all individuals, but more tumours were shown to

express the enzyme than normal samples, suggesting a role for CYP1B1 in malignant transformation of the lung No correlation could be found between CYP1B1 expression and gender, smoking history and/or tumour histology These results were confirmed more recently when expression of CYP1A1 and CYP1B1 was analysed by a novel qualitative specific RT-PCR and also by Western blotting on a set of paired tumour and normal tissue samples from 45 patients (Spivack *et al*, 2003) CYP1B1 was detected at both mRNA and protein levels in all female tumour samples and about 90% of male tumour samples, whereas expression in normal tissue ranged from 72 to 78% in both male and female samples, furthermore, expression of CYP1B1 was correlated with smoking status and estrogen receptor β expression Staining for CYP1A1 was infrequent and did not appear to be differentially regulated in tumours with respect to normal tissue samples

Low and infrequent expression of CYP1A1 in normal lung tissue could be explained by methylation status, indeed, complete or partial methylation of the CYP1A1 promoter was found in 98% of non-smokers, 71% of light smokers and 33% of heavy smokers involved in this study (Anttila *et al*, 2003) Quitting smoking resulted in methylation of the CYP1B1 promoter as early as after 1-7 days The lack of methylation found in heavy smokers was reflected in the higher levels of EROD activity found in pulmonary tissue

On the other hand, CYP1A1 expression was found to be in the same levels for smokers as ex-smokers in a small study (Kim *et al*, 2004) Indeed, quitting smoking did not appear to decrease expression of CYP1A1, while protein levels in non-smokers were significantly lower Expression of CYP1B1 was also low in non-smokers, slightly (albeit not significantly) increased in smokers and dramatically increased in ex-smokers, suggesting that induction of this P450 by tobacco smoking has lasting effects

In contrast with the previous results showing that methylation of the CYP1B1 promoter in peripheral white blood cells was unaffected by smoking (Anttila *et al*, 2003), another study showed that peripheral leukocytes from cancer patients displayed higher levels of CYP1A1 and CYP1B1 mRNA than leukocytes from non-cancer subjects (Wu *et al*, 2004), indicating that P450 expression in peripheral blood cells might be affected by the presence of malignancies, if not by smoking

Expression of P450s may also be dependent on tumour type, it was found that CYP1B1 expression was more common in adenocarcinomas than in squamous cell carcinomas of the lung, with over 50% of non-small cell lung carcinomas displaying positive immunohistochemical staining In contrast, only 3 out of 19 samples of normal lung expressed CYP1B1 (Lin *et al*, 2003)

A study performed on peripheral blood lymphocytes from 87 patients with non-small cell lung cancer found that CYP2E1 expression correlated with better survival, but only in those patients presenting the wild-type allele (Haque *et al*, 2004) This is consistent with the association of CYP2E1 with better prognosis in hepatocellular carcinoma

Oesophagus

A recent study demonstrated expression of CYP1A2, CYP3A4, CYP2E1 and CYP2C9/10 in normal oesophageal squamous mucosa, but not in cells within the basal proliferative zone (Hughes *et al*, 1999) This suggests that P450 expression is linked with the differentiated state of oesophageal cells Analysis of Barret's oesophageal metaplasia, considered an intermediate step in tumour development, showed prominent expression of the same proteins in the basal glandular regions, containing actively proliferating cells Furthermore, double staining showed co-localization of P450s and PCNA (proliferating cell nuclear antigen) expression in Barret's metaplasia, indicating that the presence of P450s is limited to proliferating cells, while the opposite is true for normal squamous epithelium RT-PCR studies confirmed the presence of P450 mRNA These results indicate an alteration in the localization pattern of P450s, but the significance of such alteration is still unclear

Ovary

Ovarian cancer usually has a poor prognosis due to the unpredictability of its response to chemotherapy In a major study involving samples from 167 patients, CYP1B1 protein was detected by immunohistochemistry in the vast majority of tumours (92%), most of them displaying moderate or strong staining (McFadyen *et al*, 2001a) Expression was found in all histological tumour subtypes and some correlation with tumour staging could be observed CYP1B1 was also detected in almost all of metastases analysed (94%), while expression in normal ovary tissue could not be demonstrated

In a recent study a novel splice variant of CYP1A1 that presents an 84bp deletion, named CYP1A1v, was detected in ovarian cancer samples (Leung *et al* 2005) This variant showed similar enzymatic activity when compared with CYP1A1, however, expression of CYP1A1v was not restricted to the endoplasmic reticulum, like that of

CYP1A1, but rather it was detected in the nucleus and in mitochondria of ovarian cancer cells Apparently, the deletion results in the creation of a translocation signal that alters intracellular distribution of CYP1A1 Expression of CYP1A1 in a series of 27 human ovarian cancers showed both nuclear and cytoplasmic staining in the majority of serous and mucinous carcinoma samples, while weaker staining was found in lower malignancy carcinomas Expression of CYP1A1 was absent in normal human ovary and in benign serous cystadenoma These results suggest that the presence of CYP1A1v, with its unique nuclear expression pattern, might be involved in the development of ovarian malignancies

Expression of 23 different P450s and also of P450R was evaluated in ovarian cancer using a panel of antibodies on an ovarian cancer tissue microarray consisting of 99 primary epithelial ovarian cancers, 22 peritoneal metastasis and 13 normal ovarian samples (Downie *et al*, 2005) Stromal cells in the normal ovary displayed weak expression of all P450s tested, with CYP2U1 and CYP3A4 being the most prominent Ovarian cancer samples also displayed positive staining for all P450s included in this study, and this staining was found to be higher and more frequent than in normal ovary for all isoenzymes excepting CYP1A1, CYP4F11, CYP24 and CYP39 Significant increases were detected for CYP1B1, CYP2A/2B, CYP2R1, CYP2U1, CYP3A5, CYP3A43, CYP4Z1, CYP26A1 and CYP51A1 Similar to the findings in normal ovary samples, the P450s with strongest and most frequent staining were CYP2U1 and CYP3A7 Samples obtained from metastatic sites displayed strong immunoreactivity for CYP2A/2B, CYP2S1 and CYP3A7, with CYP2S1 and P450R being the only enzymes significantly upregulated in samples of metastatic origin as compared to primary tumour samples From this analysis, strong staining for CYP2A/2B and no staining for CYPAZ1 were found to be significantly correlated with better survival, emerging as independent prognostic markers for ovarian cancers

Prostate

P450 subfamilies CYP1A, CYP2C and CYP3A were detected in prostate tumours in an early report (Murray *et al*, 1995b) More recently, a study has been conducted to analyse CYP1B1 expression in prostate carcinoma, premalignant prostatic intraepithelial neoplasia and other non-cancerous tissues (benign prostatic hypertrophy (BPH), metaplastic prostatic urothelium and hyperplastic prostatic urothelium) CYP1B1 was detected in 75% of prostate carcinomas, always located in the cytoplasm and absent from surrounding stromal tissue It was also present in all of

the non-cancerous premalignant samples, except for those of benign prostatic hyperplasia, where the enzyme was found in 82% of the samples CYP1B1 was not detected in normal prostate tissue, suggesting a link between its expression and malignant transformation (Carnell *et al*, 2004)

It should be taken into account that expression of P450s may depend on the different origin of samples within the prostate, it is known that prostate cancer arises mainly from the peripheral zone, while BPH is more common in the transitional zone CYP1B1 expression was found to be 2 to 6-fold higher in the peripheral zone as compared to the transition zone in twelve human prostate tissue sets, with staining in epithelial and stromal cells (Ragavan *et al*, 2004) Expression of CYP1B1 was extremely variable among individuals (up to 10-fold) This study also detected quantifiable CYP1A1 mRNA expression in 75% of samples, while CYP1A2 mRNA, although detectable, was unquantifiable

An explanation for the elevated expression of CYP1B1 may be found in increased transcription due to promoter hypomethylation, a recent report also found immunostaining for CYP1B1 to be significantly stronger in prostate cancer samples than in those coming from BPH patients (Tokizane *et al*, 2005) The authors also analysed 175 prostate cancer samples and 96 BPH samples for methylation using specific primers, they found that, while CpG sites were unmethylated in cancer samples, CpG sites, transcription factor Sp1 binding sites and dioxin responsive elements were all methylated in BPH samples, thus pointing to a probable mechanism for CYP1B1 increased expression

No detectable CYP1A1 expression was found in either normal and primary tumour prostate cells (Sterling *et al*, 2004), however, treatment with benzo[a]pyrene induced expression of this enzyme in both cell types In contrast, while CYP1A2 constitutive expression was detected in normal cells and was largely unaffected by benzo[a]pyrene treatment, CYP1A2 was absent from untreated tumour cells and yet showed significant induction after hydrocarbon treatment These data suggest differential regulation of CYP1A2 after malignant transformation

Stomach

Stomach cancer is one of the commonest tumours of the gastrointestinal tract, and it has a limited response to chemotherapy It is thought that environmental factors play a relevant role in tumour development and since the stomach is in contact with a

number of toxic substances of dietary origin, P450s are likely to be induced in this type of tumours

Indeed, this has been shown by an immunohistochemical study where a total of 39 tumours were analysed, all of them primary adenocarcinomas with different TNM (tumour, nodes, metastasis) staging CYP1A and CYP3A were found in 51% and 28% of tumours respectively, while expression of CYP2E1 could not be detected in any of the samples (Murray *et al*, 1998) Staining was always present in the cytoplasm of tumour cells, and there was no correlation with histological type or staging On the other hand, no P450 expression could be demonstrated in normal tissue, although CYP3A was present in mast cells within the stomach walls Samples of intestinal metaplasia, an intermediate stage between normal and malignant transformation, were also analysed to gain insight into the changes undergone by cells during the transformation process These samples also displayed CYP3A expression, suggesting that P450 induction accompanies tumorigenesis Another study (Yokose *et al*, 1998) also demonstrated CYP3A4 expression in the foveolar epithelium of the human stomach with intestinal metaplasia by immunohistochemistry, Western blotting and RT-PCR This enzyme was not detected in other sections of the stomach or in foveolar epithelium without intestinal metaplasia, which again suggests a role for CYP3A4 in stomach carcinogenesis

A higher proportion of samples expressing CYP1A1 was recently described this enzyme was detected by immunohistochemistry in 86% of gastric cancers, 57% of atrophic gastritis and intestinal metaplasia and in 7 7% of non-cancerous mucosa in 43 gastric cancer cases (Zhang *et al*, 2004), results were confirmed by RT-PCR and Western blot Moreover, EROD activity was also increased in cancerous or premalignant tissues as compared to normal tissue ones These results support the notion of a role for CYP1A1 in carcinogenesis

Urinary bladder

This is a very common malignant tumour and it is usually recurrent, there is scant information about its response to anticancer drugs

An early study performed on 25 samples of tumoral tissue showed CYP1A, CYP2C and CYP3A immunoreactivity in 68%, 28% and 68% of tumours, respectively (Murray *et al*, 1995a) No P450 expression was detected in normal bladder, but only 5 samples were available for analysis The staining was again uniformly cytoplasmic, and a correlation was established between CYP1A expression and tumour grade

A more recent report (Carnell *et al*, 2004) detected CYP1B1 expression in all bladder carcinoma samples tested (n=22) Protein expression was heterogeneous and cytoplasmic, present exclusively in tumour cells P450R was also evaluated in tumour specimens from 92 bladder cancer patients and correlated with response to Mitomycin C, a bioreductive drug know to be activated by this enzyme (Basu *et al*, 2004), most tumours presented moderate to high immunoreactivity, but this did not correlate with drug response, suggesting that other factors are more relevant for chemotherapy outcome

Other tissues

CYP3A4/5 expression in osteosarcomas, the most common paediatric primary bone tumours, was shown to be significantly higher in patients with more aggressive cancers, and was associated with development of distant metastases and poor prognosis (Dhaini *et al*, 2003) These particular isoforms could therefore be used as biomarkers to predict clinical outcome

Expression of CYP1A1, CYP1A2, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1 and CYP3A4 was analysed in a group of pancreatic samples, including normal tissue, chronic pancreatitis and pancreatic cancer specimens (Standop *et al*, 2003) Immunoreactivity for all P450s except CYP1A2 was detected in cancer samples at a higher frequency than in normal tissue, while a similar pattern was observed for the pancreatitis samples, suggesting a link between inflammation and P450 induction

Enzymatic activity is detectable in primary cultures from human head and neck squamous epithelium (Farin *et al*, 1995) A recent study analysed expression of CYP1A1, CYP3A4 and CYP2E1 in samples from squamous cell cancer of the head and neck (SCCHN) and compared the results to those obtained from paediatric tonsillitis samples (Ali *et al*, 2004), immunohistochemistry revealed increased expression of CYP1A1 and CYP3A4 in cancer samples compared to samples from patients with tonsillitis, while no difference in CYP2E1 expression was detected However, expression of CYP1A1 and CYP3A4 was much lower in the tumour tissue than in adjacent normal tissue Interestingly, expression of CYP1A1 and CYP3A4 was significantly correlated, even though these two enzymes have very different induction pathways

Tissue type	Upregulated P450s	Dowregulated P450s
Breast	CYP1B1	CYP1A1
	CYP2E1	CYP1A2
	CYP3A4	CYP1B1
	CYP3A5	CYP2E1
	P450R	CYP3A4
		CYP3A5
Colon	CYP1B1	
	CYP2S1	
	CYP2U1	
	CYP3A5	
Kidney	CYP1B1	CYP3A4
Liver	CYP2C19	CYP2E1
Lung	CYP1B1	
Ovary	CYP1A1v	CYP1A1
	CYP1B1	
	CYP2A/2B	
	CYP2R1	
	CYP2U1	
	CYP3A5	
	CYP3A7	
Prostate	CYP1B1	
Stomach	CYP1A1	CYP2E1
	CYP3A4	
Bladder	CYP1A	
	CYP2C	
	CYP3A	
Head and neck	CYP1A1	
	CYP3A4	
General	CYP1B1	
	CYP2J2	
	CYP2W1	

Table 1 4 3 Summary of reported changes in P450 expression in tumour tissue as compared to normal tissue

1.5 Glutathione

1 5 1 Role of Glutathione

All aerobic organisms need oxygen to obtain maximal amounts of energy from metabolic reactions, but the use of oxygen generates dangerous by-products and highly reactive intermediates, such as hydrogen peroxide, superoxide and free radicals (Anderson *et al*, 1980) All these can induce serious damage to the cell, usually by oxidation of lipids in the cell membranes

On the other hand, low levels of reactive oxygen species (ROS) can actually stimulate cell growth, although the mechanism by which they can do this is still unknown, S-thiolation of growth regulatory proteins by oxidated glutathione (GSSG), or direct chemical modifications of reactive cysteines have been suggested as possible intermediate steps It appears that the way a cell responds to ROS - whether it stimulates proliferation, stress response and damage repair, or apoptosis - depends on the nature and extent of the damage received (Tew *et al*, 1999)

Glutathione (GSH) is the major soluble antioxidant present in mammalian cells and as such, it is usually the first line of a defensive antioxidant system developed in aerobic organisms throughout evolution But GSH plays more roles in cell physiology than that of a simple antioxidant it can be attached (conjugated) to endogenous compounds or xenobiotics as means of detoxifying them, it can mediate signalling pathways that modulate cell proliferation, differentiation and apoptosis and ultimately can be one of the multiple factors involved in the development of resistance to chemotherapy

1 5 2 Biochemistry

GSH is a tripeptide produced naturally in the organism, formed by the amino acids glutamic acid, cysteine and glycine It is the most abundant non-protein molecule in the cell, with a concentration of 1-10 mM in mammalian cells (Balendiran *et al*, 2004) GSH concentrations can be increased by exogenous administration of precursors (glutamine and/or cysteine), glutathione esters or glutathione itself It should be taken into account that not only is the absolute concentration of GSH important to cell physiology, but also the capacity to synthesise it resistance to Adriamycin depends on the ability of the cell to resynthesise GSH rather than on a steadily increased

concentration (Vallis *et al*, 1997, Tipnis *et al*, 1999) Regulation of GSH levels is usually cell type-specific (McLellan *et al*, 1999)

GSH is synthesised intracellularly in two sequential steps (Fig 1 5 1) First, the γ-glutamyl cysteine synthetase (GCS) enzyme catalyses the formation of the dipeptide γ-glutamyl cysteine in an ATP-consuming step, this is the rate-limiting step for the *de novo* synthesis of GSH Secondly, glycine is incorporated to the dipeptide by the GSH synthetase enzyme in another ATP-dependent reaction

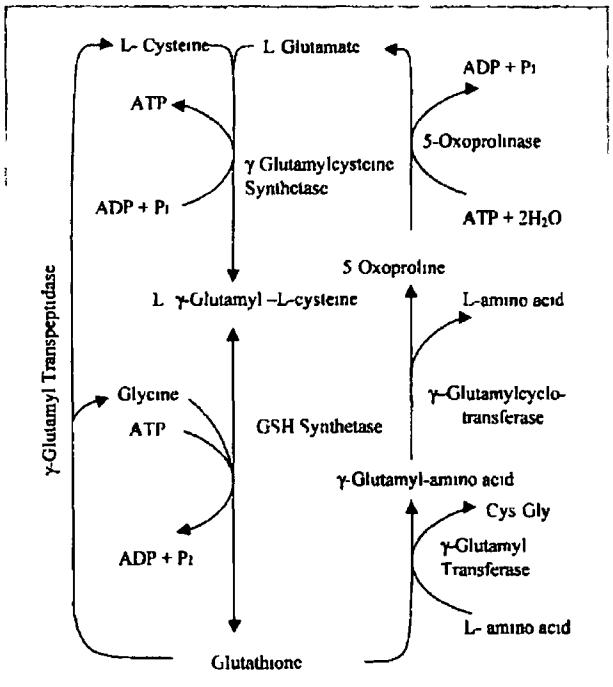


Figure 1 5 1 Glutathione metabolism (from Balendiran *et al*, 2004)

GCS is a heterodimer composed of heavy (GCSH) and light (GCSL) subunits coded by separate genes The heavy subunit contains the active site which catalyses the synthesis of γ-glutamyl cysteine and is feedback-inhibited by GSH, the light subunit appears to exert a regulatory function (Rahman *et al*, 2000)

γ-glutamyl transpeptidase (GGTP) is a membrane-bound enzyme prevalent in cells that are specialised in absorption and excretion, such as epithelial cells It is the only enzyme able to break down the γ-glutamyl linkage and liberate cysteinyl glycine, this peptide is subsequently cleaved by dipeptidase, releasing the free amino acids into the extracellular space The extracellular concentration of GSH is very low, but it is believed that it can still protect cell membranes from oxidative damage It is known that GSH can be transported from the liver, usually to the kidneys, which have a high transpeptidase activity

While GGTP transfers the γ-glutamyl residue to free amino acids, γ-glutamyl cyclotransferase converts the residue into 5-oxo-L-proline, releasing the acceptor amino acid 5-oxo-L-prolinase (5-OPase)-mediated catalysis then converts 5-oxo-L-proline into L-glutamate, which can act as a GSH substrate and start the cycle again (McLellan *et al*, 1999)

GSH is a highly reactive molecule and it can donate its γ-glutamyl radical to form amino acid derivatives or react via its sulphydryl moiety (Fig 1 5 2) Under normal conditions, the majority of GSH is in reduced form, it can react directly with free radicals, or indirectly through antioxidant enzymes to render the oxidized form, GSSG Although direct reaction with free radicals exists, GSH usually acts as a cofactor for enzymes such as Se-dependent or independent GSH peroxidases (GPxs) and phospholipid hydroperoxide GSH peroxidases Also, GSSG is formed as a by-product of nucleotide synthesis

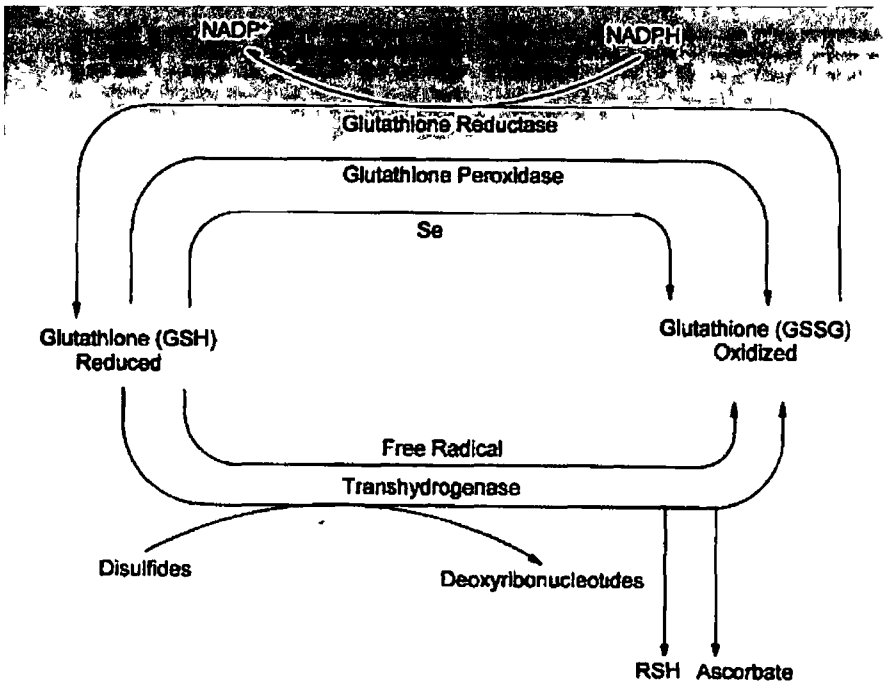


Figure 1 5 2 Glutathione catabolism (from Balendiran *et al*, 2004)

GSSG can be reduced back to GSH by NADPH-dependent glutathione reductase, which is a highly efficient enzyme, keeping extremely low GSSG intracellular concentrations under normal conditions (Kosower *et al*, 1978) The GSSG/GSH ratio is an indicator of cellular redox state (Schafer *et al*, 2001)

Protein and non-protein sulphydryl radicals can be conjugated with GSH, and this molecule can also be found in the form of GSH thioesters Xenobiotics can also be conjugated with GSH as means of detoxification this function is carried out by GSH transferases (GSTs) Conjugates are later exported from the cell by ATP-dependent transport

1 5 3 Regulation of expression

The transcription factor Nrf2 appears to mediate constitutive and inducible expression of GSH transferases, enzymes present in the GSH biosynthetic pathway and other enzymes able to mediate chemotherapy resistance, such as quinone reductase (Itoh *et al*, 1997 and 1999) Indeed, Nrf2 knock out mice displayed reduced expression of GSTs α, μ and π, and also of GCSH, however, GSH synthetase levels were not affected (Chanas *et al*, 2002)

Nrf2, together with small Maf transcription factors, binds to antioxidant responsive elements (ARE) in the DNA, which share a remarkable similarity to those sites that are AP-1 responsive Oxidative stimuli, such as UV irradiation, GSH depletion, or exposure to cisplatin, cause Nrf2 to dissociate from its inhibitor Keap 1 and migrate to the nucleus to initiate transcription AP-1 can be activated through the SAP kinases by the same stimuli Response to stress can be inhibited by treatment with antioxidants

Nrf2 is not the only stress sensor in the cytoplasm, certain proteins homologous to members of the GST P family are able to migrate to the nucleus in response to oxidative stress The NF-κB pathway is also activated by stressful conditions and inhibited by antioxidants (McLellan *et al*, 1999) More recently, BRCA1 was also shown to stimulate transcription of antioxidant responsive elements, inducing the expression of several redox state related genes including GSTs and oxidoreductases (Bae *et al*, 2004)

1 5 4 Functions

GSH is the major antioxidant present in the cell and it is essential for maintaining the intracellular redox balance It is also involved in detoxification and in the immune response

Antioxidation GSH is the major soluble antioxidant present in mammalian cells it can protect cells by directly scavenging free radicals or as a cofactor of antioxidant enzymes GSH peroxidase and phospholipid hydroperoxide GSH peroxidase can convert oxidised lipids back into harmless fatty acids

The redox state of a particular cell can affect a number of signalling pathways controlling proliferation, differentiation and morphogenesis Among the signalling molecules activated by the redox state – either by GSH or by free radicals – are AP-1, AP-2, c-jun kinase (JNK), stress-activated protein kinase (SAPK), protein kinase C (PKC) and tyrosine kinases (Janssen *et al* , 1993, Hayes *et al* , 1999) A decrease in the level of GSH causes activation of NF-κB, and this activation is inhibited in the presence of other antioxidants (Fernandez *et al* , 1999)

Detoxification conjugation with GSH is the major Phase II reaction in mammalians, particularly for electrophilic substrates like epoxides, alkenes, halides and heavy metals (Kaplowitz *et al* , 1985) High levels of GSH can be found in the liver, kidney and lungs, tissues that are particularly exposed to exogenous toxins

GSH conjugates are then excreted by an ATP-dependent pump, which explains the synergy observed between GSTs and MRPs in several studies (see below)

Immune system modulation there is evidence that the intracellular concentration of GSH modulates T-cell functions, such as cytotoxicity, activation, proliferation and differentiation (Balendiran *et al* , 2004)

Prooxidation it is known that reduction of certain metal ions by GSH can lead to the formation of superoxide anions, which can in turn oxidise a number of different molecules This could constitute a novel signalling mechanism (Pompella *et al* , 2003, Paolicchi *et al* , 2002)

1 5 5 Glutathione and proliferation

Recent studies suggest a link between redox state/GSTs and stress activated kinases such as JNK and apoptosis signalling kinase (ASK) These enzymes are kept in an inactive mode in normal, non-stressful conditions GST P1, the most abundant isoform of GSTs in non-hepatic tissues, has been recently described as a JNK inhibitor (Adler *et al* , 1999), apparently, stress causes GST P1 to dissociate from JNK, which is then activated by phosphorylation (and subsequent phosphorylation and binding of c-jun)

and is able to initiate transcription of several stress-response related genes This interaction has been further confirmed by studies involving transgenic mice null for GST P1 high levels of JNK activity in basal conditions were detected in the transgenic tissues (Henderson *et al*, 1998) Mouse embryo fibroblasts from GST P1 null mice also have a much shorter doubling time than their wild-type counterparts Furthermore, the specific GST P inhibitor TER199 was able to activate JNK in human HL60 cells (Ruscoe *et al*, 2001) The inhibitor TER199 has also been reported to act as a myeloproliferative agent (Kauvar *et al*, 1998)

It is generally believed that the role of GSTs in drug resistance depends on their conjugating activity, namely, that their primary function is to inactivate the drug by conjugation with GSH followed by export out of the cell However, a study performed on Adriamycin resistant MCF-7 cells failed to detect any form of GSH-Adriamycin conjugates An interesting finding of the study was that Adriamycin tended to localise in the Golgi apparatus of resistant cells, while it was usually detected in the nucleus of sensitive cells (Gaudiano *et al*, 2000)

These findings suggest that there might be a function for GSTs beyond that of simple detoxification enzymes, and this is of particular importance for cancer therapeutics, since the GST P family has been the one most often linked to malignancy and to increased resistance to anticancer agents (Tew *et al*, 1994, Lee, *et al*, 1994) Supporting this theory, overexpression of GSTs is found even in cells resistant to compounds that are not detoxified by these enzymes Moreover, another family of redox state-dependent proteins – thioredoxins – has been linked to regulation of cell proliferation and sensitivity to chemotherapy (Tew *et al*, 1999), further supporting the connection that seems to exist between these two events

But GSH could also have a direct role in signalling pathways, regardless of GSTs indeed, it has been shown to act as a negative modulator of protein kinase C (PKC) (Ward *et al*, 1998) p53 function might also be redox sensitive, as shown by *in vitro* studies (Rainwater *et al*, 1995, Liu *et al*, 1998)

1 5 6 Glutathione and apoptosis

GSH depletion occurs in cells that are undergoing apoptosis, for example, those that have been exposed to an anti-Fas antibody (van den Dobbelsteen *et al*, 1996) In this case, apoptosis can be delayed by treating the cells with glutathione precursors or GSH itself Another way of preventing apoptosis is by overexpression of GSH peroxidase (GPx) T47D breast cancer cells transfected with GPx1 were resistant to apoptosis triggered by an anti-Fas antibody Resistance was developed without a

concomitant increase in the expression of antiapoptotic proteins, and could be reverted by depletion of GSH Similar events were observed *in vivo*, with transgenic mice overexpressing GPx1 displaying resistance to anti-Fas induced apoptosis (Gouaze *et al*, 2002)

A similar scenario is also found for p53-dependent apoptosis, it has been shown that oxidative damage is a consequence of p53 activation and treatment with antioxidant compounds can prevent or revert this damage and inhibit the subsequent apoptotic process (Johnson *et al*, 1996) The p53 response is complex and still not totally understood, but an important subset of genes activated by this protein is related to the maintenance of redox balance within the cell, GSTs and GPxs are some of the enzymes induced by p53 (Polyak *et al*, 1997)

Increased levels of GSH were detected after upregulation of Bcl-2 expression (Mirkovic *et al*, 1997, Wright *et al*, 1998) Bcl-2 overexpressing cells are resistant to apoptosis, but their sensitivity can be restored by GSH depletion, indicating that glutathione plays a pivotal role in resistance It has also been shown that upregulation of this protein affects compartmentalisation of GSH, since it can actually stimulate its transport to the nucleus (Voehringer *et al*, 1998) The presence of GSH in the nuclear compartment can inhibit activation of the serine protease AP24, ultimately responsible for DNA fragmentation, thus preventing the nuclear alterations characteristic of apoptosis (Wright *et al*, 1998), exogenous addition of GSH to cells caused reversible AP24 inhibition Similar, though not as potent, inhibition was displayed by other reducing agents such as N-acetyl cysteine

As stated in the previous section, GST P1 can act as a JNK inhibitor, since JNK is a pro-apoptotic signalling molecule, increased expression of GST P1 could constitute another mechanism of resistance to apoptosis

It seems therefore that increased levels of GSH and GSH-related enzymes could contribute to chemotherapy resistance not only by inactivating anticancer drugs or reverting their oxidative effects, but also by making tumour cells resistant to apoptosis

1 5 7 Glutathione, cancer and chemotherapy resistance

The role of GSH in cancer is a dual one, which has not been totally clarified on the one hand, it protects cells from oxidative agents, which can cause DNA damage and induce carcinogenesis On the other hand, when present in high levels in tumour cells, it can render them resistant to chemotherapy and radiotherapy

There is evidence that GSH levels are elevated in tumours found in bone marrow, breast, colon, larynx and lungs Increased expression of GCS has also been found in colon, lung, breast and liver among other types of cancer (Balendiran *et al*, 2004) A study performed in tumours and paired normal tissue from 41 breast cancer patients found that reduced and total GSH contents were dramatically increased in tumoral tissue, while several enzymes involved in GSH metabolism displayed elevated activity (Perquin *et al*, 2000) Ovarian tumoral biopsy samples from patients who had developed resistance to alkylating agents after treatment presented GSH levels 10-fold higher than those found in the samples before treatment (Britten *et al*, 1992) Depletion of these levels markedly increases tumour sensitivity to anticancer agents

1 5 7 1 Studies in cell lines

Several studies have been carried out to measure differences in GSH metabolism between resistant cell lines and their sensitive counterparts High intracellular levels of GSH have long been known to correlate with resistance to Cyclophosphamide, Melphalan, Mechlorethamine, Nitrosourea, quinone-containing drugs and sulphydryl-reactive chemotherapeutic drugs Increased levels of GSH and GSTs were found in K562 cells that had developed resistance to Adriamycin (Kalinina *et al*, 2001) Similarly, increased levels of GSH were detected in squamous lung carcinoma cell lines after combination chemotherapy treatment (Kawai *et al*, 2002)

It has been demonstrated that by reducing intracellular GSH levels cells become more sensitive to anticancer agents (Chen *et al*, 1998) The most commonly used inhibitor of GSH synthesis is buthionine sulfoximine (BSO) Early studies on cell lines using this compound showed changes in the response to chemotherapy after coadministration with BSO No effects were observed with a combination of anticancer drugs and oxothiazolidine-4-carboxylate (OTZ), a chemical that stimulates GSH synthesis in normal cells (Russo *et al*, 1986) However, treatment of MCF-7 cells with OTZ actually decreased GSH levels and rendered the cells more sensitive

to treatment with Melphalan (Chen *et al*, 1998), indicating a differential regulation of GSH levels in normal and transformed cells

Treatment with BSO was shown to revert resistance developed by ovarian cancer cells to cyanomorpholino adriamycin, an Adriamycin metabolite (Sheehan *et al*, 2000), and also that of neuroblastoma cells to Melphalan (Anderson *et al*, 2000) The most exciting fact about this line of treatment is that it appears that the enhancement in cytotoxicity observed after GSH depletion affects only cancerous cells, with no additional toxicity for normal cells (Chen *et al*, 1998)

It should be noted that the measure of steady-state levels of enzymes or substrates does not always give an accurate picture of GSH involvement in resistance As an example, Gamcsik *et al* (2002) found that both Vincristine-resistant and Adriamycin-resistant MCF-7 cells had lower steady-state levels of GSH than parental cells, while 4-hydroperoxycyclophosphamide (4-HCP) resistant MCF-7 cells had increased GSH metabolism and steady-state levels When the GSH metabolic rate was analysed in the Vincristine and Adriamycin-resistant cell lines, it was found to be higher than that of parental cells and similar to that of 4-HCP resistant cells

As for the enzymes involved in GSH turnover, enhanced GCS activity has been found in many resistant cell lines, while the role of GSH synthetase in the development of resistance remains to be determined (McLellan *et al*, 1999)

Studies performed on GCS have usually been focused on the catalytic (heavy) subunit (GCSH), expression of this protein seems to be upregulated in Cisplatin- and/or Melphalan-resistant tumour cell lines, usually correlating with increased GSH levels An additional feature of Cisplatin-resistant cell lines is that a concomitant increase in MRP levels is usually observed This could be explained by a joint regulating mechanism, as suggested by the studies of Iida *et al* (2001) A hammerhead ribozyme against GCSH was developed and transfected into Cisplatin resistant colon cancer cells not only did transfection decrease GSH levels and increase sensitivity to Cisplatin, but it also suppressed expression of MRP1, MRP2 and MDR1, indicating that drug resistance related proteins are co-ordinately expressed (MDR1 is not usually found upregulated concomitantly with GSH turnover enzymes, it has actually been reported to be downregulated by ROS (Wartenberg *et al*, 2001)) This theory is further supported by the positive correlation in the expression of GST P1 and MRP found by Yang *et al* (2000) in bladder carcinoma Not only coordinated expression, but also functional synergy between these two proteins has been reported melanoma cells transfected with GST P1 displayed an increased resistance to Vincristine, which was abolished by treating the cells with the MRP1 inhibitors Sulfinpyrazone and Verapamil (Depeille *et al*, 2004) A similar effect

was observed when treating GST P1-transfected HepG2 cells – rich in MRP2 – with the MRP inhibitors Sulfinpyrazone or Cyclosporin A (Morrow *et al*, 2000)

Little is known about the role of the regulatory (light) subunit (GCSL), though it has been shown to be co-ordinately upregulated with its heavy counterpart Whether increased expression of both subunits is needed for chemotherapy resistance remains unknown (McLellan *et al*, 1999) Transfection of GCSL in HeLa cells resulted in an increase in GSH synthesis and conferred a modest resistance to Adriamycin, but not to Melphalan or Cisplatin (Tipnis *et al*, 1999), suggesting that GCSL may play a secondary, drug-specific role in chemotherapy resistance

Involvement of GSH peroxidase in anticancer drug resistance has been reported several times (Black *et al*, 1991, Kramer *et al*, 1988) There are at least four different isoforms of Selenium-dependent peroxidases, of which GPx1 is the most widely studied Transfection of this enzyme into a breast cancer cell line resulted in increased resistance to hydrogen peroxide (H_2O_2) and the redox cycling drug Menadione (Mirault *et al*, 1991)

Reduction of peroxides or peroxidation products by GSH generates high levels of GSSG, which can then be transported out of the cell by members of the MRP family, or reduced to GSH by the NADPH-dependent GSH reductase Slight increases in the expression of this enzyme have been observed in Cisplatin and quinone resistant cell lines (Black *et al*, 1991, Hosking *et al*, 1990, Colinas *et al*, 1996) Inhibition of GSH reductase activity by *N,N*-bis (2-chloroethyl)-*N*-nitrosourea resulted in decreased GSH levels, reduced MRP activity and overall increase in Adriamycin toxicity (Vanhoefer *et al*, 1997)

The GGTP-mediated breakdown of glutathione serves as transport of GSH constituents between cells, and increased enzymatic activity could result in higher levels of circulating substrates for GSH synthesis, and ultimately increased GSH levels Selected cell lines display an increase in GGTP activity that correlates with elevated intracellular GSH concentration Also, increased levels of GGTP activity have been measured in an ovarian cell line resistant to Cisplatin, Chlorambucil and 5-FU (Black *et al*, 1991) Contradictory reports have arisen after transfection of prostate cells with GGTP Bailey *et al* (1994) found no change in intracellular GSH levels or in sensitivity to Melphalan, Cisplatin or Adriamycin in transfected cells However, a more recent study (Hanigan *et al*, 1999) performed on a different prostate cell line reported increased resistance to Cisplatin after transfection

Much as with GGTP, increased activity of 5-oxo-L-prolinase (5-OPase) can result in higher levels of GSH precursors and augmented synthesis However, the importance of 5-OPase for cancer treatment lies in its ability to release free cysteine from the

prodrug L-2-oxothiazolidine-4-carboxylate (OTC), the effect of this drug is to increase intracellular GSH levels of cells subjected to oxidative stress The interesting fact is that this increase only occurs in normal cells, whereas tumour cells see their GSH levels depleted An explanation for this selective effect has been provided by the study of 5-OPase expression in normal and tumoral tissue by Western blotting and immunohistochemistry enzyme levels were found to be considerably lower in tumours than in paired normal tissue (Chen *et al* , 1998)

1 5 7 2 Role of GSTs

GST polymorphisms have been correlated with an increased susceptibility to lung, bladder, gastric, colorectal, skin, breast, kidney and liver cancers (Balendiran *et al* , 2004), suggesting a causative and/or associative relationship between GST activity and carcinogenesis

Results obtained in polymorphism-carrying patients depend on the particular isoform and type of cancer Low or null expression of GST M1 and 3 is linked with an increased risk of bladder and lung cancer (Cartwright *et al* , 1982, Nakajima *et al* , 1995, Inskip *et al* , 1995) Similarly, higher risk of tumour development has been found in patients with low GST P activity in gastrointestinal mucosa (Peters *et al* , 1990) However, no association was found between GST P1 polymorphisms and lung cancer (Harris *et al* , 1998) Lack of expression of GST T1 was found to correlate with a higher incidence of basal cell carcinomas (Strange *et al* , 2001), and also with bladder, gastro-intestinal tract and smoking-related tumours (Guengerich *et al* , 1995) On the other hand, individuals expressing normal GST T1 were found to be at a higher risk of developing kidney and liver tumours (Landi *et al* , 2000)

It is usually not just the presence of a single polymorphism, but rather the combination of several ones, which gives a more accurate picture of the risk a certain person is at, therefore, it is not unusual to obtain contradictory results about the association of a particular polymorphism with the development of disease

Involvement of GSH and GSTs in the development of resistance to chemotherapy is highlighted by the fact that increased levels of GSH and/or GST expression, together with increased excretion of GSH conjugates, correlates with a decreased sensitivity to a number of anticancer agents This can arise as a result of direct conjugation of drugs to GSH, or by antioxidant prevention of damage caused by ROS, the latter mechanism is of particular importance against alkylating agents such as Cisplatin and also redox cycling drugs such as Adriamycin (McLellan *et al* , 1999)

Enhanced conjugation of anticancer drugs to GSH has been reported in numerous occasions and in different cancerous tissues (Dirven *et al*, 1994, Hayes *et al*, 1995, Cnubben *et al*, 1998), while a negative correlation between expression of GSTs and patient survival has been suggested GST expression is commonly upregulated in drug-resistant cell lines, cancerous and pre-cancerous tissue, with GST P1 being the isoform most often found overexpressed in human tumours such as lung (Mattern *et al*, 2002) and bladder (Yang *et al*, 2000) Moreover, the presence of GST P1 can act as a resistant marker positive staining for GST P1 in breast cancer tumour samples was correlated with resistance to chemotherapy in a study performed on 42 female patients (Su *et al*, 2003) Association of increased GST P1 expression and resistance to anticancer drugs – Cisplatin, in this case – was also found in head and neck carcinoma cell lines and tumours (Cullen *et al*, 2003) Indeed, deletion of GST P1 in mice dramatically enhanced tumour sensitivity to redox cycling drugs like Adriamycin (Henderson *et al*, 2005)

Contrasting with these findings, expression of GST – and particularly of GST P1 - was measured in a panel of 12 human glioma cell lines unexpectedly, it was found that higher enzyme levels did not translate into resistance to several anticancer agents Furthermore, increased expression of GST P1 was correlated with enhanced sensitivity to Vincristine (Winter *et al*, 2000)

In a different approach, a vector containing antisense GST P1 was transfected into a human cholangiocarcinoma cell line, resulting in sensitisation to Adriamycin, Cisplatin and Melphalan (Nakajima *et al*, 2003) Combination assays were performed in the same cell line with Adriamycin and the GST P1 specific inhibitor O^1-hexadecyl-γ-glutamyl-*S*-(benzyl)cysteinyl-D-phenyl glycine ethyl ester the inhibitor was shown to have a synergic effect on Adriamycin toxicity

Not just presence, but also intracellular localisation of GSTs could be of importance to determine the relative sensitivity of tumour cells to chemotherapy recent studies have highlighted the fact that cells positive for nuclear GST P were more resistant to Adriamycin than those with negative nuclear staining (Goto *et al*, 2001) Further studies showed that GST P accumulated in the nucleus in response to several anticancer drugs, and that the levels of resistance to Cisplatin and Adriamycin - but not to Etoposide or 5-FU - were reduced by an inhibitor of nuclear transport (Goto *et al*, 2002) These results show a drug-specific inhibitory effect of nuclear GST P

GST P1-mediated modulation of cell proliferation was also showed in knock out mice animals carrying a GST P1 deletion had an increased ERK1/ERK2 kinase activity, and they also had a higher number of circulating white blood cells, as compared to the wild-type mice Administration of the GST P1 inhibitor γ-glutamyl-*S*-(benzyl)cysteinyl-

R-phenyl glycine diethyl ester (TLK199) stimulated proliferation of bone marrow cells only in wild-type mice (Ruscoe *et al* , 2001) In an interesting turn, deletion of GST P1 in human lung fibroblasts caused the cells to undergo apoptosis, suggesting a protective role for GST P1 in this type of tissue (Ishii *et al* , 2003)

Those anticancer drugs that are inactivated through a GSH-dependent mechanism are listed in Table 1 5 1 The mechanisms of resistance to anticancer drugs that involve GSH and GSH-related enzymes are listed in Table 1 5 2

Mechanism	**Substrates**	**Genes involved**
Conjugation	Melphalan, Cyclophosphamide, Mitoxantrone, Thiotepa, Chlorambucil, Nitrosoureas	GST (α, μ, π), GCS, MRPs
Chelation	Platinum drugs	GCS, MRP2
Free radical scavenging and peroxide inactivation	Adriamycin, Daunorubicin, Mitomycin C, Tirapazamine, Bleomycin	GSH peroxidases, non-Se GSH peroxidases, GCS, MRP1
Detoxification of lipid peroxidation products	Anthracyclines and other redox cycling agents (same as above)	GST A4-4, GSH peroxidases
GSH-dependent transport	Vincristine, Daunorubicin	MRPs, GCS

Table 1 5 1 Anticancer agents that are substrates of GSH-related inactivation (adapted from McLellan *et al* , 1999)

Mechanism	GSH-dependent protection	Genes involved
Conjugation with GSH and export	Conjugation is of particular importance to inactivate and facilitate export of alkylating agents Nitrosoureas can be denitrosated by GSTs	GSTs (α and π for alkylating agents, μ for nitrosoureas)
Free radical scavenging	Radicals generated by redox cycling drugs such as anthracyclines are inactivated by GSH	GSH synthesis enzymes, GSH peroxidases
Reduction of peroxides and lipid peroxidation products	Peroxide (OH) and lipid hydroperoxides are reduced and/or conjugated	GSTs (non Se GSH peroxidases), Se-dependent GSH peroxidases, GST A4-4
GSH-dependent export of unconjugated drugs	Vincristine is transported out of the cell by a GSH-dependent mechanism Cisplatin could be complexed with GSH, then exported	MRP1, MRP2, GCS, GSTs
Regulation of signalling pathways	GSH is modulated by redox status and can mediate direct or indirect modulation of JNK, MAPK, ras, NF-κB, SAPK, AP-1	GST P1, AP endonuclease, Keap 1, CL 100
Inhibition of apoptosis	GSH inhibits serine protease AP24 Bcl-2 over expression increases GSH levels and promotes nuclear localisation Inhibition of GSH efflux during apoptosis prevents cell death	GSH transporters

Table 1 5 2 Summary of GSH-related chemotherapy resistance mechanisms (adapted from McLellan *et al*, 1999)

1 6 Conclusion

Several *in vitro* studies suggest that enzymes involved in drug metabolism play a role in determining tumour sensitivity to anticancer agents, indeed, they have the potential to inactivate anticancer drugs and thus reduce therapeutic efficacy However, enzymatic inactivation is not a rapid process, or at least is not as effective as efflux Also, enzymes do get saturated and this reduces the efficiency with which they inactivate drugs This poses a question as to whether drug metabolism enzymes are capable of detoxifying anticancer drugs inside tumours and contribute to the development of drug resistance

At the same time, the drug metabolism machine is a vastly complex network of enzymes working simultaneously and able to affect the expression and/or metabolic capacity of each other It is rarely found that a single gene conditions the response to a certain stimulus or event, it is often the coordinated effect of a number of different genes that eventually determines the outcome of a process A clear example of this is shown by the contradictory reports on the influence of P450 polymorphisms in tumorigenesis It is known that some of their metabolites are active carcinogens, but this is clearly not enough to point them as the sole cause of cancer And as the factors that contribute to the development of cancer are multiple, it is difficult to find a correlation between overexpression or enhanced activity of a certain enzyme and carcinogenesis The same is probably true of anticancer drug resistance both are such complex and multi-stepped processes that the influence of a certain enzyme is unlikely to cause noticeable effects

The presence of drug metabolism enzymes in hepatic and most extrahepatic tissues has been established, albeit with significant differences in the proportion of positive samples for each isoform The disparities found between reports that evaluate mRNA and those that analyse protein can be easily explained by posttranslational regulatory events afftecting particular enzymes, while interstudy inconsistencies in mRNA expression could be attributed to the use of different primers and the existence of alternative splicing variants The differences in immunohistochemical studies could be ascribed to the use of different antibodies, cross-reaction between different isoforms, presence of posttranslational modifications that alter recognition by antibodies or loss of antigens due to sample treatment

It should also be taken into account that levels of enzymes involved in drug metabolism are deeply affected by environmental agents, as large interindividual differences in expression show, diet, exposure to pollution and medication can significantly alter the expression of drug metabolism enzymes Race, gender and

levels of female hormones (e g , in premenopausal or postmenopausal women) all appear to affect their expression to a certain extent as well, particularly in cytochromes P450 The presence of a tumour also seems to influence the expression of these enzymes in surrounding tissue, as shown by several studies, it appears that the inflammatory reaction generated by the tumour, or perhaps the tumour itself, are able to modulate expression of different enzymes in the normal adjacent tissue For this reason, it should always be noted whether expression in tumour samples is compared to normal adjacent tissue or from cancer-free samples

Overall, great interindividual differences in enzymatic levels are to be expected due to the large number of factors affecting their expression Also, the use of different techniques contributes to these divergences, adding extra factors that will affect the results Nevertheless, most studies report overexpression of drug metabolism enzymes in tumour as compared to normal tissue, indicating that they are either involved in malignant transformation or serve as markers of the carcinogenic process Either way, the more we understand about cytochromes P450 and GSH-related enzymes, the more we will gain insight into the origins of cancer and the development of specific therapies to combat it

Drug metabolism enzymes indeed appear to play a role in the development of drug resistance, but the nature of this role is still obscure These enzymes certainly have the potential to constitute a major resistance mechanism but further studies need to be carried out before their significance is revealed

Aims of thesis

1 The main aim of this project was to establish the role that enzymes involved in drug metabolism play in the development of resistance to chemotherapy The first thing to examine was whether these enzymes were expressed in tumour tissue and also whether their expression patterns were altered in cancer as compared to normal tissue Analysis of a database obtained from a whole genome microarray experiment was performed and the presence of genes of interest investigated

2 The second aim of this project was to determine the role of cytochromes P450 in chemotherapy resistance Cytochromes P450 are the most relevant enzymes to carry out Phase I drug metabolism in humans, that is, they usually transform active drugs into less or non-toxic derivatives (although some agents undergo P450-mediated activation) Recombinant cytochrome 3A4 (CYP3A4), CYP1B1 and P450 NADPH reductase (P450R) were used to study the direct metabolism of anticancer drugs by these enzymes The toxicity profile of CYP3A4 and P450R-transfected cell lines was also studied in order to find out whether this enzyme is able to confer resistance to anticancer drugs Transient transfection of CYP1B1 into MCF-7 cells and induction of CYP1B1 expression in HL60 cells were also used to investigate possible changes in resistance Finally, expression of CYP1B1 was knocked down using siRNA technology in MCF-7 cells pulse-selected with Taxotere

3 The third aim of this project was to investigate alterations in the expression of enzymes involved in glutathione (GSH) turnover and correlate them with resistance to drugs commonly used in cancer chemotherapy Similar to P450s, GSH transferases play a prominent role in Phase II metabolism and GSH is one of the most important antioxidant molecules present in the cell For this purpose, expression of key enzymes involved in GSH turnover was studied in a panel of pulse-selected cell lines and their parent counterparts, expression patterns were then correlated with the toxicity profiles of each cell type

4 Finally, to gain insight into the changes undergone by tumour cells after exposure to chemotherapy, two metabolically competent cell lines were exposed to Taxol, Taxotere and Cisplatin weekly over a period of 6 weeks Resistance profiles were then determined for a number of anticancer agents and expression of CYP3A4 and CYP3A5, as well as that of MDR1 and GSH-related enzymes was studied

Section 2.0

Materials and Methods

2 1 Ultrapure water

Ultrapure water (UHP) was used in the preparation of all media and solutions This water was purified to a standard of 12-18 MΩ/cm resistance by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP)

2 2 Glassware

The solutions used in the various stages of cell culture were stored in sterile glass bottles All sterile bottles and other glassware required for cell culture related applications were prepared as follows glassware and lids were soaked in a 2% RBS-25 (AGB Scientific) for 1 hour After this time, they were cleansed and washed in an industrial dishwasher, using Neodisher detergent and rinsed twice with UHP The resulting materials were sterilised by autoclaving as described in Section 2 3

2 3 Sterilisation Procedures

All thermostable solutions, water and glassware were sterilised by autoclaving at 121°C for 20 minutes at 15 p s i Thermolabile solutions were filtered through 0 22µm sterile filters (Millipore, Millex-GV SLGV025BS) Larger volumes (up to 10 litres) of thermolabile solutions were filter sterilised through a micro-culture bell filter (Gelman, 12158)

2 4 Preparation of cell culture media

Basal media used during cell culture was prepared as follows 10X media was added to sterile UHP water, buffered with HEPES (N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) and $NaHCO_3$ as required and adjusted to pH 7 5 using sterile 1 5 N NaOH or 1 5 N HCl The media was then filtered through sterile 0 22µm bell filters (Gelman, 12158) and stored in sterile 500ml bottles at 4°C Sterility checks were performed on all bottles of media by inoculation of media samples on to Colombia blood agar (Oxoid, CM217), Sabauraud dextrose (Oxoid, CM217) and Thioglycolate broths (Oxoid, CM 173) All sterility checks were then incubated at both 25°C and

37°C These tests facilitated the detection of bacterial, yeast and fungal contamination

Basal media were stored at 4°C for up to three months The HEPES buffer was prepared by dissolving 23 8g of HEPES in 80ml UHP water and this solution was then sterilised by autoclaving Then 5ml sterile 5N NaOH was added to give a final volume of 100ml $NaHCO_3$ was prepared by dissolving 7 5g in 100ml UHP water followed by autoclaving Complete media was then prepared as follows supplements of 2mM L-glutamine (Gibco, 11140-0350) for all basal media and 1ml 100X non-essential amino acids (Gibco, 11140-035) and 100mM sodium pyruvate (Gibco, 11360-035) were added to MEM Other components were added as described in Table 2 1 Complete media was stored at 4°C for a maximum of one month

Cell Line	**Basal Media**	**FCS (%)**	**Additions**
A549	DMEM Ham's F12 1 1	5	N/A
Caco2	MEM	10	Sodium pyruvate, non-essential amino acids, $NaHCO_3$, HEPES
HepG2	MEM	10	Sodium pyruvate, non-essential amino acids, L-glutamine
MDA 231	RPMI 1640	10	Sodium pyruvate, non-essential amino acids
MCF-7	MEM	10	Sodium pyruvate, non-essential amino acids, L-glutamine
BEAS-2B	LHC-9	Serum -free	Retinoic acid, Epinephrin
HL60	RPMI 1640 Phenol red free	10	L-glutamine
H1299	RPMI 1640	5	Sodium pyruvate
SKLU-1	MEM	5	Sodium pyruvate, non-essential amino acids, L-glutamine

Table 2 1 Additional components in media N/A no additions

2 5 Cells and Cell Culture

All cell culture work was carried out in a class II laminar airflow cabinet (Holten LaminAir) All experiments involving cytotoxic compounds were conducted in a cytogard laminar airflow cabinet (Holten LaminAir Maxisafe) Before and after use the laminar airflow cabinet was cleaned with 70% industrial methylated spirits (IMS) Any items brought into the cabinet were also swabbed with IMS Only one cell line was used in the laminar air-flow cabinet at a time and upon completion of work with any given cell line the laminar air-flow cabinet was allowed to clear for at least 15 minutes before use to eliminate any possibilities of cross-contamination between the various cell lines The cabinets were cleaned weekly with industrial disinfectants (Virkon or TEGO) and these disinfectants were alternated every month Details pertaining to the cell lines used for the experiments detailed in this thesis are provided in Table 2 2 All cells were incubated at 37°C and where required, in an atmosphere of 5% CO_2 Cells were fed with fresh media or subcultured (see Section 2 5 1) every 2-3 days in order to maintain active cell growth All of the cell lines listed in Table 2 2 are anchorage-dependent cell lines except for HL60, which grow in suspension

BCMV Neo and BCMV 3A4 cells were kindly provided by Dr Katherine Mace, Nestec, Switzerland, while MDA EV and MDA R4 cells were a kind gift of Dr Ian Stratford, Manchester University, UK

Cell Line	Details	Source
Caco2	Colorectal carcinoma	ATCC
HepG2	Liver carcinoma	ATCC
HL60	Acute promyelocytic leukaemia	ECACC
BCMV 3A4	Immortalised normal bronchial cells transfected with human CYP3A4 cDNA	Dr Katherine Mace, Nestec
BCMV Neo	Immortalised normal bronchial cells transfected with empty vector	Dr Katherine Mace, Nestec
MCF-7	Breast adenocarcinoma	ATCC
MDA R4	Breast adenocarcinoma transfected with human P450 NADPH reductase cDNA	Dr Ian Stratford, Manchester University
MDA EV	Breast adenocarcinoma transfected with empty vector	Dr Ian Stratford, Manchester University
A549	Lung adenocarcinoma	ATCC
H1299	Large cell lung carcinoma	ATCC
SKLU-1	Lung adenocarcinoma	ATCC

Table 2 2 Cell lines used in this thesis

2 5 1 Subculturing of cell lines

The cell culture medium was removed from the tissue culture flask and discarded into a sterile bottle The flask was then rinsed out with 1ml of trypsin/EDTA solution (0 25% trypsin (Gibco, 043-05090), 0 01% EDTA (Sigma, E9884) solution in PBS (Oxoid, BRI4a)) to ensure the removal of any residual media Trypsin was then added to the flask, which was then incubated at 37°C, for approximately 5 minutes, until all of the cells detached from the inside surface of the flask The amount of trypsin used varies with flask size, i e , 1ml for T25cm^2, 2ml for T75cm^2 and 5ml for T175cm^2 The

trypsin was deactivated by adding an equal volume of complete media to the flask The cell suspension was removed from the flask and placed in a stenle universal container (Sterilin, 128a) and centrifuged at 1000rpm for 5 minutes The supernatant was then discarded from the universal and the pellet was suspended in complete medium A cell count was performed and an aliquot of cells was used to re-seed a flask at the required density

Cells growing in suspension were subcultured in a similar way, except that no trypsin was used Cells were mixed to ensure homogeneity and a cell count was performed An aliquot of cells was then used to re-seed a flask at the required density, topping the flask up with fresh medium

2 5 2 Assessment of cell number and viability

Cells were trypsinised, pelleted and resuspended in media An aliquot of the cell suspension was then added to trypan blue (Gibco, 525) at a ratio of 5 1 The mixture was incubated for 3 minutes at room temperature A 10µl aliquot of the mixture was then applied to the chamber of a glass coverslip enclosed haemocytometer Cells in the 16 squares of the four grids of the chamber were counted The average cell numbers per 16 squares were multiplied by a factor of 10^4 and the relevant dilution factor to determine the number of cells per ml in the original cell suspension Non-viable cells stained blue, while viable cells excluded the trypan blue dye as their membrane remained intact, and remained unstained On this basis, percentage viability was calculated

2 5 3 Cryopreservation of cells

Cells for cryopreservation were harvested in the log phase of growth and counted as described in Section 2 5 2 Cell pellets were resuspended in a suitable volume of FCS An equal volume of a 10 –20% DMSO/FCS solution was added dropwise to the cell suspension A total volume of 1ml of this suspension was then placed in cryovials (Greiner, 122278) These vials were then placed in the vapour phase of a liquid nitrogen container, which was equivalent to a temperature of –80°C After a period of four hours, vials were removed from the vapour phase and transferred to the liquid phase for storage (- 196°C)

2 5 4 Thawing of cryopreserved cells

A volume of 5ml of fresh warmed growth medium was added to a sterile universal The cryopreserved cells were removed from the liquid nitrogen and thawed at 37°C The cells were removed from the vials and transferred to the aliquoted media The resulting cell suspension was centrifuged at 1,000 rpm for 5 minutes The supernatant was removed and the pellet resuspended in fresh culture medium An assessment of cell viability on thawing was then carried out (Section 2 5 2) Thawed cells were then added to an appropriately sized tissue culture flask with a suitable volume of growth medium and allowed to recover overnight The following day, flasks were fed with fresh media to remove any non-viable cells

2 5 5 Monitoring of sterility of cell culture solutions

Sterility testing was performed in the case of all cell culture media and cell culture related solutions Samples of prepared basal media were incubated at 37°C for a period of seven days This ensured that no bacterial or fungal contamination was present in the media

2 5 6 Serum Batch Testing

Batch to batch variation is a major problem associated with the use of FCS in cell culture In extreme cases this variation may result in a lack of cell growth, whereas in more moderate cases growth may be retarded To avoid the effects of the above variation, a range of FCS batches were screened for growth of each cell line A suitable FCS was then purchased in bulk for a block of work with each particular cell line in use Screening involved seeding cells in 96 well plates and determining growth as a percentage of a serum with known acceptable growth rate Logarithmically growing cells were seeded into a 96 well plate (Costar, 3599) from a single cell suspension at a density of 10^3 cells/well in 100µl of medium without FCS 100µl volumes of medium containing 10% or 20% FCS was added to respective wells on the 96 well plate, resulting in final dilutions of the FCS to 5% or 10%, respectively The

first column of each plate was maintained as a control where FCS resulting in a known acceptable growth rate was used Plates were placed at 37°C in 5% CO_2, for 5 days, after which growth was assessed (see section 2 7 4)

2 6 *Mycoplasma* analysis of cell lines

Cell lines were tested for possible *mycoplasma* contamination in house by Ms Aine Adams and Mr Michael Henry The protocol used is detailed in the following Sections 2 6 1 and 2 6 2

2 6 1 Indirect staining procedure for *Mycoplasma* analysis

Mycoplasma-negative NRK (Normal rat kidney fibroblast) cells were used as indicator cells for this analysis The cells were incubated with a sample volume of supernatant from the cell lines in question and then examined for *Mycoplasma* contamination A fluorescent Hoechst stain was used in this analysis The stain binds specifically to DNA and so stains the nucleus of the cell in addition to any *Mycoplasma* present *Mycoplasma* infection was indicated by fluorescent bodies in the cytoplasm of the NRK cells

2 6 2 Direct staining procedure for *Mycoplasma* analysis

Direct staining for *Mycoplasma* analysis involved inoculating samples on to a *Mycoplasma* culture broth (Oxoid, CM403) This was supplemented with 16% serum, 0 002% DNA (BDH, 42026), 2µg/ml fungizone (Gibco, 042 05920), $2x10^3$ units penicillin (Sigma, Pen-3) and 10ml of a 25% yeast extract solution Incubation was carried out at 37°C for a period of 48 hours Samples of this broth were then streaked onto plates of *Mycoplasma* agar base (Oxoid, CM401) that had been supplemented as described above The plates were incubated for three weeks at 37°C while exposed to CO_2 The plates were examined microscopically every 7 days The appearance of small oval shaped colonies indicated the presence of *Mycoplasma* infection

2 7 *In vitro* toxicity assays

Adherent cells in the exponential phase of growth were harvested by trypsinisation as described in section 2 5 1 Cell suspensions containing $1x10^4$ cells/ml were prepared in cell culture medium Volumes of 100µl/well of these cell suspensions were added to 96-well plates (Costar, 3599) using a multichannel pipette Plates were agitated gently in order to ensure even dispersion of cells over a given well Cells were then incubated overnight at 37°C in an atmosphere containing 5% CO_2 Cytotoxic drug dilutions were prepared at 2X their final concentration in cell culture medium Volumes of the drug dilutions (100µl) were then added to each well using a multichannel pipette Plates were then mixed gently as above Cells were incubated for a further 6-7 days at 37°C and 5% CO_2 until the control wells had reached approximately 80-90% confluency Assessment of cell survival in the presence of drug was determined by the acid phosphatase assay (section 2 7 2) The concentration of drug which caused 50% cell kill (IC50 of the drug) was determined from a plot of the % survival (relative to the control cells) versus cytotoxic drug concentration using Calcusyn software

In the case of non-adherent cells, a cell suspension containing $5x10^4$ cells/ml was prepared in cell culture medium 100µl/well of this suspension were added to 96-well plates using a multichannel pipette Plates were agitated gently to ensure even dispersion of cells over a given well After an overnight incubation at 37°C in an atmosphere containing 5% CO_2, cytotoxic drug dilutions were added, as explained above Cells were then incubated for a further 72 hours and assessment of cell survival determined by the XTT assay (section 2 7 3) IC50 values for each drug were calculated as above

2 7 1 Combination toxicity assays

Cells were harvested in the exponential phase of growth as described in section 2 5 1 Cell suspensions containing $1x10^4$ cells/ml were prepared in cell culture medium Volumes of 100µl/well of this cell suspension were added into 96-well plates (Costar, 3599) using a multichannel pipette Plates were agitated gently in order to ensure even dispersion of cells over a given well Cells were then incubated overnight at 37°C in an atmosphere containing 5% CO_2 Dilutions of cytotoxic drugs and other agents were prepared at 4X their final concentration in media Volumes of 50µl of the drug dilution and 50µl of the combination drug dilution were then added to each

relevant well so that a total final volume of 200µl was present in each well All potential toxicity-enhancing agents were dissolved in DMSO, ethanol or media Stock solutions were prepared at approximately 15mg/10ml media, filter sterilised with a 0 22µm filter (Millex-GV, SLGV025BS) and then used to prepare all subsequent dilutions Cells were incubated for a further 6 days at 37°C in an atmosphere containing 5% CO_2 At this point the control wells would have reached approximately 80-90% confluency Cell number was assessed using the acid phosphatase assay (section 2 7 2) or the XTT assay (section 2 7 3)

2 7 2 Assessment of cell number - Acid Phosphatase assay

Following the incubation period of 6-7 days, media was removed from the plates Each well on the plate was washed twice with 100µl PBS This was then removed and 100µl of freshly prepared phosphatase substrate (10mM *p*-nitrophenol phosphate (Sigma 104-0) in 0 1M sodium acetate (Sigma, S8625), 0 1% triton X-100 (BDH, 30632), pH 5 5) was added to each well The plates were then incubated in the dark at 37°C for 2 hours The enzymatic reaction was stopped by the addition of 50µl of 1N NaOH The plate was read in a dual beam plate reader (Synergy HT, Bio-Tek, USA) at 405nm with a reference wavelength of 620nm

2 7 3 Assessment of cell number - XTT assay

50 µl of a phenazine methosulphate solution (PMS, Sigma, P9625, 2 5 mM in PBS) were added to 5ml of XTT reagent (sodium 3'-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene sulphonic acid hydrate, Sigma, X4251, 1 mg/ml in complete culture medium) immediately before addition to cells This volume was enough for one plate After a 72 hour incubation period, 50 µl of this mixture were added to each well The plates were then incubated in the dark at 37°C for 4 hours After this incubation period, absorbance was read in a plate reader at 450 nm

2 8 Safe handling of cytotoxic drugs

Cytotoxic drugs were handled with extreme caution at all times in the laboratory, due to the potential risks in handling these drugs Disposable nitrile gloves (Medical Supply Company Ltd) were worn at all times and all work was carried out in cytotoxic cabinets (Holten LaminAir Maxisafe) All drugs were stored in a safety cabinet at room temperature or in designated areas at 4°C The storage and means of disposal of the cytotoxic drugs used in this work are outlined in Table 2 3

Cytotoxic Agent	Storage	Disposal
Adriamycin	4°C in dark	Incineration
Daunorubicin	4°C in dark	Incineration
Taxol	Room temperature in dark	Incineration
Taxotere	Room temperature in dark	Incineration
Vincristine	4°C in dark	Incineration
VP-16	Room temperature in dark	Incineration
Irinotecan	Room temperature in dark	Incineration
Cisplatin	Room temperature in dark	Incineration
5-Fluorouracil	Room temperature in dark	Incineration
Mitoxantrone	Room temperature in dark	Incineration
Mitomycin C	4°C in dark	Incineration

Table 2 3 Storage and disposal details for chemotherapeutic agents

2 9 Pulse selection of parent cell lines

A number of drug resistant variants were established from the cell lines Caco2 and HepG2 by pulse-selection with Taxol, Taxotere and Cisplatin

2 9 1 Determination of drug concentration for pulse selection

Cells were seeded into twelve 25cm^2 flasks at 1 5 x 10^5 cells per flask and allowed to attach overnight at 37°C The following day media was removed from the flask and a

range of concentrations of appropriate drug was added to the flasks in duplicate Complete media was added to two flasks as a 100% survival control Flasks were returned to the 37°C incubator for a 4 hour incubation Drug was then removed and the flasks were rinsed and fed with fresh complete media The flasks were then incubated for 5-7 days until the cells in the control flasks had reached approximately 80% confluency At this point, medium was removed from the flasks and cells were trypsinised and counted in duplicate as described in section 2 5 2 The concentration of drug that caused appropriate cell kill was determined from a plot of the percentage survival relative to the control cells versus cytotoxic drug concentration
Concentrations chosen for pulse selection of Caco2 and HepG2 gave a 70% kill, i e IC70 (Table 2 4)

Cell Line	Taxotere (ng/ml)	Taxol (ng/ml)	Cisplatin (ng/ml)
Caco2	160	2560	
HepG2	160	1280	2500

Table 2 4 Concentrations used in pulse-selection

2 9 2 Pulse selection

Cells at low confluency in 75cm^2 flasks were exposed to the chosen concentration of Taxol, Taxotere or Cisplatin for 4 hours After this period, the drug was removed and the flasks were rinsed and fed with fresh complete media The cells were then grown in drug-free media for 6 days, refeeding every 2-3 days This was repeated once a week for six weeks If the cells had not recovered sufficiently from the previous pulse, a week was skipped, but all cell lines received six pulses

2 10 Western blotting

2 10 1 Whole cell extract preparation

Cells were grown to 80-90% confluency in 75cm^2 flasks Media was removed and cells were harvested as described in section 2 5 1 Cells were washed twice with ice-cold PBS All procedures from this point forward were performed on ice Cells were

resuspended in 100-200µl of NP-40 lysis buffer and incubated on ice for 30 minutes Table 2 5 provides the details of the lysis buffer Immediately before use, 10µl of the 100X stocks listed in Table 2 6 were added to 1ml of lysis buffer

Addition required per 500ml stock	Final concentration
425ml UHP water	-
25ml 1M Tris-HCl (pH 7 5)	50mM Tris-HCl (pH 7 5)
15ml 5M NaCl	150 mM NaCl
2 5ml NP-40	0 5% NP-40

Table 2 5 NP-40 lysis buffer

100X stock	Preparation instructions
100mM DTT	154mg in 10ml UHP
100mM PMSF	174mg in 10ml 100% ethanol
100X Protease inhibitors	2 5 mg/ml leupeptin, 2 5 mg/ml aprotinin, 15 mg/ml benzamidine and 1mg/ml trypsin inhibitor in UHP water

Table 2 6 NP-40 lysis buffer 100X stocks

After incubation on ice, lysates were centrifuged on a bench centrifuge at 14000rpm for 15 minutes at 4°C Supernatant containing extracted protein was transferred to a fresh chilled eppendorf tube Protein concentration was quantified using the Biorad assay as detailed in section 2 10 2 Samples were then stored in aliquots at -80°C

2 10 2 Protein quantification

Protein levels were determined using the Bio-Rad protein assay kit (Bio-Rad, 5000006) as follows A 2mg/ml bovine serum albumin (BSA) solution (Sigma, A9543) was prepared freshly in lysis buffer A protein standard curve (0, 0 2, 0 4, 0 6, 0 8 and 1 0mg/ml) was prepared from the BSA stock with dilutions made in lysis buffer 5µl of each sample or BSA dilution were pipetted in duplicate into a 96-well plate The Bio-Rad reagent was diluted 1 5 in UHP water and 200µl of this dilution were added to

each well After a 5 minute incubation, absorbance was assessed at 595nm The concentration of the protein samples was determined from the plot of the absorbance at 595nm versus concentration of the protein standard

2 10 3 Gel electrophoresis

Proteins for analysis by Western blotting were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) The Atto dual mini slab kit was used (AE 6450) The stacking and resolving gels were prepared as illustrated in Table 2 7 The gels were set in clean 9cm x 8cm gel cassettes, which consisted of 2 glass plates separated by a rubber gasket to a width of 1mm The resolving gel was added to the gel cassette and allowed to set Once the resolving gel had set, stacking gel was poured on top A comb was placed into the stacking gel after pouring, in order to create wells for sample loading (maximum sample loading volume of 15-20µl)

Components	10% Resolving Gel	5% Stacking Gel
Acrylamide stock	4 6 ml	670µl
UHP water	5 6ml	2 7ml
1 875 M Tris-HCl pH 8 8	3 5ml	-
1 25 M Tris-HCl pH 6 8	-	500µl
10% SDS	140µL	40µL
10% NH_4- persulfate	140µL	40µL
TEMED	5 6µL	4µL

Table 2 7 Preparation protocol for SDS-PAGE gels (2 x 0 75mm gels)

The acrylamide stock in Table 2 7 consists of a 30% (29 1) ratio of acrylamide bis-acrylamide (Sigma, A2792) In advance of samples being loaded into the relevant sample wells, 20µg of protein was diluted in 10X loading buffer Molecular weight markers (Sigma, C4105) were loaded alongside samples The gels were run at 250V and 45mA until the bromophenol blue dye front was found to have reached the end of the gel, at which time sufficient resolution of the molecular weight markers was achieved

2 10 4 Western blotting

Western blotting was performed by the method of Towbin *et al* (1979) Once electrophoresis had been completed, the SDS-PAGE gel was equilibrated in transfer buffer (25mM Tris (Sigma, T8404), 192mM glycine (Sigma, G7126), pH 8 3-8 5) for approximately 30 minutes Five sheets of Whatman 3mm filter paper were soaked in freshly prepared transfer buffer These were then placed on the cathode plate of a semi-dry blotting apparatus (Bio-rad) Air pockets were then removed from between the filter paper Nitrocellulose membrane (Amersham Pharmacia Biotech, RPN 303D), which had been equilibrated in the same transfer buffer, was placed over the filter paper on the cathode plate Air pockets were once again removed The gels were then aligned on to the membrane and covered by five additional sheets of transfer buffer soaked filter paper, again removing all air pockets The proteins were transferred from the gel to the membrane at a current of 34mA at 15V for 30-40 minutes, until all colour markers had transferred

Following protein transfer, membranes were stained using Ponceau red (Sigma, P7170) to ensure efficient protein transfer The membranes were then blocked for 2 hours at room temperature using 5% milk powder (Cadburys, Marvel skimmed milk) in PBS

Membranes were treated with primary antibody overnight at 4°C and a negative control where the membrane was exposed to antibody diluent was also performed All antibodies were prepared in 1% Marvel in PBS and are listed in Table 2 8 Primary antibody was removed after this period and the membranes rinsed 3 times with PBS containing 0 5% Tween 20 (Sigma P1379) for a total of 15-30 minutes

The secondary antibody (anti mouse IgG, Sigma, A6782, used 1 2000, or anti rabbit IgG Sigma, A4914, used 1 1000) was then added for 1 5 hour at room temperature The membranes were washed thoroughly in PBS containing 0 5% Tween for 15 minutes

Primary Antibody	Dilution
CYP3A4 (Chemicon, USA, AB1254)	1 1000
CYP3A5 (Chemicon, USA, AB1279)	1 500
CYP1B1 (Gentest, USA, 458211)	1 500
β-actin (Sigma, USA, A5441)	1 10,000
MDR1 (Santa Cruz, USA, SC-13131)	1 200
GSTP1 (Calbiochem, UK, 354212)	1 200
GSTA1 (Calbiochem, UK, 354206)	1 200
GCSH (Santa Cruz, USA, SC-28965)	1 200
GCSL (Santa Cruz, USA, SC-28966)	1 200
GGTP (Santa Cruz, USA, SC-20639)	1 200
P450R (Santa Cruz, USA, SC-25270)	1 200

Table 2 8 List of Primary antibodies and dilutions

2 10 5 Enhanced chemiluminescence (ECL) detection

Immunoblots were developed using an Enhanced Chemiluminescence kit (Amersham, RPN2109), which facilitated the detection of bound peroxidase-conjugated secondary antibody Following the final washing membranes were subjected to ECL A volume of 3ml of a 50 50 mixture of ECL reagents was used to cover the membrane The ECL reagent mixture was completely removed after a period of one minute and the membrane wrapped in clingfilm, carefully removing all excess air bubbles The membrane was then exposed to autoradiographic film (Kodak, X-OMATS) for various times (from 10 seconds to 30 minutes depending on the signal) The exposed autoradiographic film was developed for 3 minutes in developer (Kodak, LX-24) The film was then washed in water for 15 seconds and transferred to a fixative (Kodak, FX-40) for 5 minutes After another 5-10 minute wash in water, the film was left to dry at room temperature

2 11 RT-PCR analysis

2 11 1 Preparation of materials for RNA analysis

Due to the labile nature of RNA and the high abundance of RNase enzymes in the environment a number of precautionary steps were followed when analysing RNA throughout the course of these studies

- General laboratory glassware and plasticware are often contaminated by RNases To reduce this risk, glassware used in these studies was baked at 180°C (autoclaving at 121°C does not destroy RNase enzymes) for at least 8hr Sterile, disposable plasticware is essentially free of RNases and was therefore used for the preparation and storage of RNA without pre-treatment Polyallomer ultracentrifuge tubes, eppendorf tubes, pipette tips etc , were autoclaved before use All spatulas which came in to contact with any of the solution components were baked, chemicals were weighed out onto baked aluminium-foil and a stock of chemicals for "RNA analysis only" was kept separate from all other laboratory agents All solutions (which could be autoclaved) that came in to contact with RNA were prepared from sterile ultra-pure water and treated with 0 1% diethyl pyrocarbonate (DEPC) (Sigma, D5758) before autoclaving (autoclaving inactivates DEPC)
- Disposable gloves were worn at all times to protect both the operator and the experiment (hands are an abundant source of RNase enzymes) This prevents the introduction of RNases and foreign RNA/DNA in to the reactions Gloves were changed frequently
- All procedures were carried out under sterile conditions where feasible

2 11 2 Total RNA extraction from cultured cell lines

Adherent cells were grown in 75cm^2 flasks until approximately 80% confluent Media was then removed and 1ml of TRI reagent (Sigma, T-9424) was added per 75cm^2 flask for 5 minutes ensuring that all cells were covered with the solution TRI reagent is a mixture of guanidine thiocyanate and phenol in a mono-phase solution, it effectively dissolves DNA, RNA, and protein on lysis of cell culture samples After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate To ensure complete dissociation of nucleoprotein

complexes, the sample was allowed to stand for 5 minutes at room temperature Subsequently, 0 2ml of chloroform (not containing isoamyl alcohol or any other additive) per ml of TRI reagent was added to the cell lysate The sample was covered tightly, shaken vigorously for 15 seconds and allowed to stand for 2-15 minutes at room temperature The resulting mixture was centrifuged at 13,000rpm for 15 minutes at 4°C Centrifugation separated the mixture into 3 phases an organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA) The aqueous phase was then transferred to a fresh tube and 0 5ml of isopropanol per ml of TRI reagent used in sample preparation added and mixed The sample was then allowed to stand for 5-10 minutes at room temperature, followed by centrifugation at 13,000rpm for 10 minutes at 4°C The RNA precipitate formed a pellet on the side and the bottom of the tube The supernatant was removed and the RNA pellet was washed by adding 1ml (minimum) of 75% ethanol per 1ml of TRI reagent The sample was vortexed and centrifuged at 8000rpm for 5 minutes at 4°C Samples can be stored in ethanol at 4°C for at least 1 week and up to one year at -20°C The RNA pellet was air-dried briefly and resuspended in approximately 50µl DEPC-treated H_2O The RNA was then stored at -80°C until required for PCR analysis

2 11 3 RNA quantification using Nanodrop

RNA was quantified spectrophotometrically at 260nm and 280nm using the Nanodrop A 1µl aliquot of suitably diluted RNA was placed on the Nanodrop The software calculated the amount of RNA present using the fact that an optical density of 1 at 260nm is equivalent to 40mg/ml RNA An A_{260}/A_{280} ratio is used to indicate the purity of the RNA, which was always between 1 8 and 2 1

2 11 4 Reverse transcription of RNA isolated from cell lines

The following components were used in the reverse transcriptase (RT) reaction for RNA isolated from cell lines 1µl oligo $(dT)_{12-18}$ primers (1µg/ml) (Promega, C1101), 2µl of total RNA (0 5µg/ml), and 2µl of DEPC-H_2O were mixed together and heated at 70°C for 10 min and then chilled on ice to remove any RNA secondary structure formation and allow oligo (dT) primers to bind to the poly $(A)^+$ tail on the mRNA 4µl of

a 5X buffer (consisting of 250mM Tris-HCl, pH 8 3, 375mM KCl and 15mM $MgCl_2$), 2µl of DTT (100mM), 1µl of dNTPs (10mM each of dATP, dCTP, dGTP and dTTP), 7µl of water and 1µl of Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (Sigma, M1302) were then added to the heat-denatured RNA complex and the mixture was incubated at 37°C for 1 hour to allow the MMLV-RT enzyme to catalyse the formation of cDNA on the mRNA template The enzyme was then inactivated and the RNA and cDNA strands separated by heating to 95°C for 2 min The cDNA was used immediately in the PCR reaction or stored at -20°C until required for analysis

2 11 5 Polymerase Chain Reaction (PCR) analysis of cDNA formed from mRNA isolated from cell lines

PCR reactions were set up as 50µl volumes using 5 µl of cDNA formed during the RT reaction (see Section 2 11 4) cDNA was amplified for varying cycle numbers but where possible amplification was carried out in the exponential phase of amplification The sequences of all primers used for PCR in this thesis are shown in Table 2 9
Each PCR reaction tube contained 5µl 10Xbuffer (100mM Tris-HCl, pH 9 0, 50mM KCl, 1% Triton X-100), 2 µl 25mM $MgCl_2$, 1µl of first strand target primer (250ng/µl), 1µl of second strand target primer (250ng/µl), 0 5µl of first strand endogenous β-actin control primer (250ng/µl), 0 5µl of second strand endogenous β-actin control primer (250ng/µl), 1µl of 10mM dNTP (Sigma, DNTP-100), 0 5µl of Taq DNA Polymerase enzyme (Sigma, D4545) and 31 5µl UHP 5µl of cDNA (pre-heated to 95°C for 3min to separate strands and remove any secondary structure if the sample had been stored at -20°C) was added to the above The mixture was then heated to 94°C for 5min (reduces non-specific binding of primers to template) and the cDNA was then amplified by PCR (Techne, PHC-3) using the following program

- 94°C for 3min (denature double stranded DNA)
- 25-35 cycles 94°C for 30sec (denature double stranded DNA),
  X°C for 30sec (anneal primers to cDNA),
  72°C for 30sec (extension),
- 72°C for 7min (extension)
- Storage at 4°C

Where X is the annealing temperature varied with primer set used

Gene	Length (bp)	Annealing Temperature (°C)	Size (bp)	Sequence
β-actin (large)	29	55	383	GAA ATC GTG CGT GAC ATT AAG -GAG AAG CT
	22			TCA GGA GGA GCA ATG ATC TTG A
β-actin (small)	23	55	142	TGG ACA TCC GCA AAG ACC TGT AC
	22			TCA GGA GGA GCA ATG ATC TTG A
CYP1B1	20	49	316	GTA TAT TGT TGA AGA GAC AG
	20			AAA GAG GTA CAA CAT CAC CT

Table 2 9 Sequences of primers used for PCR

A 10µl aliquot of tracking buffer, consisting of 0 25% bromophenol blue (Sigma, B5525) and 30% glycerol in water, was added to each tube of amplified cDNA products 20µl of cDNA products from each tube were then separated by electrophoresis at 100mV through a 2% agarose (Sigma, A9539) gel containing ethidium bromide (Sigma, E8751), using TBE (22 5mM Tris-HCl, 22 5mM boric acid (Sigma, B7901), 0 5mM EDTA) as running buffer Molecular weight markers "ϕ-X174" Hae III digest (Sigma, P0672) were run simultaneously as size reference When the gels were placed on a transilluminator (UVP Transilluminator) the resulting product bands were visualised as pink bands due to the intercalation of the cDNA with the ethidium bromide The gels were photographed and subjected to densitometric analysis

2 12 Determination of CYP3A4 activity by HPLC

2 12 1 Testosterone 6ß-hydroxylase assay

The detection of Testosterone 6ß-hydroxylase activity in the microsomal fraction of recombinant insect cells (microsomes) expressing CYP3A4 and P450 NADPH reductase (P450R) (Gentest, 456202) or P450R alone (Gentest, 456244) was performed according to Baron *et al* (2001) 50 µl of a 520 µM solution of Testosterone

(Sigma, T1500) were prewarmed at 37° C in a total incubation mixture volume of 250 µl containing potassium phosphate buffer (PBS, pH 7 4), $MgCl_2$ (25 mM, 25 µL) and NADPH (6 5 mM, 75 µl) The reaction was initiated by the addition of 100 µl of a 1mg/ml solution of ice-cold microsomes to the incubation mixture, as recommended by the manufacturer After 15 minutes incubation, the reaction was stopped by the addition of 750 µl of ethyl acetate containing the internal standard Corticosterone (Sigma, C2505) A control reaction was carried out using microsomes from cells transfected with P450R alone (Gentest, 456244)

The detection of Testosterone 6β-hydroxylase activity in cultured cells was also performed according to Baron *et al* (2001) $5x10^5$ cells were seeded per $75mm^2$ flask and incubated in standard medium for 24 hs at 37° C Hemin (Sigma, H5533) was then added to a final concentration of 5 µg/ml and cells cultured for a further 24 hs, it provides a heme group and increases the levels of P450 expression The Hemin-containing medium was then replaced with 4 ml of fresh medium and samples incubated for 1 hr at 37° C After this period, Testosterone was added directly into the culture medium to a final concentration of 100 µM Following a 3 hr-incubation, approximately 5 ml of medium were removed, transferred to a polypropylene tube and mixed with 1 ml of ethyl acetate containing the internal standard Corticosterone

Extraction of the samples was performed with a method adapted from Baltes *et al* (1998) The combination of reaction mixture or medium and ethyl acetate was mixed for 5 minutes on a blood-tube mixer and then centrifuged at 4000 rpm for 5 minutes After removing 0 75 ml of the ethyl acetate extract, 1 ml of fresh solvent was added to the sample, which was again mixed and centrifuged in the same conditions A further 1 ml of the ethyl acetate extract was removed and pooled with the first 0 75 ml, the resulting mixture was pipetted into a glass vial and the solvent evaporated under a stream of nitrogen The residue was reconstituted with 80 µl of mobile phase prior to HPLC analysis Testosterone and 6β-hydroxy testosterone standards of known concentration were processed in the same way

2 12 2 Separation of metabolites by HPLC

A Hypersil BDS C18, 3 µM (10 cm x 0 46 cm) analytical column maintained at room temperature (Phenomenex, USA) was used for analysis (Whalley *et al*, 2001) For each sample, a linear mobile phase (1 25 ml/min) gradient [tetrahydrofuran-acetonitrile-water (10 10 80, v/v, mobile phase A) changing to tetrahydrofuran-

acetonitrile-water (14 14 72, v/v, mobile phase B)] was run over 10 min, with a further 3 min run isocratically The column was re-equilibrated with mobile phase A for 2 min before injection of the next sample Detection of metabolites was performed by in-line UV detection at 255 nm These conditions allow for a total cycle time of 15 min, including the 2 min re-equilibration

Mass of 6β-hydroxy testosterone produced was then calculated by interpolation in a standard curve CYP3A activity was then calculated as the measured amount of 6β-hydroxy testosterone (in pmoles), divided by the total protein content of the sample (in milligrams) and by the total reaction time (in minutes)

2 13 Determination of EROD activity by fluorescence

Ethoxyresorufin O-deethylase (EROD) activity was determined in the microsomal fraction of recombinant insect cells (microsomes) expressing CYP1B1 and P450 NADPH reductase (Gentest, 456220) with a method adapted from Yu *et al* (2004) An incubation mixture containing 100 nM PBS buffer, pH 7 4, 5 nM $MgCl_2$, 1 5 nM EDTA and 0 2 μM 7-ethoxyresorufin (Sigma, E3763) was prewarmed at 37°C, reaction was initiated by the addition of 100 μl of a 1mg/ml solution of ice-cold microsomes to the incubation mixture, as recommended by the manufacturer After 30 minutes incubation, 100 μl of reaction mixture were transferred to an opaque 96-well plate and fluorescence determined at 530nm excitation and 590nm emission in a fluorescence plate reader (Synergy HT, Bio-Tek, USA) The sample measurements were compared to a resorufin (Sigma, R3257) standard curve A control reaction was carried out using microsomes from cells transfected with P450 NADPH reductase alone (Gentest, 456244)

EROD activity of cells in culture was determined as described in Bandiera *et al* (2005) Briefly, cells were incubated with a 400 nM solution of 7-ethoxyresorufin in PBS pH 7 4 at 37°C/5% CO_2 for 30 minutes After incubation, 200 μl of the cell supernatant were transferred to an opaque 96-well plate and fluorescence determined as before

Resorufin standards of known concentration were included in the analysis and processed in the same way EROD activity was then calculated as the measured amount of resorufin (in pmoles), divided by the total protein content of the sample (in milligrams) and by the total reaction time (in minutes)

2 14 Determination of P450R activity with a colorimetric assay

P450R activity was determined in cell extracts by measuring the amount of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) converted to formazan with a colorimetric assay Briefly, cells were scrapped from a confluent T75 flask and washed with ice-cold PBS, they were then resuspended in PBS pH 7 4 containing 1 mM PMSF, 1 mM DTT, 25 µg/ml leupeptin, 25 µg/ml aprotinin, 150 µg/ml benzamidine and 10 µg/ml trypsin inhibitor The cell suspension was sonicated on ice with 4 cycles of 30 seconds each, leaving 10 seconds in between cycles, cells were then spun down at 14000 rpm for 15 minutes at 4°C 250 µl of supernatant were transferred to a 24-well plate and 25 µl of 100 µM MTT were added 25 µl of 0 5 mM NADPH were then added to each well and the absorbance at 610 nm read immediately every 30 seconds during a 5 minute-interval in a plate reader (Synergy HT, Bio-Tek, USA) The response was linear for at least 5 minutes (see section 3 5 2) A standard curve of formazan standards of known concentration was measured at the same time, and the absorbance values obtained interpolated in the standard curve to obtain the respective concentration values P450R activity was expressed as nmol formazan produced per mg of total protein per min

2 15 Determination of NADPH content in cell extracts

NADPH levels were determined in cell extracts by measuring the absorbance at 340 nm using a spectrophotometer Briefly, cells were scrapped from a confluent T75 flask and washed with ice-cold PBS, they were then resuspended in PBS pH 7 4 containing 1 mM PMSF, 1 mM DTT, 25 µg/ml leupeptin, 25 µg/ml aprotinin, 150 µg/ml benzamidine and 10 µg/ml trypsin inhibitor The cell suspension was sonicated on ice with 4 cycles of 30 seconds each, leaving 10 seconds in between cycles, cells were then spun down at 14000 rpm for 15 minutes at 4°C The supernatant contains both NADH and NADPH, both of which absorb at 340 nm For that reason, the NADPH present in the supernatant is converted to $NADP^+$, which does not absorb at this wavelength, by reaction with glutathione reductase With this purpose, an aliquot of the supernatant (100 µl) was incubated in 0 1 M phosphate buffer pH 7 6 with 0 05 mM EDTA, 0 05 % Triton X, 5 µM oxidated glutathione (GSSG) and 5 IU of

glutathione reductase for 5 minutes at 25°C The absorbance at 340 nm of the supernatant was measured (this value represents the total NADPH and NADH contents) and the absorbance value of the post-incubation aliquot (representing total NADH content) subtracted to determine the total NADPH content A standard curve of NADPH standards of known concentration was measured at the same time, and the absorbance values obtained interpolated in the curve to obtain the respective concentration values NADPH levels were expressed as nmoles NADPH per mg of total protein

2 16 Determination of reactive oxygen species (ROS) by fluorescence

2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) is a non-fluorescent compound that easily enters the cell by free diffusion through the plasmatic membrane, once inside the cytoplasm, this compound loses the diacetyl radical by reaction with cellular esterases and remains inside the cell The resulting product, 2',7'-dichlorodihydrofluorescein (DCFH) is not fluorescent *per se*, but upon reaction with reactive oxygen species (ROS), particularly hydroxyl radical and superoxide, it is converted to the fluorescent compound dichlorofluorescein For this assay, 1 875 x 10^5 cells were seeded per well in a 24-well plate and incubated for 48 hours at 37°C After this incubation, the appropriate concentrations of drug were added in quadruplicate and the cells incubated a further 4 hours DCFHDA was then added to two of the four wells treated with each drug at a final concentration of 30 µM and incubated for 40 minutes The plate was then washed three times with PBS Fluorescence was then read at 485 nm excitation and 535 nm emission in a fluorescence plate reader (Synergy HT, Bio-Tek, USA) The wells containing drug but not DCFHDA acted as blanks for each sample ROS production was expressed as % increase in fluorescence relative to untreated control cells

2 17 Drug metabolism by cells and microsomes

Drug metabolism was investigated in the microsomal fraction of recombinant insect cells (microsomes) expressing CYP3A4 and P450 NADPH reductase (P450R) (Gentest, 456202), CYP1B1 and P450R (Gentest, 456220) or P450R alone (Gentest, 456244) 50 µl of a stock solution of the appropriate chemotherapy drug were

prewarmed at 37° C in a total incubation mixture volume of 250 µl containing potassium phosphate buffer (PBS, pH 7.4), $MgCl_2$ (25 mM, 25 µL) and NADPH (6.5 mM, 75 µl). The reaction was initiated by the addition of 100 µl of a 1mg/ml solution of ice-cold microsomes to the incubation mixture, as recommended by the manufacturer. After an incubation period of either 30 or 60 minutes, the mixture was either filter sterilised and used for toxicity assays, or stored at–80°C until extracted for LC/MS analysis.

2.17.1 Extraction of Adriamycin-containing samples for LC/MS analysis

The frozen microsomal preparation was thawed. 20 µl of 33% silver nitrate were then added to the extraction tube, followed by 100 µl of Daunorubicin, which acted as an internal standard. The pH of the mixture was brought to appropriate levels by the addition of 100 µl of ammonium formate buffer (6.3 % ammonium formate, pH 8.5) and 700 µl of ice-cold isopropanol were then added to each tube. The mixture was then extracted with 1.4 ml chloroform, mixed on a blood tube mixer for 5 minutes and centrifuged at 4000 rpm for another 5 minutes. The liquid was then separated into two layers, of which the bottom layer contains the drug. Approximately 1.1 ml of liquid from the bottom layer was removed using a glass Pasteur pipette and transferred to a glass vial and the solvent evaporated under a stream of nitrogen. Adriamycin standards of known concentrations were processed in the same way. Samples were reconstituted with 80 µl of mobile phase and injected into the LC/MS for analysis.

2.17.2 LC/MS analysis

The LC-MS instrument used was a GE Healthcare (Amersham Biosciences) Ettan™ Multi Dimensional Liquid Chromatographic system (MDLC) interfaced to ThermoFinnigan™ PDA detector and a LTQ™ Mass Spectrometer. It was integrated into and operated from Thermo Electron's Xcalibur™ software. The chromatographic separation was performed on a Phenomenex® Prodigy ODS(3) 100 Å, 5µm, 150 x 2.1 mm column with a mobile phase of 72:28:0.1 (v/v/v) water-acetonitrile-formic acid delivered isocratically at a flow-rate of 200 µl/min. A 20 ul injection volume was used automatically through the MDLC autosampler.

Samples were analysed on the mass spectrometer using electro spray ionisation (ESI). The sheath gas, auxiliary gas and sweep gas flow rate (arbitrary units) for the ESI unit were set to 30, 10, and 1.9 units. The ESI Spray Voltage was 4.5 kV. The

capillary temperature was 300 °C and the capillary voltage was 39 V Mass spectral data were collected in the scan range 200-700 m/z Selected reaction monitoring (SRM) mode was used for quantification using the transition ion m/z 544→397, the $[M+H^+]$ Adriamycin adduct and 528→363, the $[M+H^+]$ Daunorubicin adduct The Adriamycin aglycone metabolite was identified by quantification of the ion with a m/z of 414

2 18 Transfection of mammalian cells with exogenous DNA

In order to understand the role of CYP1B1 more clearly, a vector containing the target cDNA sequence was transiently transfected into MDA R4 and MCF-7 cells to exogenously increase the level of expression of this gene A plasmid containing the CYP1B1 cDNA sequence was obtained from Dr Thomas Friedberg (Fig 2 1) and a control empty vector was generated from it (see Section 2 18 1) A plasmid containing the cDNA sequence for green fluorescent protein (GFP) was purchased from Open Biosystems (pEGFP-C1), this plasmid was used to optimise transfection conditions and check for efficiency of transfection Stock plasmid was produced by transforming competent JM109 cells, growing up a large stock of these and isolating the plasmid from them The isolated plasmid was then transfected into the chosen cell line

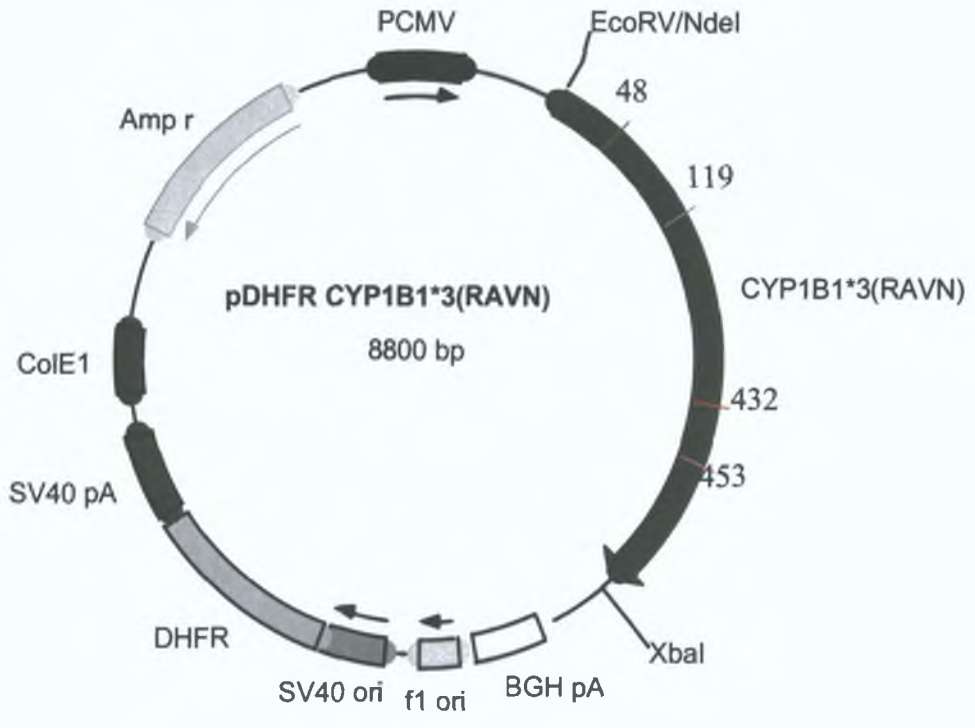


Figure 2.1: Structure of the CYP1B1 plasmid.

2.18.1 Generation of an empty vector plasmid

Digestion of the CYP1B1 plasmid was performed with several restriction enzymes in order to find two that would cut the DNA sequence at one site only, linearising the plasmid. Generation of linearised plasmid was checked by TAE low melt 1% gel electrophoresis of the digestion fragments using the appropriate markers. The restriction enzymes Xba I and Acc 65 I were chosen for the next step, where 5 µg of the CYP1B1 were digested with both endonucleases for 1 hour at 37°C to remove the gene coding sequence. The restriction digest was again analysed by electrophoresis and revealed the presence of the expected 7.2 kb and 1.6 kb fragments, corresponding to the vector backbone and the released CYP1B1 cDNA, respectively. The restriction digest was heated at 72°C for 10 minutes and placed on ice for 1 minute. After this step, 1 µl of Klenow enzyme was added, together with 20 µl Klenow buffer and 1 µl DNTPs. This mixture was incubated for 10 minutes at room temperature and then heated at 72°C and placed on ice as before. 2 µl of ligase were added to the previous solution and combined with 20 µl ligation mix. The mixture was incubated overnight at 4°C. Ligation of the plasmid was checked by electrophoresis, comparing against undigested and linearised plasmid.

2 18 2 Plasmid preparation

2 18 2 1 Transformation of JM109 cells

A bacterial cell suspension (100 µl) of competent JM109 (Promega, L2001) was mixed with 20 ng DNA and placed on ice for 40 min after which the mixture was heat-shocked at 42°C for 90 seconds and then placed on ice for 3 minutes LB broth (1 ml) ((10 g Tryptone (Oxoid, L42), 5 g Yeast Extract (Oxoid, L21) 5 g NaCl (Merck, K1880814))/litre LB, autoclaved before use) was added to the competent cell suspension and incubated at 37°C for 1 hour Different volumes of this suspension were spread on selecting agar plates (LB agar containing 100 mg/ml Ampicillin) and incubated overnight at 37°C Single colonies that grew on these selecting plates were incubated into 5 ml LB broth containing Ampicillin and grown for 8 hours with shaking at 37°C, cells were then subject to a DNA mini preparation (see section 2 18 2 2) or large scale preparation (Section 2 18 2 3) For a large scale DNA preparation this culture was then inoculated into 270 ml LB broth containing Ampicillin (100mg/ml) in a 1 L baffled flask with shaking overnight at 37°C

2 18 2 2 DNA Miniprep of plasmid DNA

The Qiagen Plasmid DNA Extraction Mini Kit (Qiagen, 12143) was used according to the manufacturer's instructions to isolate plasmid DNA from a 5 ml culture grown overnight in LB broth containing selection antibiotic with shaking at 37°C After incubation, the cells were pelleted by centrifugation at 8,500 rpm for 3 minutes Pelleted cells were resuspended in 250 µl of buffer P1 and the cell suspension was transferred to a microcentrifuge tube To this solution, 250 µl buffer P2 were added and mixed gently A volume of 350 µl buffer N3 was then added to the solution and the tube was mixed immediately This mixture was centrifuged for 10 minutes at 13,000 rpm The supernatant was applied to a QIAprep spin column and centrifuged for 60 seconds The flow-through was discarded The column was washed by adding 500 µl buffer PB and centrifuging for 60 seconds Again, the flow through was discarded This step was repeated with 750 µl buffer PB The column was centrifuged for a further 60 seconds to remove the remaining wash buffer and was then placed in a clean microcentrifuge tube To elute the DNA, 50 µl of buffer EB were added to the column The column was let stand for 60 seconds and then centrifuged for 60

seconds The DNA concentration of the eluate was determined by measuring the OD_{260nm} Plasmid was stored at –20°C until required

2 18 2 3 DNA Maxiprep of plasmid DNA

Plasmid DNA was purified using an Endofree Plasmid Maxi kit (Qiagen, 12362) Following growth of plasmid culture in 270 ml Ampicillin-containing LB broth in 1L baffled flask with shaking overnight at 37°C, the culture was split into 2 x 250 ml centrifuge bottles and spun at 3,500 rpm for 15 minutes at 4°C Pellets were processed immediately or stored at –20°C until required

The bacterial pellet was resuspended in 10 ml buffer P1 To this suspension, 10 ml buffer P2 was added, mixed gently and incubated at room temperature for 5 minutes A volume of 10 ml chilled buffer P3 was then added to the lysate and mixed gently and immediately The lysate was poured into the barrel of a QIAfilter cartridge and incubated at room temperature for 10 minutes The cap from the QIA filter outlet nozzle was then removed and the plunger gently inserted into the cartridge The cell lysate was filtered into a 50 ml tube To the lysate, 2 5 ml buffer ER were added and mixed by inverting repeatedly This mixture was incubated on ice for 30 minutes A Qiagen-tip 500 column was equilibrated by applying 10 ml buffer QBT and the column was allowed to empty by gravity flow The filtered lysate was then applied to the Qiagen-tip and also allowed to enter the resin by gravity flow The Qiagen-tip was washed twice with 30 ml buffer QC The DNA was then eluted by addition of 15 ml buffer QN and the eluate was collected in a 30 ml tube The DNA was precipitated by adding 10 5 ml isopropanol to the DNA solution, which was then mixed and centrifuged at ~10,000 rpm for 30 minutes at 4°C The DNA pellet was washed with 5 ml of 70% ethanol and again centrifuged at ~10,000rpm for 10 minutes The pellet was then air-dried and dissolved in 500 µl buffer TE The DNA concentration was determined by measuring the OD_{260nm}

2 18 3 Optimisation of plasmid transient transfection

Transient transfection was initially optimised for MDA R4 cells Cell suspensions of different concentrations (5 x 10^4, 1 x 10^5 and 2 x 10^5 cells/ml) were prepared in complete growth medium, 5 ml of these suspensions were seeded in a T25 flask Lipofectamine 2000 (Invitrogen, 11668019) and GFP plasmid DNA were prepared

with the volumes of Lipofectamine 2000 and cDNA being varied Different amounts of plasmid DNA and Lipofectamine 2000 were tested in order to ascertain the most efficient transfection conditions, as follows,

- 4 μg GFP cDNA 10 μl Lipofectamine 2000
- 8 μg GFP cDNA 20 μl Lipofectamine 2000

Briefly, the plasmid was incubated at room temperature in serum-free medium (OptiMEM) for 5 minutes The appropriate volume of Lipofectamine 2000 was also incubated in OptiMEM in the same conditions Both solutions were combined, mixed gently and allowed to form complexes over a 20-minute incubation at room temperature A 1 ml aliquot of this complex was added to each T25 flask and mixed gently The cells were trypsinised 48 hours after transfection, diluted to a concentration of 200 cells/μl and loaded onto a Guava flow cytometer (Guava Technologies, USA) A total of 5000 cells per sample were counted and plotted with the Guava Express program as green fluorescence versus forward scatter The proportion of green fluorescent cells was calculated by the Guava software and expressed as % of the total number of cells The conditions chosen were 1 x 10^5 cells/ml, 4 μg GFP cDNA 10 μl Lipofectamine 2000, which gave over 50% transfection efficiency

The transient transfection protocol was also optimised for MCF-7 cells To this end, cell suspensions of different concentrations were prepared at different concentrations (1 x 10^5, 2 x 10^5 and 4 x 10^5 cells/ml) in complete growth medium, 2 ml of these suspensions were seeded 24 hours before transfection in each well of a 6-well plate Again, different amounts of plasmid DNA and Lipofectamine 2000 were tested in order to ascertain the most efficient transfection conditions, as follows,

- 2 5 μg GFP cDNA 9 μl Lipofectamine 2000
- 5 μg GFP cDNA 18 μl Lipofectamine 2000

A 500 μl aliquot of these complexes was added to each well of a 6-well plate and mixed gently The cells were trypsinised 48 hours after transfection and loaded onto a Guava flow cytometer, as before The proportion of green fluorescent cells was calculated by the Guava software and expressed as % of the total number of cells The conditions chosen were 2 x 10^5 cells/ml, 2 5 μg GFP cDNA 9 μl Lipofectamine 2000, which gave over 50% transfection efficiency

2 19 RNA interference (RNAi)

RNAi using small interfering RNAs (siRNAs) was carried out to silence the expression of specific genes The siRNAs used were chemically synthesized and purchased from Ambion Inc These siRNAs were 21-23 bps in length and were introduced to the cells via reverse transfection with the transfection agent siPORT™ *Neo*FX™ (Ambion inc , 4511)

2 19 1 Transfection optimisation

In order to determine the optimal conditions for siRNA transfection, an optimisation with an siRNA for kinesin (Ambion Inc , 16704) was carried out for each cell line Cell suspensions were prepared at 5 x 10^4 and 7 5 x 10^4 cells per ml Solutions of negative control and kinesin siRNAs at a final concentration of 30 nM were prepared in optiMEM (Gibco™, 31985) NeoFX solutions at a range of concentrations were prepared in optiMEM in duplicate and incubated at room temperature for 10 minutes After incubation, either negative control or kinesin siRNA solution was added to each NeoFX concentration, these solutions were mixed well and incubated for a further 10 minutes at room temperature Replicates of 10 µl of the siRNA/NeoFX solutions were added to a 96-well plate The cell suspensions were then added to each plate at a final cell concentration of 5 x 10^3 and 7 5 x 10^3 cells per well The plates were mixed gently and incubated at 37°C for 24 hours After this period, the transfection mixture was removed from the cells and the plates were fed with fresh medium The plates were then assayed for changes in proliferation at 72 hours using the acid phosphatase assay Optimal conditions for transfection were determined as the combination of conditions which gave the greatest reduction in cell number after kinesin siRNA transfection and also the least cell kill in the presence of transfection reagent The optimised conditions for the cell lines are shown in Table 2 10

Cell line	Seeding density per well	Volume NeoFX per well (µl)
MCF-7 Txt	7.5×10^3	0.6

Table 2.10 Optimised conditions for siRNA transfection

2.19.2 Toxicity assays on siRNA-transfected cells

To assay for changes in sensitivity to chemotherapeutic agents, siRNA experiments in 96-well plates were set up using the optimised conditions and the appropriate siRNAs, as listed in Table 2.11. Transfection medium was removed after 24 hours and replaced with fresh growth medium. Concentrations of the chemotherapeutic agent (2X) were added to the plates in replicates of four 48 hours after transfection. The plates were assayed for changes in proliferation at 72 hours using the acid phosphatase assay.

Target name	Ambion IDs
Scrambled	Negative control #2
CYP1B1.1	2253
CYP1B1.2	105952
CYP1B1.3	112548

Table 2.11 List of siRNAs used

2 20 Statistics

Analysis of the significance of the difference in the mean IC50 value calculated from toxicity assays was performed using an unpaired, two-tailed student t-test assuming unequal variances, which was run on Microsoft Excel

A p value > 0 05 was deemed not significant
A p value < 0 05 was deemed significant
A p value < 0 005 was deemed highly significant

Section 3.0

Results

3.1 Expression of drug metabolism–related genes in normal and tumour breast tissue

Expression of enzymes involved in drug metabolism has been readily detected in every different type of tissue studied to date, both normal and tumour, techniques employed for detection include RT-PCR, Western blot, immunocytochemistry and immunohistochemistry Enzymatic activity measurements have also been performed in tissue homogenates and/or isolated microsomes, which are cell fractions mainly composed of endoplasmic reticulum membranes Although the presence of metabolic enzymes in both normal and tumour tissue has been demonstrated, reports attempting to compare the relative expression of these enzymes in malignant versus normal tissue have generated contradictory results

Microarray technology constitutes a more recent approach for the detection and quantification of gene expression in a particular type of tissue This technique allows for direct analysis of the expression of thousands of different genes at the same time, it is therefore a powerful tool for the study of differential patterns of expression in normal and tumour tissue

3 1 1 Analysis of drug metabolism-related genes in normal and tumour breast tissue using microarrays

Whole genome expression microarrays were used in our centre in an attempt to identify differences in the expression profiles of normal and malignant breast tissue samples This study involved the analysis of 106 breast cancer biopsies, of which 84 were classified as invasive ductal carcinoma, 17 were invasive lobular and 5 were tumours of special type (2 tubular and 3 mucinous) Samples were removed prior to any treatment with Tamoxifen or chemotherapeutic agents Information was gathered regarding tumour size, grade, presence of lymph node metastasis, oestrogen receptor staining and age of patient at diagnosis, and the follow-up information was also recorded Microarray analysis was performed on all 106 cases and also on 20 normal breast tissue specimens Following preliminary analysis, 2 tumour specimens and 3 normal specimens were removed from the study Extraction of mRNA from tissue samples was performed by Dr Lorraine O'Dnscoll and Dr Elaine Kenny, while microarray testing was carried out by Dr Patrick Gammell, Dr Padraig Doolan and

Helena Joyce Data analysis was performed by Mr Jai Prakash Mehta and Mr Eoin Ryan Correlation analysis was performed by Dr O'Driscoll

The presence of several different drug metabolism-related enzymes was detected in both normal and tumour tissue, as shown in Tables 3 1 1, 3 1 2 and 3 1 3

Gene ID	Gene name	Normal (%)	Tumour (%)
NM_000499	CYP1A1	23 5	6 7
NM_000761	CYP1A2	88	96
NM_000104	CYP1B1	100	100
NM_000762	CYP2A6	35 3	30 5
NM_000767	CYP2B6	0	4 8
NM_000770	CYP2C8	11 8	4 8
NM_030878	CYP2C8	5 9	31 4
NM_000771	CYP2C9	17 6	5 7
NM_000772	CYP2C18	41 2	15 2
NM_000106	CYP2D6	11 8	52 4
AF182276	CYP2E1	47	77
NM_000775	CYP2J2	35 3	79
NM_024514	CYP2R1	11 8	33 3
BC012027	CYP2U1	0	1
NM_017781	CYP2W1	47	33 3
NM_017460	CYP3A4	58 8	61 9
NM_022820	CYP3A43	11 8	9 5
NM_000777	CYP3A5	58 8	13 3
NM_000765	CYP3A7	23 5	30 5
NM_000941	NADPH cytochrome P450 oxidoreductase	82	95

Table 3 1 1 Expression of members of the P450 superfamily expressed in breast tissue samples as percentage of samples positive for each particular gene

Gene ID	Gene name	Normal (%)	Tumour (%)
NM_000846	Glutathione transferase A1	23 5	18 1
NM_000847	Glutathione transferase A3	5 9	10 5
NM_001512	Glutathione transferase A4	100	100
NM_015917	Glutathione transferase K1	100	100
NM_000561	Glutathione transferase M1	94 1	76 2
NM_000848	Glutathione transferase M2	94 1	95 2
A1459140	Glutathione transferase M3	94	81
NM_000850	Glutathione transferase M4	52 9	21 9
NM_000851	Glutathione transferase M5	100	92 4
NM_004832	Glutathione transferase O1	100	100
NM_000852	Glutathione transferase P1	100	100
NM_000853	Glutathione transferase T1	76 5	80
NM_000854	Glutathione transferase T2	23 5	39
NM_001498	Gamma glutamyl cysteine synthetase, heavy subunit	100	98 1
NM_002061	Gamma glutamyl cysteine synthetase, light subunit	100	98 1
NM_005265	Gamma glutamyl transpeptidase	88 2	97 1
NM_000581	Glutathione peroxidase 1	100	100
NM_002083	Glutathione peroxidase 2	23 5	50 5
NM_002084	Glutathione peroxidase 3	94 1	98 1
NM_002085	Glutathione peroxidase 4	100	100
NM_003996	Glutathione peroxidase 5	5 9	1 9
NM_000637	NADPH Glutathione reductase	47 1	61

Table 3 1 2 Expression of glutathione-related genes in breast tissue samples expressed as percentage of samples positive for each particular gene

Gene ID	Gene name	Normal (%)	Tumour (%)
NM_001621	Aryl hydrocarbon receptor	100	98 1
NM_014862	Aryl hydrocarbon receptor nuclear translocator protein 2	17 6	75 2

Table 3 1 3 Expression of drug metabolism-related transcription factors in breast tissue samples expressed as percentage of samples positive for each particular gene

As seen in the above tables, presence of drug metabolism–related genes is a common event in both normal and tumour tissue Some of these genes also show differential regulation, their expression levels significantly changed in malignant as compared to normal tissue Amongst the P450s, CYP2C8, CYP2D6, CYP2E1 and CYP2J2 show increased frequency of expression in tumour as compared to normal tissue, on the other hand, CYP1A1, CYP2C18, CYP2W1 and CYP3A5 are more commonly found in normal than in tumour tissue As for the glutathione (GSH)-related genes, only two appear to be differentially regulated in tumour and normal tissue Expression of GSH transferase (GST) M4 is less frequent in tumour than in normal samples, while that of GSH peroxidase 2 is more common in tumour tissue The aryl hydrocarbon receptor nuclear translocator protein 2 is also considerably more frequent in tumour than in normal samples, suggesting that it could be used as a biomarker of disease

Alterations in expression can be seen in a different aspect in Table 3 1 4, which shows significant changes in the expression of genes involved in drug metabolism as detected in normal and tumour tissue In this table, changes in expression are stated as fold change between normal and tumour sample values, these values are related to the intensity of microarray probe binding and are expressed in arbitrary units For every gene of interest, the microarray platform used has 11 different probes, Table 3 1 4 refers to the results obtained with specific probes, hence the existence of more than one entry for a single gene

Probe set	Gene name	Normals	Tumours	Fold change	p value
202435_s_at	CYP1B1	649	973	1.5	0.018
214320_x_at	CYP2A6	37	472	12.8	0.008
1494_f_at	CYP2A6	37	394	10.7	0.023
200736_s_at	Glutathione peroxidase 1	4152	1867	-2.2	<0.001
214091_s_at	Glutathione peroxidase 3	2159	169	-12.8	<0.001
201348_at	Glutathione peroxidase 3	3189	210	-15.2	<0.001
201106_at	Glutathione peroxidase 4	5192	2084	-2.50	<0.001
225609_at	Glutathione reductase	259	494	1.9	<0.001
235405_at	Glutathione S-transferase A4	58	173	3.0	<0.001
202967_at	Glutathione S-transferase A4	314	204	-1.5	0.005
217751_at	Glutathione S-transferase kappa 1	1294	953	-1.4	0.027
227163_at	Glutathione S-transferase omega 2	60	323	5.3	<0.001
203815_at	Glutathione S-transferase theta 1	550	242	-2.3	0.019
201415_at	Glutathione synthetase	273	491	1.8	<0.001

Table 3.1.4: List of drug metabolism-related genes with significant changes in expression levels in tumour relative to normal breast tissue. Fold change is calculated as intensity values of nomal/intensity values of tumours.

As can be seen in list of genes in Table 3.1.4, frequency of expression does not necessarily correlate with changes in the expression levels of a particular gene; many of the genes listed in Tables 3.1.1-3.1.3 are not differentially expressed in malignant as compared to normal tissue. The P450s in this list are upregulated in tumour tissue, in accordance with the general tendency observed in P450 expression studies. The GSH-related genes, on the other hand, appear to be preferentially downregulated in tumour tissue, with the exception of GSH reductase, GST omega 2, GSH synthetase and one of the probes of GST A4.

For those genes that were significantly expressed (i e , present in 15-85% of samples), the possibility of a correlation between level of expression and overall survival, relapse-free survival, relevance as a positive (good) or negative (bad) prognostic factor, age at diagnosis, oestrogen receptor status, lymph node spread, tumour type, tumour size and tumour grade was analysed The results are shown in Tables 3 1 5 and 3 1 6

mRNA	OS	G/B	RFS	G/B	Age at Diagnosis	ER Status	Lymph Node Spread	Tumour Type	Tumour Size	Tumour Grade
CYP2A6	N	N/A	N	N/A	N	N	N	N	N	N
CYP2C8	N	N/A	N	N/A	N	N	N	N	N	N
CYP2C18	N	N/A	N	N/A	N	N	N	N	N	N
CYP2D6	N	N/A	N	N/A	N	Y (ER+)	N	N	N, but p=0.053 (p α smaller)	N
CYP2E1	N	N/A	N	N/A	N	N	N	N	N	N
CYP2J2	N	N/A	N	N/A	N	N	N	N	--------	N
CYP2R1	N (but p=0.0814)	P ~ good	N	N/A	N	N	N	N	N	N
CYP2W1	N	N/A	N	N/A	N	N	N	N	N	N
CYP3A4	N	N/A	N	N/A	N	N	N	N	N	N
CYP3A7	N	N/A	N	N/A	N	N	N	N	N	N
ARNT2	Y (p=0.0018)	G	Y (p=-0.0094)	G	N	N but p=0.052 (p α ER+)	N	N	---------	p α lower grade

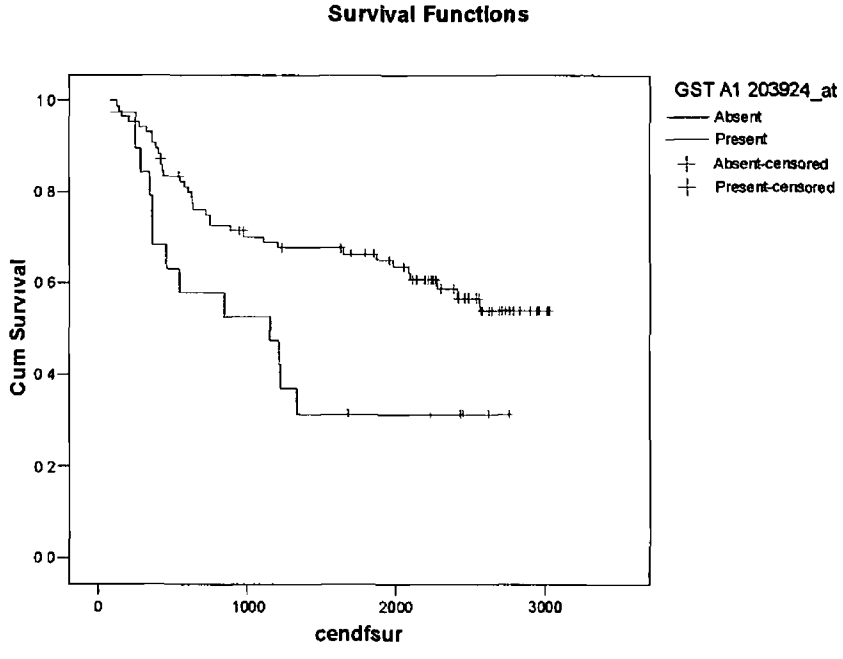
Table 3.1.5: Correlation between mRNA expression of P450s and ARNT2 and overall survival (OS), relapse-free survival (RFS), age at diagnosis, oestrogen receptor (ER) status, lymph node spread, tumour type, tumour size and tumour grade. G: good prognostic factor. B: bad prognostic factor. α: directly proportional to. p: probability value. Y: yes. N: no. N/A: not analysed.

mRNA	OS	G/B	RFS	G/B	Age at Diagnosis	ER Status	Lymph Node Spread	Tumour Type	Tumour Size	Tumour Grade
GST A1	Y (p=0.0028)	B	Y (p=0.0131)	B	N	N	----------	N	N	N
GST M1	N	N/A	N	N/A	N	N	N	N	N	N
GST M3	N	N/A	N	N/A	N	N	N	N	N	N
GST M4	N	N/A	N	N/A	Y (p=0.030; older age)	N	----------	N	N	N
GST T1	N	N/A	N	N/A	N	N	N (but p=0.054)	N	N	N
GST T2	N	N/A	N	N/A	Y (p α older patients)	N	N	N	N	N
Glutathione Peroxidase 2	N	N/A	N	N/A	----------	N	N	N	N	N
NAPDH Glutathione Reductase	N	N/A	N	N/A	N	N	N	N	N	N

Table 3.1.6: Correlation between GSH-related gene expression and overall survival (OS), relapse-free survival (RFS), age at diagnosis, oestrogen receptor (ER) status, lymph node spread, tumour type, tumour size and tumour grade. G: good prognostic factor. B: bad prognostic factor. α: directly proportional to. p: probability value. Y: yes. N: no. N/A: not analysed.

As seen in the previous tables, both ARNT2 and GST A1 significantly correlate with patient survival Patient disease-free survival was plotted relative to the presence or absence of GST A1 and ARNT2 (Figs 3 1 1 A and B)

A



B

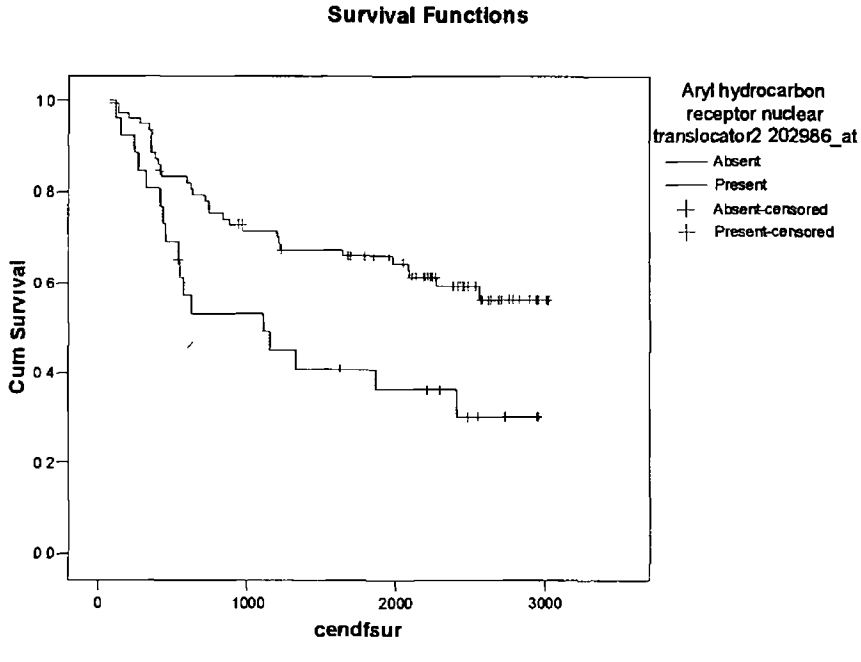


Figure 3 1 1 Censored disease-free survival data of patients with positive (present) or negative (absent) expression of GST A1 (A) and ARNT2 (B) Cum survival cumulative survival Cendfsur censored disease-free survival

Fig 3 1 1 shows graphs that present "censored" data this means that the data have been corrected by taking into account those patients for whom complete follow-up information was not available This is a common event in this type of studies some patients move to a different place, or no longer attend follow-up clinics Their data are therefore included in the study, but only up to the last time that information was available on their status After that, they're no longer considered for the statistics calculations

As seen in these graphs, presence of GST A1 is a strong predictor of poor prognosis, while ARNT2 is associated with a longer period of disease-free survival

3 2 Anticancer drug metabolism by cytochromes P450

A number of chemotherapeutic drugs are metabolised by cytochromes P450, among them, Adriamycin has been reported to undergo metabolism by CYP3A4 to generate less active compounds (Baumhakel *et al*, 2001) In contrast, 5-fluorouracil is metabolised by enzymes involved in thymidine synthesis and catabolism and is not a substrate of P450s Even though expression of CYP1B1 has been reported to confer resistance to Taxotere, no CYP1B1 metabolites of this drug have been described to date

Insect cells are widely used to express recombinant human proteins, as they can perform post-translational modifications in a similar – albeit not identical - way to mammalian cells In these cells, recombinant baculovirus particles are used as vectors to transfect foreign cDNA

Recombinant insect cells have been developed, which are infected with baculovirus carrying human cytochrome P450 and P450 NADPH reductase (P450R) or P450R cDNA alone, control cells are transfected with wild type baculovirus containing no foreign cDNA The microsomal fractions of these cells, which consists of fragments of endoplasmic reticulum, display high levels of P450 activity and are commercially available These fractions, termed microsomes, were used to study the metabolism of anticancer drugs by cytochromes P450 and P450R

3 2 1 Drug metabolism by CYP3A4

3 2 1 1 Measurement of testosterone β-hydroxylase activity in CYP3A4 microsomes

An HLPC method was adapted (from Whalley *et al*, 2001, see Section 2 12) and optimised for the detection of CYP3A4 activity This method is based on the metabolism of testosterone, which is converted to 6ß-hydroxy testosterone by members of the CYP3A subfamily Testosterone and its metabolite can then be separated by reverse-phase HPLC and quantified by UV absorbance An internal standard, corticosterone, is added to each sample at a known concentration as means of reference In the optimised conditions, 6β-hydroxy testosterone elutes at around four minutes, while intact testosterone does so at eleven minutes, corticosterone elutes at about nine minutes (Fig 3 2 1)

Microsomes expressing CYP3A4 and P450R or P450R alone were incubated with testosterone for 15 minutes at 37°C The reaction mixture was then extracted and testosterone and its metabolites separated by HPLC

As seen in Fig 3 2 1 and Table 3 2 1, CYP3A4-expressing microsomes possess significant testosterone 6β-hydroxylase activity The presence of metabolite was undetectable in P450R microsomes

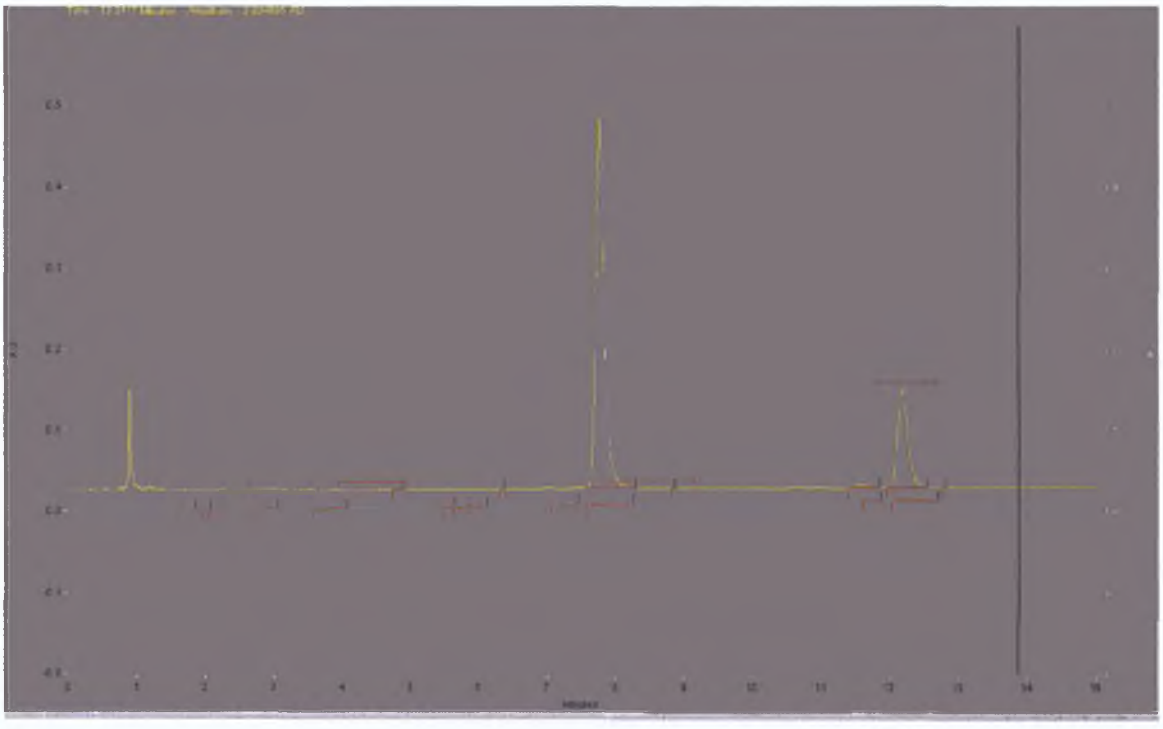


Figure 3.2.1: Determination of CYP3A activity on CYP3A4 microsomes by HPLC. Testosterone elutes at approximately 12 minutes, while 6β-hydroxy testosterone does so at approximately 4 minutes. corticosterone, used here as internal standard, elutes at approximately 8 minutes. Yellow line: P450R microsomes. Blue line: CYP3A4+P450R microsomes.

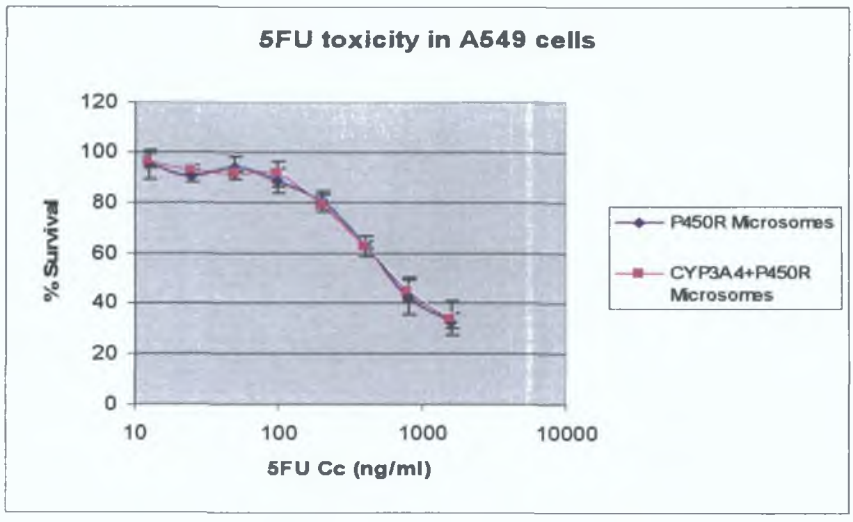
	CYP3A activity (pmol/mg x min)
P450R microsomes	n.d.
CYP3A4 + P450R microsomes	0.353+/-0.086

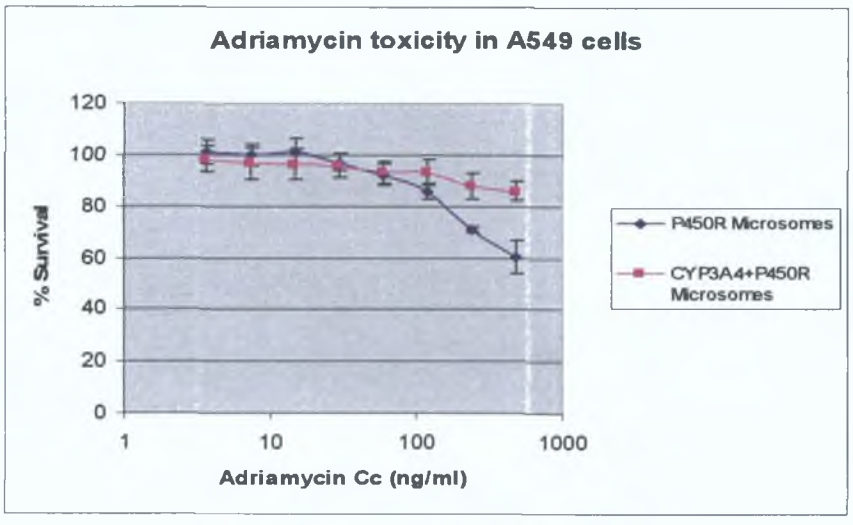
Table 3.2.1: Measurement of testosterone 6β-hydroxylase activity on CYP3A4 microsomes by HPLC. Activity is expressed as pmoles of 6ß-hydroxy testosterone produced per minute per mg of total protein. Microsomes were incubated for 15 minutes at 37°C in a reaction mixture containing testosterone, NADPH, $MgCl_2$ and microsomal protein in PBS, pH 7.4. Results are expressed as mean +/- SD, representing the average of three independent experiments. n.d.: not detectable.

3 2 1 2 CYP3A4 metabolism and drug toxicity in A549 cells

In order to study the degree to which P450 activity affects the toxicity of anticancer drugs, *in vitro* toxicity assays were performed on A549 cells, where the drug in question was incubated with recombinant insect cell microsomal fractions before being added to the cells (see Section 2 17) A549 cells were chosen for these assays since they display no measurable testosterone 6β-hydroxylase activity as measured by HPLC, even after treatment with inducers such as Phenobarbital (data not shown) These cells were exposed to different concentrations of Adriamycin, Vincristine and 5-fluorouracil, which had been preincubated with the microsomal fractions of insect cells infected with either human CYP3A4 and P450R or P450R cDNA alone Independent experiments were carried out, in which the drug was incubated with microsomes for either 30 minutes or 1 hour

As seen in Figs 3 2 2 and 3 2 3, preincubation of 5-fluorouracil with CYP3A4-expressing microsomes or with microsomes expressing P450R alone yielded the same toxicity curve In the case of Vincristine and especially Adriamycin, a time-dependent decrease in drug toxicity was observed compared to the effect of preincubation with P450R microsomes, as the decrease was more evident after 1 hour incubation This could be attributed to CYP3A4-mediated drug inactivation





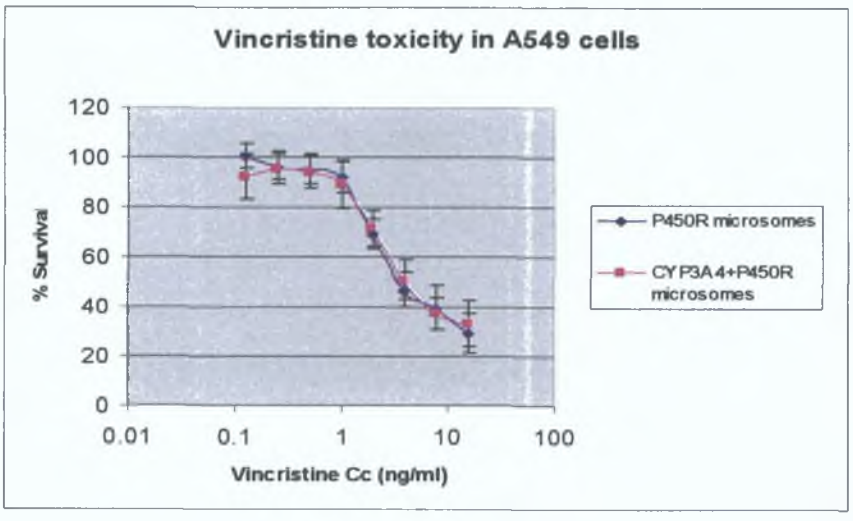
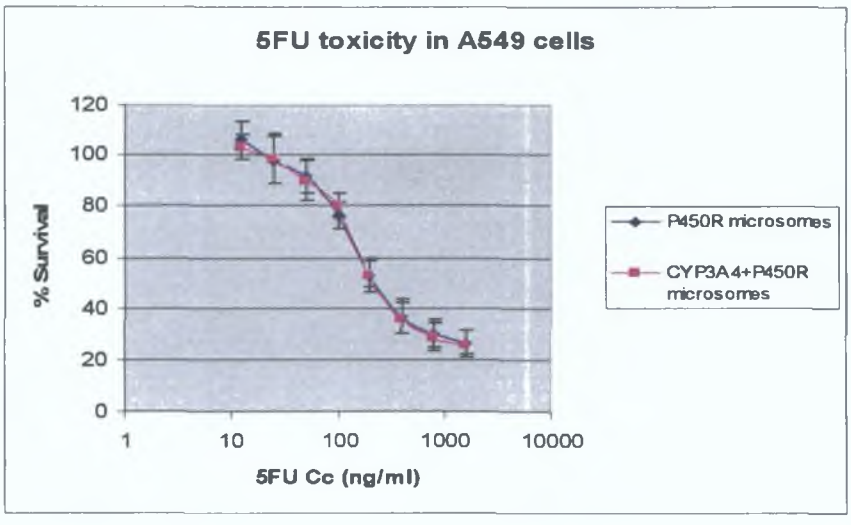
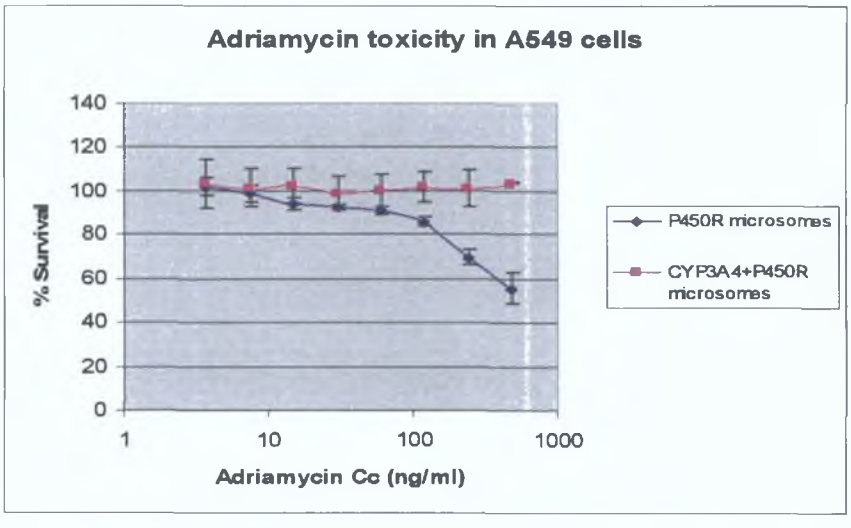


Figure 3.2.2: Drug toxicity on A549 cells as determined by the acid phosphatase assay. A known concentration of either 5-fluorouracil, Adriamycin or Vincristine was incubated with insect cell microsomes containing either CYP3A4 and P450R or P450R alone at 37°C for 30 minutes prior to addition to cells. The reaction mixture also contained 25 mM $MgCl_2$ and 6.5 mM NADPH in potassium phosphate buffer (PBS, pH 7.4). Results are the average of three independent experiments.





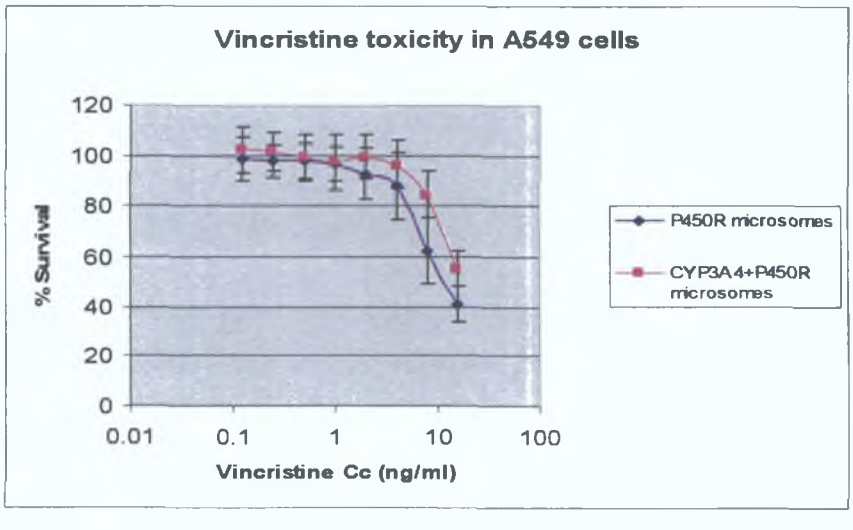


Figure 3.2.3: Drug toxicity on A549 cells as determined by the acid phosphatase assay. A known concentration of either 5-fluorouracil, Adriamycin or Vincristine was incubated with insect cell microsomes containing either CYP3A4 and P450R or P450R alone at 37°C for an hour prior to addition to cells. The reaction mixture also contained 25 mM $MgCl_2$ and 6.5 mM NADPH in potassium phosphate buffer (PBS, pH 7.4). Results are the average of three independent experiments.

3 2 2 Drug metabolism by CYP1B1

3 2 2 1 Measurement of EROD activity in CYP1B1 microsomes

Members of the CYP1 family, CYP1A1, CYP1A2 and CYP1B1, are capable of metabolising 7-ethoxy resorufin into the fluorescent compound resorufin, this reaction is referred to as ethoxy resorufin O-deethylation (EROD) EROD activity of CYP1B1 and P450R-containing microsomes was determined by fluorescence at various time points and subsequently compared to that of microsomes containing P450R alone (see section 2 13)

As shown in Fig 3 2 4 and Table 3 2 2, CYP1B1 microsomes display readily measurable EROD activity

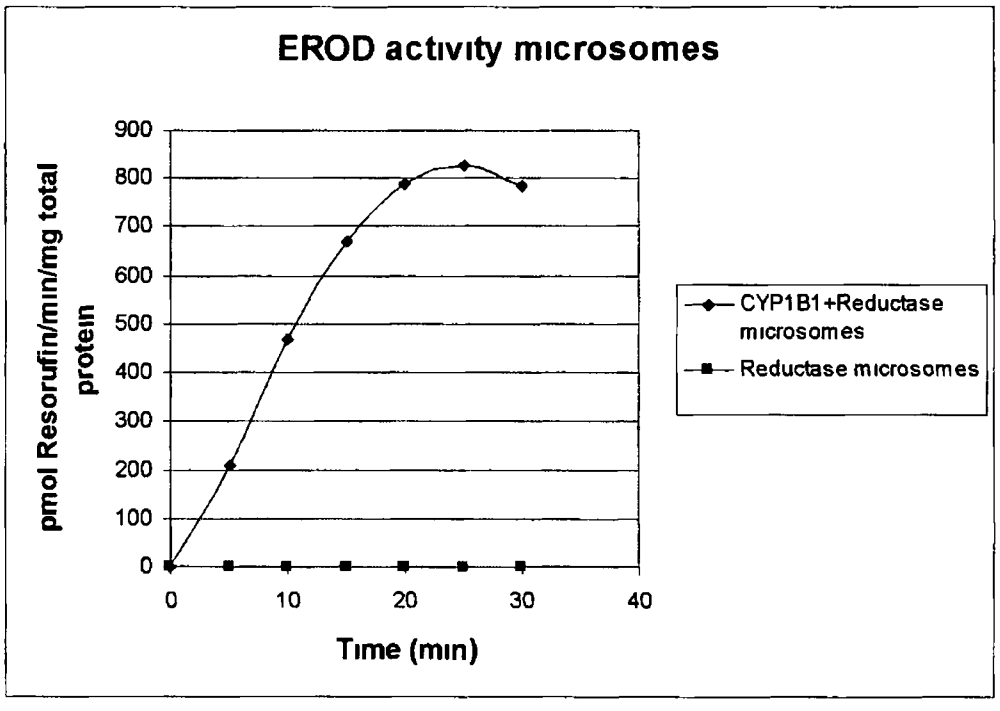


Figure 3 2 4 EROD activity measured at various time points and expressed as pmoles of resorufin produced per mg of total protein After addition of 7-ethoxy resorufin, microsomes were incubated at 37°C for the indicated periods of time The reaction mixture also contained 25 mM $MgCl_2$ and 6 5 mM NADPH in potassium phosphate buffer (PBS, pH 7 4) The graph represents the average of two independent experiments

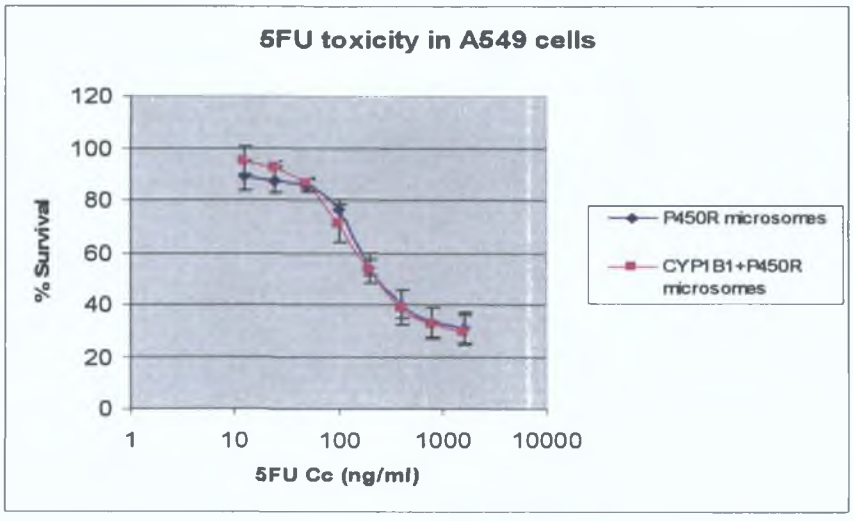
	EROD activity (pmol resorufin/min/mg total protein)
P450R microsomes	n d
CYP1B1 microsomes	1203

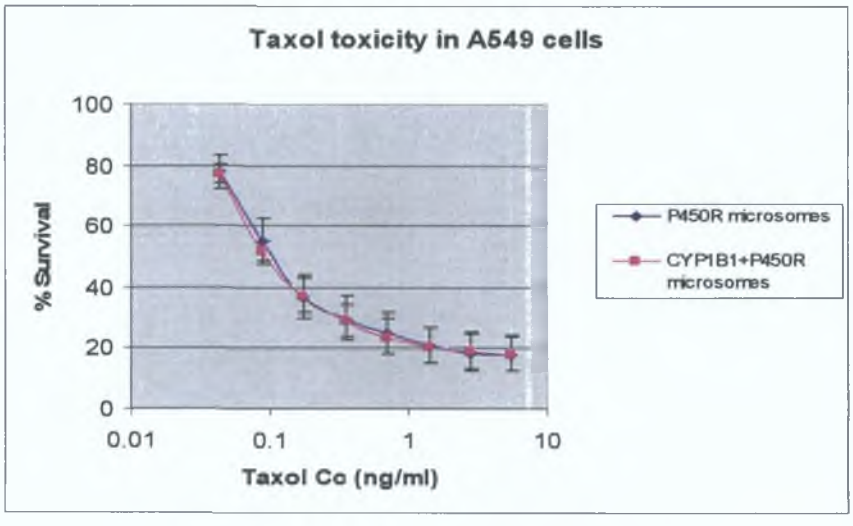
Table 3 2 2 Measurement of EROD activity on CYP1B1 microsomes by fluorescence Activity is expressed as pmoles of resorufin produced per minute per mg of total protein Microsomes were incubated for 25 minutes at 37°C in a reaction mixture containing 7-ethoxy resorufin, NADPH, $MgCl_2$ and microsomal protein in PBS, pH 7 4 n d not detectable Results are expressed as mean and represent the average of two independent experiments

3 2 2 2 CYP1B1 metabolism and drug toxicity in A549 cells

A similar approach to that of CYP3A4 was taken with CYP1B1 on A549 cells Although transfection of CYP1B1 has been reported to confer resistance to Taxotere, no Taxotere metabolites could be detected after incubation of the drug with CYP1B1 in a number of different conditions (Bournique *et al*, 2002) Toxicity of Taxol - a structurally and mechanistically related drug - was also tested, while 5-fluorouracil again acted as control

Figures 3 2 5 and 3 2 6 show that preincubation with CYP1B1 does not affect the toxicity of any of the drugs tested on A549 cells, at any of the two time points analysed (in independent experiments) In the case of 5-fluorouracil this is to be expected, since the drug does not undergo metabolism by this cytochrome Similarly, Taxol has not yet been confirmed as a CYP1B1 substrate As for Taxotere, the inability of CYP1B1 to inactivate the drug is consistent with the fact that no CYP1B1-generated Taxotere metabolite has been detected to date Altogether, these results suggest that Taxotere resistance arising from CYP1B1 transfection is not due to direct inactivation of the drug by this enzyme





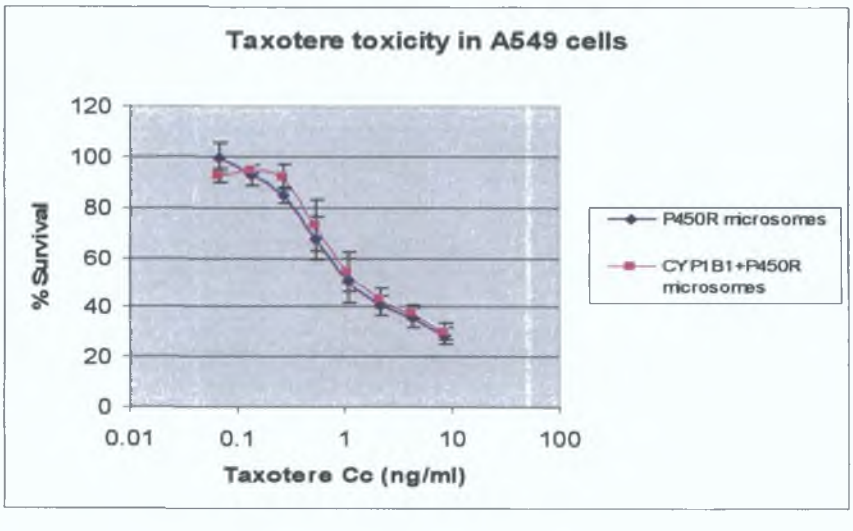
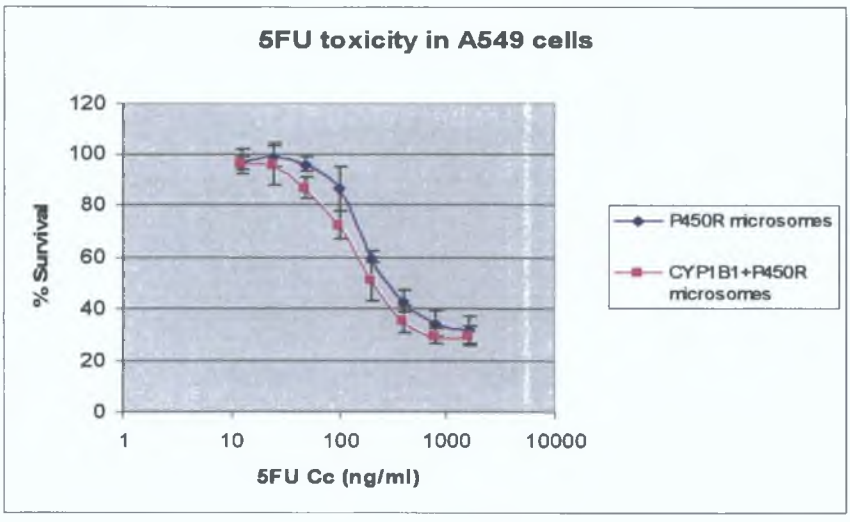
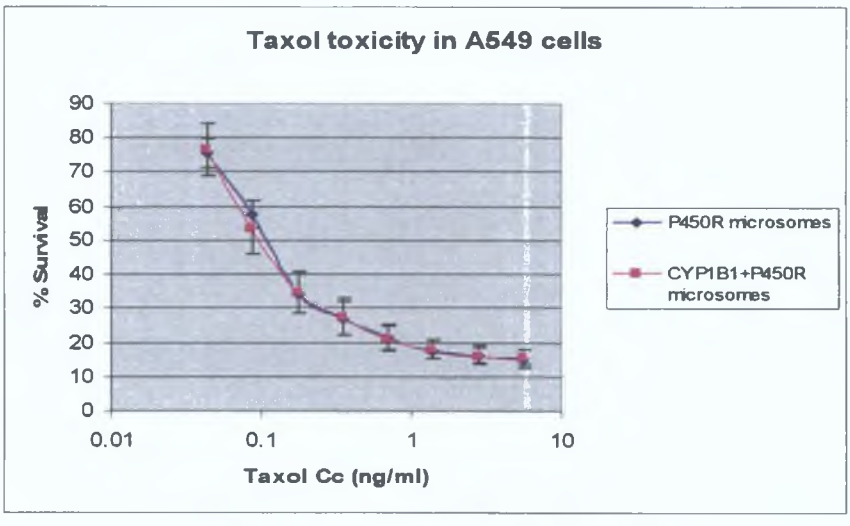


Figure 3.2.5: Drug toxicity on A549 cells as determined by the acid phosphatase assay. A known concentration of either 5-Fluorouracil, Taxol or Taxotere was incubated with insect cell microsomes containing either CYP1B1 and P450R or P450R alone at 37°C for 30 minutes prior to addition to cells. The reaction mixture also contained 25 mM $MgCl_2$ and 6.5 mM NADPH in potassium phosphate buffer (PBS, pH 7.4). The results represent the average of three independent experiments.





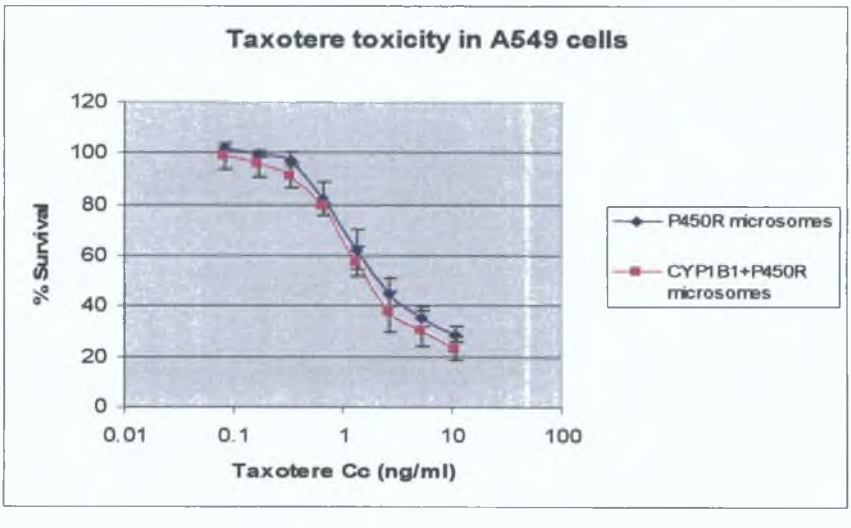


Figure 3.2.6: Drug toxicity on A549 cells as determined by the acid phosphatase assay. A known concentration of either 5-Fluorouracil, Taxol or Taxotere was incubated with insect cell microsomes containing either CYP1B1 and P450R or P450R alone at 37°C for 1 hour prior to addition to cells. The reaction mixture also contained 25 mM $MgCl_2$ and 6.5 mM NADPH in potassium phosphate buffer (PBS, pH 7.4). The results represent the average of three independent experiments.

3.2.3 Drug metabolism by P450R

3.2.3.1 P450R metabolism and drug toxicity in MDA 231 cells

In order to study the degree to which P450R activity affects anticancer drug-induced toxicity, *in vitro* toxicity assays were performed on MDA 231 cells transfected with P450R (MDA R4) or an empty vector (MDA EV), where the drug in question was preincubated with recombinant insect cell microsomal fractions expressing P450R or control microsomes, which display no measurable enzymatic activity.

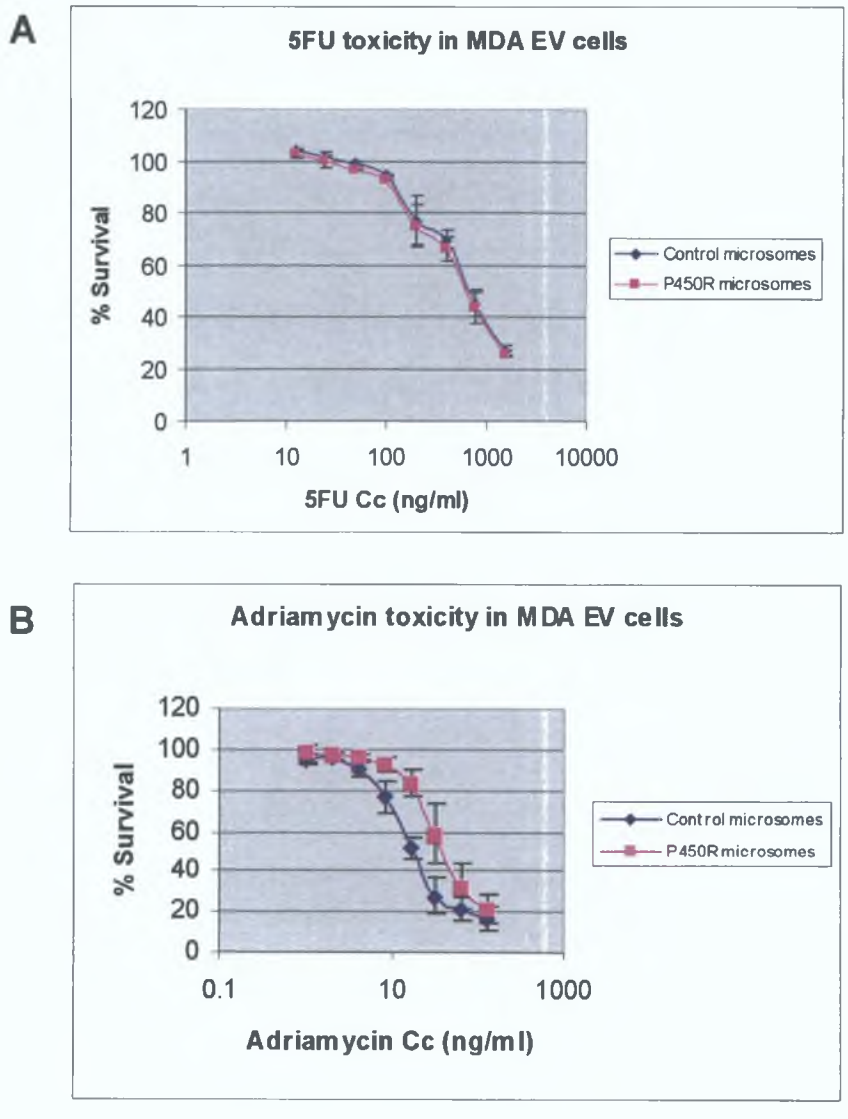
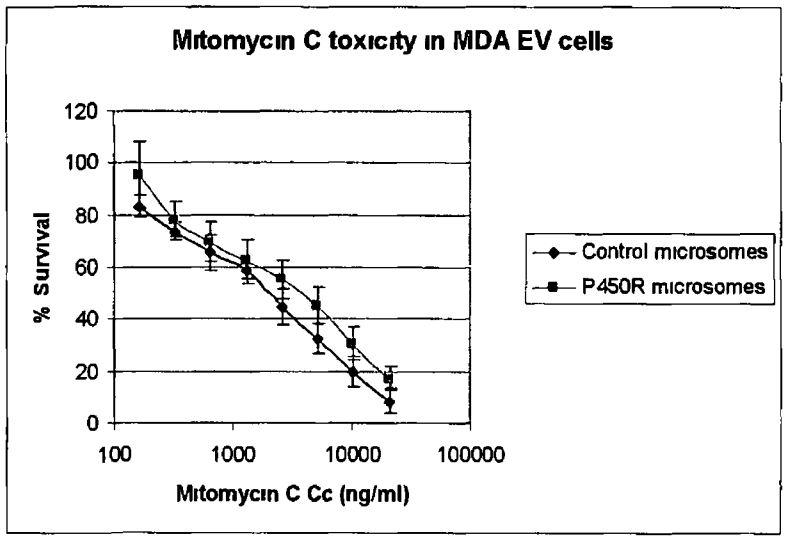


Figure 3.2.7: Toxicity induced by 5-fluorouracil (A) and Adriamycin (B) preincubated for 30 minutes with microsomes expressing P450R or control microsomes in MDA 231 EV cells. Control microsomes display no measurable P450R enzymatic activity. The results represent the average of three independent experiments.

A



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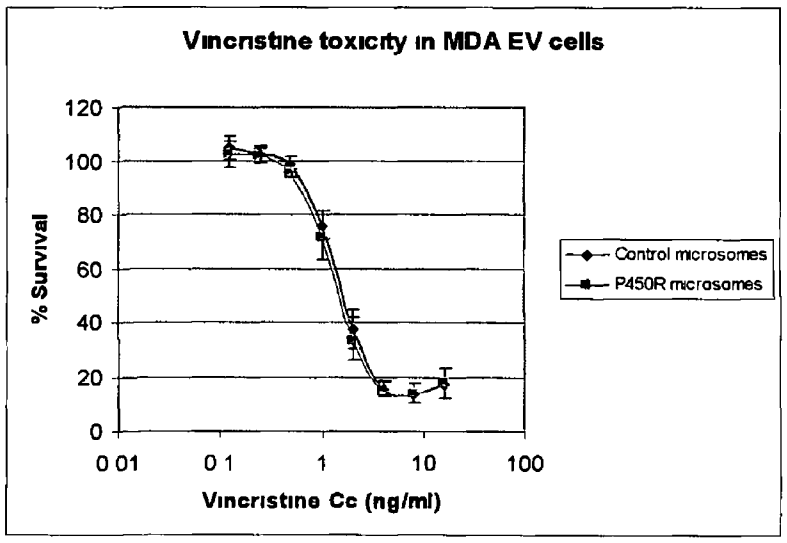


Figure 3 2 8 Toxicity induced by Mitomycin C (A) and Vincristine (B) preincubated for 30 minutes with microsomes expressing P450R or control microsomes in MDA 231 EV cells Control microsomes display no measurable P450R enzymatic activity The results represent the average of three independent experiments

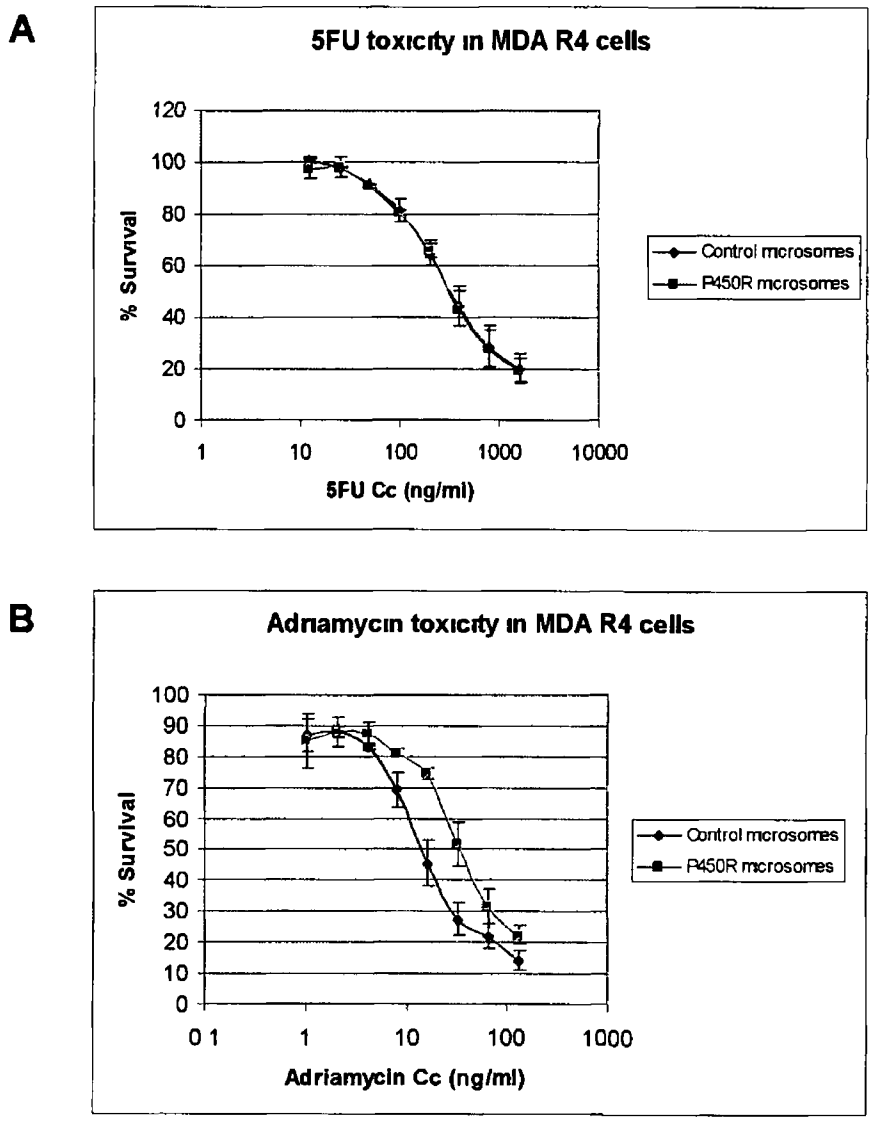
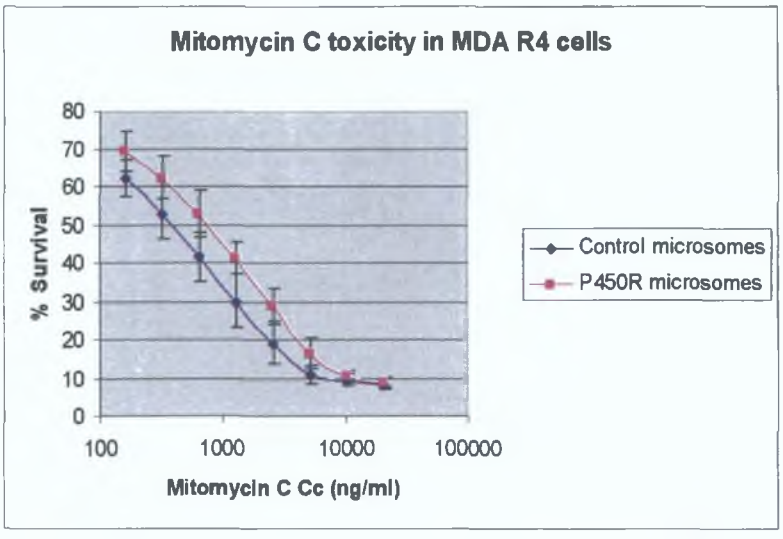


Figure 3 2 9 Toxicity induced by 5-fluorouracil (A) and Adriamycin (B) preincubated for 30 minutes with microsomes expressing P450R or control microsomes in MDA 231 R4 cells Control microsomes display no measurable reductase *enzymatic* activity The results represent the average of three independent experiments

A



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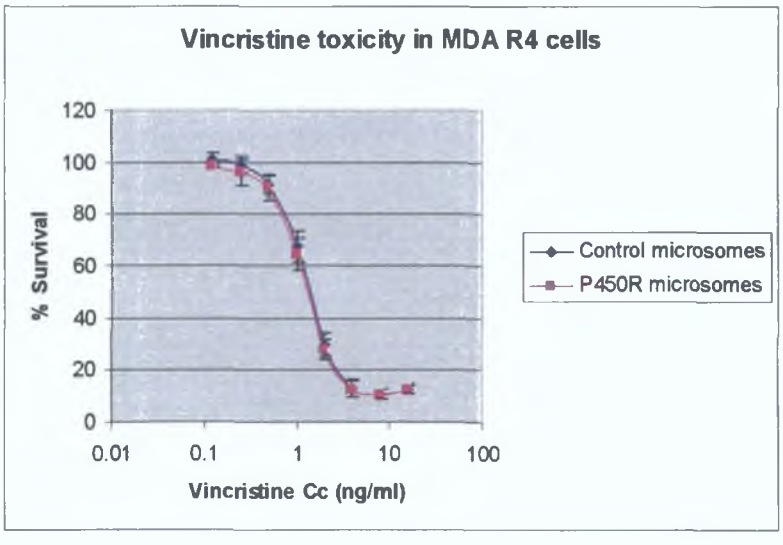


Figure 3.2.10: Toxicity induced by Mitomycin C (A) and Vincristine (B) preincubated for 30 minutes with microsomes expressing P450R or control microsomes in MDA 231 R4 cells. Control microsomes display no measurable reductase enzymatic activity. The results represent the average of three independent experiments.

Preincubation with P450R-expressing microsomes substantially decreased Adriamycin-induced toxicity in both MDA 231 EV and R4 cells (Figs. 3.2.7 to 3.2.10), indicating that the effect is independent of the expression of endogenous P450R. Mitomycin C also appeared to have less toxicity on A549 cells after incubation with P450R. Toxicity of Mitomycin C was higher in MDA R4 cells, but it should be noted that this drug is activated by cellular P450R to more toxic metabolites. On the other hand, toxicity of Vincristine and 5-fluorouracil was unaffected by preincubation with the enzyme. These results suggest that Adriamycin is inactivated to some extent by P450R before the drug is added to the cells, thereby reducing its cytotoxic effects.

3 2 3 3 Adriamycin metabolism by P450R microsomes

In order to study the metabolism of Adriamycin by P450R, this drug was incubated for 1 hour with either P450R-expressing or control microsomes The reaction mixture was then extracted and analysed by liquid chromatography followed by mass spectrometry (LC/MS) LC/MS operation was carried out by Dr Robert O'Connor Fig 3 2 11 shows the position of the standard peaks in the conditions used for the analysis, Adriamycin eluted at approximately 4 5 minutes, while Daunorubicin (used as internal standard) did so at approximately 10 minutes Adnamycin aglycone, the main metabolite of Adriamycin generated by P450R (Cummings *et al*, 1992), eluted at approximately 13 minutes

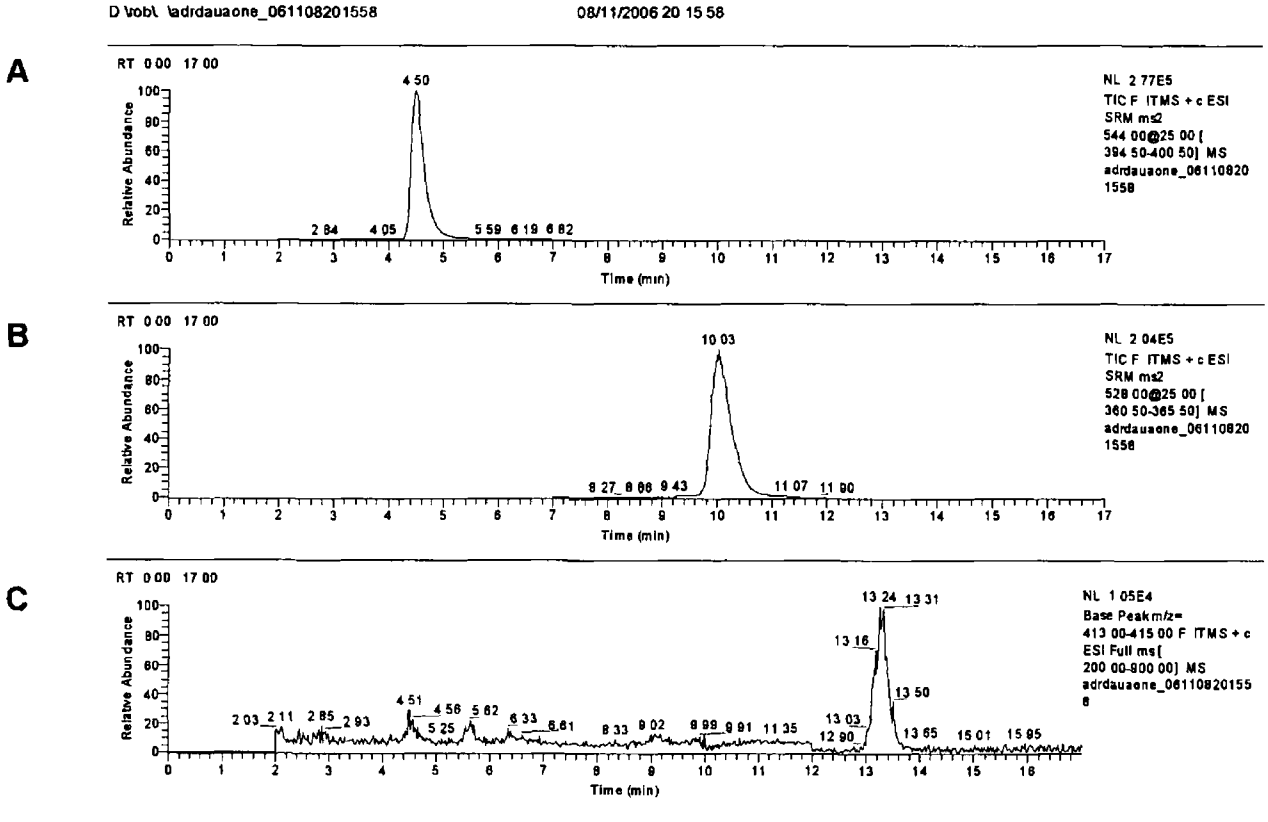


Figure 3 2 11 LC/MS analysis of a mixture of Adnamycin (A), Daunorubicin (B) and Adnamycin aglycone (C) standards Adriamycin eluted at approximately 4 5 minutes, Daunorubicin (used as internal standard) at approximately 10 minutes and Adriamycin aglycone at approximately 13 minutes

No Adriamycin aglycone was detected after incubation of Adriamycin with control microsomes obtained from empty vector-transfected cells, as expected (Fig 3 2 12) However, this metabolite was not detected after incubation of P450R-expressing microsomes either (Fig 3 2 13) This result is unexpected and suggests that either no aglycone metabolite was generated under the experimental conditions used or that the amount produced was below the limit of detection of the LC/MS method

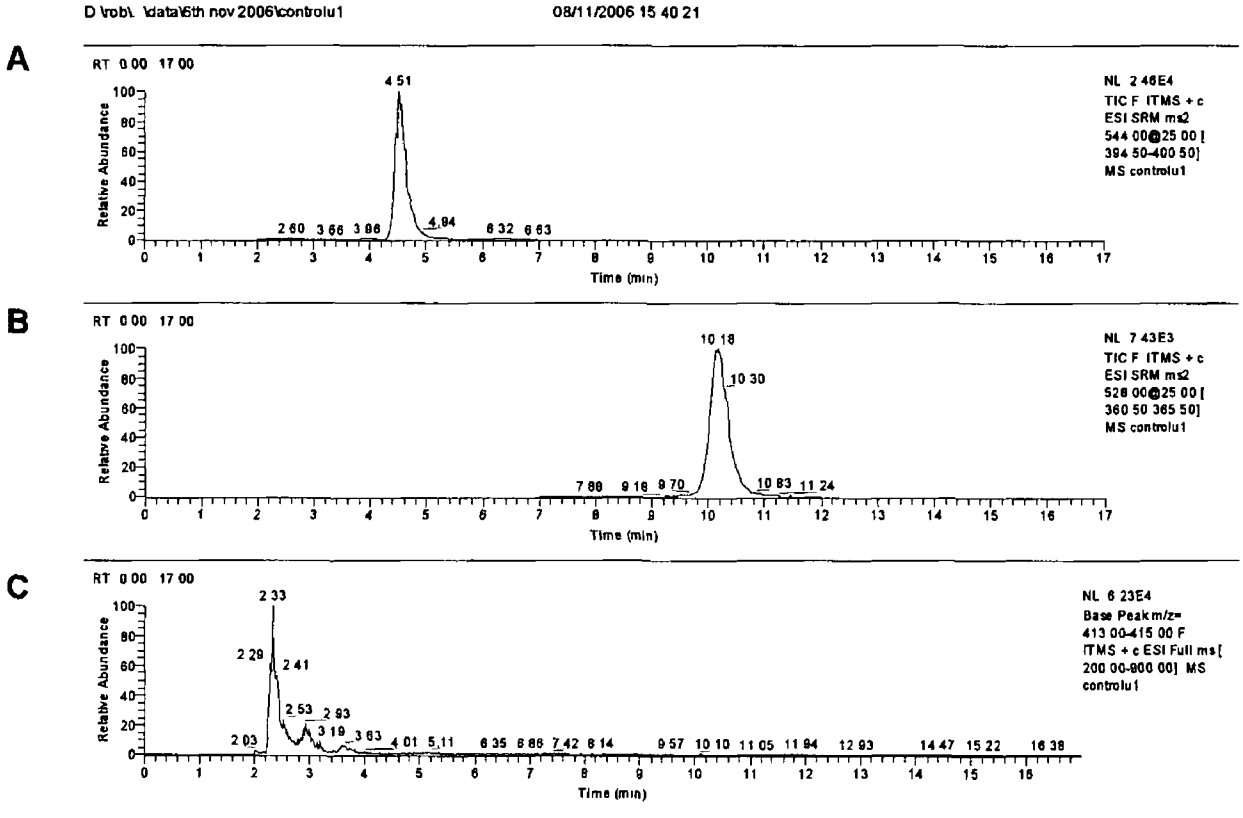


Figure 3 2 12 LC/MS analysis of Adriamycin samples after a 1 hour incubation with control microsomes Adriamycin (A) eluted at approximately 4 5 minutes Daunorubicin (B, used as internal standard) at approximately 10 minutes and Adriamycin aglycone (C) at approximately 13 minutes The results shown here are representative of three experiments

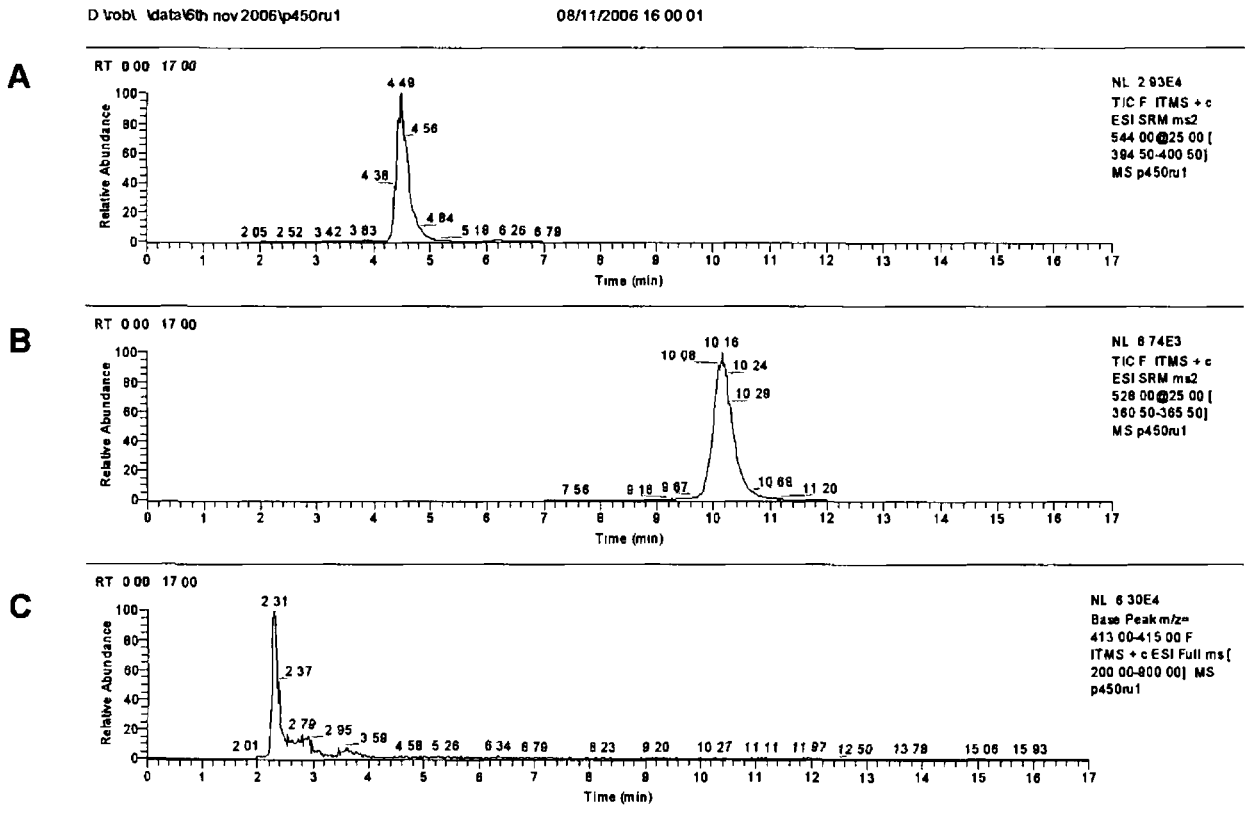


Figure 3.2.13 LC/MS analysis of Adriamycin samples after a 1 hour incubation with P450R-expressing microsomes. Adriamycin (A) eluted at approximately 4.5 minutes, Daunorubicin (B, used as internal standard) at approximately 10 minutes and Adriamycin aglycone (C) at approximately 13 minutes. The results shown here are representative of three experiments.

3 3 Role of CYP3A4 in chemotherapy resistance

CYP3A4 is by far the most relevant cytochrome P450 involved in drug metabolism, responsible for the inactivation of about half of all known drugs Substrates of CYP3A4 include several anticancer agents, such as Taxol, Ifosfamide, Tamoxifen, VP-16 and Vinca alkaloids

Expression of CYP3A4 is very low in cell lines, the mechanism underlying this effect has not been completely elucidated, but decreased levels of specific transcription factors have been suggested as a possible cause (Martinez *et al*, 2002) The resultant low CYP3A4 activity makes it difficult to find a suitable model for the study of this P450, transfection of CYP3A4 cDNA can bring the enzyme levels closer to the values found in normal body cells, generating useful models for the study of this particular protein

3 3 1 Expression of CYP3A4 in BCMV cells

BEAS-2B are normal human bronchial cells immortalized with the SV40 virus BCMV cells are BEAS-2B variants that have been transfected with a vector containing either the cDNA of CYP3A4 under the control of the CMV early promoter, or an empty vector They are denominated as BCMV 3A4 or BCMV Neo, respectively These cells were a gift from Dr Katherine Mace, from the Department of Nutrition, Nestle Research Center They constitute an interesting model to study the effects of CYP3A4 overexpression on the resistance to anticancer agents

BCMV 3A4 cells express high levels of CYP3A4 mRNA and protein, as shown by RT-PCR (Fig 3 3 1) and Western blotting (Fig 3 3 2)

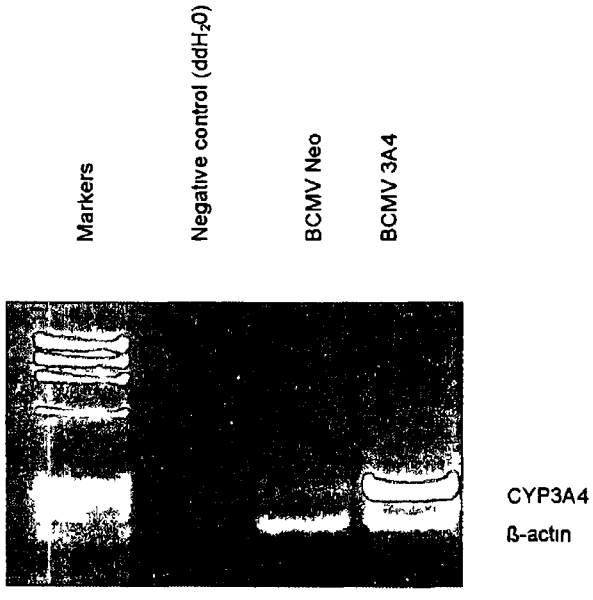


Figure 3 3 1 RT-PCR showing CYP3A4 mRNA expression in BCMV Neo and BCMV 3A4 cells

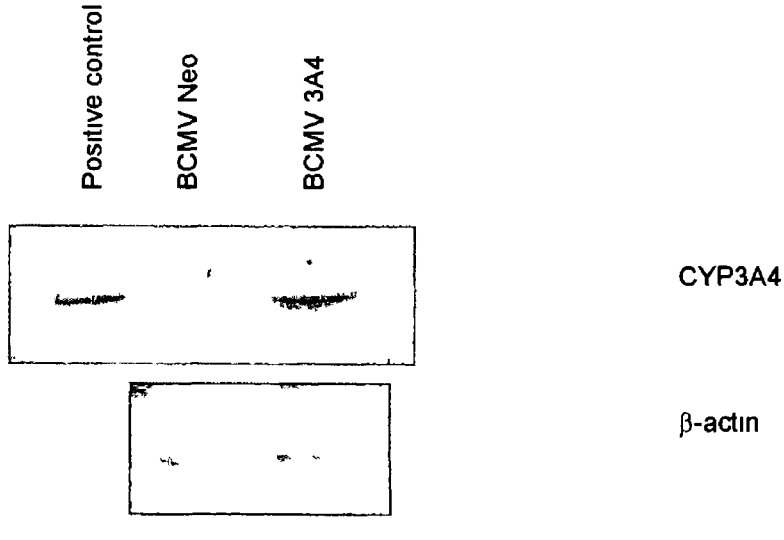


Figure 3 3 2 Western blot showing CYP3A4 protein expression in BCMV Neo and BCMV 3A4 cells CYP3A4-expressing microsomes were used as positive control

3 3 2 Testosterone 6β-hydroxylase activity in BCMV cells

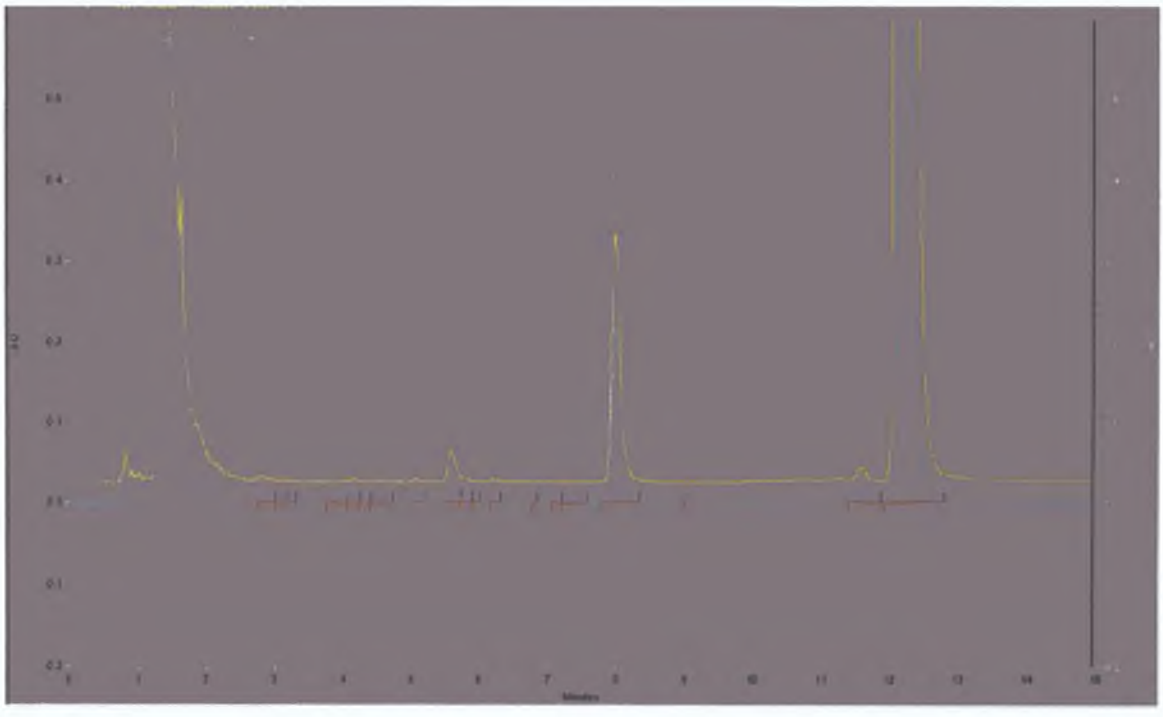
CYP3A activity was measured in BCMV cells by HLPC using the testosterone 6ß-hydroxylation assay (see Section 2 12 Testosterone metabolites were separated by reverse-phase HPLC and quantified by UV absorbance An internal standard, corticosterone, was added to each sample at a known concentration as means of reference In the optimised conditions, 6β-hydroxy testosterone elutes at four minutes, while unmodified testosterone does so at ten minutes, corticosterone elutes at nine minutes

As it can be seen in Table 3 3 1 and Fig 3 3 3, BCMV 3A4 cells exhibit readily measurable testosterone 6β-hydroxylase activity In contrast, no testosterone metabolite could be detected in BCMV Neo cells

	CYP3A activity (pmol/mg x min)
BCMV Neo	n d
BCMV 3A4	0 066

Table 3 3 1 CYP3A activity expressed as pmol of metabolite (6ß-hydroxy testosterone) per mg of total protein per minute of incubation Cells were incubated for one hour at 37°C in a reaction mixture containing testosterone, NADPH, $MgCl_2$ and total cell protein in PBS, pH 7 4 n d not detectable Results represent the average of two independent experiments

A



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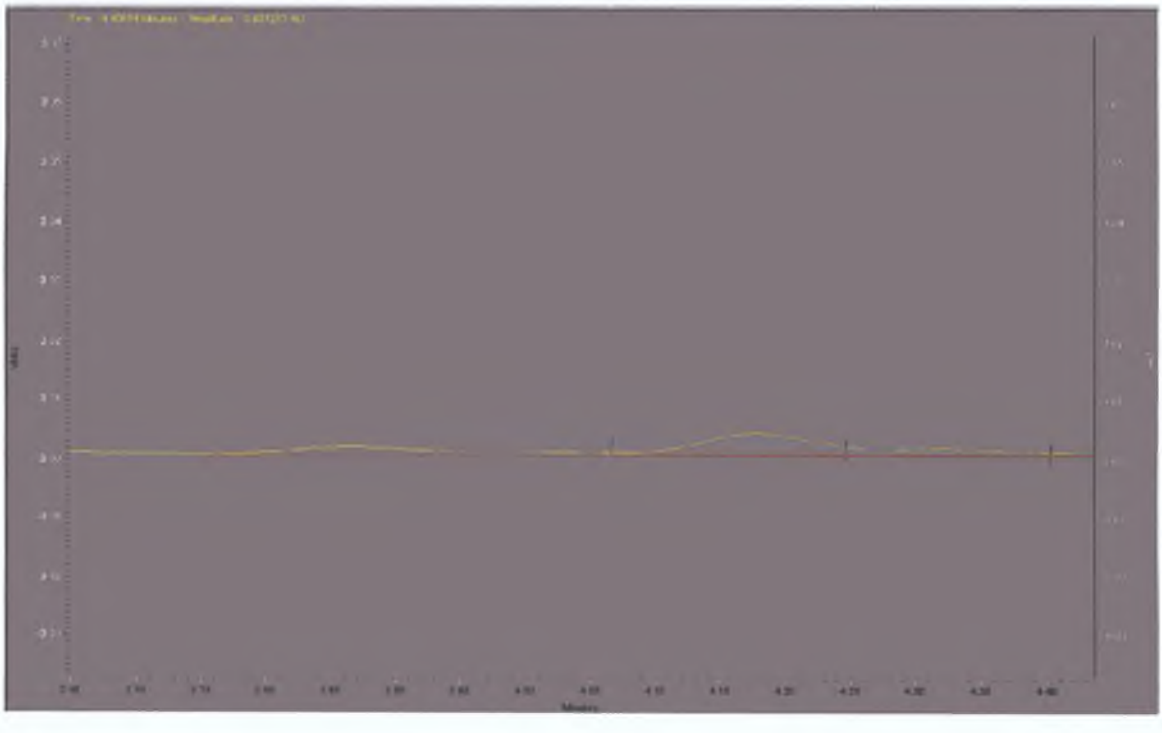


Figure 3.3.3: Testosterone metabolites detected by HPLC in BCMV cells. A, complete chromatogram. B, zoom in at approximately 4 minutes. Yellow line: BCMV Neo cells. Blue line: BCMV 3A4 cells. Testosterone elutes at approximately 12 minutes, while 6β-hydroxy testosterone does so at approximately 4 minutes. Corticosterone, used here as internal standard, elutes at approximately 8 minutes.

3.3.3 Anticancer drug-induced toxicity in BCMV cells

BCMV Neo and BCMV 3A4 cells were exposed to increasing concentrations of anticancer drugs to determine the drug IC50 (i.e., the concentration that results in 50% of cell death as compared to an untreated control). The anticancer agents used for these experiments were Adriamycin, 5-fluorouracil and Cisplatin. Adriamycin is known to be detoxified by CYP3A4; 5-fluorouracil and Cisplatin, on the other hand, are not metabolised by P450s.

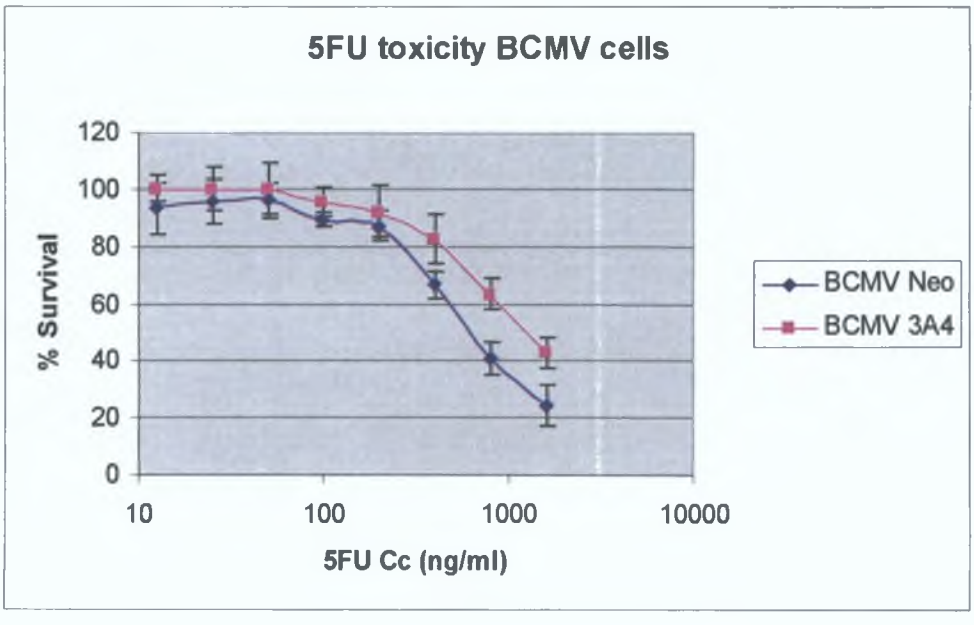


Figure 3.3.4: 5-fluorouracil IC50 as determined by the acid phosphatase assay in BCMV Neo and BCMV 3A4 cells. Results represent the average of three independent experiments.

Cell line	5-FU IC50 (ng/ml)	Fold resistance	t-test (p value)
BCMV Neo	674±112	1	
BCMV 3A4	1233±182	1.83	0.016

Table 3.3.2: 5-fluorouracil fold resistance in BCMV 3A4 as compared to empty vector transfected cells. Results are expressed as mean +/- SD and represent the average of three independent experiments.

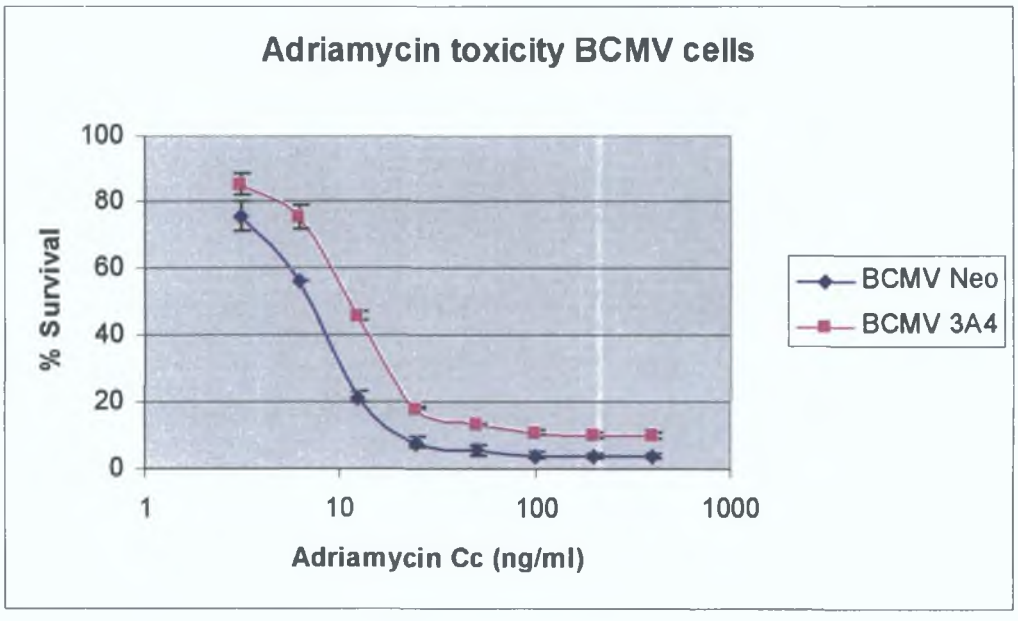


Figure 3.3.5: Adriamycin IC50 as determined by the acid phosphatase assay in BCMV Neo and BCMV 3A4 cells. Results represent the average of three independent experiments.

Cell line	Adriamycin IC50 (ng/ml)	Fold resistance	t-test (p value)
BCMV Neo	6300±400	1	
BCMV 3A4	11400±900	1.8	0.004

Table 3.3.3: Adriamycin fold resistance in BCMV 3A4 as compared to empty vector transfected cells. Results are expressed as mean +/- SD and represent the average of three independent experiments.

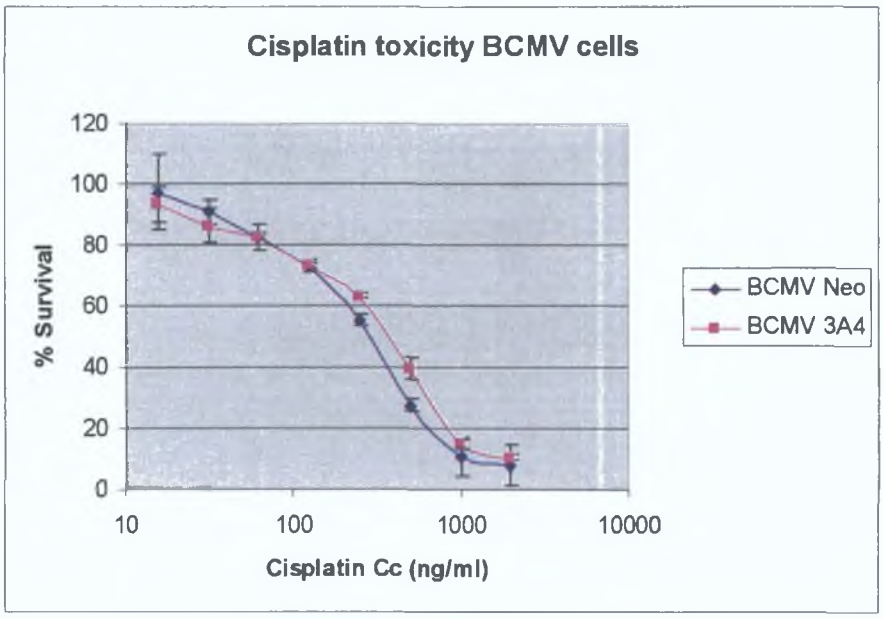


Figure 3.3.6: Cisplatin IC50 as determined by the acid phosphatase assay in BCMV Neo and BCMV 3A4 cells. Results represent the average of three independent experiments.

Cell line	Cisplatin IC50 (ng/ml)	Fold resistance	t-test (p value)
BCMV Neo	240±30	1	
BCMV 3A4	260±40	1.07	0.545

Table 3.3.4: Cisplatin fold resistance in BCMV 3A4 as compared to empty vector transfected cells. Results are expressed as mean +/- SD and represent the average of three independent experiments.

As expected, transfection of CYP3A4 enhanced cell resistance to the toxic effects of Adriamycin, presumably by protecting cells by drug inactivation (Table 3.3.3). However, BCMV 3A4 cells also displayed significant resistance to 5-fluorouracil when compared to BCMV Neo cells (Table 3.3.2). This is unexpected, since the literature shows no relationship between 5-fluorouracil and CYP3A4. Resistance was more evident at intermediate concentrations of drug.

Cisplatin toxicity was unaffected by CYP3A4 transfection (Table 3.3.4), suggesting that the increase in resistance is not general for all types of anticancer drugs.

3.3.4 Inhibition of testosterone 6β-hydroxylase activity by 17 AEE

17α-ethynyl estradiol (17 AEE) causes non-competitive inactivation of members of the CYP3A subfamily (Guengerich, 1990). Inhibition of CYP3A by 17 AEE results from an irreversible modification of the P450 active site by the binding of this chemical compound. 17 AEE inhibited testosterone 6β-hydroxylase activity of CYP3A4 + P450R microsomes in a concentration-dependent fashion (Fig. 3.3.7), as measured by HPLC (see section 2.12). Inhibition, though readily measurable, was not as strong as that of ketoconazole, used as positive control.

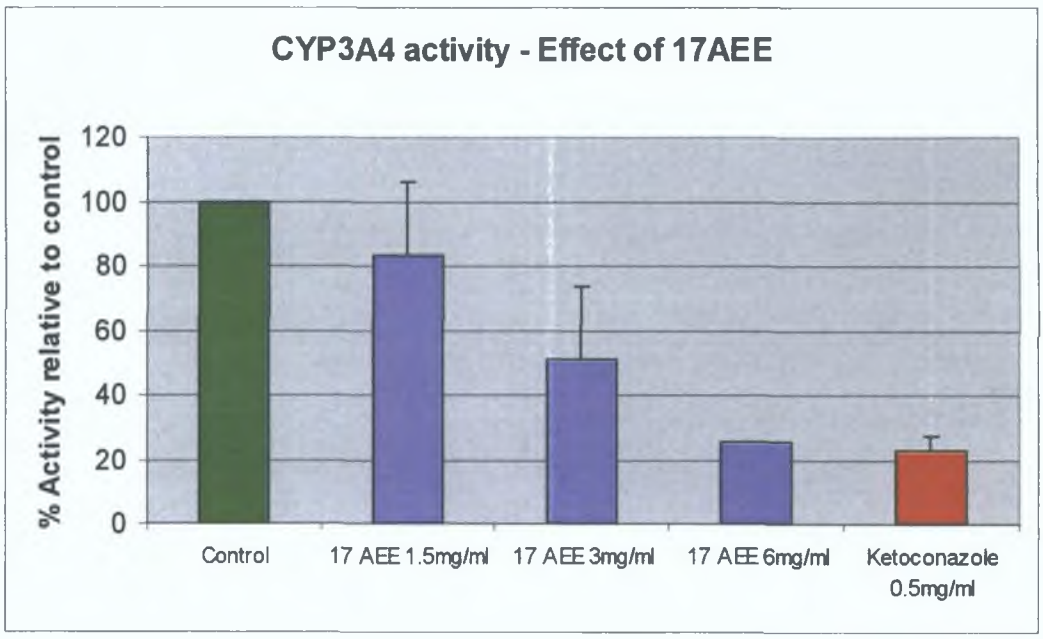


Figure 3.3.7: Dose-dependent inhibition of CYP3A4-mediated testosterone 6β-hydroxylase activity by 17 AEE on CYP3A4 and P450R-expressing microsomes. Microsomes were incubated for 15 minutes at 37°C in a reaction mixture containing testosterone, NADPH, $MgCl_2$ and microsomal protein in PBS, pH 7.4, as well as different concentrations of 17 AEE (replaced with PBS for the control). CYP3A activity was then measured by HPLC and expressed as % relative to control. Ketoconazole was used as a positive control. Results represent the average of two independent experiments.

3.3.4 Effect of CYP3A4 inhibition on anticancer drug-induced toxicity

CYP3A4-transfected cell lines did show an increase in resistance to Adriamycin and 5-fluorouracil. However, stable transfection requires the integration of foreign DNA into the cell's genome; integration can take place anywhere in the genome and can disrupt the normal function of endogenous genes. In order to confirm that increased CYP3A4 activity was responsible for the observed increase in resistance, BCMV 3A4 cells were exposed to Adriamycin, Cisplatin and 5-fluorouracil in the presence of the CYP3A inhibitor 17 AEE.

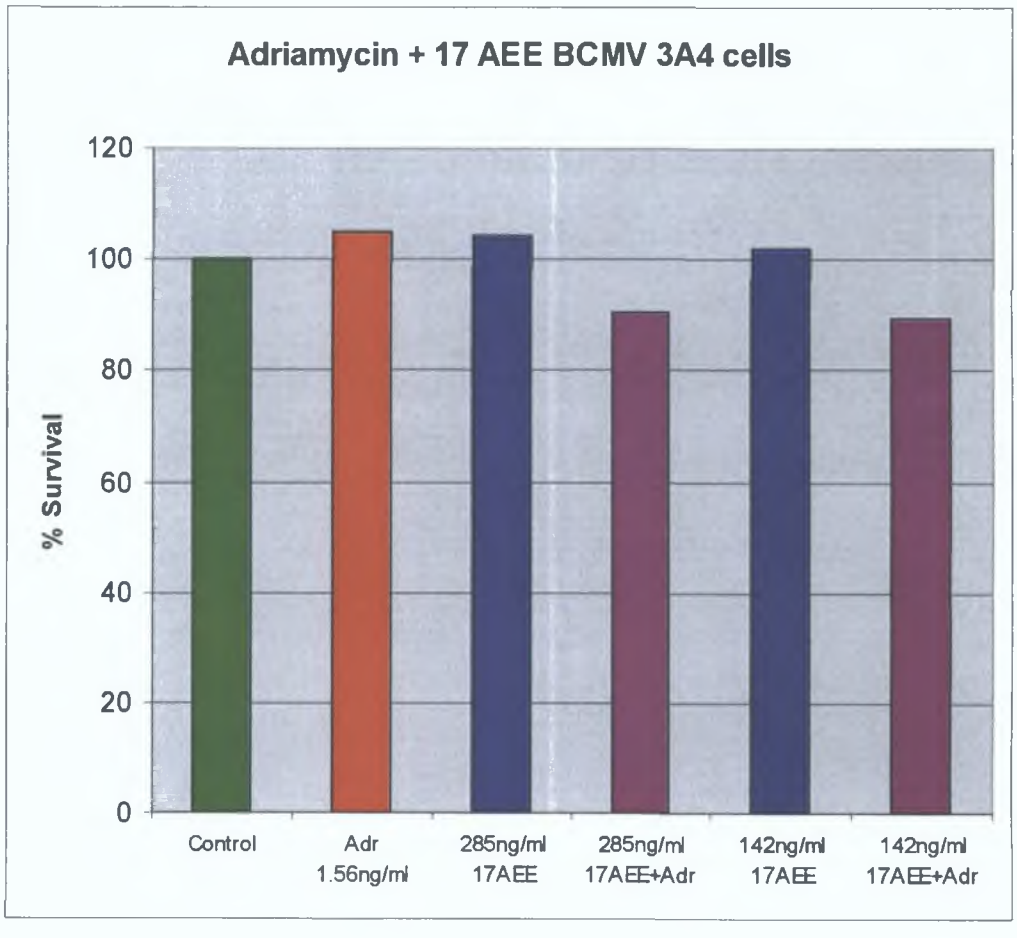


Figure 3.3.8: Effect of Adriamycin and 17 AEE combination on the viability of BCMV 3A4 cells as determined by the acid phosphatase assay. Results represent the average of two independent experiments.

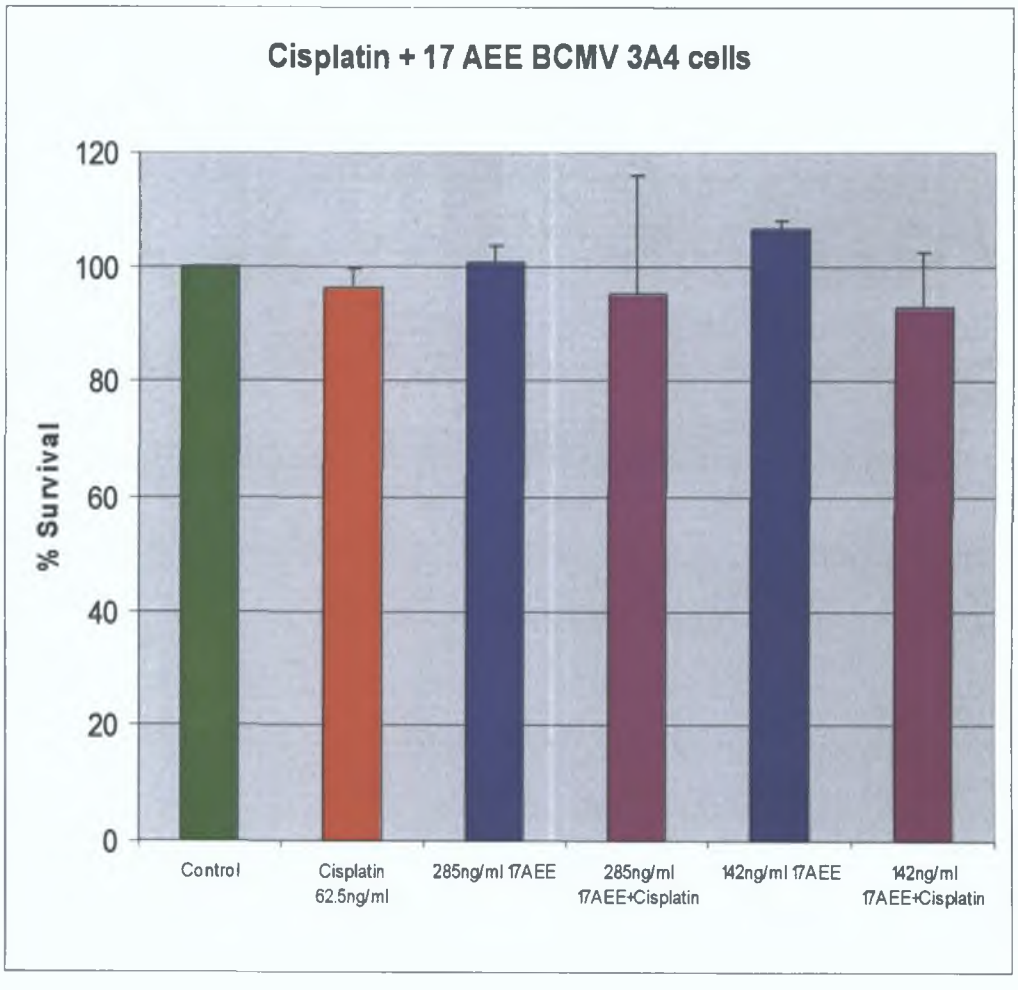


Figure 3.3.9: Effect of Cisplatin and 17 AEE combination on the viability of BCMV 3A4 cells as determined by the acid phosphatase assay. Results represent the average of three independent experiments.

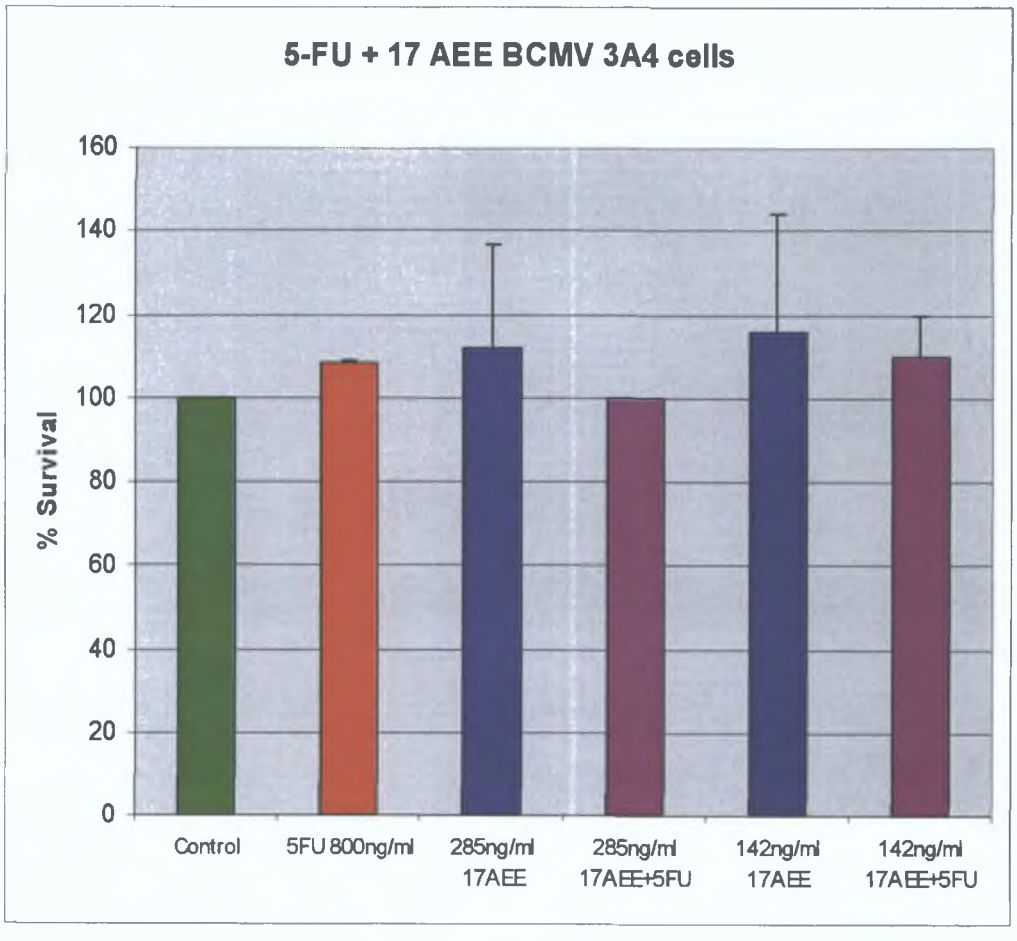


Figure 3.3.10: Effect of 5-fluorouracil and 17 AEE combination on the viability of BCMV 3A4 cells as determined by the acid phosphatase assay. Results represent the average of three independent experiments.

As shown in Fig. 3.3.8, the combination of non-toxic concentrations of Adriamycin and 17 AEE resulted in a decrease of about 10% in cell viability. This effect was not observed with Cisplatin (Fig. 3.3.9). In the case of 5-fluorouracil, there is no apparent change in viability, but the observed trend is similar to that of Adriamycin (Fig. 3.3.10).

3.4 Role of CYP1B1 in chemotherapy resistance

Recent studies have raised great interest on CYP1B1 and its role in malignant transformation and drug resistance This protein is consistently overexpressed in tumour cells as compared to their normal counterparts (see section 1 4 5), although its precise role in cancer development is yet to be defined Similarly, even though transfection of CYP1B1 resulted in an increased resistance to Taxotere in cultured cell lines, no actual metabolite could be detected when the drug was incubated with recombinant CYP1B1 enzyme in all different conditions tested (Bournique *et al*, 2002), these results were confirmed by experiments performed with CYP1B1 microsomes (see section 3 2 2 2) Thus, even though there seems to be a clear connection between CYP1B1 and drug resistance, the nature of this connection remains obscure

3 4 1 Expression of CYP1B1 mRNA in HL60 cells

HL60 cells were originally obtained from a patient suffering from acute promyelocytic leukaemia, they display myeloblast-like morphology and have been shown to express CYP1B1 (Nagai *et al*, 2002) Expression levels of this enzyme are increased in response to treatment with differentiating agents such as retinoic acid and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (Fig 3 4 1) U937 lymphoma cells, however, do not appear to express CYP1B1, even after treatment with the inducer

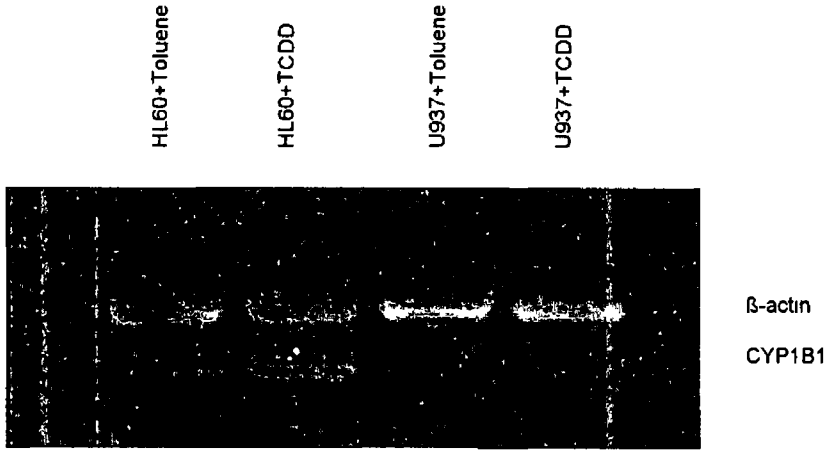


Figure 3 4 1 CYP1B1 mRNA expression as detected by RT-PCR in HL60 and U937 cell lines Cells were exposed to 3 22 ng/ml TCDD or vehicle (toluene) for 72 hours prior to mRNA extraction

3 4 2 EROD activity in HL60 cells

EROD activity was measured in HL60 cells with a protocol similar to the one used for the CYP1B1 microsomes activity assay (see section 2 13) Cells were resuspended in 7-ethoxy resorufin-containing PBS and incubated at 37°C Supernatant samples were removed at various time points and the amount of resorufin produced was measured by fluorescence against a blank of HL60 cells in PBS As seen in Fig 3 4 2, the cells displayed low but measurable activity

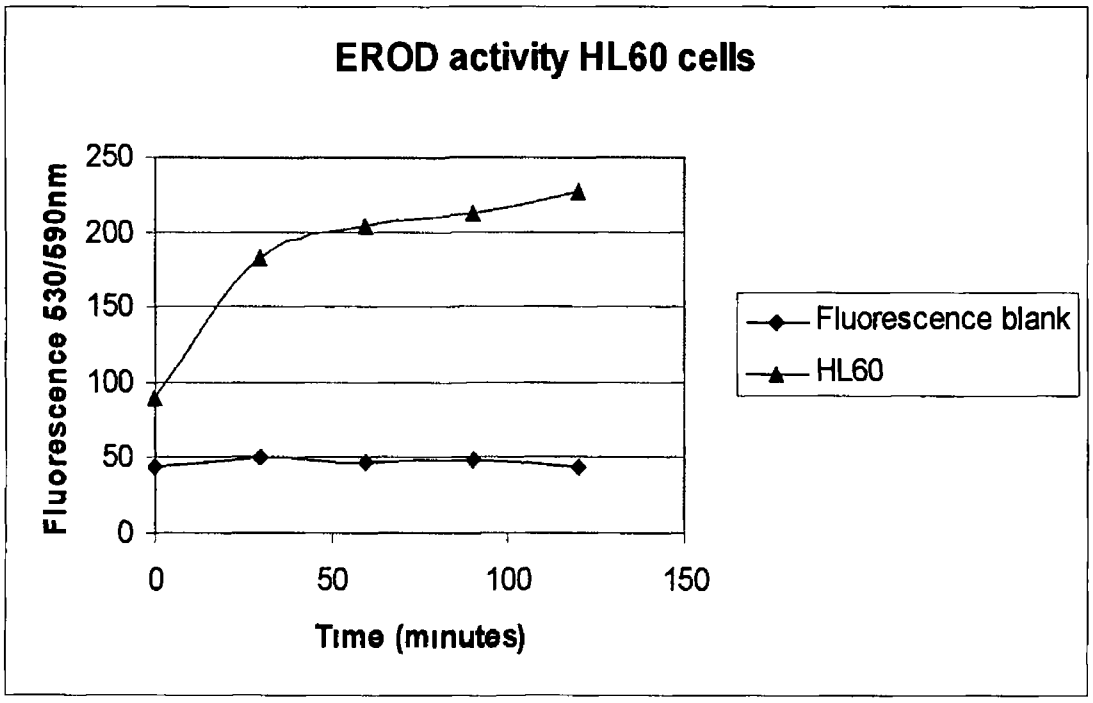


Figure 3 4 2 EROD activity measured at various time points and expressed as fluorescence detected at 530nm (excitation)/590nm (emission) After addition of 7-ethoxy resorufin, cells were incubated at 37°C for the indicated periods of time Samples were removed and spun down, and the amount of Resorufin present in the supernatant determined by fluorescence HL60 cells without Resorufin were used as fluorescence blank

3.4.3 Taxotere-induced toxicity In HL60 cells in the presence and absence of ANF

EROD activity, displayed by members of the CYP1A and CYP1B subfamilies, can be inhibited by the addition of α-naphthoflavone (ANF). In an attempt to establish the effect of CYP1 inhibition in drug resistance, HL60 cells were exposed to a non-toxic concentration of either Taxotere or ANF, or to a combination of both doses. The resulting effect on cell survival was quantified by the XTT assay (see section 2.7.3).

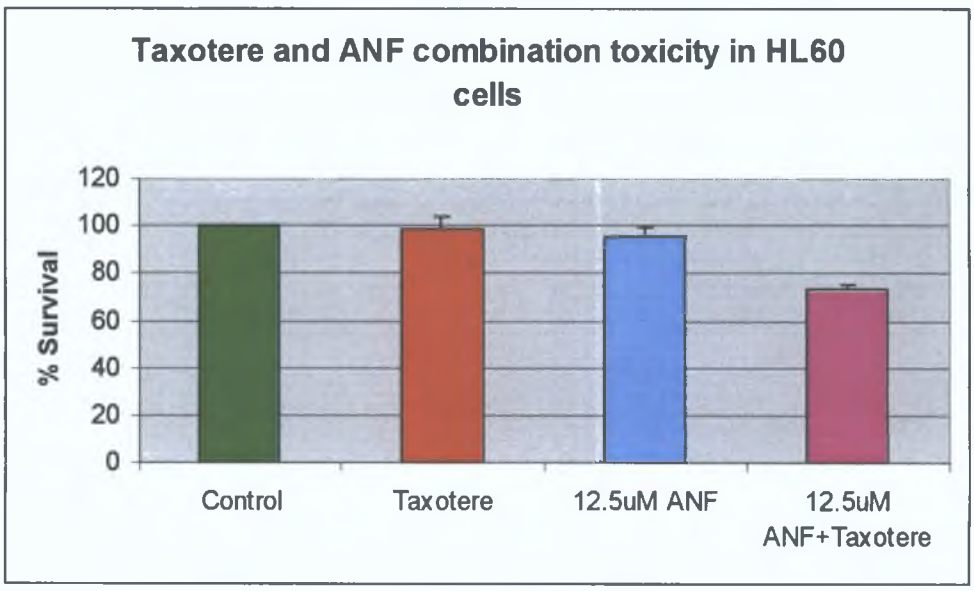


Figure 3.4.3: Taxotere-induced toxicity in HL60 cells in the presence or absence of 12.5 μM ANF as measured by the XTT assay. The Taxotere concentration used was 1.07 ng/ml. The results represent the average of three independent experiments.

As seen in Fig. 3.4.3, a slightly (about 5% kill) toxic concentration of ANF synergises with a non-toxic concentration of Taxotere to give a cell kill value of approximately 30%. These results suggest that CYP1 inhibition could enhance Taxotere toxicity in HL60 cells.

3.4.4 Drug-induced cytotoxicity in the presence and absence of ANF

In an effort to better understand the effect on ANF in anticancer drug resistance, HL60 cells were exposed to different concentrations of 5-fluorouracil, Adriamycin, Taxol and Taxotere in the presence and absence of 12.5 μM ANF.

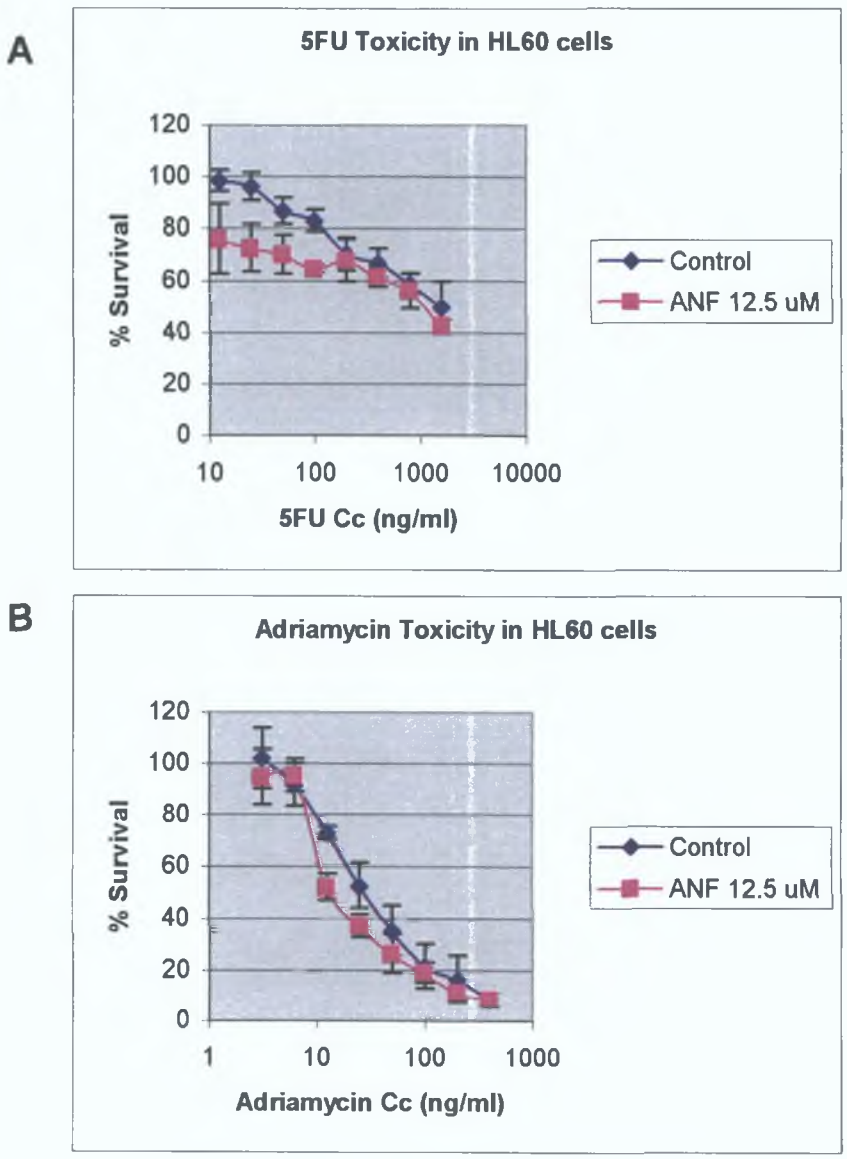


Figure 3.4.4: 5-fluorouracil (A) and Adriamycin (B)-induced cytotoxicity in HL60 cells as determined by the XTT assay in the presence and absence of 12.5 μM ANF. Results represent the average of at least three independent experiments.

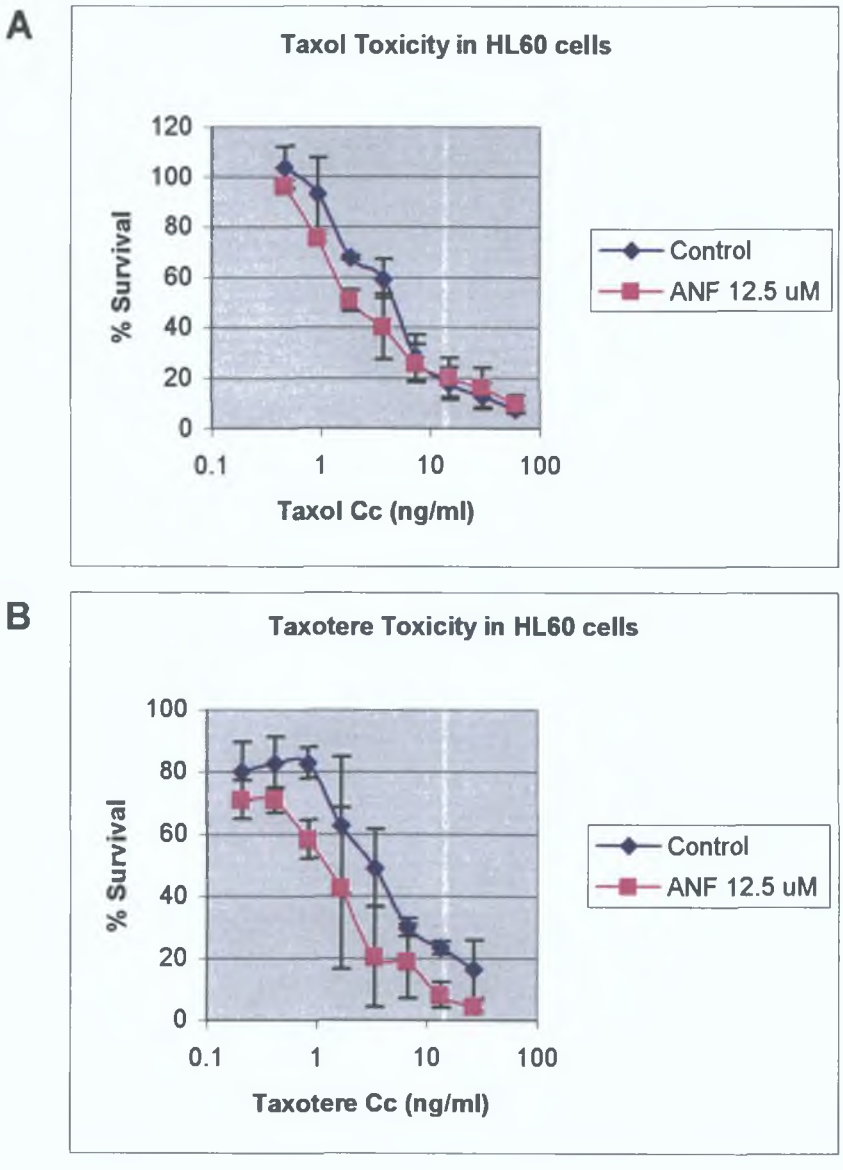


Figure 3.4.5: Taxol (A) and Taxotere (B)-induced cytotoxicity in HL60 cells as determined by the XTT assay in the presence and absence of 12.5 μM ANF. Results represent the average of at least three independent experiments.

A tendency towards increased sensitisation in the presence of ANF is observed in Figs. 3.4.4 and 3.4.5. The fact that ANF appears to increase the toxicity of all the drugs tested suggests that enhanced sensitivity arises from a general toxic effect of ANF, rather than from inhibition of CYP1B1. It should be noted that out of the drugs tested only Taxotere has been reported as a CYP1B1 substrate.

3 4 5 Determination of CYP1B1 induction in HL60 cells by TCDD

TCDD is a well-known ligand of the aryl hydrocarbon receptor (AhR) Upon entrance into the cell, TCDD binds the receptor, which translocates into the nucleus and stimulates transcription of its target genes These genes include members of the CYP1 family, such as CYP1A1 and CYP1B1 Induction of CYP1B1 expression by TCDD has been well documented in a number of different cell lines

A time-dependent increase in CYP1B1 expression was detected in HL60 cells by RT-PCR after treatment with 3 22 ng/ml TCDD, as shown in Fig 3 4 6 EROD activity was also measured in vehicle (toluene) and TCDD-treated HL60 cells (see section 2 13) and revealed that an approximately 10-fold raise in CYP1 activity paralleled the increase in mRNA expression (Fig 3 4 7 and Table 3 4 1)

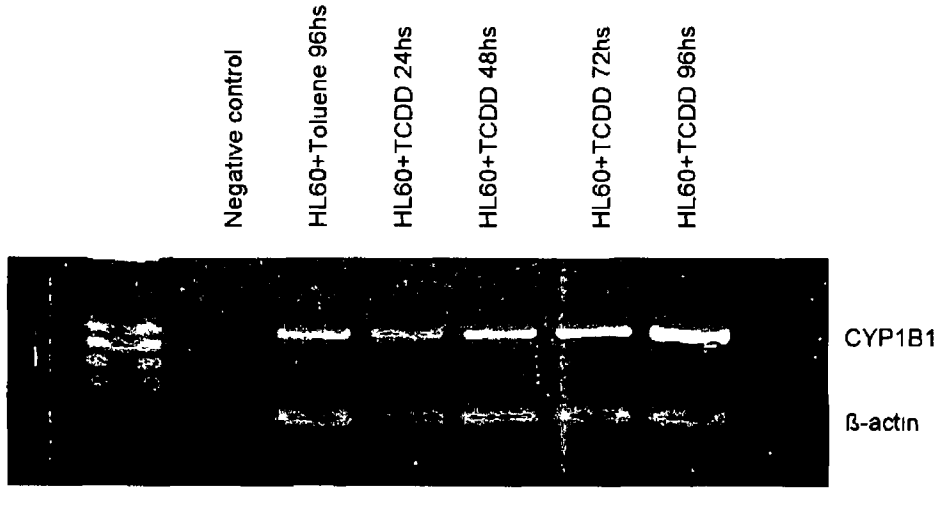


Figure 3 4 6 Induction of CYP1B1 expression by 3 22 ng/ml TCDD in HL60 cells Cells were treated with TCDD for 24, 48, 72 or 96 hours, or with toluene for 96 hours before mRNA extraction

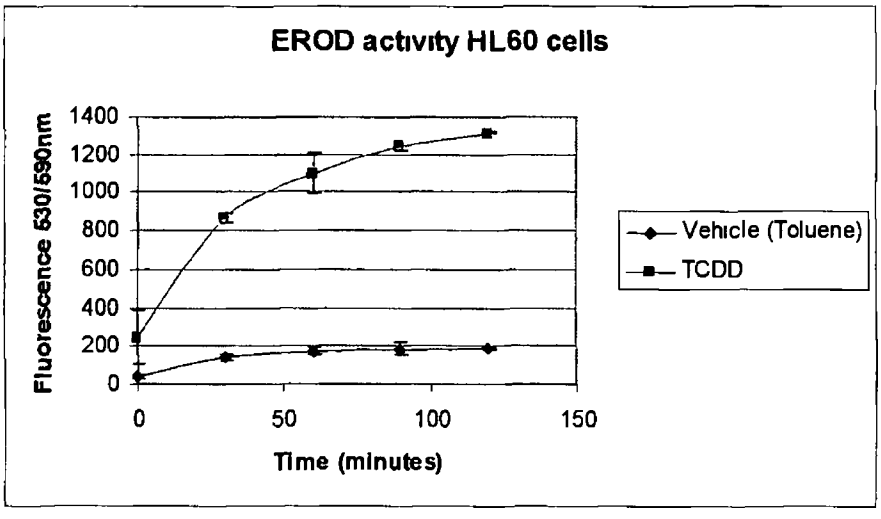


Figure 3 4 7 EROD activity in HL60 cells after treatment with either vehicle or 10 nM TCDD for 72 hs Activity was measured at various time points and expressed as fluorescence 530/590nm After addition of 7-ethoxy resorufin, cells were incubated at 37°C for the indicated periods of time Samples were removed and spun down, and the amount of resorufin present in the supernatant determined by fluorescence (530 nm excitation 590 nm emission) Results represent the average of three independent experiments

	EROD activity (pmoles resorufin/mg total protein/minute)
HL60 + toluene	0 079+/-0 071
HL60 + TCDD	0 725+/-0 772

Table 3 4 1 EROD activity of HL60 cells treated with TCDD or vehicle (toluene) for 72 hours Activity is expressed as pmoles resorufin per mg of total protein per minute Results are expressed as mean +/- SD and represent the average of three independent experiments

3 4 6 Determination of anticancer drug toxicity after treatment with TCDD

Toxicity assays were carried out on HL60 cells that had been treated with 3 22 ng/ml TCDD or an equal volume of vehicle (toluene) for 72 hours The drugs used in these assays were 5-fluorouracil, Cisplatin, Taxol and Taxotere

As can be seen in Figs 3 4 8 and 3 4 9, treatment with TCDD did not appreciably affect cell sensitivity towards any of the drugs tested, suggesting that a substantial increase in CYP1B1 mRNA expression and activity does not alter anticancer drug toxicity in HL60 cells

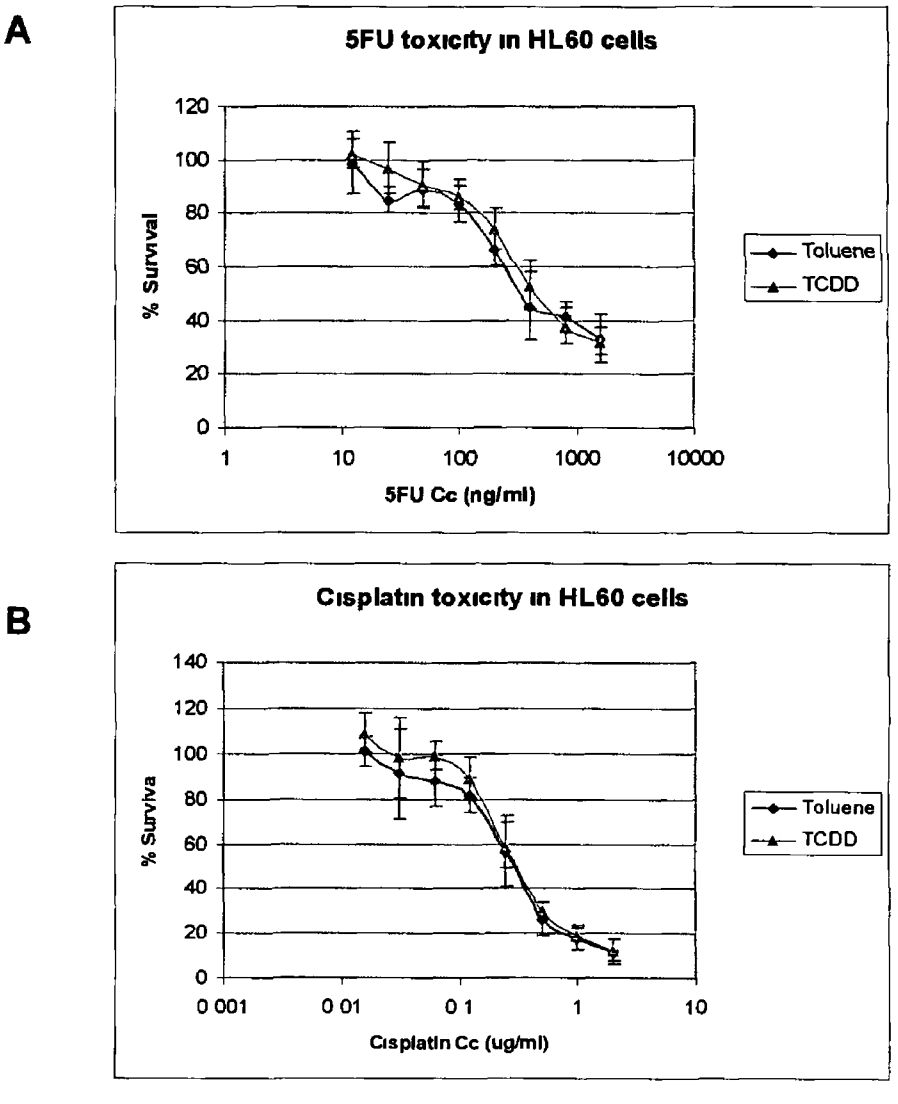


Figure 3 4 8 5-fluorouracil (A) and Cisplatin (B)-induced toxicity in HL60 cells as determined by the XTT assay after treatment with either vehicle (toluene) or 3 22 ng/ml TCDD for 72 hours Results represent the average of at least two independent experiments

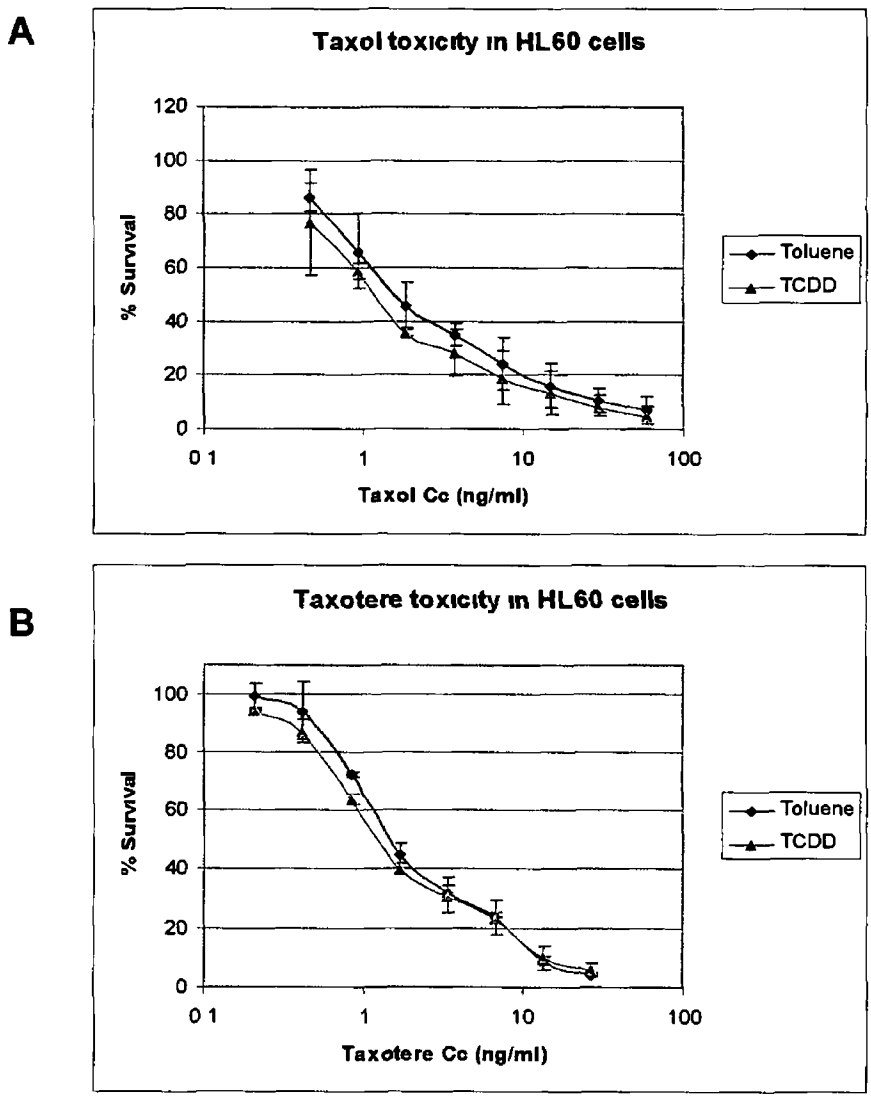


Figure 3 4 9 Taxol (A) and Taxotere (B)-induced toxicity in HL60 cells as determined by the XTT assay after treatment with either vehicle (toluene) or 3 22 ng/ml TCDD for 72 hours Results represent the average of at least two independent experiments

3 4 7 Expression of CYP1B1 in MCF-7 cells and their resistant variants

CYP1B1 expression is abundant in hormone-dependent tissues such as uterus, ovary and breast (Tsuchiya *et al*, 2005) A breast cell line, MCF-7, was then chosen as a model in which to study expression of CYP1B1

MCF-7 cells were originally derived from breast carcinoma and were pulse-selected by Dr Yizheng Liang with Adriamycin (4 pulses, once a week for 4 hours, with 250 ng/ml Adriamycin), Cisplatin (9 pulses, once a week for 4 hours, with 800, 100, 300, 300, 300, 300, 350, 350 ng/ml Cisplatin) and Taxotere (6 pulses, once a week for 4 hours, except for the first one which was for a half hour, with 50, 10, 10, 13, 13, 13 ng/ml Taxotere) to generate the MCF-7 Adr, MCF-7 CisPt and MCF-7 Txt variants, respectively Toxicity assays were carried out in these cells with the acid phosphatase assay to establish their resistance profile (Tables 3 4 2 and 3 4 3) MCF-7 Adr were 4 9-fold more resistant to Adriamycin than parent MCF-7 cells, similarly, MCF-7 CisPt were 3 6-fold more resistant to Cisplatin and MCF-7 Txt displayed a 2 75-fold increase in resistance against Taxotere as compared to MCF-7 cells Some cross-resistance was observed in MCF-7 CisPt cells with Taxotere, but it was not important with other drugs or in any of the other cell lines Interestingly, MCF-7 Adr cells were more sensitive to 5-fluorouracil, Cisplatin and Taxotere than parent cells

IC50s (ng/ml)	MCF-7	MCF-7 CisPt	MCF-7 Txt	MCF-7 Adr
5-FU	336+-/87	234+/-15	270+/-27	151+/-81
Adriamycin	43 1+/-2 7	47 0+/-9 5	58 0+/-5 9	211+/-158
Cisplatin	580+/-120	2084+/-237	489+/-219	92 3+/-35 4
Taxotere	1 1+/-0 2	2 1+/-0 3	3 1+/-0 7	0 3+/-0 2

Table 3 4 2 5-fluorouracil, Adnamycin, Cisplatin and Taxotere IC50 values in MCF-7 cells and their pulse-selected variants as determined by the acid phosphatase assay Results are expressed as mean +/- SD and represent the average of at least two independent experiments

Fold resistance (relative to parent)	MCF-7	MCF-7 CisPt	MCF-7 Txt	MCF-7 Adr
5-FU	1	0 7	0 8	0 4
Adriamycin	1	1 1	1 3	4 9
Cisplatin	1	3 6	0 8	0 2
Taxotere	1	1 8	2 8	0 2

Table 3 4 3 Fold resistance of MCF-7 pulse-selected variants as compared to parent cells

Expression of CYP1B1 was measured in MCF-7 cells and their resistant variants by RT-PCR (Fig 3 4 10) The mRNA levels of this enzyme were dramatically upregulated in MCF-7 Txt cells as compared to parent cells, this increase in CYP1B1 expression was not observed in MCF-7 Adr cells

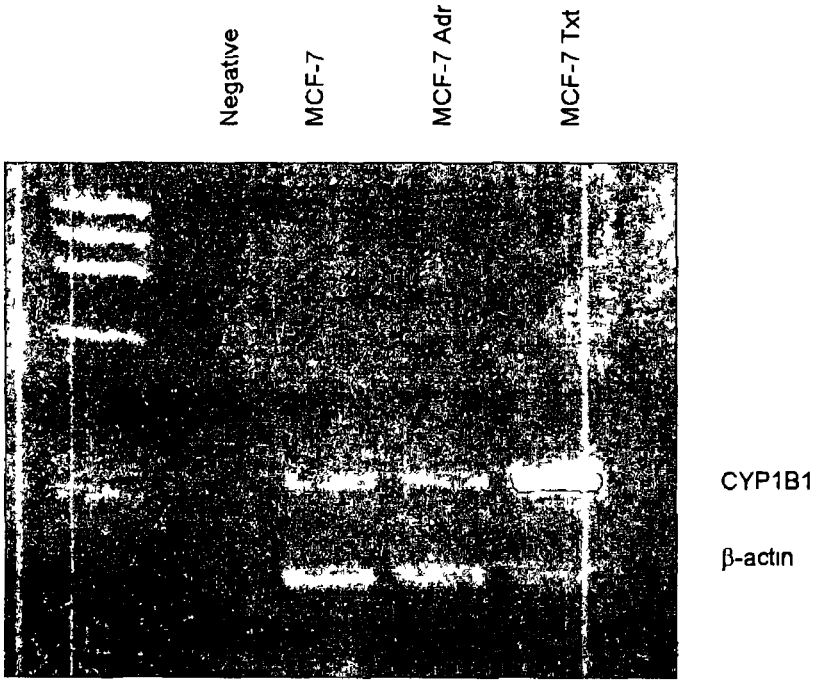


Figure 3 4 10 Expression of CYP1B1mRNA in MCF-7 cells and their resistant variants by RT-PCR

3.4.8 EROD activity in MCF-7 and MCF-7 Txt cells

In order to confirm that the increase in CYP1B1 transcription resulted in enhanced enzymatic activity, EROD activity was measured in MCF-7 and their Taxotere-selected variants. Activity was low but indeed detectable, while no measurable activity could be detected in parent MCF-7 cells (Fig. 3.4.11 and Table 3.4.4).

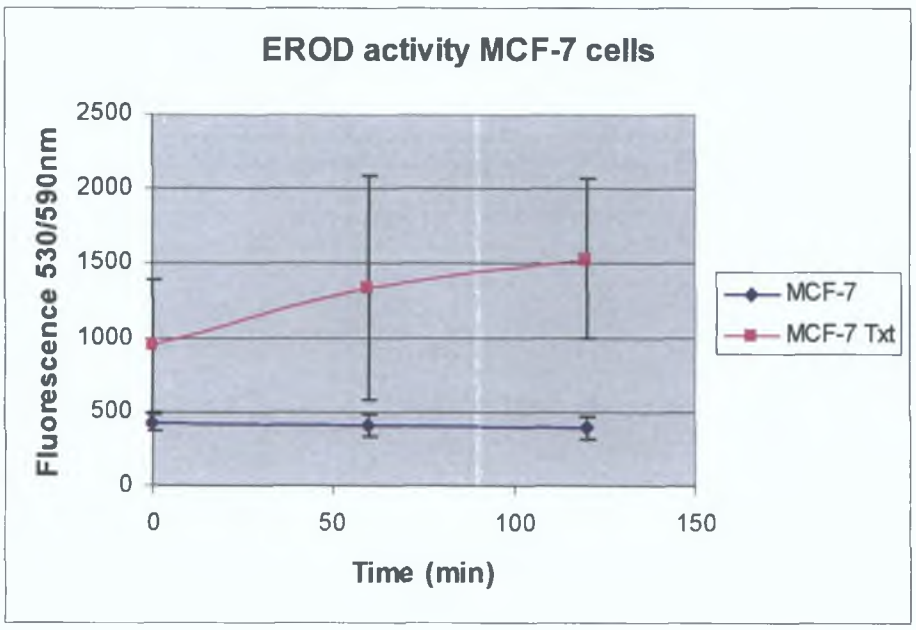


Figure 3.4.11: EROD activity of MCF-7 cells. Activity was measured at various time points and expressed as fluorescence 530/590nm. After addition of 7-ethoxy resorufin, cells were incubated at 37°C for the indicated periods of time. Samples were removed and spun down, and the amount of resorufin present in the supernatant determined by fluorescence (530 nm excitation; 590 nm emission). Results represent the average of at least two independent experiments.

	EROD activity (pmoles resorufin/mg total protein/minute)
MCF-7	n.d.
MCF-7 Txt	0.026

Table 3.4.4: EROD activity of MCF-7 cells and their Taxotere-selected variant. Activity is expressed as pmoles resorufin per mg of total protein per minute. n.d.: not detectable. Results are expressed as mean and represent the average of at least two independent experiments.

3 4 9 Expression of CYP1B1 in a panel of pulse-selected cells of various origins

In order to find out whether increased expression of CYP1B1 was a common effect of Taxotere pulse selection, a panel of cell lines – colon carcinoma Caco2, squamous cell lung carcinoma SK-MES-1, pancreatic carcinoma BxPc-3 and hepatoma cell line HepG2 – and their Taxotere-selected variants were analysed for CYP1B1 mRNA expression (Fig 3 4 12) Like MCF-7 cells, HepG2 cells displayed an increase in CYP1B1 expression after pulse selection with Taxotere However, no CYP1B1 mRNA was detected in any of the other cell lines In the case of HepG2 cells, only the Taxotere-selected variants showed increased expression of CYP1B1 mRNA, while Cisplatin and Taxol-selected HepG2 cells displayed no detectable levels of CYP1B1 mRNA

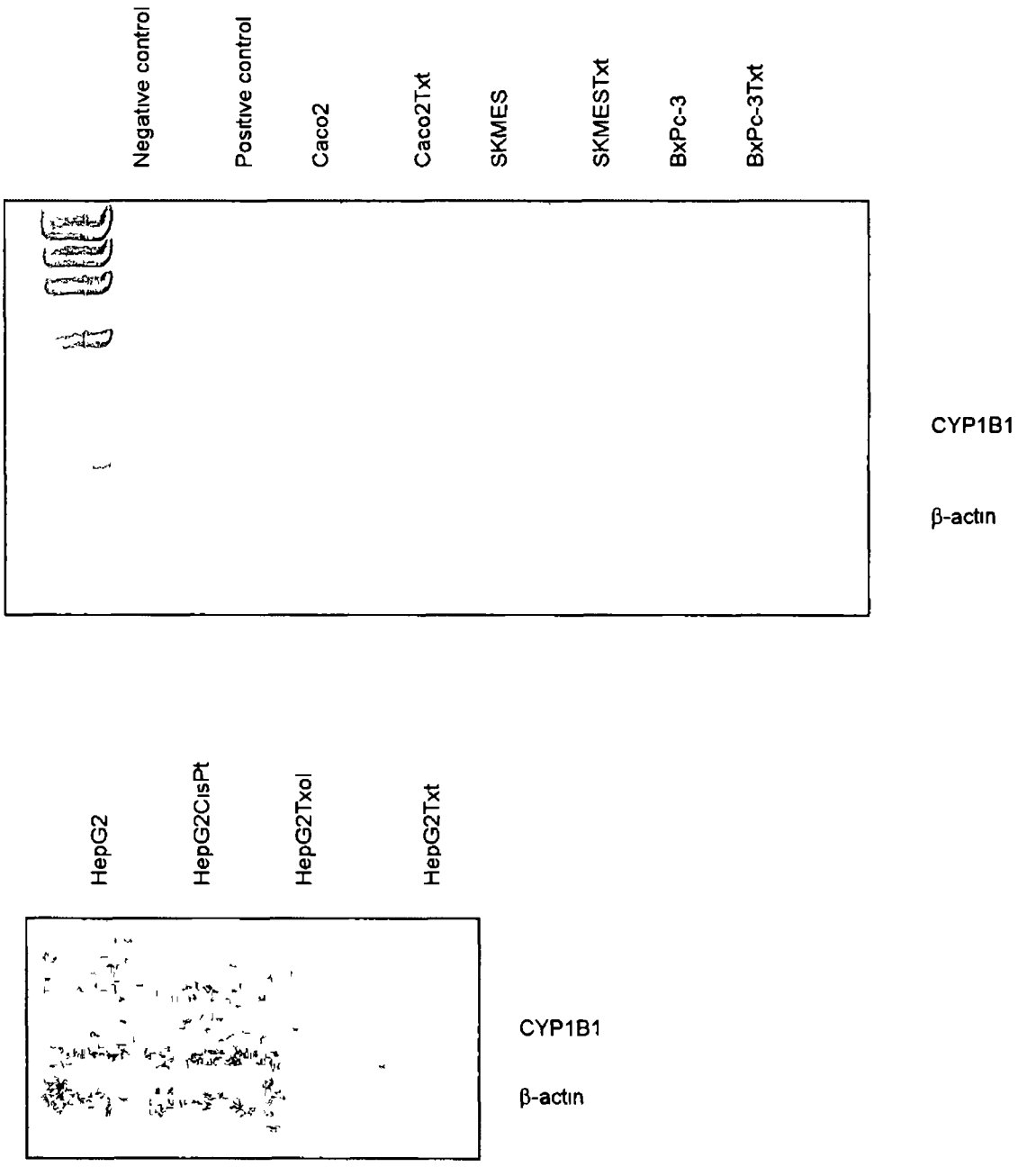


Figure 3 4 12 Expression of CYP1B1 mRNA in a panel of cell lines pulse-selected with Taxotere and their respective parent cells by RT-PCR HL60 cells treated with TCDD were used as positive control UHP water was used as negative control

3 4 10 Effect of Taxotere on CYP1B1 expression in a panel of breast cell lines

CYP1B1 is commonly expressed in hormone-dependent tissue, such as prostate, breast and endometrium, and its expression can be affected by the oestrogen receptor (ER) status of cells (see section 1 4 3 1) In order to find out whether Taxotere is able to induce CYP1B1 expression, a panel of breast cell lines – MCF-7, MDA 453, BT-20 and MDA 231 – was selected for analysis MCF-7 and MDA 453 cells are ER positive, while BT-20 and MDA 231 cells are ER negative These cells were exposed to 80 ng/ml of Taxotere for 4 hs and expression of CYP1B1 was then measured by RT-PCR and compared to that of untreated cells

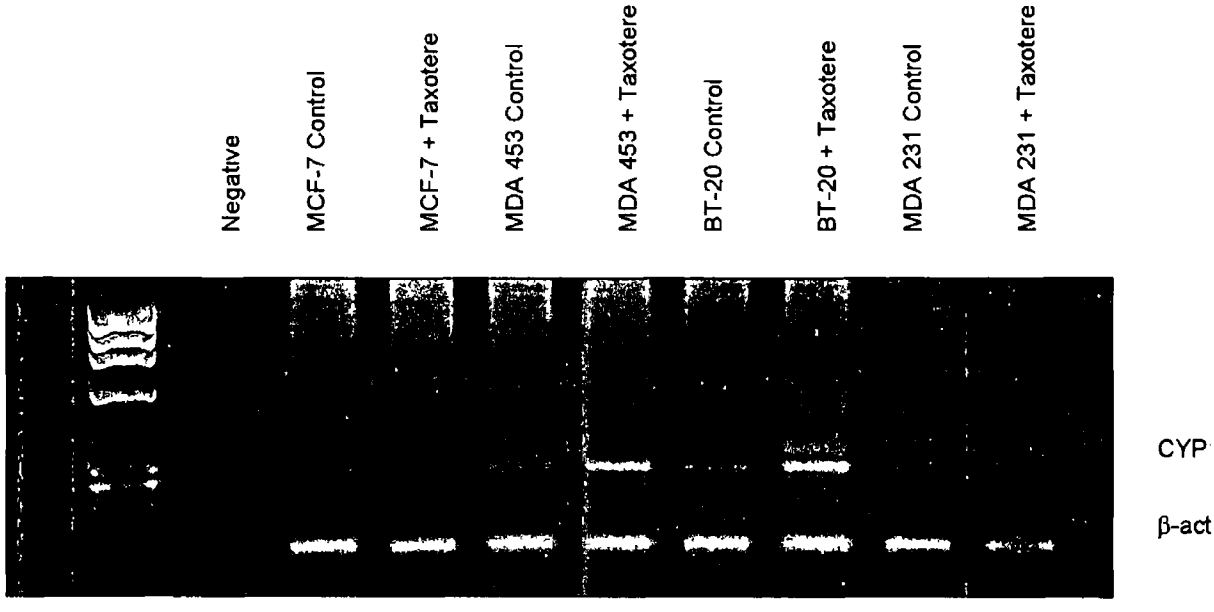


Figure 3 4 13 Expression of CYP1B1 by RT-PCR in a panel of breast cell lines after Taxotere treatment Cells were exposed to 80 ng/ml Taxotere or medium for 4 hours before mRNA extraction

As can be seen in Figure 3 4 13, a single 4 hour pulse of Taxotere did appear to increase expression of CYP1B1 in MDA 453 and BT-20 cells No apparent effect on expression was observed in MCF-7 or MDA 231 cells after Taxotere treatment In the case of MCF-7, this might suggest that the observed increase in CYP1B1 expression in MCF-7 Txt cells is associated with the increase in resistance observed in cells that had been pulse-selected for a longer period of time On the other hand, expression of CYP1B1 is regulated by the AhR, the lack of CYP1B1 induction by Taxotere could be attributed to the fact that this receptor is only present at low levels or is non-functional

3 4 11 Effect of TCDD on CYP1B1 expression in a panel of breast cell lines

In order to find out whether activation of the AhR can induce CYP1B1 expression in the panel of cell lines studied, cells were treated with TCDD, a well-known AhR agonist, for 4 hours Expression of CYP1B1 mRNA was then analysed and revealed upregulation of this enzyme in all cell lines tested in response to TCDD treatment (Fig 3 4 14) This result suggests that activation of the AhR by TCDD is able to induce CYP1B1 mRNA expression in the panel of breast cell lines, independently of ER status

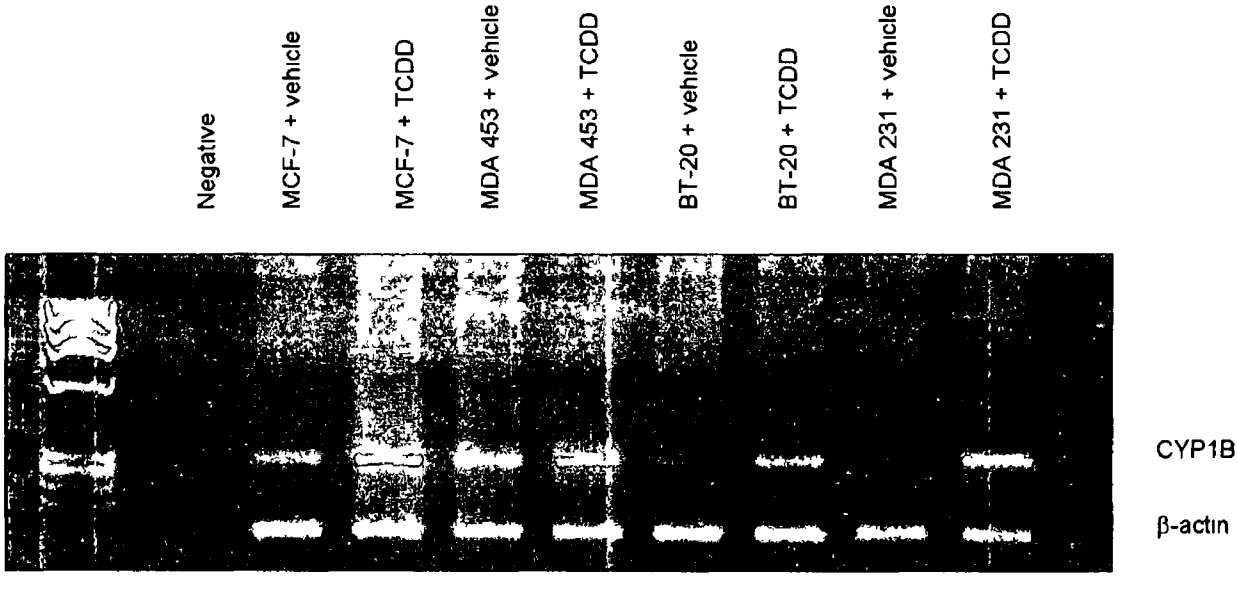


Figure 3 4 14 Expression of CYP1B1 by RT-PCR in a panel of breast cell lines after TCDD treatment Cells were treated with 3 22 ng/ml TCDD or vehicle (toluene) for 4 hours before mRNA extraction

3.4.12 CYP1B1 siRNA transfection in MCF-7 Txt cells

CYP1B1 is upregulated in MCF-7 Txt cells, which show an approximate 3-fold increase in Taxotere resistance (see section 3.4.7). In order to investigate the role of CYP1B1 in Taxotere resistance in these cells, siRNA transfections were carried out in order to knock-down the expression of the gene of interest. Three pre-designed siRNAs were used for CYP1B1; cells were transfected and then exposed to different concentrations of Taxotere (see section 2.19).

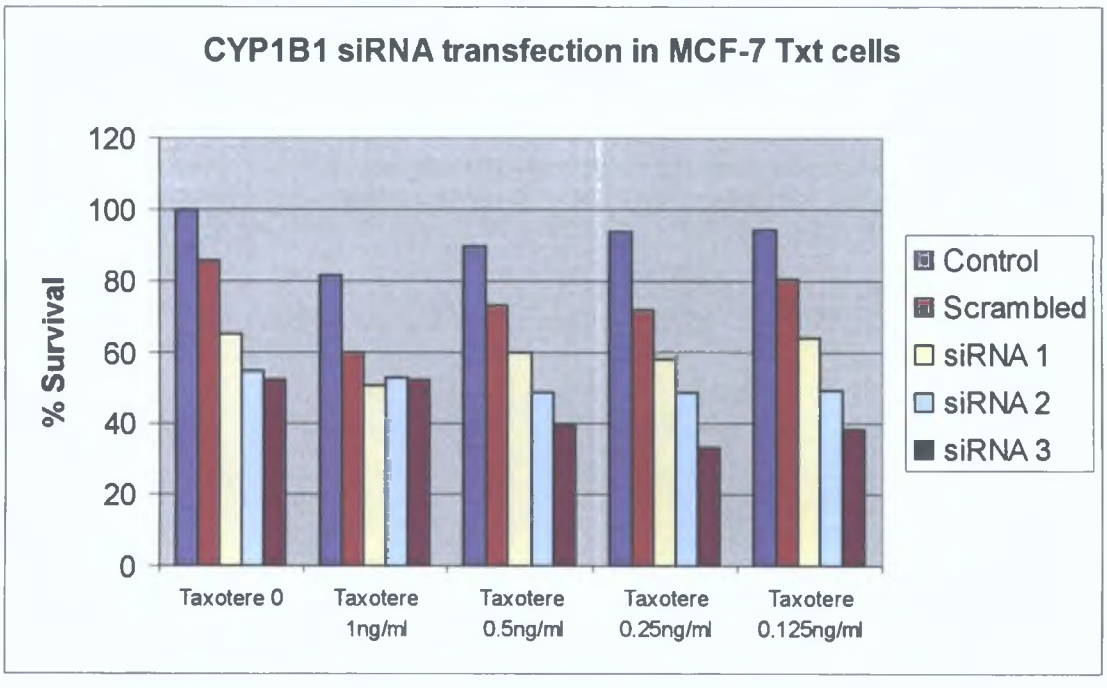


Figure 3.4.15: Effect of CYP1B1 siRNA transfection on Taxotere toxicity in MCF-7 Txt cells. Control cells received only fresh medium. Scrambled siRNA-transfected cells received a nonsense siRNA sequence. Results represent the average of two independent experiments.

As seen in Fig. 3.4.15, all three CYP1B1 siRNAs decreased the cell survival value in the absence of Taxotere, suggesting that knock-down of this enzyme is detrimental for cell viability. Transfection of CYP1B1 siRNAs 1 and 2 did not result in appreciable enhancement of Taxotere toxicity, but siRNA 3 appeared to increase the cytotoxic effect of this drug, particularly at low concentrations.

3 4 13 Transfection of CYP1B1 into mammalian cells

In order to further investigate the role of CYP1B1 in anticancer drug resistance, it was decided to transiently transfect a cell line with this enzyme and then evaluate possible changes in the resistance profile associated with the overexpression of CYP1B1 The chosen cell line for these assays was MDA 231 R4, since it had been transfected before (with the P450R cDNA) and also expressed high levels of P450R, which are needed for optimal CYP1B1 function

A range of different conditions were tested for transfection, changing the cell density, amount of cDNA and transfection reagent (Lipofectamine 2000) used, these assays were carried out using a GFP plasmid (see section 2 18 3) GFP-transfected cells emit green fluorescence that is easily visible under a fluorescence microscope and can be readily quantified by flow cytometry

48 hours after GFP transfection using the optimised conditions, cells readily displayed green fluorescence, as seen in Figs 3 4 16 A and B The transfection efficiency obtained with these conditions was approximately 55%, as measured by flow cytometry (Fig 3 4 17)

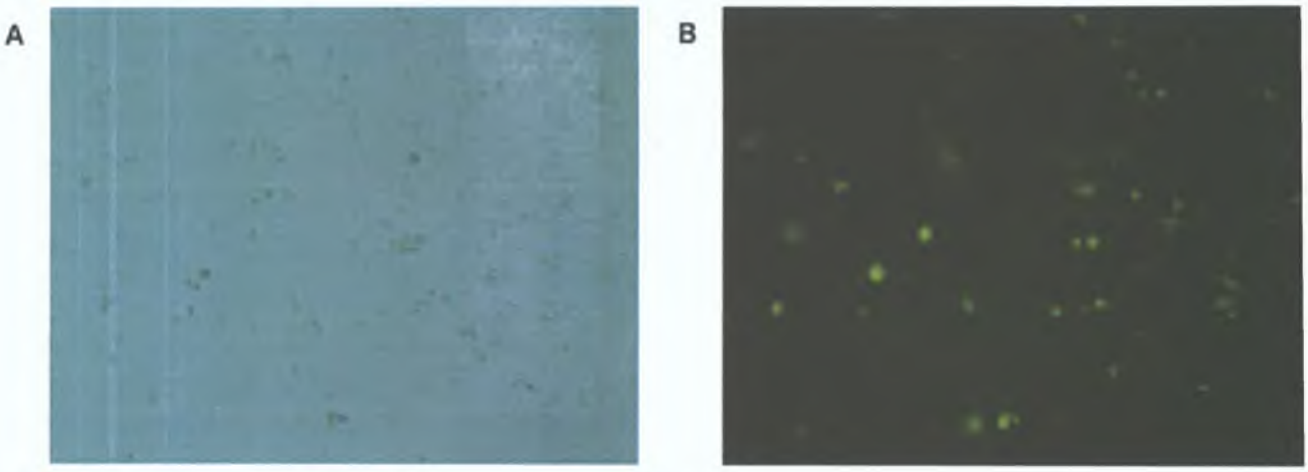


Figure 3.4.16: GFP transfection of MDA R4 cells. A, transfected cells visualised under normal light. B, transfected cells visualised under fluorescent light.

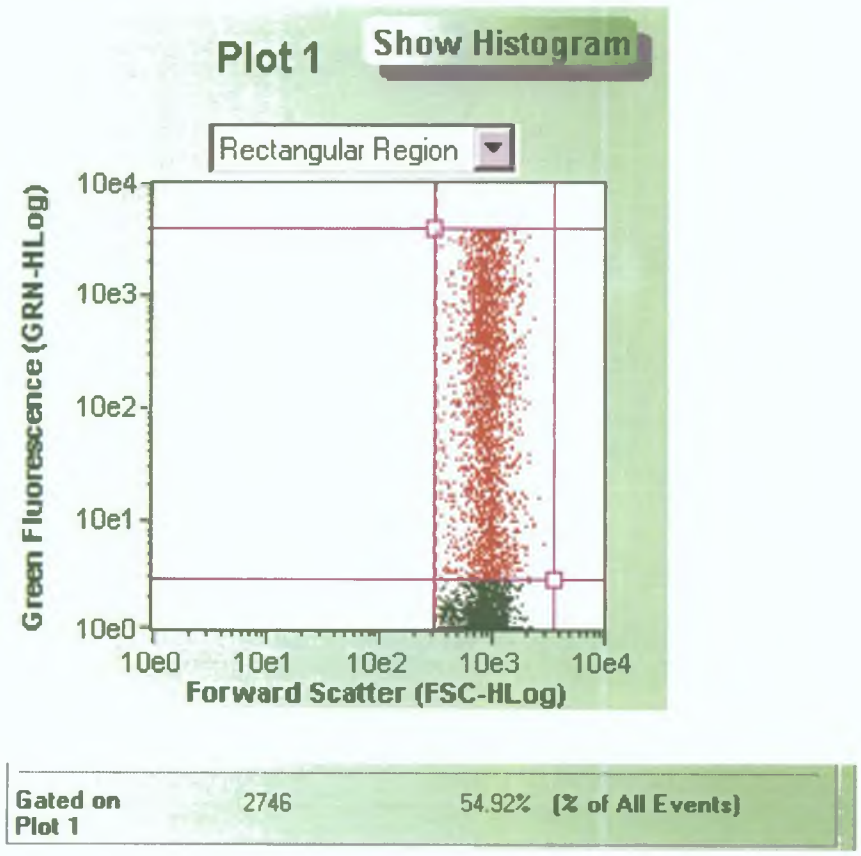


Figure 3.4.17: Efficiency of GFP transfection as measured by flow cytometry (Guava). Transfected cells display high values of green fluorescence and are shown in red. Non-fluorescent cells are shown in green. Pink lines represent gating of fluorescence values. Transfected cell efficiency is calculated as percentage of green fluorescent cells in the total number of cells in the sample (determined as a measure of forward light scatter).

After optimising transfection conditions, MDA R4 cells were transfected with a CYP1B1 plasmid obtained from Dr Thomas Friedberg This plasmid had previously been used to transiently transfect COS cells An empty vector (EV) plasmid was generated by releasing the CYP1B1 cDNA from the backbone with restriction enzymes (see section 2 18 1) and used as control for transfections

Expression of CYP1B1 protein was analysed by Western blot As shown in Fig 3 4 18, no detectable protein was found in CYP1B1-transfected cells

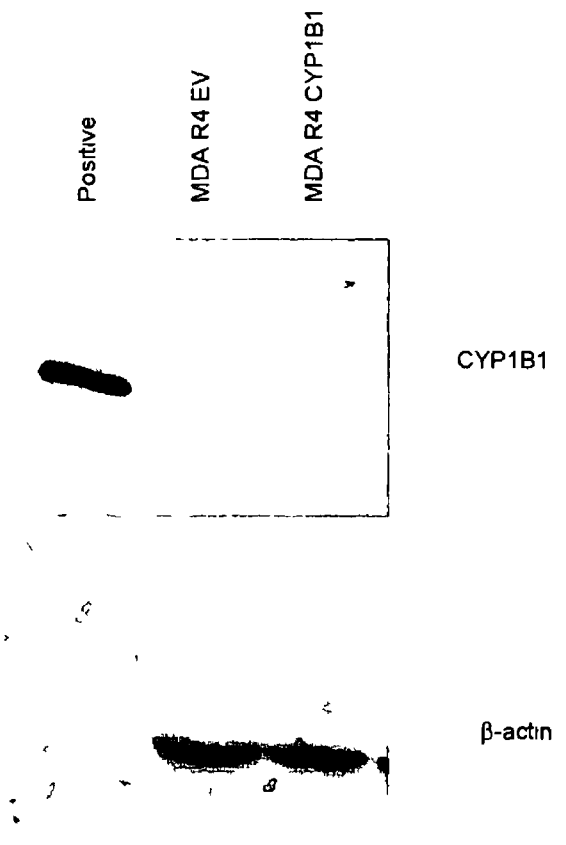


Figure 3 4 18 Expression of CYP1B1 protein in transfected MDA R4 cells by Western blot CYP1B1 microsomes were used as positive control

Since transfection of MDA R4 cells had been unsuccessful, it was decided to transfect MCF-7 cells instead. These cells express measurable levels of P450R that can support CYP1B1 function (Chen *et al.*, 1995). Transfection conditions were again optimised for this cell line, this time using 6-well plates (see section 2.18.3). Transfection of GFP into MCF-7 cells using the optimised conditions gave approximately 52% transfection efficiency, as measured by flow cytometry (Fig. 3.4.19).

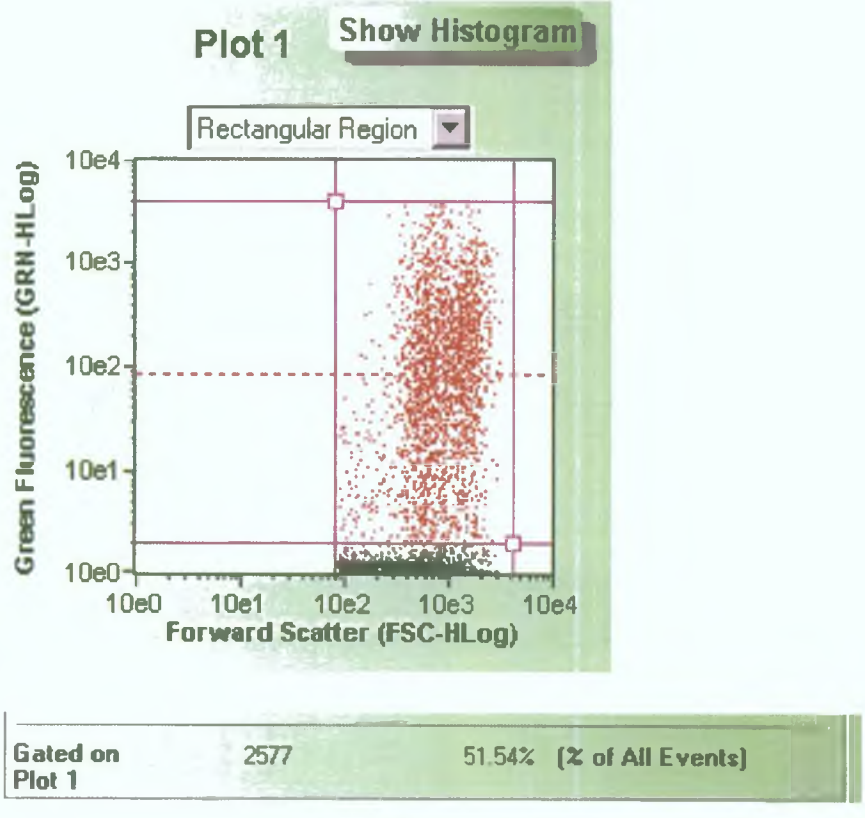


Figure 3.4.19: Efficiency of GFP transfection as measured by flow cytometry (Guava). Transfected cells display high values of green fluorescence and are shown in red. Non-fluorescent cells are shown in green. Pink lines represent gating of fluorescence values. Transfected cell efficiency is calculated as percentage of green fluorescent cells in the total number of cells in the sample (determined as a measure of forward light scatter).

Expression of CYP1B1 was analysed in CYP1B1 and empty vector-transfected MCF-7 cells by Western blot As shown in Fig 3 4 20, no apparent difference in CYP1B1 levels was found between MCF-7 cells transfected with the empty vector or the CYP1B1 plasmid

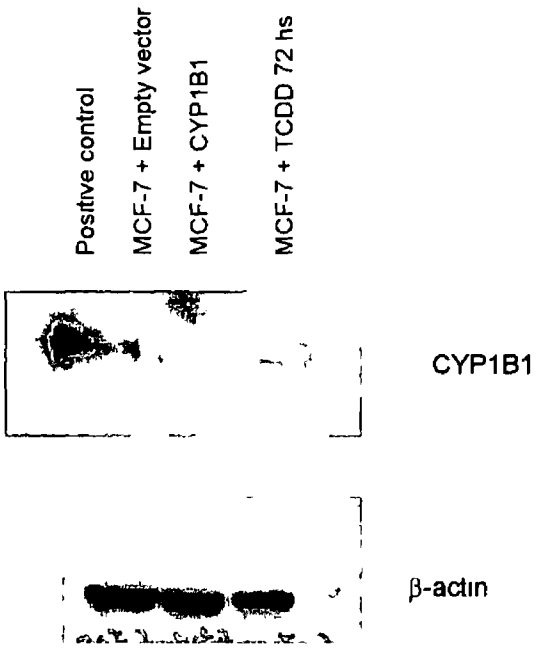


Figure 3 4 20 Expression of CYP1B1 protein in transfected MCF-7 cells by Western blot CYP1B1 microsomes were used as positive control

3 5 Role of P450 NADPH reductase in chemotherapy resistance

Activity of cytochromes P450 is highly dependent on P450 NADPH reductase (P450R), this enzyme is needed for the electron donation process that is the core of the P450 oxidation cycle P450R can also metabolise drugs on its own, independently of cytochromes P450, the best-studied example of a drug biotransformed in this way is Mitomycin C, although Adriamycin is also known to undergo metabolism by P450R (Cummings *et al*, 1998, Bartoszek *et al*, 1992) Reduction of these drugs generates more toxic compounds and also free radicals, the contribution of free radicals to the toxic effects of these drugs remains undetermined

3 5 1 P450R expression in MDA 231 cells

MDA 231 cells were originally derived from breast adenocarcinoma Human P450R cDNA-transfected MDA 231 cells (MDA R4) were a generous gift from Dr Ian Stratford, from Manchester University P450R-transfected cells show a substantial increase in P450R expression as compared to empty vector transfected MDA 231 cells (MDA EV) (Fig 3 5 1) These cells were used as a model to investigate the role of P450R in the development of drug resistance

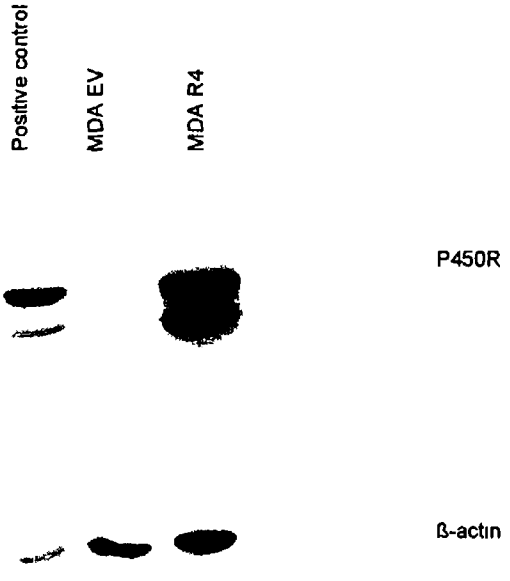


Figure 3 5 1 Expression of P450R protein as detected by Western blot on MDA 231 cells HepG2 cells were used as positive control

3.5.2 P450R activity in MDA 231 cells

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) is converted by P450R to formazan, a blue compound that absorbs at 610nm. The increase in absorbance at 610nm can then be directly correlated to the conversion of MTT to formazan by P450R. Formazan production by MDA EV and MDA R4 cell extracts was measured spectrophotometrically and expressed as nmoles formazan produced per mg of total protein (see section 2.14). As can be seen in Fig. 3.5.2, the increase in absorbance was linear up to 5 minutes. MDA R4 cells showed over a 7-fold increase in formazan production compared to MDA EV cells (Table 3.5.1).

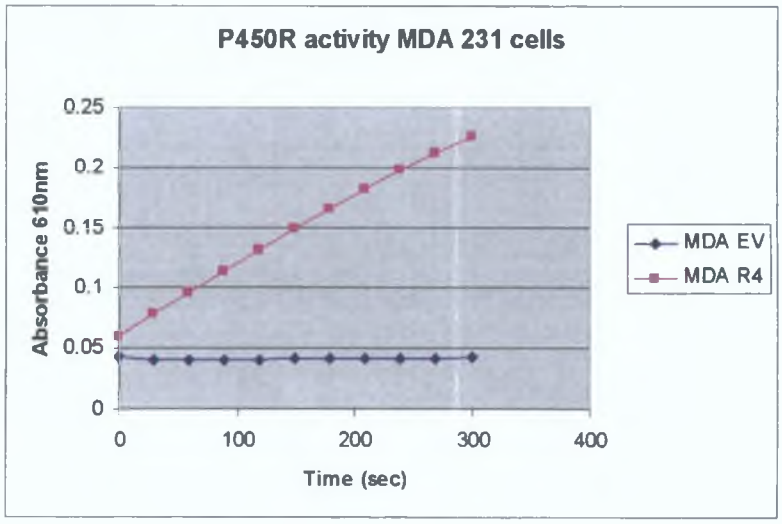


Figure 3.5.2: Increase in absorbance at 610 nm observed in MDA EV and MDA R4 cells. Total cell extracts were incubated at 37°C with MTT and NADPH for 5 minutes. Readings were taken every 30 seconds.

	P450R activity (nmoles formazan/mg total protein/minute)
MDA EV	303+/-25
MDA R4	2169+/-220

Table 3.5.1: P450R activity in MDA 231 cells expressed as nmoles of formazan produced per mg of total protein per minute. Results are expressed as mean +/- SD and represent the average of five independent experiments.

3 5 3 Anticancer drug-induced toxicity in MDA 231 cells

In order to find out more about the role of P450R expression in anticancer drug resistance, MDA EV and MDA R4 cells were exposed to a series of commonly used drugs and their IC50s determined by the acid phosphatase assay

It is known that P450R activates Mitomycin C by reacting with this drug to form a quinone intermediate that alkylates DNA, causing cell damage and inducing apoptosis (Rooseboom *et al*, 2004) As expected, MDA R4 cells were more sensitive to Mitomycin C exposure than MDA EV cells, suggesting that increased P450R activity results in enhanced Mitomycin C activation and greater cytotoxicity (Table 3 5 2)

Drug	MDA 231 EV IC50 (ng/ml)	MDA 231 R4 IC50 (ng/ml)	Fold sensitisation (R4/EV)	t-test (p value)
5-fluorouracil	1039+/-169	375+/-31	2 8	0 018
Adriamycin	21 1+/-0 2	9 0+/-2 4	2 4	0 012
Cisplatin	421+/-55	374+/-19	1 1	0 272
Mitomycin C	356+/-52	53 9+/-10 3	6 6	0 008
Mitoxantrone	3 5+/-0 5	4 0+/-1 4	0 8	0 723
Taxol	1 1+/-0 2	1 2+/-0 3	0 9	0 656
Taxotere	0 34+/-0 05	0 25+/-0 04	1 4	0 065
Vincristine	1 4+/-0 1	1 3+/-0 3	1 1	0 460
VP-16	130+/-30	172+/-11	0 8	0 126

Table 3 5 2 IC50 values and fold resistance of MDA EV cells as compared to MDA R4 cells A p value of <0 05 was deemed significant Results are expressed as mean +/- SD and represent the average of six independent experiments

As shown in Table 3 5 2, the toxicity Mitomycin C was dramatically increased by overexpression of P450R, as expected from known activation of this drug by P450R Increased sensitivity to Adriamycin was also observed in MDA R4 cells, this drug appears to share a similar activation mechanism with Mitomycin C, although contradictory reports exist on the subject

The toxicity of Cisplatin, Mitoxantrone, Taxol, Vincristine and VP-16 did not exhibit any changes after P450R transfection Overexpression of P450R appeared to confer a slight sensitisation to Taxotere, albeit non-significant, this was unexpected, since Taxotere-mediated cytotoxicity is largely due to interferences with microtubule dynamics and has not been reported as influenced by a reductive environment The most unexpected result was that of 5-fluorouracil, which was significantly more toxic to MDA R4 than to MDA EV cells This increase in toxicity is hard to explain, for this drug does not appear to interact directly with P450R (see section 3 2 3 1) P450R is a reductase and, as such, is associated with the oxidative status of the cell, the observed alterations in drug toxicity might be attributed to changes in this status

3 5 4 NADPH levels in MDA 231 cells

NADPH is an essential cofactor of P450R, apart from being an antioxidant molecule that can protect cells from various sources of oxidative stress Overexpression of P450R is expected to deplete cellular levels of NADPH, since increased enzymatic activity will be accompanied by the exhaustion of NADPH from cellular pools Indeed, this is what was observed when NADPH levels were measured in MDA EV and MDA R4 cells by a spectrophotometric method (see section 2 15) As seen in Fig 3 5 3 A and B and Table 3 5 3, absolute NADPH levels were extremely variable in MDA EV and MDA R4 cells, however, when NADPH contents were expressed relative to MDA EV levels, a consistent decrease of NADPH was shown for MDA R4 as compared to MDA EV cells

A

NADPH levels (nmoles NADPH/mg total protein)	Experiment 1	Experiment 2	Experiment 3
MDA EV	0.167	0.791	0.050
MDA R4	0.076	0.442	0.017

B

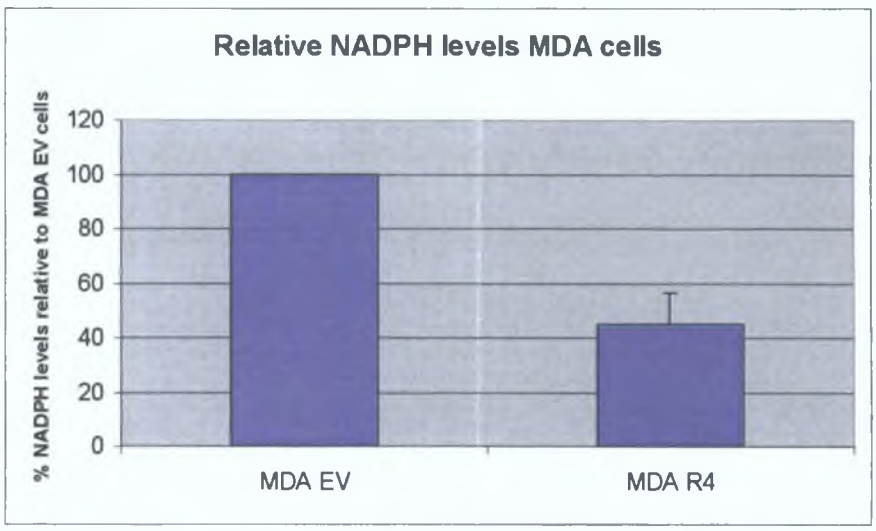


Figure 3.5.3: NADPH levels as determined by absorbance at 340 nm in MDA EV and MDA R4 cells. NADPH levels are expressed as nmoles NADPH per mg of total protein (A) or as % relative to MDA EV levels (B). Results represent the average of three independent experiments.

	NADPH levels (nmoles/mg total protein)	NADPH levels (% relative to MDA EV cells)
MDA EV	0.336+/-0.399	100
MDA R4	0.178+/-0.230	45+/-11

Table 3.5.3: NADPH levels in MDA 231 cells expressed as nmoles NADPH per mg of total protein, or as % NADPH content relative to that of MDA EV cells. Results are expressed as mean +/- SD and represent the average of four independent experiments.

3 5 5 Glutathione-related protein expression in MDA 231 cells

As seen in section 3 5 4, MDA R4 cells display decreased levels of NADPH as a consequence of P450R overexpression A major reduction in NADPH levels would mean that these cells have decreased antioxidant defences and are therefore more sensitive to reactive oxygen species (ROS)-mediated damage In this situation, it might be expected that the cells increase the levels of other antioxidants to protect themselves from the detrimental effects of ROS Glutathione (GSH) is the most abundant non-protein antioxidant present in the cell, it is synthesised by γ-glutamyl cysteine synthetase (GCS), which consists of two subunits, one heavy, termed GCSH, and one light, termed GCSL GSH is then conjugated to toxic agents by GSH transferases (GSTs), of which the most important is GST P1 Expression of GCSH, GCSL and GST P1 was analysed in MDA EV and MDA R4 cells by Western blot
Figs 3 5 4 and 3 5 5 show upregulation of GST P1, GCSH and GCSL in MDA R4 cells as compared to MDA EV cells It is likely that MDA R4 cells increase the expression of these enzymes in order to reduce the toxic effects of ROS generated by P450R overexpression

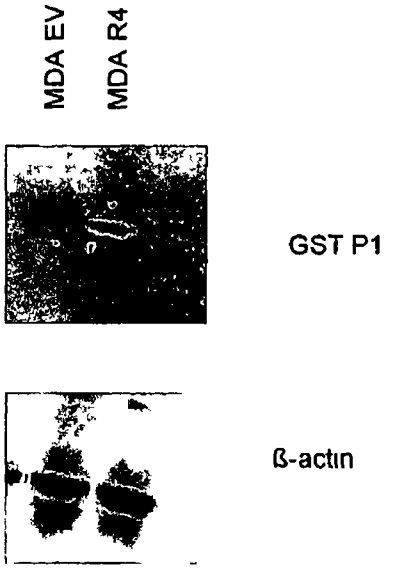


Figure 3 5 4 Western blot analysis of GST P1 protein expression in MDA 231 cells

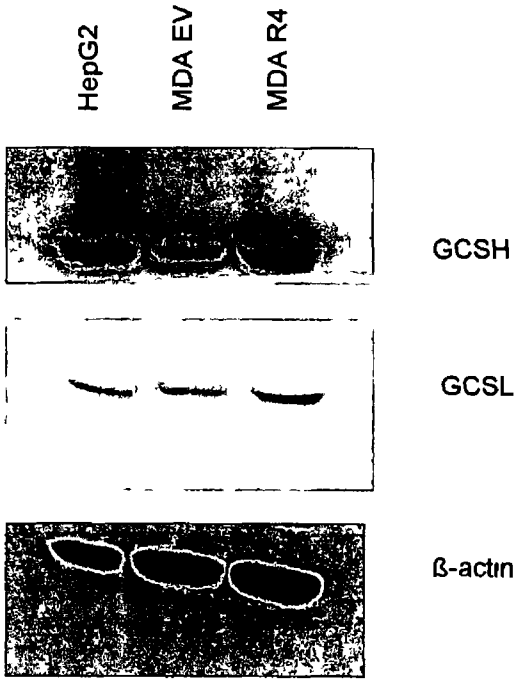


Figure 3 5 5 Western blot analysis of GCSH and GCSL protein expression in MDA 231 cells HepG2 cells were used as positive control

3 5 6 Oxidative stress in MDA 231 cells

One of the possible explanations for the increased sensitivity of MDA R4 cells to anticancer drugs is that oxidative stress levels would be increased in these cells, making them more susceptible to substances that can affect their already altered redox equilibrium These cells have lower levels of the antioxidant NADPH, also, GSH-related enzymes are upregulated, suggesting that cells have been exposed to oxidative stimuli To test this theory, oxidative stress levels were determined in MDA EV and MDA R4 cells using the ROS-sensitive probe dichlorofluorescein diacetate (DCFHDA) This is a non-fluorescent compound that diffuses freely through the cell membrane and is hydrolysed in the cytoplasm to dichlorofluorescein (DCFH), a more polar compound that remains inside the cell DCFH is not fluorescent either, but reacts with ROS present in the intracellular compartment to generate a fluorescent compound, the observed increase in fluorescence after a certain incubation period is directly proportional to the amount of ROS present in the cell (see section 2 16)

As seen in Fig 3 5 6, ROS are increased in MDA R4 cells at basal levels, consistent with the decrease in antioxidant NADPH The levels of ROS are also increased in response to Adriamycin and Mitomycin C, which undergo redox cycling by P450R, generating free radicals in the process, the generation of these free radicals could explain the observed increase in ROS However, this effect is also seen after 5-fluorouracil treatment This drug has several reported mechanisms of action, but none of them involve changes in the oxidative status of cells

Resveratrol is a polyphenol with antioxidant properties, although it has also been reported to induce the generation of ROS in tumour cell lines (Tinhofer *et al* , 2001) The opposing effects of this drug on ROS levels can be seen in Fig 3 5 6 treatment with Resveratrol reduced ROS from their basal levels in MDA R4 cells, but it increased the levels of ROS in MDA EV cells

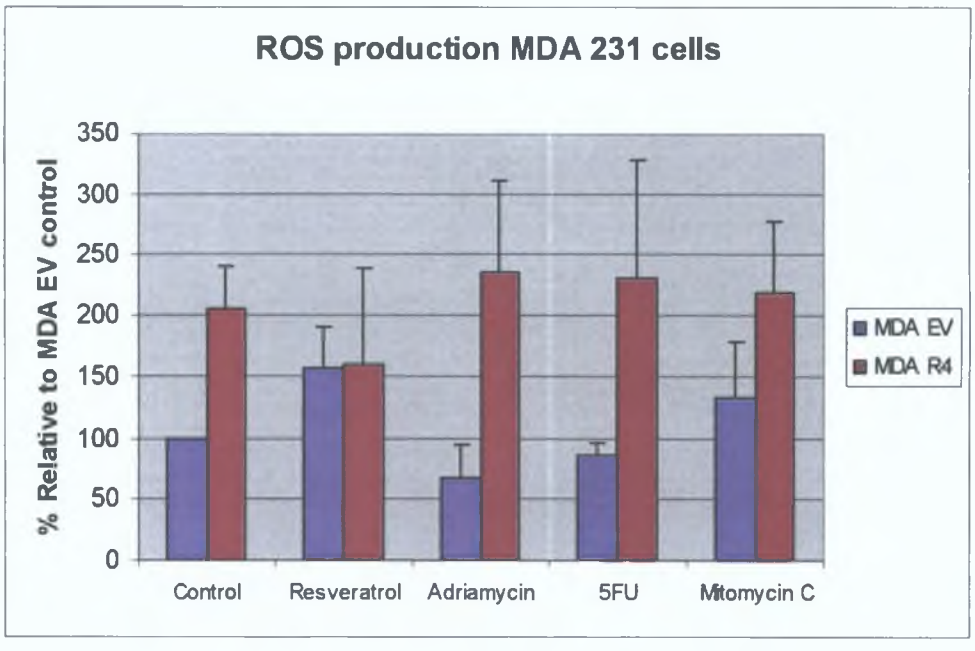


Figure 3.5.6: Oxidative stress levels before and after treatment with different anticancer drugs measured by labelling with DCFHDA and expressed as percent fluorescence relative to basal MDA EV cell levels. The concentrations used were 22.8 μg/ml Resveratrol, 1.16 μg/ml Adriamycin, 1.3 μg/ml 5-fluorouracil and 0.7 μg/ml Mitomycin C. Results represent the average of three independent experiments.

ROS levels (% relative to basal MDA EV levels)	MDA EV	MDA R4
Control	100	205+/-36
Resveratrol	157+/-33	159+/-80
Adriamycin	68+/-27	235+/-76
5-fluorouracil	85+/-11	231+/-97
Mitomycin C	133+/-45	219+/-59

Table 3.5.4: Oxidative stress levels before and after treatment with different anticancer drugs as measured by labelling with DCFHDA and expressed as percent fluorescence relative to basal MDA EV cells levels. Results are expressed as mean +/- SD and represent the average of three independent experiments.

3.6 Role of glutathione turnover in chemotherapy resistance

In-depth investigations have been carried out on glutathione (GSH)-related enzymes (GSH transferases, GSH synthetases and other metabolic enzymes such as GSH peroxidases) in order to define their role in the development of resistance to chemotherapy Techniques employed included overexpression of these enzymes by transfection and also inhibition of their expression by antisense or dominant negative transfection A number of studies report increased expression of GSH-related enzymes in resistant cell lines as compared to their parental counterparts Several reports have also shown enhanced cell resistance to different chemotherapy drugs as a consequence of transfection of these enzymes, however, the levels of resistance achieved after transfection of a single gene are usually modest This suggests that resistance does not depend on the expression of a single enzyme, but rather on a coordinated system that includes several enzymes and also different kinds of proteins, such as efflux pumps (especially members of the MRP family)

In order to study the changes in GSH turnover that are associated with the resistant phenotype, the expression of several GSH-related enzymes was analysed by Western blot in a panel of cancer cell lines and their pulse-selected counterparts Changes in GSH metabolism are usually associated with the development of resistance to platinum compounds and to anthracyclins such as Adnamycin, based on this concept, cell lines were chosen which had been previously pulse-selected with these drugs Parental (non-selected) cells were also tested, while cells that had been pulsed with the same protocol but using a different drug (such as Taxotere) were included in the analysis as well

3 6 1 Drug resistance profiles of chosen cell lines

A number of cell lines have been developed in our centre by repeated pulsing with anticancer drugs Dr Laura Breen generated a number of resistant variants by pulse selection of lung carcinoma cells with Taxol and Carboplatin She then determined their toxicity profiles by the acid phosphatase assay and calculated the IC50 values for a number of drugs, as well as the fold resistance

A549 lung carcinoma cells were pulsed with Carboplatin (100 µg/ml) and Taxol (150 ng/ml) to generate the A549 Cpt and A549 Tax variants, respectively Their drug resistance profile is shown in Table 3 6 1

IC50 (ng/ml)	A549	A549-Tax	Fold resistance	A549-Cpt	Fold resistance
Adriamycin	17 3 ± 0 2	31 9 ± 4 9	1 8	13 3 ± 1 8	0 8
Taxol	1 1 ± 0 1	6 ± 0 5	5 5	0 5 ± 0 1	0 5
Taxotere	0 8 ± 0 9	1 5 ± 0 2	1 8	0 3 ± 0 1	0 3
Carboplatin	5900 ±200	7300 ±300	1 3	9600 ± 2200	1 6
Cisplatin	584 ± 32	518 ± 22	0 9	1377 ± 427	2 4
5-FU	151 ± 14	290 ± 10	1 9	205 ± 26	1 4
VP-16	117 ± 18	484 ± 81	4 1	106 ± 12	0 9
Vincristine	3 7 ± 0 5	6 0 ± 0 2	1 6	4 4 ± 0 9	1 2

Table 3 6 1 Drug resistance profile of A549 cells and their pulse-selected variants IC50 values are expressed as mean +/- SD and represent the average of three independent experiments Fold resistance is calculated as average IC50 selected variant/average IC50 parent (from Dr Laura Breen, Ph D thesis)

H1299 lung carcinoma cells were also pulse-selected with Taxol (150 ng/ml) and Carboplatin (50 μg/ml) Their toxicity profile is shown in Table 3 6 2

IC50 (ng/ml)	H1299	H1299 Tax	Fold resistance	H1299 Cpt	Fold resistance
Adriamycin	30 3+/-0 9	29 8+/-1 3	1	27 4+/-1 7	0 9
Taxol	4 0+/-0 3	17 7+/-0 7	4 4	4 7+/-0 3	1 2
Taxotere	0 8+/-0 1	1 9+/-0 2	2 5	0 5+/-0 2	0 6
Carboplatin	3700+/-600	6200+/-500	1 7	7500+/-900	2
Cisplatin	437+/-30	655+/-196	1 5	673+/-86	1 5
5-FU	152+/-15	270+/-10	1 8	148+/-2	1
VP-16	111+/-12	122+/-19	1 1	156+/-10	1 4
Vincristine	2 4+/-0 1	5 4+/-1	2 3	1 8+/-0 3	0 8

Table 3 6 2 Drug resistance profile of H1299 cells and their pulse-selected variants IC50 values are expressed as mean +/- SD and represent the average of three independent experiments Fold resistance is calculated as average IC50 selected variant/average IC50 parent (from Dr Laura Breen, Ph D thesis)

Finally, the SKLU-1 lung carcinoma cell line was pulse-selected with Taxol (10 ng/ml) to generate the SKLU-1 Tax variant or with Carboplatin (50 μg/ml) to generate SKLU-1 Cpt cells Their toxicity profiles are shown in Table 3 6 3

IC50 (ng/ml)	SKLU-1	SKLU-1 Tax	Fold resistance	SKLU-1 Cpt	Fold resistance
Adriamycin	27 7+/-0 4	55 8+/-5 1	2 1	78 0+/-10 8	2 9
Taxol	1 2+/-0 1	6 2+/-0 5	5	10 5+/-0 6	8 5
Carboplatin	2800+/-200	3700+/-900	1 3	4000+/-200	1 4
Cisplatin	406+/-21	566+/-46	1 4	1177+/-75	2 9
5-FU	1223+/-74	1210+/-215	1	553+/-8	0 5
VP-16	116+/-6	162+/-17	1 4	240+/-13	2 1
Vincristine	0 7+/-0 3	1 3+/-0 1	1 8	1 0+/-0 3	1 4

Table 3 6 3 Drug resistance profile of SKLU-1 cells and their pulse-selected variants IC50 values are expressed as mean +/- SD and represent the average of three independent experiments Fold resistance is calculated as average IC50 selected variant/average IC50 parent (from Dr Laura Breen, Ph D thesis)

MCF-7 breast carcinoma cells were pulse-selected by Dr Yizheng Liang with Adriamycin, Cisplatin and Taxotere to generate the MCF-7 Adr, MCF-7 CisPt and MCF-7 Txt variants The protocol used for their selection as well as their resistance profile can be seen in section 3 4 7

3 6 2 Selection of proteins involved in GSH metabolism

To gain insight into the alterations of GSH metabolism that occur in cancer cells, a series of key proteins and enzymes were selected for analysis

γ-glutamyl cysteine synthetase (GCS) is the key enzyme in the synthesis of GSH It is composed of two subunits a heavy one, named the catalytic subunit or GCSH, and a light one, termed the regulatory subunit or GCSL Increased expression of GCS, especially GCSH, leads to higher levels of GSH, and this event is associated with enhanced drug resistance, especially against platinum compounds and anthracyclines The involvement of GCSL in the development of resistance remains to be fully explained

γ-glutamyl transpeptidase (GGTP) is a membrane protein and the main enzyme responsible for GSH breakdown In spite of this, high levels of expression of GGTP have been shown to correlate with drug resistance, since increased turnover of GSH also results in higher levels of substrates for its synthesis

GSH transferases play a central role in drug metabolism attachment of a GSH moiety to the drug greatly facilitates its excretion The most widely expressed of these enzymes is GST P1, which is also the most studied This enzyme has a demonstrated role in drug resistance, as shown by a number of studies (see section 1 5 7 2) overexpression results in increased resistance to chemotherapy drugs such as Cisplatin and Adriamycin GST A1 is also relevant to drug metabolism, although its role in chemotherapy resistance has not been completely elucidated

Expression of these enzymes was analysed by Western blot in our panel of resistant cell lines and their parental counterparts

3 6 3 GCSH expression in a panel of pulse-selected cell lines by Western blot

Expression of GCSH was quite even across the cell line panel, with few exceptions (Fig 3 6 1) MCF-7 Adr displayed a slightly increased expression, suggesting that pulse selection with Adriamycin can induce GCSH protein expression On the contrary, MCF-7 Txt showed a marked decrease in GCSH levels Expression of GCSH was largely unchanged in A549, H1299 and SKLU-1 cells

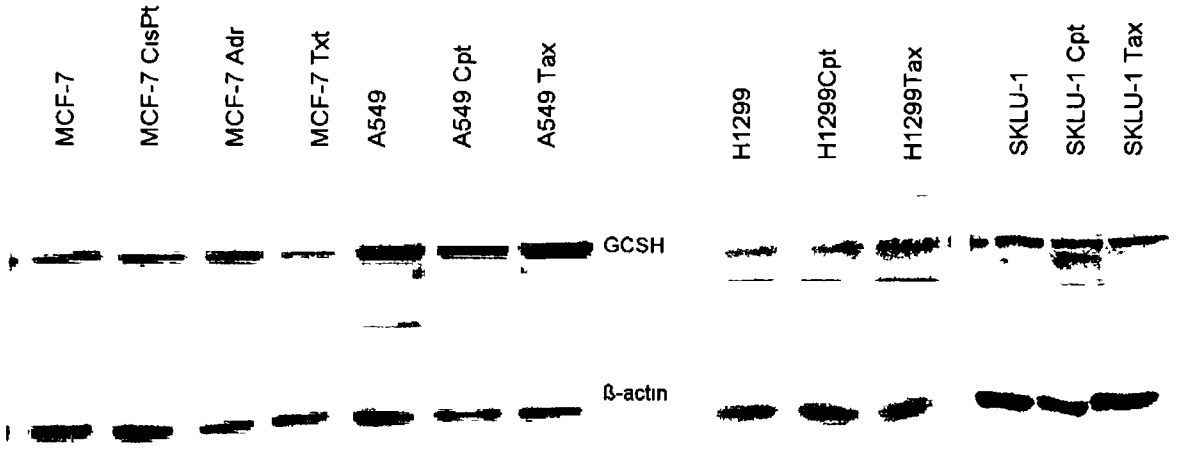


Figure 3 6 1 Expression of GCSH in some of the cell lines from our panel by Western blot

3 6 4 GCSL expression in a panel of pulse-selected cell lines by Western blot

Expression of GCSL did not always parallel that of GCSH, even though they are two subunits of the same enzyme (Fig 3 6 2) GCSL levels were not appreciably changed in any of the resistant variants analysed as compared to parent cells

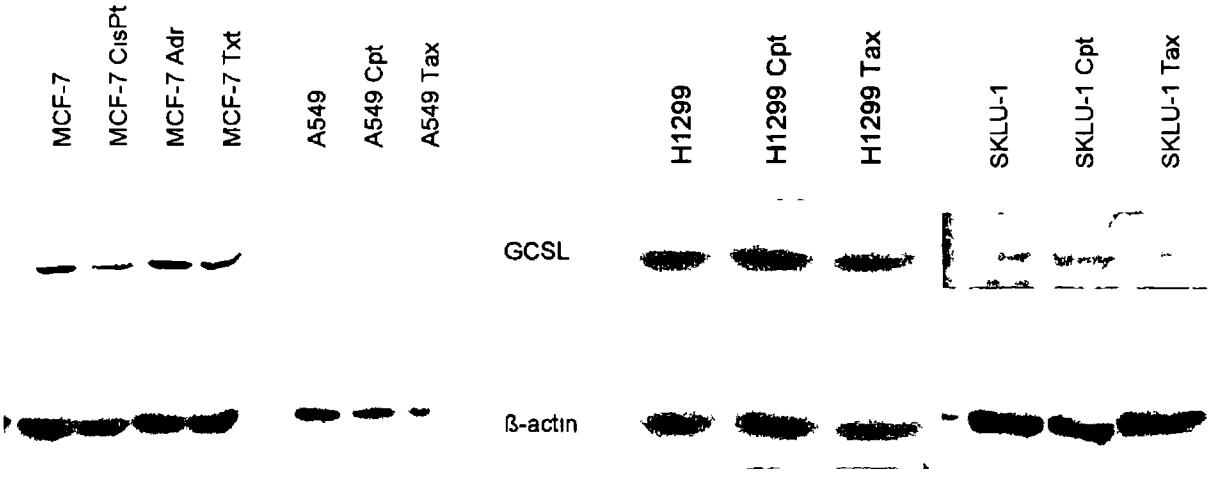


Figure 3 6 2 Expression of GCSL in the cell panel by Western blot

3 6 5 GGTP expression in a panel of pulse-selected cell lines by Western blot

Expression of GGTP was not measurable all across the panel MCF-7 parent cells and resistant vanants displayed no detectable levels of this enzyme (Fig 3 6 3) On the other hand, Taxol-selected H1299 cells showed decreased GGTP expression, while H1299 Cpt did not show any appreciable differences in GGTP levels as compared to parent cells, a similar profile was also observed in A549 parent and pulse-selected cells Expression of GGTP was slightly increased in both SKLU-1 Cpt and SKLU-1 Tax as compared to parent cells

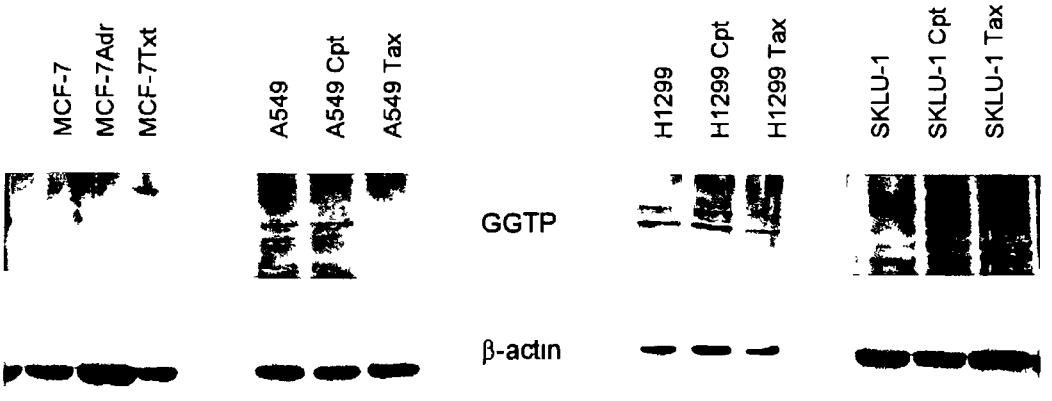


Figure 3 6 3 Expression of GGTP in the cell panel by Western blot

3 6 6 GST P1 expression in a panel of pulse-selected cell lines by Western blot

Expression of GST P1 was not measurable in all the cell lines analysed similar to the case of GGTP, no staining was detected in MCF-7 parent or pulse-selected cells (Fig 3 6 4) On the contrary, GST P1 levels were readily detectable A549 and SKLU-1 variants and also in parent cells Both resistant variants showed no major changes in expression as compared to parent cells

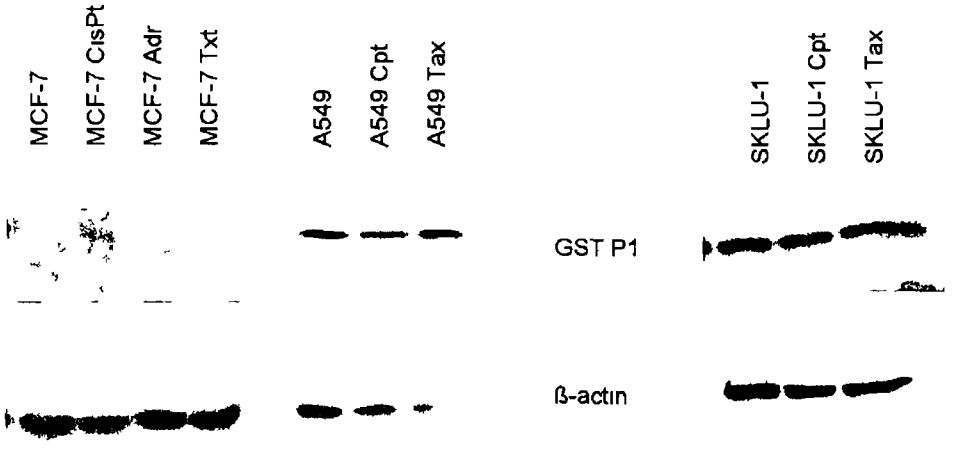


Figure 3 6 4 Expression of GST P1 in the cell panel by Western blot

3 6 7 GST A1 expression in a panel of pulse-selected cell lines by Western blot

Expression of GST A1 was largely unchanged in all MCF-7 resistant variants as compared to the parent cells (Fig 3 6 5) In contrast, A549 Cpt cells displayed a slight decrease in the expression of this enzyme, while expression of GST A1 in A549 Tax cells appeared somewhat increased GST A1 levels were increased in SKLU-1 variants as compared to parent cells, especially in SKLU-1 Tax cells

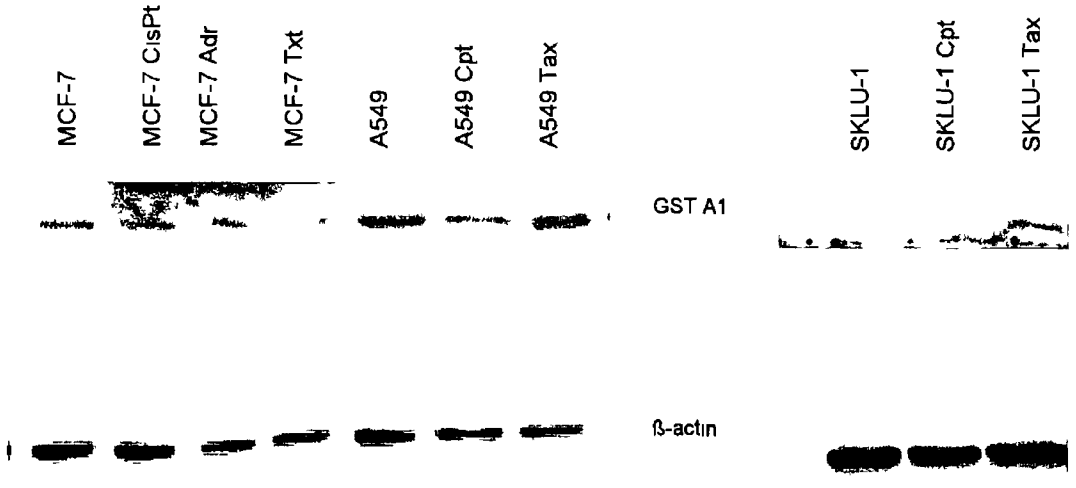


Figure 3 6 5 Expression of GST A1 in the cell panel by Western blot

3 7 Pulse selection of cell lines

When cells are exposed to toxic compounds, they activate a series of defence mechanisms aimed at decreasing the compound concentration (i e by active efflux or deactivation) and/or inhibiting its toxic effects (i e increased expression of antiapoptotic proteins or DNA repair enzymes) A single toxin usually activates several different mechanisms, which is to be expected, since multiple means of defence can inhibit toxic effects more effectively

These phenomena are observed in normal and also in cancer cells Exposure of cancer cells to chemotherapeutic drugs and the subsequent study of the defence mechanisms activated in response to this treatment can shed light on how these cells become resistant to the drugs and also how this resistance might be reverted

3 7 1 Pulse selection of Caco2 and HepG2 cells

Caco2 and HepG2 cell lines were exposed to IC70 concentrations (i e , the drug concentration which causes 70% cell death as compared to an untreated control) of different chemotherapeutic drugs in order to study which defensive mechanisms are activated by cells upon exposure to cytotoxic drugs As it can be seen in Tables 3 7 1 and 3 7 2, the concentrations used for pulse selection are considerably lower than the reported achievable plasma concentrations (Alberts *et al* , 1997, Ricotti *et al* , 2003, Scripture *et al* , 2005) Caco2 cells are derived from a colon carcinoma, and were chosen because of their metabolic competence, literature shows they are able to produce metabolites similar to those found in the body (Lampen *et al* , 1998) HepG2 cells are derived from a hepatic carcinoma and have been reported as useful models to study regulation of drug metabolism enzymes (Wilkening *et al* , 2003) These cells therefore have the potential to inactivate several different chemotherapy drugs by biotransformation

Cell line	Drugs used for pulse selection	Concentration used (ng/ml)
Caco2	Taxol	2560
	Taxotere	160
HepG2	Taxol	1280
	Taxotere	160
	Cisplatin	2500

Table 3 7 1 Cell lines and drugs used in pulse selection experiments

Drug	Achievable plasma concentration (ng/ml)
Taxol	1200-5300
Taxotere	3670
Cisplatin	10000-100000

Table 3 7 2 Achievable drug concentrations as measured in patient plasma samples during pharmacokinetic studies

3 7 2 Toxicity profiles of pulse-selected cell lines

After the six-pulse treatment was completed, IC50 values of pulse-selected cell lines were determined by *in vitro* toxicity assays and compared to those of the parent (untreated) cells The drugs chosen for these experiments were Cisplatin, Irinotecan, Taxol, Taxotere and Vincristine Taxol, Taxotere and Vincristine are CYP3A and MDR1 substrates, whereas Cisplatin is neither, being biotransformed largely through other metabolic routes Irinotecan constitutes a special case, since it is inactivated by CYP3A4 but the contribution of this pathway to the overall detoxification is low (Kehrer *et al* , 2002)

IC50s (ng/ml)	Cisplatin	Irinotecan	Taxol	Taxotere	Vincristine
Caco2	420+/-30	2040+/-440	29 7+/-2 9	11 4+/-2 2	2 4+/-0 4
Caco2Txt	330+/-40	2170+/-10	384+/-44	51 5+/-8 9	81 4+/-8 9
Caco2Txol	480+/-60	2230+/-440	306+/-8	41 6+/-7 2	103+/-28

Table 3 7 3 IC50s of Caco2 cells and their pulse-selected variants Results are expressed as mean +/- SD and represent the average of at least three experiments

IC50s (ng/ml)	Cisplatin	Irinotecan	Taxol	Taxotere	Vincristine
HepG2	810+/-150	580+/-50	9 1+/-4 0	1 1+/-0 2	12 4+/-1 9
HepG2Txt	869+/-130	530+/-100	91 6+/-29 0	4 2+/-1 2	18 9+/-2 8
HepG2Txol	920+/-150	560+/-360	41 9+/-5 4	2 6+/-0 7	30 4+/-1 2
HepG2CisPt	820+/-20	470+/-80	10 9+/-0 5	0 8+/-0 3	10 4+/-1 7

Table 3 7 4 IC50s of HepG2 cells and their pulse-selected variants Results are expressed as mean +/- SD and represent the average of at least three experiments

Fold resistance	Cisplatin	Irinotecan	Taxol	Taxotere	Vincristine
Caco2	1	1	1	1	1
Caco2Txt	0.8	0.9	12.9	5	33.6
Caco2Txol	1.2	0.9	10.3	5.3	42.5

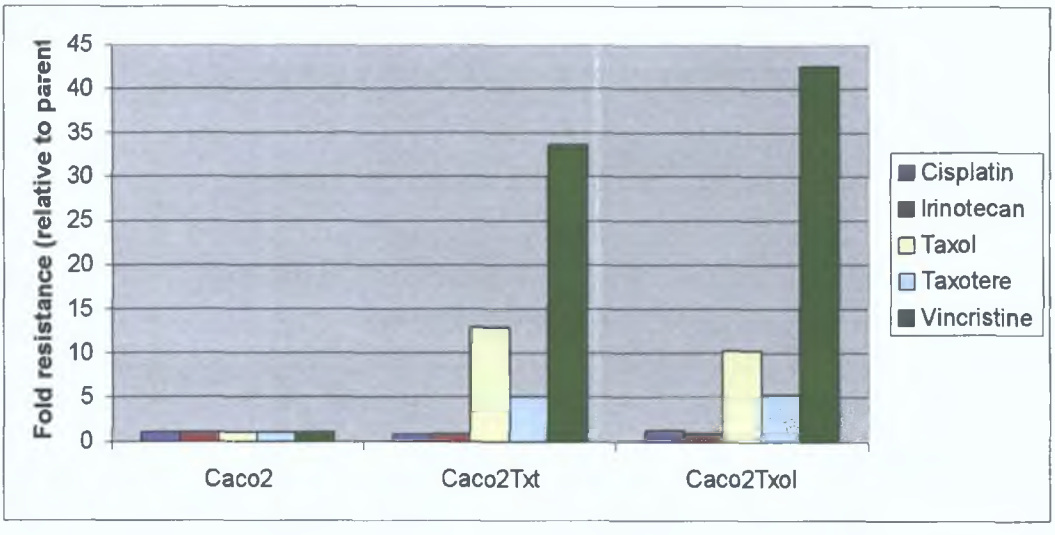


Figure 3.7.1: Fold resistance of pulse-selected Caco2 cell lines relative to parental cells.

Fold resistance	Cisplatin	Irinotecan	Taxol	Taxotere	Vincristine
HepG2	1	1	1	1	1
HepG2Txt	1	1	6.4	3.8	1.5
HepG2Txol	1.1	1.4	2.6	5.9	2.5
HepG2CisPt	1	0.8	1.2	0.7	0.8

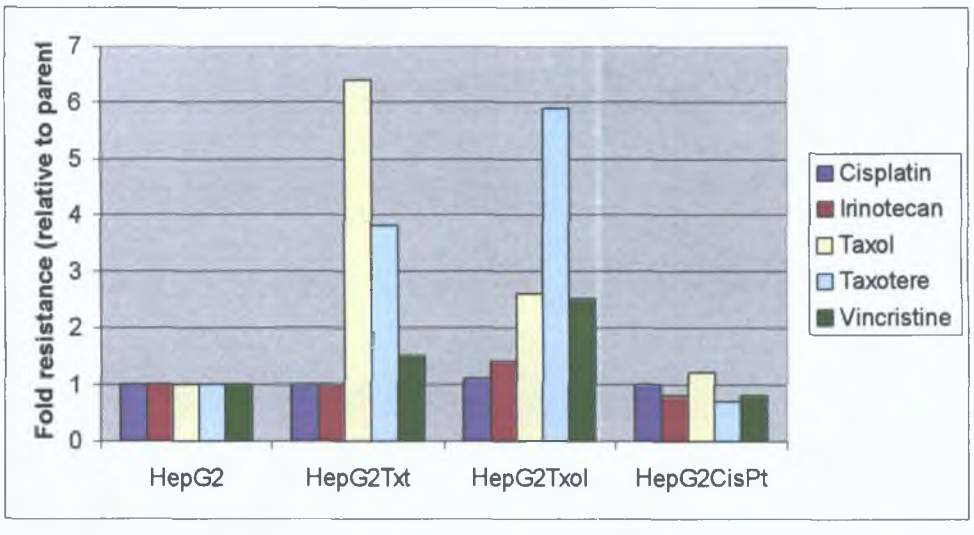


Figure 3.7.2: Fold resistance of pulse-selected HepG2 cell lines relative to parental cells.

As seen in Table 3.7.3 and Fig. 3.7.1, Caco2 cells acquired a substantial level of resistance following pulse selection with Taxol and Taxotere. Remarkable resistance was developed against the drug that cells were pulsed with and cross-resistance was also observed against taxanes and Vincristine; all three drugs are both cytochrome P450 and MDR1 substrates. No appreciable resistance was developed against either Cisplatin or Irinotecan.

In a similar way, HepG2 pulse-selected cells also developed resistance to the taxanes and to Vincristine, while no substantial change in sensitivity was observed against Cisplatin or Irinotecan (Table 3.7.4 and Fig. 3.7.2). Cisplatin pulse-selected HepG2 cells did not display any apparent changes in sensitivity to any of the tested drugs.

3.7.3 CYP3A4 expression in Caco2 pulse-selected cells

Expression of CYP3A4 in both parent and Taxol and Taxotere-selected Caco2 cells as detected by Western blot is shown below. An increase in CYP3A4 protein levels was observed in both pulse-selected variants as compared to parent Caco2 cells (Fig. 3.7.3).

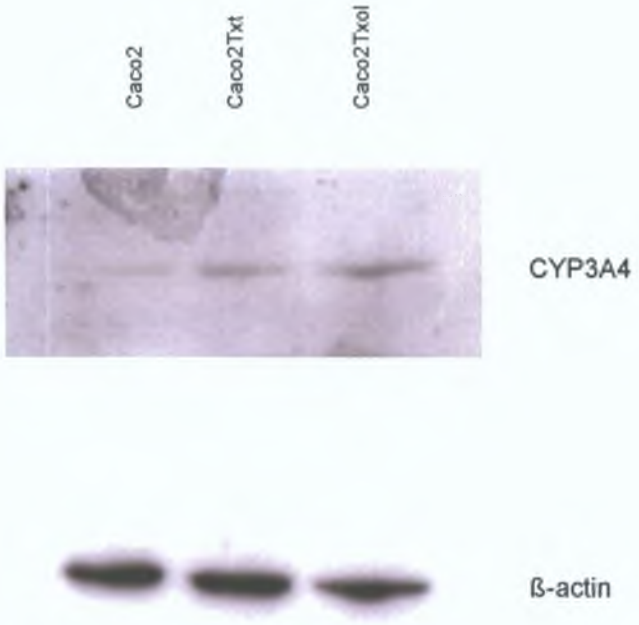


Figure 3.7.3: Expression of CYP3A4 in Caco2 cells and pulse-selected variants by Western blot.

3.7.4 CYP3A4 expression in HepG2 pulse-selected cells

Expression of CYP3A4 in both parent and selected HepG2 cells as detected by Western blot is shown in Fig. 3.7.4. A slight increase in CYP3A4 protein levels was observed in all pulse-selected variants as compared to parent HepG2 cells.

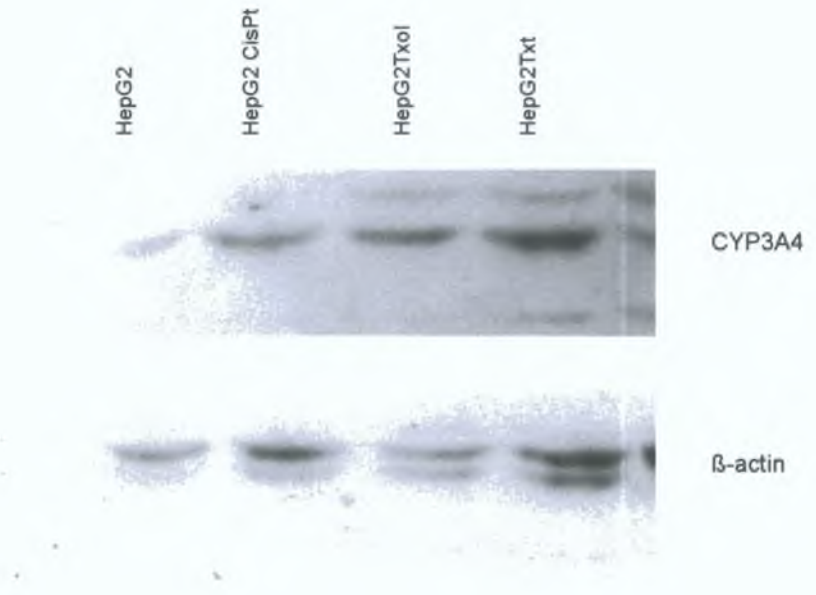


Figure 3.7.4: Expression of CYP3A4 in HepG2 cells and pulse-selected variants by Western blot.

3.7.5 CYP3A5 expression in Caco2 pulse-selected cells

Expression of CYP3A5 in both parent and Taxol and Taxotere-selected Caco2 cells as detected by Western blot is shown in Fig. 3.7.5. As seen in the densitometry graph, Caco2Txt cells only display a modest increase in CYP3A5 expression, while the effect on Taxol variants is more patent.

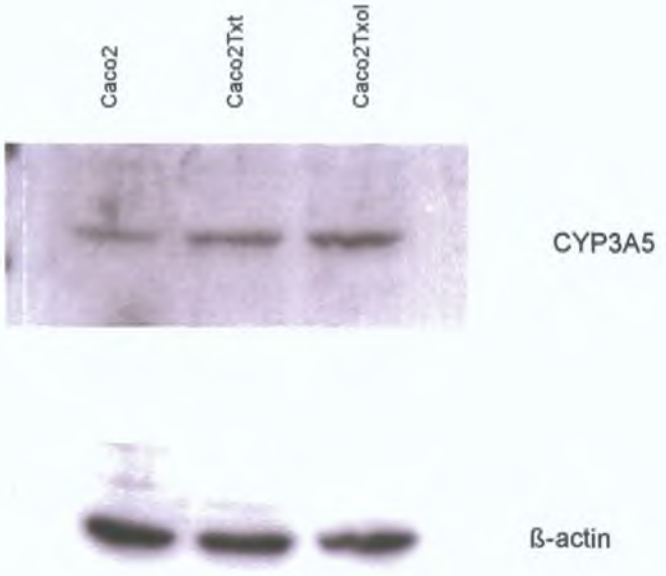


Figure 3.7.5: Expression of CYP3A5 in Caco2 cells and pulse-selected variants by Western blot.

3.7.6 CYP3A5 expression in HepG2 pulse-selected cells

Expression of CYP3A5 in both parent and pulse-selected HepG2 cells as detected by Western blot is shown in Fig. 3.7.6. HepG2CisPt and HepG2Txol cells displayed a modest increase in CYP3A5 levels, while HepG2Txt cells do not show altered CYP3A5 expression as compared to HepG2 cells.

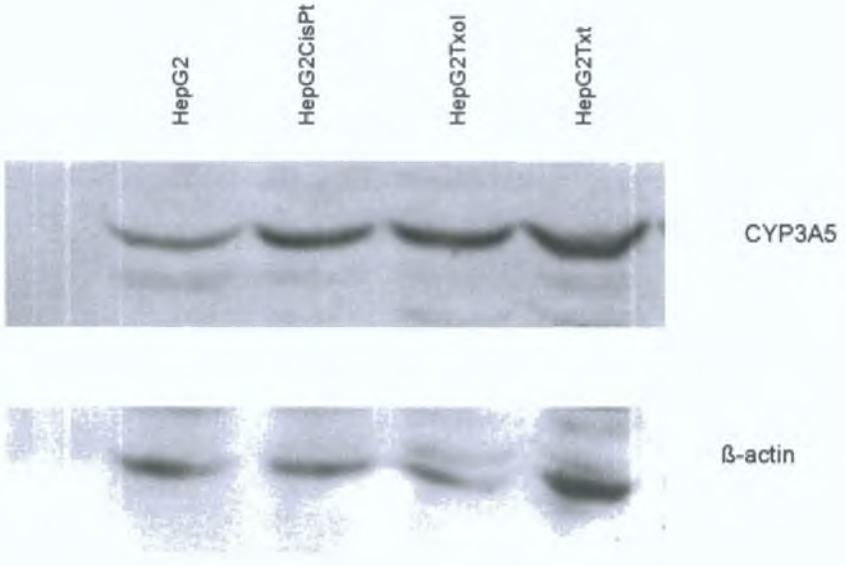


Figure 3.7.6: Expression of CYP3A5 in HepG2 cells and pulse-selected variants by Western blot.

3.7.7 Expression of CYP3A4 and CYP3A5 in other pulse-selected cell lines

Expression of CYP3A4 and CYP3A5 was also analysed in H1299 parent and Carboplatin and Taxol-selected cells, as well as on MCF-7 parent cells and resistant variants developed by pulse selection with Adriamycin and Taxotere. As seen in Fig. 3.7.7, pulse-selected MCF-7 cells did not show any change in CYP3A4 or CYP3A5 expression relative to MCF-7 cells, while the levels of these proteins were actually decreased in H1299 as compared to parent cells.

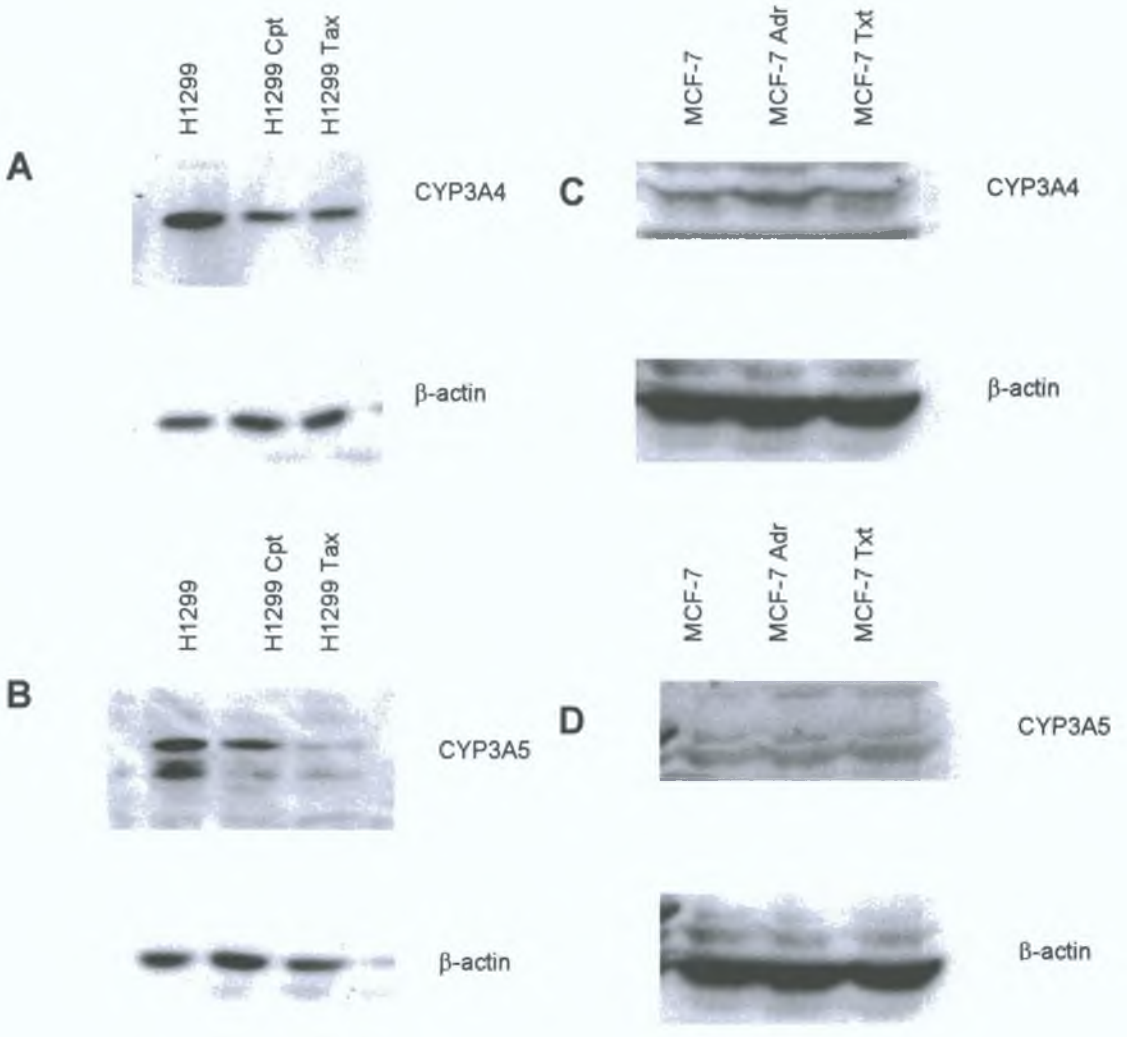


Figure 3.7.7: Expression of CYP3A4 (A and B) and CYP3A5 (C and D) proteins in H1299 cells (A and C) and MCF-7 cells (B and D) and pulse-selected variants by Western blot.

3.7.8 MDR1 expression in Caco2 pulse-selected cells

Expression of MDR1 in both parent and Taxol and Taxotere-selected Caco2 cells as detected by Western blot is shown in Fig. 3.7.8. Both selected variants displayed a marked increase in MDR1 expression.

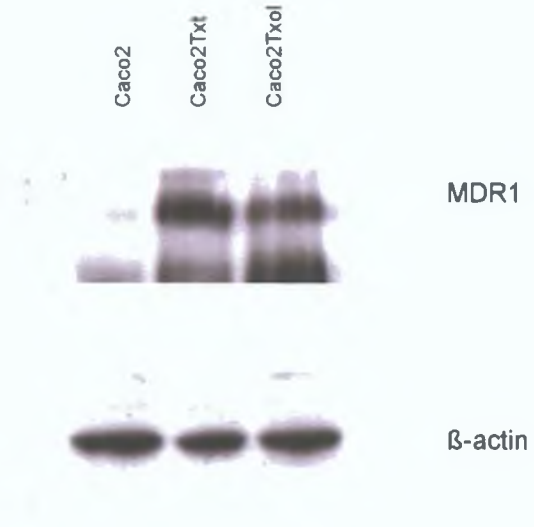


Figure 3.7.8: Expression of MDR1 in Caco2 cells and pulse-selected variants by Western blot.

3.7.9 MDR1 expression in HepG2 pulse-selected cells

MDR1 expression was analysed by Western blot in parent HepG2 cells and their selected variants. As seen in Fig. 3.7.9, MDR1 expression was clearly upregulated in pulse-selected HepG2 variants, especially HepG2Txol and HepG2Txt. MDR1 was not detectable in parent HepG2 cells.

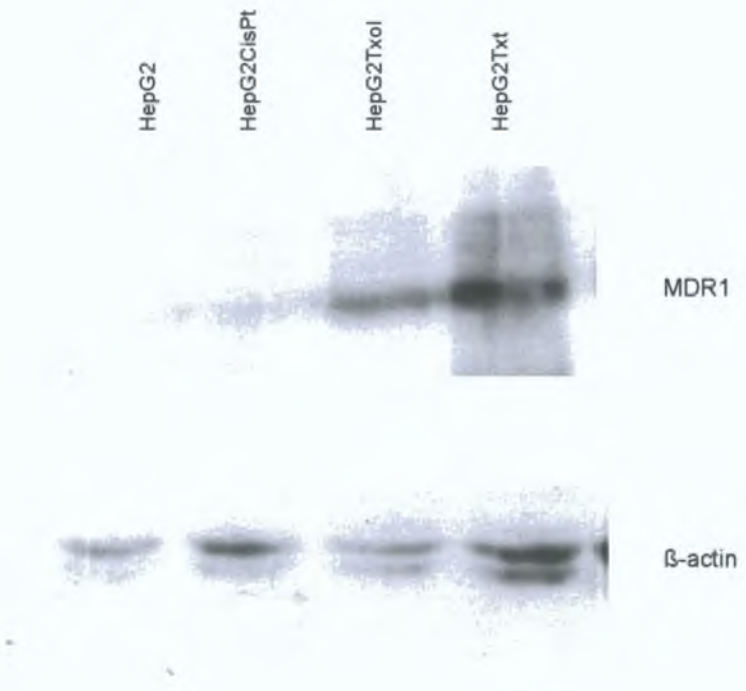


Figure 3.7.9: Expression of MDR1 in HepG2 cells and pulse-selected variants by Western blot.

3.7.10 Expression of GSH-related proteins in Caco2 pulse-selected cells

Pulse-selected Caco2 cells displayed much higher levels of resistance than HepG2 cells. Expression of key enzymes related to GSH turnover was therefore analysed in Caco2 parent and pulse-selected variants by Western blot in an attempt to investigate whether GSH turnover was affected in these cells by repeated drug exposure. Fig. 3.7.10 A shows increased expression of GCSH in both Caco2 variants with respect to parent cells. This increase in expression of GCSH is paralleled by higher levels of GCSL in pulse-selected cells as compared to their parental counterparts (Fig. 3.7.10 B).

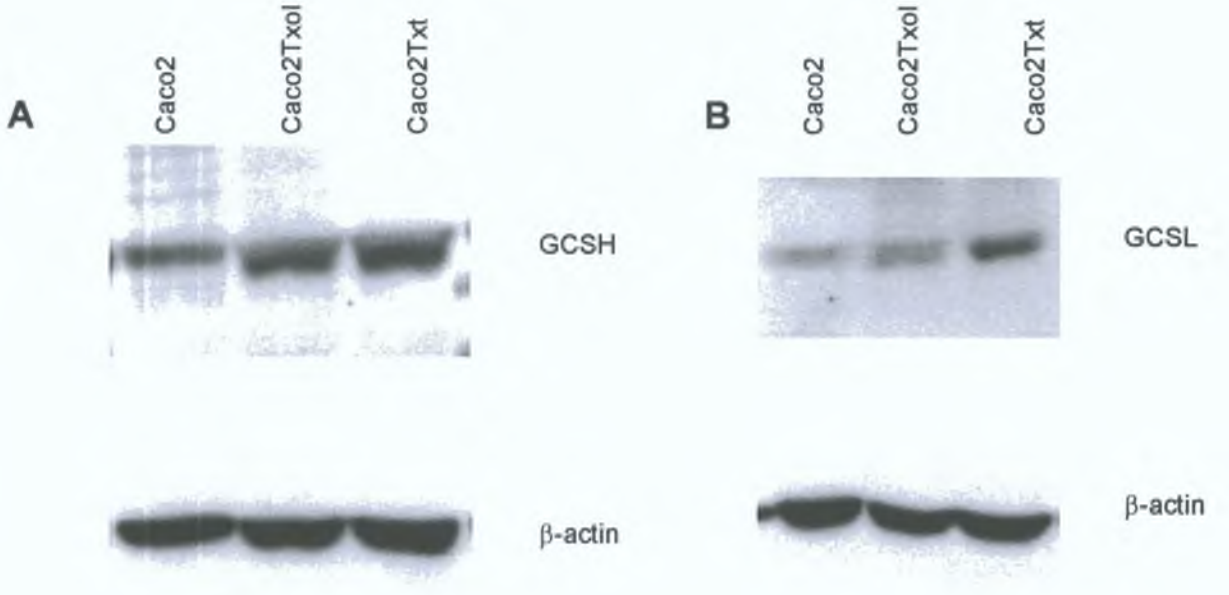


Figure 3.7.10: Expression of GCSH (A) and GCSL (B) in Caco2 cells and pulse-selected variants by Western blot.

As shown in the previous figures, expression of GSH synthetase subunits is increased, which suggests that the levels of GSH are higher in pulse-selected cells. GSH can either be transferred to toxic compounds by GSTs to facilitate their excretion or be broken down by GGTP and its components reused to synthesise new GSH molecules. Expression of GGTP is remarkably increased in Caco2 pulse-selected cells, especially in Caco2Txt cells (Fig. 3.7.11 A); this enzyme is almost absent in parent Caco2 cells. A similar increase was also observed for GST P1, the most important of the GSTs (Fig. 3.7.11 B); again, the increase in expression was more patent in Caco2Txt cells. These results suggest altered GSH turnover in pulse-selected Caco2 cells.

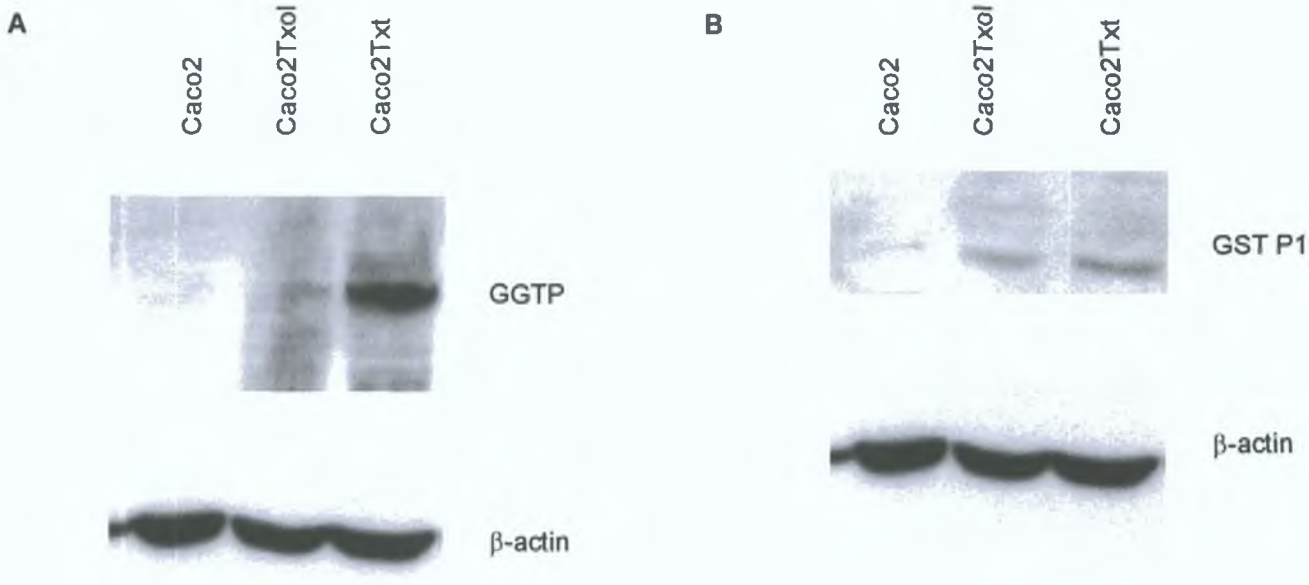


Figure 3.7.11: Expression of GGTP (A) and GST P1 (B) in Caco2 parent and pulse-selected variants by Western blot.

3.7.11 Reversion of resistance in Caco2 and HepG2 pulse-selected cells by GF120918A

Several inhibitors of MDR1 have been identified, including Verapamil, Cyclosporin A and GF120918. Most of these inhibitors act as competitors for MDR1 substrates, thus decreasing the amount of drug that is actively pumped out of the cell and subsequently enhancing the toxic effects; GF120918 is an example of a non-competitive inhibitor. Taxotere and Taxol are MDR1 substrates; cells that express high levels of MDR1 become resistant to these drugs, but the combination of Taxotere or Taxol with an MDR1 inhibitor greatly enhances the toxicity of a slightly or even non-toxic concentration of anticancer drug. In these cells, non-MDR1 substrate drugs such as Cisplatin show no increase in toxicity after simultaneous treatment with an MDR1 inhibitor, which is to be expected.

The Caco2 and HepG2 pulse-selected variants displayed an increased resistance to Taxol, Taxotere and Vincristine, all of them MDR1 substrates; this pointed to a prominent role for MDR1 in the development of resistance. In an effort to find out more about the role of this pump in the pulse-selected cells, combination assays were performed using GF120918 and either Taxotere or Cisplatin at low or non-toxic concentrations.

As can be seen in Fig. 3.7.12, inhibition of MDR1 increased Taxotere-induced toxicity in parent Caco2 cells; this effect was much more pronounced in both selected variants, suggesting an increased dependence on MDR1 activity in these cells (Fig. 3.7.13). As expected, enhancement of toxicity was not observed with Cisplatin in Caco2 parent (Fig. 3.7.14) or pulse-selected variants (Fig. 3.7.15).

GF120918 also synergised with Taxotere to induce greater toxicity in HepG2 parent cells (Fig. 3.7.16). However, contrary to what was observed for Caco2 cells, this effect was similar, or even decreased, in pulse-selected variants (Fig. 3.7.17). No enhancement of Cisplatin toxicity was observed in HepG2 parent (Fig. 3.7.18) or selected variants (Fig. 3.7.19) after simultaneous treatment with GF120918, suggesting an MDR1-exclusive phenomenon.

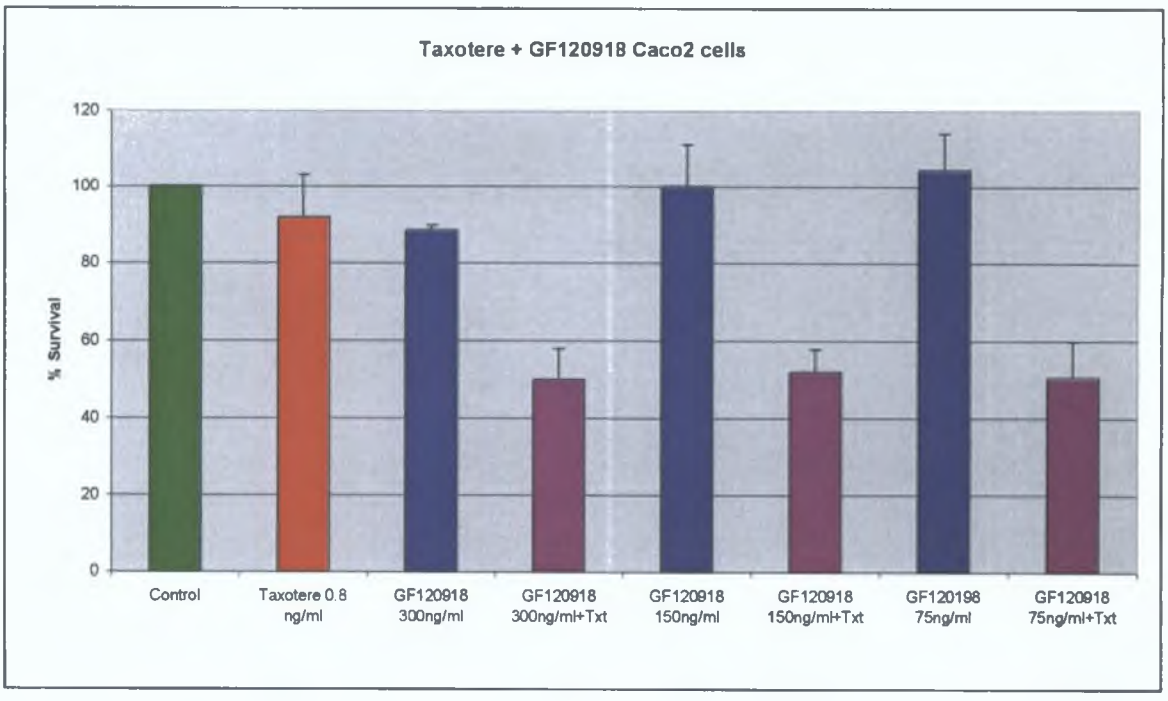
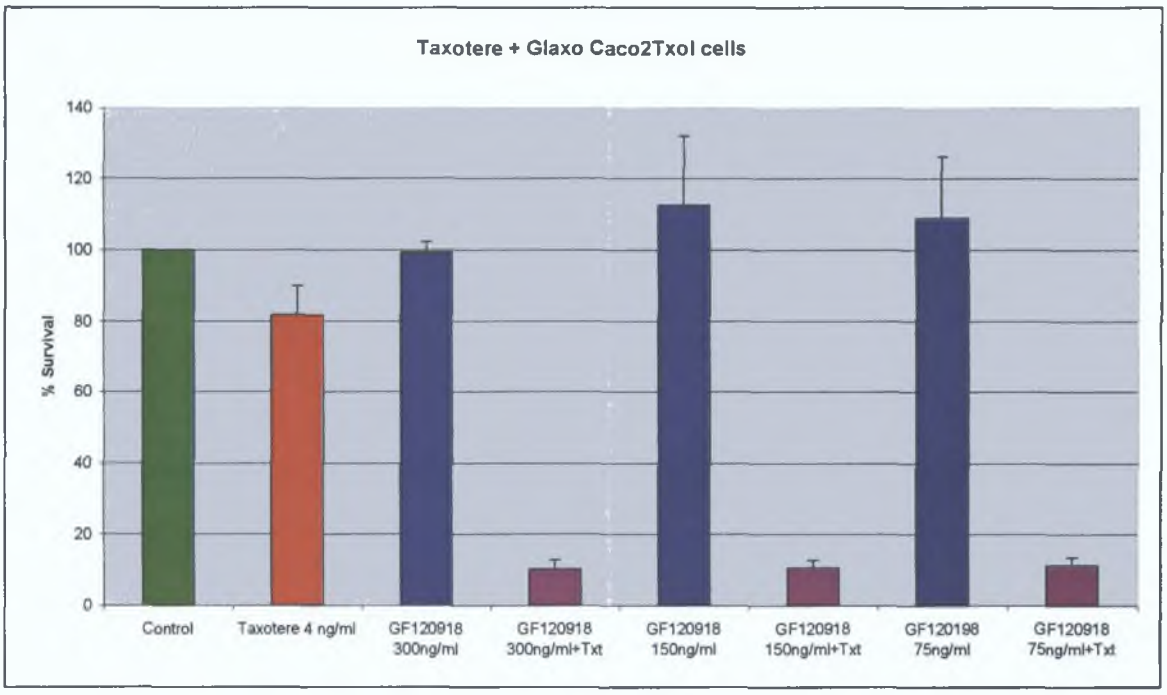


Figure 3.7.12: Modulation of Taxotere toxicity in Caco2 cells by GF120918. Results represent the average of at least three experiments.

A



B

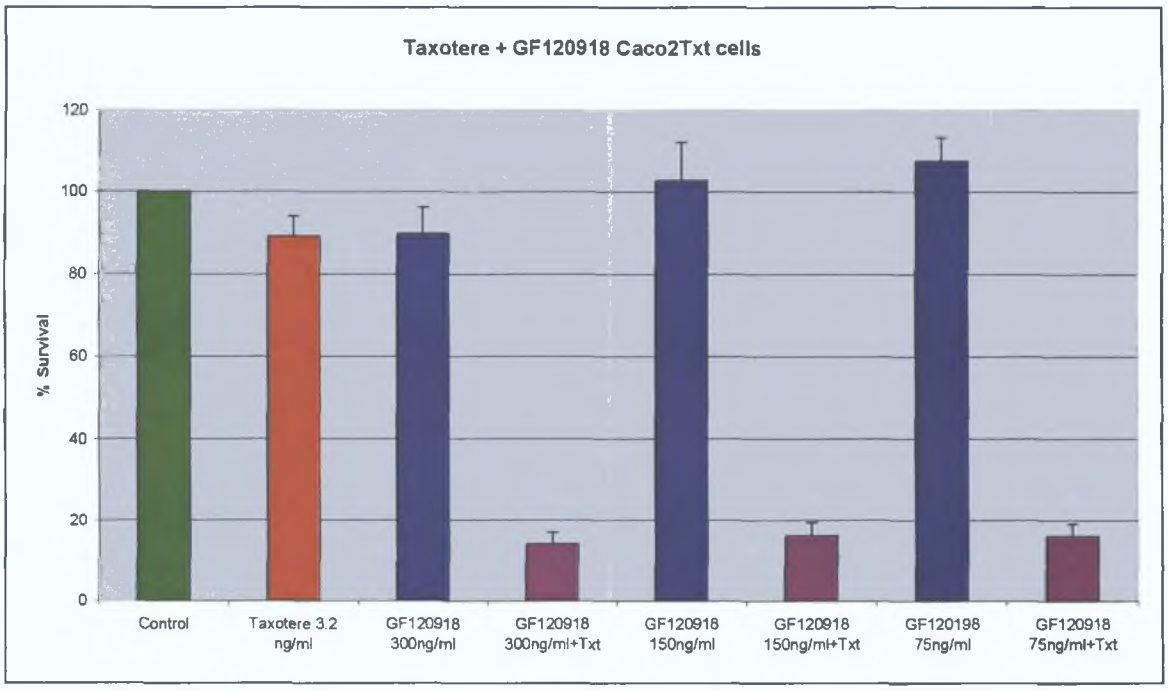


Figure 3.7.13: Modulation of Taxotere toxicity in Caco2 Taxol (A) and Taxotere (B) pulse-selected cells by GF120918. Results represent the average of at least three experiments.

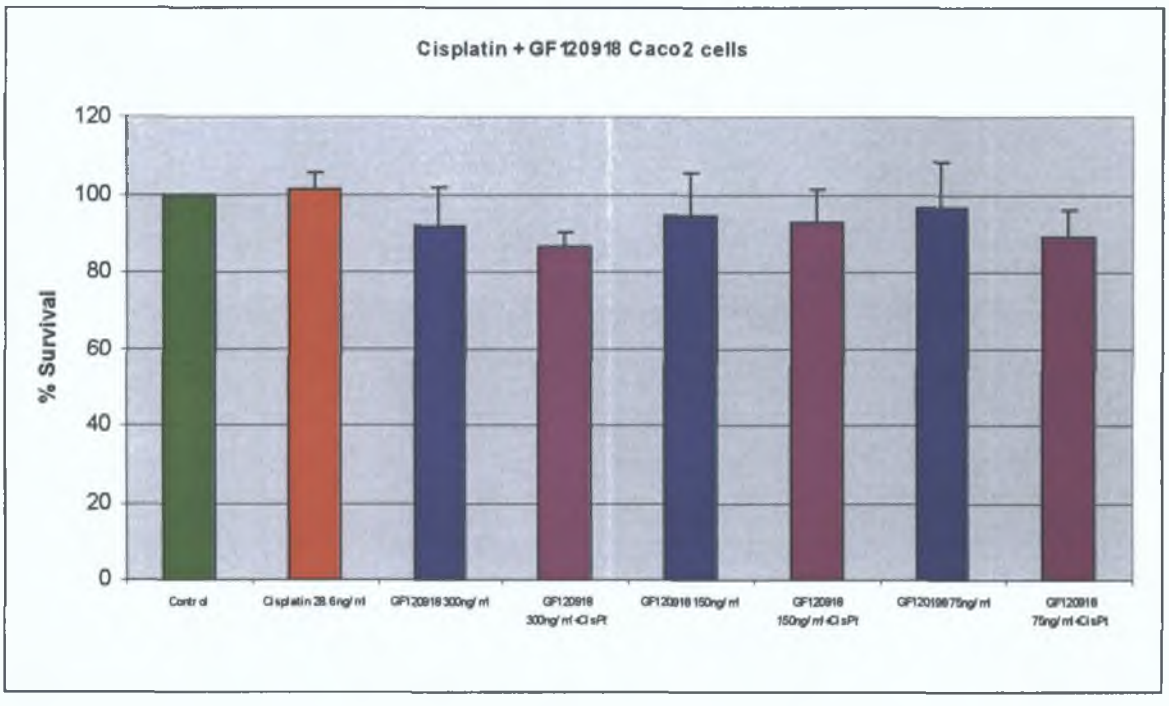
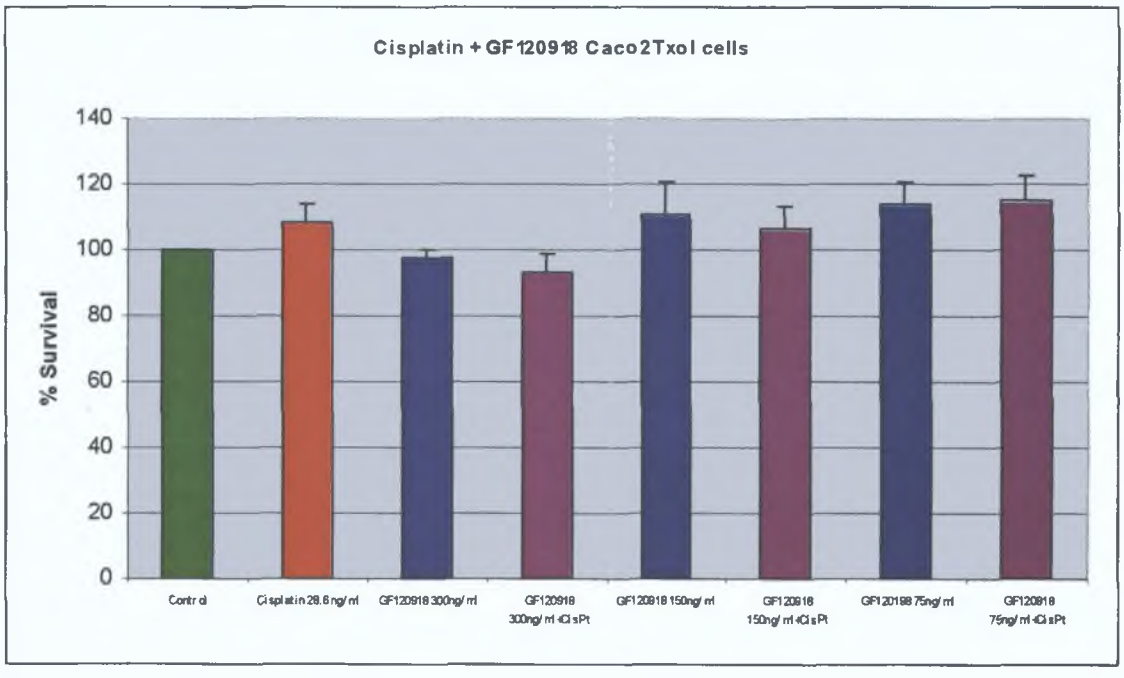


Figure 3.7.14: Modulation of Cisplatin toxicity in Caco2 cells by GF120918. Results represent the average of at least three experiments.

A



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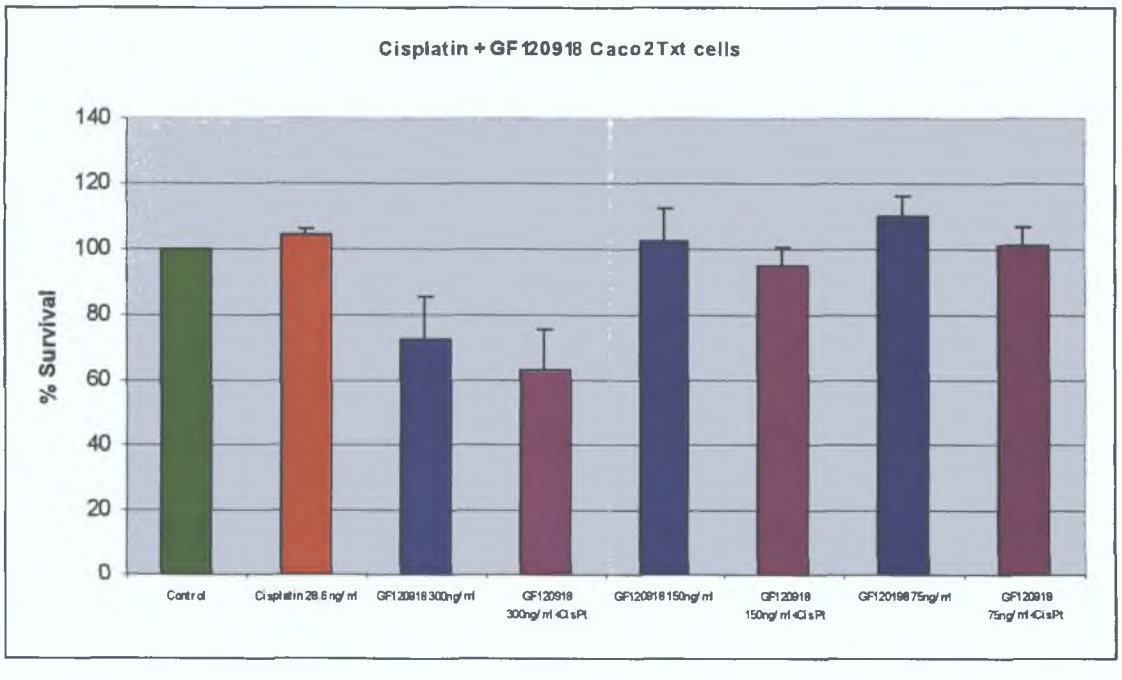
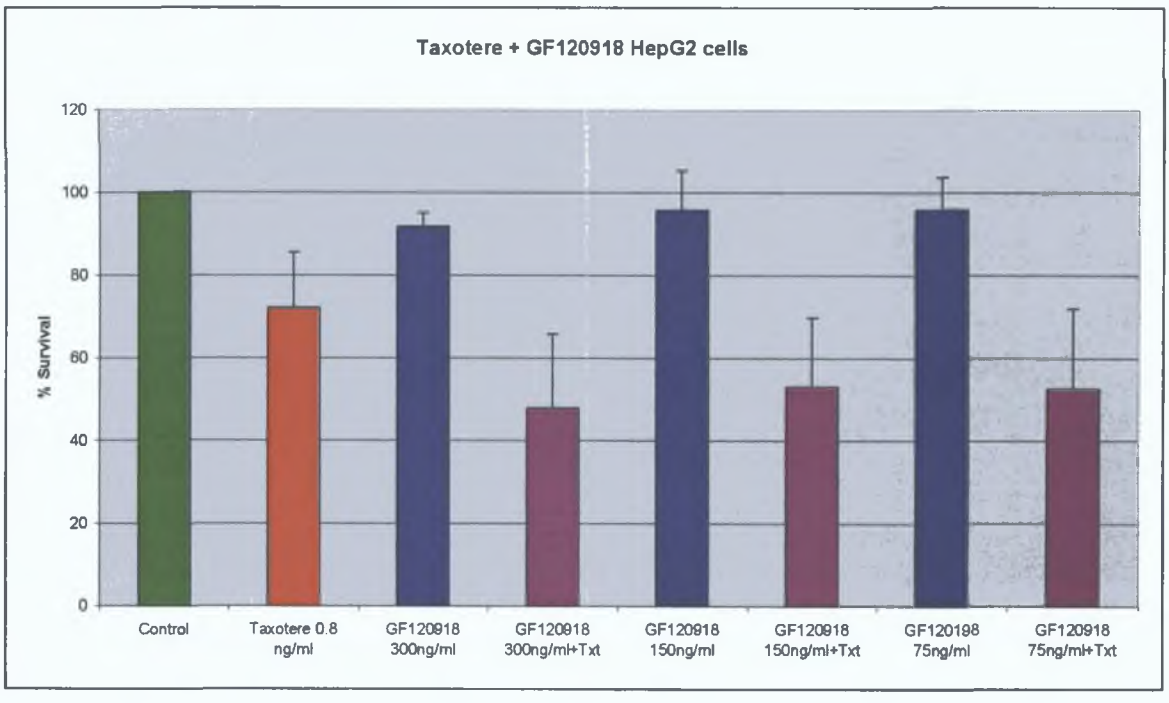


Figure 3.7.15: Modulation of Cisplatin toxicity in Caco2 Taxol (A) and Taxotere (B) pulse-selected cells by GF120918. Results represent the average of at least three experiments.

A



B

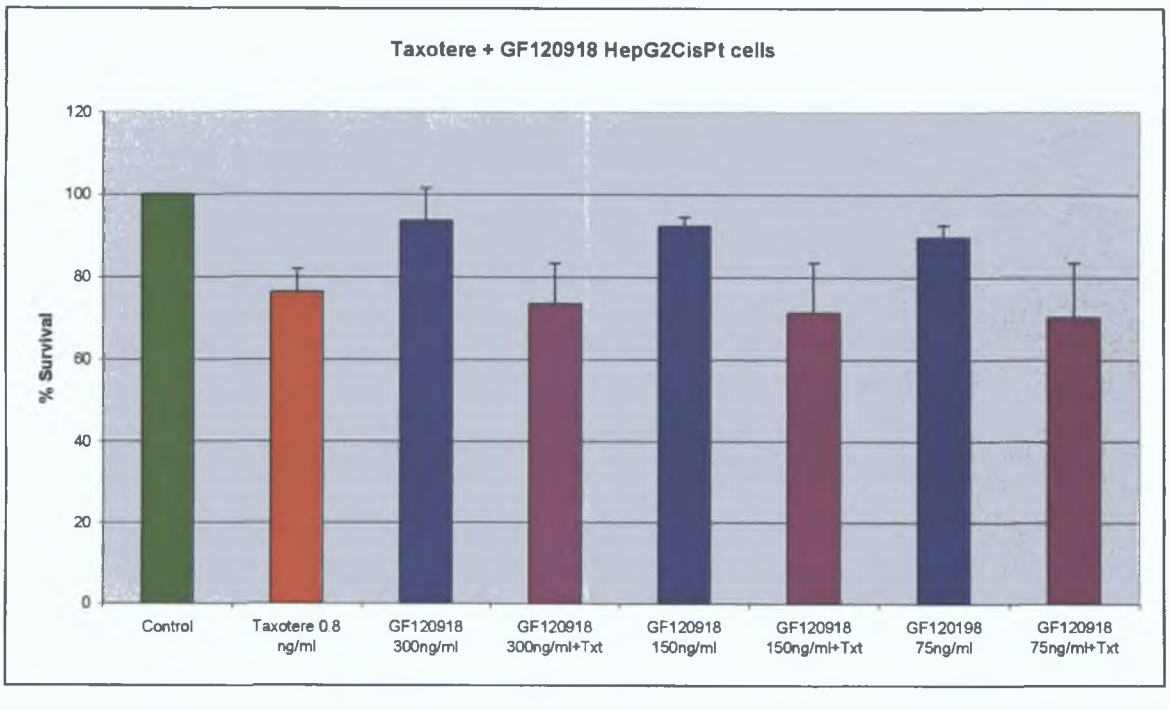
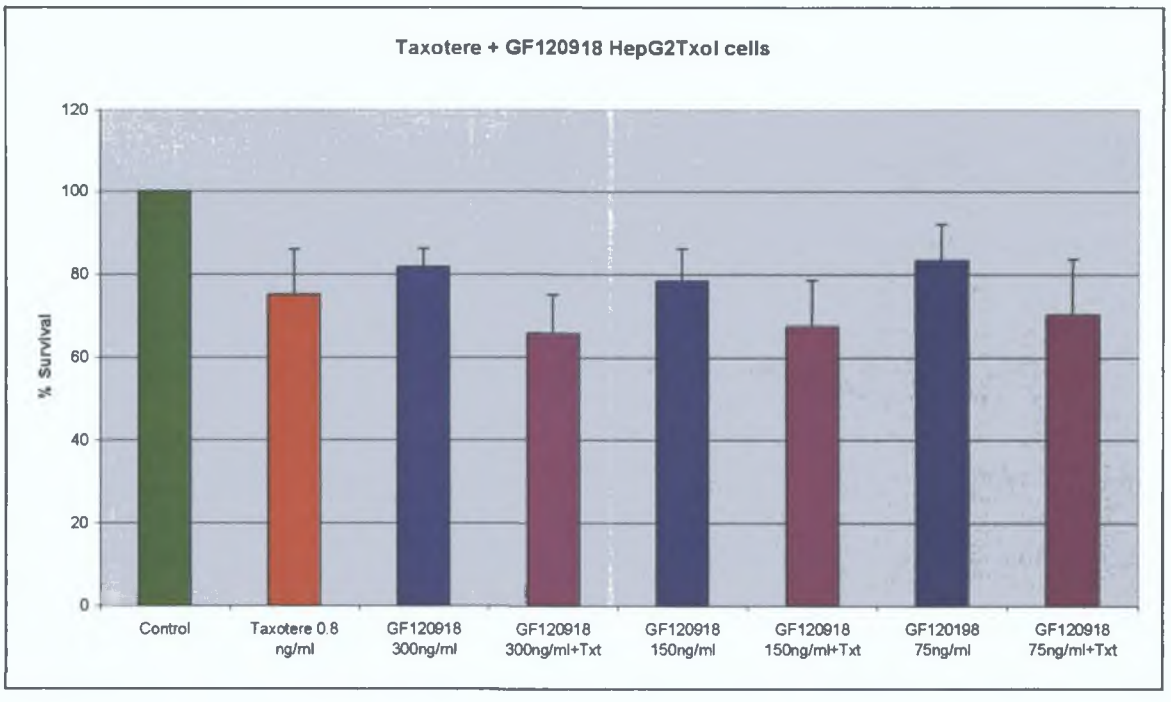


Figure 3.7.16: Modulation of Taxotere toxicity in HepG2 parent (A) and Cisplatin pulse-selected cells (B) by GF120918. Results represent the average of at least three experiments.

A



B

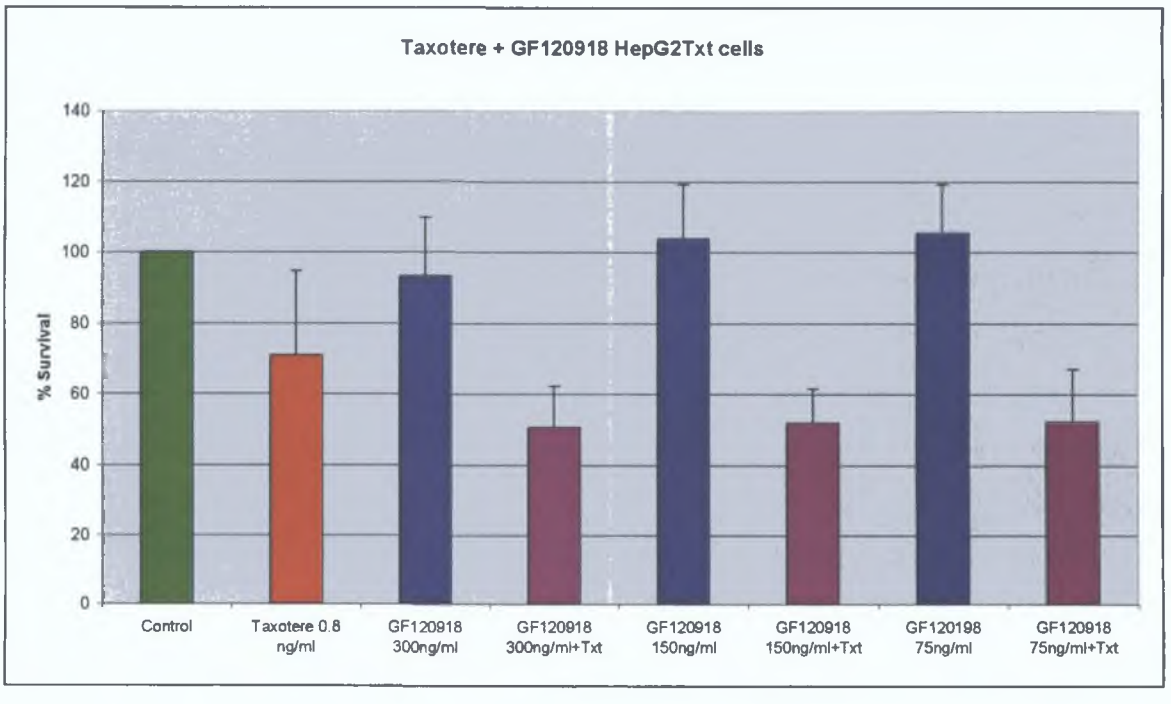
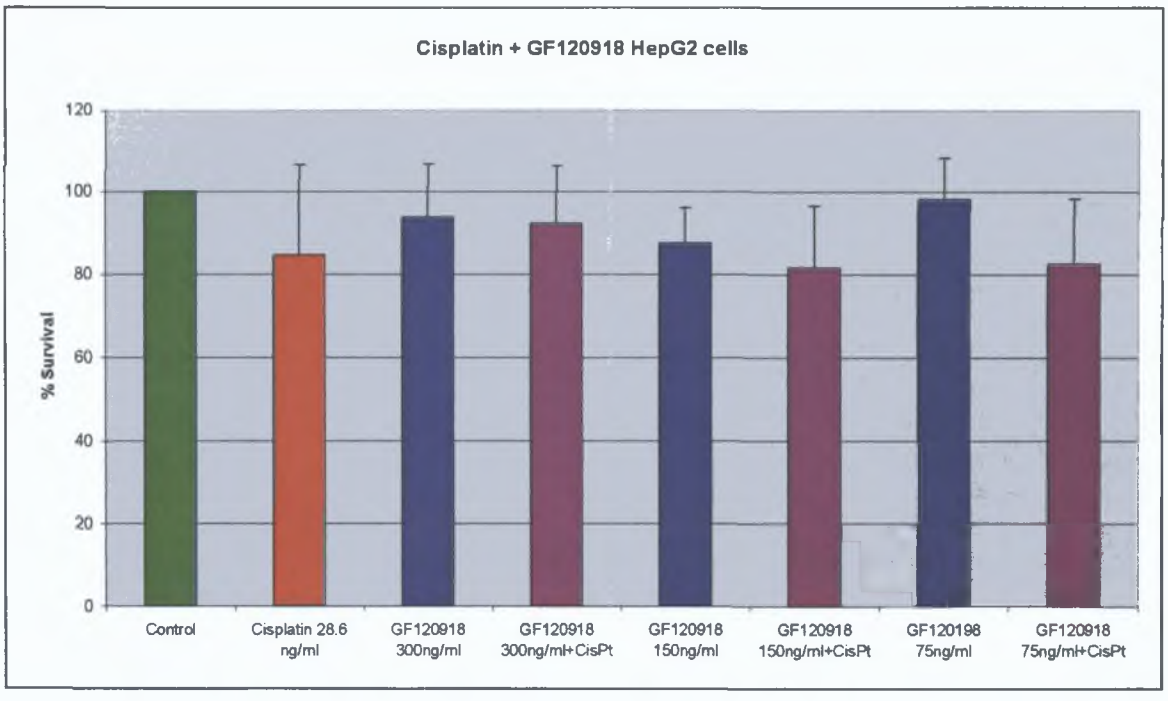


Figure 3.7.17: Modulation of Taxotere toxicity in HepG2 Taxol (A) and Taxotere (B) pulse-selected cells by GF120918. Results represent the average of at least three experiments.

A



B

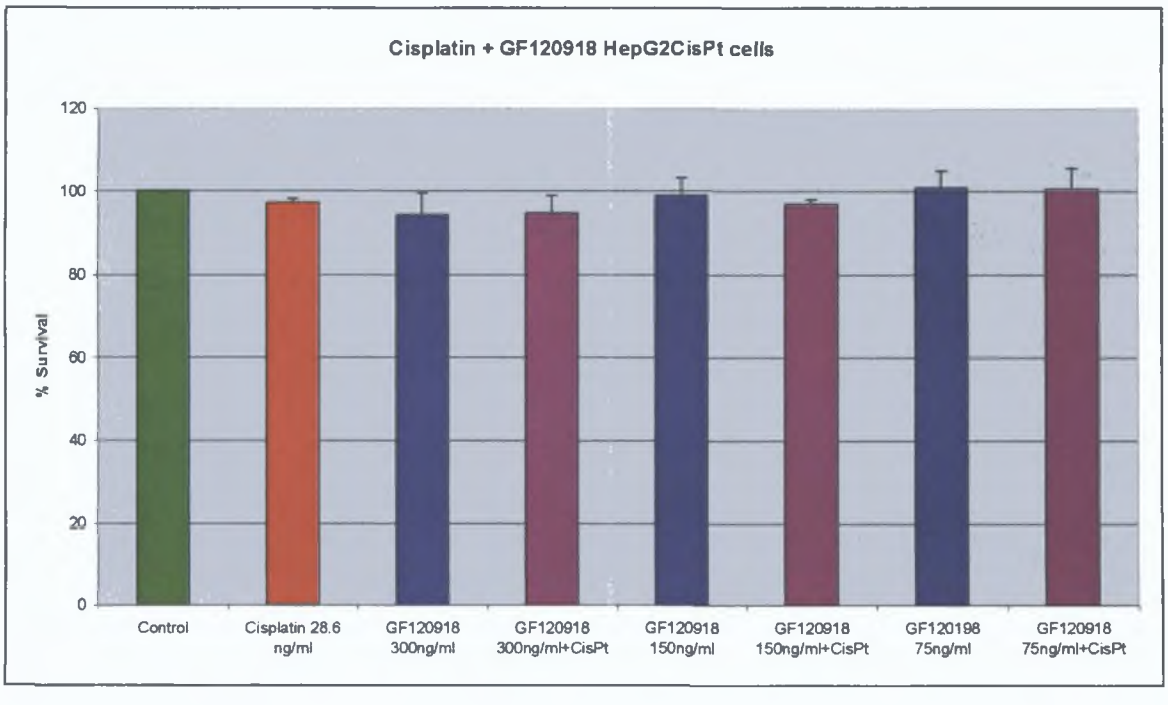
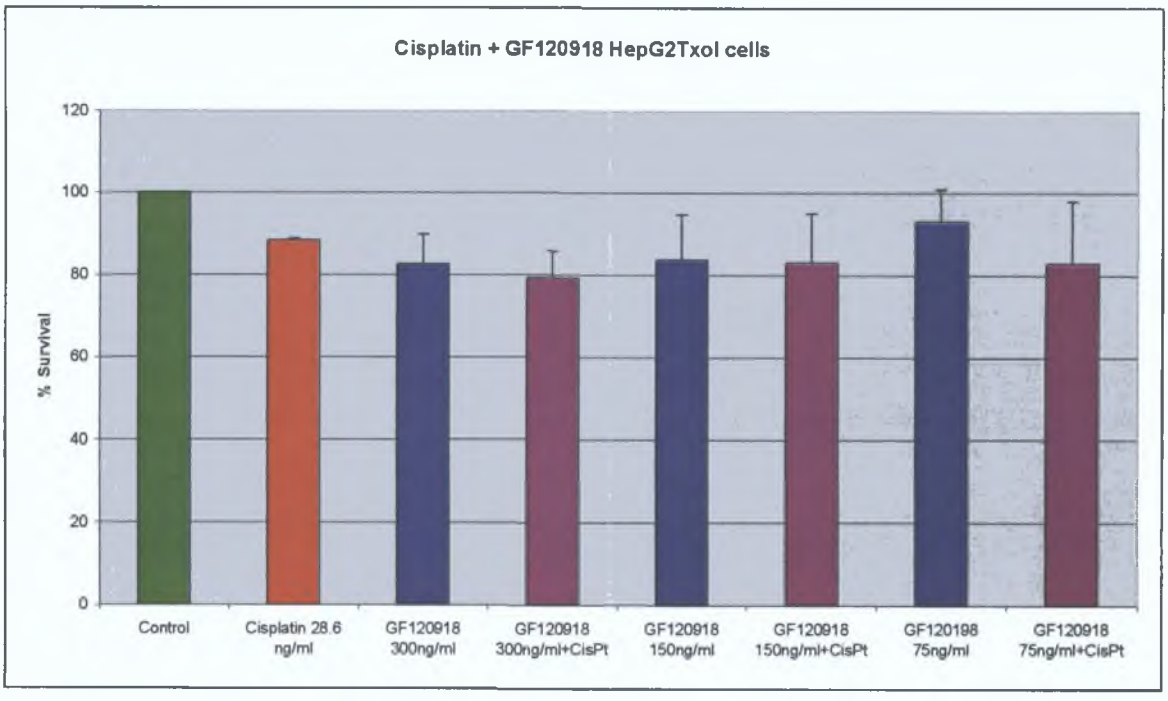


Figure 3.7.18: Modulation of Cisplatin toxicity in HepG2 parent (A) and Cisplatin pulse-selected (B) cells by GF120918. Results represent the average of at least three experiments.

A



B

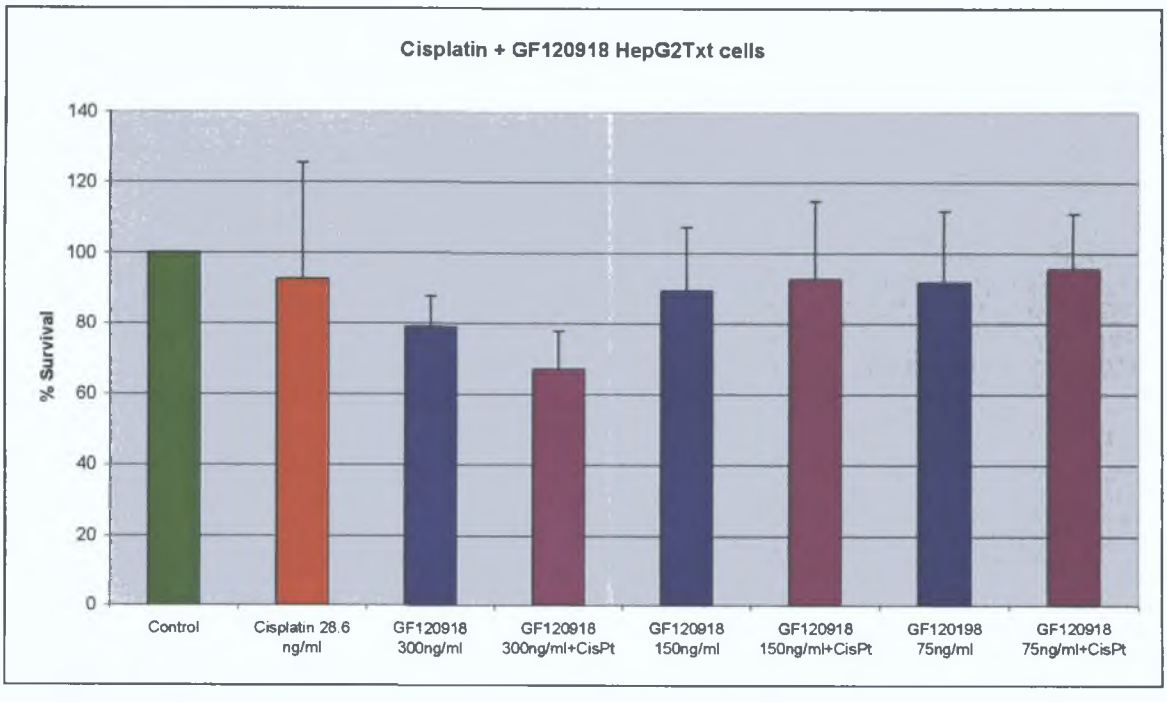


Figure 3.7.19: Modulation of Cisplatin toxicity in HepG2 Taxol (A) and Taxotere (B) pulse-selected cells by GF120918. Results represent the average of at least three experiments.

3.7.12 Reversion of resistance in Caco2 and HepG2 pulse-selected cells by Sulindac

Sulindac is a well-known inhibitor of MRP1 (O'Connor *et al.*, 2004). Simultaneous incubation of Sulindac with Adriamycin, an MRP1 substrate, did not result in a significant increase in toxicity in either Caco2 or Caco2Txol cells (Figs. 3.7.20 and 3.7.21 A); however, some synergy was observed between these two drugs in Caco2Txt cells, especially at the highest concentration of Sulindac (Fig. 3.7.21 B). No major effects in toxicity resulted from combination with Sulindac in HepG2 parent or selected cells, suggesting that MRP1 does not play a major role in the observed increase in resistance (Figs. 3.7.22 and 3.7.23).

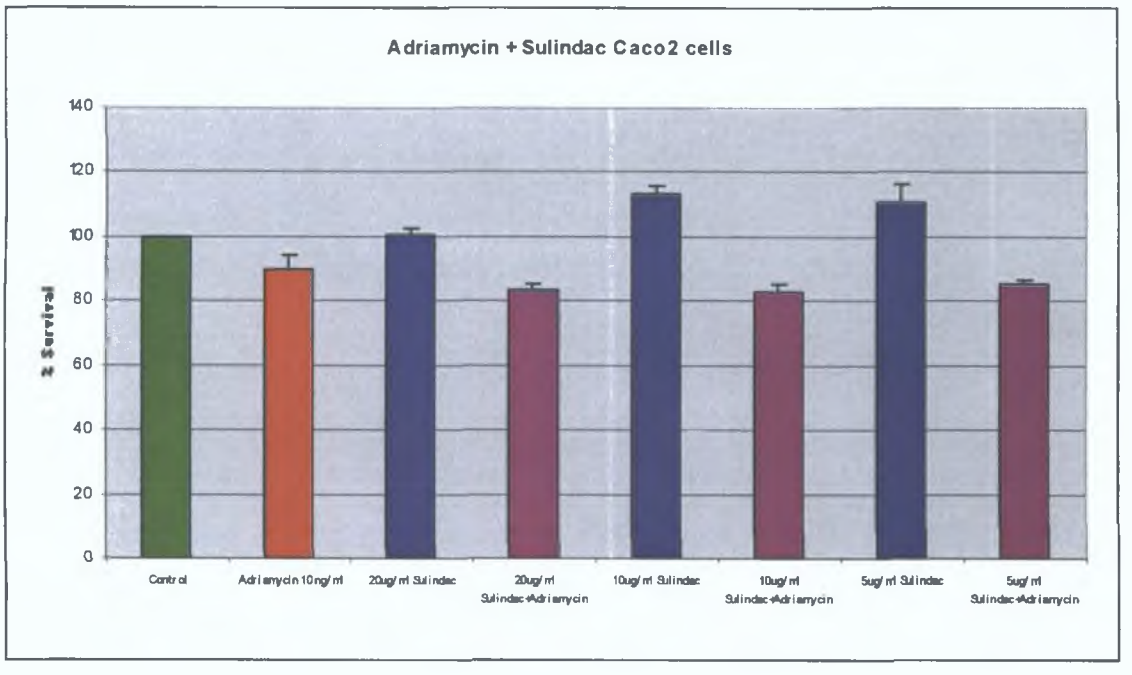
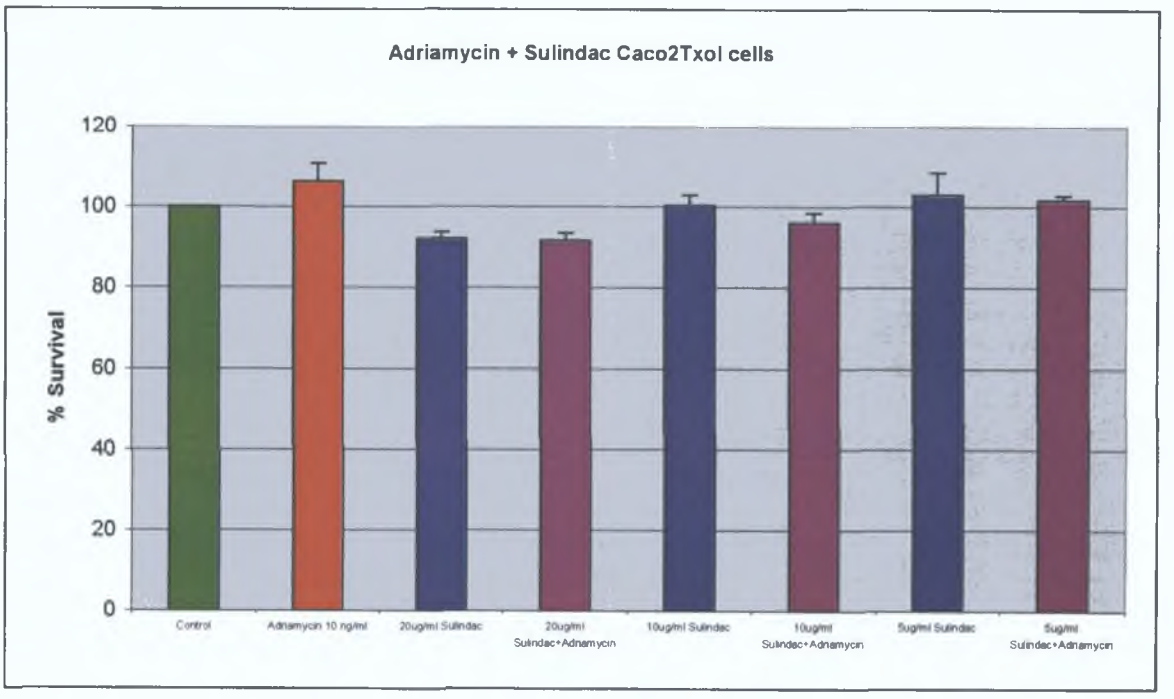


Figure 3.7.20: Modulation of Adriamycin toxicity by Sulindac in Caco2 cells. Results represent the average of three independent experiments.

A



B

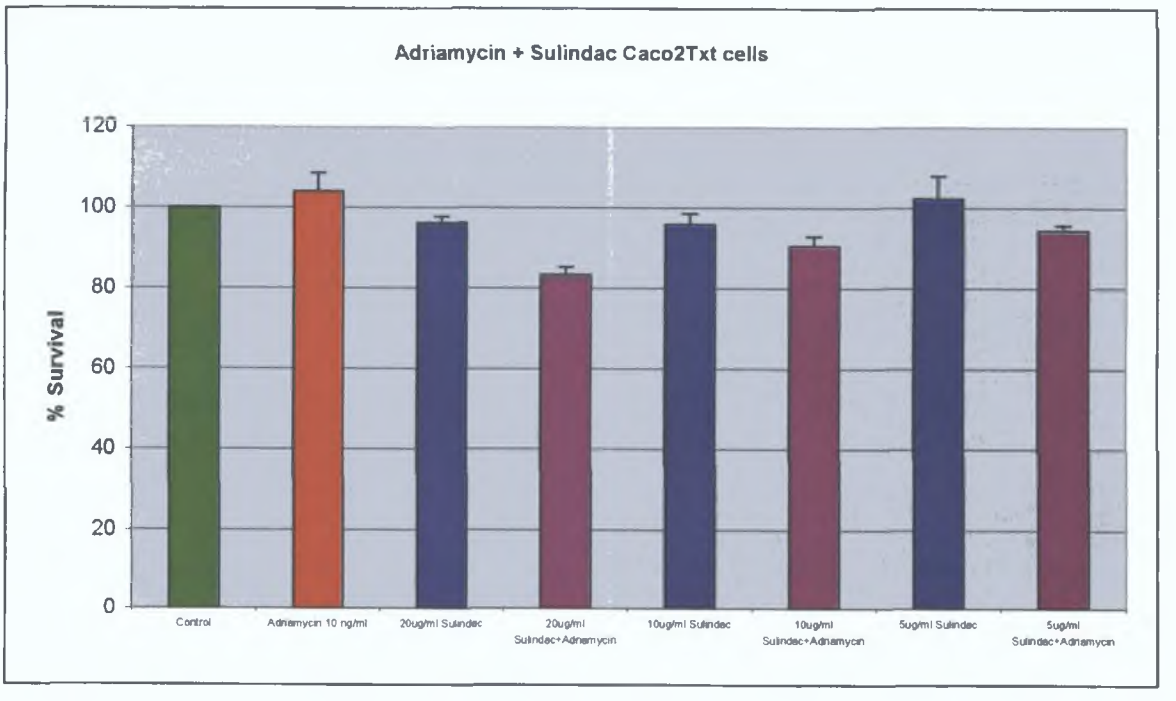
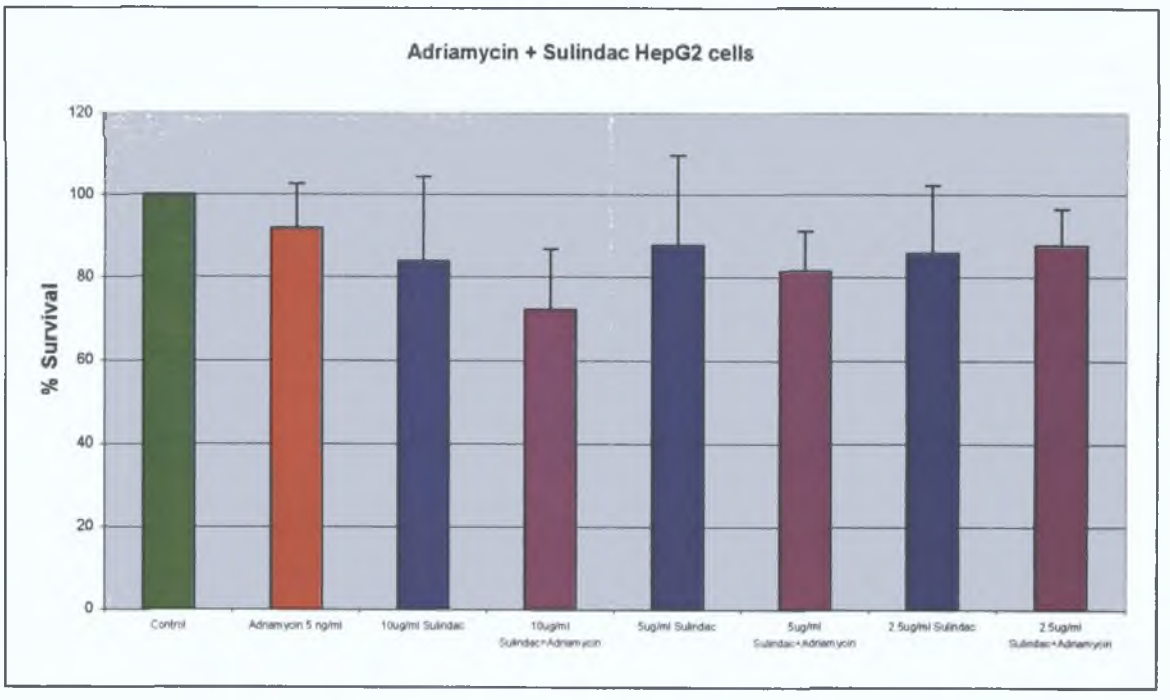


Figure 3.7.21: Modulation of Adriamycin toxicity by Sulindac in Caco2 Taxol (A) and Taxotere (B) pulse-selected cells. Results represent the average of three independent experiments.

A



B

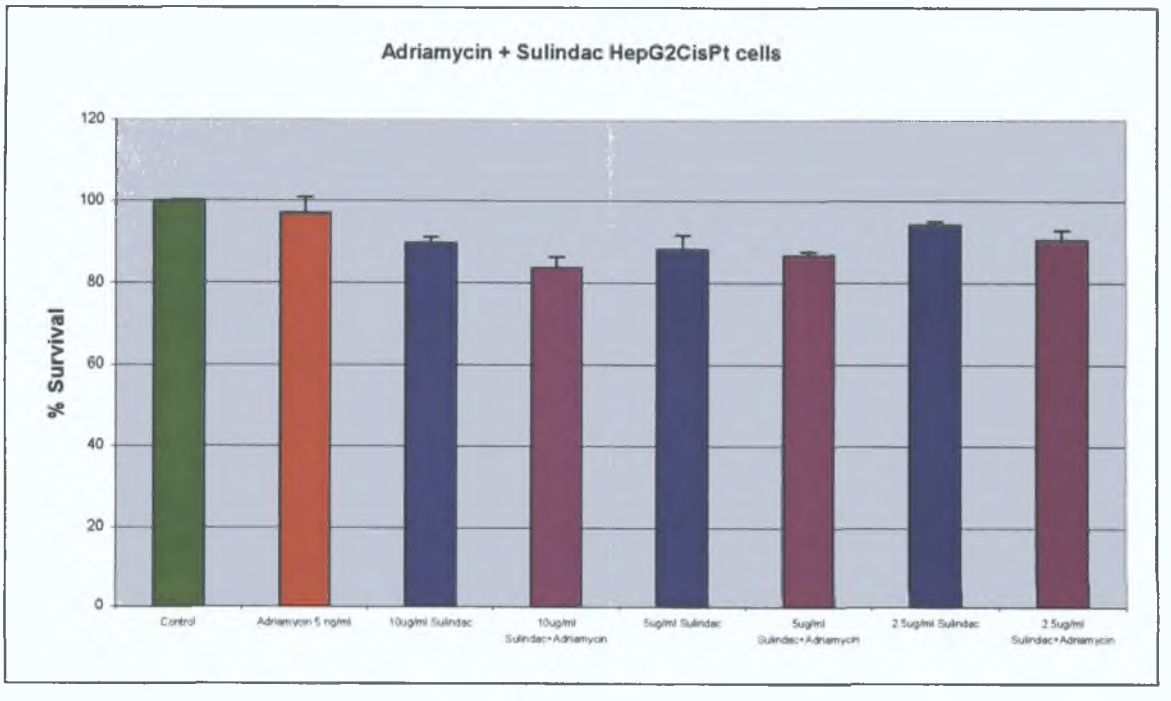
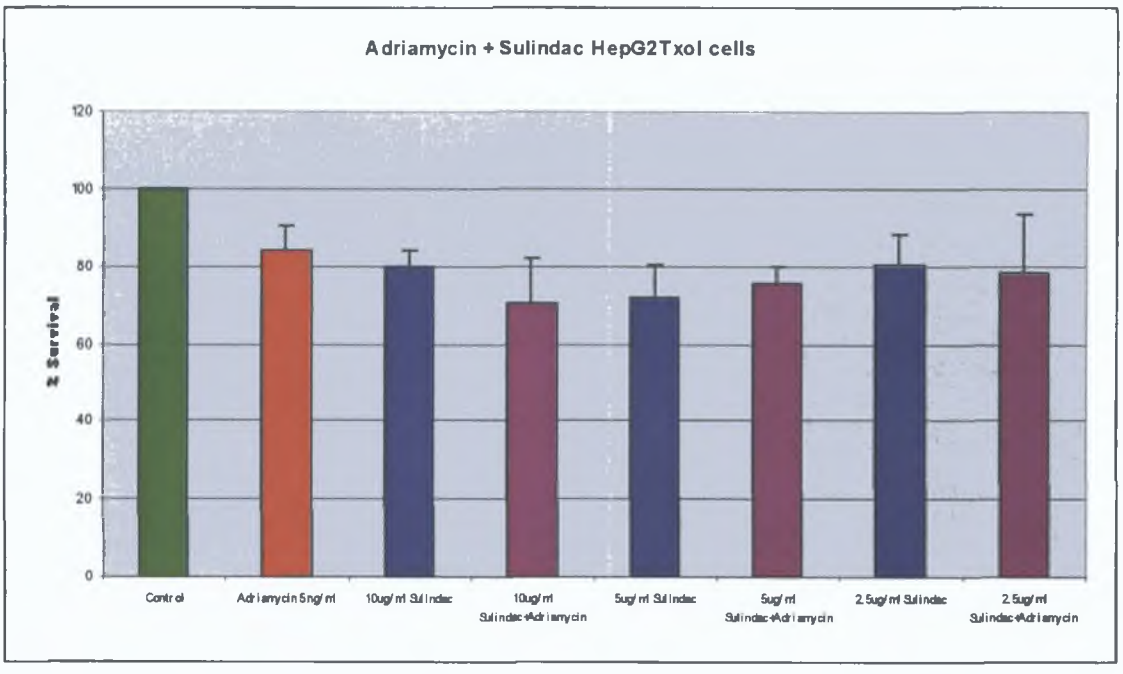


Figure 3.7.22: Modulation of Adriamycin toxicity by Sulindac in HepG2 parent (A) and Cisplatin pulse-selected (B) cells. Results represent the average of three independent experiments.

A



B

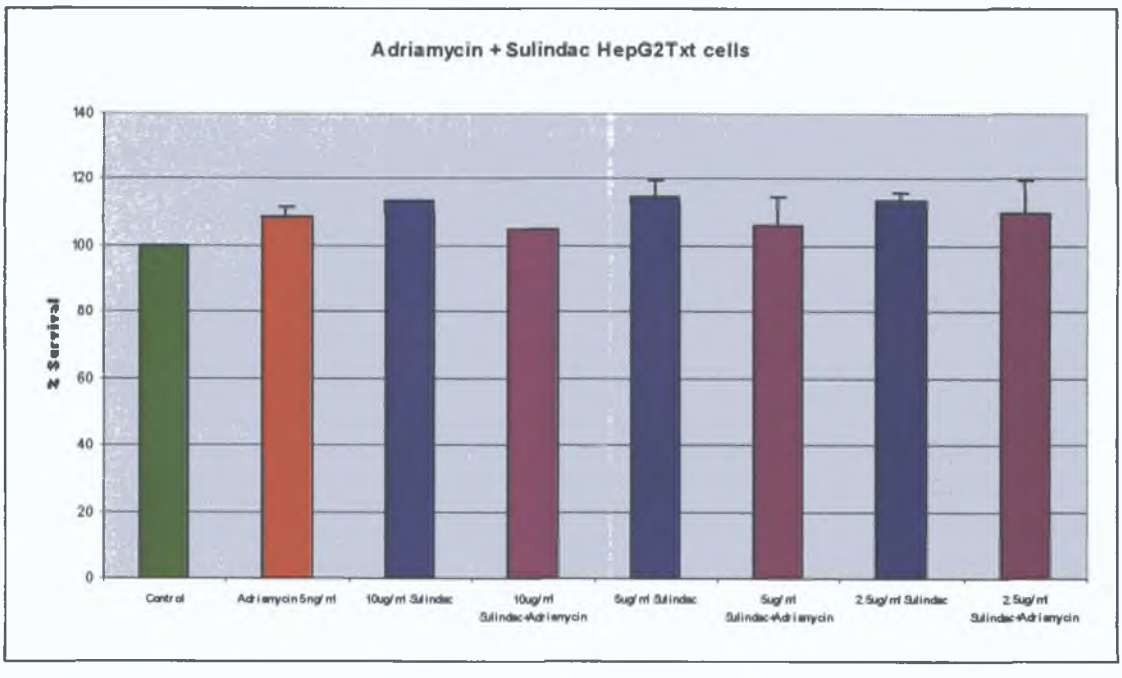


Figure 3.7.23: Modulation of Adriamycin toxicity by Sulindac in HepG2 Taxol (A) and Taxotere (B) pulse-selected cells. Results represent the average of three independent experiments.

3.7.13 Reversion of resistance in Caco2 and HepG2 pulse-selected cells by 17 AEE

In order to find out whether the increased expression of CYP3A could protect pulse-selected cells against the toxic effects of anticancer drugs, cells were treated with Taxol, a CYP3A4 substrate, in the presence or absence of 17 AEE. As shown in Figs. 3.7.25, 3.7.28 and 3.7.29, combination of 17 AEE and Taxol, a CYP3A substrate, did not appear to substantially enhance the effect of the anticancer drug in any of the pulse-selected cell lines tested, even at the highest concentrations of 17 AEE. This suggests that CYP3A does not play a relevant role in the resistance developed by these variants. A similar result was obtained when treating cells with Cisplatin in the presence or absence of 17 AEE (Figs. 3.7.26, 3.7.27, 3.7.30 and 3.7.31). A slight non-dose-dependent enhancement of Taxol toxicity was observed in Caco2 cells in the presence of 17 AEE (Fig. 3.7.24), indicating that CYP3A expression might at least partially protect these cells against Taxol-induced toxicity; however, this effect was not observed in HepG2 cells (Fig. 3.7.28 A).

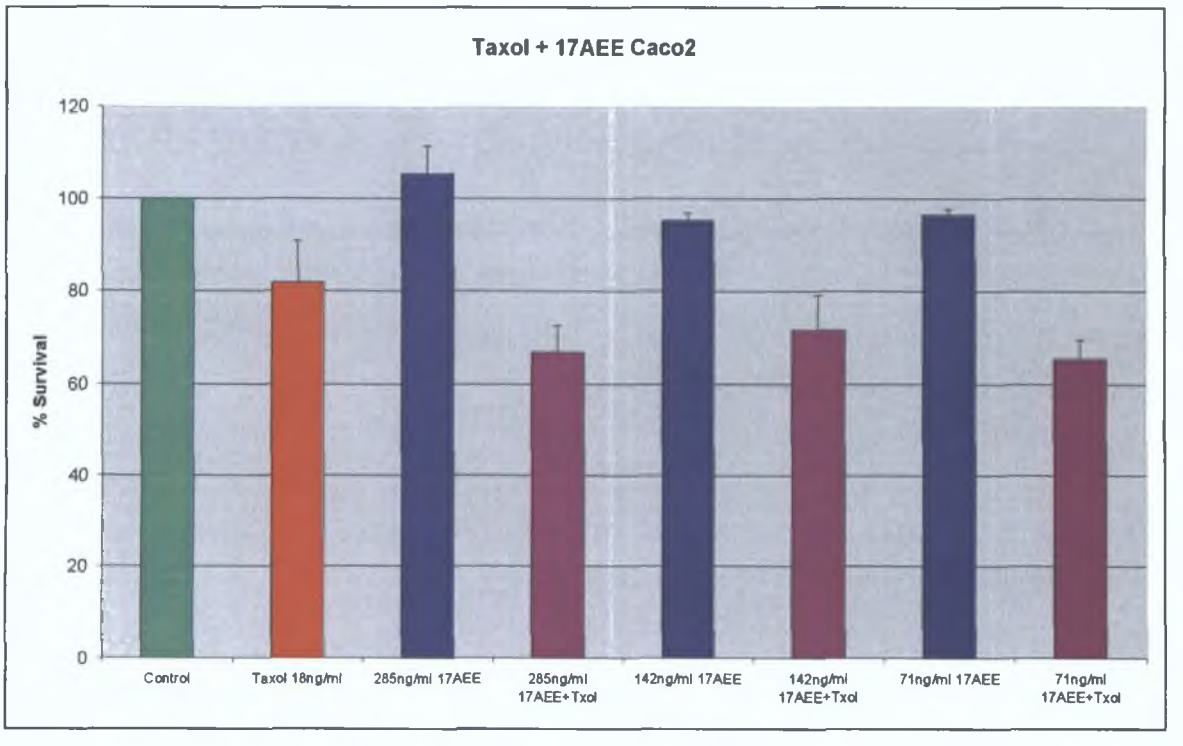
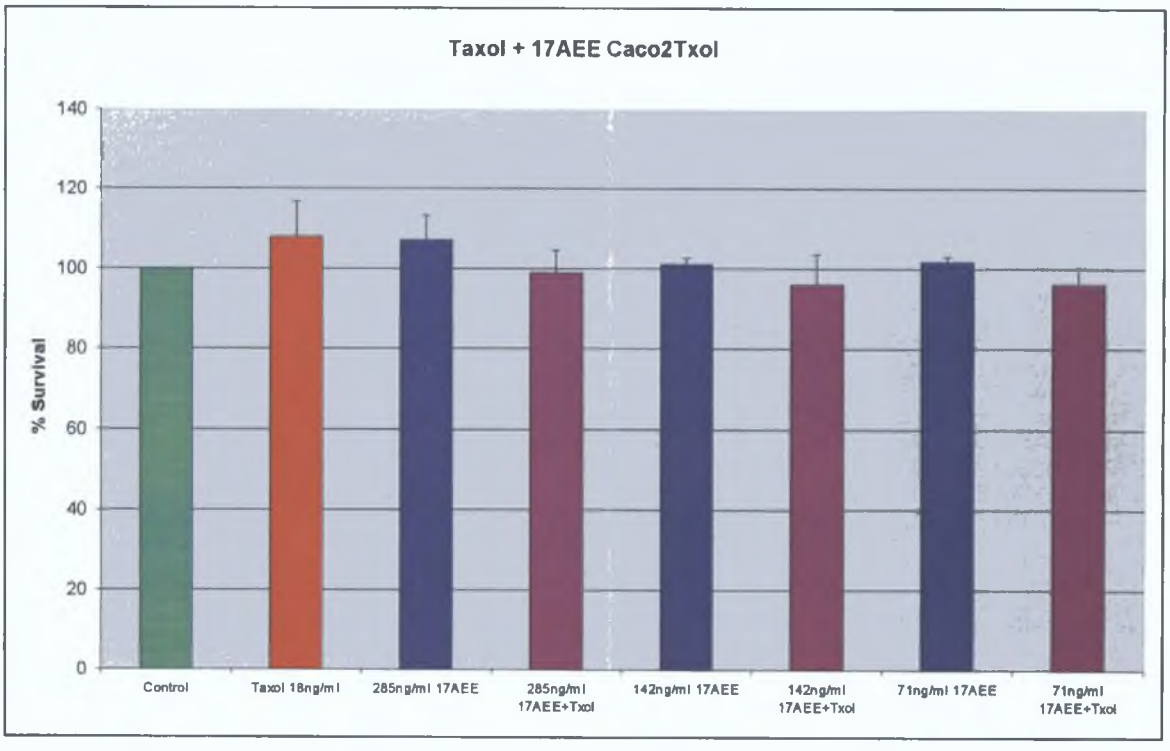


Figure 3.7.24: Modulation of Taxol toxicity by 17 AEE in Caco2 cells. Results represent the average of four independent experiments.

A



B

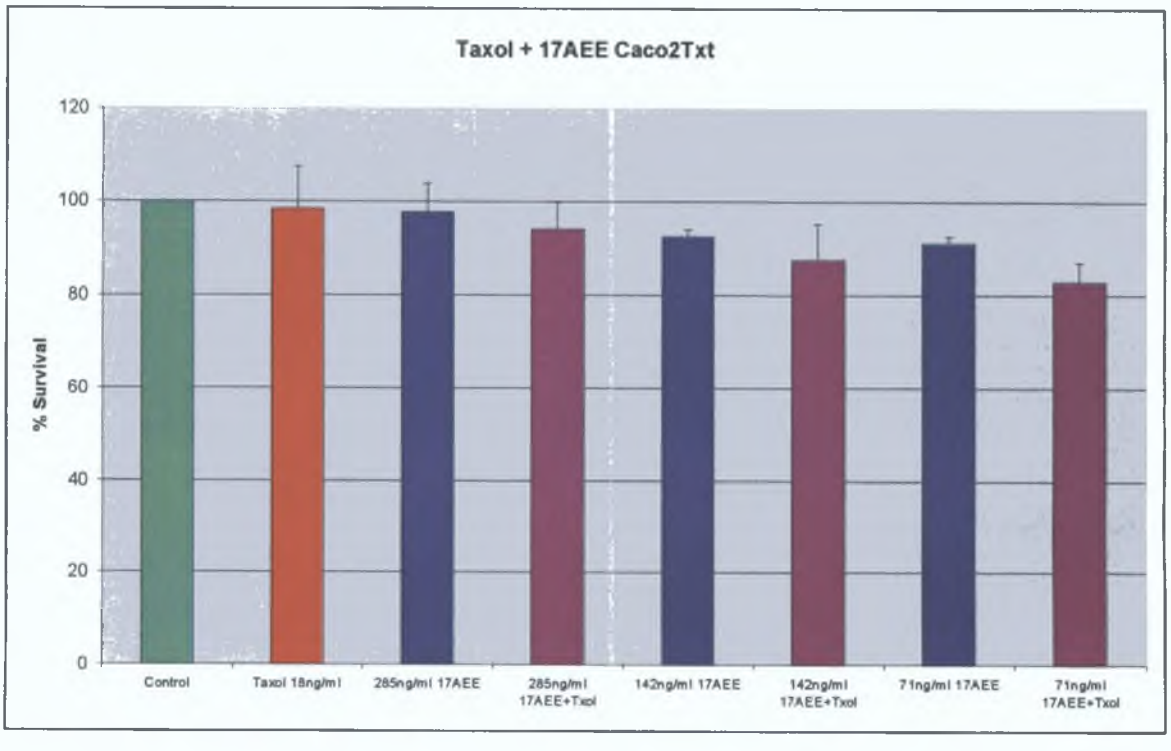


Figure 3.7.25: Modulation of Taxol toxicity by 17 AEE in Caco2 Taxol (A) and Taxotere (B) pulse-selected cells. Results represent the average of four independent experiments.

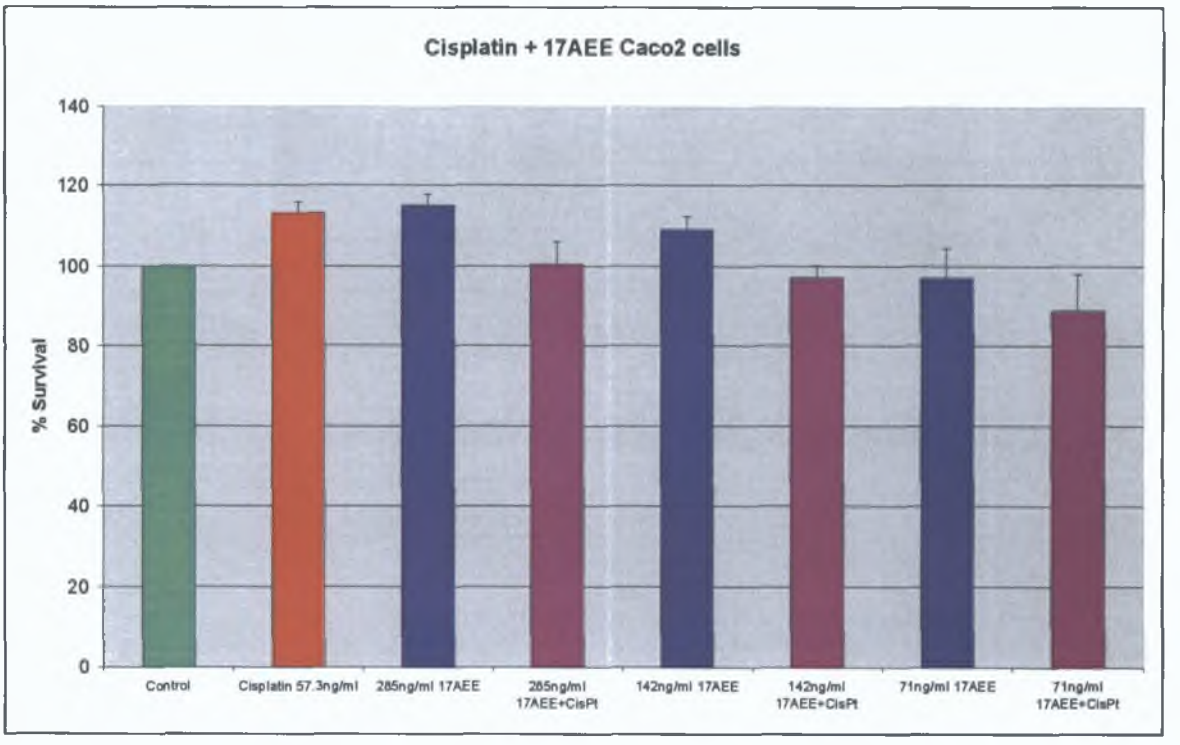
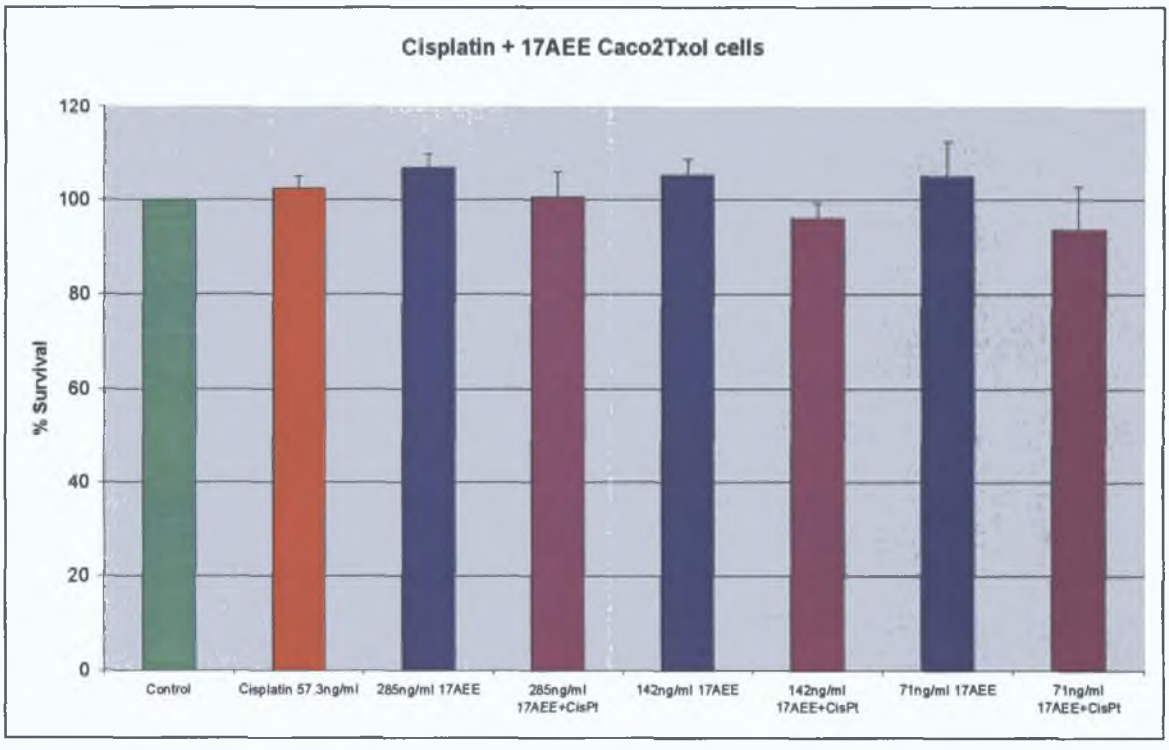


Figure 3.7.26: Modulation of Cisplatin toxicity by 17 AEE in Caco2 cells. Results represent the average of four independent experiments.

A



B

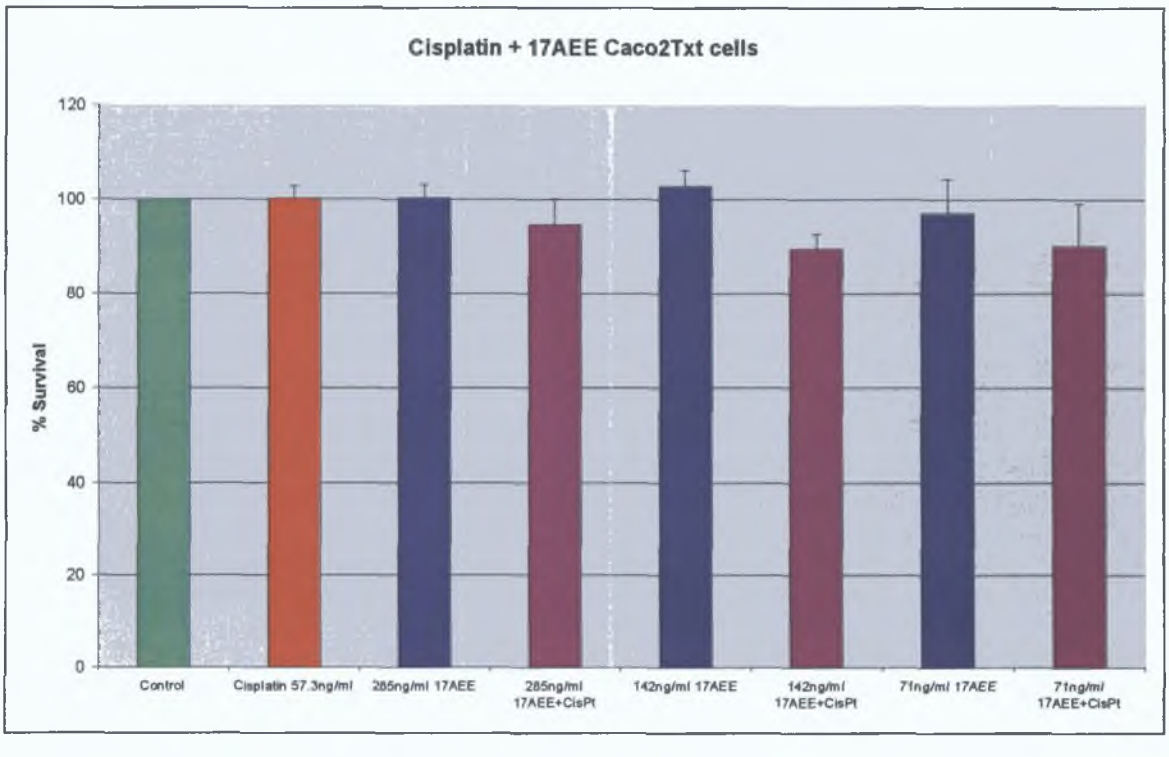
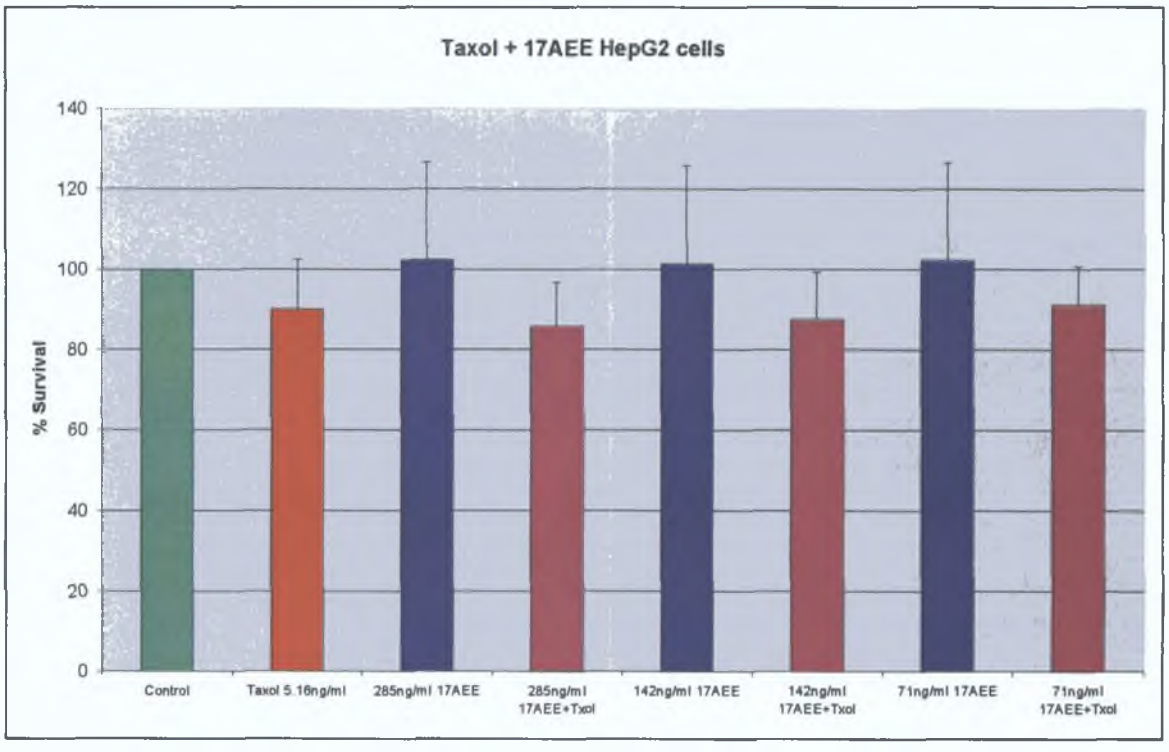


Figure 3.7.27: Modulation of Cisplatin toxicity by 17 AEE in Caco2 Taxol (A) and Taxotere (B) pulse-selected cells. Results represent the average of four independent experiments.

A



B

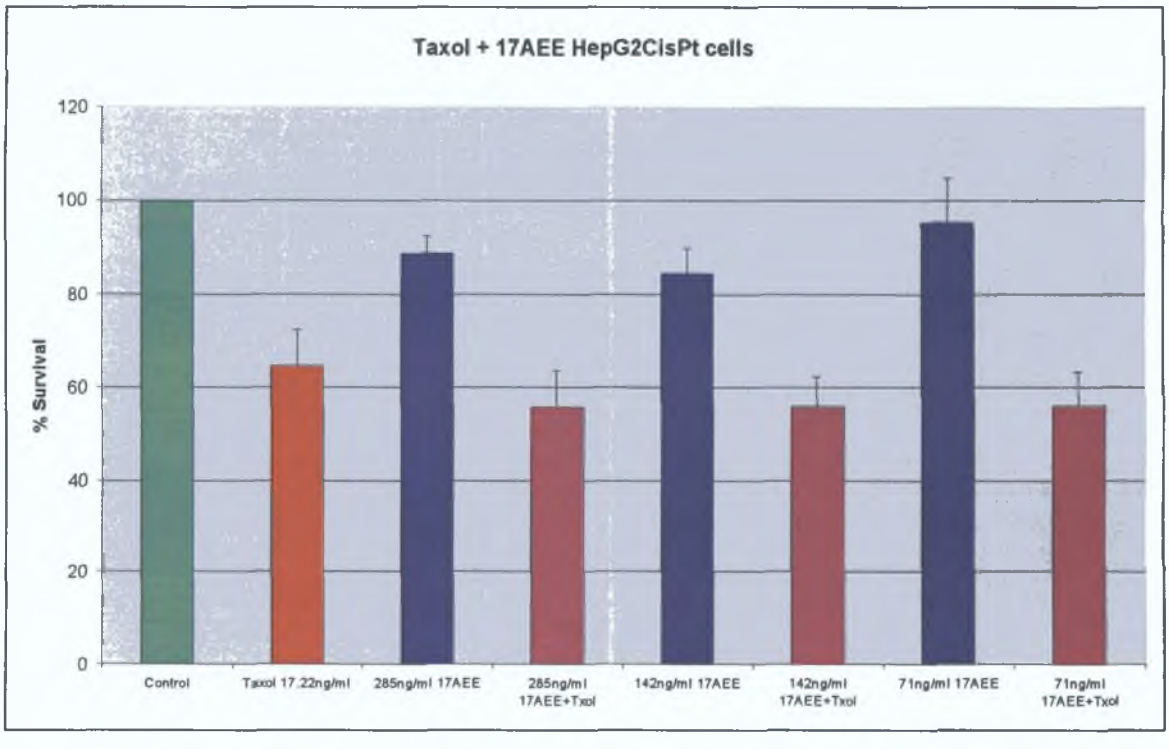
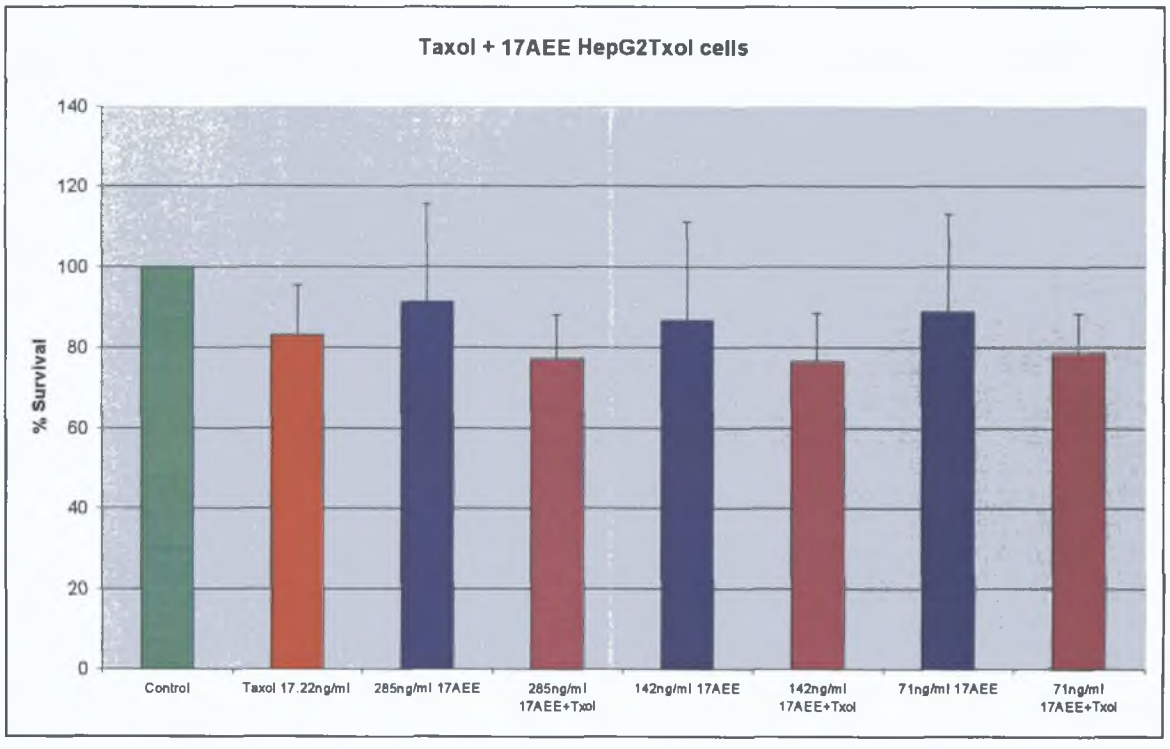


Figure 3.7.28: Modulation of Taxol toxicity by 17 AEE in HepG2 parent (A) and Cisplatin pulse-selected (B) cells. Results represent the average of at least three independent experiments.

A



B

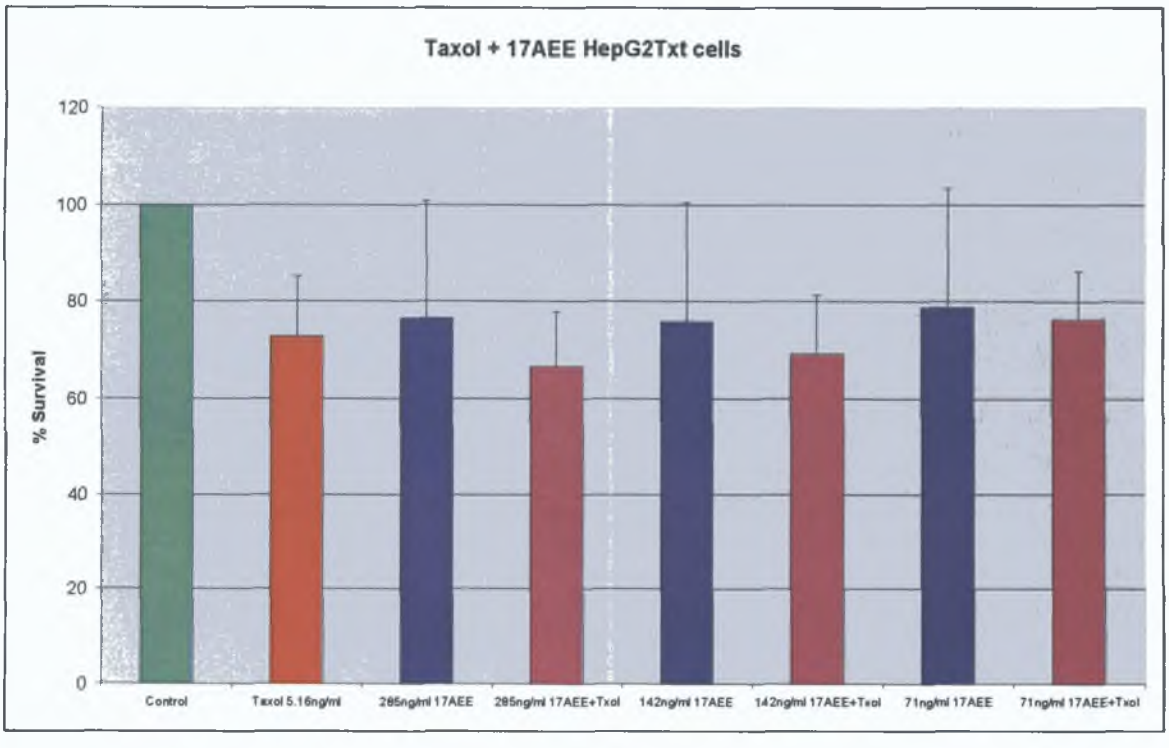
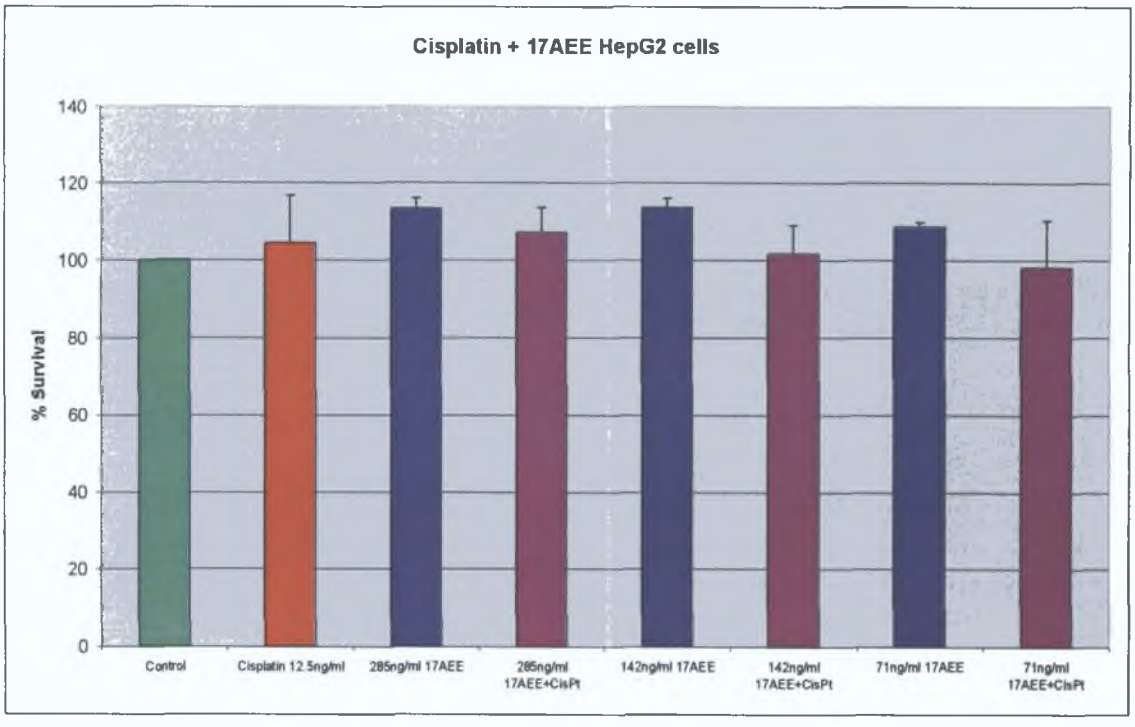


Figure 3.7.29: Modulation of Taxol toxicity by 17 AEE in HepG2 Taxol (A) and Taxotere (B) pulse-selected cells. Results represent the average of at least three independent experiments.

A



B

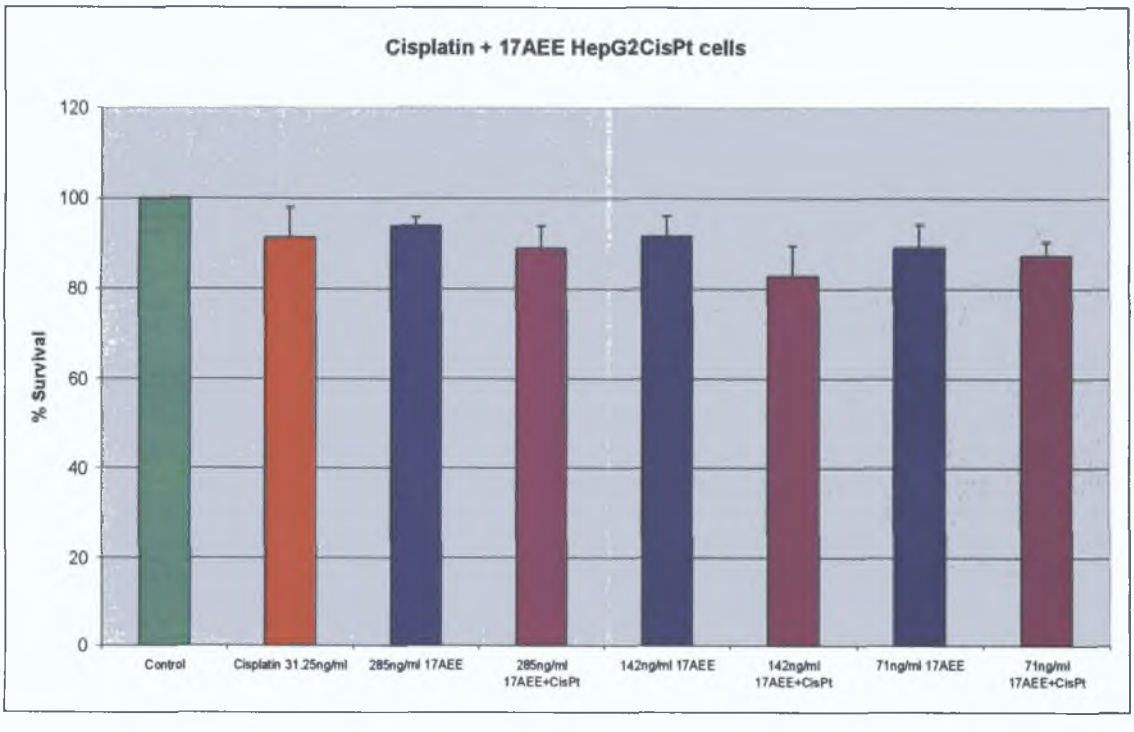
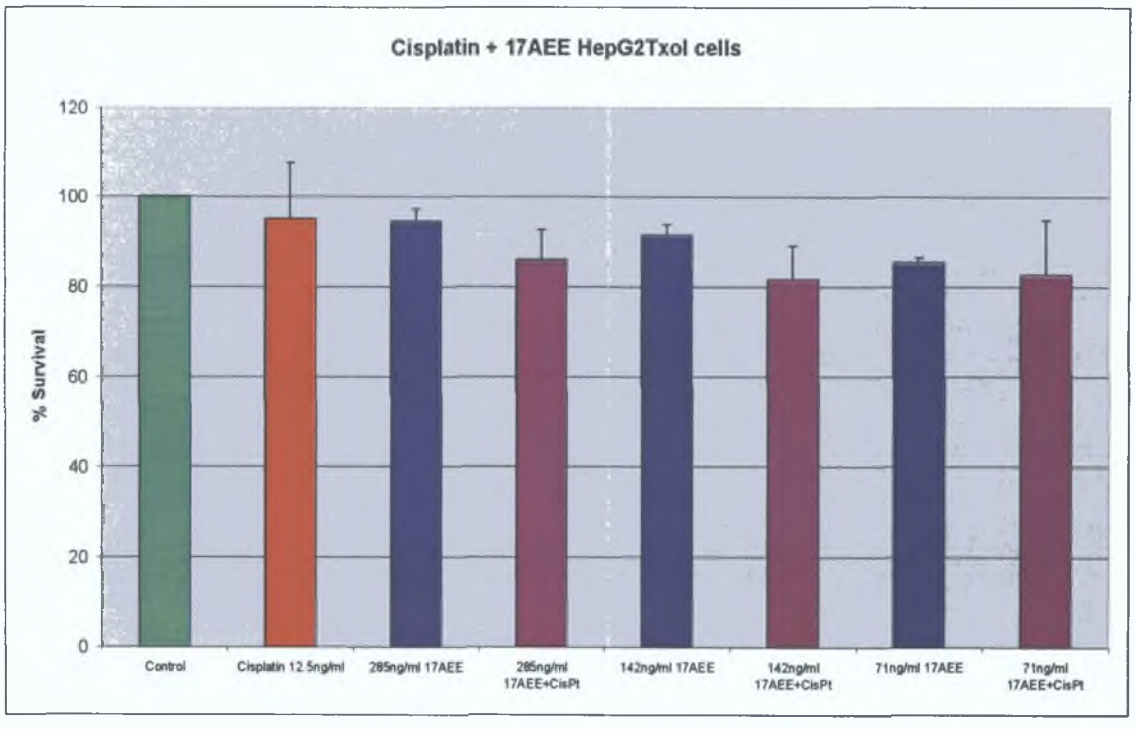


Figure 3.30: Modulation of Cisplatin toxicity by 17 AEE in HepG2 parent (A) and Cisplatin pulse-selected (B) cells. Results represent the average of at least three independent experiments.

A



B

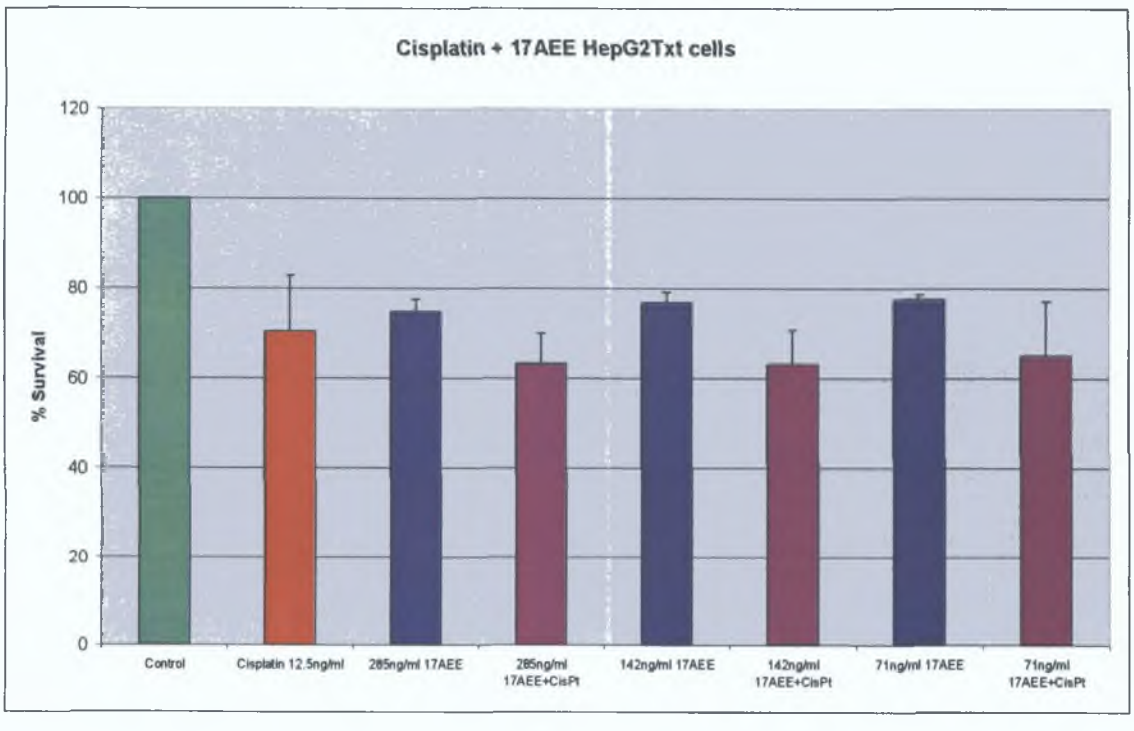


Figure 3.7.31: Modulation of Cisplatin toxicity by 17 AEE in HepG2 Taxol (A) and Taxotere (B) pulse-selected cells. Results represent the average of at least three independent experiments.

3 7 14 Reversion of resistance in Caco2 pulse-selected cells by simultaneous treatment with 17 AEE and GF120918

Previous results obtained with GF120918A and 17 AEE alone suggested that the increased expression of MDR1 in pulse-selected cells played a far more important role in the development of resistance than that of P450s Indeed, MDR1 appeared to be the main mechanism of resistance in these cells P450s may then be considered as a second line of defence against cytotoxic drugs, relevant only where MDR1 is inactive or as and adjuvant to MDR1 activity In order to test this hypothesis, Caco2 pulse-selected and parent cell lines were exposed to Taxol in the presence of either GF120918A, 17 AEE or both As shown in Fig 3 7 33 B, 17 AEE treatment of Caco2Txt cells in the presence of GF120198A did not further increase Taxol toxicity, suggesting that CYP3A activity is not an important resistance mechanism in these cells, even after inhibition of MDR1 However, 17 AEE did enhance Taxol toxicity in the presence of GF120918A in Caco2Txol cells (Fig 3 7 33 A), suggesting that CYP3A might act as a second-order defence in these cells Caco2 parent cells only showed a slight increase in toxicity at the highest concentration of 17 AEE (Fig 3 7 32)

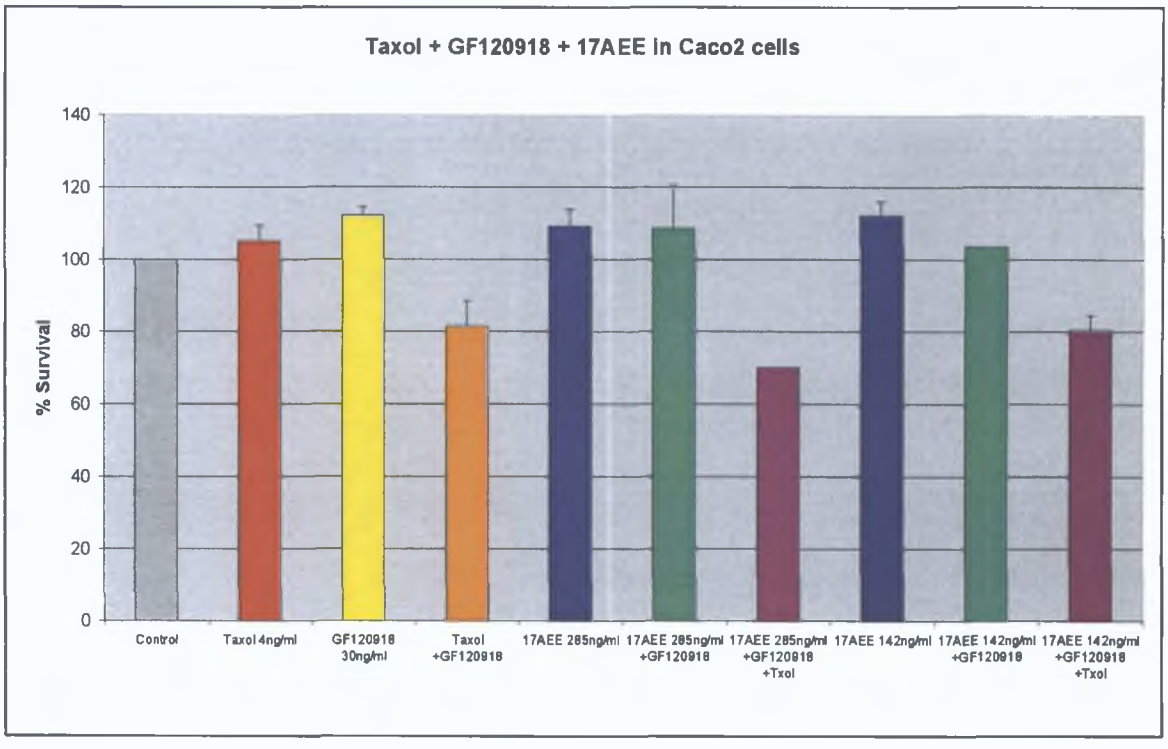
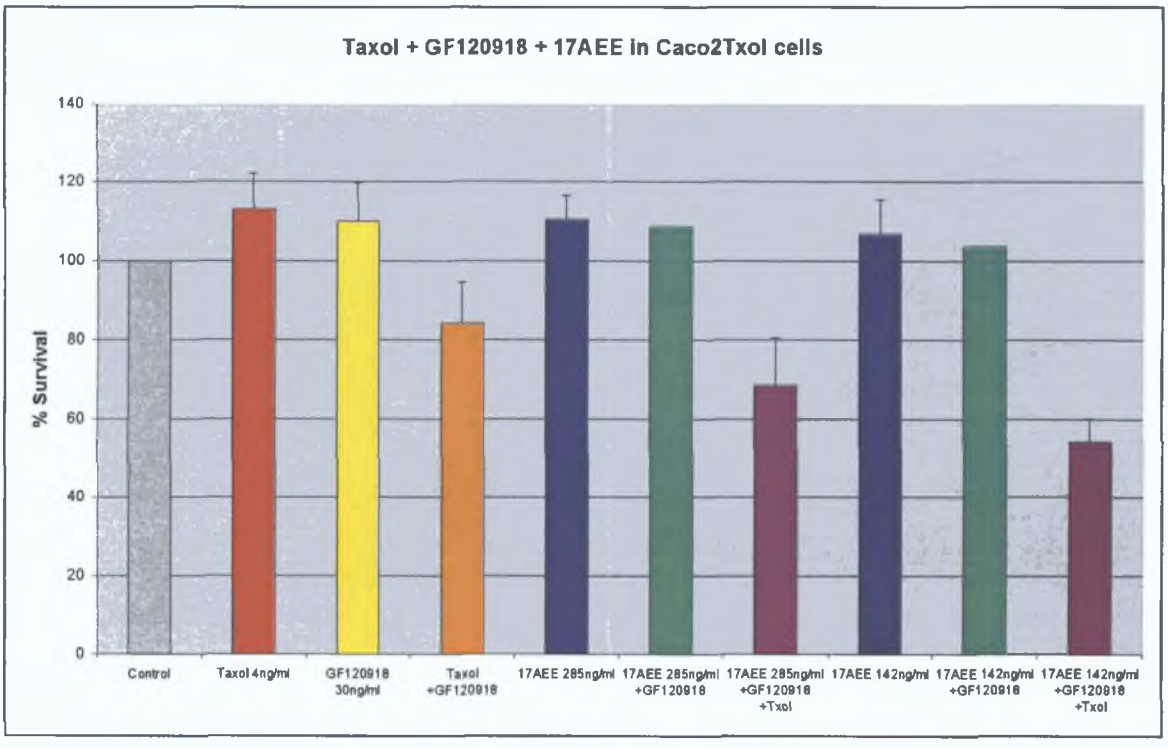


Figure 3.7.32: Triple combination assay showing modulation of Taxol toxicity by 17 AEE in the presence of GF120918A in Caco2 cells. Results represent the average of at least four independent experiments.

A



B

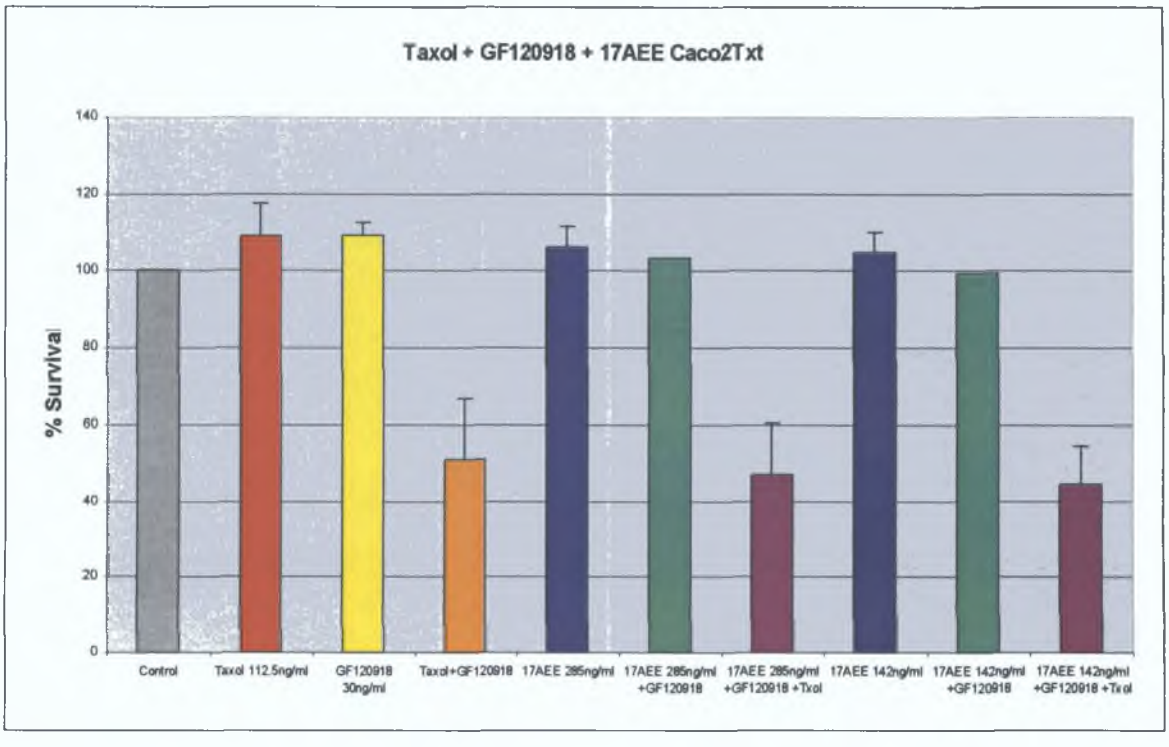


Figure 3.7.33: Triple combination assay showing the effect of Taxol and 17 AEE in the presence of GF120918A in Caco2 Taxol (A) and Taxotere (B) pulse-selected cells. Results represent the average of at least four independent experiments.

Section 4.0

Discussion

The work described in this thesis attempted to increase our understanding of the role of drug metabolism enzymes in anticancer drug resistance using several different approaches

- Analysis of drug metabolism-related gene expression using whole genome gene expression microarray technology
- Use of recombinant P450s to study the direct inactivation of anticancer drugs
- Use of transfected and/or inducer-treated cell lines overexpressing cytochromes P450 or NADPH P450 reductase
- Use of siRNA to knock-down the expression of CYP1B1
- Comparative analysis of glutathione-related enzyme expression in parent and pulse-selected cells
- Study of resistance mechanisms developed by pulse selection of cells

These results are now discussed in the context of previously published reports

4.1 Expression of drug metabolism-related genes in normal and tumour breast tissue

4 1 1 Expression of drug metabolism-related genes in breast samples by microarray analysis

A number of studies have clearly demonstrated that cytochromes P450 and other metabolic enzymes are commonly expressed in both normal and tumour tissue (see section 1 4 5) Literature also suggests a differential regulation of these enzymes in malignant cells, although the nature of these differences is still a matter of controversy

The usual techniques employed to analyse expression of genes of interest in tissue samples are RT-PCR, Western blot and immunohistochemistry, these traditional techniques are widely used and highly efficient, although they do require optimisation Their main disadvantage is that they only allow for the analysis of one or a few genes at a time It is believed that the expression of hundreds or even thousands of genes is altered in cells that have undergone malignant transformation, analysis of these changes therefore requires techniques with higher throughput capacity In recent years, the use of gene expression microarrays has emerged as a remarkably powerful tool that allows for the simultaneous measurement of expression of thousands of

genes. Microarrays are now widely used to identify genes involved in a particular process, such as malignant transformation or development of drug resistance.

Van't Veer *et al.* (2002) recently carried out a DNA microarray analysis on 117 breast tumour samples with the aim of finding a signature gene expression that could accurately detect those breast tumours with a high chance of developing metastasis in lymph node negative patients. These samples were analysed with a microarray platform containing some 25,000 genes, of which 5,000 were found to be significantly up or downregulated. Of these, 231 genes were associated with disease outcome; further analysis of the genes present in the latter group allowed for the detection of a subset of 70 genes that could correctly predict disease outcome in 83% of cases. No genes related to drug metabolism made it to the final subset, which suggests that their involvement in breast tumour progression is limited. However, changes in the expression of a number of cytochromes P450 and GSH-related proteins associated with prognosis were reported. It was found that expression of CYP3A7, CYP1A1, GSH peroxidase 5 and aryl hydrocarbon receptor interacting protein was increased in those samples with better prognosis (lower chance of developing metastasis).

Analysis of a large number of breast tumour samples was performed in our centre by Dr. Lorraine O'Driscoll *et al.* using whole genome gene expression microarrays; the results obtained are predominantly in agreement with previous findings (see section 1.4.5). A large proportion of the samples studied expressed a variety of drug metabolism-related genes. In many cases, significant alterations in gene expression were found in tumour as compared to normal samples.

It is known that the expression of CYP2J2 increases cell proliferation and inhibits apoptosis (Jiang *et al.*, 2005), so it is not unexpected that CYP2J2 mRNA was more often expressed in tumours than in normal tissue. CYP2W1, however, has recently been hailed as a tumour-specific P450 (Karlgren *et al.*, 2006), with no or very low levels of CYP2W1 mRNA and protein found in normal adult tissues, so it was unexpected to find that mRNA expression of this enzyme in normal tissue was more frequent than in tumour samples. The low prevalence of CYP1A1 mRNA expression found in this analysis is in correlation with the results of El-Rayes *et al.* (2003), who found lower frequency of CYP1A1 expression in tumour as compared to normal tissue. Iscan *et al.* (2001) also found very low levels of this enzyme in both breast tumour samples and their surrounding normal tissue by RT-PCR analysis. However, these authors found decreased expression of CYP2E1 in tumour samples by immunoblotting, which is in contradiction with the observed mRNA expression frequencies in our results. These inconsistencies could be attributed to

posttranscriptional events, since mRNA levels do not always correlate with protein levels.

GSH transferase M4 (GST M4) mRNA expression was found to be more common in normal than tumour tissue. The role played by this enzyme in cancer remains obscure, but a recent study suggested an association between GST M4 polymorphisms and lung cancer risk, implying that the activity of this enzyme might be involved in tumour development (Liloglou *et al.*, 2002). Expression of GSH peroxidase 2 mRNA was also found to be more frequently expressed in tumour as compared to normal breast tissue. This enzyme is believed to act as an anticarcinogenic factor, since knock-out mice for GSH peroxidase 2 show enhanced gastrointestinal tumour formation (Brigelius-Flohe *et al.*, 2006). Expression of GSH peroxidase 2 has been established in breast tissue (Chu *et al.*, 1999) and increased expression of this enzyme has recently been reported in lung adenocarcinomas and in normal alveolar epithelium from smokers as compared to normal lung tissue from non-smokers (Woenckhaus *et al.*, 2006). This enzyme can protect cells from free radicals and also regulate proliferation and apoptosis through redox signalling (Brigelius-Flohe *et al.*, 2001) and thus its increased expression may confer a survival advantage to tumour cells.

4.1.2 Potential biomarkers identified in the microarray study

Differential mRNA expression of ARNT2 was one of the major findings in our study, with expression in tumour tissue being much more prevalent than in normal tissue samples and significantly correlating with better prognosis. To date, no correlation has been reported in the literature between expression of this gene and cancer progression or survival.

Upon binding a specific ligand, the aryl hydrocarbon receptor (AhR) dimerises with the AhR nuclear translocator protein (ARNT) and activates the transcription of its target genes. ARNT2 is a homolog of ARNT with a more restricted pattern of expression, commonly found in the central nervous system (Maltepe *et al.*, 2000), although its expression has also been reported in other developing organs such as kidney (Freeburg *et al.*, 2004). The main function of ARNT2 appears to be in brain development, since mice deficient in this gene showed defects in their secretory neurons (Hosoya *et al.*, 2001). It has also been suggested that ARNT2 plays a wider role in development: disruption of ARNT2 expression in fertilised zebrafish eggs cause severe defects in brain, eyes, heart and gut development (Hsu *et al.*, 2001).

Increased expression of ARNT2 in tumour tissue could explain the low levels of CYP1A1 observed: it has been reported that a zebrafish homologue of ARNT2 repressed 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-dependent CYP1A transcription (Wang *et al.*, 2000). Moreover, it was shown that ARNT2 dimerises with the hypoxia-inducible factor-1 (HIF-1) to induce the expression of genes in response to oxygen deprivation (Maltepe *et al.*, 2000). Formation of ARNT2/HIF-1 complexes in teratocarcinoma cells increased VEGF expression and tumour angiogenesis, as well as enhancing cell proliferation. The presence of ARNT2 therefore seems to improve growth efficiency in tumours, which might explain why tumour samples showed increased expression of this factor. However, this does not explain why ARNT2-positive tumours are associated with better prognosis. Indeed, statistical analysis revealed that ARNT2 expression correlates with overall survival and disease-free survival.

It should be noted that Van't Veer *et al.* (2002) also found increased mRNA expression of an AhR-related element, the aryl hydrocarbon interacting protein, in samples from patients that had a better prognosis. This points to the AhR receptor pathway as a commonly altered target in breast cancer; its deregulation, however, appears to be associated with good prognosis.

The AhR receptor pathway was initially considered to be involved mainly in the metabolism of polycyclic aromatic hydrocarbon and halogenated hydrocarbons; more recent studies have shown that the AhR is implicated in such essential cell functions as growth, proliferation, apoptosis and epithelial-mesenchymal transition (Schlezinger *et al.*, 2006). The role of the AhR in these phenomena remains undefined, as there is evidence that it can both increase and inhibit cell proliferation, as well as protect cells from apoptosis or induce cell death. One of the best characterised mechanisms of growth inhibition by the AhR involves repression of oestrogen receptor (ER) signalling; it is then not unreasonable to believe that ER+ tumours would display impaired growth and decreased proliferation rates. It should also be noted that even though ER expression did not significantly correlate with ARNT2 expression, the P value was almost at the limit of significance (P=0.052). This would suggest that many of the tumours expressing ARNT2 were also ER+. AhR signalling, helped by ARNT2, might then slow down the progression of these tumours. This model would explain the better prognosis observed in patients with ARNT2-overexpressing tumours.

It should be noted that ER+ tumours are associated with better survival; since many of the tumours expressing ARNT2 are also ER+, the better survival rates associated with ARNT2 might just be due to the fact that this protein is linked with ER+ status, which in turn is associated with better survival. Increased ARTN2 mRNA expression might

thus not directly impair tumour progression but rather be associated with the better prognosis-related ER+ status Even if this were the case, ARNT2 would still be useful in prediction of prognosis and might also be used as a marker of disease, since it is considerably upregulated in tumour as opposed to normal tissue (see Table 3 1 3)

Expression of GST A1 was associated with decreased overall and relapse-free survival This might be related to therapy efficacy, since expression of GST A1 in cell lines is associated with an increase in resistance to Carboplatin and Cyclophosphamide (Tanner *et al*, 1997) Increased expression of GST A1 in tumour tissue would mean enhanced inactivation of anticancer drugs that could result in treatment failure

Several reports have tried to establish a correlation between GST A1 expression in tumours and disease prognosis, sometimes generating contradictory results Early studies carried out in breast cancer patients failed to establish a correlation between GST expression and clinicopathological features such as disease-free and overall survival (Peters *et al*, 1993, Alpert *et al*, 1997) Confirming these results, Wrigley *et al* (1996) failed to find an association between the expression of any of the GSTs analysed and response to treatment, overall survival and disease-free survival in ovarian cancer Similarly, another study carried out in ovarian cancer samples reported lack of correlation between GST expression and resistance to Cyclophosphamide and/or Carboplatin (Tanner *et al*, 1997) However, mean plasma concentrations of GST A1 were found to be significantly increased at the time of relapse in dogs with lymphoma (Hahn *et al*, 1999), suggesting that this isozyme might be of use as a marker of disease progression

A more recent analysis performed by Sweeney *et al* (2003) found that patients homozygous for a GST A1 polymorphism that results in reduced expression of the enzyme showed a significant difference in 5-year survival after breast cancer treatment when compared to heterozygous patients, indeed, low expression of GST A1 correlated with reduced risk of death in the 5 years following diagnosis Lower expression of GST A1 in the liver would mean that drugs such as Cyclophosphamide are inactivated in a slower manner, leaving more drug available in the circulation and improving therapy efficacy

In conclusion, gene expression microarray analysis makes possible the study of alterations in the expression of thousands of genes during cancer development and progression, providing an accurate picture of what is happening inside the cell As shown by our results, it can also help to elucidate the connection between gene

expression and cancer phenotype or therapy outcome, thereby improving our knowledge of the disease and providing biomarkers for diagnosis and characterisation

4.2 Anticancer drug metabolism by cytochromes P450

4 2 1 Anticancer drug metabolism by CYP3A4

The microsomal fractions of insect cells infected with recombinant baculovirus carrying human cytochrome P450 cDNAs are a useful tool for the study of P450-mediated drug metabolism They display higher levels of activity, as shown in section 3 2 1 1 by measuring testosterone 6β-hydroxylase (CYP3A) activity However, testosterone 6β-hydroxylase activity of CYP3A4 microsomes, calculated as 0 353±0 086 pmol substrate/mg total protein/minute, is still substantially lower than that reported for human liver (0 5-9 nmol/mg/min, Draper *et al*, 1998) or small intestine microsomes (0 4-2 4 nmol/mg/min, Obach *et al*, 2001) Still, they constitute valuable models to investigate the effect of CYP3A4 activity on anticancer drugs
Incubation of Vincristine and especially Adriamycin, but not 5-fluorouracil, with CYP3A4 microsomes in the presence of the appropriate cofactors appreciably decreased drug-induced toxicity in A549 cells As mentioned before, both Adriamycin and Vincristine are CYP3A4 substrates (Lewis *et al*, 1992, Yao *et al*, 2000), whereas 5-fluorouracil does not interact directly with P450s A549 cells display no measurable endogenous CYP3A activity, so the contribution of endogenous anticancer drug metabolism is negligible The observed decrease in toxicity was time-dependent, which suggests it is related to enzymatic activity Indeed, CYP3A4 is known to convert most of its substrates into less or non-toxic derivatives, mainly by increasing their hydrophillicity and thus facilitating conjugation and excretion The reduced toxicity of these drugs after preincubation with CYP3A4-containing microsomes could then be explained by enzymatic inactivation These results demonstrate that CYP3A4 has the potential to reduce the toxicity of certain anticancer drugs

4 2 2 Anticancer drug metabolism by CYP1B1

Contrasting with the results obtained with CYP3A4, preincubation of 5-fluorouracil, Taxol or Taxotere with CYP1B1-containing microsomes did not affect the toxicity induced by these drugs on A549 cells Ethoxyresorufin-O-deethylase (EROD) activity measured in CYP1B1 microsomes was substantially (about three orders of magnitude) higher than that previously reported from human renal tumours, which ranged from 62 to 995 fmol/mg of microsomal protein/min (McFadyen *et al*, 2004)

Transfection of CYP1B1 cDNA has been reported to confer Taxotere resistance to Chinese hamster V79 cells (McFadyen *et al*, 2001), because this enzyme is involved in the metabolism of endogenous factors, it was thought that it would inactivate Taxotere, transforming it into less-toxic derivatives However, incubation of Taxotere with CYP1B1 microsomes did not produce any metabolites, even after addition of enzyme activators and changes in 10 different incubation factors that might affect the reaction (Bournique *et al*, 2002) CYP1B1 reaction with 17β oestradiol, a known endogenous substrate, generated the expected metabolite in most conditions tested by the authors The study could only show that Taxotere effectively bound CYP1B1 and acted as an effector of this enzyme These results are in agreement with our findings

Our results would suggest that CYP1B1 does not mediate the direct inactivation of Taxotere and that other factors are responsible for the observed increase in Taxotere resistance in CYP1B1-transfected cells

4 2 3 Anticancer drug metabolism by P450R

It is known that upon reaction with P450R, Adriamycin loses its sugar moiety to generate an aglycone metabolite (Fig 4 2 1) This metabolite is generally considered as less toxic than the parent drug, although several studies have shown that it still retains some toxicity, including induction of cytochrome C release from isolated mitochondria (Clementi *et al*, 2003) and alteration of sugar metabolism in erythrocytes (Misiti *et al*, 2003)

Preincubation of P450R-expressing microsomes reduced the toxic effects of Mitomycin C and especially Adriamycin, two well-known substrates of P450R, on MDA 231 cells This effect was not observed with 5-fluorouracil or Vincristine, which is to be expected, since these drugs are not known to be directly inactivated by P450R The effect was observed on both MDA EV (empty vector-transfected) and MDA R4 (P450R-transfected), indicating that the decrease in toxicity is independent of endogenous P450R expression These results suggest that P450R activity can decrease the cytotoxicity of Adriamycin and Mitomycin C in tumour cells

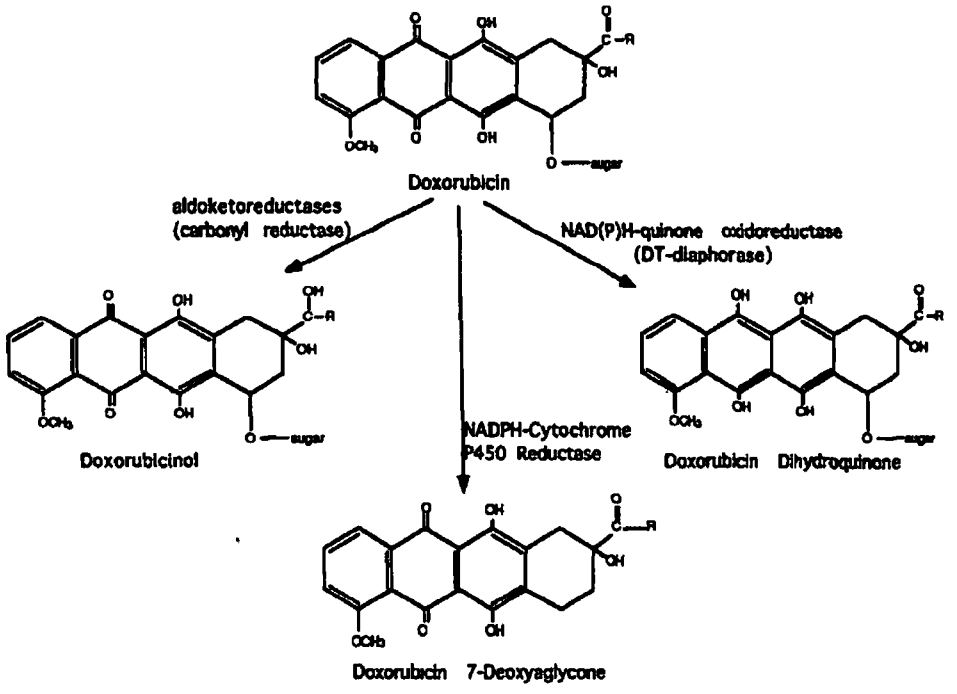


Figure 4 2 1 Metabolism of Adriamycin (also known as Doxorubicin) to less toxic derivatives (from Shairoz Ramji, M Sc thesis)

However, the aglycone metabolite could not be detected by LC/MS after incubation of Adriamycin with P450R-expressing microsomes for 1 hour Given that preincubation of P450R microsomes with Adriamycin reduced the toxicity of the drug on MDA 231 cells, it appears that the drug is indeed being converted to less toxic metabolites by reaction with P450R This suggests that either the method is not appropriate for detection of Adriamycin aglycone or that the aglycone is unstable and is degraded before it can be detected Direct injection of Adriamycin aglycone resulted in a readily detectable peak (see Fig 3 2 11), so it seems unlikely that this metabolite is degraded inside the mass spectrometer, unless it is present at extremely low concentrations Also, it should be taken into account that the method used for analysis was designed for the detection of anthracyclines and not their metabolites, the same is true of the extraction method This method has not been optimised for the detection of the Adriamycin aglycone and thus it could be that the metabolite is either lost during extraction or degraded during sample processing

4.3 CYP3A4 and drug resistance

One of the major difficulties in the study of cytochromes P450 is the extremely low levels of enzyme expression displayed by cell lines CYP3A4 activity is undetectable in many cell lines, which makes it very hard to find a suitable model to study the role of this enzyme in the development of drug resistance

Transfection of cell lines with P450 cDNAs provides a solution to this problem Indeed, CYP3A4-transfected BCMV 3A4 cells showed remarkable expression of this enzyme as shown by RT-PCR and Western blotting CYP3A activity could also be measured in this cell line, although it was considerably (about 6 times) lower than that found in CYP3A4 microsomes It should be noted, though, that metabolite levels were measured in culture medium from a confluent flask, so activity refers to total protein rather than to microsomal protein, as is the case in CYP3A4 microsomes The two values are therefore not directly comparable

Toxicity assays carried out on these cells show a modest (1 8-fold) increase in Adriamycin resistance displayed by BCMV 3A4 as compared to BCMV Neo (empty vector-transfected) cells, this is to be expected, since this drug is a CYP3A4 substrate and can therefore be inactivated by the overexpressed enzyme Indeed, reduction in Adriamycin toxicity was observed after preincubation of this drug with CYP3A4 microsomes (see section 3 2 1 2) However, the magnitude of this increase was small, suggesting that even though CYP3A4 can protect BCMV 3A4 cells from Adriamycin-induced toxicity, the overall contribution of this enzyme to drug resistance is low Another explanation for these results would be that P450R activity is too low in BCMV cells to support CYP3A4 metabolism, however, testosterone 6β-hydroxylase activity was readily detected in these cells, suggesting that P450R activity levels are not hindering CYP3A4 metabolism Furthermore, BCMV 3A4 cells were shown to efficiently activate the procarcinogen aflatoxin B1 (Van Vleet *et al*, 2002) Cisplatin toxicity was unaffected by CYP3A4 transfection, which again was anticipated, as this enzyme does not appear to play a role in Cisplatin metabolism 5-fluorouracil metabolism is not related to P450s and no direct inactivation could be shown by preincubation of the drug with CYP3A4 microsomes (see section 3 2 1 2), however, BCMV 3A4 cells also showed a modest (1 8-fold) yet significant (p=0 016) increase in resistance to this drug when compared to empty vector-transfected cells Furthermore, the magnitude of this increase was about the same as that observed for Adriamycin

In order to confirm that the observed increase in Adriamycin resistance was due to enhanced CYP3A4 activity, BCMV cells were treated with a non-toxic dose of this drug in the presence of the CYP3A inhibitor 17 α-ethynyl oestradiol (17 AEE) 17 AEE showed strong and dose-dependent inhibition of testosterone 6β-hydroxylase activity in CYP3A4-expressing microsomes Combination assays showed a slight enhancement of cell kill in BCMV 3A4 cells after simultaneous treatment with these drugs, the effect, though modest (about 10% kill), was reproducible and was not observed with Cisplatin, which was expected Combination of a non-toxic dose of 5-fluorouracil with 17 AEE did not result in increased cell kill, although it resulted in a similar trend as that observed with Adriamycin

No association between CYP3A4 activity and 5-fluorouracil toxicity has ever been reported in the literature Transfected cell lines were selected with neomycin during the cloning process and were also cultured in neomycin-containing medium for two passages after thawing, to ensure the presence of the plasmid A possible explanation for the results obtained could be that prolonged culture in selecting medium has affected cell sensitivity to toxic drugs, increasing cell resistance However, toxicity of Cisplatin was not altered in CYP3A4-transfected cells Furthermore, both BCMV Neo and BCMV 3A4 would have been cultured in the same conditions, including passaging in selecting medium, and thus should display similar responses to 5-fluorouracil-induced toxicity The CYP3A4 cDNA could have integrated anywhere in the genome of stably transfected cells, there is a possibility that integration could have disrupted the expression of genes that affect 5-fluorouracil toxicity It could also be speculated that CYP3A4 overexpression generates increased levels of an endogenous metabolite that increases resistance to 5-fluorouracil, this, however, is only a conjecture and would need much further analysis in order to be confirmed The mechanism by which CYP3A4 transfection enhances resistance to 5-fluorouracil in BCMV 3A4 cells remains to be explained

4.4 CYP1B1 and drug resistance

4 4 1 Effect of CYP1B1 inhibition on anticancer drug toxicity in HL60 cells

Cytochrome P450 1B1 (CYP1B1) has been the object of intensive research in past years After a number of studies found the expression of this P450 to be upregulated in malignant as compared to normal tissue (see section 1 4 5 2), further investigations revealed that CYP1B1 transfection increased Taxotere resistance in V79 cells (McFadyen *et al*, 2001) The link between CYP1B1 expression and Taxotere resistance is still unexplained

In agreement with previous findings, incubation of Taxotere with CYP1B1 did not affect the toxicity induced by this drug on A549 cells (see section 3 2 2 2) Indeed, it has been reported that no Taxotere metabolite could be detected after incubation of this drug with CYP1B1 in a number of different conditions (Bournique *et al*, 2002) The toxicity induced by 5-fluorouracil and Taxol was also unaffected by this treatment, which is to be expected, since none of them have been reported to undergo metabolism by CYP1B1 (see section 3 2 2 2)

Treatment of HL60 cells with a non-toxic concentration of Taxotere in the presence of the CYP1 inhibitor α-naphthoflavone (ANF) enhanced the anticancer drug toxicity by about 30% (see section 3 4 3) This result suggested that inhibition of CYP1B1 by ANF was enhancing the cytotoxicity of Taxotere, presumably by impairing its inactivation HL60 cells express CYP1B1 mRNA, as shown by RT-PCR, and their levels of EROD activity, catalysed by the CYP1 family, are low but readily measurable It should be noted that CYP1A1 and CYP1A2 also display EROD activity, so the levels of activity measured cannot be attributed solely to CYP1B1

With the aim of investigating whether the observed increase in toxicity in the presence of ANF was a Taxotere-exclusive effect, toxicity of 5-fluorouracil, Adriamycin and Taxol was also evaluated in the presence of ANF in HL60 cells A similar enhancement of toxicity was observed with all of these drugs, particularly with Taxotere and 5-fluorouracil ANF is generally considered non-hazardous, but an early report showed that treatment with this compound induced liver carcinogenesis in rats (Liehr *et al*, 1991), ANF-DNA adducts were later identified in the liver of treated animals

Enhancement of Taxotere toxicity by CYP1B1 inhibition is to be expected, considering the existent link between enzyme transfection and Taxotere resistance However, increased resistance to 5-fluorouracil, Adriamycin or Taxol by CYP1B1-transfected

cells has not been reported Enhancement of the toxicity of drugs with such different structures and mechanisms of action suggests that the effect observed in HL60 cells is due to ANF toxicity synergising with the effect of anticancer drugs, rather than due to inhibition of drug metabolism by CYP1B1 Indeed, DNA damage could sensitise cells against anticancer drugs, explaining the general increase in their toxicity It should also be noted that even though EROD activity was measurable in HL60 cells, the observed levels were very low, thereby resting importance to the effect of CYP1B1 in drug resistance

4 4 2 Effect of CYP1B1 induction by TCDD on anticancer drug toxicity in HL60 cells

TCDD is a classic agonist of the AhR Upon binding this ligand, the activated receptor translocates to the nucleus and induces the transcription of a number of genes involved in drug metabolism, but also in proliferation, cell growth and apoptosis
Induction of CYP1B1 expression in HL60 cells by TCDD treatment was confirmed by RT-PCR Increased CYP1B1 mRNA levels were paralleled by a significant raise in EROD activity, which was readily detected and measured as approximately 10-fold increase over vehicle (toluene)-treated cells Again, it should be noted that this increase might also occur as a consequence of CYP1A1 and/or CYP1A2 induction by TCDD
Toxicity of 5-fluorouracil, Cisplatin, Taxol and Taxotere was determined in HL60 cells after treatment with TCDD or vehicle for 72 hours Although treatment with TCDD did significantly increase EROD activity in HL60 cells, it did not appear to alter the toxicity induced by any of the drugs tested These results suggest that a substantial increase in CYP1 activity does not affect anticancer drug toxicity in HL60 cells It could be argued that the levels of CYP1B1 activity, though considerably increased as compared to untreated HL60 cells, were still too low to produce a significant effect However, it should be noted that the levels of activity measured are well within the range of activity values measured in tumours (62 to 995 fmol/mg of microsomal protein/min (McFadyen *et al* , 2004), while EROD activity in HL60 cells was measured in total cell lysates and is then expressed as pmol/mg of total protein/min) The relatively high (as compared to values reported in the literature) levels of EROD activity observed in HL60 cells would suggest that P450R activity in these cells is high enough to support the activity of CYP1 enzymes These results therefore may reflect in vivo events

It should be noted that TCDD treatment results in a number of alterations in gene expression, i.e., it does not only result in CYP1B1 upregulation. In a recent report, Sarioglu *et al.* (2006) describe 89 proteins that are up- or downregulated in rat hepatoma 5L cells in response to TCDD treatment, many of them involved in cell cycle regulation, growth factor signalling and apoptosis.

4.4.3 Effect of Taxotere on CYP1B1 expression in MCF-7 cells

CYP1B1 is responsible for the metabolism of a number of endogenous substrates and is involved in steroid hormone synthesis and turnover. It is not unexpected then that CYP1B1 expression is constitutive and found at high levels in steroidogenic tissues such as breast, uterus and placenta (Dasmahapatra *et al.*, 2002; Tsuchiya *et al.*, 2005). A connection has been shown between the ER and the AhR pathways (Spink *et al.*, 1998); indeed, oestrogen is required for maximal expression of CYP1B1 in MCF-7 cells (Spink *et al.*, 2003). Furthermore, oestrogen has been shown to induce CYP1B1 expression in ER positive MCF-7 cells (Tsuchiya *et al.*, 2004). This effect of oestrogen appears to be tissue-specific, for ER negative cells do not appear to induce CYP1B1 expression after treatment with oestrogen.

CYP1B1 is expressed at high levels in hormone-dependent tissues such as breast, which constitutes a suitable model to study the regulation of CYP1B1 expression. MCF-7 parent and pulse-selected cells were chosen for further studies on CYP1B1 expression.

It is a common feature of cytochromes P450 that the expression of a particular enzyme is induced by its substrate (see section 1.4.1); expression of CYP1B1 was then analysed in MCF-7 parent cells and also in resistant variants developed in this centre by pulse selection with Adriamycin and Taxotere. CYP1B1 mRNA levels were dramatically increased in MCF-7 Txt cells as compared to parent and Adriamycin-selected cells; the increase in mRNA levels was paralleled by higher levels of EROD activity, which was undetectable in MCF-7 parent cells. MCF-7 Txt cells also showed an approximately 3-fold increase in resistance to Taxotere as compared to parent MCF-7 cells.

In order to find out whether pulse selection with Taxotere had a general effect on CYP1B1 expression, a panel of Taxotere-selected cell lines – hepatoma cell line HepG2, colon carcinoma Caco2, pancreatic carcinoma BxPc-3 and squamous cell lung carcinoma SK-MES-1 – and their parental counterparts were subjected to RT-PCR analysis. CYP1B1 mRNA expression was increased in Taxotere-selected

HepG2 cells, but no CYP1B1 was detected in Caco2, BxPc-3 or SK-MES-1 cells, suggesting that the effect of Taxotere on CYP1B1 expression is tissue-specific. Parent HepG2 cells or variants pulse-selected with Cisplatin and Taxol did not display CYP1B1 mRNA expression, suggesting a Taxotere-specific effect. CYP1B1 expression is not induced in HepG2 cells after treatment with TCDD (Spink *et al.*, 1994), so it is unexpected that the levels of this enzyme are increased after treatment with Taxotere. However, it should be noted that only acute changes in expression were evaluated after TCDD treatment, while MCF-7 Txt and HepG2 Txt cells have been exposed to Taxotere on a regular basis and for a much longer period of time. These cells also display changes in their resistance profile, so in this case the induction of CYP1B1 mRNA expression could be associated with the observed increase in resistance.

The effect of Taxotere on CYP1B1 mRNA expression was further analysed in a panel of breast cell lines, ER-positive MCF-7 and MDA 453 cells and ER negative BT-20 and MDA 231 cells. These cells were exposed to a high concentration of Taxotere for 4 hours before extracting mRNA for RT-PCR analysis. RT-PCR results showed that a short pulse of Taxotere induced a modest increase in the expression of CYP1B1 in MDA 453 and BT-20 cells, suggesting that this effect is not dependent on ER status; an increase in CYP1B1 mRNA expression was not observed in MDA 231 cells. Curiously, the effect was not observed in MCF-7 cells either, suggesting that the increase in CYP1B1 expression in MCF-7 Txt cells was a consequence of repeated exposure to Taxotere, perhaps associated with the development of a resistant phenotype.

CYP1B1 expression is usually regulated through the AhR pathway. In order to confirm the functionality of this pathway, the panel of breast cell lines was also exposed to TCDD for 4 hours. Induction of CYP1B1 mRNA was observed in all cell lines irrespective of ER status, suggesting that disruption of the AhR pathway is not a cause of the observed lack of acute CYP1B1 mRNA induction by Taxotere in MCF-7 and MDA 231 cells.

4.4.4 Effect of CYP1B1 siRNA transfection on Taxotere resistance in MCF-7 Txt cells

Both expression and activity of CYP1B1 were increased after pulse selection of MCF-7 cells with Taxotere; resistance to this drug was also considerably increased. siRNA

gene expression knock-down was carried out in MCF-7 Txt cells in order to determine the role of CYP1B1 in the observed increase in resistance in these cells.

Transfection of three different CYP1B1 siRNAs directed to three different sites in the CYP1B1 mRNA molecule resulted in decreased cell survival in basal conditions (i.e., in the absence of drug). This suggests that CYP1B1 expression can somehow promote cell survival, and that reduced mRNA levels of this enzyme are detrimental to cells. The effect was not pronounced, but it was appreciable. Furthermore, this result would also explain the sensitisation to anticancer drugs of HL60 cells after CYP1B1 inhibition by ANF (see Section 4.4.1).

In contrast, no major effect on Taxotere toxicity was detected in CYP1B1 siRNA-transfected MCF-7 Txt cells. Only a slight enhancement was observed with siRNA 3, which was significant at a Taxotere concentration of 0.25 ng/ml ($p=0.038$). This could be due to the low levels of CYP1B1 activity measured in these cells (see section 3.4.8). It should be noted that the Taxotere concentrations used for this experiment were quite low and caused little change in cell survival in control cells; however, when siRNA experiments were optimised in this centre it was observed that drug concentrations resulting in approximately 20% kill or lower showed best the effect of gene expression knock-down. Taxotere concentrations were then chosen for these experiments with this in mind.

4.4.5 CYP1B1 cDNA transfection in MCF-7 cells

In spite of the growing interest in CYP1B1 and the uncertainties that still exist about its precise role in drug resistance, transfection of this enzyme into mammalian cells has been successfully attempted very few times. Indeed, CYP1B1 transfection has only been reported for V79 hamster ovary cells (Luch *et al.*, 1998) and COS-1 green monkey kidney cells (Bandiera *et al.*, 2005). V79 cells were stably transfected and showed high EROD activity levels (1-10 pmol resorufin/mg total protein/min), while COS-1 cells were only transiently transfected and displayed much lower EROD activity (about 0.05 pmol resorufin/mg total protein/min without cotransfection with P450R and 0.1 pmol resorufin/mg total protein/min with cotransfection with P450R. These values were also normalised by the authors to transfection efficiency). To date, no successful transfection of a human cell line has been reported.

MDA R4 cells are MDA 231 breast carcinoma cells transfected with P450R cDNA (Patterson *et al.*, 1997). These cells had already been successfully transfected and also expressed high levels of P450R, which is essential to support CYP1B1 activity,

so they were chosen as recipients for transient transfection with a CYP1B1 cDNA plasmid Optimisation of transfection conditions was performed using a GFP plasmid and it was shown that more than 50% of MDA R4 cells readily expressed GFP as confirmed by fluorescence microscopy and flow cytometry However, no measurable CYP1B1 protein could be detected by Western blot in cells transfected with the CYP1B1 plasmid using the optimised conditions It was thought then that transfection had been unsuccessful because MDA R4 cells could not support two large plasmids Taxotere-selected MCF-7 cells showed increased levels of CYP1B1 mRNA These cells have been shown to express P450R at high enough levels to support CYP1B1 activity (Chen *et al*, 1995) Optimisation of transfection conditions on MCF-7 cells was again performed with a GFP plasmid, again, transient transfection of GFP resulted in more than 50% of cells expressing high levels of green fluorescence as measured by flow cytometry However, no CYP1B1 protein could be detected by Western blot in the cells transfected with optimised conditions Transfection of CYP1B1 was also carried out on MDA 453 cells, with the same results (data not shown)

It appears from transfection optimisation experiments that cells are readily transfected, yet for some reason CYP1B1 is not expressed The CYP1B1 plasmid is larger than the GFP plasmid, but not to the point where it could affect transfection efficiency It could be argued that the plasmid containing the CYP1B1 cDNA might have integrated at a site in the chromosome which would not have allowed its transcription, however, it is unlikely that this would have happened in three different experiments and with three different cell lines

Regulation of CYP1B1 expression is very complex and can occur at different sites, indeed, it is known that CYP1B1 mRNA levels do not always correlate with protein expression Several reasons for this lack of correlation have been suggested among them is the belief that a certain level of mRNA should be reached before transcription can occur (McFadyen *et al*, 2003) Another possible explanation could be the degradation of CYP1B1 newly synthesized protein (Bandiera *et al*, 2003) These results could suggest that there is an endogenous factor in breast cell lines that prevents the overexpression of CYP1B1 exogenous protein

Recent studies have reported regulation of CYP1B1 expression by methylation (Nakajima *et al*, 2003, Widschwendter *et al*, 2004, Tokizane *et al*, 2005) It is possible that the CYP1B1 promoter might have been methylated upon entrance into the cell, thereby inhibiting its transcription The presence of endogenous inhibiting factors could also silence the expression of the CYP1B1 cDNA However, the expression of plasmid CYP1B1 is under the control of the CMV promoter, a strong enhancing element, which makes it quite unlikely that transcription of the plasmid

cDNA would be affected by endogenous factors However, the presence of an endogenous inhibiting factor might explain why no human cell line has been successfully transfected to date

A possible explanation for the lack of CYP1B1 expression in these cells would be that the gene is transcribed but the mRNA is unstable and is degraded before any translation can occur A variety of CYP1B1 polymorphisms have been described and differences do exist in the stability of the various alleles, however, these differences refer to the translated protein and not to mRNA levels

In conclusion, transfection of CYP1B1 cDNA was attempted in three different breast cell lines and although the plasmid appears to have been taken up by the cells, no CYP1B1 protein could be detected These results could suggest that an endogenous inhibitory molecule is preventing exogenous CYP1B1 protein expression, although the nature of this molecule and the mechanism it uses to silence expression remain unknown It is possible that a number of different factors are at play at the same time and that the combination of events results in silencing of CYP1B1 plasmid expression Further studies are needed in order to identify the factor or factors responsible for lack of exogenous CYP1B1 expression in these cells

In summary, CYP1B1 expression appears to be connected with resistance to certain anticancer drugs, most notably Taxotere It is possible that the increased levels of CYP1B1 reported in tumour as compared to normal tissue are simply due to the fact that expression of this enzyme is associated with carcinogenesis, or with the resistant phenotype, rather than being directly involved in drug resistance However, knock-down and/or inhibition of CYP1B1 appears to be detrimental to some cells, this suggests that CYP1B1 is not a simple resistance-associated marker but an enzyme with a role in promotion of survival CYP1B1 does not appear to enhance Taxotere resistance by direct inactivation of the drug, so if this enzyme does affect cell sensitivity to this drug the effect is likely to be exerted through an indirect mechanism It could be speculated that an endogenous metabolite of this enzyme is responsible for the promotion of cell survival, but further studies are needed to clarify this

4.5 P450 NADPH reductase and drug resistance

4 5 1 Effect of P450R transfection on anticancer drug-induced toxicity in MDA 231 cells

P450 NADPH reductase (P450R) function is essential for cytochrome P450 activity This enzyme provides the electrons used in all P450-mediated reactions using NADPH as a cofactor Given that P450R is always present in tissues expressing cytochromes P450 and also that it is able to metabolise drugs by itself, its possible involvement in drug resistance was investigated

MDA 231 R4 cells are breast adenocarcinoma cells transfected with human P450R cDNA They express high levels of this enzyme when compared to MDA 231 EV empty-vector transfected cells, as confirmed by Western blot and activity assays These cells were used as models to study how P450R affects drug resistance For this, toxicity assays were performed with a panel of commonly used anticancer drugs on P450R and empty vector-transfected cells

MDA R4 cells were significantly more sensitive to Mitomycin C than MDA EV cells Mitomycin C requires activation by P450R to generate metabolites that are much more toxic than the parent drug (Cummings *et al* , 1998), it is easy to see then why MDA R4 cells are more susceptible to the effects of this drug It is likely that Mitomycin C undergoes metabolic activation in P450R-overexpressing cells, generating toxic compounds and enhancing the cytotoxicity of the anticancer drug Conversely, transfection of P450R did not seem to affect the sensitivity of MDA 231 cells to Cisplatin, Mitoxantrone, Taxol, Vincristine or VP-16, which was expected, since none of these drugs have a reported interaction with P450R A slight increase in sensitivity to Taxotere was observed following transfection, although it was not significant Still, sensitisation of MDA cells following P450R transfection was an unexpected finding, for Taxotere exerts its toxic effects through disruption of microtubule dynamics, no apparent connection exists between microtubules and redox cell status Overexpression of P450R increases oxidative stress in MDA R4 cells (see Section 4 5 2) and this might increase their sensitivity to cytotoxic drugs, however, it is difficult to explain why this effect was observed with Taxotere and not with other drugs, even those that, like Taxol, have a very similar mechanism of action

Another noteworthy result was the observed sensitisation of MDA R4 cells to Adriamycin The increased toxicity of Adriamycin is in opposition with the results

obtained in the P450R microsome preincubation assays (see section 3 2 3) in this case, preincubation with P450R-expressing microsomes decreased drug toxicity on MDA 231 cells This would suggest that P450R decreases Adriamycin-mediated toxicity, presumably through partial drug inactivation However, P450R-mediated activation of Adriamycin can also occur

The planar nucleus of Adriamycin is a quinone and as such can undergo one-electron reduction by P450R to generate the semiquinone form (Bartoszek *et al*, 1992) The semiquinone is an unstable intermediate that can react with molecular oxygen, donating an electron to regenerate the parent quinone and produce superoxide anion in the process The superoxide anion is not very toxic *per se*, but can undergo successive reactions to generate hydrogen peroxide and hydroxyl radical, which is considered as the most toxic of the reactive oxygen species (ROS) Furthermore, metabolism of Adriamycin by P450R appears to generate an alkylating metabolite that was detected in the culture medium but not in cell lysates (Bartoszek *et al*, 1992), the authors concluded that it must be unstable in the intracellular environment This metabolite could be detected by HPLC but could not be identified and it seemed to be responsible for the observed increase in toxicity, as the presence of several ROS scavengers did not prevent the enhanced cytotoxic effect of Adriamycin High doses of GSH, however, did completely abrogate the increase in Adriamycin toxicity GSH is the most abundant non-protein antioxidant present in the cell, but it is also a cofactor of GSTs, which are responsible for the detoxification of a number of drugs, including Adriamycin (McLellan *et al*, 1999) The lack of effect of other free radical scavengers would indicate that GSH prevents the increase in cytotoxicity by neutralising a toxic compound rather than by acting as an antioxidant

In summary, preincubation of Adriamycin with P450R leads to partial inactivation of the drug, but treatment of P450R overexpressing cells with Adriamycin means that alkylating agents and toxic free radicals are generated inside the cell and contribute to the cytotoxicity of the drug The results obtained suggest that the increase in sensitivity observed in P450R-transfected cells is due to the generation of short-lived species during Adriamycin redox cycling, while preincubation of the drug with P450R most probably generates inactive or less active metabolites It should also be noted that there was no change in Mitoxantrone toxicity after P450R transfection Mitoxantrone was initially developed as an Adriamycin analogue and both drugs are similar in many ways However, Mitoxantrone has a reduced ability to generate free radicals compared to the anthracyclines (Hande *et al*, 1998), suggesting that free radical generation could be responsible for the differential toxicity observed with the two drugs

Our results are in agreement with those of Bartoszek *et al.* (1992), who found that incubation of MCF-7 cells with Adriamycin in the presence of exogenous recombinant P450R significantly increased the toxic effects of the drug. This increase in cytotoxicity was not observed when the drug was preincubated with P450R prior to addition to the cells, suggesting that the enhanced toxicity was mediated by short-lived species generated *in situ*.

However, Ramji *et al.* (2003) failed to detect any changes in Adriamycin toxicity in MDA R4 as compared to MDA EV cells. These authors had confirmed reduction of Adriamycin by P450R by NADPH consumption assays with recombinant enzyme; they also found that the rate of Adriamycin reduction correlated with P450R activity in 17 samples of human liver microsomes. These results suggested that P450R had the potential to increase Adriamycin cytotoxicity by reductive activation in transfected cell lines, but toxicity assays showed that the sensitivity of MDA R4 cells was similar to that of empty vector-transfected MDA EV cells. The results reported by these authors are in direct contradiction with the findings of this thesis. However, an explanation for this discrepancy can be found in the different methods used to assess toxicity: while the acid phosphatase assay was employed to assay Adriamycin toxicity in MDA 231 cells, Ramji *et al.* used the MTT assay. This assay is based on the reduction of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) by cellular reductases to the coloured compound formazan. As can be seen in section 3.5.2, P450R readily converts MTT to formazan, to such an extent that this reaction was used to quantify enzymatic activity. It is not unreasonable to assume then that the same number of MDA R4 cells would convert more MTT to formazan than that of MDA EV cells, thereby making it difficult to ascertain any low-grade increase in sensitivity. Indeed, toxicity assays were performed in parallel in MDA R4 and MDA EV cells using two different endpoint assays, the acid phosphatase and XTT, an equivalent of MTT with a water-soluble product (formazan has little solubility in water and needs to be dissolved in DMSO before absorbance readings). Results obtained with the XTT assay suggested an increase in resistance to all the drugs tested for MDA R4 as compared to MDA EV cells (data not shown), while acid phosphatase assays showed either sensitisation or no change in toxicity. This suggests that the XTT and MTT assays do not provide reliable results when used with P450R-transfected cells.

Sensitisation to 5-fluorouracil after P450R transfection was another unexpected finding. This effect was not due to direct inactivation of the drug by P450R, as shown by the P450R microsome preincubation assays (see section 3.2.3.1); this is to be

expected, as no interaction of this drug with P450R has been reported The observed enhancement of 5-fluorouracil toxicity must then be due to a pharmacodynamic effect NADPH is a key compound required for 5-fluorouracil catabolism, this drug is a pyrimidine analogue, and as such undergoes the same metabolic reactions as endogenous pyrimidines (Fig 4 1) Reduction of these compounds by NADPH is followed by opening of the pyrimidine ring and generation of a beta aminoacid, which is later excreted

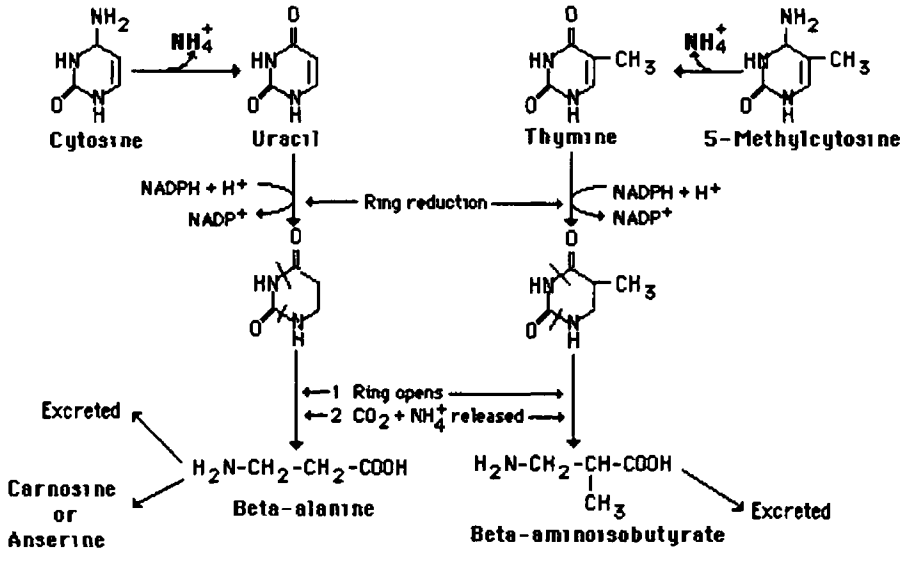


Figure 4 5 1 Pyrimidine catabolism (from www-medlib med utah edu/NetBiochem/pupyr)

Overexpression and consequent increased activity of P450R would result in depletion of NADPH from the cell pool An important decrease in NADPH levels could impair 5-fluorouracil catabolism, leaving additional drug available to exert its toxic effects This could explain the increase in sensitivity shown by MDA R4 cells Incidentally, NADPH is also required as a thioreductase cofactor for the *de novo* synthesis of deoxynucleotides Decreased levels of NADPH could then result in diminished DNA synthesis and subsequent inhibition of DNA damage repair

Two papers have been recently published (Hwang *et al*, 2001, Liu *et al*, 2002) about the role of ferredoxin reductase in drug resistance Ferredoxin reductase is the only mammalian mitochondrial cytochrome P450 NADPH reductase, which means that both ferredoxin reductase and P450R have exactly the same role, differing only in

their subcellular localisation (Hwang *et al.*, 2001). The aforementioned studies have found that high levels of ferredoxin reductase sensitise cells against oxidative stress induced by p53 signalling after 5-fluorouracil and Adriamycin treatment. Depletion of NADPH levels has been pointed as the mechanism by which this enzyme exerts its sensitising effects. The model proposed by the authors involves p53, which is activated by anticancer drugs and in turn induces expression of ferredoxin reductase; increased levels of this enzyme impair ROS detoxification, generating mitochondrial and DNA damage and enhancing p53 function, thereby entering a positive feedback loop.

In summary, transfection of P450R sensitises MDA R4 cells to 5-fluorouracil cytotoxicity, presumably by depleting NADPH cell levels, which in turn impair drug catabolism and DNA repair and also increase oxidative stress by decreasing antioxidant reserves in the cell. Increased oxidative stress due to NADPH depletion could also be partly responsible for the observed sensitisation to Adriamycin.

4.5.2 Oxidative stress in MDA 231 cells

P450R uses NADPH as a source of electrons and transfers them to diverse acceptors, so it was expected that reduced NADPH levels would be decreased in MDA R4 cells as compared to MDA EV cells. Indeed, even though the levels of NADPH in MDA 231 cells were extremely variable, MDA R4 NADPH levels were consistently about half of those measured in MDA EV cells. This suggests that even though there might be large variations in NADPH levels, MDA R4 cells show a definite decrease of this antioxidant.

NADPH is an essential antioxidant and contributes to ROS detoxification (Liu *et al.*, 2002). The decrease in NADPH levels would be expected to result in an increase in other antioxidant defences, in an attempt to reduce oxidative damage. GSH is the most important non-protein antioxidant present in the cell and both subunits of the enzyme responsible for its synthesis, GCSH and GCSL, are upregulated in MDA R4 as compared to MDA EV cells. This increase in GSH synthesis is likely to result in increased GSH levels, although the drop in NADPH might mean that GSH is used up to detoxify ROS. GST P1 was also induced in MDA R4 cells, suggesting that conjugation with GSH is increased as a detoxification mechanism.

Finally, production of ROS was measured in MDA EV and MDA R4 cells at basal and drug-induced conditions. ROS levels were increased in MDA R4 cells to about double the values measured in MDA EV cells in basal conditions, suggesting that the

depletion of NADPH levels increases cellular oxidative stress by impairing ROS detoxification The levels of ROS were not significantly affected in MDA EV cells after treatment with 5-fluorouracil, although they did appear to increase slightly with Mitomycin C Surprisingly, administration of Adriamycin slightly decreased ROS levels in MDA EV cells, suggesting that overexpression of P450R is necessary for ROS generation in these cells

The levels of ROS in MDA R4 cells were dramatically increased after treatment with Mitomycin C, Adriamycin and 5-fluorouracil These results suggest that the increased sensitivity to these drugs observed in MDA R4 cells is at least in part due to increased ROS production and subsequent cellular damage In the case of Mitomycin C, it has been reported that ROS production, though it occurs, does not contribute to the overall toxic effects of the drug (Cummings *et al*, 1998) In the case of Adriamycin, the role of ROS in cytotoxicity remains to be elucidated, while 5-fluorouracil appears to exert its toxic effect in part through a p53-mediated increase in ROS production

In the case of Resveratrol, a dual effect was observed in MDA cells while this compound increased ROS levels in MDA EV cells, it decreased the basal levels of ROS in MDA R4 cells, though not to those measured in MDA EV cells This is consistent with the dual role reported for Resveratrol in the oxidative status of cells while this compound is generally considered to be an antioxidant and a free radical scavenger (Sgambato *et al*, 2001, Leonard *et al*, 2003, Lorenz *et al*, 2003), it has also been found that it induces ROS-mediated apoptosis in CEM-C7H2 leukaemia cells (Tinhofer *et al*, 2001) Indeed, treatment of these cells with Resveratrol significantly increased ROS levels after only 30 minutes This compound also induced the production of ROS in blood platelets (Olas *et al*, 1999) The effect of Resveratrol depended on the concentration at which it was used at the lowest concentrations tested (up to 12 5 µg/ml), this compound caused an increase in superoxide production, while it inhibited it at higher concentrations (up to 100 µg/ml)

It has recently been reported that transfection of P450R in V79 hamster cells resulted in increased oxidative DNA damage even in the absence of any exogenous compound (Heine *et al*, 2006) The addition of buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, increased the levels of DNA damage, indicating that cells are heavily reliant on GSH production to counteract the increase of harmful oxidative species Moreover, transfection of CHLA-20 neuroblastoma cell lines with GFP increased cell sensitivity to Carboplatin, VP-16 and Melphalan, as well as to high concentrations of Adriamycin (Goto *et al*, 2003) GFP-transfected cells were found to have much higher GSH levels than untransfected and vector-transfected cells

Simultaneous treatment of GFP-transfected cells with VP-16 and the antioxidant N-acetyl cysteine abrogated the observed increase in sensitivity The authors concluded that ROS generated by GFP could only enhance VP-16 toxicity in cooperation with endogenous ROS It was also shown that sensitisation was dependent on p53 expression, as p53 non-functional cells did not increase their GSH levels upon transfection with GFP

As seen in previous results, oxidative stress levels are increased in MDA R4 as compared to MDA EV cells This means that MDA R4 cells are subject to constant oxidative damage due to a decrease in their antioxidant defences It is not unexpected then that these cells also display enhanced sensitivity to anticancer agents, especially those that can increase ROS levels either directly through redox cycling or indirectly via p53-mediated induction

In summary, P450R plays a role in drug resistance independently of cytochromes P450 This enzyme can partially inactivate drugs, as in the case of Adriamycin, but it can also enhance their effect by redox cycling and generation of more toxic compounds (ROS and also alkylating metabolites), or through depletion of NADPH levels, which decreases cellular defences against oxidative damage Expression of P450R in tumour cells (which has been reported in many occasions, see section 1 4 5) might therefore modulate the toxicity of certain drugs, this makes it an attractive target for the enhancement of chemotherapy efficacy

4 6 Glutathione-related genes and drug resistance

Deregulation of glutathione (GSH) metabolism as a consequence of malignant transformation has been thoroughly studied in the past years, and has been shown to be associated with enhanced drug resistance in tumours (Hao *et al*, 1994, Perquin *et al*, 2000) Alterations observed usually include elevated GSH levels, increased expression of GSH transferases (GSTs) and also enhanced expression of partner proteins such as MRP1 These alterations are the consequence of changes in the expression of a number of GSH-related genes, including GSH synthetases, peroxidases and also enzymes involved in GSH turnover The contribution of each one of these genes has been analysed in cell lines by transfection and knock-out of expression Each gene appears to play only a modest role in resistance, however, co-transfection of two or more of these genes results in dramatic increases in resistance (O'Brien *et al*, 2000)

These results suggest that it is the overall deregulation of GSH metabolism, rather than the involvement of a single gene, which ultimately confers cells increased drug resistance For this reason, the expression of a number of key genes involved in GSH metabolism was analysed in a panel of four pulse-selected cell lines and their parental counterparts

GSH synthesis is mainly mediated by γ-glutamyl cysteine synthetase (GCS), an enzyme composed of two subunits a heavy or catalytic subunit (GCSH) and a light or regulatory one (GCSL) Increased activity of GCS resulted in higher rates of GSH synthesis that correlated with VP-16 and Adriamycin resistance in MCF-7 cells (Gamcsik *et al*, 2002) Inhibition of GCS with buthionine sulfoximine (BSO) reversed Cisplatin resistance in a panel of renal carcinoma cell lines (Asakura *et al*, 2005) GCSH has been shown to play a significant role in resistance, even on its own transfection of a ribozyme against GCSH decreased GSH levels and increased sensitivity to Cisplatin in human colon cancer cells (Iida *et al*, 2001) In addition, knock-down of GCSH also suppressed the expression of MRP1, MRP2 and MDR1, once more pointing to a coordinated regulation of defence mechanisms against toxic insults The role of GCSL is less clear, as most studies have only analysed GCSH expression and the levels of GCSL do not always correlate with those of GCSH, this suggests a differential regulation of the two subunits However, ribozyme knock-down of GCSL in A549 cells decreased the levels of Cisplatin resistance in these cells more efficiently than knock-down of GCSH (Inoue *et al*, 2003)

Expression of GCSH did not appear to be significantly affected by pulse selection in some of the cell lines analysed, indeed, A549 Carboplatin and Taxol-selected variants showed no change in GCSH expression as compared to parent cells Similarly, H1299 and SKLU-1 pulse-selected cells displayed no major changes in expression as compared to parent cells The most significant results were observed in MCF-7 cells Adriamycin-selected MCF-7 cells showed a modest increase in the expression of this enzyme, while GCSH levels were decreased by half in MCF-7 Txt cells These results are in agreement with previous findings increased GCSH expression usually correlates with increased GSH levels, which have been reported to confer resistance mainly to drugs such as the anthracyclines and platinum derivatives Enhanced synthesis of GSH could therefore be pointed as a possible cause of the increase in resistance observed in MCF-7 Adr cells However, it is difficult to explain why the levels of GCSH were not also increased in Cisplatin-selected MCF-7 cells
Expression of GCSL did not always correlate with that of GCSH, suggesting that both subunits are independently regulated in these cells The levels of this enzyme were largely unaffected by pulse-selection in all the resistant variants as compared to parent cells

The membrane-bound γ-glutamyl transpeptidase (GGTP) is the enzyme responsible for degrading extracellular GSH to its component aminoacids An interesting feature of this process is that GGTP-expressing cells may extract GSH from the extracellular environment, generating free aminoacids that then enter the cell and are reused for intracellular GSH synthesis Indeed, this mechanism has been demonstrated in non-small cell lung cancers, which are able to extract circulating GSH to increase intracellular synthesis and GSH levels (Blair *et al*, 1997) Overexpression of GGTP usually correlates with high GSH levels (Black *et al*, 1991) Moreover, GGTP activity and GSH levels were significantly higher in high grade and metastatic soft tissue sarcomas than in less aggressive tumours (Hochwald *et al*, 1997)
Expression of GGTP was decreased in the Taxol-resistant variants of A549 and H1299 cells as compared to parent and Carboplatin-selected cells This is consistent with the fact that increased GSH levels confer protection particularly to drugs such as platinum compounds However, both SKLU-1 variants showed slightly increased levels of this enzyme Expression of GGTP was not detectable in MCF-7 parent or pulse-selected cells

GSTs are responsible for the generation of GSH-conjugates and play a prominent role in the detoxification of a number of compounds Several families of GSTs exist (see

section 1.3.2.1) and each is composed of one or more isoforms, of which the best studied is GST P1. Transfection of a cholangiocarcinoma cell line with a GST P1 antisense vector resulted in significant increase of Adriamycin, Cisplatin and Melphalan toxicity (Nakajima *et al.*, 2003); similar results were obtained when treating cells with a specific GST P1 inhibitor. On the other hand, increased expression of GST P1 was associated with resistance to anticancer agents in breast (Su *et al.*, 2003) and head and neck cancer (Cullen *et al.*, 2003). No correlation between GST P1 expression and response to chemotherapy was found in non small cell lung cancer patients (Unsal *et al.*, 2003). However, acquired resistance to Cisplatin and Adriamycin in a small cell lung cancer cell line was correlated with increased GST P1 levels (Hao *et al.*, 1994).

Expression of GST P1 was common across the lung cell lines, showing no apparent changes in expression in pulse-selected as compared to parent cells. Expression of GST P1 could not be detected in MCF-7 parent or pulse-selected cells. These results suggest that regulation of GST P1 expression is tissue and/or cell specific.

GST A1 has also been associated with anticancer drug resistance: transfection of GST A1 protected endothelial cells from arrest mediated by the alkylating agent Busulfan (Ritter *et al.*, 2002). Several studies have also shown that GST A1 and MRP1 or MRP2 work synergistically to increase anticancer drug resistance in tumour cells (Morrow *et al.*, 1998; Paumi *et al.*, 2001; Smitherman *et al.*, 2004).

Expression of GST A1 was again largely unchanged in all MCF-7 and A549 resistant variants as compared to parent cells, although A549 Tax cells - but not A549 Cpt cells – appeared to show a slight increase in GST A1 levels when compared to parent A549 cells. SKLU-1 cells, however, showed dramatic upregulation of this enzyme particularly in the Taxol-selected cells.

Overall, alterations in the expression of GSH-linked enzymes were readily detected in pulse-selected cells as compared to their parent counterparts; however, these alterations were not substantial nor consistently associated with resistance to a certain drug or group of drugs, or with the level of resistance. For example, GCSH levels were increased in Adriamycin-resistant MCF-7 cells as compared to parent MCF-7 cells, but no change with respect to parent cells was observed in Cisplatin-resistant MCF-7 cells. These results suggest that higher GSH levels associated with increased expression of this enzyme are not always a limiting factor in protecting these cells against platinum compounds.

Expression of GST P1, which is considered as the most important of the GSTs and also the one that has a clearer association with drug resistance, was not majorly affected by pulse selection in the two cell lines where it was present. However,

expression of GST A1 was upregulated in SKLU-1 pulse-selected cell lines, suggesting that higher levels of this enzyme are associated with increased drug resistance in these cells These results are in agreement with the findings discussed in section 4 1, where GST A1 expression in tumour samples was correlated with poor prognosis

In summary, alterations in the expression of GSH-linked enzymes are a common event in the panel of pulse-selected cell lines studied However, no correlation was found between the expression of one or a set of these enzymes and the levels or resistance to certain drugs or groups of drugs These results suggest a complex and cell-specific regulation of the expression of GSH-related enzymes

4.7 Pulse selection of cell lines

4.7.1 Toxicity profiles of pulse-selected cells

Exposure of cancer cells to anticancer drugs can affect patterns of gene expression and ultimately result in the development of resistance. Cells that have been repeatedly exposed to drugs commonly used in chemotherapy constitute a good model to identify changes undergone by tumour cell lines after drug treatment and to investigate the association of these changes with the resistant phenotype.

Two protocols have been used to study this process: the continuous growth of cells in the presence of drug, and the periodic exposure of cells to high doses of drugs for a brief period of time, separated by regular intervals. This second method resembles the treatment given to patients, who usually receive chemotherapy treatment once every 1-3 weeks, allowing for recovery in between cycles.

The aim of these studies was to analyse changes in the expression of drug-metabolism enzymes, especially cytochromes P450, caused by pulse selection. Even though biopsy samples usually display readily measurable P450 activity, most cell lines have lost P450 expression and/or ability of induction. This is a common phenomenon and appears to be due to alterations in specific transcription factors (Rodriguez-Antona *et al.*, 2002). Pulse selection was therefore performed on two metabolically competent cell lines, that is, cells that still retain P450 expression and/or induction capacity.

Caco2 cells were originally derived from colon adenocarcinoma. They express several enzymes related to drug metabolism, as well as transporters and efflux pumps; they have been reported to generate metabolites similar to those observed *in vivo* and are thus considered metabolically competent (Lampen *et al.*, 1998). HepG2 cells are derived from a hepatoma and as such express a number of enzymes usually found in liver. This cell line has been deemed as useful to study the regulation of drug-metabolism enzymes (Wilkening *et al.*, 2003).

Caco2 and HepG2 cells were exposed to pharmacologically achievable levels of Taxol, Taxotere or Cisplatin in order to examine the resistance mechanisms activated upon exposure to chemotherapy drugs. Pulse selection with Taxol or Taxotere resulted in a marked increase in resistance for both cell lines; cross-resistance was also observed against both taxanes and also to Vincristine. No significant change in sensitivity was observed against either Cisplatin or Irinotecan. In the case of Cisplatin-

selected HepG2 cells, no change in sensitivity was observed against any of the drugs tested, even Cisplatin.

The levels of resistance to Taxol, Taxotere and Vincristine achieved for Caco2 pulse-selected cells ranged from about 5 to 40-fold as compared to parent cells, while for HepG2 cells they were between approximately 1.5 and 6-fold. These levels of resistance were stable after weeks in culture and relate to observed *in vivo* levels of resistance, which are usually about 5 to 10-fold (Simon *et al.*, 1994). Taxol, Taxotere and Vincristine are all substrates of CYP3A, so the observed increase in resistance could be attributed to enhanced inactivation by this subfamily of enzymes. However, the three drugs are also substrates for the efflux pump MDR1. Furthermore, resistance to agents that affect microtubule dynamics, as is the case for these drugs, can also arise from alterations in drug binding affinity and changes in tubulin subtype or expression (Fojo *et al.*, 2005). Any of these phenomena – or a combination of the three – might explain the observed increase in resistance to the three drugs, as well as the lack of change in Cisplatin and Irinotecan toxicity; neither of these drugs undergoes significant metabolism by CYP3A (this enzyme can inactivate Irinotecan, but this is only a minor pathway of drug detoxification) or are substrates of MDR1 or affect microtubule dynamics.

For Caco2 cells, the levels of Taxol resistance were higher than those observed for Taxotere, even in cells that had been Taxotere-selected. In HepG2 cells, curiously, even though cross-resistance to the other taxane was observed, resistance to Taxotere was higher in cells that had been pulse-selected with Taxol and viceversa. This suggests differential regulation of Taxol and Taxotere resistance in these two cell lines. It also suggests that response to drugs of such similar structure and mechanism of action as the two taxanes is also quite different. Furthermore, cross-resistance to Vincristine was of much higher magnitude than resistance to taxanes in Caco2 cells; once again, this phenomenon was not observed in HepG2 cells, where resistance to Vincristine, although significant, was still lower than that observed for the taxanes. Altogether, these results highlight the cell type-specificity of the response to drug exposure.

4.7.2 Changes in protein expression in pulse-selected cells

Analysis of P450 expression by Western blot showed modest (about 1.5 to 3-fold) induction of CYP3A4 and CYP3A5 protein expression as a result of the selection process in almost all of the pulse-selected cell lines tested. Increase in CYP3A protein

expression in Taxol and Taxotere-selected cells was expected, as P450s are usually induced by their substrates. Expression of CYP3A4 has been reported to increase in response to Taxol treatment in human hepatocytes (Nallani *et al.*, 2004); this study did not find substantial changes in CYP3A4 expression after Taxotere treatment. CYP3A4 levels were also increased after selection with Cisplatin, which is not a P450 substrate; this is an unexpected result and points to an increase in CYP3A expression as part of a general defence system against toxic compounds. The levels of induction of CYP3A4 and CYP3A5 in both Caco2 and HepG2 pulse-selected cells were similar, suggesting a common response to drug exposure.

On the other hand, expression of CYP3A4 and CYP3A5 was unchanged, or even decreased, after pulse-selection in H1299 and MCF-7 cells. These results highlight the importance of using metabolically competent cells for P450 expression analysis. Metabolically competent cells retain expression of cytochromes P450 and, more importantly, inducible regulation of this expression.

Expression of MDR1 was also induced in selected cell lines and at much higher levels than those of CYP3A. Parent Caco2 and HepG2 cells displayed only very low levels of this efflux pump, which was significantly upregulated upon pulse selection with Taxol, Taxotere and also Cisplatin, albeit at much lower levels of induction than those observed with the taxanes. This latter result is unexpected for, as stated before, Cisplatin is not an MDR1 substrate. However, induction of both CYP3A4 and MDR1 expression has been reported to occur in response to Cisplatin treatment (Masuyama *et al.*, 2005). Again, this suggests that cells activate a number of different defence mechanisms upon exposure to toxic compounds, some of which would be useful for the detoxification of the specific toxin and also some that would not.

Supporting this observation, analysis of GSH-related enzymes in Caco2 variants – the most resistant cells obtained after pulse selection – revealed that the two subunits of the key enzyme involved in GSH synthesis were upregulated in the selected variants as compared to parent cells. This was true especially of GCSH, which showed a modest increase in expression, while levels of GCSL not always paralleled this increase. Higher levels of induction were observed for GGTP and GST P1, especially in Caco2Txt cells, indicating that GSH turnover is altered in these cells in response to pulse selection.

Modulation of Taxol toxicity by GSH is evident, for treatment with the GCS inhibitor BSO sensitises MCF-7 and A549 cells to the toxic effects of this drug (Liebmann *et al.*, 1993). However, transfection of lung cancer cells with GST P1 did not cause an appreciable effect in Taxol toxicity (Miyara *et al.*, 1996). In the case of Taxotere, GST P1 appears to be at least partially responsible for the inactivation of the drug, since

inhibition of this enzyme enhanced Taxotere cytotoxicity in human gastrointestinal cancer cell lines (Park *et al.*, 2002). Moreover, Taxotere induced ROS production and GSH depletion in hepatocellular carcinoma SMMC-7721 cells (Geng *et al.*, 2003). Finally, a recent study has found that expression of genes involved in regulation of the redox environment, such as GSTs, is elevated in patients with poor response to Taxotere treatment (Iwao-Koizumi *et al.*, 2005). These results suggest that GSH-related genes can modulate taxane-induced cytotoxicity and that the increase in their expression might be at least partially responsible for the enhanced resistance of pulse-selected Caco2 cells.

4.7.3 Role of MDR1 in the observed increase in resistance in pulse-selected cells

Given the fact that MDR1 has a proven association with drug resistance, combination assays were performed with GF120918, a well-known non-competitive inhibitor of MDR1. GF120918, also known as Elacridar, can efficiently inhibit MDR1 without any noticeable effect on MRP1 function (Evers *et al.*, 2000).

This compound elicited a remarkable synergistic response when used together with Taxotere on Caco2 parent cells and resistant variants, suggesting that MDR1 plays a relevant role in the development of resistance in these selected cells. Sensitisation to Taxotere was more patent in pulse-selected variants than in parent cells, indicating that resistant cells are more dependent on MDR1 function. Such an effect was not observed when Cisplatin was used in combination with the inhibitor; this is to be expected, since this drug is not an MDR1 substrate.

The effect of GF120918 was also observed in parent HepG2 cells, even though these cells displayed almost no measurable expression of MDR1 by Western blot. Some studies have suggested that even a low level of MDR1 might be enough to protect cells from the toxic effects of drugs (Allen *et al.*, 2000). Sensitisation was also observed in HepG2Txol and HepG2Txt in the presence of GF120918, although the effect was modest in magnitude. Only a minimum effect was observed in HepG2CisPt variants, even though Western blots showed induction of MDR1 in these cells. Again, Cisplatin showed negligible synergy with GF120198. The synergy between Taxotere and GF120918 was much stronger in Caco2 cells, consistent with the fact that these cells developed much higher levels of resistance than HepG2.

In conclusion, inhibition of MDR1 dramatically reverts the observed increase of resistance in Caco2 and, to a lesser extent, in HepG2 pulse-selected cells. These

results indicate that MDR1 is at least partly responsible for the resistant phenotype of these cells.

4.7.4 Role of MRP1 in the observed increase in resistance in pulse-selected cells

Sulindac belongs to the non-steroid anti-inflammatory drug group and, as many other members of this group, has been shown to inhibit MRP1 without any noticeable effect in MDR1 activity (O'Connor *et al.*, 2004). Inhibition of MRP1 by Sulindac did not result in major enhancement of Adriamycin toxicity in any of the cell lines tested, whether parent or pulse-selected, indicating that MRP1 activity is not heavily involved in the observed increase in resistance. This is not unexpected, since the taxanes are poor MRP1 substrates.

A slight sensitisation to Adriamycin toxicity was observed in Caco2 and especially Caco2Txt cells, but not in Caco2Txol. The decrease in cell survival after simultaneous treatment with Adriamycin and Sulindac was about 20%, as compared to the effect of Adriamycin alone.

As for HepG2 parent cells, a similar level of sensitisation was observed in the parent cell line, while none of the resistant variants displayed any appreciable increase in Adriamycin toxicity in the presence of Sulindac.

4.7.5 Role of CYP3A in the observed increase in resistance in pulse-selected cells

The estrogen 17 AEE is used in oral contraception and is also a well-known, non-competitive CYP3A inhibitor. Indeed, incubation of CYP3A4-expressing microsomes with increasing concentrations of this compound resulted in dose-dependent inhibition of testosterone 6β-hydroxylase activity (see section 3.3.4).

Treatment of pulse-selected cells with Taxol, a CYP3A substrate, in the presence of 17 AEE did not significantly affect the toxicity of the anticancer drug, suggesting that increased CYP3A activity is not a main protective mechanism against Taxol in these cells. A slight increase in Taxol toxicity was observed in parent Caco2 cells, suggesting that CYP3A activity might be a second-line mechanism of defence against Taxol in non-pulsed cells. However, this effect was not observed in parent HepG2 cells.

Taxol is metabolised by both CYP2C8 and CYP3A4; it is generally considered that the main detoxification pathway is that involving CYP2C8, while CYP3A4 constitutes only a minor pathway. However, the main metabolic pathway of Taxol detoxification in colorectal cancer microsomes was carried out by CYP3A (Martinez *et al.*, 2002). Induction of CYP3A4 expression has been reported to modify Taxol pharmacokinetics (Monsarrat *et al.*, 1998), indicating that this enzyme might play a more prominent role in Taxol metabolism than previously thought. Furthermore, it was found that expression of tumour CYP3A4 mRNA might predict the response to Taxol treatment in breast cancer patients (Miyoshi *et al.*, 2002). These results suggest that CYP3A is a relevant enzyme for Taxol metabolism, particularly in colorectal cancer.

No significant effect in toxicity was observed when treating any of the cell lines simultaneously with Cisplatin and 17 AEE as compared to Cisplatin alone, as expected.

4.7.6 Role of CYP3A in the observed increase in resistance in pulse-selected cells after MDR1 inhibition

Pulse-selected cells showed increased expression of CYP3A4 and CYP3A5. This increase, however, did not appear to be directly related to the observed enhancement in drug resistance. Indeed, MDR1 seems to be the predominant mechanism of resistance in these cell lines; however, it was hypothesised that other mechanisms might take over if MDR1 is inhibited.

In order to investigate the possibility of P450s acting as a second-line defence mechanism to MDR1, Caco2 parent and pulse-selected cells were simultaneously exposed to Taxol, GF120918 and 17 AEE. These cells showed the highest MDR1 activity and also the highest levels of resistance. Simultaneous treatment with non-toxic concentrations of Taxol and GF120918 resulted in an increase in Taxol toxicity in these cells, as expected. Addition of 17 AEE resulted in a further 20% increase in toxicity in Caco2Txol cells. This effect was not seen in Caco2Txt cells, although it is difficult to assess any further increase in toxicity due to the low cell survival obtained with the combination of Taxol and GF120918. Caco2Txol cells are less resistant to Taxol than Caco2Txt cells (10-fold versus 13-fold); increased resistance to Taxol might be related to the lack of synergy observed between Taxol, GF120918 and 17 AEE. A slight increase in Taxol toxicity was observed in Caco2 parent cells at the highest concentration of 17 AEE.

Efflux pumps are usually considered as a low affinity, high capacity detoxification mechanism, while enzymes involved in drug metabolism exhibit high affinity and low capacity. When cells are exposed to high concentrations of drug, it is likely that the cell will rely on quick detoxification of large amounts of toxic compounds; thus it is not unexpected that efflux pumps such as MDR1 are more relevant to pulse-selected cell resistance than drug metabolism enzymes. However, these enzymes, although dealing with low amounts of drug at a time, have a high affinity for their substrates and also a high processing efficiency. Therefore, they might constitute a solid second-line defence mechanism against toxic insults.

However, it should also be noted that expression of MDR1 was increased to much higher levels than that of CYP3A4 or CYP3A5; it is therefore not unlikely to assume that the levels of CYP3A-mediated metabolism were simply not high enough to compare with MDR1-mediated efflux. It might also be the case that CYP3A4 and MDR1 work in synergy to increase resistance. Indeed, it has been known for some time that GSTs and certain members of the MRP family work in synergy, that is, MRP1 can pump a GSH-conjugated drug easier than the drug alone. Consistent with this, transfection of both proteins confers a synergistic resistance that is larger than that expected based on the toxicity profiles resulting from the transfection of each protein alone. The multidrug resistance pump MDR1 and CYP3A4 have overlapping substrates and inducers; thus they appear to be connected in some way. Some studies show that MDR1 can pump out CYP3A4 metabolites (Hochman *et al.*, 2001), while others have indicated that the presence of MDR1 enhances CYP3A4 activity (Baron *et al.*, 2001; Chan *et al.*, 2004). The extent of this interaction and its potential impact in chemotherapy resistance have not been fully elucidated.

In summary, it appears that, upon exposure to toxic insults, tumour cells activate a number of defence mechanisms, including cytochromes P450, GSH-related enzymes and efflux pumps such as MDR1. This appears to be a general response, independent of the characteristics of the toxin (as exemplified by the increase in CYP3A and MDR1 expression caused by Cisplatin pulse selection in HepG2 cells), although not every single mechanism is induced for every drug (as seen in the lack of MRP1 involvement in some pulse-selected cells). MDR1 appears to be the main resistance mechanism for drugs that are substrates of this pump in pulse-selected cells, possibly due to the fact that this protein can pump considerable amounts of drug out of the cell in a short period of time and thus is more efficient than drug-metabolism enzymes in detoxification. However, CYP3A appears to be a second-line defence

mechanism against certain drugs for some cells, more relevant when MDR1 is inhibited.

Section 5.0

Conclusions and future work

5.1 Conclusions

The main aim of this thesis was to investigate the role played by drug metabolism enzymes in the development of resistance to anticancer drugs. With this purpose, a number of different approaches were taken, which aimed to show different aspects of the relationship between drug metabolism and drug resistance.

The first aim of this thesis, to find whether drug metabolism enzymes were expressed in tumour tissue and whether their expression was altered relative to normal tissue, was achieved through analysis of whole genome expression microarray data. Microarrays revealed that these enzymes were readily expressed in tumour tissue and that frequency and levels of expression were differentially altered in tumour as compared to normal samples.

The second aim of this thesis, to investigate the role of cytochromes P450 in anticancer drug resistance, was achieved by using different approaches. Experiments with microsomes showed that CYP3A4 and P450R are able to reduce the toxic effects of anticancer drugs, while the same could not be shown for CYP1B1. Transfection of cells with CYP3A4 and P450R revealed that overexpression of these enzymes affects the resistance profile of transfected cells. Transfection of CYP1B1 could not be accomplished, presumably due to the existence of intracellular regulatory molecules. Knock-down of CYP1B1 expression did show a new role for this enzyme, namely the promotion of cell survival.

The third aim of this project, to investigate changes in the expression of glutathione-related enzymes in pulse-selected cells, was achieved by Western blotting analysis. Changes were detected but could not be correlated to cell type, resistance to a certain drug or group of drugs or with levels of resistance.

The final aim of this project was to analyse the changes undergone by cancer cells after repeated exposure to anticancer drugs. Changes in the expression of drug metabolism enzymes and efflux pumps were detected, as were functional alterations resulting from these changes in pulse-selected cells.

Other important findings in this thesis are presented here as a summary.

1. Analysis of microarray data indicates that the mRNA of enzymes involved in drug metabolism is indeed expressed at significant levels in both normal and tumour tissue. Examination of these results also shows that the expression of several of these mRNAs is

altered in tumour as compared to normal tissue samples. Two genes of interest emerged from the microarray data obtained in this centre as possible markers of prognosis: ARTN2 and GST A1 were revealed as gene markers of potential clinical interest. ARNT2 was associated with better prognosis, while GST A1 expression correlated with poor survival.

2. Vincristine and especially Adriamycin were partially inactivated in a time-dependent fashion by incubation with the microsomal fraction of recombinant insect cells overexpressing CYP3A4; this effect was not observed with 5-fluorouracil, which unlike the other two drugs mentioned is not a P450 substrate.
3. CYP1B1 does not appear to directly inactivate any of the drugs tested, including Taxotere.
4. Transfection of CYP3A4 conferred modest (about 1.8-fold) levels of resistance against Adriamycin and, unexpectedly, 5-fluorouracil to lung epithelial cells. The increase in Adriamycin resistance can be explained by the inactivation of the drug by CYP3A4, since simultaneous treatment with a CYP3A inhibitor abrogated the decrease in sensitivity. The enhanced resistance to 5-fluorouracil developed by these cells is harder to explain; it does not appear to be due to a general increase in cell resistance as a consequence of the long-term exposure of cells to a selecting agent, for the toxicity of Cisplatin remained unaffected.
5. Simultaneous treatment of HL60 cells with 5-fluorouracil, Adriamycin, Taxol and Taxotere and a CYP1 inhibitor increased the cytotoxic effects of these drugs by about 20%. This appears to be a general, non-specific toxic effect, since it was observed with all of the drugs tested, even those that are not known to interact with P450s.
6. Treatment of HL60 cells with TCDD greatly induced the expression and activity of CYP1B1; however, this induction did not result in changes in the toxicity profile of these cells. This could be due to the diverse effects of TCDD on protein expression.
7. Expression and activity of CYP1B1 were significantly increased in MCF-7 cells pulse-selected with Taxotere as compared to parent cells. Knock down of CYP1B1 expression by siRNA did not have a major effect on Taxotere toxicity, but it did decrease cell viability (by

over 20%), suggesting that CYP1B1 expression can promote survival in MCF-7 Txt cells.

8. Transfection of CYP1B1 into three different breast cell lines was unsuccessful due to the apparent existence of regulatory controls that impede expression of CYP1B1 cDNA.

9. Mitomycin C and Adriamycin were inactivated by incubation with the microsomal fraction of recombinant insect cells overexpressing P450R; this effect was not observed with 5-fluorouracil or Vincristine.

10. MDA 231 cells transfected with P450R (MDA R4) were more sensitive to Mitomycin C, Adriamycin and, unexpectedly, 5-fluorouracil (about 3, 2 and 7-fold, respectively). It has long been known that Mitomycin C is activated by P450R to more toxic compounds, while Adriamycin metabolism by this enzyme produces alkylating agents that cause more damage than the parent compound; the latter reaction also generates ROS. MDA R4 cells appeared to be more sensitive to oxidative stress than their empty vector-transfected counterparts, providing a plausible explanation for their increased sensitivity to anticancer agents.

11. Alterations in the expression of GSH-related enzymes were common in a panel of pulse-selected cell lines. Taxol and Taxotere-selected Caco2 cells showed the most dramatic changes, with increased expression of GCSH, GCSL, GST P1 and GGTP, while Carboplatin and Taxol-selected SKLU-1 cells showed modest increases in the expression of GCSL, GGTP, GST P1 and GST A1. Changes in expression patterns were dependent on the drug used for pulse selection, although no clear correlation could be established between level of resistance to a particular drug and a particular enzyme.

12. Pulse selection of Caco2 and HepG2 cells with Taxol or Taxotere generated resistant variants that showed resistance to the drug they had been pulsed and also cross-resistance to taxanes and Vincristine. HepG2 cells pulse-selected with Cisplatin showed no appreciable change in their toxicity profile. Expression of CYP3A4 and CYP3A5 was modestly increased in these variants, as was that of GCSH, GGTP and GST P1, suggesting that cells have a general, non-specific response against toxic compounds, activating the

expression of several different genes involved in xenobiotic defence. MDR1 levels were dramatically increased.

13. Simultaneous treatment of parent and pulse-selected cells with Taxol and a CYP3A inhibitor did not enhance the toxicity of the anticancer agent, suggesting that CYP3A activity is not required to sustain the resistant phenotype.

14. Treatment of pulse-selected cells with Taxotere in the presence of an MDR1 inhibitor caused almost complete reversion of the observed resistance to this drug, indicating that cells depend on the activity of this efflux pump to resist the toxic effects of Taxotere.

15. Simultaneous treatment of pulse-selected cells with Taxol and an MDR1 inhibitor in the presence of a CYP3A inhibitor did increase Taxol toxicity by about 20% in Taxol-selected Caco2 cells, suggesting that drug inactivation by CYP3A is a second line of defence that might become relevant to these cells when MDR1 is inhibited. MDR1 therefore appears to be the main mechanism of resistance in taxane pulse-selected cells.

The study of drug metabolism in cancer and its possible contribution to chemotherapy resistance is complicated by the difficulty in finding a suitable model; this is especially true of cytochromes P450. As mentioned before, most commonly used cell lines have lost expression of P450s, to the point that no enzymatic activity or even protein expression can be detected. Induction of P450 expression by treatment with chemical compounds can increase P450 levels to values similar or equivalent to those found *in vivo*. However, the high redundancy of the P450 system means that chemical inducers are rarely specific. Also, pharmacodynamic effects other than cytochrome P450 induction exerted by these compounds cannot be ruled out. P450-transfected cell lines represent therefore the best model to study drug metabolism in cancer cell lines. Yet regulation of the expression of some P450 isoenzymes appears to be tightly controlled and this makes it difficult for these enzymes to be expressed in transfected cell lines. This difficulty in finding an appropriate model that accurately reflects *in vivo* events makes it hard to appreciate the real contribution of cytochromes P450 to drug resistance. Models available at the moment usually display P450 activities that are still several orders of magnitude below levels measured in liver or intestine microsomes. Yet it should be noted that these tissues are specialized in detoxification, particularly the liver, and that P450 activity values in other tissue types are very likely not as high.

Microsomes from recombinant insect cells transfected with cytochromes P450 and P450R are also good models to study drug metabolism *in vitro*. They display high levels of activity and are available commercially for most of the P450 isoenzymes with a known involvement in drug metabolism.

Drug metabolism enzymes appear to be involved in the development of anticancer drug resistance: their expression is generally increased in resistant cell lines, particularly in the case of P450s, and their transfection into drug-sensitive cells increases the levels of resistance. However, their involvement seems limited, as even when they are overexpressed they only appear to confer modest levels of resistance to tumour cell lines. The high-affinity, high-efficiency and low-capacity characteristics of the xenobiotic metabolism system cannot provide detoxification as effectively as efflux pumps can, so the level of resistance conferred by drug metabolism enzymes is much smaller than that conferred by efflux pumps.

Overall, it appears that individual enzymes involved in drug metabolism have little or no impact in the development of resistance against anticancer agents. It seems more likely that a general defence system is activated when cells are faced with environmental toxins, which includes several different types of drug metabolism enzymes as well as efflux pumps and antioxidant compounds; it is also likely that this system includes a number of antiapoptotic proteins as well.

A recent approach to enhance the efficacy of chemotherapy uses efflux pump inhibitors as adjuvants to decrease the amount of drug that is being pumped out of the cell. Inhibition of P450s or GSTs could also increase intratumoral drug levels and thus amplify the efficacy of treatment; indeed such an approach has already been tested by using Ketoconazole, a well-known P450 inhibitor, in conjunction with anticancer drugs. However, because these enzymes are responsible for detoxification, this would also increase the general toxic effects of the drug; for drugs with such a narrow therapeutic window as anticancer drugs, co-administration with inhibitors of their metabolism could be highly dangerous or indeed fatal. It is therefore unlikely that P450 or GST inhibitors will gain widespread use as chemotherapy adjuvants.

In spite of a number of disparities, most studies report increased expression of enzymes involved in drug metabolism in tumour as compared to normal tissue, indicating that these enzymes are either involved in malignant transformation or serve as markers of the carcinogenic process. Xenobiotic-related enzymes could then be useful as biomarkers of disease, progression and also therapy outcome, as has been previously shown. The existence of tumour-specific isoforms is particularly promising, for they would be excellent markers of malignancy. The existence of these tumour-specific enzymes would also be used for the design of pro-drugs that can be

specifically activated at the site of the tumour, thereby reducing toxicity and side effects

To conclude, it should be noted that cancer is a multifactorial disease and this explains its many features The fact that a particular molecule or pathway might be relevant to the development of resistance to anticancer drugs does not necessarily mean that this sole molecule or pathway is responsible for the cell's fate It is the interaction of complex regulatory pathways, rather than the contribution of single molecules, that will eventually lead cells to express a certain phenotype In this context, drug metabolism enzymes can certainly confer tumour cells a survival advantage, but they are only one of many factors that will influence the cell's fate in the balance between death and survival

5 2 Future work

In order to complete the work on microarrays, real time PCR (qRT-PCR) analysis will be carried out on the breast samples to validate the microarray results

Transfection of cell lines of different origin with CYP3A4 cDNA would provide new models to study the effect of this enzyme on 5-fluorouracil-induced toxicity These models could provide an explanation for the increased resistance to 5-fluorouracil observed in BCMV 3A4 cells BCMV cells, on the other hand, could also provide a useful model to study CYP3A4 and MDR1 interactions and possible cooperation in the development of resistance BCMV Neo and BCMV 3A4 could be transfected with an MDR1 plasmid and toxicity assays performed on BCMV MDR1, BCMV 3A4 and BCMV 3A4-MDR1 to analyse the contribution of each gene to drug resistance and possible synergistic effects

Expression of CYP1B1 is regulated by methylation in prostate cell lines and treatment with the methylase inhibitor 5-aza-2'-deoxycytidine (AzaC) can induce mRNA expression in these cells (Tokizane *et al* , 2005) The panel of breast cell lines used to study CYP1B1 mRNA expression could be treated with AzaC to investigate whether CYP1B1 expression is also regulated by methylation in breast cell lines

CYP1B1 siRNA transfection was toxic to MCF-7 Txt cells, transfection could also be carried out in different cell lines, derived from breast and also other hormone-responsive tissues such as ovary, to find out whether this is a general effect Also, toxicity profiles of CYP1B1 inhibitors such as α-napthoflavone in MCF-7 Txt and also other cell lines would expand our knowledge of the toxic effects of CYP1B1 inhibition in cancer cell lines

It appears that Taxotere can induce CYP1B1 mRNA expression, this could be confirmed by transfecting cells with a plasmid containing the luciferase gene under the control of the CYP1B1 promoter Treatment of transfected cells with Taxotere would then confirm whether this drug can directly induce the expression of this enzyme It would also be interesting to investigate wheter Taxotere has an effect on CYP1A1 and CYP1A2 expression Breast cells could also be incubated with Taxotere and other anticancer drugs in the presence of exogenous recombinant CYP1B1 to find out whether exogenously generated metabolites of this enzyme can promote cell survival

Overexpression of P450R can sensitise cells against anticancer drugs such as Adriamycin and 5-fluorouracil Combination assays with a P450R inhibitor such as diphenyliodonium chloride on P450R-expressing cells would reveal more about the relevance of this enzyme in drug resistance Also, ferredoxin reductase was reported as an essential gene for survival, transfection of cells with ferredoxin reductase siRNA caused cell death (Hwang *et al*, 2001) Transfection of P450R-expressing cells HepG2 with P450R siRNA could reveal whether the same is true of P450R

ROS production is increased in MDA R4 cells as compared to MDA EV cells after treatment with Mitomycin C, Adriamycin and 5-fluorouracil The study of ROS production after treatment with other drugs such as Taxotere or Vincristine would reveal more about ROS generation in response to anticancer drugs

Section 6.0

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