Enhancement of the Cytotoxicity of MRP Substrates by Indomethacin and Related Compounds

A thesis submitted for the degree of PhD

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 PhD, 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

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Certain non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin and sulindac, at non-toxic concentrations, were found to enhance the toxicity of a range of chemotherapeutic drugs, such as doxorubicin, epirubicin, vincristine and VP-16. This effect appeared to be most significant in MRP-expressing cell lines such as DLKP and A549, and was not evident in Pgp-overexpressing cell lines such as DLKPA. Analogues of indomethacin were subsequently generated to investigate the structure-activity relationship (SAR) of indomethacin-mediated toxicity enhancement. An important goal of this research was to identify an analogue of indomethacin, capable of potentiating the toxicity of anticancer drugs to the same degree as indomethacin but without the toxic side effects observed after prolonged use of indomethacin. It is believed that these side effects are mediated through inhibition of the constitutively expressed form of the Cyclooxygenase enzyme, Cyclooxygenase-1 (COX-1). A number the positive indomethacin analogues (BRI 138/1, BRI 153/1 and BRI 60/1) were found to have the ability to potentiate the toxicity of a number of anticancer drugs while having little or no COX-1 inhibitory activity rendering these compounds less likely to cause gastrointestinal toxicity. BRI 60/1 was also found to be a good COX-2 inhibitor. These results for BRI 60/1 suggest a potential clinical application due to reduced toxic side effects and in addition, increased ability as a tumour suppresser due to inhibition of COX-2.

Most of the active indomethacin analogues were found to have very little Glutathione S-transferase inhibitory activity and hence their mode of action was not by inhibiting the conjugation of glutathione to the anticancer drug. Inside-out Membrane Vesicles (IOVs) were utilised to demonstrate the ability of the analogues to directly inhibit the MRP pump by measuring the uptake of

the MRP substrate, LTC_4 in to the vesicle. Surprisingly, BRI 138/1, which was quite active in the combination toxicity assay, was a weak inhibitor of LTC_4 transport as compared to indomethacin and other positive indomethacin analogues suggesting, due to structural variations, reduced ability of BRI 138/1 to bind to the active site on the MRP molecule and compete with LTC_4.

Results from drug efflux studies suggested that the active NSAIDs are competitive substrates for MRP1. Several of these analogues are as effective as indomethacin at potentiating the toxicity of certain anticancer drugs but some are less potent (on a molar basis) than indomethacin. An analogue of indomethacin (e.g. BRI 138/1, BRI 153/1, BRI 60/1) with similar potentiation ability, but without the side effects caused by the inhibition of COX-1, may be a promising candidate for future cancer therapy.

The ability of indomethacin, indomethacin analogues and sulindac to potentiate the toxicity of chemotherapeutic drugs in cell lines expressing MRP2-6 has not previously been investigated. The results from the combination toxicity assays in the ovarian carcinoma cell line, 2008, transfected with MRP2 or MRP3, suggest that indomethacin may have the ability to potentiate adriamycin toxicity in both 2008 MRP2 and MRP3. However, a basal level of MRP1was found in all the 2008 cell lines which makes it difficult to distinguish if the potentiation was simply as a result of the expression of MRP1 in the cells. The toxicity of methotrexate was not potentiated in the 2008 MRP2-transfected cell line (which is MTX-resistant in short-term toxicity assays) suggesting that indomethacin is not active in MRP2-overexpressing cell lines. In contrast, sulindac had a small, but significant, potentiation effect on methotrexate in the 2008 MRP2 cells.

The toxicity of taxol and taxotere was potentiated by indomethacin and sulindac in a number of cell lines and the effect appears to be MRP-related. However, the synergy between piroxicam (which was unable to enhance the toxicity of other MRP1 substrate chemotherapeutic drugs) and taxol suggests an alternative or additional mechanism of taxane toxicity enhancement may also be present. Enhancement of taxol and taxotere toxicity was not observed in A549 cells which overexpress MRP1 but were also found to highly overexpress MRP4.

In contrast, the toxicity of cisplatin was decreased in the presence of indomethacin in a number of cell lines including DLKP, DLKPC 14, HepG2 and the 2008 cell lines. BRI 138/1 did not potentiate the toxicity of cisplatin but the effect was additive not antagonistic. Therefore, it is possible that the antagonistic effect on cisplatin toxicity is indomethacin specific. Perhaps indomethacin actually enhances the efflux (or inhibits efflux) of certain anticancer drugs, including cisplatin, from particular cancer cell lines.

Pulse selecting DLKP cells with 300μg/ml indomethacin increased the resistance of the cells to adriamycin, vincristine, VP-16, cisplatin, indomethacin and, in particular, 5-FU. RT-PCR analysis demonstrated an increase in MRP1, 2 and 4 mRNA expression in the pulsed cells relative to the parental DLKP cell line.

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1.0 Introduction

1.1 Multidrug resistance in cancer

Multidrug resistance (MDR) is a major impediment to the success of cancer chemotherapy. MDR may be observed in primary therapy (inherent resistance) or be acquired during or after treatment (acquired resistance) (Yu *et al.,* 1999). *In vitro*, MDR can appear after exposure of cells to a single drug, which is almost always a natural product, and is characterised by resistance to structurally unrelated compounds with different subcellular targets (Lautier *et al.,* 1996). There are numerous mechanisms of drug resistance observed in clinical settings and the spectrum of drugs to which resistance may be conferred is very broad and encompasses all classes of chemotherapeutic agents including alkylating agents, antimetabolites, hormones, platinum containing drugs and natural products (Grant *et al.,* 1994).

One major mechanism of such resistance is linked to decreased cellular accumulation of anticancer drugs through enhanced cellular efflux of the anti-tumour compounds. Such multidrug resistance can be conferred *in vivo* and *in vitro*, by a number of proteins including the M_r 170,000 P-glycoprotein (encoded by the *MDR*1 gene) (Gottesman *et al.,* 1993), and the more recently identified 190kDa MRP (Cole *et al.,* 1992 and Grant *et al.,* 1994). Both of these proteins are members of the ABC (ATP-binding cassette) transporter family and function as ATP-dependent active transporters (Sarkadi *et al.,* 1997).

Both proteins have been demonstrated to contain two functional nucleotide binding domains connected to large membrane-bound regions (Higgins, 1992; Hipfner *et al.,* 1997 and 1999). It has been established that both proteins can transport a wide variety of chemically unrelated, large, amphiphilic, uncharged or slightly positively charged molecules in an ATP-dependent fashion (Sarkadi *et al.,* 1997), while MRP also transports some negatively charged compounds, especially glutathione conjugates (Loe *et al.,* 1996b). The compounds most frequently applied in cancer chemotherapy but effectively extruded by MDR1 and MRP from the tumour cells are of natural and semi-synthetic origin. These include the anthracyclines (e.g. adriamycin, daunorubicin and idarubicin), mitoxantrone, *vinca* alkaloids (vincristine, vinblastine), epipodophyllotoxins (e.g. VP-16), the taxanes (e.g., taxol and taxotere) and actinomycin D (Hipfner *et al.,* 1999).

Pgp is localised mainly in the plasma membrane and confers drug resistance by functioning as an ATP-dependent drug efflux pump which is able to transport anticancer drugs against a substrate concentration gradient, causing both decreased drug uptake and increased drug efflux (Germann *et al.,* 1997). The basis for MRP-mediated drug resistance is less well defined. In addition to drug accumulation defects, differences in subcellular drug distribution have been observed in a number of drug-selected MRP-expressing cell lines or MRP cDNA-transfected cells (Cole *et al.,* 1992; Zaman *et al.,* 1993; Loe *et al.,* 1996 and Varsenvoort *et al.,* 1995). Therefore, MRP may also function by sequestering some of its substrates in intracellular membrane compartments.

It is generally believed that the preferential development of multidrug resistance is dependent upon the types of tumour and drugs used (Choi *et al.,* 1999). The authors have also suggested that the overexpression of MRP or Pgp is dependent upon drug concentrations and reported that in experiments with adriamycin in acute myelogenous leukaemia, MRP was first expressed with exposure to low concentrations of adriamycin followed by an overexpression of Pgp by increasing the drug concentration.

Other biochemical mechanisms associated with acquired resistance to broad groups of cytotoxic drugs in laboratory systems include overexpression of the human major vault protein, LRP (Izquierdo *et al.,* 1996a), cytosolic detoxification due to overexpression of glutathione S-transferase and/or related enzymes (resulting in resistance to anthracyclines and alkylating agents including cisplatin) and nuclear changes including modified activity of topoisomerase II (resulting in resistance to anthracyclines and epipodophyllotoxins) (Twentyman *et al.,* 1997). In addition other biochemical determinants, including mutations of the p53 gene or overexpression of the bcl-2 gene product have been associated with protecting the cell against programmed cell death or apoptosis (Twentyman *et al.,* 1997).

The ultimate goal of MDR research is to improve treatment outcome in patients with cancer by devising strategies that are able to prevent the emergence of MDR or to circumvent existing resistance. It appears that drug resistance in many cancers is multifactoral (Larsen *et al.,* 2000 and Lehnert, 1996), and modulating a single resistance mechanism, such as MRP, may not sufficient in several cancer types. However, in a number of cancers, where a particular mechanism is the dominant factor for clinical MDR, and has been shown to be of prognostic

significance, modulation of a single resistance mechanism could prove to have a significant impact on treatment outcome.

1.1.1 Multidrug transporters from bacteria to man.

Toxic compounds have always been part of the natural environment in which all organisms dwell. The development of strategies for life in this habitat has been crucial for survival of the cell. As a result organisms, from bacteria to mammalian cells, have developed versatile mechanisms to resist the effects of xenobiotics, including antibiotics and other cytotoxic drugs. Examples of such mechanisms include the enzymatic degradation or inactivation of drugs and the alteration of drug targets. In addition, many cells have been shown to possess membrane proteins which can actively efflux drugs, and hence, are able to overcome cell toxicity by lowering the cytoplasmic drug concentration.

Some of these drug transporters are specific for a given drug or class of drugs, but the multidrug transporters have specificity for compounds with very different chemical structures and cellular targets (van Veen *et al.,* 1997). These transport systems can be divided into two main groups on the basis of bioenergetic and structural criteria: **1**. Secondary transporters, which mediate the extrusion of drugs from the cell in a coupled exchange with ions (Paulsen *et al.,* 1996), and **2**. ATP-binding cassette [ABC] transporters, which utilise the release of phosphate bond-energy by ATP hydrolysis to pump drugs out of the cell (Higgins, 1992).

1.2 Secondary multidrug transporters

The study of multidrug transporters is rapidly developing. Secondary multidrug transporters belong to one of three distinct families of transport proteins: The Major Facilitator Superfamily (MFS), The Resistance-Nodulation-Cell division (RND) family, and the Small Multidrug Resistance (SMR) family (Ma *et al.,* 1994; Saier *et al.,* 1998; Williams, 1996) as described in Table 1.2.1.

Table 1.2.1: Properties of transport protein families that include multidrug resistance (MDR) efflux pumps.

A. <u>The ATP binding cassette (ABC) superfamily</u>

1. 28 families specific for sugars, amino acids, ions, drugs, antibiotics, vitamins, iron complexes, peptides, proteins, complex carbohydrates etc.
2. Driven by ATP hydrolysis.
3. Multicomponent, multidomain systems; total size, > 1000 residues; Usually 12 (6+6) membrane spanning domains.
4. Found in bacteria, archaea, and eukaryotes.
5. >500 sequenced members.
6. Well characterised members: MalEFGK, MDR1, MRP1, and CFTR.
7. Drug efflux pumps are found in a few (3 - 4) of the many (about 3 dozen) recognised families.

B. <u>The major facilitator superfamily (MFS)</u>

1. 17 families specific for sugars, drugs, metabolites, anions etc.
2. Consists of symporters, antiporters and uniporters.
3. Size, ~400 residues or larger; 12 or 14 membrane spanning domains.
4. Found in bacteria, archaea and eukaryotes.
5. Approx. 500 sequenced members.
6. Well characterised members: LacY, Glut1 and TetB.
7. MDR pumps are found in 3 of the 17 recognised families.

C. <u>The small multidrug resistance (SMR) family</u>

1. Two subfamilies specific for drugs and unknown substrates, respectively
2. Catalyze drug:H^+ antiport.
3. Probably homooligomeric; subunit size ~100 residues with 4 spanners.
4. Found only in prokaryotes.
5. 16 sequenced members.
6. Well characterised members: Smr and EmrE.
7. MDR pumps are found in one of the two subfamilies.

D. <u>The resistance/nodulation/division (RND) family</u>

1. Three subfamilies specific for drugs, metal ions and lipopolysaccharides respectively.
2. Catalyze drug:H^+ antiport.
3. Subunit size ~1000 residues; 12 spanners.
4. Found only in prokaryotes.
5. 16 sequenced members.
6. Well characterised members: AcrB and MexB.
7. MDR pumps are found in one of three subfamilies.

(Table taken from Saier *et al.*, (1998))

Secondary multidrug transporters have been detected in rat kidney cells (OCT1) (Grundeman *et al.*, 1994), and synaptic vesicles of presynaptic neurons (VMAT) (Schuldiner *et al.*, 1995), in pathogenic yeasts such as *Candida albicans* (caMDR1p) (Ben-Yaacov *et al.*, 1994; Walsh *et al.*, 1997), and pathogenic bacteria, such as methicillin resistant *Staphylococcus aureus* (QacC) (Williams, 1996), *Mycobacterium smegmatis* (LfrA), *Neisseria gonorrhoeae* (MtrD) (van Veen *et al.*, 1997), *L. lactis* (LmrP) and *Pseudomonas aeruginosa* (MexB) (Nikaido *et al.*, 1994). Some of the most common secondary multidrug transporters are listed in Table 1.2.2.

Table 1.2.2: Examples of most common secondary multidrug transporters.

Transporter	Organism	Gene location	Substrate
Major facilitator in Gram Positive Bacteria			
OtrB	*Streptomyces rimosus*	Chromosome	Oxytetracycline
Tel(L)	Various cocci, *Bacillus subtilis*	Plasmid	Tetracycline
Mmr	*Streptomyces coelicolor*	Chromosome	Methylenomycin
ActII	*Streptomyces coelicolor*	Chromosome	Actinorhodin
TcmA	*Streptomyces glaucescens*	Chromosome	Tetracinomycin
NorA	*Staphylococcus aureus*	Chromosomal	Fluoroquinolones, Basic dyes, puromycin chloramphenicol, tetraphenylphosphonium.
QacA*	*Staphylococcus aureus*	Plasmid	Quarternary ammonium compounds
Bmr	*Bacillus subtilis*	Chromosome	Basic dyes, chloramphenicol puromycin, fluoroquinolones.
Major facilitator in Gram Negative Bacteria			
TetA	*Eschericia coli*	Plasmid	Tetracycline
CmlA	*Pseudomonas aeruginosa*	Plasmid	Chloramphenicol
Bcr	*Eschericia coli*	Chromosome	Bicyclomycin
EmrB	*Eschericia coli*	Chromosome	CCCP**, nalidixic acid, tetrachlorosalicyanilide, phenylmercury acetate.
EmrD	*Eschericia coli*	Chromosome	CCCP**, phenylmercury acetate.

Transporter	Organism	Gene location	Substrate
RND family			
AcrE (AcrB)‡	*Eschericia coli*	Chromosome	Basic dyes, SDS,† erythromycin, novobiocin, fusidic acid, tetracycline, mitomycin C.
EnvD	*Eschericia coli*	Chromosome	Basic dyes, SDS,† erythromycin, fusidic acid, tetracycline, mitomycin C and others.
MexB	*P.areuginosa*	Chromosome	Tetracycline, chloramphenicol, , fluoroquinolones β-lactams, pyoverdine.
Smr family			
Smr(QacC)‡	S.aureus	Plasmid	Quarternary ammonium compounds, basic dyes.
QacE	Klebsiella aerogenes	Plasmid	Quarternary ammonium compounds, basic dyes.
MvrC(EmrE)‡	E.coli	Chromosome	basic dyes. Methyltriphenyl- -phosphonium

* QacB is very similar to QacA; ** CCCP, carbonyl cyanide m-chlorophenyl-hydrazone;

† SDS, sodium dodecylsulfate;

‡ alternative names are shown in parentheses;

(Table taken from Nikaido *et al.,* (1994).)

Members of the MFS and SMR families tend to have specificity for an exceptionally wide range of amphiphilic, cationic drugs including several types of antibiotics, quaternary ammonium compounds, aromatic dyes, and phosphonium ions. Transporters in the RND family tend to confer resistance to amphiphilic

anionic compounds such as antibiotics, basic dyes and detergents. In Gram negative bacteria, transport of drugs from the interior of the cell to the external medium requires the translocation of solutes across the cytoplasmic outer membrane. Therefore, there are a number of drug transporters (e.g., MexB) in such organisms, which are found in association with an accessory protein, which spans the periplasmic sphere and interacts with a porin in the outer membrane. These auxiliary proteins belong to the Membrane Fusion Protein family and Outer Membrane Factor family (van Veen *et al.,* 1997).

1.3 ABC Transporters

The ATP-binding cassette (ABC) transporters, present in organisms ranging from bacteria to man, are involved in the ATP-dependent transport of a wide variety of compounds, ranging from inorganic ions to large polypeptides (Tusnady *et al.,* 1997), and are involved in the tolerance to a wide diversity of cytotoxic agents in both prokaryotes and eukaryotes (Higgins, 1992).

In most cases these proteins have been demonstrated to function as transport ATPases, that is, hydrolysing ATP in conjunction with transporting substrate molecules through cellular or intracellular membranes. Included in this transport family are the human multidrug resistance P-glycoprotein, MDR1 (Pgp) (Gottesman *et al.,* 1993), and human multidrug resistance associated protein MRP1 (MRP) (Cole *et al.,* 1992). Both are plasma membrane transporters which catalyse the extrusion of anti-tumour drugs from drug-exposed cancer cells. Other characteristic eukaryotic ABC transporters include the yeast pheromone transporter, STE6, LtPgpA (a *Leishmania tarentolae* protein) and the human cystic fibrosis transmembrane conductance regulator (CFTR), the mutations of which are causative of cystic fibrosis.

The ABC transporters, form one of the largest known protein families. They are built from combinations of conserved domains, that is, ATP- (nucleotide-) binding ABC units and characteristic membrane bound regions (Varadi *et al.,* 1998). The ABC units contain the highly conserved 'Walker A' and 'Walker B'

sequences, most probably directly responsible for the interaction with ATP or other nucleotides. These Walker sequences are separated by a stretch of about 120-170 amino acids, including a short peptide motif, 12-13 amino acids in length, called the ABC transporter 'signature' region, which is in fact characteristic for these proteins. The membrane bound domains of the ABC transporters in most cases are predicted to contain six transmembrane (TM) helices. In some bacterial and in all eukaryotic ABC transporters at least one ABC unit and one transmembrane domain are encoded by a single gene (Tusnady *et al.*, 1997). They may exist as a single unit or be fused, as in the case of Pgp, so that they contain 2 non-identical sets of transmembrane domains and nucleotide binding sites (Higgins *et al.*, 1997).

1.4 Pgp, LRP and MRP in Multidrug Resistance

Remarkable advances in cancer treatment have been made since the introduction by Farber and associates, in 1948, of the folate antagonists for the treatment of leukemia (Farber *et al.*, 1948).

During the last few decades, new cytotoxic agents have been developed that have improved the outcome of many cancers, including solid tumours. Among these drugs, the natural product isolates and their derivatives - including anthracyclines, vinca alkaloids, epipodophyllotoxins, and taxanes - have proved to be effective in inducing remissions and cures in many malignancies (Beck *et al.*, 1997).

The development of resistance to multiple drugs used in cancer chemotherapy is a serious limitation to this form of treatment and is considered to be one of the most significant obstacle to curing cancer (Hipfner *et al.*, 1999 and deVita, 1989). *In vitro*, broad resistance to multiple structurally and functionally unrelated drugs is observed intrinsically or after intermittent or prolonged exposure of tumour cells to only a single agent. This "classical" multidrug resistance is typically characterised by cross-resistance to four classes of commonly used natural product drugs, the anthracyclines, *Vinca* alkaloids, taxanes and epipdophyllotoxins (Hipfner *et al.*, 1999). Elucidation of the mechanisms determining inherent or chemotherapy - induced resistance in human tumours to

many cancer agents, is of great interest to researchers and of great importance to patients.

The nature of drug resistance is complex. To date, multidrug resistance in model systems is known to be conferred by two different integral membrane proteins, the 170kDa P-glycoprotein (Pgp) (Riordan *et al.,* 1985), and the 190-kDa multidrug resistance-associated protein (MRP) (Cole *et al.,* 1992). These proteins belong to the ATP binding cassette (ABC) (Higgins, 1992), or traffic ATPase superfamily of transport proteins (Ames, 1992).

In 1993, Scheper *et al.,* identified a 110kDa ATP-dependent cytoplasmic transporter protein in a number of non-P-glycoprotein-mediated MDR tumour cell lines, normal cells and tissues by the use of a p110-specific monoclonal antibody LRP-56. This protein, known as the Lung Resistance Protein (LRP), was identified by Scheper *et al.,* (1995), as the major vault protein. Isquierdo *et al.,* (1996a), describes these vaults as cellular organelles in search of a function. They are complex ribonucleoprotein particles containing at least three minor proteins and a small RNA molecule in addition to the 110-kDa major vault protein. Vaults are mainly located in the cytoplasm and current speculation sees vaults as part of the bidirectional transport system between nucleus and cytoplasm (Borst *et al.,* 1997). Izquierdo *et al.,* (1996), have also suggested that vaults might somehow be involved in the transport of drugs into cytoplasmic vesicles or directly out of the cell, but, at present, there is no evidence to back this suggestion.

1.5 P-glycoprotein (Pgp)

Over-expression of the transmembrane transport protein, P-glycoprotein, (P-gp), has been detected in many multidrug - resistant tumour cell lines and in a variety of tumours from patients with both acquired and inherent drug resistance. This protein, encoded by the human MDR1 gene, is a 170 kDa membrane protein and from its structure, appears to be a member of the ABC family of transporters (Gottesman *et al.,* 1993). It was originally found on the surface of multidrug resistant tumour cells. *In vitro* studies have shown that it confers resistance to a range of natural product xenobiotics that are used as chemotherapeutic drugs

(Vinca alkaloids, anthracyclines, etoposide, taxol), calcium channel blockers (verapamil, diltiazem, azidopine), immuno- modulators (cyclosporin A, FK-506), cardiac glycoside (digoxin), fluorescent dyes (rhodamine 123, fluo-3), and steroids (cortisol, aldosterone) (Naito M *et al.,* 1998; Clynes *et al.,* 1993 and Sharom *et al.,* 1999).

The basis of this MDR appears to be a decrease in drug accumulation in the cells due to increased expulsion of drug from the cytosol by this transmembrane transport protein. It is believed that Pgp is a transmembrane pore forming protein with an energy dependent drug effluxing ability. It has also been suggested that Pgp can lower intracellular drug concentrations by regulating the plasma membrane pH gradient and electrical membrane potential (Roepe *et al.,* 1994). Three classes of Pgp gene products exist; the class I and II isoforms are multidrug transporters, whereas the class III isoform appears to be a lipid flippase, moving phosphatidylcholine (PC) from the inner to the outer leaflet of the bile canalicular plasma membrane (Sharom *et al.,* 1999). Photoaffinity labelling experiments with a photoactive analogue of vinblastine have demonstrated the ability of Pgp to bind cytotoxic drugs directly. Mutations in critical transmembrane regions can interfere with this binding (Cornwell *et al.,* 1986). Plasma membrane vesicles from Pgp- overexpressing cells have the ability to transport [^{3}H]-labelled drug in an ATP-dependent manner (Horio *et al.,* 1988).

Pgp has been identified not only in tumour cells *in vitro,* but also in a number of different types of cancers where, in a number of cases, it has been shown to be associated with the clinical manifestations of resistance (Barrand *et al.,* 1997). In many cases, Pgp-expression in tumours has been shown to be associated with a poor therapeutic prognosis (Fisher and Sikic, 1995).

1.5.2 Structure of the gene encoding P-glycoprotein

The Pgp protein in humans is encoded by the MDR gene, which is present on the long arm of chromosome 7, band q21.1 (Chen *et al.*, 1986). This gene possesses 27 exons in the protein coding region of the gene with 14 of these exons coding for the left and 13 for the right half of the Pgp molecule (Roninson, 1991). Two promoter regions for this gene have been identified, one of which is upstream and the other is downstream (Ueda *et al.*, 1987a, b). The majority of resistant cells *in vitro* preferentially use the downstream promoter (Ueda *et al.*, 1997).

1.5.3 P-glycoprotein protein structure

The mdr 1 gene product is a 170kDa membrane-associated protein of 1280 amino acids containing 12 transmembrane domains with 6 extracellular loops and two cytoplasmic ATP domains (Gros *et al.*, 1986). Pgp is expressed at the apical surface of intestinal epithelium, where it is responsible for the low bioavailability of many drugs in the gut, and in the endothelial cells of capillaries in the brain, where it is a major contributor to the blood-brain barrier (Sharom *et al.*, 1999). The Pgp protein is predominately localised in the plasma membrane of MDR cells (Isquierdo, 1996a), but low levels of expression have been shown to be present in association with the endoplasmic reticulum and Golgi apparatus (Germann, 1996).

P-glycoprotein is a phosphorylated glycoprotein. Increased phosphorylation of P-glycoprotein has been shown to cause an increase in Pgp-mediated drug efflux activity whilst inhibition of phophorylation decreases this activity (Chambers *et al.*, 1990). It is now believed that phophorylation may have an indirect role in certain aspects of Pgp activity including the kinetics of drug transport, Pgp protein stability and drug resistance pattern of Pgp-overexpressing cells (Elliott, 1998).

1.6 Multidrug Resistance Protein – MRP

A number of human tumour cell lines show decreased cellular accumulation and increased resistance to drugs associated with the MDR phenotype, but do not possess Pgp (Twentyman *et al.*, 1996). A novel 96-kDa membrane protein has been characterised in the human breast cancer MCF-7/AdrVp subline, which does not over-express Pgp. This protein has been correlated with drug resistance to doxorubicin (Chen *et al.*, 1990). Similarly, a 42-kDa and 85-kDa membrane bound protein have been identified by a polyclonal antibody against the putative ATP binding domain of Pgp in MCF/MX cells selected by mitoxantrone. These cells are cross resistant to doxorubicin and etoposide but without Pgp-over expression. (Nakagawa *et al.*, 1992).

It was known that members of the ATP-dependent family of transporters shared certain structural and sequence homologues and that, particularly in the region of the nucleotide binding domains, there were highly conserved amino-acid sequences. Marquardt *et al.*, (1990), raised antibodies to a number of different sequences along the length of the Pgp molecule and found that one particular antibody, (ASP14), raised against one of these highly conserved domains was able to recognise a protein, over-expressed in certain of these non-Pgp cell lines (Barrand *et al.*, 1997).

While searching for such alternative molecules, many investigators analysed the HL60 human leukaemia cell line and its drug-selected resistant variants. It was discovered that HL60 cells selected for resistance to doxorubicin exhibited MDR but did not contain detectable levels of P-glycoprotein (McGrath, 1987). Comparison of membrane proteins from the HL60 and HL60/ADR cell lines, using antibodies raised against synthetic peptides derived from P-glycoprotein, revealed the presence of a 190-kDa ATP-binding protein that was primarily localised in the endoplasmic reticulum of the HL60/ADR cells. This protein was not detectable in the membranes of parental cells and could easily bind 8-azido[alpha-^{32}P] ATP (Marquardt *et al.*, 1990).

Investigators proceeded to study gene expression in various cell systems to further characterise this unique 190kDa protein. One of the most extensively

characterised non-Pgp multidrug resistant cell line is H69AR, a small cell lung cancer cell line which was derived from the drug-sensitive parental H69 line by stepwise selection in doxorubicin (Mirski *et al.*, 1987). H69AR displayed a drug resistance pattern typical of that associated with increased amounts of Pgp. The cells displayed moderately high levels of resistance (10-100-fold) to the *Vinca* alkaloids, epipdophyllotoxins, doxorubicin and mitoxantrone. In addition, these cells were highly resistant to daunorubicin, epirubicin and colchicine (Mirski *et al.*, 1987 and Cole *et al.*, 1992). A number of biochemical alterations occurred during selection, including reduced levels of DNA topoisomerase II α and β (Giaccone *et al.*, 1992), increased expression of annexin II, a decrease in reduced glutathione (GSH), and altered levels of GSH-associated drug detoxification enzymes. However, as with most clinical specimens of SCLC, H69AR does not over-express Pgp protein or its cognate mRNA. Consistent with this observation, major differences in net drug accumulation or efflux did not appear to be part of the resistant phenotype of the H69AR cell line (Cleary, 1995). Another feature that distinguishes H69AR from cell lines that over-express Pgp is the inability of cyclosporin A and several other chemosensitising agents to reverse doxorubicin (Cole *et al.*, 1992).

As a result of these findings, a search was undertaken for evidence of other alterations in gene expression using a differential hybridisation approach to identify mRNA species that are over-expressed in H69AR relative to those present in parental H69 cells. Using this technique a 7.8 - 8.2kb mRNA was identified, which was expressed in the resistant cell lines at levels approximately 100 - 200 fold higher rate than the sensitive parental cell line. Sequencing of cDNA clones derived from this mRNA revealed that it had the potential to encode a 1531 amino acid protein that was predicted to be a member of the ABC transporter superfamily and was subsequently named the multidrug resistance protein, or MRP (Loe *et al.*, 1996). This protein is now recognised as MRP1 (Hipfner *et al.*, 1999).

Though MRP1 and Pgp both belong to the ABC transporter family, they share only 15% amino acid homogeneity (Cole *et al.*, 1992). Nevertheless, both proteins confer resistance to a broad range of cytotoxic xenobiotics including doxorubicin, vincristine and VP-16 (etoposide), drugs that are widely used in the treatment of

many human cancers. However, growing evidence suggests that the mechanisms by which MRP1 and Pgp reduce cellular drug accumulation are not the same, indicating that there are major differences in the drug protein interactions of these two molecules (Loe *et al.*, 1996).

1.6.1 Homologues of MRP

When the sequence of MRP was first analysed in 1992, the most closely related members of the ABC superfamily were the CFTR chloride channel (19% identity) and LtPgpA (30% identity) (Cole *et al.*, 1992). The first transport protein to be recognised as a member of the MRP family was cMOAT (Taniguchi *et al.*, 1996). The authors specifically targeted the ATP-binding domain conserved in MDR1, MRP, and CFTR genes and compared the difference in mRNA from both cisplatin resistant and sensitive cells. They isolated a full length human cMOAT cDNA which was highly homologous to rat cMOAT and found that human cMOAT was a homologue of MRP. Sequencing of cMOAT revealed an open reading frame coding for 1545 amino acids that showed 46% similarity to that of human MRP. Taniguchi *et al.*,(1996), estimated the size of cMOAT to be approx. 4.5kb, similar to that of MRP mRNA but larger than the human MDR 1 mRNA.
Since this time many proteins more closely related to MRP1 have been identified in a wide variety of eukaryotic organisms, ranging from plants and yeast to mammals (Klein *et al.*, 1999). The Yeast family contains one well characterised MRP homologue, the Yeast Cadmium Resistance Factor, YCFI, shown by Li *et al.*, (1996), to be a vacuolar GS-X pump. YCFI mutants can be complemented by MRP1 (Borst *et al.*, 1997). At least 4 MRP homologues are expressed in *Caenorhabditis elegans* (Kool *et al.*, 1997), and it was therefore possible that the human MRP gene family would have more than two members. To date five human MRP1-related proteins, designated MRP2, MRP3, MRP4, MRP5 and MRP6 have been discribed (Kool *et al.*, 1997; Taniguchi *et al.*, 1996; Paulusma *et al.*, 1996; Konig *et al.*, 1999; Kool *et al.*, 1999a and 1999b and Kiuchi *et al.*, 1998). A search of the human Expressed Sequence Tag (EST) database by Allikmets *et al.*, (1996), yielded 21 new ABC genes, including genes for transporters related to MRP1. In an independent search, Kool *et al.*, (1997), found

four transporters related to MRP1 and cMOAT and analysed the expression of these genes, called MRP3-6, in normal tissues and tumour cell lines. A fifth homologue of MRP1 was also identified as the human SUR (sulfonyl urea receptor) gene. The percentages of homology for the COOH-terminal 124 amino acids are shown in Table 1.6.1. The highest homology is found between MRP1 and MRP3 (83% similarity) and the lowest between SUR and any of the MRPs (≤ 59% similarity).

	MRP1	**cMOAT**	**MRP3**	**MRP4**	**MRP5**	**MRP6**	**HSUR**
MRP1	100						
cMOAT	73	100					
MRP3	83	73	100				
MRP4	69	65	64	100			
MRP5	66	65	62	66	100		
MRP6	69	64	67	62	57	100	
HSUR	59	57	57	58	57	46	100

Table 1.6.1: Homology between the COOH terminal 124 amino acids of the six human MRP homologues and human SUR. Percentages of identity were determined using the GAP program of GCG (Kool *et al.*, 1997).

The existence of a seventh family member, MRP7, has only been inferred from a database search and so far there is no other information available (Borst *et al.*, 1999). Some of the MRPs are known by other names as summarised in table 1.6.2.

The Mammalian MRP family

MRP1	=	ABCC1	=	MRP				
MRP2	=	ABCC2	=	cMOAT	=	cMRP	=	EBCR (rabbit)
MRP3	=	ABCC3	=	MOAT-D	=	cMOAT-2	=	MLP-2
MRP4	=	ABCC4	=	MOAT-B				
MRP5	=	MOAT-C	=	MOAT- C	=	pABC11	=	sMRP*
MRP6	=	ABCC6	=	MLP-1	=	ARA*		
MRP7	=	ABCC10						

*only 3'end.

Table 1.6.2: Overview of the MRP family and the alternative names used in the literature for the individual members (Borst *et al.*, 1999).

The sMRP has been reported by Suzuki *et al.*, (2000), to be a spliced variant of the MRP5 gene, expressed in various human tissues. ARA represents the 3'end of the MRP6 gene that is incidentally co-amplified with MRP1 in cells selected for adriamycin resistance (Kool *et al.*, 1999). Within the group of mammalian ABC transporters the MRPs form a cluster that is clearly demarcated from the other known groups, such as Pgp, CFTR and the sulphonylurea receptors.

1.6.2 Chromosome location of MRP and homologues

The newly identified MRP homolgues MRP3-5 are all located on other chromosomes other than those containing MRP1 or cMOAT genes (Kool *et al.*, 1997; Cole *et al.* 1999). This confirms that MRP3, MRP4, and MRP5 are indeed new genes and not alternative splice products of MRP1 or cMOAT. The MRP1 gene has been mapped to chromosome 16 at band p13.13-13.12, the cMOAT gene was mapped to chromosome 10, band q24, MRP3, MRP4 and MRP5 were found on chromosomes 17, 13 and 3 respectively (Kool *et al.*, 1997). MRP6 is located on chromosome 16, band 16p13.11, next to MRP1 (Kool *et al.*, 1999).

1.6.3 Cellular location of MRP

Although MRP was initially believed to be predominantly located in the endoplasmic reticulum of resistant cells (Krishnamachary and Centre, 1993), significant levels are now known to be predominantly localized to the plasma membrane (Flens *et al.*, 1994; Muller *et al.*, 1994 and Zaman *et al.*, 1994), with detectable levels present in intracellular membrane compartments of some cell types (Almiquist *et al.*, 1995 and Hipfner *et al.*, 1999). Immunohistochemical studies show that MRP1 in normal tissue is predominantly cytoplasmic, whereas, in malignant tissue, it is mainly plasma membrane located with some granular cytoplasmic staining observed (Flens *et al.*, 1996). However, Flens *et al.*, (1996), and Zaman *et al.*, (1994), have reported that resistant cell lines show predominantly plasma-membrane staining.

MRP2 is localised to the canalicular membrane of rat and human hepatocytes (Paulusma *et al.*, 1997 and Keppler *et al.*, 1996), as well as to the apical membrane of rat hepatoma cells (Konig *et al.*, 1999a). MRP3 was found in the basolateral membrane of hepatocytes but was not detectable in the canalicular membrane domain of hepatocytes. In polarised monolayers of kidney cells, MRP 1, 3 and 5 are routed to the basolateral membrane and MRP2 is the apical MRP isoform (Borst *et al.*, 1999). The subcellular locations of MRP4 and MRP6 have not yet been analysed (Borst *et al.*, 1999). Using specific monoclonal antibodies, MRP2 has been located in apical and MRP1 and MRP3 in basolateral membranes of tissues (Kool *et al.*, 1999, Flens *et al.*, 1996 and Konig *et al.*, 1999b).

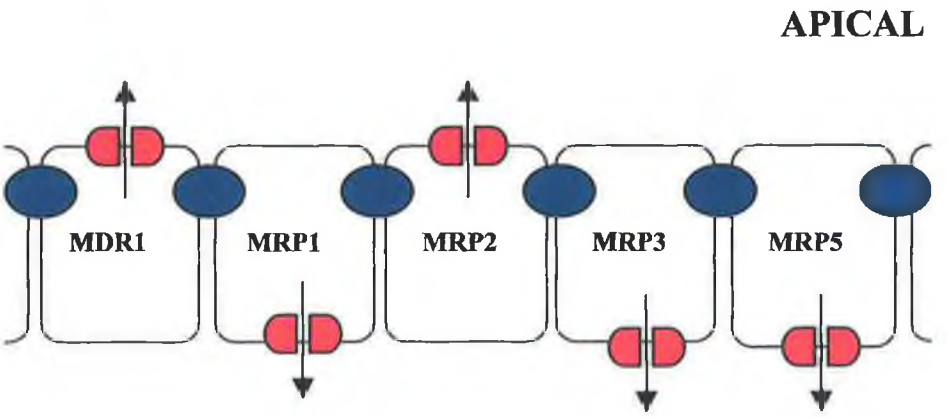


Figure 1.6.1: Schematic presentation of the localisation of MDR1 Pgp, MRP1, MRP2, MRP3 and MRP5 in transfected polarized epithelial cells. The physical barrier between the apical and basolateral plasma membrane is formed by tight junctions.

1.6.4 MRP expression in cell lines

Since the discovery of MRP1 in the small cell lung cancer cell line, H69AR, MRP1 has been identified in non-Pgp multidrug resistant cell lines from a variety of tumour types, including leukemias, fibrosarcoma, non-small cell lung, human small cell lung, breast, cervix, prostate, and bladder carcinomas (Table 1.6.3) (Izquierdo *et al.,* 1996). Although many of these cell lines have been selected in doxorubicin or other anthracyclines, others have been selected in etoposide (VP-16) or vincristine. (Loe, 1996). Recently cell lines have been identified which co-express both MRP1 and Pgp but the relative contribution of each protein to the overall multidrug resistance phenotype has yet to be determined. (Brock *et al.,* 1995).

Tumour Type	Cell line	Selecting drug
Human small cell lung	H69AR	Doxorubicin
	GLC_4/ADR	Doxorubicin
	POGB/DX	Doxorubicin
	H69/VP	VP-16
	UNCC-VP	VP-16
Non-small cell lung	MOR/R	Doxorubicin
	CORL23/R	Doxorubicin
Cervical	HelaJ2	Doxorubicin
	KB/C-A	Doxorubicin*
	KB/7d	VP-16
Fibrosarcoma	HT1080/DR4	Doxorubicin
Leukaemia	HL60/ADR	Doxorubicin
	U-937/A	Doxorubicin
	CEM/E	Doxorubicin
Breast	MCF7/VP	VP-16
	MCF7/GL	Geldanamycin
Bladder	T24/ADM	Doxorubicin
	KK47/ADM	Doxorubicin
	5637/DR5.5	Doxorubicin
Prostate	P/VP20	VP-16
Mouse Erythroleukemia	PC-V	Vincristine
	WEH1-3B/NOVO	Novobiocin

*With cepharanthine and mezerine. (Taken from: Loe *et al.*, 1996a)

Table 1.6.3 MRP1 expressing tumour cell lines.

1.6.5 MRP expression in tissues

MRP1 has been detected either at the protein or mRNA level in normal human tissues including lung, stomach, colon, peripheral blood macrophages, thyroid, testis, nerve, bladder, adrenal, ovary, pancreas, gall-bladder, duodenum, heart, muscle, placenta, brain, kidney, liver and spleen (Sugawara *et al.*, 1997; Loe *et al.*, 1996a; Zaman *et al.*, 1993; Cole *et al.*, 1992 and Kool *et al.*, 1997).

MRP2 (cMOAT) is found predominantly in the liver, duodenum and, in low levels, in the kidney (Kool *et al.*, 1997; Schaub *et al.*, 1997). Kool *et al.*, (1997) and Kiuchi *et al.*, (1998), reported that MRP3 mRNA is mainly expressed in the liver, colon, intestine and adrenal gland, and to a lesser extent in several other tissues.

MRP3, like MRP2, is found predominantly in the liver. High levels of MRP3 mRNA in human liver have also been reported by other authors including Kiuchi *et al.*, (1993); Belinsky *et al.*, (1998) and Fromm *et al.*, (1999). However, results with new MRP3 antibodies show that there is little MRP3 protein in normal human liver (Kool *et al.*, 1999a). Hirohashi *et al.*, (1998), reported that MRP3 mRNA levels are low or undetectable in normal rat liver but that the level is increased in rats made cholestatic by bile duct ligation. Therefore, it appears that a high level of MRP3 is detected in diseased liver cells and only a very low expression is detected in normal liver cells.

Kool *et al.*, (1997), reported that MRP4 was found only in a small number of tissues at very low levels. However, Lee *et al.*, (1998), demonstrated, using RNA blot analysis, the expression of MRP4 in a wide range of tissues, with particularly high levels in prostate, but almost undetectable levels in the liver. MRP5, like MRP1, is readily detected in several tissues with highest levels in skeletal muscle, intermediate levels in kidney, testis, heart and brain and low levels in most other tissues, including lung, liver, spleen, thymus, prostate, ovary and placenta (Belinsky *et al.*, 1998).

Recent investigations have shown that MRP6 is predominantly expressed in liver and kidney cells and to a lesser extent in other tissues (Kool *et al.*, 1999b). Table 1.6.4 shows the levels of RNA transcripts of MRP1, cMOAT, MRP3, MRP4, MRP5 and MRP6.

	MRP1[1]	cMOAT[1]	MRP3[1]	MRP4[1]	MRP5[1]	MRP6[2]
Lung	●●●●	○	●	●	●●	●●
Kidney	●●●	●	●	●	●●	●●●●
Bladder	●●●●	○	●	●	●●	○
Spleen	●●●●	○	●	○	●●	○
Mammary gland	ND	○	○	ND	ND	ND
Salivary gland	ND	○	○	○	●●	●●
Thyroid	●●●●	○	○	○	●	●
Testis	●●●●	○	○	○	●●	○
Nerve	●	●	○	○	●●	○
Stomach	●●●	○	●	○	●●	○
Liver	○	●● ● ●	●●●●	○	●	●●●●
Gall bladder	●●●	ND	ND	●	●●	○
Duodenum	●●	● ●	●●●	ND	ND	●●
Colon	●●●	○	●●●	○	●●	●●
Adrenal gland	●●●●	○	●●●	○	●	○
Skeletal muscle	●●	○	○	○	●●●●	○
Heart	●	○	○	○	●●	○
Brain	●	○	○	○	●●●	○
Placenta	●●	○	○	○	●	○
Ovary	●●	○	○	○	●	○
Pancreas	●	○	●	○	●	○
Tonsil	ND	○	●	●	●●	○

[1]Kool *et al.*, (1997); [2]Kool *et al.*, (1999b). ND, not determined; ○, no expression; ● - ●●●●, low to high expression.

Table1.6.4: Levels of mRNA transcripts of MRP1, cMOAT (MRP2), MRP3, MRP4, MRP5 and MRP6 in human tissues.

1.6.6 MRP Protein structure

Both of the known human multidrug resistance transporters, P-glycoprotein and MRP, together with several other bacterial and eukaryotic transporters, are members of the ABC transporter protein family. In most cases these proteins have been shown to function as transport ATPases, hydrolysing ATP in conjunction with transporting their substrate molecules through cellular or intracellular

membranes. These proteins share a common molecular architecture - they are built from combinations of conserved domains, that is ATP- (nucleotide-) binding ABC units, and characteristic membrane- embedded regions (Varadi *et al.*, 1998).

Most ABC transporters are comprised of two polytopic MSDs (Membrane Spanning Domain) and two NBDs (Nucleotide Binding Domain) (Higgins, 1992). In proteins such as Pgp, CFTR, and STE6, all four domains are contained within a single polypeptide but others, such as bacterial transporters and the maltose and histidine permeases, consist of four polypeptides each encoding a single domain. Mammalian TAP1 and TAP2, which are involved in antigen presentation, each contain one MSD and one NBD, and heterodimerise to form a functional complex. It is thought that ABC transporters such as Pgp, CFTR, and STE6 evolved by duplication or fusion of genes encoding half-transporters with domain organisations similar to those of TAP1 and TAP2 (Gao *et al.*, 1998). The predicted topology of MRP1 is inconsistent with the typical four-domain structure. It was the first example of a subgroup of the ATP-binding cassette superfamily whose members have three membrane spanning domains and two nucleotide binding domains (Hipfner *et al.*, 1997 and 1999). Mutagenesis of potential N-glycosylation sites has recently shown that the NH_2 terminus of MRP1 is extracellular so it appears most likely that MSD1 spans the membrane five times (Varadi *et al.*, 1998). This additional hydrophobic domain is not present in ABC transporters such as Pgp and CFTR. Thus, it is a characteristic feature of members of the MRP branch of the ABC transporter superfamily (Hipfner *et al.*, 1997).

More recently identified ABC transporters with a third NH_2 terminal MSD include cMOAT (MRP2), MRP3 and MRP6 the yeast cadmium resistance factor (YCF1), and the sulfonylurea receptors (SUR), as well as several less well characterised MRP-related proteins (Lautier *et al.*,1996; Hipfner *et al.*, 1999; Tusnady *et al.*, 1997 and Bakos *et al.*, 1996). Comparisons of protein primary structure and gene organisation suggest that the MRP-related proteins share a common, four-domain ancestor with the CFTR. It is also apparent from such comparisons that the NH_2 terminal MSDs of the MRP-related proteins are poorly conserved relative to the other four domains in these proteins. Gao *et al.*, (1998), investigated the possible role of the third MSD of MRP1 and its related

transporters and their results demonstrated that two truncated molecules (MRP229-1531 and MRP281-1531) lacking MSD1 can be expressed in Sf21 cells as efficiently as the full length protein. They then examined the ability of various MRP1 fragments, expressed individually and in combination, to transport the MRP substrate, Leukotriene C_4 (LTC_4). It was found that elimination of the entire NH_2 terminal MSD, or just the first putative transmembrane helix, or substitution of the MSD with the comparable region of the functionally and structurally related transporter, the canalicular multispecific organic anion transporter (cMOAT/MRP2), had little effect on protein accumulation in the membrane. However, all three modifications decreased LTC_4 transport activity by at least 90%. Transport activity could be reconstituted by co-expression of the NH_2-terminal MSD with a fragment corresponding to the remainder of the MRP molecule, but this required both the region encoding the transmembrane helices of the NH_2 terminal MSD and the cytoplasmic region (L_0) linking it to the next MSD. In contrast, a major part of the cytoplasmic region linking the NH_2-proximal nucleotide binding domain of the protein to the COOH-proximal MSD was not required for active transport of LTC_4.

In most ABC transporters, the binding and the subsequent hydrolysis of ATP by the NBDs is believed to be coupled to, and provide the energy for substrate transport (Hipfner *et al.*, 1999). These domains are highly conserved, typically showing 30-40% identity among different superfamily members in a core region of about 200 amino acids. The NBDs of ABC superfamily members share two sequence motifs, designated "Walker A" and "Walker B", with many other nucleotide binding proteins (Walker *et al.*, 1982). Mutational analysis of a number of ABC proteins indicates that these two regions are critical for ATPase function (Hipfner *et al.*, 1999). Another feature that distinguishes MRP1-like transporters from other ABC superfamily members is a difference in the structure of the NH_2-proximal NBD (NBD1). Alignment of the primary sequences of MRP1, LtPgpA, and CFTR with the human Pgp encoded by the MDR1 gene revealed that, in comparison to P-glycoprotein, these transporters all contain a "deletion" of 13 amino acids located between the Walker A and B motifs of NBD1.

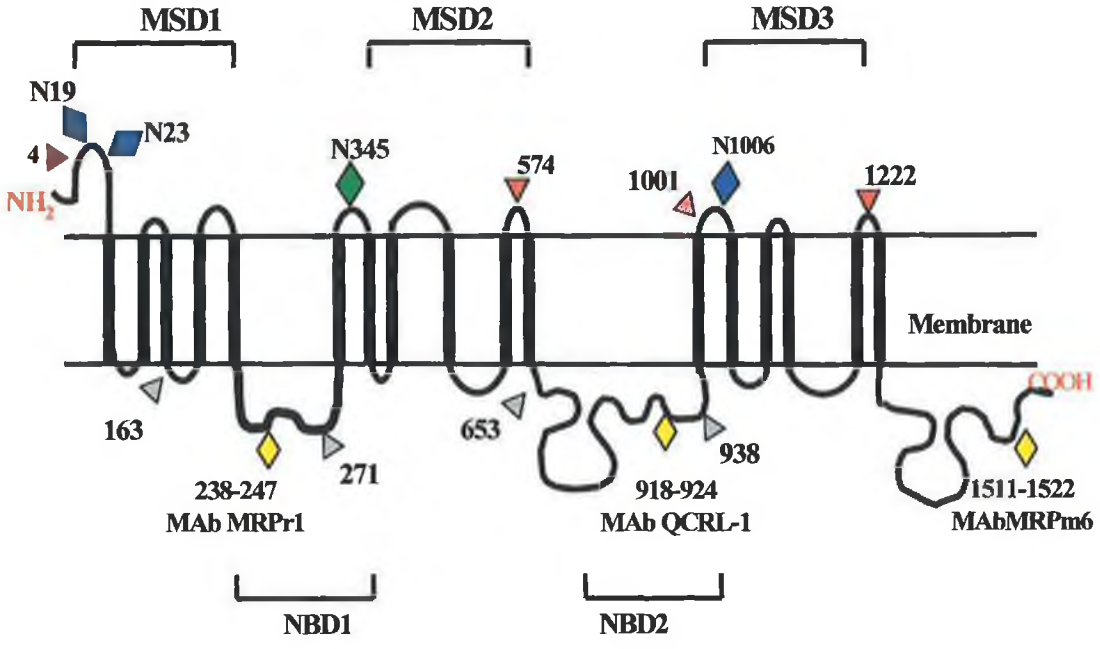


Figure 1.6.2: Proposed membrane topology of MRP1. Models of the membrane topology of MRP1 are shown with amino acids experimentally determined to be localised to the cytosolic or extracytosolic side of the membrane indicated. Six amino acids have been localised to the extracytoplasmic side of the membrane by N-glycosylation site utilization analysis (N19, N23 and N1006 ◆) or HA epitope insertion (amino acid positions 4, 574, 1001 and 1222 ▲). Additional amino acids or regions have been localised to the cytosolic side of the membrane by epitope mapping of MRP1-specific mAbs MRPr1, QCRL-1 and MRPr6 (◇) or by HA epitope insertion (at amino acid positions 163, 271, 653, 938 ▲). A glycoylation site that is not utilised but predicted to be extracytosolic is indicated ◆) (Hipfner *et al.*, 1999).

Within the MRP family, homolgy is highest between MRP1, 2, 3 and 6. MRP 2, MRP3 and MRP6 have a similar structure to MRP1 and are characterised by the presence of MSD1 connected to a Pgp-like core by cytoplasmic linker, L_0. However, both the MRP4 and the MRP5 cDNA sequences predict a structure more similar to CFTR and MDR1 than MRP1 because of the lack of the NH_2-terminal membrane-bound extension of about 280 amino acids. Although this segment is absent in these MRP homologues, it is reported that they still have the basic structure that seems to be required for GS-X pump activity in MRP1 (Bakos *et al.*, 1998), i.e. the P-glycoprotein-like core structure and the L_0 loop.

The amino acid sequence of MRP1 contains various sites known to be relevant for ATP binding and post-translational modification (Loe *et al.*, 1996a). MRP1 has been detected immunologically as a 190 kDa N-glycosylated phosphoprotein which binds ATP. Various studies have shown that the unmodified MRP polypeptide has an apparent mass of 170 kDa and is processed into a mature 190 kDa form by addition of N-linked oligosaccharides (Almiquist *et al.*, 1995). Only three of the twelve potential sites for N-linked glycosylation, contained in the human MRP1 gene, are external to the plasma membrane and glycosylated (Almiquist *et al.*, 1995). The effect of glycosylation on MRP1 activity is not fully known but it has been demonstrated that tunicamycin-induced inhibition of glycosylation has little effect on the cellular drug accumulation characteristics of MRP1 expressing cells (Almiquist *et al.*, 1995). Hipfner *et al.*, (1997), also report that although MRP1 expressed in Sf21 cells is underglycosylated, it displays transport kinetics similar to those of fully glycosylated MRP1.

MRP1 is highly phosphorylated with phosphate groups contained in at least 9 tryptic peptides (Ma *et al.*, 1995). All phosphate groups are present at serine residues and it has also been observed that certain protein kinase inhibitors such as chelerythrine and staurosporine are capable of reducing P190 phosphorylation and reversing drug resistance (Ma *et al.*, 1995).

It is believed that phosphorylation of MRP1 occurs mainly in the linker region of the protein but the precise location and functional role of these phosphorylated residues on MRP1 are unclear.

1.6.7 Function /Transport by MRP1

Lutzky *et al.*, (1989), demonstrated that inhibition of Glutathione (GSH) synthesis by buthionine sulfoximide (BSO), an inhibitor of γ-glutamylcysteine synthetase, resulted in significant sensitization to daunorubicin and suggested that changes in the intracellular distribution of GSH/Glutathione-S transferase (GST) and/or increased drug retention may have been involved in mediating this effect. Increased intracellular GSH levels were shown to correlate with intrinsic anthracycline resistance (Russo *et al.*, 1985). Increased activity of GSH-dependent enzymes was associated with acquired resistance (Lutzky *et al.*, 1989). In 1994 two groups independently demonstrated that MRP1 is able to transport glutathione (GSH) conjugates of drugs (Leier *et al.*, 1994 and Jedlitschly *et al.*, 1994). Leier *et al.*, (1994), demonstrated, using isolated plasma membrane vesicles with an inside out orientation prepared from HeLa cells transfected with MRP1 expression vectors, that the MRP1 gene encodes a primary-active ATP dependent export pump that can transport the cysteinyl leukotriene, LTC_4 and glutathione conjugates such as glutathione disulphate (GSSG). Elevated levels of ATP-dependent transport of LTC_4 and certain other GSH conjugates were demonstrated by Jedlitschly *et al.*, (1994), in membrane vesicles prepared from drug selected HL60/ADR cells. The findings that MRP1 can transport cysteinyl leukotrienes (e.g. LTC_4) as well as other GSH conjugates suggest that this protein may be a GSH conjugate/organic anion transporter, a GSH-X pump. Therefore, it appears that MRP1 is a transporter of multivalent organic anions, preferably glutathione S-conjugates (Loe *et al.*, 1996b; Jedlitsky *et al.*, 1996; Muller *et al.*, 1994), but also of sulphate conjugates (Jedlitsky *et al.*, 1996; Renes *et al.*, 1999). MRP1 confers resistance to heavy metals that interact with GSH and transports oxidised GSH (GSSG), steroid glucuronides and bile salt derivatives (Loe *et al.*, 1996; Cole *et al.* 1994), and complexes of reduced glutathione (GSH) with arsenite (Zaman *et al.*, 1995). Moreover, because of the MRP-mediated export of

glutathione disulfide from cells (Leier *et al.*, 1996), MRP family members are reported to play a role in the defence against oxidative stress (Keppler *et al.*, 1999a).

The mechanism of MRP-mediated transport of chemotherapeutic drugs is presently unclear. Although MRP1 has been shown to reduce cellular accumulation of at least some of the drugs to which it confers resistance, such as adriamycin, daunorubicin, vincristine and VP-16, it has previously not been possible to demonstrate direct transport of these compounds or other unmodified chemotherapeutic drugs by MRP1-enriched membrane vesicles (Cole *et al.*, 1998; Jedlitschky *et al.*, 1996; Loe *et al.*, 1996 and Muller *et al.*, 1994), and contrary reports claiming to have shown direct transport of these compounds have recently been retracted (Cole *et al.*, 1998). However, Vezmar *et al.*, (1998), reported the direct binding of the antimalarial drug, chloroquine, to MRP1 and the transport of this compound in an ATP-dependent manner.

Because MRP1 has the ability to transport glutathione conjugates, it was postulated that the transporter confers multidrug resistance by pumping the glutathione conjugates of anionic metabolites of lipophilic cytotoxic drugs (Paul *et al.*, 1996). However, glutathione conjugates of the natural drug products for which MRP1 confers resistance have not been isolated (Tew, 1994), and MRP transfectants do not exhibit increased resistance to alkylating agents, a class of drugs for which glutathione conjugation is known to occur (Grant *et al.*, 1994; Paul *et al.* 1996; Loe *et al.*, 1996 and Renes *et al.*, 1999). There are also other reasons why the ability to transport drug conjugates is unlikely to provide a general explanation of the ability of MRP1 to confer resistance to a structurally diverse spectrum of anti-neoplastic agents in such a wide variety of cell types. Most interesting is the observation that Phase II conjugation plays a relatively minor role in the *in vivo* and *in vitro* metabolism of these compounds (Loe *et al.*, 1998). In addition, Phase I and Phase II biotransformation reactions are known to occur primarily in the liver (and to a lesser extent in other tissues), and it is highly unlikely that all of the cell types in which MRP1 overexpression causes resistance are competent to carry out these reactions with the required efficiency and completeness (Cole *et al.*, 1998; Hipfner at al., 1999).

A number of authors have also reported the ability of the MRP protein to mediate the active transport of neutral (etoposide (VP-16)) and cationic (adriamycin, vincristine, daunorubicin and rhodamine 123) lipophilic drugs as well as anions (e.g. dinitrophenyl-S-glutathione and calcein) (Broxterman *et al.*, 1996; Renes *et al.*, 1999). Therefore, the need for extensive conjugation in tumour cells of such a variety of compounds makes this hypothesis even more unlikely.

However, a number of authors have reported that depletion of cellular GSH (reduced glutathione) levels by treatment with BSO (buthionine sulfoximine), improved the efficacy of some natural product drugs both in cultured cells and in mice bearing tumours that express elevated levels of MRP1 (Loe *et al.*, (1998); Lautier *et al.*,1996 and Schneider *et al.*, 1995). Recently Benderra *et al.*, (2000), have reported that BSO was able to increase nuclear accumulation of daunorubicin in cells overexpressing MRP1 (MCF7/VP) but had no effect in cells that overexpressed Pgp.

These findings together with the observation that MRP1 increased drug efflux from intact cells but was apparently unable to transport the same compounds in isolated plasma membrane vesicles, suggested that either the efflux of certain drugs might require the activation of MRP1 by GSH or that some form of co-transport mechanism might be involved (Cole *et al.*, 1998). Loe *et al.*, (1996), reported that physiological concentrations of GSH significantly enhanced the ability of vinblastine and vincristine to inhibit MRP1-mediated ATP-transport of LTC_4 although GSH or vincristine alone are very poor inhibitors of MRP-mediated ATP-transport of LTC_4. They also demonstrated the direct uptake of unmodified VCR by MRP1-enriched vesicles in an ATP and GSH-dependent manner and that the tripeptide structure of GSH is a requirement for stimulation of VCR transport to occur. Renes *et al.*, (1999), demonstrated, using membrane vesicles isolated from *in vitro* selected multidrug resistant cell lines overexpressing MRP1 (GLC4/ADR) (which did not express MRP2, MRP3, MRP4 or MRP5 (Kool *et al.*, 1997)) and a MRP1-transfected cell line (S1(MRP)), that MRP1 transports vincristine and daunorubicin in an ATP-and GSH-dependent manner. This transport can be inhibited by the MRP1 inhibitor, MK571, and the MRP1-specific monoclonal antibody, QCRL-3. Previously, Leier *et al.*, (1996), reported that GSH was not transported by MRP1. But Loe *et al.*, (1998), reported that VCR stimulates the ATP-dependent transport of GSH in a

concentration-dependent manner although in the absence of VCR, they detected no ATP-dependent GSH transport. Therefore, the information provided by Loe *et al.*, (1996) and (1998), and Cole *et al.*, (1998), indicates that MRP1 can actively cotransport GSH and unmodified vincristine and that these compounds probably interact, either with LTC_4 binding site(s) on the protein or with a mutually exclusive site.

Additional evidence favouring a drug/GSH co-transport mechanism has been obtained from studies using murine MRP double-knockout ($MRP^{-/-}$) cell lines. The export of GSH from murine wild type ($MRP^{+/+}$) embryonic stem cells, but not from the knockout ($MRP^{-/-}$) cell lines, has been reported to be increased in the presence of VP-16 (Cole *et al.*, 1998; Rappa *et al.*, 1997; Wijnholds *et al.*, 1997 and Loe *et al.*, 1998). In addition expression of MRP1 in some cell lines, but not all, is associated with a significant (2- to 6-fold) decrease in intracellular GSH levels (Loe *et al.*, 1998). Recently, Rappa *et al.*, (1999), reported in studies of murine MRP knockout models that MRP, besides being capable of exporting certain glutathione conjugates, may also catalyse the co-transport of GSH and drugs or endogenous metabolites. A recent publication by Poirson -Bichat *et al.*, (2000), reported that most, if not all, human tumours are dependent on methionine for growth and that depletion of methionine resulted in a reduction of the ATP pool and glutathione content, resulting in increased efficiency of a number of chemotherapeutic drugs including adriamycin, cisplatin and carmustine. This further indicates the involvement of GSH and ATP in chemoresistance and drug efflux mechanisms.

However, this co-transport mechanism still can not fully explain all MRP-mediated resistance mechanisms, in particular, with respect to the anthracycline antibiotics, since GSH displays little or no ability to enhance either their transport directly or their ability to inhibit ATP-dependent, MRP1 mediated LTC_4 transport (Loe *et al.*, 1996b). These authors also found that treatment of either drug-selected or transfected cells with BSO restored sensitivity to vincristine far more effectively than to adriamycin. It appears that GSH is of far greater importance to MRP1-mediated transport of *Vinca* alkaloids compared to anthracyclines.

However, recently several GSH-independent interactions of MRP1 have have also been reported. The MRP1-mediated transport of antifolates and the induction of MRP1 ATPase activity by the (iso)flavonoids, both in the absence of GSH,

showed that the presence of GSH was not absolutely required for MRP1 function (Hooijberg *et al.*, 1999 and 2000).

A number of cell lines, which over-express MRP1, have been developed through a process of drug selection. Because these cell lines are obtained by a stepwise selection in drug it is difficult to obtain information on the potential drug transporting abilities of MRP1 as it is more than likely that a variety of drug resistance mechanisms are co-expressed in the cell lines. MRP- transfected cell lines may be more useful models for defining the profile of drugs transported by MRP as the use of cell systems such as these should avoid the interference from multiple resistance mechanisms found in drug selected cell lines. However, as most cells contain endogenous (organic anion) transporters, resulting in background transport activity (Borst *et al.*, 1999), it has been difficult to generate cell lines which express one transport protein only. Therefore, MRP-transfected cell lines may only be useful when the problem of endogenous transporters is resolved.

Cole *et al.*, (1994), found that MRP1-transfected cell populations were 6.9-fold, 6.3-fold, 8.6-fold, 11.6-fold, 10.3-fold more resistant to adriamycin, daunorubicin, epirubicin, vincristine and VP-16, respectively, than negative control transfectant. These transfectants were only slightly resistant to taxol, vinblastine, and colchicine (1.7-fold, 3-fold, 2.1-fold respectively). In addition, antifolates, such as the anticancer agent methotrexate, have recently been described as MRP1 substrates (Hooijberg *et al.*, 1999 and Kool *et al.*, 1999a). In addition to methotrexate, several additional amphiphilic organic anions which are not conjugated have been shown to be transported by MRP1 directly in a GSH-dependent manner (Keppler *et al.*, 1999 and Konig *et al.*, 1999). Although the resistance profiles of drug-selected MRP or Pgp-overexpressing cell lines were similar, considerable differences existed particularly with regard to taxol, mitoxantrone and colchine resistance (Cole *et al.*, 1994). These three highly lipophilic drugs are normally included as members of the resistance spectrum of Pgp. However, a number of authors have reported low level resistance to taxol in MRP-overexpressing cell lines (Zaman *et al.*, 1994; Breuninger *et al.*, 1995 and Vanhoefer et al., 1996 and 1997). The MRP transfected cells were also resistant to a number of heavy metal anions including arsenite, arsenate and trivalent and

pentavalent antimonials but were not resistant to cadmium chloride. MRP1 transfected cells were not resistant to 9-alkyl anthracyclines, or cisplatin (Loe *et al.*, (1996a). It appears that MRP1 has a preference for more hydrophilic xenobiotics.

The drug accumulation characteristics of MDR cell lines with high levels of MRP, vary significantly. Cell lines which over-express MRP following drug selection, can accumulate less drug and/or have higher rates of efflux than the sensitive cells from which they were derived. A number of MRP expressing cell lines also appear to sequester drug into cytoplasmic vesicles (Marquardt *et al.* 1992) which may lead to the diversion of the drug from the relevant cellular target without affecting total cellular accumulation levels.

It is unclear why a cell develops MRP over Pgp-mediated resistance during drug exposure but it is believed that overexpression of MRP1 may confer initial levels of resistance, while Pgp overexpression develops as higher levels of resistance are required for survival (Elliott, 1997 and Choi *et al.*, 1999). Brock *et al.*, (1995) showed that in the small cell cancer cell line, H69, MRP1 was over-expressed during selection in low concentrations of VP-16. Following further selection in higher concentrations of drug, MRP1 expression remained relatively constant, but Pgp expression developed.

1.6.8 MRP1 and clinical multidrug resistance

There is considerable interest in determining the potential involvement of MRP1 in clinical multidrug resistance and a number of different MRP1-specific monoclonal antibodies (mAbs) including mAbs QCRL-1, QCRL-3, MRPr1 and MRPm6 have been used in a wide variety of immunoassays for the analysis of MRP1 expression and localization in both normal and malignant tissues (Flens *et al.*, 1994). The expression of MRP1 protein and/or mRNA has been detected in almost every tumour type examined, including both solid tumours (lung, gastrointestinal and urothelial carcinomas, neuroblastoma, glioma, retinoblastoma, melanoma, cancers of the breast, endometrium, ovary, prostate and thyroid) (Ito *et al.*, 1998; Canitrot *et al.*, 1998; Chan *et al.*, 1997; Nanashima *et al.*, 1999; Hipfner *et al.*, 1999; Oshika *et al.*, 1998; Loe *et al.*, 1996), and

hematological malignancies (Filipits *et al.*, 1997; Abbaszadegan *et al.*, 1994; Loe *et al.*, 1996). Among the common tumour types, expression of high levels of MRP1 is particularly frequent in the major histologic forms of non-small cell lung cancer (Nooter *et al.*, 1996; Giaconne *et al.*, 1996; Hipfner *et al.*, 1999). There are difficulties in establishing a causative role for MRP1 (and other resistance-associated proteins such as Pgp) in clinical multidrug resistance. There are a number of reasons for this. The broad spectrum of drugs encompassed by clinical multidrug resistance indicates that multiple resistance mechanisms are likely involved. Due to differences in the methods used to quantify MRP in clinical samples discrepant results have been reported by different investigators. The design and execution of more informative studies to address the role of resistance proteins in chemotherapy failure has been hindered by difficulties in obtaining suitable patient samples (e.g. pre-and post-chemotherapy samples from the same patient) (Hipfner *et al.*, 1999).

However, a number of authors have reported that the expression levels of MRP1 are of prognostic significance. Chan *et al.*, (1997), reported that MRP1 expression in retinoblastoma (RB) was associated with the rare failures of chemotherapy in RB. Canitrot *et al.*, (1998), and Campling *et al.*, (1997), reported that the expression of MRP1 mRNA was a negative determinant of the chemotherapeutic response of untreated Small Cell Lung Cancer (SCLC). MRP1 was also reported by Ito *et al.*, (1999), to have prognostic value in primary breast cancer and might be used as one of the markers for poor prognosis in patients with this disease (Huang *et al.*, 1998). Oda *et al.*, (1996), also suggested a link between MRP1 expression and poor prognosis in Ewings sarcoma and malignant peripheral neuroectodermal tumour of bone (MPNT).

Norris *et al.*, (1997), and Bordow *et al.*, (1994), have reported that amplification of the N-*myc* oncogene is a powerful indicator of poor response to chemotherapy and poor outcome in neuroblastoma and is central to the malignant phenotype of this disease. Expression of the MRP1 gene is common in both primary neuroblastoma tumours and cultured cell lines and was found to correlate with amplification and overexpression of the N-*myc* oncogene (Bordow *et al.*, 1994). Norris *et al.*, (1997), have hypothesised that the N-*myc* oncogene influences neuroblastoma outcome by regulating *MRP1* gene expression.

It has also been reported that mutant p53 gene significantly correlated with MRP overexpression in a number of cell lines derived from different tumour types including NSCLC (Oshika *et al.*, 1998 and Wang *et al.*, 1998), colorectal cancer (Fukishima *et al.*, 1999), acute myeloblastic leukemia (Turzanski *et al.*, 2000), leukaemia and ovarian carcinoma (Wang *et al.*, 1998). Wang *et al.*, (1998), have suggested that it may be feasible to overcome drug resistance due to MRP1 overexpression by restoring the wild type p53 status of those cells bearing mutant p53.

1.6.9 Function/Transport properties of the MRP analogues

1.6.9.1a MRP2 (cMOAT)

The discovery that MRP1 is a GS-X pump also raised the question as to whether other GS-X pumps, known to exist in human tissues might be involved in some forms of drug resistance. The first of these pumps to be recognised as a member of the MRP family was the canalicular multispecific organic anion transporter gene, cMOAT (MRP2) (Taniguchi *et al.*, 1996).

The liver converts many endogenous and xenobiotic lipophilic compounds into anionic conjugates with glutathione, glucuronate or sulfate. These conjugates are transported across the canalicular (apical) membrane into bile by the 190kDa membrane glycoprotein, MRP2 (Jansen *et al.*, 1985; Taniguchi *et al.*, 1996). Defects in MRP2 are known to cause Dubin-Johnson syndrome (DJS) (Mayer *et al.*, 1995; Paulusma *et al.*, 1996). Koike *et al.*, (1997), reported that MRP2 activity mediates the ATP-dependent transport of various hydrophobic anionic compounds in liver canalicular membranes and other tissues. Studies with mutant rats (TR/GY or EHBR), which lack the MRP2 protein in the canalicular membrane of hepatocytes, have shown that the substrate specificity of MRP2 is very similar to that of MRP1 (Oude Elferink *et al.*, 1995; Keppler *et al.*, 1997; Roelofsen *et al.*, 1999). Paulusma *et al.*, (1999), demonstrated a role for cMOAT in the excretion of GSH both *in vivo* and *in vitro*. In several independent cMOAT transfectants, the level of GSH excretion correlated with the expression level of

the protein (Paulusma *et al.*, (1999). The authors further demonstrated, using membrane vesicles isolated from cMOAT-expressing MDCKII cells, that GSH is a low-affinity substrate for the transporter and that its excretion is reduced upon ATP depletion.

Like MRP1, MRP2 transports bilirubin glucuronides in an ATP dependent manner. Bilirubin is secreted from the liver into bile mainly as glucuronosyl and bisglucoronsyl conjugates. Jedlitschy *et al.*, (1997), demonstrated that bilirubin glucuronides were better substrates for MRP2 than MRP1. Keppler *et al.*, (1998,1999a and 1999b), and Konig *et al.*, (1999), have also reported that the K_m value (the Michaelis Menton constant (the dissociation constant for the complex)) of human MRP2 for LTC_4 was 10-fold higher than that for MRP1. Moreover, the K_m of human MRP2 for 17β-glucuronosyl estradiol was 4.8-fold higher than for MRP1. MRP2 is also reported to be the predominant export pump responsible for hepatobiliary excretion of the amphiphilic anion Fluo-3 (Keppler *et al.*, 1999 and Konig *et al.*, 1999). In addition, the quinoline-based LTD_4 analog, MK571, more potently inhibits MRP1-mediated transport than the rat MRP2-mediated transport (Keppler *et al.*, 1998).

1.6.9.1 b: Proposed working model for MRP1 and MRP2

Borst *et al.*, (1999), proposed a working model for MRP1 and MRP2 with two drug binding sites: one with a relatively high affinity for GSH (G-site) and a low affinity for drug, and one with a relatively high affinity for drug and a low affinity for GSH (D-site). The authors propose that in the absence of drugs, both binding sites are occupied by GSH resulting in a slow export of GSH. At low drug concentrations, the G-site remains occupied by GSH and the D-site becomes occupied by drug, resulting in co-transport of both compounds. The authors infer that both sites show positive cooperativity i.e. the activity of MRP requires substrate binding to both binding sites and the binding of a substrate to to the G or D-site can affect the transport of the substrate on the other binding site (for example, GSH and vincristine). Borst *et al.*, (1999), also suggest that at high drug concentrations some (negatively charged) drugs appear to be able to occupy both

the G- and the D- site resulting in transport of drug alone. Evers *et al.*, (1998), and Jedlitschky *et al.*, (1996) and (1997), reported that MRP1 and MRP2 do not require free GSH for the transport of compounds that are conjugated to glutathione, glucuronide or sulfate. Therefore, Borst *et al.*, (1999), suggest that these substrates have a relatively high affinity for both the G- and the D-site and are therefore transported efficiently without requiring GSH or stimulating GSH export.

1.6.9.1c MRP2 and drug resistance in cancer

Cancer cells that overexpress Pgp or MRP1 do not show cross-resistance to platinum-containing compounds, alkylating agents and anti-metabolites (Taniguchi *et al.*, 1996; Koike *et al.*, 1997). MRP1-transfected cells show resistance to anthracyclines, vinca alkaloids, epipodophyllotoxins and heavy metal anions but are sensitive to platinum-containing compounds. It was first suggested by Tanaguchi *et al.*, (1996), that human cMOAT may function as a cellular cisplatin transporter, as expression of human cMOAT was enhanced in cisplatin resistant human cell lines with decreased cellular cisplatin accumulation, but was not enhanced in cell lines with normal levels of drug accumulation. Human cMOAT was overexpressed 4.0-6.0 fold in the three cell lines analysed by Tanaguchi *et al.*, (1996), as compared to their parental drug-sensitive counterparts but did not overexpress MRP1. The cell lines analysed were the cisplatin resistant human (CRH) head and neck cancer KB cell line KB/KCP4, the CRH prostatic cancer PC-3 cell line P/CDP5 and the CRH bladder cancer T24 cell line T24 DDP10.

HepG2, a human hepatic cancer cell line, was found to express high levels of cMOAT mRNA (Narasaki *et al.*, 1997), and protein (Koike *et al.*, 1997). Koike *et al.*, (1997), transfected an expression vector containing cMOAT antisense cDNA into the HepG2 cell line and observed a reduction in the cMOAT protein as well as an enhanced level of glutathione in the antisense transfectants but increased expression of MRP and MDR1 was not observed. The transfectants displayed an increased sensitivity to cisplatin, vincristine, doxorubicin and the

camptothecin derivatives but not to etoposide (VP-16), mitomycin C and 5-FU (5'Fluorouracil). The results suggested that cMOAT levels are inversely correlated with those of glutathione and that cMOAT and it's related genes may be involved in the sensitivity of cells to certain anticancer agents. Kool *et al.*, (1996), examined a large number of cisplatin and doxorubicin resistant cell lines and showed a correlation between cMOAT transcript levels and sensitivity to cisplatin but not to doxorubicin. Increased resistance to vincristine and etoposide was observed in MDCKII cells transfected with MRP2. HEK292, transfected with MRP2, showed increased resistance to etoposide, cisplatin, epirubicin and doxorubicin (Konig *et al.*, 1999). MRP2 was also shown to confer resistance to the antifolate drug, methotrexate (Hooijberg *et al.*, 1999).

Thus there is indirect evidence to suggest that an organic anion pump, notably cMOAT, could contribute to cisplatin resistance by exporting the cisplatin – GSH complex. Elevated GSH levels and synthesis may be required to drive formation of the complex if contact with cisplatin is extended, as is usually the case with cell lines selected for resistance *in vitro*. However, even in the limited set of cell lines analysed by Kool *et al.*, (1996), all chosen for lowered cisplatin accumulation, there was no simple quantitative correlation between cisplatin resistance and the combination of raised GSH and cMOAT (Borst *et al.*, 1997).

Kauffmann *et al.*, (1997), demonstrated the inducibility of cMOAT gene expression in rat hepatocytes treated with 2-AAF (acetylaminofluorene), cisplatin or cyclohexamide. This group of researchers put forward the theory that expression of the cMOAT transporter may be part of an adaptive response mechanism aimed at preventing further cell damage. They also postulated that the stress-activated kinases, a subgroup of the mitogen-activated protein kinase family, are involved in the regulation of cMOAT. Additional candidates that may participate in cMOAT regulation include other protein kinases or factors inducible by cDNA damage such as gadd45 and p53.

It would seem, from the evidence to date that cMOAT may be induced by a variety of cytotoxic, carcinogenic and chemotherapeutic agents which is likely to be of relevance for the acquisition of multidrug resistance during chemotherapy. Matsunga *et al.* (1998), reported that the enhanced expression of MRP or cMOAT in childhood liver tumours was more common and higher, especially in advanced cases, with poor outcome, than that observed in normal liver or in 9 hepatocellular

carcinomas from adult patients. The author suggests that the enhanced expression of these genes might be characteristic of childhood malignant tumours and related to their clinical chemoresistance. However, there is no direct evidence to date for the involvement of cMOAT in clinical drug resistance, particularly the cisplatin resistance of MDR (Borst *et al.*, 1997).

1.6.9.2 MRP3

MRP3, like MRP1 and MRP2 has also been established as an organic anion transporter and is a GS-X pump (Kool *et al.*, 1999a). Among the current members of the MRP family, MRP3 has the highest sequence homology to MRP1. The most striking difference between MRP1 and MRP3 is that 2008 or MDCKII MRP3-transfected cells, overexpressing MRP3, do not detectably excrete more GSH than the parental cells, in contrast to cells transduced with either MRP1 or MRP2 constructs (Paulusma *et al.*, 1999; Kool *et al.*, 1999a). Kool *et al.*, (1999a), also suggested that cells with high levels of MRP3 did not secrete GSH. If the MRP model, proposed in section 1.6.8.1b, is correct, then it appears that the G-site in this transporter may have a very low affinity for GSH. Zeng *et al.*, (1999), reported that MRP3 did not appear to transport natural product agents in a GSH-dependent manner in human embryonic kidney 293 cells transfected with MRP3. The authors also reported that the transport of glutathione conjugates such as LTC_4, which is an excellent substrate for MRP1, was not detected. Hirohashi *et al.*, (1999), reported that cloning of rat MRP3 and determination of its substrate specificity in inside-out membrane vesicles from transfected cells indicated that, unlike MRP1 and MRP2, it preferentially transported glucuronosides, such as 17 β-glucuronosyl estradiol, but that glutathione S-conjugates were relatively poor substrates. Konig *et al.*, (1999a), observed a particularly strong expression of the MRP3 protein in the basolateral hepatocyte membrane of two patients with Dubin-Johnson syndrome who are deficient in MRP2. They concluded that MRP3 might be upregulated when the canalicular secretion of anionic compounds is impaired.

Early studies of MRP3 did not find any correlation between expression of this transporter and drug resistance (Kool *et al.*, 1997). A more recent survey of a

panel of lung cancer cell lines showed, however, a strong correlation between MRP3 and doxorubicin resistance and a weaker, but still highly significant correlation with resistance to vincristine, etoposide and cisplatin (Young *et al.*, 1999). MRP3 was also found to transport methotrexate (Hirohashi *et al.*, 1999 and Kool *et al.*, 1999a), and was also reported by Kool *et al.*, (1999a), to confer low levels of resistance on etoposide and teniposide. Uchiumi *et al.*, (1998), analysed cisplatin resistant human head and neck cancer and human prostatic cancer cell lines over their parental counterparts for the overexpression of MRP3. They did not observe any specific change in cellular levels of the MRP3 mRNA in cisplatin resistant cancer cell lines with decreasing drug accumulation.

As it is difficult to generate transfected cells with high concentrations of MRP3 in the plasma membrane (Kool *et al.*, 1999a and Borst *et al.*, 1999), it remains possible that higher levels of MRP3 will also result in resistance to adriamycin and other drugs as suggested by the correlation study by Young *et al.*, (1999).

mRNA levels of MRP3 have been reported to be higher in NSCLC (non-small cell lung cancer) cell lines than in SCLC cell lines (Young *et al.*, 1999), hence, this MRP-transporter may contribute to the multifactoral multidrug resistance phenotype of lung cancer cells, particularly NSCLC.

1.6.9.3 MRP4

Although MRP4 is expressed in a wide range of tissues (Lee *et al.*, 1998), and the similarity between MRP1 and MRP4 suggested that it may share a similar substrate specificity, characterisation of MRP4 substrates remains to be elucidated. Borst *et al.*, (1999), reported that the structure of MRP4 differed from that of MRP1, MRP2, MRP3 and MRP6 in that the NH_2 terminal MSD was absent from MRP4. However, Bakos *et al.*, (1998), reported that MRP4 still had the basic structure required for GS-X pump activity in MRP1. Kool *et al.*, (1997), screened a large number of human cell lines derived from various tissues and their resistant sublines selected with either adriamycin, cisplatin, tetraplatin or $CdCl_2$. They reported that MRP4 was expressed only at low or very low levels in the cells lines they analysed and no overexpression of MRP4 was detected in resistant

sublines. Schuetz *et al.*, (1999), carried out a study of alternative or additional mechanisms of resistance operating during antiviral therapy and discovered that the overexpression and amplification of the MRP4 gene correlated with the ATP-dependent efflux of PMEA (9-(2-phosphonylmethoxyethyl)adenine) and azidothymidine mono-phosphate from cells and, thus, with resistance to these drugs. Overexpression of MRP4 mRNA and MRP4 protein severely impaired the antiviral efficiency of PMEA, azidothymidine and other nucleoside analogues in the human T-lymphoid cell line, CEM-r1. The authors reported that increased resistance to PMEA and amplification of the MRP4 gene correlated with enhanced drug efflux; transfer of chromosome 13 containing the amplified MRP4 gene also conferred resistance to PMEA. Therefore, these results appear to link expression of MRP4 with the efflux of nucleoside monophosphate analogs from mammalian cells.

1.6.9.4 MRP5

Little is known as yet about MRP5 (Borst *et al.*, 1995). Suzuki *et al.*, (2000), reported that MRP5 mRNA was detected in a large number of human tissues but also that the spliced variant of MRP5, sMRP, was preferentially expressed in the liver and placenta and that this sMRP may also have a physiological role.

Kool *et al.*, (1997), reported that MRP5 was expressed in all of the cell lines analysed with the highest levels in MOR/P and 2008, but MRP5 was not highly overexpressed in any of the resistant cell lines. The authors reported that MRP5 was only slightly overexpressed in three cell lines selected for cisplatin resistance (T24/DDP10, HCT8/DDP and KCP-4(-)), but many other cisplatin-selected cell lines showed no overexpression. Therefore, it is questionable whether this low level of MRP5 overexpression has anything to do with cisplatin resistance. Recently, Wijnholds *et al.*, (1999), reported a possible connection between MRP5 and resistance to thio-purines although this remains to be substantiated by drug accumulation and vesicular transport studies (Borst *et al.*, 1999).

1.6.9.5 MRP6

Within the MRP family, homology is highest between MRP1,-2,-3, and -6. Using 3' and 5' derived MRP6 probes, Kool *et al.*, (1999b), determined that MRP6 was highly expressed in liver and kidney cells and to a low or very low extent in a few other tissues including the colon, gall bladder and ovary. The authors reported that their analysis of a number of MDR and cisplatin resistant cell lines provided no evidence for the involvement of MRP6 in drug resistance. They found overexpression of MRP6 only in those cell lines with high expression or amplification of the MRP1 gene. It seems that MRP6 does not play a role in the resistance of the cells analysed and that MRP6 is only co-amplified with MRP1 because of it's location, immediately next to it, on the same chromosome. However, Belinsky *et al.*, (1999), suggested that MRP6 might participate in hepatobiliary and renal excretion of organic anions. While cMOAT (MRP2) is a major pump for organic anions in liver, the hepatobiliary excretion of organic anions is not completely abolished in cMOAT-deficient rat strains, suggesting the existence of other organic anion transporters. MRP1 is expressed in hepatocytes at low levels, but it is localised at the lateral membrane which does not communicate with bile canaliculi (Mayer *et al.*, 1995). Belinsky *et al.*, (1999), suggested that MRP6 may function as an alternative system to cMOAT for the hepatobiliary excretion of organic anions. The authors also suggest that MRP6 may also serve a different function in the liver and suggests also that due to the abundant expression of MRP6 in the kidney, it may function as an ATP-dependent transporter of organic anions into urine.

1.6.10 Circumvention of chemotherapeutic drug resistance

In attempting to find modulators of multiple drug resistance, where membrane changes were clearly involved, a range of membrane active compounds were investigated by Tsuruo *et al.*, (1981). This investigation led to the development of the calcium channel blocker, verapamil, and the calmodulin inhibitor, trifluoperazine as specific modulators of Pgp in a mouse leukaemia cell line. A

large number of other calcium antagonists were subsequently examined and compared with verapamil for effectiveness as Pgp modulators. However, it was not possible to achieve sufficient plasma levels of these early compounds, which could be predicted from *in vitro* studies to produce adequate reversal of Pgp (Twentyman *et al.*, 1996). Subsequently, research began on the immunosuppressive cyclic peptide, cyclosporin A (Twentyman *et al.*, 1987). Because of its potent immunosuppression and also because adequate plasma levels could not be achieved in patients, cyclosporin A still left much to be desired as a clinical modifier (Twentyman *et al.*, 1996). Gaveriaux *et al.*, (1991), reported a modifier of MDR, SDZ-PSC-833, which was non-suppressive and 10-20 fold more potent than cyclosporin A. Other compounds that have also been extensively studied as modifiers of Pgp-mediated multidrug resistance include quinine and quinidine, the anti-oestrogen, tamoxifen, dexniguldipine, and the acridone carbocamide derivative, GF120918 (Ford, 1995). These compounds have generally been shown to restore the defective drug accumulation seen in Pgp-mediated MDR cells and to displace binding of the photoactive calcium antagonist, azidopine, from a 170kD band on protein gels prepared from membranes of MDR cells. It is, therefore, assumed that their primary mode of action is competition for drug-binding sites on the Pgp molecule. Photoactive analogues of verapamil and cyclosporin A have been shown to bind directly to the 170kD band (Safa, 1988). In 1997, Germann *et al.*, evaluated the ability of VX-710 (Biricodar), a novel non-macrocyclic ligand of the FK506-binding protein, FKBP12, to reverse Pgp-mediated MDR *in vitro.* The authors demonstrated a direct, high affinity interaction of VX-710 with Pgp, preventing efflux of cytotoxic drugs by the MDR1 gene product in multidrug resistant tumour cells. Safa *et al.*, (1999), reported that VX-710, modulated both Pgp as well as MRP mediated resistance. VX-710 reversed Pgp-mediated MDR at concentrations of 0.5-2.5μm by direct interaction with P-glycoprotein and inhibition of its efflux activity. Moreover, at 0.5-5.0μm, it restored the sensitivity of HL60/ADR cells, known to express MRP1, to the cytotoxic action of adriamycin, VP-16 and vincristine. VX-710 was about two-fold more effective than verapamil and cyclosporin A in circumventing MRP-mediated MDR.

In 1989, studies of two cell lines, H69AR and HT1080/DR4, now known to over-express MRP1, demonstrated that both verapamil and cyclosporin A had only a

modest effect as modulators of resistance and that verapamil was the most effective of the two (Cole *et al.*, 1989). Twentyman *et al.*, (1996), reached the same conclusions using the MRP-overexpressing cell lines, CORL23 (R) and MOR(R). However, studies performed with MRP-transfected HeLa-T5 cells showed that the cytotoxic effects of vincristine and adriamycin could be enhanced in a dose-dependent fashion by co-administration of verapamil (Cole *et al.*, 1994). In addition, it was found that cyclosporin A also increased vincristine toxicity but that it had less of an effect on adriamycin toxicity. A further study by Brueninger *et al.*, (1995), showed that verapamil increased the sensitivity of MRP-transfected HeLa-pSRα-MRP16 cells relative to control transfectants, to adriamycin and VP-16.

Barrand *et al.*, (1993), and Aszalos *et al.*, (1999), reported that clinically optimal plasma levels of the Pgp blockers, verapamil, SDZ-PSC-833 and cremophor, which are capable of completely blocking the functioning of Pgp in Pgp-overexpressing cell lines, only partially blocked the function of MRP. Combinations of these optimal concentrations acted antagonistically in MRP-overexpressing cells whereas these combinations resulted in synergistic effects in Pgp-overexpressing cells. The reasons for this antagonism were difficult to explain but Aszalos *et al.*, (1999), reported that verapamil, SDZ-PSC-833 and cremophor reduced membrane "fluidity" in the MRP-expressing MCF-7 cells than in the Pgp-expressing 3T3 cells and this reduction might effect the uptake of the chemotherapeutic drug in to the cell. Membrane fluidity is determined by the degree of unsaturation of the fatty acid residues in the component phospholipids and by the cholesterol content (Davies *et al.*, 1999).

Nakamura *et al.*, (1999), demonstrated, by measuring [^{3}H] leukotriene C_4 uptake into membrane of cells and intracellular calcein and [^{3}H]vincristine accumulation, that MS-209, a novel quinoline derivative capable of reversing P-glycoprotein-mediated multidrug resistance (MDR) is also effective at reversing intrinsic and acquired MRP-mediated MDR of gastric cancer cells by interacting directly with MRP. MK571 is also reported in the literature as an anionic quinoline LTD_4 receptor antagonist MRP inhibitor which does not significantly inhibit Pgp (Gekeler *et al.*, 1995 and Renes *et al.*, 1999). A complete reversal of vincristine resistance was achieved by co-incubation with a non-toxic level (40μM) of MK571 in an MRP-expressing cell line (HL60/AR). This compound was found to

have no effect in a Pgp-overexpressing multidrug resistant HL60 subline. MK571 has been shown to completely inhibit LTC_4 transport by MRP in membrane vesicles prepared from MRP-overexpressing cells and has also been shown to totally inhibit photoaffinity labelling of MRP by [^{3}H]-LTC_4 (Jedlitschky *et al.*, 1994). Gekeler *et al.*, (1995b), showed that the specific bisindolylmaleimide protein Kinase C inhibitor GF 109203X totally reversed vincristine resistance in one MRP-overexpressing cell line, but only partially reversed adriamycin and vincristine resistance in a second MRP-overexpressing cell line.

Nakano *et al.*, (1998), demonstrated that ONO-1078, a new class of peptide leukotriene receptor antagonist, modulated multidrug resistance and inhibited LTC_4 efflux in lung cancer cells (NCI-H520) by inhibition of MRP function. This resulted in the enhanced sensitivity of these cells to vincristine, doxorubicin and etoposide. Marbeuf-Gueye *et al.*, (2000), reported that 2-[4-(diphenylmethyl)-1-piperazinyl]ethyl-5-(trans-4,6-dimethyl-1,3,2-dioxaphos-phorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P oxide (PAK-104P) was a non-competitive inhibitor of the of both Pgp and MRP1-mediated efflux of anthracycline derivatives and calcein acetoxymethyl ester, at low concentrations. The authors suggested the existence of two very different mechanisms for the inhibition by PAK-104P of the MRP1-mediated efflux of molecules: the first mechanism, involving a low-affinity site for PAK-104P, and which affects molecules such as calcein, LTC_4 etc. whose efflux does not seem to depend on glutathione. The second mechanism involves a high-affinity site for PAK-104P and which interacts with molecules such as the anthracyclines and calcein acetoxymethyl ester whose efflux depends on the presence of glutathione.

Curtin *et al.*, (1999), reported that the nucleoside transport inhibitor, dipyridamole (DP), caused chemosensitisation to VP-16, methotrexate and adriamycin in MRP1-overexpressing cell lines, CORL23(R). However, this effect appears to have been caused by a depletion of cellular GSH rather than a direct effect of DP on MRP-mediated drug accumulation and efflux.

Hipfner *et al.*, (1999), reported that MRP-specific monoclonal antibodies, QCRL-2, -3, -4 and -6 can inhibit the transport of several MRP substrates by interfering with substrate binding or by trapping MRP in a conformation that does not allow transport to occur.

In addition, a number of authors (Bennet *et al.*, 1982; Hall *et al.*, 1989; Maca *et al.*, 1991; Hollo *et al.*, 1996; Draper *et al.*, 1997; Kobayashi *et al.*, 1997; Duffy *et al.*, 1998 and Roller *et al.*, 1999), have described the ability of a number of Non-Steroidal Anti-Inflammatory Drugs (NSAIDS), at non-toxic concentrations, to enhance the toxicity of chemotherapeutic drugs, including adriamycin, daunorubicin, epirubicin, vincristine, methotrexate and VP-16. The spectrum of drugs, on which the NSAIDs exerted their potentiation effect, were recognised as being MRP substrates (Duffy *et al.*, 1998), and the enhancement effect was observed in cell lines which overexpressed MRP (Duffy *et al.*, 1998 and Hollo *et al.*, 1996). These authors also reported that the enhancement effect was independent of Pgp expression and Duffy *et al.*, (1998) and Roller *et al.*, (1999), demonstrated the effect to be independent of COX-1 and COX-2 inhibition. The potentiation of cytotoxic drug toxicity by the NSAIDs is discussed in detail in section 1.9. It appears that these NSAIDs are specific modulators of MRP-mediated resistance and may be of clinical significance as the potentiation effects were evident when these compounds were used at concentrations readily achievable in the blood.

These results suggest that the identification of the specific mechanism of drug resistance is important for the selection of chemotherapeutic strategies to block the efflux pump on the cancer cell. In addition, MRP and Pgp are reported to have protective functions in normal tissues (Twentyman *et al.*, 1997). Therefore, effective clinical application of resistance reversal strategies will depend on optimisation of therapeutic benefit *versus* increased toxicity.

1.6.11 Influence of Drug influx and accumulation on multidrug resistance

Before the chemotherapeutic drug reaches its intracellular target, it has to be taken up in to the cell by some mechanism of drug uptake. In addition to increased drug efflux, it is possible that decreased drug uptake may also contribute to the drug resistance mechanism of a particular cell line. A reduction in drug influx has been identified in some MDR cell sublines as the only factor involved in drug resistance (Pallares-Trujillo *et al.*, 2000). Both the plasma membrane lipid

composition (Pallares-Trujillo *et al.*, 1993), and the pH of the extracellular medium (Gerweck *et al.*, 1999, Larsen *et al.*, 1998 and Larsen *et al.*, 2000), have been reported to affect drug influx.

Many anticancer agents, such as the anthracyclines, adriamycin and daunorubicin, vincristine, vinblastine and mitoxantrone, are weak lipophilic bases with p*K*s between 7 and 9 (Larsen *et al.*, 2000). A substantial fraction of these molecules are uncharged (non-ionized) at normal intracellular pH and in their non-ionized forms the lipophilicity of these drugs is increased, thereby enhancing their diffusion through the cell membrane to an intracellular site of action (Gerweck *et al.*, 1999). Although the intracellular pH of tumour and normal tissues are similar, the extracellular pH of human tumours is more acidic than normal tissues, giving rise to substantially different cellular pH gradients in these tissues (Gerweck *et al.*, 1999). Adriamycin accumulation and toxicity in Chinese Hamster ovary cells was reported by Gerweck *et al.*, (1999), to increase with increasing medium pH and that the pH gradient across the cell membrane was the major determinant of adriamycin uptake. It appears that passive diffusion of the non-ionized form of the drug is the most likely explanation for the pH-dependent modification of cellular drug uptake (and by implication, cytotoxicity).

Several studies suggest that vesicular trafficking may be involved in the uptake, distribution and efflux of many cancer agents (Hindenburg *et al.*, 1989; Coley *et al.*, 1993). Larsen *et al.*, (2000), reported that when the drug encounters an acidic environment, such as the interior of acidic vesicles, it is converted to a charged form that is unable to cross internal membranes. This results in the sequestering and accumulation of such anticancer agents in cytoplasmic organelles, followed by transport to the cell surface and extrusion in to the extracellular environment. Since the equilibrium between the charged and the uncharged forms of the drug is pH-dependent, drug accumulation in acidic vesicles is favoured by a large pH gradient between the cytoplasm and the acidic compartments, whereas acidification of the cytoplasm and/or alkalization of the acidic vesicles decreases drug accumulation in these organelles.

MRP is thought to cause multidrug resistance by decreasing the intracellular concentration of the cytotoxic drugs, and although most MRP-expressing cell lines show a defect in accumulation (Gaj *et al.*, 1998; Zaman *et al.*, 1993; Krishnamachary *et al.*, 1993; Zaman *et al.*, 1994), others do not (Gaj *et al.*, 1998).

There is no clear evidence in the literature to date to suggest a correlation between the MDR-transporters, Pgp and MRP, and drug uptake. However, Colin *et al.*, (1997), suggested that a reduced influx of drugs could be a major defect in MDR cells. Pgp modulators such as verapamil, sodium orthovanadate, chlorpromazine or triperazine induced an enhanced influx in CEM/VLB100 and also suggested a possible role for Pgp-membrane lipids in drug influx.

Protein Kinase C (PKC) may also be involved in the reduction of drug influx (Palleres-Trujillo *et al.*, 2000). Sachs *et al.*, (1995), reported that the PKC-β I isoenzyme, which could reduce drug influx, was also shown to inhibit the Pgp ATPase and drug binding activity through phosphorylation which seemed to suggest an involvement between Pgp and drug uptake. However, Drew *et al.*, (1996), Bergman *et al.*, (1997) and Sedlak *et al.*, (1997), have reported the induction of drug uptake by PKC inhibitors by a mechanism that does not involve Pgp or MRP. This mechanism of drug uptake has yet to be fully elucidated.

Decreased intracellular accumulation of cisplatin and carboplatin has been associated with resistance to these chemotherapeutic drugs (Shen *et al.*, 2000). The mechanism(s) by which cisplatin enters the cell, and by which decreased accumulation occurs in resistant cells have yet to be determined. It has generally been believed that cisplatin enters cells largely through passive diffusion, however, evidence provided by Shen *et al.*, (2000), suggest the involvement of a novel active transport process in the uptake of cisplatin and carboplatin in to the human liver carcinoma cell line, BEL-7404. This uptake was significantly reduced in the cisplatin–resistant derivative 7404-CP20. Shen *et al.*, (2000), demonstrated decreased MRP1 and MRP2 protein expression in this cisplatin resistant cell line making it highly unlikely that MRP1 or cMOAT is involved in reducing cisplatin or carboplatin influx or efflux in this particular cell line.

Nucleoside transporters are involved in the uptake of nucleosides and provide the route of entry in to cells for many cytotoxic nucleoside analogues used in cancer and viral chemotherapy (Baldwin *et al.*, 1999). Nucleoside transport into cells is divided into two categories. In equilibrative transport, the flux of nucleoside molecules across the membrane is driven solely by the concentration gradient, whereas in concentrative transport, the flux is coupled to that of sodium ions such that the electrochemical ion gradient can drive cellular uptake of nucleosides against their concentration gradient (Baldwin *et al.*, 1999). It is possible that this

mechanism is involved in the cellular uptake of chemotherapeutic agents such as 5-FU, Gemcitabine and AZT.

1.7 Nonsteroidal anti-inflammatory drugs (NSAIDs)

The history of aspirin can be traced to ancient Egypt, where an extract of willow bark was used to treat inflammation. Willow bark contains the glucoside of salicyl alcohol, which can be converted to the actual anti-inflammatory agent salicylic acid through stepwise hydrolysis and oxidation (Vaino *et al.*, 1997). The development of aspirin in 1897 was a significant landmark in the history of medicine because it stimulated the development of a family of medicines that are collectively called NSAIDs, which are amongst the most prescribed therapeutic drug class.

NSAIDs such as sulindac, indomethacin and piroxicam are effective in alleviating pain, inflammation and fever, and they are commonly prescribed for the treatment of rheumatoid arthritis (Vaino *et al.*, 1997). In the USA the estimated number of NSAID prescriptions rose from 27.5 million in 1973 to 100 million in 1983., In 1984 it was estimated that nearly one in seven Americans were treated with an NSAID, although since then the prescription trends have levelled off (Pace *et al.*, 1995 and Brooks *et al.*, 1991). Over 20% of Australians over the age of 65 years are exposed to these drugs and in the UK over 20 million prescriptions for NSAIDs are issued each year (Pace *et al.*, 1995). NSAIDs are the principle therapy for the majority of arthritis patients. It has been estimated that more than 15 million people with arthritis take these drugs daily. This use is predicted to increase greatly, not only as a result of an aging population, with the consequent increase in the prevalence of arthritis, but also because NSAIDs may prove to have a role in decreasing colonic neoplasma and in reducing the likelihood of conditions such as Alzheimers disease (Silverman, 1998).

NSAIDs come from a variety of chemical classes (Table 1.7.1). Their physiochemical properties determine their distribution in the body, and thus

differences in these properties may lead to variable therapeutic performance. NSAIDs are planar, anionic molecules that partition into lipid environments, such as the lipid bilayers of plasma membranes, and the more acidic the pH, as at inflammatory sites, the greater the lipophilicity (Abramson *et al.*, 1989). The more lipid soluble NSAIDs penetrate the central nervous system more effectively and may have greater central effects. The central effects of NSAIDs include mild changes of mood and cognition which are more common with the more lipid soluble NSAIDs. The vast majority of these drugs are weakly acidic, with ionizing constants (pKa) ranging from 3 to 5 (Verbeeck *et al.*, 1990). The proportion of an NSAID that is not ionised at a particular pH is of importance as it influences the distribution of these drugs in tissue (Brooks *et al.*, 1991). More acidic NSAIDs become sequestered preferantially in the synovial tissue of inflammed joints, which may be of potential advantage during episodes of arthritis. Some NSAIDs are pro-drugs (such as sulindac), with the active drug being produced *in vivo* by the normal processes of metabolism.

Table 1.7.1 : Chemical Classification of NSAIDs

Carbo- and heterocyclic acetic acids:	
Etodolac	Zomepirac
Indomethacin	Acemetacin
Sulindac	Tolmetin
Phenylacetic acids:	
Diclofenac	Aclofenac
Fenamates:	
Niflumic acid	Flufenamic acid
Mefenamic acid	Tolfenamic acid
Oxicams:	
Isoxicam	Piroxicam
Tenoxicam	Sudoxicam
Propionic acid derivatives:	
Benoxaprofen	Carprofen
Fenbufen	Fenoprofen
Flurbiprofen	Ibuprofen
Ketoprofen	Naproxen
Oxaprozin	Pirprofen
Suprofen	Tiaprofenic acid
Infoprofen	Acetaminophen (paracetamol)
Pyrazoles:	
Azapropazone	Phenylbutazone
Oxyphenbutazone	
Salicylates:	
Aspirin (ASA)	Fendosal
Diflunisal	Salicylic acid
Acetamidophenol	Acetaphenetidin
Salicylamide	
Non-Acidic compounds:	
Nabumetone	Proquazone
Bufecamic	

Compiled from Brooks *et al.*, (1991); Lu *et al.*, (1995) and Verbeeck *et al.*, 1990.

The increase in NSAID use has been accompanied by an increase in adverse effects reports. Brooks *et al.*, (1988), reported that the adverse reactions due to treatment with NSAIDs could be produced by a number of different mechanisms:

i. consequences of inhibition of prostaglandin synthesis,

ii. idiosyncratic reactions, dose-related effects

iii. production of reactive intermediate metabolites.

The most common toxic effects of NSAIDs are gastrointestinal pathology (gastrophy), renal dysfunction, liver function abnormalities and hypersensitivity reactions (Alhava, 1994).

It is generally believed that NSAIDs function pharmacologically primarily by reducing the synthesis of prostaglandins (Vane *et al.*, 1991; Vainio *et al.*, 1997; Vane *et al.*, 1996; Levy *et al.*, 1997 and Abramson *et al.*, 1989). At sites of inflammation, prostaglandins are produced in excess amounts and exert pro-inflammatory effects. It is postulated that NSAIDs reduce the production of prostaglandins by inhibiting the enzyme prostaglandin endoperoxide synthase, which exists in two isoforms 1, and 2, and which transforms arachidonic acid, liberated by phospholipase A_2, to prostaglandins. Vane *et al.*, (1998), reported that the NSAIDs inhibit the binding of the prostaglandin substrate, arachidonic acid, to the active site of the enzymes. This enzyme is commonly referred to as cyclooxygenase (COX-1 and COX-2). COX is a dual function enzyme, incorporating both a cyclooxygenase and a peroxidase activity (Vane *et al.*, 1996).

PGE_2 is the predominant eicosanoid detected in man in inflammatory conditions ranging from experimental acute oedemas and sunburn through to chronic arthritis. It is a potent dilator of vascular smooth muscle which causes the characteristic vasodilation and erythema (redness) seen in acute inflammation. PGE_2 also acts synergistically with other mediators to produce inflammatory pain. Additionally, PGE_2 is a potent pyretic agent and its production, stimulated by the release of IL-1, in bacterial and viral infections contributes to the associated fever. Other COX products have been found in inflammatory lesions including $PGF_2\alpha$, PGD_2, prostacyclin (PGI_2 as 6-oxo-$PGF_1\alpha$) and thromboxane A_2 (TXA_2) (Vane *et al.*, 1996), but usually they are present at less than a quarter of the concentrations of PGE_2.

The inhibition by aspirin is due to the irreversible acetylation of the cyclooxygenase component of COX, leaving the peroxidase activity of the enzyme unaffected. In contrast to the irreversible action of aspirin, other NSAIDs such as indomethacin or ibuprofen produce reversible COX inhibition by competing with the substrate, arachidonic acid, for the active site of the enzyme (Vane *et al.*, 1996; 1998). The inhibition of prostaglandin synthesis by NSAIDs has been demonstrated in a wide variety of cell types and tissues, ranging from whole animals and man to microsomal enzyme preparations.

Therefore NSAIDs prevent the pathological over-production of prostaglandins which contribute to the inflammatory process (therapeutic effects) and the physiological formation of prostanoids (which results in the characteristic side effects). Several of the NSAIDs inhibit the production of prostacyclin, an important cytoprotective product of the gastric mucosa, potentialy leading to the formation of ulcers (Fosslien *et al.*, 1996). NSAIDs also damage the gastrointestinal tract via other mechanisms including effects on neutrophil function, altering gastric mucosal blood flow in a non-prostaglandin dependent manner, direct irritant effects including ion trapping and interference with growth factors and ulcer healing mechanisms (Donnelly *et al.*, 1997).

1.7.1 COX-1 and COX-2

It is now known that the cyclooxygenase enzyme exists as two distinct isoforms, Cyclooxygenase 1 (COX-1) and Cyclooxygenase 2 (COX-2). COX-1 is constitutively produced and is believed to be involved in regulating normal cellular processes, such as gastro intestinal (GI) cytoprotection, vascular homeostasis, and renal function. The concentration of the enzyme remains largely stable, but small increases of expression of two-to four-fold can occur in response to stimulation with hormones or growth factors (DeWitt *et al.*, 1991). In contrast, COX-2, identified by Fu *et al.*, (1990), as an inducible synthase and a distinct isoform of cyclooxygenase encoded by a different gene from COX-1, is undetectable in most normal tissue. However, the expression of COX-2 can be increased dramatically after exposure of

fibroblasts, vascular smooth muscle or endothelial cells to growth factors, hypoxia, phorbol esters or cytokines and by lipopolysaccharides (LPS) in monocytes/macrophages (Bolten 1998 and Vane *et al.*, 1996). In chronic inflammation, levels of COX-2 protein also increase in parallel with the over-elaboration of prostaglandins in many cells and tissues. The family of primary response genes, of which COX-2 is a member, also includes the gene for inducible nitric oxide synthase which is induced during inflammation and cell growth. Conversely, COX-2 seems to play a physiological role in some situations. It is believed to be involved in uterine contraction and appears to be present in the renal medulla. Recently, Hinz *et al.*, (1999), reported that COX-2 is expressed constitutively in the brain, spinal cord and kidney as well as numerous other organs. Hence COX-2 inhibition may lead to adverse effects in these tissues (Donnelly *et al.*, 1997).

Human COX-1, a 22kb gene, is a membrane bound hemo-and glyco-protein with a molecular weight of 71kD. It is found in greatest amounts in the endoplasmic reticulum of prostanoid forming cells. It functions by cyclizing arachidonic acid and then adding the 15-hydroperoxy group to form PGG_2. The hydroperoxy group of PGG_2 is reduced to the hydroxy group of PGH_2 by a peroxidase (in the same enzyme protein). PGH_2 is then coverted to PGE_2, $PGF_{2\alpha}$. $PGD_{2\alpha}$, PGI_2 and thromboxane A_2. The three-dimentional structure, determined by Picot *et al.*, (1994), shows that COX-1 comprises three independent folding units: an epidermal growth factor like domain, a membrane binding motif and an enzymatic domain. The sites for peroxidase and COX activity are adjacent but spatially distinct. Three of the helices of the structure form an entrance channel to the active site and it is postulated that their insertion into the membrane could allow arachidonic acid to gain access from the interior of the lipid bilayer. The COX active site is a long hydrophobic channel. Tyrosine at position 385 and serine 530 are at the apex of this active site. Aspirin irreversibly inhibits COX-1 by acetylation of serine 530 and as a result excludes access by arachidonic acid. Other subsites for drug binding may exist in the channel to explain the interactions of reversible COX inhibitors such as indomethacin and ibuprofen (Vane *et al.*, 1996).

The human COX-2 gene is 8.3kb in size, smaller than COX-1 but with a similar molecular weight. The amino acid sequence of its cDNA shows only a 60%

homology with COX-1. COX-2 and COX-1 also have similar active sites for the attachment of arachidonic acid or NSAIDs, although the active site of COX-2 is larger than that of COX-1 and can accept a wider range of structures as substrates (Meade *et al.*, 1993). Both enzymes have similar Km and Vmax values for the metabolism of arachidonic acid. (Meade *et al.*, 1993).

1.7.2 NSAIDs and inhibition of COX-1 and COX-2.

NSAID inhibit the synthesis of cytoprotective prostaglandins by COX-1 in the gastrointestinal tract leading to the accumulation of surplus arachidonic acids. The surplus enhances the generation of leukotrienes via the lipoxygenase pathway inducing neutrophil adhesion to endothelium and vasoconstriction. The NSAIDs containing a carboxyl group also inhibit oxidative phosphorylation (OXPHOS) lowering adenosine-triphosphate (ATP) generation, leading to loss of mucosal cell tight junctions and increased mucosal permeability (Fosslien, 1998). Inhibition of COX-2 by NSAIDs reduces synthesis of pro-inflammatory prostaglandins and produces analgesia (Fosslien, 1998). Evidence, therefore, suggests that much of the GI toxicity associated with NSAID use is primarily the result of inhibition of COX-1 and anti-inflammatory effects are largely due to the inhibition of COX-2. An NSAID, which selectively inhibits COX-2, and has little effect on COX-1, would be ideal. However, on the basis of data obtained in several laboratories by means of the "human whole blood assay", there is now convincing evidence that none of the currently available NSAIDs is a selective COX-2 inhibitor (Hinz B *et al.*, 1999). A number of COX-2 specific inhibitors are presently being tested world wide in phase III clinical trials on patients with rheumatoid arthritis and osteoarthrits (Hinz B *et al.*, 1999; Lefkowith et al., 1999).

Aspirin, indomethacin, piroxicam, tolmetin and sulindac are slightly more potent against COX-1 than COX-2 but are generally considered as non-selective NSAIDs (Riendeau *et al.*, 1997). These drugs are also known for their propensity to cause gastric damage. Meloxicam has been shown by Engelhardt *et al.*, (1996), to possess a high preference in intact cells for inhibition of the COX-2 rather than COX-1 isoenzyme. Two case control studies carried out in 1994 and data obtained from the UK Committee on the Safety of Medicines showed that ibuprofen and diclofenac

were associated with the least number of adverse effects, while piroxicam was associated with the most (Bateman, 1994).

1.7.3 Non-prostaglandin-dependent properties of NSAIDs

NSAIDs exert biological effects other than the inhibition of PG synthesis. It is suggested that this broad spectrum of effects results from their physiochemical properties, which may disrupt protein-protein interactions of many kinds within biological membranes (Abramson *et al.*, 1989).

NSAIDs have been found to inhibit a variety of membrane-associated processes, including that of superoxide anion generation by a cell-free NADPH oxidase system of neutrophils, mononuclear cell phospholipase C activity, and the 12-hydroperoxyeicosatetraenoic acid peroxidase of the lipoxygenase pathway in platelets. Aspirin-like drugs uncouple oxidative phosphorylation via effects with in the mytochondrial membrane. Aspirin also alters the uptake of precursor arachidonate and its insertion into the membrane of cultured human monocytes and macrophages. Salicylates inhibit anion transport across a variety of cell membranes including human erythrocyte and renal tubular epithelium. (Abramson *et al.*, 1989).

Acetaminophen, an anti-pyretic and analgesic, has little anti-inflammatory activity. Salicylate is an order of magnitude less active than aspirin on the crude COX-enzyme prepared from lung tissue, yet it is reported that salicylate is as potent as aspirin in suppressing arthritis (Vane *et al.*, 1996 and Mitchell *et al.*, 1994).

1.7.4 Initiation of carcinogenesis by Prostaglandin H synthases

Prostaglandin H synthases (PHS) (COX-1 and COX-2) may be involved in the initiation of carcinogenesis by activation of carcinogens to DNA-binding forms. The peroxidase activity of PHS has a broad specificity. Other hydroperoxides, other than the primary substrate PGG, can be reduced by PGH peroxidase. Among the classes of carcinogens that can be used as electron donors for "co-oxidation", and thereby activated by PHS, are polycyclic aromatic hydrocarbons, aflatoxins, halogenated pesticides, aromatic amines and phenols (Levy *et al.*, 1997). PHS may be also

involved in initiation of carcinogenesis by the generation of malondialdehyde (MDA), a direct acting mutagen in bacterial and mammalian test systems and also carcinogenic in rats. MDA is produced by non-enzymatic as well as enzymatic breakdown of PGH. The enzyme Thromboxane Synthase is particularly active in MDA production. MDA formation from PGH may be inhibited by NSAIDs (Marnett, 1992). A third suggested mechanism for the involvement of PHS in tumour initiation involves peroxyl radicals as reactive intermediates (Levy *et al.*, 1997). Peroxyl radicals may be formed by PHS and can epoxidize double bonds of pro-carcinogens such as benzopyrene-7,8-diol or dihydroxydihydrobenzo-anthracene to produce the carcinogens of these compounds (Levy *et al.*, 1997).

1.7.5 NSAIDs and Cancer

Chemoprevention is defined as the use of natural or pharmacological agents to disrupt the process of carcinogenesis (Garay et al., 1999).

The origin of the hypothesis that NSAIDs have chemopreventative properties, dates back to 1975 when Bennet and Del Tacca (1975), observed that certain human cancers, including breast cancer and colorectal cancers contain more prostaglandin E_2 than surrounding normal mucosa. They hypothesized that tumours that overproduce prostaglandin E_2 might promote their own growth and /or spread (Bennet *et al.*, 1975; Hwang *et al.*, 1998; Vainio *et al.*, 1997).

Because NSAIDs reduce the synthesis of prostaglandins, a series of experimental studies in rodents were carried out to assess whether NSAIDs would inhibit or prevent the growth of coloretal cancer and various other forms of cancer. Most of the NSAIDs tested (aspirin, sulindac, piroxicam and indomethacin) effectively inhibited colorectal tumours in rats and mice. Oesophageal tumours in mice treated with N-nitrododiethylamine were reduced by indomethacin administered either with the carcinogen or 4 months after exposure (Rubio *et al.*, 1984). Pancreatic tumours induced by N-nitrosobis(2-oxopropyl)amine were reduced in hamsters receiving indomethacin, aspirin or phenylbutazone beginning five weeks after initiation of carcinogenesis (Takahashi *et al.*, 1990). Narisawa *et al.*, (1981), demonstrated that rats exposed to methylnitrosourea developed colonic tumours but that their

incidence could be significantly lowered by indomethacin. However, in rats given the carcinogen and then indomethacin, cessation of treatment led to rapid development of tumours. Thus, indomethacin treatment inhibited the development of tumours but did not kill the initiated cells. Piroxicam was shown by Reddy *et al.*, (1987), to inhibit azoxymethane-induced colon cancer in rats in a dose-dependent manner. Sulindac was shown by Moorghen *et al.*, (1988), to have a protective effect against dimethylhydrazine-induced colonic tumours in mice. Sulindac administered with the carcinogen caused a significant reduction in tumour incidence and tumour burden but sulindac administered 17 weeks after the carcinogen, had no inhibitory effect, thus demonstrating protection against initiation of tumours but no effect on regression of established tumours in this model. The incidence of adenocarcinomas was reduced 69% in rats with dimethylhydrazine-induced colonic carcinogensis, by aspirin received one week before and after the carcinogen (Craven *et al.*, 1992). Aspirin also had no effect on tumour incidence when started 4 weeks after carcinogen exposure.

1.7.6 NSAIDs in Human Cancer.

Colorectal cancer is a major cause of death in the United States where it accounts for approximately 57,000 deaths per year (Garay *et al.*, 1999). Differences in dietary habits and lifestyles among populations in different geographic locations have been associated with an altered risk for developing colorectal cancer (Smalley *et al.*, 1997).

In 1988, Kune *et al.*, reported an observational study in which a negative association was found between the incidence of colon cancer and the use of aspirin. This Australian study demonstrated that people who used aspirin had a 40-50% reduction in colon cancer incidence than those who reported no aspirin use and the reductions in colon cancer were of similar magnitude in men and women. Reduction of a lesser magnitude was noted with the use of other NSAIDs. No reduction of risk was observed with the use of steroids, oral contraceptives, tranquilizers or sedatives. Thun *et al.*, (1991), reported that in a study of over one million persons, men who used aspirin more than 16 times a month had a relative risk of developing colorectal cancer of 0.48 (0.30-0.76) and women a relative risk of 0.53 (0.32-0.87) compared

to those who didn't regularly use aspirin. Giovannucci *et al.*, (1994), reported similar results from a study involving persons between the ages of 40 -70 who regularly and consistently took aspirin. Analysis of the duration of aspirin use showed that the protective effect of aspirin did not become statistically apparent unless intake had been for ten years or greater. This was attributed to the idea that adenomas take approximately 10 years to evolve into invasive carcinomas. (Giovannucci *et al.*, 1995). In contrast to the results for aspirin use, no association of change in fatal colon cancer risk with acetominophen use was found (Levy *et al.*, 1997).

1.7.7 NSAIDs and Familial Adenomatous Polyposis

Further evidence for the anti-carcinogenic effect of NSAIDs is provided by studies of familial adenomatous polyposis (FAP), an autosomal dominant disorder characterised by the formation of hundreds to thousands of colorectal adenomas/polyps in adults, usually below the age of thirty, and subsequent elevated risks of development of colorectal cancer (Levy *et al.*, 1997 and Owen *et al.*, 1998). Clinical trials using the NSAID sulindac have shown dramatic regression of colonic adenomas in patients with FAP (Wadell *et al.*, 1983; Tonelli *et al.*, 1994; Piazza *et al.*, 1997 and Ahnen, 1998). D'Alteroche *et al.*, (1998), reported the complete remission of a mesenteric fibromatosis in a male patient, after taking sulindac for periods of 1 to 8 months for 6 years, 11 years after having familial adenomatous polyposis coli treated by total colectomy. Hirata *et al.*, (1994), demonstrated the regression of rectal polyps by indomethacin suppositories in FAP although it had previously been reported that oral indomethacin failed to reduce the number of rectal adenomas (Hirota *et al.*, 1996).

Boolbal *et al.*, (1996), used a mouse model of FAP in which a strain containing a dominant mutation in the APC (adenomatous polyposis coli) tumour suppressor gene was developed. This strain, known as MIN mice, developed gastrointestinal adenomas by 110 days of age. It was found that the MIN mice produced increased levels of COX-2 and PgE_2 compared to wild type mice. Treatment with sulindac inhibited tumour formation and decreased $PGHS_2$ and PgE_2 levels to baseline as well as restoring normal levels of apoptosis (normally at a level of 27-47% of the

levels of wild-type mice) (Giardiallo *et al.,* 1998; and Vainio et al., 1997). In a study using piroxicam in the MIN mouse, Jacoby *et al.*, (1996), showed a dose-dependent decrease in the intestinal adenomas and aberrant crypt foci as well as a parallel decrease in serum levels of thromboxane B_2. Watson (1998), reported that mutation of APC caused the up-regulation of COX-2 whose products cause further tumour progression. Treatment of APC-mutated mice, with an NSAID selective for COX-2 reduced tumour formation significantly.

However, Watson (1998), also raised the possibility that inhibition of submucosal COX-1 by NSAIDs could reduce the mutation frequency in colonic epithelium and prevent polyp initiation and this could explain how NSAIDs, such as aspirin and sulindac, which have greater potency against COX-1 than COX-2, can inhibit polyp formation.

Koki *et al.*, (1999), characterised the expression of COX-1/-2 in biopsies of human lung, colorectal, oesophageal, breast, pancreatic and prostate cancers to assess the importance of COX enzymes in tumourigenesis. In human cancers, COX-2 was consistently detected in the angiogenic vessels, neoplastic epithelium and in inflammatory cells. COX-2 was either not expressed, or detected at very low levels in normal, compared to hyperplastic or neoplastic regions. In contrast COX-1 was ubiquitously expressed throughout the malignant and non-malignant areas. (Langman *et al.*, 1998; Koki *et al.*, 1999 and Gilhooly *et al.*, 1999).

A recent study found a statistically significant elevation of PGE_2 in 21 surgically excised colorectal cancers compared to the accompanying normal colorectal mucosa. (Sheng *et al.*, 1998).

1.7.8 NSAIDs and Apoptosis.

Another common property of NSAIDs and related drugs that may explain their anti-neoplastic effect on colorectal cancer is their ability to induce apoptosis of colonocytes (Levy *et al.*, 1997).

This explanation for the antineoplastic properties of NSAIDs was first suggested by Aldolphie *et al.*, (1972), who reported that certain NSAIDs were capable of inhibiting the proliferation of cultured HeLa cells by causing cell cycle arrest.

Programmed cell death (Apoptosis) is an intrinsic part of organismal development and aging (Lu *et al.*, 1995). It is an orderly process of internal cellular disintegration which is associated with membrane blebbing, structural condensation and the maintenance of some organelle integrity (Samaha *et al.*, 1997).

Until recently, reports in the literature attributed the anti-neoplastic activity of sulindac and other NSAIDs to their ability to inhibit protaglandin synthesis. This rationale was based on studies showing that the levels of PGE_2 and mRNA for COX-2 are elevated in a number of human cancers (Hubbard *et al.*, 1988). Prostaglandins stimulate the proliferation of cancers (Qiao *et al.*, 1995), and PGE_2 may interfere with host anti-tumour immunologic functions (Sheng *et al.*, 1998).

1.7.9 COX-2 and apoptosis.

Colonic neoplasms are believed to develop through a series of sequential steps over 15-20 years that reflect the progressive accumulation of mutations. Early in the transition from normal colonic epithelium to adenoma, mutations of key genes occur, which have been implicated in the transformation process. One such gene is the APC gene. Inactivating mutations of this gene are known to cause colorectal cancer in patients with familial adenomatous polyposis syndrome, and truncation mutations in the APC gene occur somatically in a large percentage of colorectal cancers that form spontaneously (Sheng *et al* 1998). Recently, it has been shown that disruption of the APC gene in mice leads to increased levels of COX-2 in intestinal tumours (Oshima *et al.*, 1996). Therefore, the increased levels of COX-2 in intestinal tumours could represent an event downstream of an early mutation in a key regulatory gene, such as APC (Oshima *et al.*, 1996). COX-2 levels are increased in human colorectal adenocarcinomas (Kargman *et al.*, 1995; Kutchera *et al.*, 1996). Forced expression of COX-2 in intestinal epithelial cells leads to inhibition of apoptosis and induction of Bcl-2 expression (Sheng *et al.*, 1998). These authors determined the effect of COX-2-derived eicosanoids, such as PGE_2 on the biology of human colon cancer cells. In addition to observing an

increase in the number of HCA-7 human colon cancer cells, in response to PGE_2 treatment, there was also a reduction in the percentage of cells undergoing apoptosis. They also determined that PGE_2 treatment led to a significant induction of Bcl-2 (the anti-apoptotic oncogene) expression but not Bax or Bcl-x. PGE_2 treatment also led to a marked activation of MAPK, which preceded induction of Bcl-2 and which may indicate a putative signal transduction pathway by which Bcl-2 expression and other genes could be induced. In the HCA-7 cell line apoptosis was found to be regulated by treatment with a highly selective COX-2 inhibitor (SC-58125), and that this is reversible upon treatment with PGE_2.

Watson *et al.*, (1998), reported that once polyp initiation had occurred, COX-2 was expressed in epithelial cells and acted to inhibit apoptosis and drive the growth of the tumour through interactions with growth factors such as TGF-β. TGF-β inhibits proliferation in normal intestinal epithelium but this anti-proliferative effect is frequently lost after malignant transformation. This change in the action of TGF-β from anti-proliferative to pro-proliferative is thought to be due to COX-2 overexpression. COX-2 also enhanced angiogenesis and cell migration and thereby aided metastasis (Watson *et al.*, 1998). This indicates that COX-derived eicosanoids are likely to play an important role in colorectal carcinogenesis. Many tumour types over-produce prostanoids and these prostanoids have been shown to increase the proliferation rate of tumour cells and related cells *in vivo* (Qiao *et al.*, 1995). NSAIDs such as indomethacin can cause a subsequent reduction in tumour cell growth by inhibition of this growth stimulatory activity (Tripathy *et al.*, 1996 and Tanaka *et al.*, 1989).

1.8 The anti-tumour activities of NSAIDs

It is widely assumed that the inhibition of PGHS activity is the main mode of action by which the NSAIDs assert their anti-neoplastic effect (Bennet and Del Tacca, 1975 and Marnett *et al.*, 1992). However, the mechanism by which NSAIDs influence tumour growth is presently unclear.

A number of recent reports indicate that there are several other possible explanations for the anti-tumour activity of NSAIDs. Suggested mechanisms include induction of apoptosis in the cancer cell by the NSAID, independent of COX inhibition, and interference with the tumour cell cycle (Shiff *et al.*, 1995; 1996 and Qiao *et al.*, 1998). It is known that the influence of NSAIDs on tumour cell growth is not entirely dependent on PGHS inhibition because of a number of factors:

i. DeMello *et al.*, (1980), demonstrated that there was no correlation between the ability of an NSAID to inhibit tumour cell growth and the potency of that NSAID as an inhibitor of PGHS.

ii. When indomethacin was used to inhibit cell growth, the inhibition could not be overcome by the addition of prostaglandins or arachidonic acid. If inhibition of PG synthesis was the mechanism of NSAID cytostasis, addition of PG would be expected to overcome this inhibition. This was not the case; in fact higher concentrations of PG enhanced the cytostatic effect of indomethacin (DeMello *et al.*, 1980). This was also demonstrated by Planchon *et al.*, (1995), with the breast cancer cell line MCF-7.

iii. Sulindac is a pro-drug that is metabolised to a pharmacologically active sulfide derivative that potently inhibits prostaglandin synthesis. Sulindac can also be reversibly oxidised to sulindac sulfone, which is devoid of any PGHS inhibitory activity. Sulindac sulfone has been shown to inhibit HT-29 colon carcinoma cell growth to an extent comparable to that achieved with sulindac sulfide (Piazza *et al.*, 1995). This inhibition was not due to inhibition of prostanoids due to the lack of any PGHS-inhibitory activity. Therefore, the mechanism of the anti-neoplastic affect of NSAIDs does not necessarily include anti-prostaglandin activity. (Piazza *et al.*, 1995; 1997a; 1997b; Levy *et al.*, 1997).

iv Haniff *et al.*, (1996), demonstrated that sulindac sulfide and piroxicam had comparable growth inhibitory effects on two cell lines: HT-29, which produces PGE_2,. $PGF_{2\alpha}$ and PGI_2 and HCT-15, a cell line which does not possess PGHS and hence is devoid of prostanoid synthesising ability. Therefore, both NSAIDs exerted their growth inhibitory effects independent of any inhibition of PGHS.

In 1995, Lu *et al.* used chicken embryo fibroblasts (CEFs) transformed with a temperature sensitive mutant of the Rous sarcoma virus (RSV) to demonstrate the ability of a large number of NSAIDs to induce programmed cell death. Of the effective drugs, indomethacin, diclofenac, carprofen, niflumic acid, mefanamic acid, flufenamic acid, diflunisal and acemetacin were the most potent in inducing apoptosis in the cells. Treatment of the CEFs with any of the above eight NSAIDs induced transglutaminase (Tgase), a molecular indicator of apoptosis. This induction was not proportional to the ability of these NSAIDs to inhibit COX-1 and/or COX-2. Piazza *et al.*, (1995), concluded that increased apoptosis was the mechanism for growth inhibition of cells by sulindac sulfide and sulfone rather than inhibition of cell proliferation, altered differentiation or necrotic cell death.

The pro-apoptotic ability of sulindac and its metabolites was further defined by Han *et al.*, (1998), who demonstrated that sulindac and its metabolites were capable of inducing apoptosis in MCF-10F and MCF-7, a normal human mammary epithelial cell line and a human mammary carcinoma cell line respectively (Han *et al.*, 1998). Treatment of MCF-10F and MCF-7 cells with sulindac sulfide, the most potent inhibitor of cell growth of the three compounds tested by Han *et al.*, (1998), resulted in accumulation of the cells in the G0/ G1 phase of the cell cycle, decreases in the percent of cells in the S and G2/M phases of the cell cycle, and induction of apoptosis. Chan *et al.*, (1998), demonstrated the ability of sulindac sulfide to induce apoptosis in two colon cancer cell lines, HCT116 and SW480.
It was recently reported by Thompson *et al.*, (1997), that sulindac sulfone inhibited the occurrence of mammary carcinomas which were classified as having either the wild type or mutant Ha-*ras* gene, but was significantly more potent in the the case of carcinomas with the mutant Ha-*ras* gene. This group suggested that the effect of sulindac sulfone on mammary carcinogenesis involved interference in the signal transduction cascade involving Ha-*ras*.

Schiff *et al.*, (1996), evaluated the effect of four structurally unrelated NSAIDs: aspirin, indomethacin, naproxen and piroxicam, on cell proliferation, cell cycle phase distribution and the development of apoptosis in the HT-29 colon adenocarcinoma cells *in vitro*. All of the NSAIDs examined reduced the proliferation and altered the morphology of these cells in a time- and concentration-

dependent manner. In addition, they increased the proportion of the cells in the G0/G1 phase and reduced the proportion in the S phase of the cell cycle. Aspirin and indomethacin also reduced the percentage of cells in the G0/M phase but naproxen and piroxicam did not. Han *et al.*, (1998), reported that sulindac sulfide treatment of the breast cancer cell lines resulted in:

i. An increase in the cyclin dependent kinase (CDK) inhibitor protein $p21^{WAF1}$, which is often induced during differentiation or apoptosis, within 24 hours after treatment, and may be mediated by wild type p53 gene;

ii. A decrease in the cyclin D1 expression. Cyclin D1/CDK4 complex regulates progression through the G1 phase of the cell cycle. However, sulindac sulfide had no effect on the expression of CDK4 or the immediate early response gene c-*jun*.

Parallel to their effect on the cell cycle, aspirin and indomethacin also reduced the levels of $p34^{cdc2}$ and $p33^{cdk2}$, two cyclin-dependent kinases that are important for cell cycle progression (Shiff *et al.*, 1996).

Schiff *et al.*, (1996), and Qiao *et al.*, (1998), reported that indomethacin, naproxen, aspirin and piroxicam induced apoptosis in HT-29 colon adenocarcinoma cells.

Chan *et al.*, (1998), hypothesised that the tumour suppressive effects of NSAIDs are not likely to be due to the reduction in prostaglandins but rather are due to the elevation of the prostaglandin precursor arachidonic acid (AA). NSAID treatment of colon tumour cells results in a dramatic increase in AA that in turn stimulates the conversion of sphingomyel into ceramide, a known mediator of apoptosis.

1.9 NSAIDs and enhancement of cytotoxicity of chemotherapeutic drugs

In 1982, Bennet *et al.*, demonstrated that indomethacin and flurbiprofen decreased cancer development and spread in mice bearing a transplanted mammary adenocarcinoma when treated in combination with methotrexate and melphalan. Indomethacin increased cytotoxicity of methotrexate to the human breast cell lines DU4475 and T47D (though the mechanism of this enhancement was unknown). Hall *et al.*, (1989), demonstrated that indomethacin enhanced the toxicity of chlorambucil (5.5-fold) in a resistant Chinese Hamster Ovary cell line.

Non-toxic concentrations of indomethacin were reported by Maca, (1991), to have the ability to potentiate the toxicity of VP-16 and methotrexate in a number of cell lines including cultured Lewis lung carcinoma (LLC) cells, mouse lymphoma cells, a human acute lymphoblasic leukemia cell line and human chronic myelogenous leukemia cells. These cell lines are relatively insensitive to VP-16 alone but indomethacin was found to enhance VP-16 toxicity in all cases and this enhancement occured by increasing the cellular concentration of VP-16. In 1995, Jimbo *et al.,* reported that the antitumour effect of a synthetic lipid A derivative, DT-5461a, which markedly inhibited the growth of various syngenic tumours in mice, was enhanced by indomethacin.

Hollo *et al.*, (1996), reported the ability of the NSAID indomethacin to inhibit MRP-mediated calcein acetoxymethylester (calcein AM) extrusion from S1MRP cells (MRP-transfected S1 cells re-selected and cloned in ADR-containing media) at IC_{50} concentrations of 10-20μM. In contrast, indomethacin was unable to inhibit MDR1 (Pgp)-mediated calcein AM extrusion by 50% at concentrations less than 800μM. This suggests that indomethacin is selectively an MRP inhibitor. Draper *et al.*, (1997), reported that indomethacin increased multidrug susceptibility of both murine and human cell lines overexpressing MRP, but not those displaying Pgp-associated resistance. Indomethacin increased the accumulation of vincristine in MRP-overexpressing cell lines and sensitised these cell lines to vincristine and adriamycin. Indomethacin had little effect on the function of P-gp as it failed to modulate the P-gp-mediated altered accumulation of vincristine and failed to alter the drug resistance of both human and murine P-gp- overexpressing cell lines. This report suggested that indomethacin acts as a specific inhibitor of MRP and so modulated drug resistance as a result of this activity (Draper *et al.*, 1997). Kobayashi *et al.*, (1997), reported that indomethacin, mefenamic acid, sulindac and tolmetin enhanced the toxicity of both adriamycin and vincristine in two pulmonary adenocarcinoma cell lines. Indomethacin was shown to enhance the toxicity of methotrexate, adriamycin, VP-16 and vincristine but not cyclophosphamide, mitomycin C, 5-fluorouracil, cisplatin, vindestine or cytarabine. Djordjevic *et al.,* (1998), reported that there was no synergistic interactions between 5-FU and indomethacin in a human colon adenocarcinoma cell line, and, in fact, this combination appeared to result in an

alleviation of the 5-FU toxicity. In 1999, Roller *et al.*, reported that indomethacin and ibuprofen specifically enhanced the cytotoxic effects of adriamycin and vincristine in T98G, human malignant glioma cells. In contrast, these NSAIDs did not alter the cytotoxic effects of cytarabine, camptothecin or cisplatin. The authors concluded that the effects of indomethacin and ibuprofen were not related to alterations in the formation or detoxicification of reactive oxygen species and was also independent of COX-1 and COX-2 expression.

A number of NSAIDs have been demonstrated by our group to have the ability to enhance the cytotoxicity of a number of anti-cancer drugs *in vitro* when co-administered to a multi-drug resistant cell line which overexpresses MRP.
The NSAIDs, indomethacin, sulindac, tolmetin, acemetacin, zomepirac (heteroarylacetic acids) and mefenamic acid, all at non-toxic levels, significantly increased the cytotoxicity of the anthracyclines (doxorubicin, daunorubicin and epirubicin), as well as teniposide, VP-16 and vincristine. The other vinca alkaloids, vinblastine and vinorelbine and other anticancer drugs including methotrexate, 5-fluorouracil, cytarabine, hydroxyurea, chlorambucil, cyclophosphamide, cisplatin, carboplatin, mitoxantrone, actinomycin D, bleomycin, paclitaxel and camptothecin displayed no synergy in combination with the NSAIDs (Duffy *et al.*, 1998). In addition, two sulindac derivatives, sulindac sulfide and sulindac sulfone were found to be active in the combination toxicity assay (Duffy *et al.*, 1998 and Elliot, 1997). As described previously, sulindac sulfone is, by definition, not an NSAID due to the fact that it is not a COX inhibitor.
This paper presents the first clear evidence that enhancement, by a range of NSAIDs, of the cytoxicity of several anti-cancer drugs was MRP related. The authors reported that the enhancement of cytotoxicity was not found in p-170-overexpressing multidrug resistant cell lines, DLKP-A (a 300-fold resistant variant of DLKP, which overexpresses P-170) and DLKP pHaMDR1/A #2 (a MDR1 transfectant of DLKP, which is 15-fold resistant to doxorubicin) but was found in HL60/ADR, CORL23R, A549 and DLKP cell lines which express MRP.

1.10 Aims of thesis.

The initial aim of the thesis was to investigate the structural requirements and aspects of the mechanism of chemotherapeutic drug enhancement by NSAIDs in MRP-overexpressing cell lines. It had been previously reported by our laboratory that indomethacin was capable of potentiating the toxicity of chemotherapeutic drugs, which were MRP substrates, in MRP-overexpressing cell lines (Duffy *et al.,* 1998). Structural analogues of indomethacin were generated in a collaboration with a synthetic organic chemistry laboratory (Dr. Anita Maguire and Dr. Steven Plunkett, Chemistry Dept., University College, Cork) and in the work described here the SAR (Structure Activity Relationship) in a series of indomethacin analogues was investigated in order to elucidate which substituents were required for the enhancement effect to occur. These analogues were analysed in a number of assays to determine if:

i. The indomethacin analogues were capable of enhancing the toxicity of chemotherapeutic drugs and if this enhancement effect was restricted to those drugs known to be MRP substrates.
ii. The mechanism of drug enhancement by the positive analogues was through an interaction with MRP and occurred only in MRP- expressing cell lines.

Most of the undesirable side-effects of NSAIDs are caused by inhibition of cyclooxygenase 1 (COX-1) but not of COX-2 (section 1.7). Furthermore inhibition of COX-2 appears to have anti-tumour effects (section 1.7.9, Sheng *et al.,* 1998). Another aim of this thesis was, therefore, to find an analogue of indomethacin which had reduced COX-1 activity (but, preferably, while retaining COX-2 activity) and to determine the SAR, in the series of indomethacin analogues, for inhibition of these activities. It would also, of course, be beneficial if this work could find an analogue of indomethacin, which had greater ability to enhance to toxicity of chemotherapeutic drugs, at lower concentrations, with less toxicity and reduced toxic side effects than indomethacin.

It has been reported that taxol is a relatively poor MRP1 substrate (Cole *et al.,* 1994). There is evidence however, that resistance to taxol (paclitaxel) and taxotere (docetaxel) can, in part, be attributed to MRP1 (Vanhoefer *et al.,* 1996 and 1997). In

previous work in our laboratory some of the positive NSAIDs were not capable of enhancing the toxicity of taxol in an MRP1-overexpressing lung carcinoma cell line, A549 (Duffy *et al.,* 1998). However, no analysis had previously been carried out on the ability of the NSAIDs to potentiate the toxicity of taxotere, another member of the taxane family of chemotherapeutic drugs. Further experimental work on the taxanes was therefore undertaken in order to assess the effects of selected NSAIDs on toxicities of these drugs in a number of cell lines and to investigate mechanisms by which NSAIDs might enhance the toxicity of taxol and taxotere.

A substantial body of work has been carried out in our laboratory (Duffy *et al.,* 1998), and elsewhere (Hollo *et al.,* 1996; Draper *et al.,* 1997; Koboyashi *et al.,* 1997 and Roller *et al.,* 1999), on enhancement of toxicity of cancer chemotherapeutic drugs in MRP1-overexpressing cell lines. However, in contrast, there have been no corresponding reports in cell lines which overexpress other (more recently discovered) MRP homologues (MRP2-6). A human hepatic cancer cell line, HepG2, which overexpresses cMOAT (MRP2), and ovarian carcinoma cell line (2008) variants, transfected with cDNA for MRP1, MRP2 or MRP3, were investigated to determine if indomethacin, indomethacin analogues or another MRP-positive NSAID, sulindac, were capable of enhancing the toxicity of a range of chemotherapeutic drugs, including taxol and taxotere, in cell lines overexpressing MRP2 or MRP3.

It has yet to be determined if indomethacin is a substrate for MRP homologues other than MRP1. To investigate this DLKP cells were pulse-selected for a period of six weeks with concentrations of indomethacin which resulted in 80-90% cell kill. The resulting proliferating cell population were assessed to determine if resistance to indomethacin cytotoxicity is associated with increased expression of specific MRP homologues.

The mechanism(s) of cisplatin resistance in cancer cells has not as yet been fully elucidated and, in particular, the relationship between cisplatin resistance and MRP overexpression remains unclear. The enhancement of the toxicity of cisplatin by NSAIDs was investigated in a number of cell lines. The aims of this were two-fold:

i. To investigate the possibility that cisplatin may be a substrate of a homologue of MRP other than MRP1.

ii. To determine if non-MRP1 substrates, such as cisplatin, were potentiated by positive NSAIDs in cell lines which overexpress MRP2 or MRP3.

2.0 Materials and Methods

2.1 Ultrapure Water

Ultrapure water, (UHP) was used for 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). A conductivity meter in the system continuously monitored the quality of the UHP.

2.2 Glassware

The solutions utilised in the various stages of cell culture were stored in sterile glass bottles. These sterile bottles and other glassware required for cell culture-related applications were prepared as follows: glassware and lids were soaked in a 2% solution of RBS-25 (AGB Scientific) for 1 hour. After this time they were cleaned and rinsed in tap water. The glassware were then washed in an industrial dishwasher, using Neodisher detergent and rinsed twice with UHP. The materials were finally 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 min at 15 p.s.i.. Thermolabile solutions were filtered through 0.22μm sterile filters (Millipore, Millex-GV SLGV025BS). Large 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

The basal media used during cell culture were 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 each bottle of media by inoculating samples of the media on to Colombia blood agar plates (Oxoid, CM217), Thioglycollate broths (Oxoid, CM173) and Sabauraud dextrose (Oxoid, CM217) and incubating the plates at 37°C and 25°C. These tests facilitated the detection of bacteria, fungus and yeast contamination.

Basal media were stored at 4°C for up to three months. The HEPES buffer was prepared by dissolving 23.8g 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) and 5% foetal calf serum (Sigma, F-7524) were, in the majority of cases, added to volumes of 100ml basal media. 1ml of 100X non-essential amino acids (Gibco, 11140-035) and 1ml of 100mM sodium pyruvate (Gibco, 11360-035) were also added to MEM. Complete media were maintained at 4°C for up to a maximum of 1 week.

2.5 Cells and Cell Culture

All cell culture work was carried out in a class II laminar air-flow cabinet (Nuaire Biological Laminar Air-Flow Cabinet). All experiments involving cytotoxic compounds were conducted in a cytogard laminar air-flow cabinet (Gelman Sciences, CG series). Before and after use the laminar air-flow cabinet was cleaned with 70% industrial methylated spirits (IMS). Any items brought into the cabinet were also cleaned with IMS. At any time, only one cell line was used in the laminar air-flow cabinet and upon completion of work with the cell line the laminar air-flow cabinet was allowed to clear for at least

15 minutes so as to eliminate any possibility of cross- contamination between the various cell lines. The cabinet was cleaned weekly with an industrial disinfectant (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 2.5.1. 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 2.5.1, except for HL60ADR, are anchorage dependent cell lines. HL60ADR cells were grown in suspension in vented 75cm^2 flasks (Costar, 3276) at 37°C in an atmosphere of 5% CO_2 in RPMI media (Gibco, 52400-025) containing 10 % serum.

2.5.1 Subculturing of cell lines

1. The waste 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.
2. 5mls of 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.
3. The trypsin was deactivated by adding an equal volume of complete media to the flask.
4. The cell suspension was removed from the flask and placed in a sterile universal container (Sterilin, 128a) and centrifuged at 1000rpm for 5 minutes.
5. The supernatant was then discarded from the universal and the pellet suspended in complete medium. A cell count was performed and an aliquot of cells was used to reseed a flask at the required density.

Cell line	Basal Medium*	Cell type	Source**
A549	ATCC	Human lung adenocarcinoma	ATCC
DLKP	ATCC	Human lung squamous carcinoma	NCTCC
DLKPSQ	ATCC	Clonal subpopulation of DLKP	NCTCC
DLKPC14	ATCC	Carboplatin-selected variant of DLKPSQ	NCTCC
HL60ADR	RPMI 1640	Adriamycin-resistant variant of HL60 human leukaemia cell line	Dr. M. Centre[1]
2008	RPMI 1640	Ovarian carcinoma	Dr. M. Kool[2]
CORL23(P)	RPMI 1640	Large cell lung cancer cell line	Dr.P.Twentyman[3]
CORL23(R)	RPMI 1640	Adr-resistant variant of CORL23(P)	Dr.P.Twentyman[3]
HepG2	MEM	Human hepatocellular carcinoma	ATCC

Table 2.5.1 Source description and media requirements of cell lines used in experiments described in this thesis

[1]University of Kansas, USA.

[2]Division of Molecular Biology and Centre of Biomedical Genetics, The Netherlands Cancer Institute, Amsterdam.

[3]MRC Clinical Oncology and Radiotherapeutics Unit, Hills Road, Cambridge CB2 2QH, U.K.

* ATCC basal media consists of a 1:1 mixture of DMEM and Hams F12.

**ATCC = American Tissue Culture Collection.

NCTCC = National Cell and Tissue Culture Centre.

RPMI 1640 media supplied as a 1X stock (Gibco, 52400-025)

2.5.2 Assessment of cell number and viability

1. 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.
2. 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.
3. 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.
4. Non-viable cells stained blue, while viable cells excluded the trypan blue dye as their membrane remained intact, and remained unstained. On this basis, % viability could be calculated.

2.5.3 Cryopreservation of cells

1. Cells for cryopreservation were harvested in the log phase of growth and counted as described in Section 2.5.2.
2. Cell pellets were resuspended in a suitable volume of serum. An equal volume of a 10 % DMSO/serum solution was added dropwise to the cell suspension.
3. A total volume of 1ml of this suspension (which should contain approximately $7x10^6$ cells) 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.
4. After a period of three 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

1. A volume of 9ml of fresh growth medium was added to a sterile universal. The cryopreserved cells were removed from the liquid nitrogen and thawed rapidly at 37°C. The cells were removed from the vials and transferred to the aliquoted media.
2. The resulting cell suspension was centrifuged at 1,000 r.p.m. for 5 minutes. The supernatant was removed and the pellet resuspended in fresh culture medium.
3. An assessment of cell viability on thawing was then carried out (Section 2.5.2).
4. Thawed cells were then added to an appropriately sized tissue culture flask with a suitable volume of growth medium and allowed to attach overnight.

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 inoculated on to Colombia blood agar plates (Oxoid, CM331), Thioglycollate broths (Oxoid, CM173) and Sabauraud dextrose (Oxoid, CM217) and incubating the plates at 37°C and 25°C. These tests facilitated the detection of bacteria, fungus and yeast contamination. Complete cell culture media were sterility tested at least four days prior to use, using Columbia blood agar.

2.6 *Mycoplasma* analysis of cell lines

Cell lines were tested for possible Mycoplasma contamination by Dr. Mary Heenan at the National Cell and Tissue Culture Centre, Glasnevin, Dublin 9. 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 an 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 culture 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) which had been supplemented as described above. The plates were incubated for three weeks at 37°C while exposed to $C0_2$. The plates were examined microscopically every 7 days. The appearance of small oval shaped colonies indicated the presence of *Mycoplasma* infection.

2.7 Miniaturised *in vitro* toxicity assays

2.7.1a "Long–term" *in-vitro* toxicity assay experimental procedure

1. Cells in the exponential phase of growth were harvested by trypsinisation as described in section 2.5.1.
2. 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.
3. Cytotoxic drug/NSAID dilutions were prepared at 2X their final concentration in cell culture medium. Volumes of the drug dilutions (100µl) were added to each well using a multichannel pipette. Plates were then mixed gently as above.
4. Cells were incubated for a further 6 days at 37°C and 5% CO_2. At this point the control wells would have reached approximately 80-90% confluency.
5. 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 (IC_{50} of the drug) was determined from a plot of the % survival (relative to the control cells) versus cytotoxic drug concentration.

2.7.1b "Short-term" *in-vitro* toxicity assay experimental procedure

1. Cells in the exponential phase of growth were harvested by trypsinisation as described in section 2.5.1.
2. Cell suspensions containing $2x10^4$ cells/ml were prepared in cell culture medium and the 96-well plates were set up and incubated overnight as described in 2.7.1a.
3. 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 and allowed to incubate at 37°C and 5% CO_2 for 4 hours.

5. After this incubation period the plates were removed from the incubator, the drug/NSAID was removed from the wells and the plates were washed out 4 times with fresh media. After washing, the wells were re-fed with fresh media.

6. Cells were incubated for a further 4 days at 37°C and 5% CO_2. At this point the control wells would have reached approximately 80-90% confluency.

5. 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 (IC_{50} of the drug) was determined from a plot of the % survival (relative to the control cells) versus cytotoxic drug concentration.

2.7.2 Assessment of cell number - Acid Phosphatase assay

1. Following the incubation period of 6 days, media was removed from the plates.

2. Each well on the plate was washed 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.

3. The plates were then incubated in the dark at 37°C for 2 hours.

4. The enzymatic reaction was stopped by the addition of 50µl of 1N NaOH.

5. The plate was read in a dual beam plate reader at 405nm with a reference wavelength of 620nm.

2.7.3a NSAID-mediated drug toxicity enhancement assays (Long-term assays)

1. Cells were trypsinised from the flask in the exponential phase of growth as described in section 2.5.1.
2. 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.
3. Cytotoxic drug dilutions and NSAID dilutions were prepared at 4X their final concentration in media. Volumes of 50µl of the drug dilution and 50µl of the NSAID 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. Solvent control experiments showed that no toxicity enhancement effects were due to the presence of DMSO or ethanol.
4. 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.
5. Cell number was assessed using the acid phosphatase assay (section 2.7.2).
6. Statistical analysis of the data was performed as detailed in Section 2.16.

2.7.3b NSAID-mediated drug toxicity enhancement assays (Short-term assays)

1. Cells were set up in 96-well plates as described in section 2.7.1b.

2. Cytotoxic drug dilutions and NSAID dilutions were prepared and added to the 96-well plates as described in section 2.7.3a.
3. Plates were then mixed gently and allowed to incubate at 37°C and 5% CO_2 for 4 hours.
4. After this incubation period the plates were removed from the incubator, the drug/NSAID was removed from the wells and the plates were washed out 4 times with fresh media. After washing, the wells were re-fed with fresh media or fresh media containing the required dilution of NSAID.
5. Cells were incubated for a further 4 days at 37°C and 5% CO_2. At this point the control wells would have reached approximately 80-90% confluency.
6. Assessment of cell survival in the presence of drug was determined by acid phosphatase assay (section 2.7.2).
7. Statistical analysis of the data was performed as detailed in Section 2.16.

2.8 Procedure for determination of COX-1 and COX-2 activity

2.8.1 ELISA for assessment of COX-2 production

The concentration of PgE_2 in cell culture supernatants was analysed as follows:

1. Cells were seeded at high density (2.5 x 10^5 cells per well) in 6 well plates (Falcon, 3046).
2. Plates were then incubated overnight in serum-containing media.
3. The media was then removed and cells washed twice with DME. The compounds of interest (such as NSAIDs or IL-1β) were then added to the cells at a concentration appropriate for the cell density present. Control wells were treated with media only.
4. After 24 hours the media was removed from the wells, placed in to appropriately labelled eppendorfs and stored at -80°C.

5. Samples were analysed using a PgE_2 enzyme immunoassay kit (Cayman Chemical, 514010). Concentrations of PgE_2 present in the samples were determined from a standard curve of absorbance at 405 nm versus PgE_2 concentration.

The PgE_2 assay kit was based on the competition between PgE_2 and a PgE_2 - acetylcholinesterase conjugate for a limited amount of PgE_2 monoclonal antibody. Because the concentration of the PgE_2 tracer was held constant while the concentration of the PgE_2 varied, the amount of the detectable PgE_2 tracer that was able to bind to the PgE_2 monoclonal antibody was inversely proportional to the concentration of the PgE_2 present.

2.8.2 Procedure for detection of COX-1 activity

The experiment used to test for the ability of the NSAIDs and the UCC compounds to inhibit COX-1 is a spectrophotometric assay in which arachidonic acid is used as the substrate for the COX-1 enzyme.
The COX -1 assay was based on that used by Boopathy *et al.,* (1984) and Piazza *et al.,* (1997):

1. 100ml of 100mM TRIS-HCL (Sigma T-1378) (pH 7.4 – 8) was prepared (1.211g in 100mls UHP).
2. The following compounds were weighed out and added to 100ml of 100mM TRIS-HCL:

0.05mM Glutathione (Sigma G-9027)	15.37mg
0.625μM Haemoglobin (Sigma H-2500)	4.03mg
0.5mM Hydroquinone (Sigma H-9003)	5.51mg
1.25mM $CaCl_2$	18.3mg

3. The reaction mixture for the COX-1 assay was prepared as follows:
   - 250 units of Prostaglandin H synthesis 1 (COX-1) (Cayman Chemicals)
   - 100μM arachidonic acid (Cayman Chemicals, 90010)

- TRIS -HCl + components (as prepared above) - added to make a final volume of 1ml in the reaction mixture.

  (Note: 250 units of COX-1 enzyme was used in the assay as it was assessed in prior trials that this amount of enzyme gave the optimum level of activity when incubated with arachidonic acid.)

4. The reaction mixture was incubated @ 37°C for 20 minutes.
5. The reaction was terminated by the addition of 0.2ml of 100% (w/v) trichloroacetic acid in 1M HCl (91.7ml H_2O + 8.3ml conc. HCl).
6. After thorough mixing, 0.2ml of 1% (w/v) thiobarbituric acid solution (in 1M NaOH) was added and the mixture was heated in a boiling water bath for 20 min.
7. After cooling to room temperature and brief centrifugation, the enzyme activity was measured by the thiobarbituric acid colour reaction of malonaldehyde formed in the reaction and determined by a spectrophotometer at 530nm.
8. To assess the ability of the NSAIDs and BRI compounds to inhibit COX-1, 12µg/ml of the compounds were added to the reaction mixture, the volume brought up to 1 ml with TRIS-HCL + components and the COX-1 assay carried out as per steps 4 - 9.

   12µg/ml of compound was used in the assay as this was found to be the concentration at which indomethacin inhibited COX-1 by approx. 50 %. The enzymatic ability of COX-1 was assessed by its ability to act on the substrate arachidonic acid to form malonaldehyde. The greater the optical density reading the greater the activity of COX-1 in the reaction mixture. Indomethacin was used as the positive control. The BRI compounds were added to the reaction mixture at the same concentration as indomethacin and their abilities to inhibit COX-1 were compared.
9. The controls used in the experiment were:
   i. Arachidonic-negative control.
   ii. Indomethacin-negative control
   iii. DMSO control.
10. In the control reaction mixture arachidonic acid or enzyme was added to the reaction mixture after the addition of the trichloroacetic acid.

2.9 Western blotting

2.9.1 Sample preparation

1. Cells were seeded at a density of $3x10^6$ cells per $175cm^2$ flask two days before the experiment.
2. Media was removed and replaced with media containing the agents of interest (such as a cytotoxic drug).
3. After an appropriate time period, media was removed and cells were trypsinised as described in section 2.5.1.
4. Cells were washed twice with ice cold PBS. All procedures from this point forward were performed on ice.
5. Cells were resuspended in 1ml of NP-40 lysis buffer. Table 2.9.1 below provides the details of the lysis buffer. Immediately before use, 10µl of the 100X stocks listed in table 2.9.2 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 2.9.1: NP-40 lysis buffer

100X stock	Preparation instructions
100mM $Na_3 VO_4$	1.83g $Na_3 VO_4$ in 100ml UHP
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 2.9.2: NP-40 lysis buffer 100X stocks

6. Cells were sonicated immediately with 9 pulses lasting 0.9 seconds at 50% power.
7. Lysed cells were transferred to an eppendorf and pelleted at 13,000 r.p.m for 10 minutes
8. Supernatant was removed and protein concentration quantified as detailed in section 2.9.2. Samples were then stored in aliquots at -80°C.

2.9.2 Protein Quantification

Protein levels were determined using the Bio-Rad protein assay kit (Bio-Rad, 5000006) as follows.

1. A 2mg/ml bovine serum albumin (BSA) solution (Sigma, A9543) was prepared freshly in lysis buffer.
2. A protein standard curve was prepared from the BSA stock with dilutions made in lysis buffer.
3. The Bio-Rad reagent was diluted 1:5 in UHP water and filtered through Whatman paper before use.
4. A 20µl volume of protein standard dilution or sample was added to 0.98ml of diluted dye reagent and the mixture vortexed.
5. After 5 minutes incubation, absorbance was assessed at 570nm.
6. The concentration of the protein samples was determined from the plot of the absorbance at 570nm versus concentration of the protein standard.

2.9.3 Gel electrophoresis

Proteins for analysis by Western blotting were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The stacking and resolving gels were prepared as illustrated in table 2.9.3. The gels were set in clean 10cm x 8cm gel cassettes, which consisted of a glass plate and an aluminium plate. These plates were separated by 0.75cm plastic spacers. The resolving gel was added to the gel cassette and allowed to set while covered with 0.1% SDS.

Once set, the 0.1% SDS was removed and the stacking gel was then added. 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)

	7.5% Resolving Gel	15% Resolving Gel	5%Stacking Gel
Acrylamide stock	3.8ml	5.0ml	0.8ml
UHP water	8.0ml	6.8ml	3.6ml
1.875 M TRIS-HCL pH 8.8	3.0ml	3.0ml	-
1.25 M TRIS-HCL pH 6.8	-	-	0.5ml
10% SDS	150µL	150µL	50µL
10% NH_4- persulfate	60µL	50µL	17µL
TEMED	9µL	10µL	8µL

Table 2.9.3: Preparation protocol for SDS-PAGE gels (2 x 0.75mm gels).

The acrylamide stock in Table 2.9.3.1 was composed of 29.1g acrylamide (Sigma, A8887) and 0.9g NN-methylene bis-acrylamide (Sigma, N7256) made up to 100ml with UHP water. In advance of samples being loaded in to the relevant sample wells, 15µg of protein was diluted in 5X loading buffer (2.5ml 1.25 M TRIS-HCL, 1.0g SDS, 2.5ml mercaptoethanol (Sigma, B6250) 5.8ml glycerol (BDH, 44305) and 0.1% bromophenol blue (Sigma, B8026) made up to 10ml with distilled water). The molecular weight markers (Sigma, P1677) and protein samples (for PGHS-2 analysis) were heated to 95°C for 2 minutes. After heating, equal amounts (15µg in 5X loading buffer in a total volume of 10µl) of protein were added in to each well. 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.

2.9.4 Western Blotting - Transfer of protein to nitrocellulose

1. 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.
2. Protein was transferred from the gel to Hybond ECL nitrocellulose membranes (Amersham, RPN 2020D) by semi-dry electroblotting. 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 from between the filter paper. Nitrocellulose was also soaked in the transfer buffer and placed over the filter paper on the cathode plate. Air pockets were once again removed. The acrylamide gel was layered gently on top of the nitrocellulose. Five additional sheets of transfer buffer-soaked filter paper were placed on top of the gel and all air pockets removed. The proteins were transferred from the gel to the nitrocellulose at a current of 34mA at 15V for 35 minutes.
3. The protocol used for MRP protein analysis is summarised in section 2.9.6.

   For analysis of PGHS-1 and PGHS-2, the membranes were blocked overnight using 5% milk powder (Cadburys; Marvel skimmed milk) in TBS (125mM NaCl, 20mM TRIS pH 7.5) at 4°C.
4. Membranes were treated with primary antibody overnight at 4°C (with prostaglandin H synthase I monoclonal antibody (Cayman Chemicals 160110) or prostaglandin H synthase 2 (human) polyclonal antibody (Cayman Chemicals, 160107) diluted at 1 in 1,000) a negative control where the gel was exposed to antibody diluent or animal pre-bleed was also performed.
5. Primary antibody was removed after this period and the membranes rinsed 5 times with TBS for a total of 30 minutes.
6. Secondary antibody (1 in 1,000 dilution of anti-mouse IgG peroxidase conjugate (Sigma, A4914) in TBS or a 1 in 1,000 dilution of anti-rabbit IgG (Sigma, A4914) in TBS) was added for 1 hour at room temperature.

7. The membranes were washed thoroughly in the wash buffer (TBS containing 0.5% Tween (Sigma, P7949)) for 15 minutes.

2.9.5 Enhanced chemiluminescence (ECL) detection

Immunoblots were developed using an Enhanced Chemiluminescence kit (Amersham, RPN2109) which facilitated the detection of bound peroxidase-conjugated secondary antibody.

1. Following the final washing, nitrocellulose filters were subjected to ECL.
2. A layer of parafilm was flattened over a glass plate and the nitrocellulose placed gently upon the plate.
3. A volume of 3ml of a 50:50 mixture of ECL reagents was used to cover the nitrocellulose.
4. The ECL reagent mixture was completely removed after a period of one minute and the membrane wrapped in clingfilm. All excess air bubbles were removed.
5. The nitrocellulose was then exposed to autoradiographic film (Kodak, X-OMATS) for various times (from 1 to 15 minutes depending on the signal).
6. The exposed autoradiographic film was developed for 3 minutes in developer (Kodak, LX-24) for 2 minutes.
7. The film was then washed in water for 15 seconds and transferred to a fixative (Kodak, FX-40) for 2 minutes.
8. The film was then washed with water for 5-10 minutes and left to dry at room temperature.

2.9.6 Western blot analysis of MRP in inside-out vesicles.

Western blotting for MRP protein detection in IOVs was performed as described in Sections 2.9.1 to 2.9.5 with the following modifications:

1. A total of 20µg protein was diluted in 5X loading buffer and loaded onto the 5% stacking gel and 7.5% resolving gel.
2. Molecular weight markers (New England Biolabs, 7708S) were heated to 95-100°C for 3-5 minutes or heated at 40°C for 1 minute.
3. Membranes were blocked using 5% milk (Cadburys: Marvel skimmed milk) in TBS (125mM NaCl, 20mM TRIS pH 7.5) for 4 hours at room temperature.
4. Anti-human MRP monoclonal primary antibody (TCS Biologicals, ZUMC-201) was added (1:50 dilution in TBS) to membranes overnight at 4°C.
5. Rabbit anti-rat secondary antibody (Dako, P04050) was added at a 1:12,000 dilution in TBS for 2 hours at room temperature. Membranes were then washed 5 times in TBS containing 0.5% Tween-20 (Calbiochem, 655205).
6. A SuperSignal ULTRA Chemiluminescence kit (Pierce, 34075) was used for the development of immunoblots. This involved mixing 1.5ml Super Signal Reagent 1 with Super Signal reagent 2 and addition of this mixture to the membranes as described in 2.9.5. After 5 minutes the reagents were removed and the membrane wrapped in cling film. This was then exposed to autoradiographic film for 30 seconds. Autoradiographed film was processed as described in Section 2.9.5.

2.10 Inside-out vesicle preparation

2.10.1 Spinner flask preparation

Spinner flasks were soaked in a solution of 2% RBS for one hour and scrubbed vigorously with a scrubbing brush. They were rinsed three times with tap water and three times in UHP water. Spinner flasks were allowed to completely dry at 37°C, and after this point were treated with 10ml of dimethylchlorosilane (Sigma, D6258). The vessel was rotated to ensure exposure of all glass surfaces to the siliconising agent. Surplus fluid was then removed and the vessel left in the

fume hood to dry. Once completely dry, the flask was rinsed three times in UHP water. The spinner flasks were then autoclaved (121°C for 20 minutes).

2.10.2 Large Scale culture of cells.

1. Cells were subcultured into 75cm^2 flasks at a density of 2x10^5 cells per flask.
2. Once cells reached 70% to 80% confluency, they were trypsinised and used to seed a 175cm^2 flask.
3. Cells were cultured for 4-5 days until they reached 70% to 80% confluency. The cells were then trypsinised, and used to seed a large 500ml spinner flask (Techne, TR174-30) containing 250ml media.
4. Spinner flasks were incubated at 37°C and placed on a spinner apparatus set at a rotational speed of 25 r.p.m.
5. After a period of 24 hours, the agitation rate was increased to 35 r.p.m.
6. Cells were fed with 100ml of media after three days of growth in the spinner flasks.
7. Cells were harvested after 7 days at which time cell number was approximately 1.5x10^8 cells per flask.
8. When IOVs were isolated from HL60-ADR, cells (in RPMI media supplemented with 10% serum) were cultured in 5 vented 75cm^2 flasks (Costar, 3276) until 70-80% confluent. The media containing the anchorage-independent HL60-ADR cell line was removed from the flasks and centrifuged at 1000r.p.m. for 5 minutes. The pellets were then resuspended in 5ml media and used to seed the roller bottle (Falcon, 3027) containing 500ml of media. Cells were cultured in the roller bottle until 60% confluent. Once this had been achieved, cells were split equally in to an additional four roller bottles and the final volume in each of the five roller bottles made up to 500ml with complete media. Cells were harvested upon reaching 70% confluency.

2.10.3 Isolation of IOVs

The isolation of IOVs from various cell lines was performed as described by Ishikawa *et al.,* (1994), and as detailed below:

1. Approximately $7x10^8$ cells were pelleted at 5,000r.p.m. (1,200g) for 10 minutes at 4°C in a Sorvall refrigerated centrifuge.
2. The supernatant was decanted and the pellets resuspended in 50ml ice cold PBS. The combined pellets were then transferred to a 50ml tube and spun at 4,000 r.p.m. for 5 minutes.
3. The resulting cell pellet was resuspended in 230ml hypotonic buffer, (Table 2.10.1). The PMSF was added to the buffer immediately before use.

Buffer constituent	Preparation instructions
0.5mM Sodium phosphate (pH 7.0)	30 mg NaP in 500ml UHP
0.1mM EGTA	19.2 mg EGTA added to NaP solution
0.1 mM PMSF	100mM stock prepared in EtOH

Table 2.10.1: Hypotonic buffer for IOV isolation

4. Cells were lysed by gentle agitation at 4 °C for 1.5 hours.
5. The cell lysate was centrifuged at 28,000 r.p.m. (100,000g) for 35 minutes at 4° C with a beckman SW28 rotor in a Beckman XL-80 ultracentrifuge.
6. The resulting pellets were then resuspended in 10ml of hypotonic buffer and then homogenised for 15 minutes at 4°C with a Braun Potter S886 homogeniser.
7. The homogenised cell extract was diluted to a final volume of 20ml with incubation buffer which was prepared as shown in table 2.10.2.

Buffer constituent	Preparation instruction
10mM TRIS-HCL (pH 7.4)	1.211g TRIS in 1L UHP water
250mM Sucrose	42.79g Sucrose in 500ml 10 mM TRIS-HCL (pH 7.4)

Table 2.10.2: Incubation buffer for IOV isolation.

8. A 100µl aliquot of this crude membrane fraction was then retained for marker enzyme assays
9. The crude membrane fraction was layered over 38% sucrose/10mM TRIS-HCL pH 7.4, (38g sucrose in 100ml 10mM TRIS-HCL pH 7.4) and centrifuged at 28,000 r.p.m (100,000g) for 35 minutes at 4°C with a SW28 rotor. A volume of 10ml crude membrane fraction was layered over 28.5ml 38% sucrose/10mM TRIS-HCL, pH 7.4. The interface was marked to specify the location of the plasma membrane band which developed after the sucrose centrifugation step.
10. A thin white band became localised at the interface after centrifugation and this was removed with a pasteur pipette and diluted to a final volume of 20ml with incubation buffer.
11. The suspension was centrifuged at 38,200 rpm (100,000g) for 35 minutes at 4°C using a Beckman 70.1 rotor.
12. The pellets were resuspended in 0.2ml incubation buffer. Vesicles were formed by passing resuspended pellets through a 27-gauge needle 20 times using a 1ml syringe.
13. A protein assay was then performed (Section 2.10.4) and the IOV preparation was then diluted to a concentration of 5mg protein /ml with incubation buffer. Volumes of 50µl IOVs were then frozen at -80° C.

2.10.4 Protein assay

This assay was performed in the same manner as described in section 2.9.2. For the IOVs and the crude membrane preparations, samples were diluted 1 in 5 before analysis.

2.11 Transport assays with IOVs.

Transport assay with IOVs were performed as described by Ishikawa *et al.*, (1994). The protocol used in these assays is as follows:

1. A number of solutions were prepared in advance of the assay. The protocol used for the preparation of the incubation buffer has already been provided in Table 2.10.2.
   An ATP/creatine phosphate/$MgCl_2$/10mM TRIS-HCL (pH 7.4) solution is prepared as detailed in Table 2.11.1. Volumes of 200µl were then frozen at -80°C.

Buffer constituent	Preparation instructions
$MgCl_2$ $6H_20$	203.3mg in 30ml Incubation buffer
ATP (Disodium salt)	6.05 mg ATP in 3 ml $MgCl_26H_20$ solution
Creatine phosphate	32.7 mg in 3ml ATP solution

Table 2.11.1: ATP/Creatine phosphate/$MgCl_2$/10mM TRIS-HCL (pH 7.4) preparation protocol.

2. For the AMP solution, 4.99mg AMP (Sigma, A1752) was substituted for the ATP (Sigma A7699). Once prepared as in Table 2.11.1 above, 100µl volumes were frozen at -80° C.

3. A creatine kinase solution (2mg/ml), (Sigma, C5755) was prepared in incubation buffer and 50µl aliquots frozen at -80 °C.
4. Prior to performing the assay, filters (Millipore, GSWP-02500) were soaked in the incubation buffer for 1 hour at 4°C. Once soaked, the filters were applied to the filter apparatus (Millipore, 12-25 Sampling Manifold) and a vacuum was applied to the system.
5. An Eppendorf thermomixer (Eppendorf, 5436) was allowed to equilibrate at 37°C and once at temperature, the ATP, AMP, creatine kinase and IOV solutions were thawed rapidly at 37°C. After thawing, solutions were immediately placed on ice.
6. An eppendorf was placed in the thermomixer and the following added sequentially: 60µL incubation buffer, 30µl ATP, 5µl creatine kinase, 5ul [^{3}H]-LTC_4 (DuPont NEN, NET-1018, 0.01 mCi/ml) and 10µl IOVs. After every sequential addition the thermomixer was adjusted to half speed mixing to allow agitation of the various components of the mixture.
7. Aliquots of 20µl were removed at appropriate time-points and added in to 1ml of ice cold incubation buffer.
8. These were then washed through the filter apparatus. The eppendorf was washed out with 1ml of ice cold incubation buffer. The filter was finally washed with 2 ml of ice cold incubation buffer.
9. Filters were removed and placed in 8ml scintillation cocktail (ICN, 882475) in a scintillation vial. After allowing 12 hours for the filters to fully dissolve, the vials were counted for [^{3}H] content using a Beckman LS-6500 scintilation counter using a 1 minute count time.
10. For an AMP negative control, the above procedure was repeated with ATP replaced by AMP. For a total negative control, neither ATP nor AMP were included but were instead replaced with 30µL incubation buffer.
11. For assessment of a compounds ability to inhibit LTC_4 transport, the compound of interest was dissolved in incubation buffer at the desired concentration. 5µl of this was added to an eppendorf in the thermomixer. 55µl of incubation buffer was then added. The standard volumes of ATP, AMP, creatine kinase, LTC_4 and IOV were then added to a total final volume of 110µl.

2.12 Glutathione-S-Transferase assay.

2.12.1 Preparation of cell extract

1. DLKP cells were grown in a 75cm^2 flask until approximately 80-90% confluent. The cells were then trypsinised and pelleted as described in section 2.5.1.
2. The pellet was washed with 5ml PBS and the cells resuspended in 2ml PBS + 2ml 100mM PMSF (prepared as in Table 2.9.2).
3. The cells were sonicated until they burst (~ 20 pulses at 0.9msec) and the supernatant was then spun at 38,000 rpm (100,000g) for 1 hour at 4°C using a Beckman 70.1 rotor.
4. A protein assay (described in section 2.9.2) was then carried out on the supernatant, which contained the cell extract.
5. The cell extract was frozen at -80°C until required.

2.12.2 GST assay

1. A number of solutions were prepared in advance of the GST assay. These are described in table 2.12.1.

Buffer constituent	Preparation instructions
30mM Glutathione	184.20 mg in 20ml UHP
30mM 1-Chloro-2,4-dinitrobenzene	121.2 mg in 20ml Ethanol
100mM Potassium Phosphate buffer	136mg Potassium dihydrogen + 174mg Potassium hydrogen in 20ml UHP

Table 2.12.1: Buffer constituents for GST assay.

2. The reaction mixture, consisting of the following components was prepared:
   - 0.1ml Glutathione stock

- 0.1ml 1-Chloro-2,4-dinitrobenzene
- 2.2ml Potassium buffer
- 0.6 ml Cell extract (prepared as in section 2.12.1)

The final volume of the reaction mixture was 3ml.

3. The increase in absorbance was monitored at 340nm for 3 minutes by a spectrophotometer. The above reaction mixture was the negative control (no test compound added) for the assay. The reference cell mixture consisted of the same components as the reaction mixture above except 0.6mls cell extract was replaced with 0.6ml PBS.

4. For assessment of the ability of a compound to inhibit GST activity, the compound of interest was dissolved in DMSO at the required concentration (5 - 10 mg/ml). An aliquot of the compound to be tested was added to 0.1ml glutathione stock, 0.1ml 1-chloro-2,4-dinitrobenzene and 2.2ml potassium buffer. A final volume of 3ml was obtained by the addition of cell extract to the reaction mixture and the addition of PBS to the reference cell mixture.

5. The increase in absorbance is monitored at 340nm for three minutes in the spectrophotometer.

6. Indomethacin was used as the positive control in the GST assay as it is a known GST inhibitor.

2.13 Quantification of adriamycin efflux in DLKP cells

1. DLKP cells were seeded into 75cm^2 flasks (Costar, 3375) at 0.5x10^6 cells per flask. Cells were incubated for 48 hours, after which time medium was removed and fresh medium containing adriamycin (10μM), indomethacin (or other NSAID/indomethacin analogues) (27.95μM), or combination of both adriamycin and indomethacin/compound of interest, was added. Flasks were incubated at 37°C for a period of two hours.

2. After this two-hour incubation, the media was removed from all flasks and replaced with fresh media, or media containing indomethacin/

indomethacin analogue or adriamycin, as the experiment required. The flasks were returned to the 37°C incubator.

3. At relevant time points the media was removed from the flasks and the flasks were washed twice with PBS. Cells were then trypsinised as described in section 2.5.1 and counted as described in section 2.5.2. Pellets were then washed with PBS and frozen at -20°C.

4. When required for HPLC analysis, the frozen pellets were thawed, resuspended in 100µl UHP and added to glass tubes (test samples).

5. Untreated DLKP cells were resuspended in 800µl UHP and 100µl aliquots of this were placed into 8 glass tubes. These were the adriamycin control tubes and were labelled as follows: 50µg/ml, 10µg/ml, 5µg/ml, 2µg/ml, 1µg/ml, 0.5µg/ml, 0.25µg/ml, 0µg/ml adriamycin.

6. 1ml of the appropriate concentration of adriamycin was then added to the adriamycin control tubes.

7. A volume of 100µl 33% aqueous silver nitrate (Sigma, S6506) was then added to the pellets (all tubes from 4 and 5), followed by mixing for 5 minutes. A quantity of 300µl of the internal standard (daunorubicin, 6ug/ml in 50mls methanol) was then added to all tubes.

8. 1.3ml HPLC grade acetonitrile (Labscan) was added to the test sample tubes only. 300µl HPLC grade acetonitrile was added to the adriamycin control tubes.

9. All tubes were maintained at 4°C for 1 hour. This was followed by centrifugation at 4000rpm for 15 minutes.

10. 1.1ml of the supernatant was removed and added to HPLC autosampler vials. All solvent was then removed under a stream of nitrogen gas.

11. The remaining solids were resuspended in 50µL of HPLC mobile phase

12. The HPLC mobile phase was prepared as follows: 64ml of 0.1M phosphoric acid (Sigma, P6560) was added to 488ml UHP. The pH was then adjusted to 2.3 with 1N potassium hydroxide (Sigma P6310). A volume of 248 ml acetonitrile was added finally and the completed mobile phase allowed to degas at 4°C overnight.

13. 20 1 of sample for analysis was automatically injected into the HPLC system (Beckman System Gold 507 autosampler, 125 pump and 166 detector). Mobile phase flow rate was set at 0.5ml per minute with a total run time of 16 minutes. The column used for HPLC analysis of adriamycin in DLKP was a C18 reversed phase Prodigy 5μm particle size ODS-3 column (Phenomenex, U.K.). Absorbance was monitored at 253nm.

14. A standard curve of adriamycin peak area /daunorubicin internal standard peak area versus adriamycin concentration was used to quantify the levels of adriamycin present in the samples. Results were finally reported as the amount of adriamycin per million cells.

2.14 Quantification of indomethacin efflux in DLKP cells

1. DLKP cells were prepared and treated as in steps 1-3 in section 2.13.
2. When required for HPLC analysis, the frozen pellets were thawed, resuspended in 1000μl UHP and added to glass tubes (test samples).
3. Untreated DLKP cells were resuspended in 8.5ml UHP and 900μl aliquots of this were placed into 8 glass tubes. These were the indomethacin control tubes and were labelled as follows: 8μg/ml, 4μg/ml, 2μg/ml, 1μg/ml, 0.5μg/ml, 0.1μg/ml, 0.05μg/ml, 0μg/ml indomethacin.
4. 100μL of the appropriate indomethacin dilution (10x) was added to the corresponding labelled indomethacin control tubes.
5. Into all of the tubes (test samples and indomethacin control samples) the following was added: 100μL mefenamic acid (internal standard) (10μg/ml), 500μL 1M citrate buffer (pH 3.0), 7ml dichloromethane.
6. All tubes were mixed for 10 minutes and were then centrifuged at 4,000 r.p.m for 15 minutes.
7. 1.1ml was removed from the dichloromethane phase (bottom phase) and added to HPLC autosampler vials. The vials were left in a fumehood overnight to allow evaporation of the solvent.

8. The remaining solids were resuspended in 50μL of HPLC mobile phase

9. The HPLC mobile phase was prepared as follows: 200ml of 0.02M sodium acetate was added to a HPLC flask and the pH was adjusted to 3.6 with acetic acid. A volume of 800ml methanol (Labscan) was added finally and the completed mobile phase allowed to degas at 4°C overnight.

10. 20 1 of samples for analysis were automatically injected into the HPLC system (Beckman System Gold 507 autosampler, 125 pump and 166 detector). Mobile phase flow rate was set at 1ml per minute with a total run time of 16 minutes. The column used for HPLC analysis of indomethacin in DLKP was a C18 reversed phase Prodigy 5μm particle size ODS-3 column (Phenomenex, U.K.). Absorbance was monitored at 320nm.

11. A standard curve of indomethacin peak area/mefenamic internal standard peak area versus indomethacin concentration was used to quantify the levels of indomethacin present in the samples. Results were finally reported as the content of indomethacin per million cells.

2.15 Preparation 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 were 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 all autoclaved before use. All spatulas which came into 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 into contact with RNA were all 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 when feasible.

2.15.1 Total RNA extraction from cultures cell lines.

1. Adherent cells were grown in 75cm^2 flasks until approximately 80% confluent.
2. Media was removed and 1ml of TRI reagent per 75cm^2 flasks (Sigma, T-9424) was added to the flask for 5 minutes ensuring that all cells are 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.
3. To ensure complete disassociation of nucleoprotein complexes, the sample was allowed to stand for 5 minutes at room temperature.
4. 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.
5. The resulting mixture was centrifuged at 12,000g 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).

6. The aqueous phase was transferred to a fresh tube and 0.5ml of isopropanol per ml of TRI reagent used in sample preparation and mixed. The sample was allowed to stand for 5-10 minutes at room temperature.

7. The sample was then centrifuged at 12,000g for 10 minutes at 4°C. The RNA precipitate formed a pellet on the side and the bottom of the tube.

8. 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 7,500g 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.

9. The RNA pellet was air-dried briefly. Approximately 50µl DEPC-treated H_2O was added to the pellet. The RNA was then stored at -80°C until required for PCR analysis.

2.15.2 RNA Quantitation

RNA was quantified spectrophotometrically at 260nm and 280nm. 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. Partially solubilised RNA has a ratio of <1.6 (Ausubel *et al.,* 1991). The yield of RNA from most lines of cultured cells is 100-200µg/90mm plate (Sambrook *et al.,* 1989).

2.15.3 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:

1. 1µl oligo $(dT)_{12-18}$ primers (1µg/ml) (promega, C1101), 1µl of total RNA (1µg/ml), and 3µl 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.

2. 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 RNasin (40U/µl) (Promega, N2511), 1µl of dNTPs (10mM each of dATP, dCTP, dGTP and dTTP), 6µl of water and 1µl of Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (Gibco; 5108025 SA) was 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 catalyse the formation of cDNA on the mRNA template.

3. 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.

2.15.4 PCR analysis of cDNA formed from mRNA isolated from cell lines

Typical PCR reactions were set up as 50µl volumes using 5µl of cDNA formed during the RT reaction (see section 2.17.2). cDNA was amplified for varying cycle numbers but, where possible, amplification was carried out on the exponential phase of amplification.

1. Each PCR reaction tube contained 26.5µl of water, 5µl 10X buffer (100mM TRIS-HCL, pH 9.0, 50mM KCl, 1% Triton X-100), 2µl 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 control primer (250ng/µl), and 0.5µl of second strand endogenous control primer (250ng/µl).

2. 5µl of cDNA (pre-heated to 95°C for 3 min. to separate strands and remove any secondary structure if the sample had been stored at -20°C)

was added to the above and heated to 94°C for 5 min (reduces non-specific binding of primers to template).

3. 8µl of 1.25mM dNTP and 0.5µl of Taq DNA Polymerase enzyme (Promega, N1862) was then added to the above. The cDNA was amplified by PCR using the following program:

   - 94°C for 1.5 minutes (denature double stranded DNA);
   - 15-35 cycles 94°C for 1.5 min. (denature double stranded DNA);

     42-62°C * for 1 min. (anneal primers to cDNA)

     72°C for 3 min (extension)
   - 72°C for 7 minutes (extension)

* Annealing temperatures for MRP primers were as follows:

MRP1:	55°C;
MRP2 (cMOAT):	53°C;
MRP3:	63°C;
MRP4:	42°C;
MRP5:	49°C.
MRP6:	68°C.

4. All reaction tubes were then kept at 4°C until analysed by gel electrophoresis followed by densitometry.

5. 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.

6. 10µl of cDNA products from each tube were separated by electrophoresis for approx. 1½ - 2 hours at 100V through a 2-4% agarose (Promega, V3122) 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 (Promega, G1761) were run, simultaneously as size reference.

7. The resulting product bands were visualised as pink bands (due to interchelation of the cDNA with the ethidium bromide) when the gels were placed on a transilluminator (UVP Transilluminator).

8. The gels were photographed and the negatives produced were analysed by densitometry (Imaging Densitometer, Bio-Rad. Model GS-670).

2.15.5 Densitometric analysis

Densitometric analysis was carried out using the MS Windows 3.1 compatible Molecular Analyst software/PC image analysis software used with the 670 Imaging Densitometer (Bio-Rad. CA) Version 1.3.

Negatives of PCR gels were scanned using transmission light and the image transferred to the computer. The amount of light blocked by the DNA band is in direct proportion to the intensity of the DNA present. A standard area is set which is scanned and a value taken for the OD of each individual pixel on the screen. The average value of this OD (within a set area) was normalised for background of an identical set area. The normalised reading was taken as the densitometric value used in analysis.

2.16 Statistical analysis.

Analysis of experiments investigating possible synergy between cytotoxic drugs and compounds such as NSAIDs were performed initially using the fractional product method. The equation used was as follows:

Equation 2.16.1: Fractional product equation.

$$(f_u)_{1,2} = (f_u)_1 \times (f_u)_2$$

where: $(f_u)_{1,2}$ = Expected fraction unaffected by combining compounds 1 and 2

$(f_u)_1$ = Actual fraction unaffected by compound 1 treatment

$(f_u)_2$ = Actual fraction unaffected by compound 2 treatment

It was found that $(f_u)_{1,2}$ was significantly greater than the actual fraction unaffected by combining compounds 1 and 2, a synergistic level of cell kill was demonstrated. Mutually exclusive drugs share a common mechanism of action, such as binding to the same target in the cell. Mutually non-exclusive drugs have independent mechanisms of action. The fractional product method is only theoretically applicable in cases involving mutually exclusive drug, and so was only used as a rapid approximation to investigate possible synergy between drugs. For this reason, results obtained from the analysis of data using the fractional method were confirmed using a computer package for multiple drug effect analysis, "Dose-Effect Analysis with Microcomputers", (Chou and Chou, 1987). The program provides combination index (CI) values which are a quantitative statistical measure of drug interaction in terms of an additive (CI = 1), synergistic (CI < 1) or antagonistic (CI > 1) effect for a given endpoint of the assay used, adapted from Chou *et al.*, (1983).

3.0 Results

Several nonsteroidal anti-inflammatory drugs, NSAIDs, have been reported by a number of authors, including Duffy *et al.*, (1998), Draper *et al.*, (1997) and Kobayashi *et al.*, (1997), to have the ability to enhance the cytotoxicity of anti-cancer drugs *in vitro* when co-administered to a multi-drug resistant cell line which overexpresses MRP (See section 1.9).

An inspection of the scientific literature indicated that more research had been carried out on indomethacin than on any of the other NSAIDs, thus it was chosen as the lead candidate for further investigation (see structure page 279). To determine the SAR (Structure Activity Relationship) of indomethacin, the structure of indomethacin was varied to generate a number of analogues of this NSAID. These analogues were subsequently analysed in a number of experiments and their activities were compared to that of indomethacin so that the importance of the various indomethacin substituents could be determined. **Note:** The chemical properties of the indomethacin analogues were thoroughly examined in the analytical laboratory in UCC, as described in Appendix D, prior to any biological testing in our laboratories.

In vitro testing was carried out, in the NCTCC, on 23 indomethacin analogues synthesised by Dr. Anita McGuire and Dr. Stephen Plunkett in the Chemistry department, University College Cork. 19 of these compounds were structural variations of indomethacin and the remaining four compounds were known PLA_2 inhibitors with structures similar to that of indomethacin.
A number of assays were carried out on each of the indomethacin analogues to try to determine their SAR. Indomethacin was found to have the ability to potentiate the toxicity of adriamycin, specifically in cell lines expressing the Multidrug Resistance Protein (MRP) (Duffy *et al.,* 1998). Combination toxicity assays were firstly utilised to give an indication as to the ability of any of the indomethacin analogues to potentiate the toxicity of adriamycin in our miniaturised *in vitro* toxicity assay as described in section (2.7). To further investigate the SAR of indomethacin and indomethacin analogues GST (Glutathione S-transferase) assays were carried out, according to section 2.12. IOV (Inside Out Vesicle) assays were subsequently carried out to determine if the indomethacin analogues were capable of inhibiting the transport of the MRP substrate, LTC_4. As Duffy *et al.,* (1998), have shown that indomethacin

inhibits the transport of this MRP substrate in IOVs, this experiment was carried out to assess if structural changes to indomethacin reduced the ability of this NSAID to inhibit LTC_4 transport. COX-1 and COX-2 assays were performed to assess if the structural changes also modified the ability of indomethacin to inhibit the activity of these cyclooxygenase enzymes.

3.1 Combination *in vitro* toxicity assays with adriamycin and indomethacin analogues.

In these preliminary assays the DLKP cell line was used. DLKP is a human lung carcinoma cell line which expresses MRP1 and was established in the NCTCC (Law *et al.*, 1992). The anti-cancer drug used in these assays was adriamycin (doxorubicin), a widely used chemotherapeutic agent, and indomethacin was used as a positive synergy control. The assays were carried out in triplicate. Prior to carrying out the combination toxicity assays, each of the indomethacin analogues were analysed in toxicity assays, according to the procedure described in section 2.7.1a, to assess the highest non-toxic concentrations of each of these compounds. The highest non-toxic concentration for indomethacin and the indomethacin analogues are listed in table 3.1.1.

Since the screening results are data-intensive and repetitive, I will start with a summary of the results. The data supporting the summary follows.

Data obtained from this testing indicated that nine of the indomethacin analogues, BRI 60/1, 88/1, 92/1, 104/2, 114/2, 115/2, 153/1, 138/1 and 203/1, were also capable of increasing the toxicity of adriamycin. It was postulated that indomethacin and the positive analogues potentiated the toxicity of adriamycin in the cancer cell by inhibiting the action of the MRP pump in the cell and, in so doing, decreasing the level of chemotherapeutic drug being pumped out of the cell. An enhancement of chemotherapeutic drug toxicity, caused as a direct result of the presence of an NSAID, was regarded as significant if the level of cell kill achieved by the combination was significantly greater than the product of the individual toxicity of the drug and NSAID as assessed by Chou and Talalay analysis (Chou *et al.*, 1983).

- The first set of indomethacin analogues analysed were BRI 13/1, BRI 15/1, BRI 17/1 and BRI 18/1 (Figures 3.1.1-3.1.4). These were analogues of indomethacin retaining the indoleacetic acid structure but with substituent variation to establish SAR. The methoxy and the methyl groups, present in indomethacin, were removed from all four compounds and the *para*-substituent was varied in BRI 15/1, 17/1 and 18/1. These were subsequently tested in the combination toxicity assay, as described previously, and were not found to potentiate the toxicity of adriamycin.

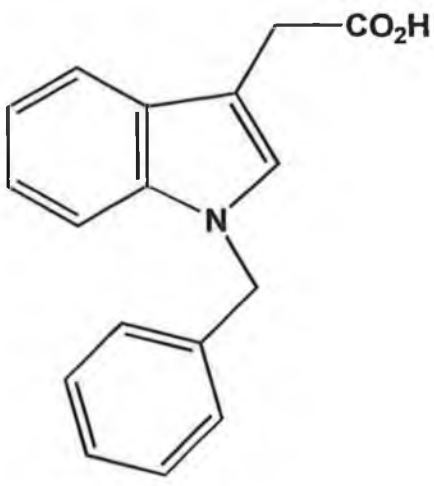


Figure 3.1.1: BRI 13/1

1–Benzylindole–3–acetic acid

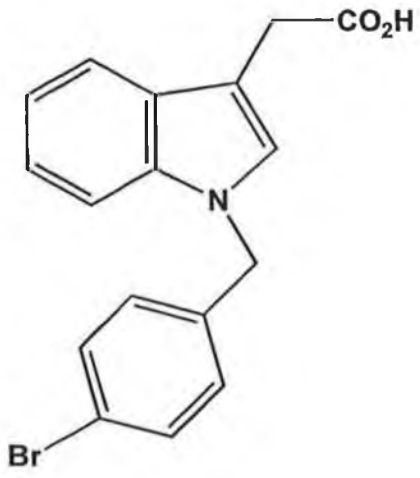
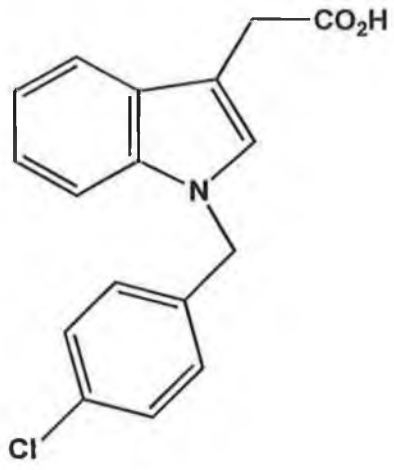


Figure 3.1.2: BRI 15/1

1–(4–Bromobenzyl)indole–3–acetic acid

Figure 3.1.3:BRI 17/1

1–(4–Chlorobenzyl)indole–3–acetic acid



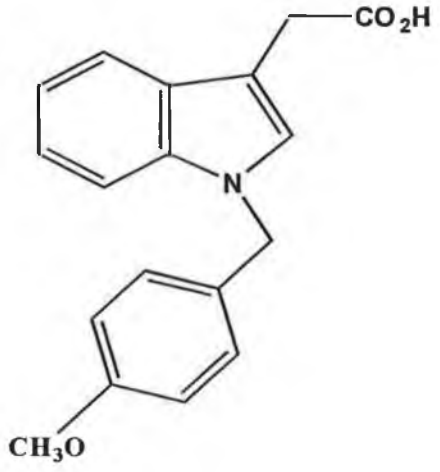


Figure 3.1.4: BRI 18/1

1–(4–Methoxybenzyl)indole–3–acetic acid

- The second set of indomethacin analogues, BRI 60/1, BRI 59/1 and BRI 69/2 were analogues of indomethacin with varied substituents. The results for these compounds are presented in Table 3.1.2. The results showed that only BRI 60/1, a N-benzyl analogue (figure 3.1.5), was positive in the combination assay. This was an interesting result as it showed that the carbonyl group was not needed for the synergistic effect of the compound to be manifested. Indomethacin was treated with dicyclohexlcarbodiimide (DCC), a well known coupling reagent widely used for the preparation of esters and amides from carboxylic acids to generate two indomethacin

analogues, BRI 59/1 (3.1.6) and BRI 69/2 (3.1.7). These analogues (N-benzoyl analogues with altered acetic acid side chains) were less soluble than BRI 60/1 and were negative in the combination toxicity assay. BRI 69/2 was more toxic to the DLKP cells than BRI 59/1 and BRI 60/1 at similar concentrations.

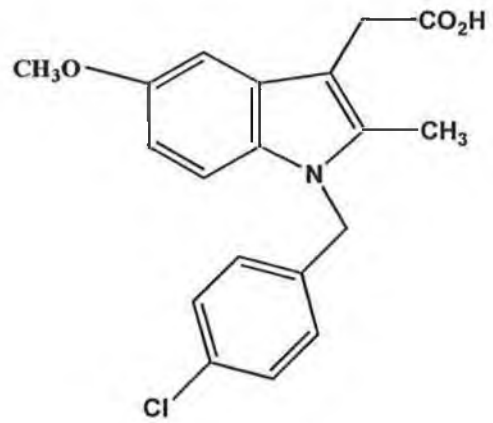


Figure 3.1.5: BRI 60/1

1–(4–Chlorobenzyl)–5–methoxy–2–methylindole–3–acetic acid

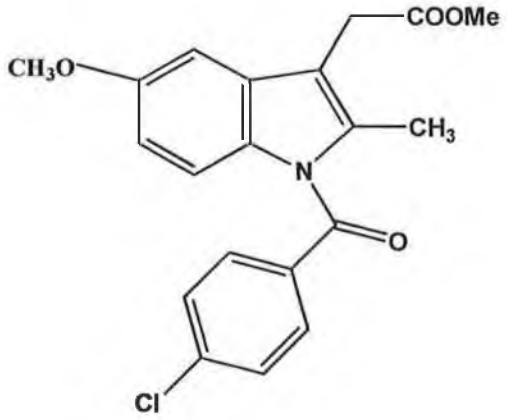


Figure 3.1.6: BRI 59/1

Methyl 1–(4–chlorobenzoyl)–5–methoxy–2–methylindole–3–acetate

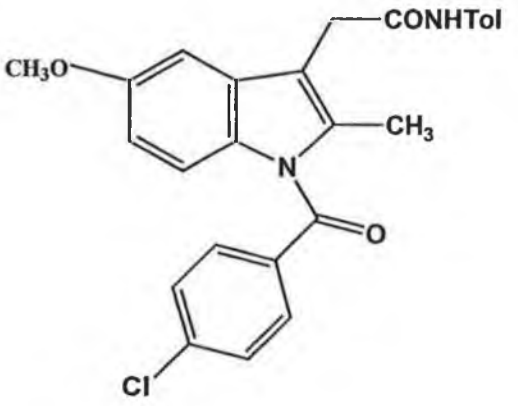


Figure 3.1.7: BRI 69/2

Methyl 1–(4–chlorobenzoyl)–5–methoxy–2–methylindole–3–acetate

- The third set of analogues analysed were N-benzoyl indomethacin analogues, BRI 88/1, BRI 92/1 and BRI 104/2. These analogues of indomethacin were generated to investigate the effect of the aryl substitution in the activity of the benzoyl derivatives of indomethacin. The highest non-toxic concentration of each compound used was 5μg/ml. Chlorine was removed from the indomethacin structure to form BRI 88/1 (Figure 3.1.8). The chlorine substituent on the indomethacin molecule was replaced with another halogen, bromine, to form BRI 92/1 (Figure 3.1.9), and the methoxy group was removed from the indomethacin structure to generate BRI 104/2 (Figure 3.1.10). In the combination toxicity assay (Table 3.1.3) all three were positive, suggesting that the substitution of the chlorine with bromine, removal of the chlorine or removal of the methoxy group did not adversely effect the efficacy of the compounds. (Note: it appears that compounds with the halogen or methoxy group removed are positive in the combination assay only when carbonyl group is present).

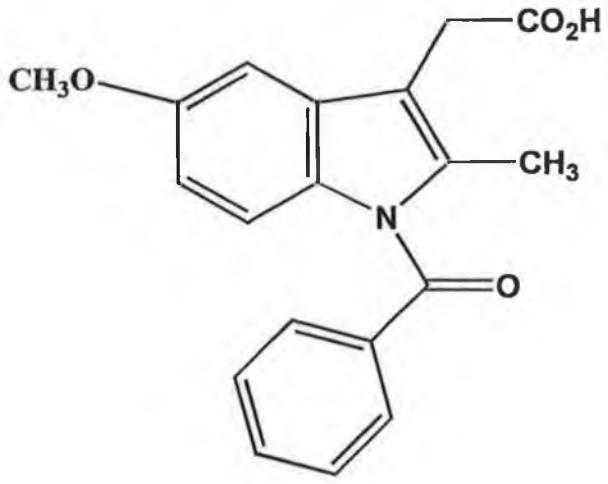


Figure 3.1.8: BRI 88/1

1-Benzoyl-5-methoxy-2-methylindole-3-acetic acid

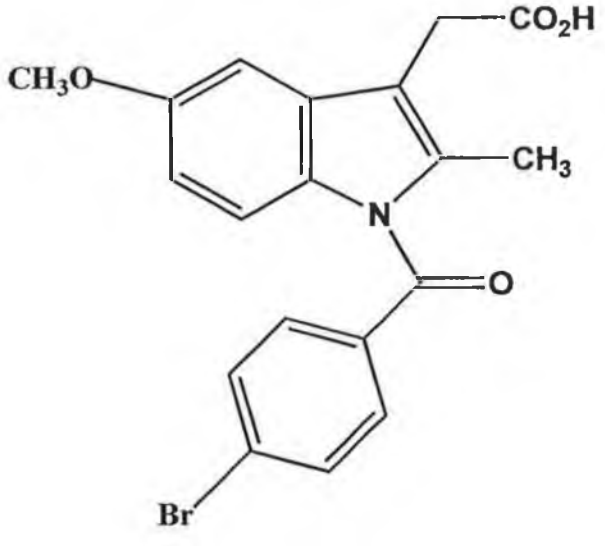


Figure 3.1.9: BRI 92/1

1-(4-Bromobenzoyl)-5-methoxy-2-methylindole-3-acetic acid

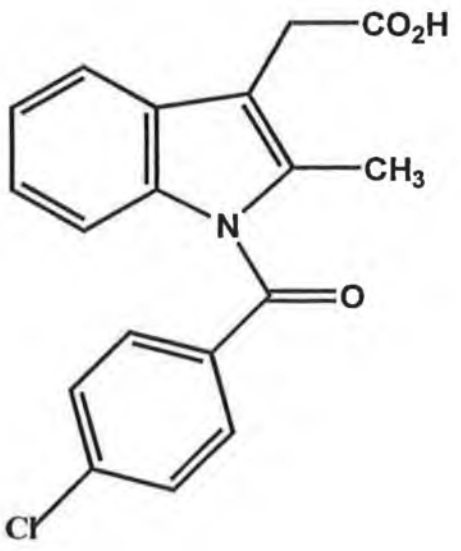


Figure 3.1.10: BRI 104/2
1–(4–Chlorobenzoyl)–2–methylindole–3–acetic acid

- The fourth set of indomethacin analogues analysed were BRI 106/1, BRI 107/1, BRI 113/1, BRI 114/2, BRI 115/2 and 124/1, N-benzyl analogues of indomethacin. These indomethacin analogues were developed to investigate the systematic variation of the position and the nature of the benzene ring substituent in benzyl derivatives of indomethacin. Chlorine is in the *para*-position on the benzene ring of the indomethacin structure. This chlorine was moved to either the *meta*- or the *ortho*-position (BRI 106/1 (Figure 3.1.11) and BRI 107/1 (Figure 3.1.12) respectively). The nature of the substituent at the *para*-position was also varied in the N-benzyl-indomethacin analogues. In BRI 113/1 (Figure 3.1.15) the substituent in the *para*-position was removed completely. Analogues of indomethacin were developed so that the chlorine was replaced with bromine (BRI 114/2, Figure 3.1.13), with fluorine (BRI 115/2, Figure 3.1.14) and with a methylthio substituent (BRI 124/1, Figure 3.1.16). Two of these compounds, BRI 114/2, and BRI 115/2, were positive in the combination assay (Table 3.1.4). The highest non-toxic concentration for these two compounds was 10μg/ml. BRI 114/2 was less soluble in DMSO than BRI 115/2. From these results we can assume that the *para*-position

of the halogen is important, as compounds BRI 106/1 and BRI 107/1, with the halogen in the *meta* and *ortho* positions respectively, were both negative.

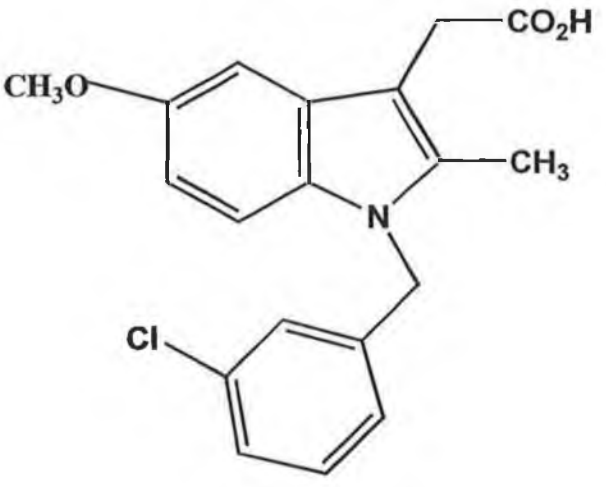


Figure 3.1.11: BRI 106/1

1-(3-Chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid

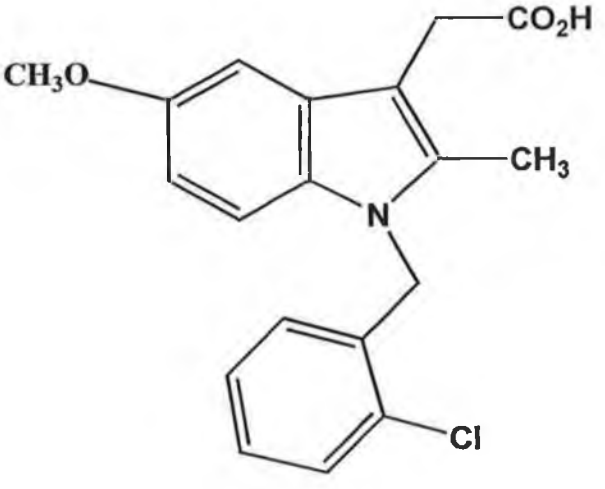


Figure 3.1.12:BRI 107/1

1-(2-Chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid

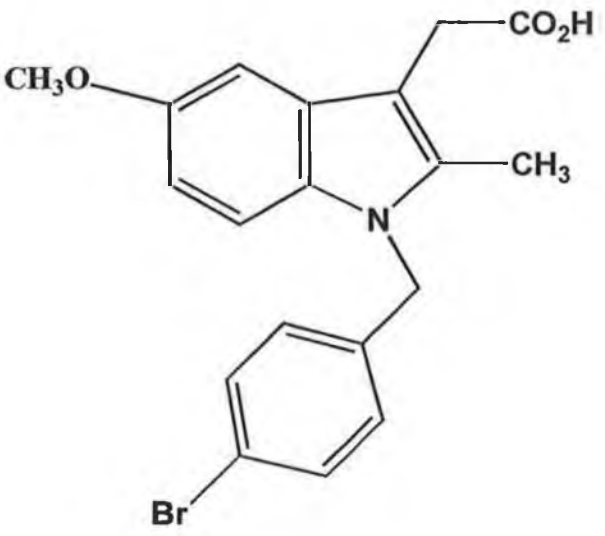


Figure 3.1.13 BRI 114/2

1-(4-Bromobenzyl)-5-methoxy-2-methylindole-3-acetic acid

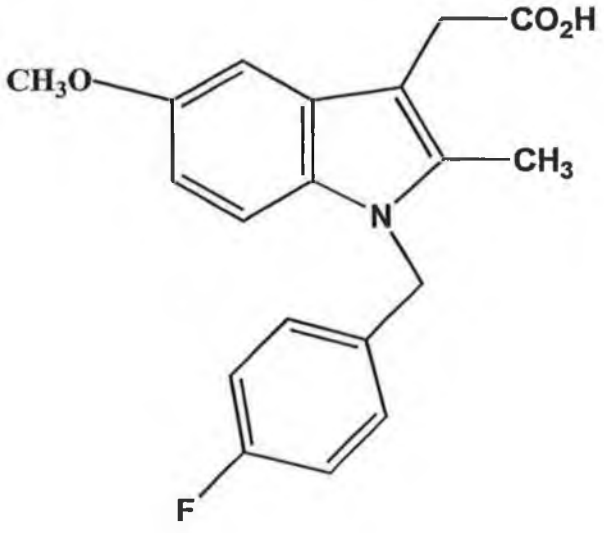


Figure 3.1.14 BRI 115/2

1-(4-Fluorobenzyl)-5-methoxy-2-methylindole-3-acetic acid

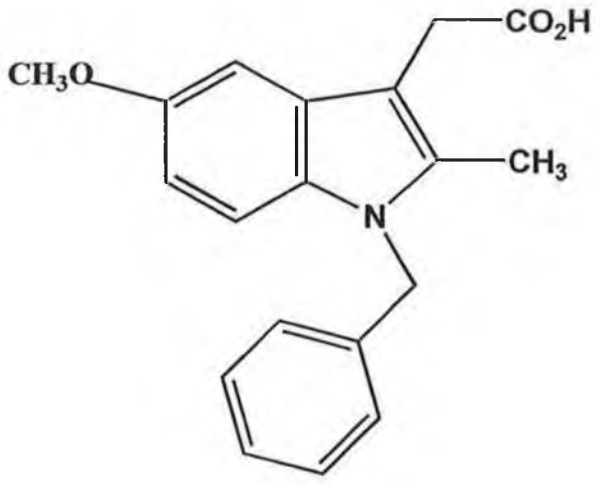


Figure 3.1.15: BRI 113/1

1-Benzyl-5-methoxy-2-methylindole-3-acetic acid

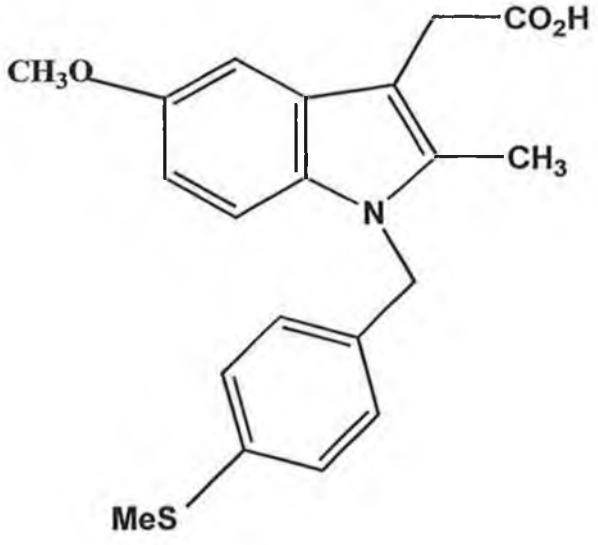


Figure 3.1.16: BRI 124/1

1-(4-Methylthiobenzyl)-5-methoxy-2-methylindole-3-acetic acid

- The position of the fluorine substituent in the fluorobenzyl-indomethacin, BRI 115/2, was then varied to assess the effect of changing the position of the fluorine from the *para*-position on the benzene ring. This resulted in the development of BRI 138/1 (figure 3.1.17). Previous results from

combination toxicity assays using *meta-* and *ortho-* forms of the indomethacin analogues were negative and it seemed that the *para-* form of the compounds was required to enhance the toxicity of the chemotherapeutic drugs. However, 138/1 proved to have almost as strong an ability to potentiate the toxicity of adriamycin as its *para-* counterpart, 115/2 (Table 3.1.8). 138/1 was less toxic in the cells as its highest non-toxic concentration in DLKP cells was 15μg/ml as opposed to 10 μg/ml for BRI 115/2.

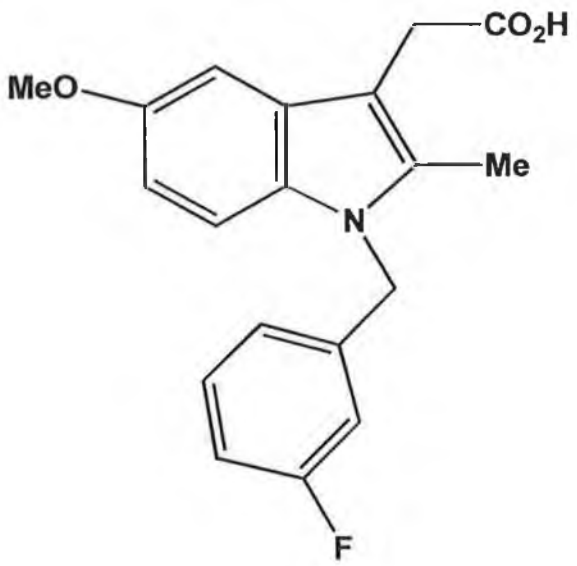


Figure 3.1.17: BRI 138/1

1–(3–Fluorobenzyl)–5–methoxy–2–methylindole–3–acetic acid

- The characterisation of N-benzyl-indomethacin was continued with the development of BRI 119/1 (figure 3.1.18) in which the methoxy substituent was removed to assess the effect of removing both the benzoyl unit and the methoxy substituent on the activity of indomethacin. To assess the effect of removing both the benzoyl unit and the methyl group on indomethacin activity, BRI 120/1 (Figure 3.1.19) was generated. These two indomethacin analogues were found to be unable to potentiate the toxicity of adriamycin in the combination toxicity assay. The results are presented in Table 3.1.5.

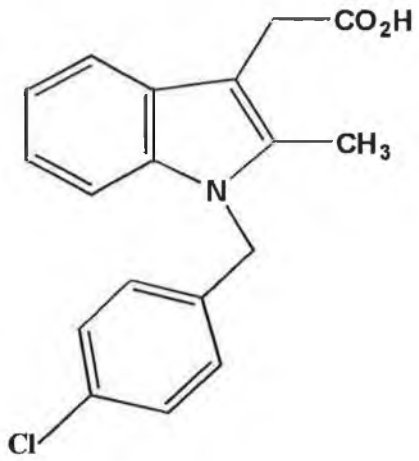


Figure 3.1.18 BRI 119/1

1-(4-Chlorobenzyl)-2-methylindole-3-acetic acid

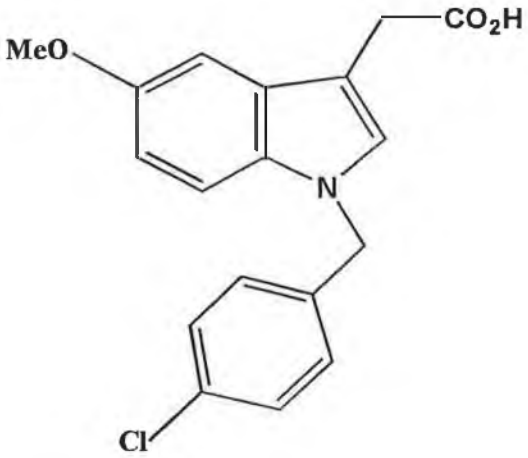


Figure 3.1.18 BRI 120/1

1-(4-Chlorobenzyl)-5-methoxyindole-3-acetic acid

As indomethacin is a well known PLA_2 inhibitor (Kaplan *et al.,* 1978), the next compound to be analysed, BRI 153/1, (4-[[3-amidomethyl)-2-ethyl-1-(phenylmethyl)-1-indol-5-yl]oxy]butanoic acid), was based on the structures of known PLA_2 inhibitors developed by Fleisch *et al.,* (1996), and Mihelich *et al.,* (1997), (figure 3.1.20). This compound was structurally similar to indomethacin. It was developed to investigate if a known PLA_2 inhibitor could potentiate the toxicity of cytotoxic drugs in a manner similar to indomethacin i.e. as well as having the ability to inhibit

PLA_2 would BRI 153/1 also have the ability to inhibit/interact with MRP1? At 50μg/ml BRI 153/1 demonstrated similar potentiation ability to indomethacin at 2.5μg/ml.

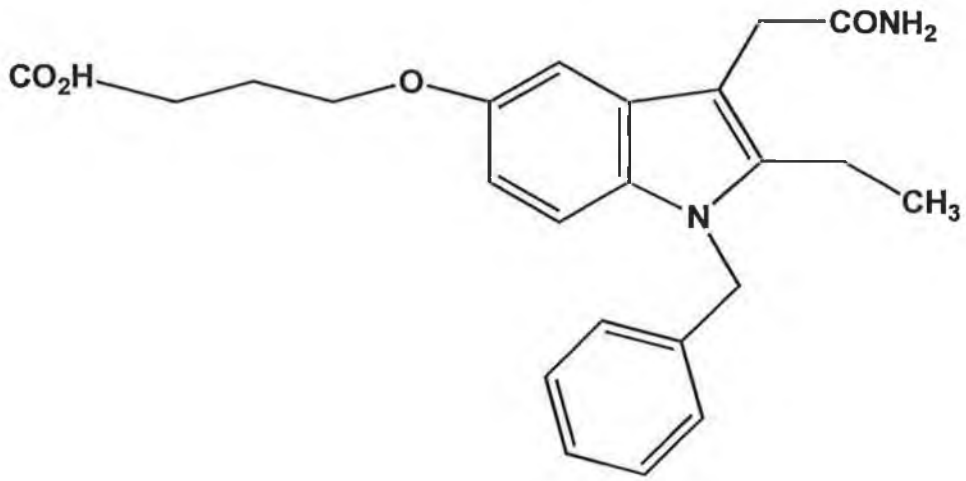


Figure 1.3.20 BRI 153/1

4-[[3-(amidomethyl)-2-ethyl-1-(phenylmethyl)-1 H-indol-5-yl] oxy]butanoic acid

- BRI 203/1 (figure 3.1.21) was subsequently developed, and was similar to BRI 153/1 except, in BRI 203/1 a methyl substituent replaced the ethyl substituent in BRI 153/1 (figure 3.1.21). These PLA_2 inhibitors were N-benzyl-2-(m)ethylindole-3-acetamides functionalised at the 5-position with a short-chain alkoxy unit terminated by a carboxylic acid. BRI 203/1 was positive in the combination toxicity assay and its highest non-toxic concentration in DLKP cells was 20μg/ml.

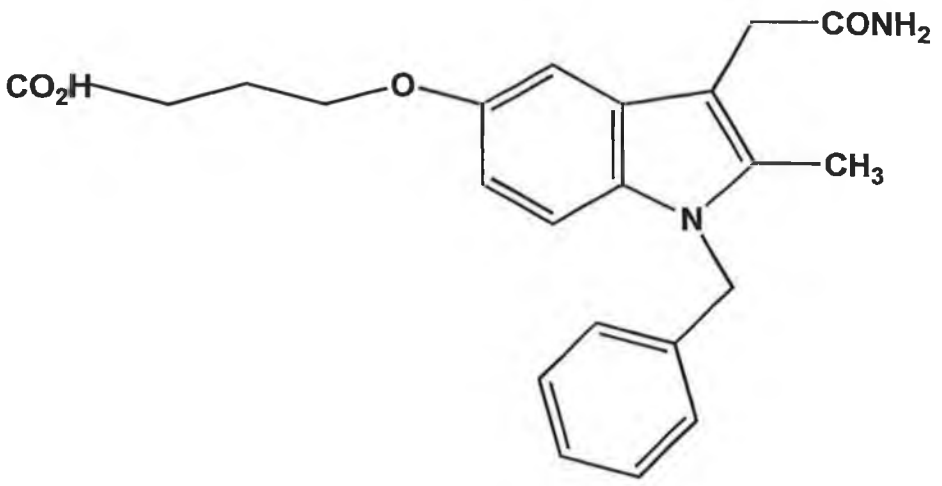


Figure 3.1.21 BRI 203/1

4-[[3-(amidomethyl)-2-methyl-1-(phenylmethyl)-1 H-indol-5-yl] oxy]butanoic acid

- BRI 205/4 (Figure 3.1.22) and BRI 215/1 (Figure 3.1.23) were then developed, the structures of which are also based on the structure of BRI 153/1. These compounds were also N-benzyl-2-(m)ethylindole-3-acetamides functionalised at the 5-position but the short-chain alkoxy unit was terminated by a phosphonic acid residue instead of a carboxylic acid residue. BRI 205/4 and BRI 215/1 are structurally identical except the methyl substituent in 205/4 is replaced with an ethyl substituent in 215/1 and the structures of which are similar to the backbone structure of indomethacin. However, BRI 205/4, at its highest non-toxic concentration of 25μg/ml, and BRI 215/1, at its highest non-toxic concentration of 50μg/ml, did not potentiate the toxicity of adriamycin in the combination toxicity assays (Table 3.1.7).

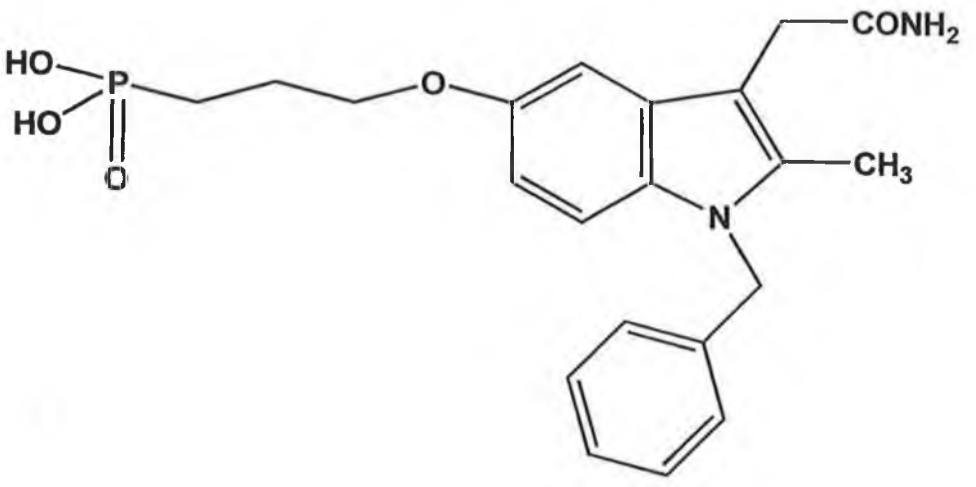


Figure 3.1.22: BRI 205/4

[3-[[3-(amidomethyl)-2-methyl-1-(phenylmethyl)-indol-5-yl]oxy] propyl]phosphonic acid

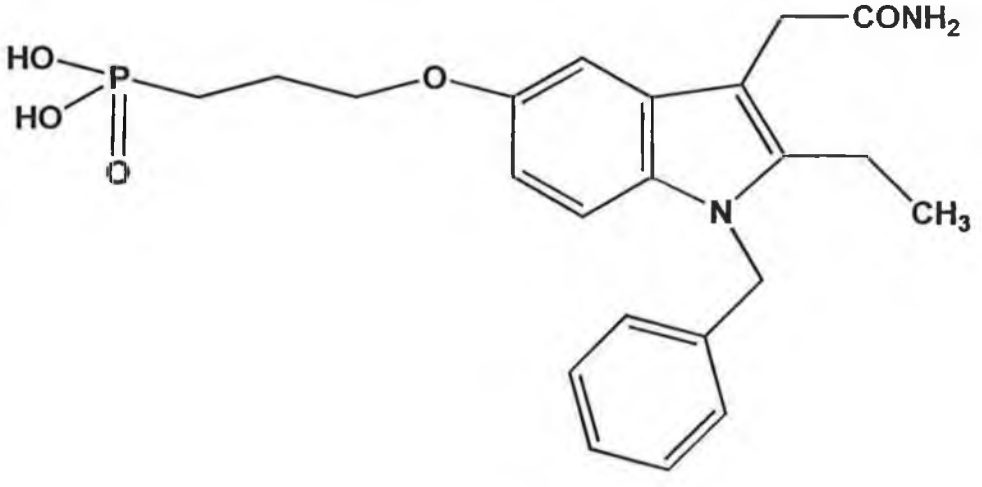


Figure 3.1.23: BRI 215/1

[3-[[3-(amidomethyl)-2-ethyl-1-(phenylmethyl)-indol-5-yl] oxy]phosphonic acid

The results of the combination toxicity assays indicate that although there are a number of indomethacin analogues that are capable of potentiating the toxicity of adriamycin *in vitro*, the potentiation ability of most of these compounds are only comparable to indomethacin at higher concentrations than those used for indomethacin (Table 3.1.9). The highest non-toxic concentration of indomethacin was 2.5μg/ml. Only six of the positive indomethacin analogues were analysed at this concentration (concentrations greater than 2.5μg/ml were required for BRI 138/1, 153/1 and 203/1 for a potentiation effect to be evident). Comparing the combination index values for indomethacin and indometahcin analogues at 2.5μg/ml indicated that of the nine positive indomethacin analogues, BRI 104/2, 92/1 and 114/2 had similar potentiation ability to indomethacin (i.e. equivalent effect at the same concentration).

Compound	μg/ml	Molar conc. (mM)
Indomethacin	2.5	0.007
BRI 60/1	5.0	0.015
BRI 59/1	5.0	0.013
BRI 69/2	2.5	0.006
BRI 88/1	5.0	0.016
BRI 92/1	5.0	0.012
BRI 104/2	5.0	0.015
BRI 106/1	10.0	0.029
BRI 107/1	5.0	0.015
BRI 114/2	10.0	0.026
BRI 115/2	10.0	0.031
BRI 113/1	10.0	0.032
BRI 119/1	5.0	0.016
BRI 120/1	5.0	0.015
BRI 124/1	5.0	0.013
BRI 153/1	50.0	0.130
BRI 203/1	20.0	0.054
BRI 205/4	25.0	0.062
BRI 215/1	50.0	0.119
BRI 138/1	15.0	0.046

Table 3.1.1: Highest non-toxic concentrations of indomethacin and indomethacin analogues used in the combination toxicity assays in DLKP cells. The non-toxic concentration of each compound was determined using data from three separate experiments.

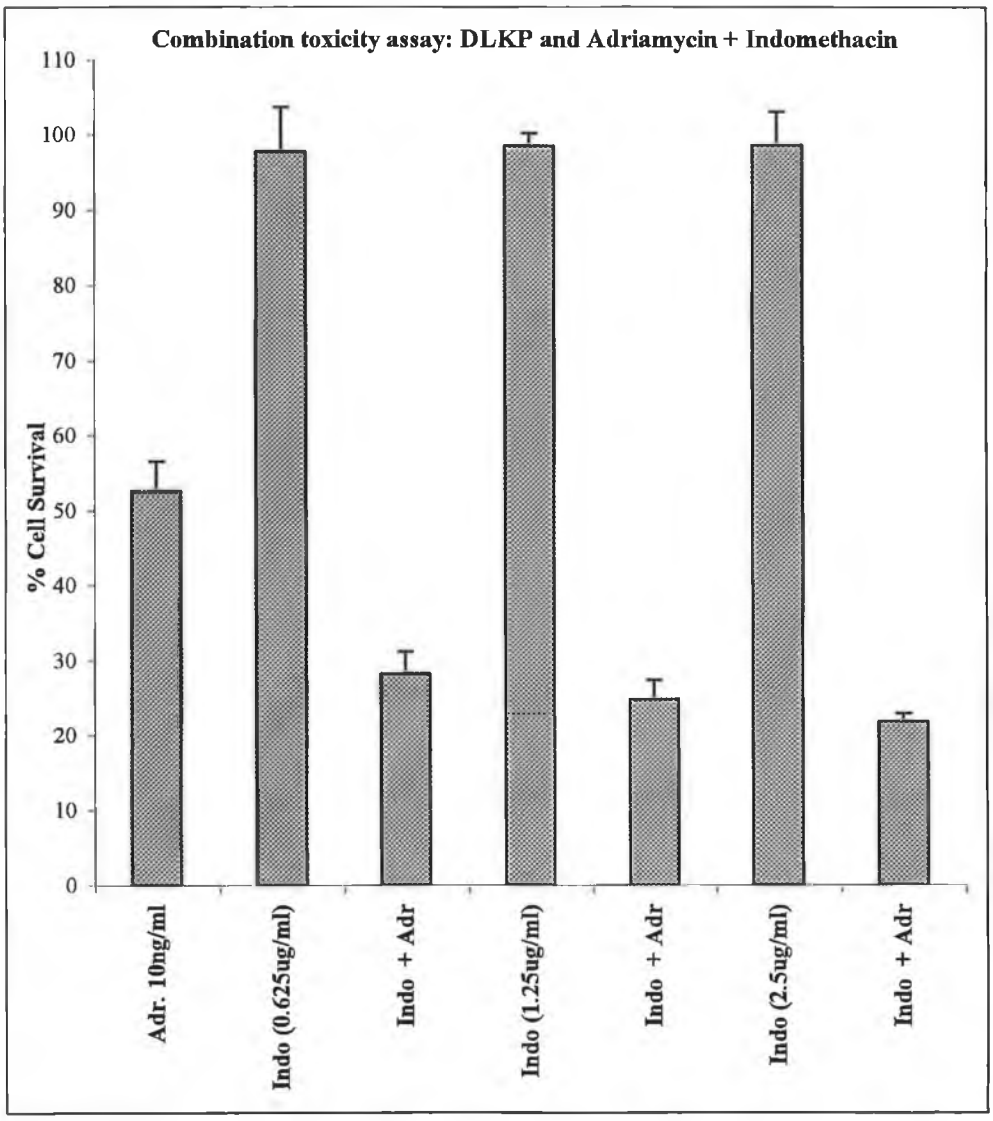


Figure 3.1.24: Combination Toxicity assay: DLKP cell survival + adriamycin in combination with non-toxic concentrations of indomethacin. The graph demonstrates the ability of indomethacin to potentiate the toxicity of adriamycin in a concentration dependent manner *in vitro* . The results are the average of triplicate determinations in three seperate experiments.

DLKP, Adriamycin and Indomethacin, BRI 60/1, BRI 59/1, BRI 69/2,		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	58.6	1.7
Indo (2.5μg/ml)	94.1	4.4
Indo + Adr	**24.5**	**1.1**
Indo (1.25μg/ml)	97.7	7.4
Indo + Adr	**30.7**	**1.7**
Indo (0.625μg/ml)	95.1	2.1
Indo + Adr	**36.3**	**1.0**
60/1 (5μg/ml)	93.8	0.9
60/1 + Adr	**20.3**	**1.8**
60/1 (2.5μg/ml)	98.6	0.8
60/1 + Adr	**31.5**	**2.8**
60/1 (1.25μg/ml)	101.0	2.3
60/1 + Adr	**40.3**	**13.5**
59/1 (5μg/ml)	101.1	5.6
59/1 + Adr	**54.1**	**3.1**
59/1 (2.5μg/ml)	101.3	0.6
59/1 + Adr	**57.5**	**4.4**
59/1 (1.25μg/ml)	97.3	5.4
59/1 + Adr	**59.0**	**5.5**
69/2 (5μg/ml)	61.8	2.5
69/2 + Adr	**24.7**	**2.7**
69/2 (2.5μg/ml)	97.9	0.9
69/2 + Adr	**59.4**	**1.6**
69/2 (1.25μg/ml)	107.7	1.2
69/2 + Adr	**63.5**	**2.6**

Table 3.1.2: % Survival of DLKP cells in the presence of various concentrations of adriamycin and indomethacin (Pos. control), BRI 60/1, 59/1 and 69/2 as found using the protocol detailed in section 2.7.3. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

DLKP, Adriamycin and Indomethacin, 88/1, 92/1 and 104/2		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	42.9	6.8
Indo (2.5μg/ml)	98.6	4.5
Indo + Adr	**21.8**	**2.5**
Indo (1.25μg/ml)	98.4	1.7
Indo + Adr	**24.8**	**2.5**
Indo (0.625μg/ml)	97.7	6.0
Indo + Adr	**28.1**	**2.9**
88/1 (5μg/ml)	94.7	2.0
88/1 + Adr	**25.9**	**1.9**
88/1 (2.5μg/ml)	99.3	4.2
88/1 + Adr	**23.7**	**0.4**
88/1 (1.25μg/ml)	99.2	1.4
88/1 + Adr	**32.2**	**4.4**
92/1 (5μg/ml)	93.7	4.7
92/1 + Adr	**20.1**	**1.6**
92/1 (2.5μg/ml)	96.8	0.9
92/1 + Adr	**21.4**	**0.3**
92/1 (1.25μg/ml)	98.7	2.1
92/1 + Adr	**23.7**	**0.4**
104/2 (5μg/ml)	96.1	0.7
104/2 + Adr	**18.5**	**2.5**
104/2 (2.5μg/ml)	99.7	2.4
104/2 + Adr	**18.3**	**2.7**
104/2 (1.25μg/ml)	99.5	2.6
104/2 + Adr	**22.4**	**3.3**

Table 3.1.3: % Survival of DLKP cells in the presence of various concentrations of adriamycin and indomethacin (Pos. control), BRI 88/1, 92/1 and 104/2 as found using the protocol detailed in section 2.7.3. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

DLKP, Adriamycin and Indomethacin, 106/1, 107/1, 114/2 and 115/2		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	44.9	5.5
Indo (2.5μg/ml)	98.6	4.5
Indo + Adr	**21.7**	**1.1**
Indo (1.25μg/ml)	98.4	1.7
Indo + Adr	**24.8**	**2.5**
Indo (0.625μg/ml)	97.7	6.0
Indo + Adr	**28.1**	**2.9**
106/1 (10μg/ml)	99.5	0.5
106/1 + Adr	**39.4**	**5.4**
106/1 (5μg/ml)	98.8	3.1
106/1 + Adr	**42.7**	**1.1**
106/1 (2.5μg/ml)	100.7	3.5
106/1 + Adr	**41.8**	**1.3**
107/1 (5μg/ml)	96.7	4.2
107/1 + Adr	**34.1**	**2.8**
107/1 (2.5μg/ml)	98.9.	4.1
107/1 + Adr	**38.0**	**1.5**
107/1 (1.25μg/ml)	99.0	4.5
107/1 + Adr	**41.0**	**1.0**
114/2 (10μg/ml))	99.2	2.5
114/2 + Adr	**10.3**	**1.2**
114/2 (5μg/ml)	104.2	2.7
114/1 + Adr	**12.3**	**1.8**
114/2 (2.5μg/ml)	103.3	3.4
114/2 + Adr	**18.4**	**2.7**
115/2 (10μg/ml)	93.1	6.7
115/2 + Adr	**18.6**	**0.5**
115/2 (5μg/ml)	93.0	7.7
115/2 + Adr	**24.3**	**2.0**
115/2 (2.5μg/ml)	94.5	3.2
115/2 + Adr	**30.4**	**3.7**

Table 3.1.4: % Survival of DLKP cells in the presence of various concentrations of adriamycin and indomethacin (Pos. control), BRI 106/1, 107/1, 114/2 and 115/2 as found using the protocol detailed in section 2.7.3. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

DLKP, Adriamycin and Indomethacin, BRI 113/1, 119/1, 120/1 and 124/1.		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	52.5	4.0
Indo (2.5μg/ml)	98.6	4.5
Indo + Adr	**21.8**	**1.1**
Indo (1.25μg/ml)	98.5	1.7
Indo + Adr	**24.8**	**2.5**
Indo (0.625μg/ml)	97.7	6.0
Indo + Adr	**28.2**	**3.0**
113/1 (10μg/ml)	98.4	1.2
113/ 1 + Adr	**37.6**	**4.7**
113/1 (5μg/ml)	97.6	2.5
113/1 + Adr	**45.3**	**0.8**
113/1 (2.5μg/ml)	100.1	2.8
113/1 + Adr	**47.3**	**2.5**
119/1 (5μg/ml)	97.4	0.6
119/1 + Adr	**50.0**	**1.8**
119/1 (2.5μg/ml)	96.5	2.1
119/1 + Adr	**57.3**	**3.5**
119/1 (1.25μg/ml)	99.0	0.5
119/1 + Adr	**55.8**	**3.1**
120/1 (5μg/ml)	98.0	5.6
120/1 + Adr	**41.0**	**3.6**
120/1 (1.25μg/ml)	100.5	4.2
120/1 + Adr	**48.1**	**2.6**
120/1 (0.625μg/ml)	96.1	2.5
120/1 + Adr	**51.7**	**3.6**
124/1 (5μg/ml)	99.0	7.7
124/1 + Adr	**42.2**	**3.3**
124/1 (2.5μg/ml)	100.3	0.9
124/1 + Adr	**47.9**	**5.3**
124/1 (1.25μg/ml)	99.3	3.1
124/1 + Adr	**50.0**	**2.7**

Table 3.1.5: % Survival of DLKP cells in the presence of various concentrations of adriamycin and indomethacin (Pos. control), BRI 113/1, 119/1, 120/1 and 124/1 as found using the protocol detailed in section 2.7.3. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

DLKP, Adriamycin and BRI 153/1		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	41.28	9.48
Indo (2.5μg/ml)	98.6	4.5
Indo + Adr	**15.9**	**6.6**
Indo (1.25μg/ml)	97.6	0.4
Indo + Adr	**19.7**	**8.28**
Indo (0.625μg/ml)	102.1	2.62
Indo + Adr	**24.5**	**11.0**
153/1 (50μg/ml)	97.8	3.1
153/1 + Adr	**13.2**	**2.9**
153/1 (25μg/ml)	99.1	1.4
153/1 + Adr	**15.7**	**3.8**
153/1 (12.5μg/ml)	99.5	2.1
153/1 + Adr	**23.2**	**8.5**

Table 3.1.6: % Survival of DLKP cells in the presence of various concentrations of adriamycin and indomethacin (Pos. control), and BRI 153/1 as found using the protocol detailed in section 2.7.3. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

DLKP, Adriamycin and Indomethacin, BRI 203/1, 205/4 and 215/4		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	57.6	1.1
Indo (5μg/ml)	**91.2**	**0.3**
Indo + Adr	**14.8**	**0.5**
Indo (2.5μg/ml)	95.4	0.5
Indo + Adr	**23.2**	**1.4**
Indo (1.25μg/ml)	94.7	0.8
Indo + Adr	**37.0**	**0.3**
203/1 (20μg/ml)	99.9	0.5
203/1 + Adr	**26.9**	**0.1**
203/1 (10μg/ml)	100.3	0.0
203/1 + Adr	**38.9**	**1.3**
203/1 (5μg/ml)	100.6	0.3
203/1 + Adr	**49.0**	**3.0**
205/4 (25μg/ml)	86.8	10.4
205/4 + Adr	**52.1**	**11.0**
205/4 (12.5μg/ml)	95.5	5.4
205/4 + Adr	**52.4**	**12.4**
205/4 (6.25μg/ml)	97.9	4.5
205/4 + Adr	**51.5**	**13.6**
215/1 (50μg/ml)	100.4	0.1
215/1 + Adr	**49.3**	**3.5**
215/1 (25μg/ml)	92.0	3.1
215/1 + Adr	**60.6**	**3.5**
215/1 (12.5μg/ml)	100.9	0.1
215/1 + Adr	**63.3**	**5.1**

Table 3.1.7: % Survival of DLKP cells in the presence of various concentrations of adriamycin and indomethacin (Pos. control), BRI 203/1, 205/4 and 215/4 as found using the protocol detailed in section 2.7.3. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

DLKP, Adriamycin and Indomethacin and BRI 138/1		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	57.6	1.1
Indo (2.5μg/ml)	91.2	0.3
Indo + Adr	**10.8**	**0.5**
Indo (1.25μg/ml)	95.4	0.5
Indo + Adr	**23.2**	**1.4**
Indo (0.625μg/ml)	94.7	0.8
Indo + Adr	**37.0**	**0.3**
138/1 (15μg/ml)	93.6	1.8
138/1 + Adr	**13.4**	**2.0**
138/1 (7.5μg/ml)	98.0	1.9
138/1 + Adr	**24.0**	**0.8**
138/1 (3.75μg/ml)	98.1	1.8
138/1 + Adr	**55.7**	**0.7**

Table 3.1.8: % Survival of DLKP cells in the presence of various concentrations of adriamycin and indomethacin (Pos. control) and BRI 138/1 as found using the protocol detailed in section 2.7.3. Survival is represented as a % of the growth of untreated cells in the same plate. Results are the average of triplicate determinations in three separate experiments.

	No Anticancer agent	+ Adriamycin (10ng/ml)	CI value*
No NSAID	100 ± 0.0	52.5 ± 4.0	0.000
Indo.(2.5μg/ml)	98.6 ± 4.5	21.8 ± 1.1	0.557
60/1 (5μg/ml)	93.8 ± 0.9	20.3 ± 1.8	0.483
59/1 (5μg/ml)	101.1 ± 0.6	54.1 ± 3.1	1.024
69/2 (2.5μg/ml)	97.9 ± 0.9	59.4 ± 1.6	1.286
88/1 (5μg/ml)	94.7 ± 2.0	25.9 ± 1.9	0.609
92/1 (5μg/ml)	93.7 ± 4.7	20.1 ± 1.6	0.566
104/2 (5μg/ml)	96.1 ± 0.7	18.5 ± 2.5	0.550
106/1 (10μg/ml)	99.5 ± 0.5	39.4 ± 5.4	1.035
107/1 (5μg/ml)	96.7 ± 4.2	34.1 ± 2.8	1.000
113/1 (10μg/ml)	98.4 ± 1.2	37.6 ± 4.7	1.020
114/2 (10μg/ml)	99.2 ± 2.2	10.3 ± 1.2	0.437
115/2 (10μg/ml)	93.1 ± 6.7	18.6 ± 0.5	0.563
119/1 (5μg/ml)	97.4 ± 0.6	50.0 ± 1.8	1.044
120/1 (5μg/ml)	98.0 ± 5.6	41.0 ± 3.6	1.074
124/1 (10μg/ml)	99.0 ± 7.7	42.3 ± 3.3	1.000
153/1 (50μg/ml)	97.8 ± 3.1	13.2 ± 2.9	0.397
203/1 (20μg/ml)	99.9 ± 0.5	26.9 ± 0.1	0.551
205/4 (25μg/ml)	86.8 ± 10.4	52.1 ± 11.0	1.385
215/1 (50μg/ml)	100.4 ± 0.1	49.3 ± 3.5	1.050
138/1 (15μg/ml)	93.6 ± 1.8	13.4 ± 2.0	0.340

***CI: Combination Index**

Table 3.1.9: Table showing results of combination of indomethacin and indomethacin analogues, at their highest non-toxic concentrations, with adriamycin in DLKP cells. Data are expressed as % cell survival ± standard deviation for a minimum of three determinations.

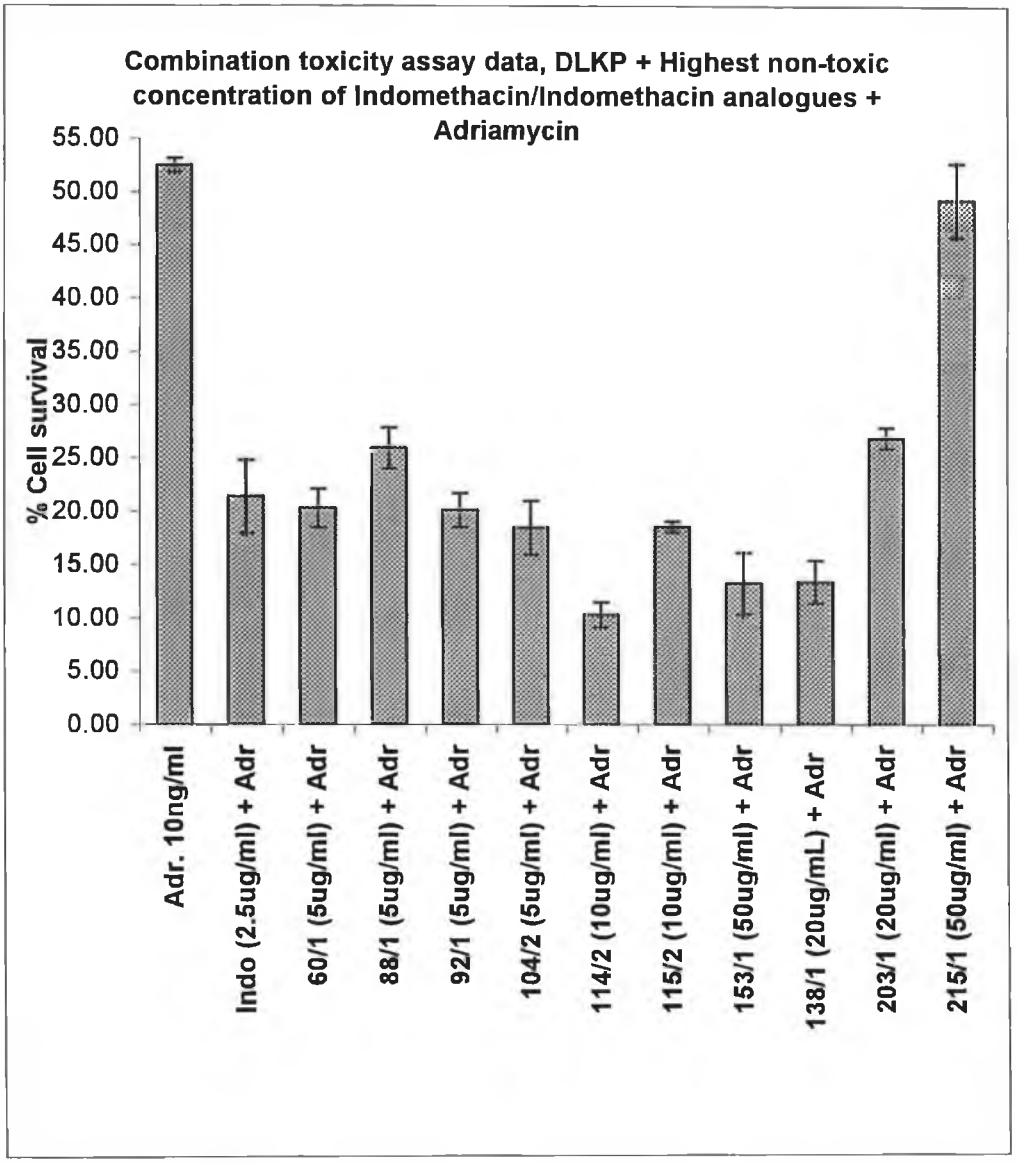


Figure 3.1.25: Combination Toxicity assay: DLKP + Adriamycin in combination with the highest non-toxic concentrations of indomethacin and positive indomethacin analogues.
The graph demonstrates the ability these compounds to potentiate the toxicity of adriamycin in DKLP cells *in vitro.*
The results are the average of triplicate determinations in three seperate experiments.

	Molar conc. in assay (mM)	No Anticancer agent	+ Adriamycin (10ng/ml)	CI value
No NSAID		100 ± 0.0	52.54 ± 4.04	0.0
Indo.(2.5μg/ml)	0.0070	98.63 ± 4.50	21.78 ± 1.08	0.557
60/1 (2.5μg/ml)	0.0075	93.77 ± 0.9	31.5 ± 2.8	0.619
88/1 (2.5μg/ml)	0.0080	94.67 ± 2.01	25.9 ± 1.9	0.619
92/1 (2.5μg/ml)	0.0062	93.68 ± 4.72	21.4 ± 0.34	0.566
104/2 (2.5μg/ml)	0.0075	96.09 ± 0.7	18.3 ± 2.7	0.544
114/2 (2.5μg/ml)	0.0064	99.1 ± 2.5	18.4 ± 2.6	0.571
115/2 (2.5μg/ml)	0.0076	94.5 ± 3.2	30.44 ± 1.7	0.754

Table 3.1.10: Table comparing synergistic combination of indomethacin and indomethacin analogues at 2.5μg/ml, with adriamycin in DLKP cells. Data are expressed as % cell survival ± standard deviation for a minimum of three determinations.

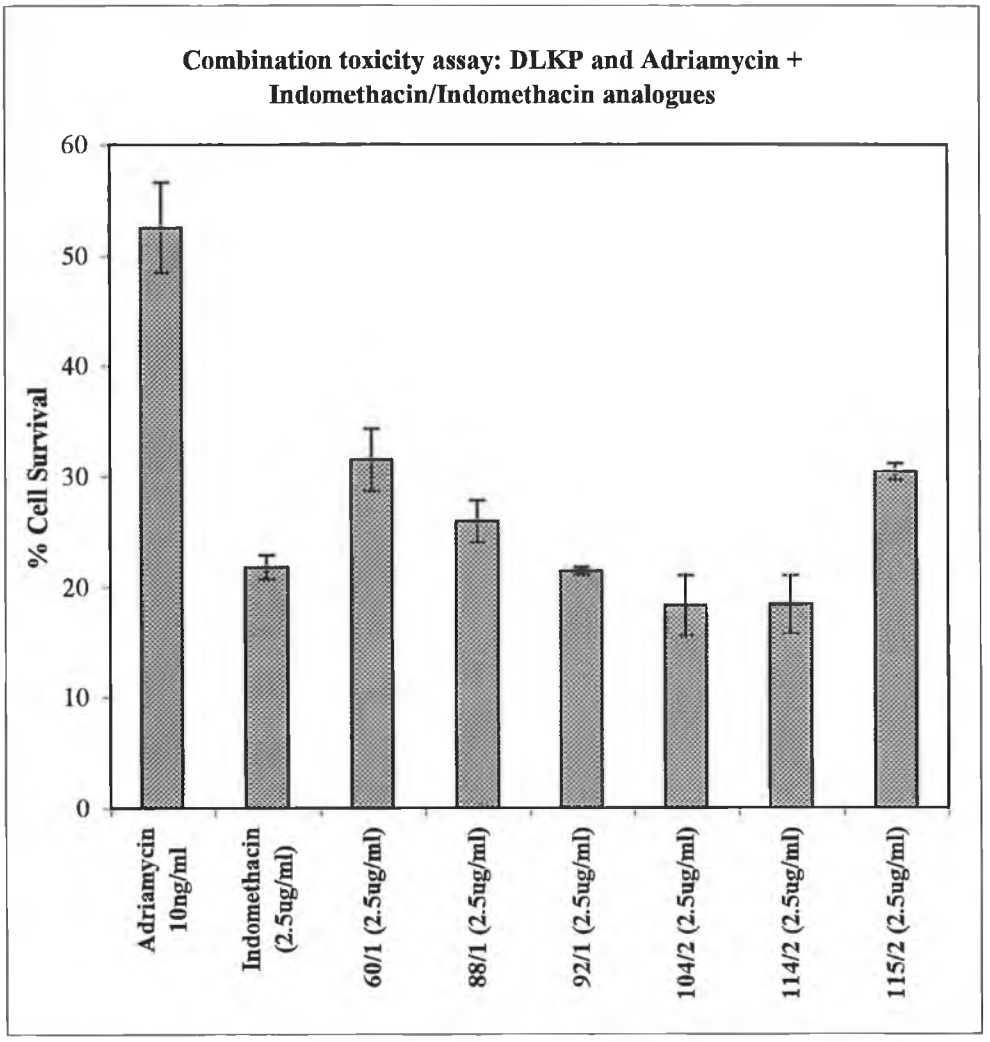


Figure 3.1.26: Combination Toxicity assay: DLKP + Adriamycin in combination with indomethacin and positive indomethacin analogues at 2.5ug/ml.
The graph compares the ability of indomethacin and analogues to potentiate the toxicity of adriamycin at identical concentrations.
The results are the average of triplicate determinations in three seperate experiments.

3.2 Glutathione S-transferase

To further determine the SAR of the compounds, Glutathione S-transferase (GST) assays were carried out for two reasons:

- One possible explanation for the mechanism by which the indomethacin analogues have their toxicity enhancing effect on the chemotherapeutic drug is through inhibiting the operation of MRP. Glutathione conjugates are transported very effectively by MRP and these glutathione conjugates are formed by the Glutathione-S- transferase enzyme (GST).
- Indomethacin is also a classic inhibitor of GST so the GST assay is carried out on the indomethacin analogues to assess if they also have this GST inhibitory activity.

The GST assay was carried out according to section 2.12 to determine if GST inhibition by the analogue was required for the synergistic activity seen in the combination toxicity assays in section 3.1. Only three of the indomethacin (N-benzoyl) analogues, BRI 88/1, 92/1 and 104/2, which were positive in the combination toxicity assay with DLKP, show comparable GST inhibitory activity to indomethacin. The results indicate that BRI 92/1 is comparable to indomethacin as an inhibitor of GST. The remainder of the indomethacin analogues that were positive in the combination toxicity assay, 60/1, 114/2, 115/2 and 153/1, 203/1 and 138/1, were not good GST inhibitors (Table 3.2.1 and Figure 3.2.1). It is understood that glutathione (GSH) is required to keep MRP in a conformational state that allows the transport of neutral or positively charged molecules – perhaps as a ternary complex. Hence, it was postulated that if GST was inhibited, less conjugates would be formed within the cell and, as a result, less molecules would be pumped out of the cell. However, the results obtained strongly suggest that the effect on the MRP pump is not through inhibition of GST. These results suggest that the ability of the compounds to enhance the toxicity of the chemotherapeutic drug *in vitro* is due to a direct interaction with the MRP pump.

Compound	Molarity of comp. in test solution (mM)	Average % Inhibition
Indomethacin	0.9	93.1 ± 3.5
BRI 60/1	1.0	7.2 ± 1.0
BRI 59/1	0.9	0.1 ± 0.1
BRI 69/2	0.8	0.1 ± 0.1
BRI 88/1	1.0	70.9 ± 4.7
BRI 92/1	0.9	94.3 ± 2.6
BRI 104/2	1.0	74.0 ± 3.8
BRI 106/1	1.0	2.0 ± 0.2
BRI 107/1	1.0	46.7 ± 0.7
BRI 113/1	1.1	17.6 ± 2.3
BRI 114/2	1.0	15.1 ± 2.7
BRI 115/2	1.0	21.2 ± 2.6
BRI 119/1	1.1	4.0 ± 0.3
BRI 120/1	1.0	0.1 ± 0.1
BRI 124/1	0.9	0.0 ± 0.0
BRI 203/1	0.8	12.1 ± 0.9
BRI 153/1	0.8	13.4 ± 1.2
BRI 205/4	0.8	9.7 ± 1.4
BRI 138/1	1.0	8.2 ± 0.8
BRI 215/1	0.8	11.7 ± 2.1
DMSO Control		0.9 ± 1.3

Table 3.2.1: GST assay results showing the % inhibition of production of glutathione conjugates by indomethacin and indomethacin analogues. Results are the average of a minimum of two readings for each compound from a minimum of three assay repeats.

Data is expressed as % inhibition relative to an untreated control (Negative control).

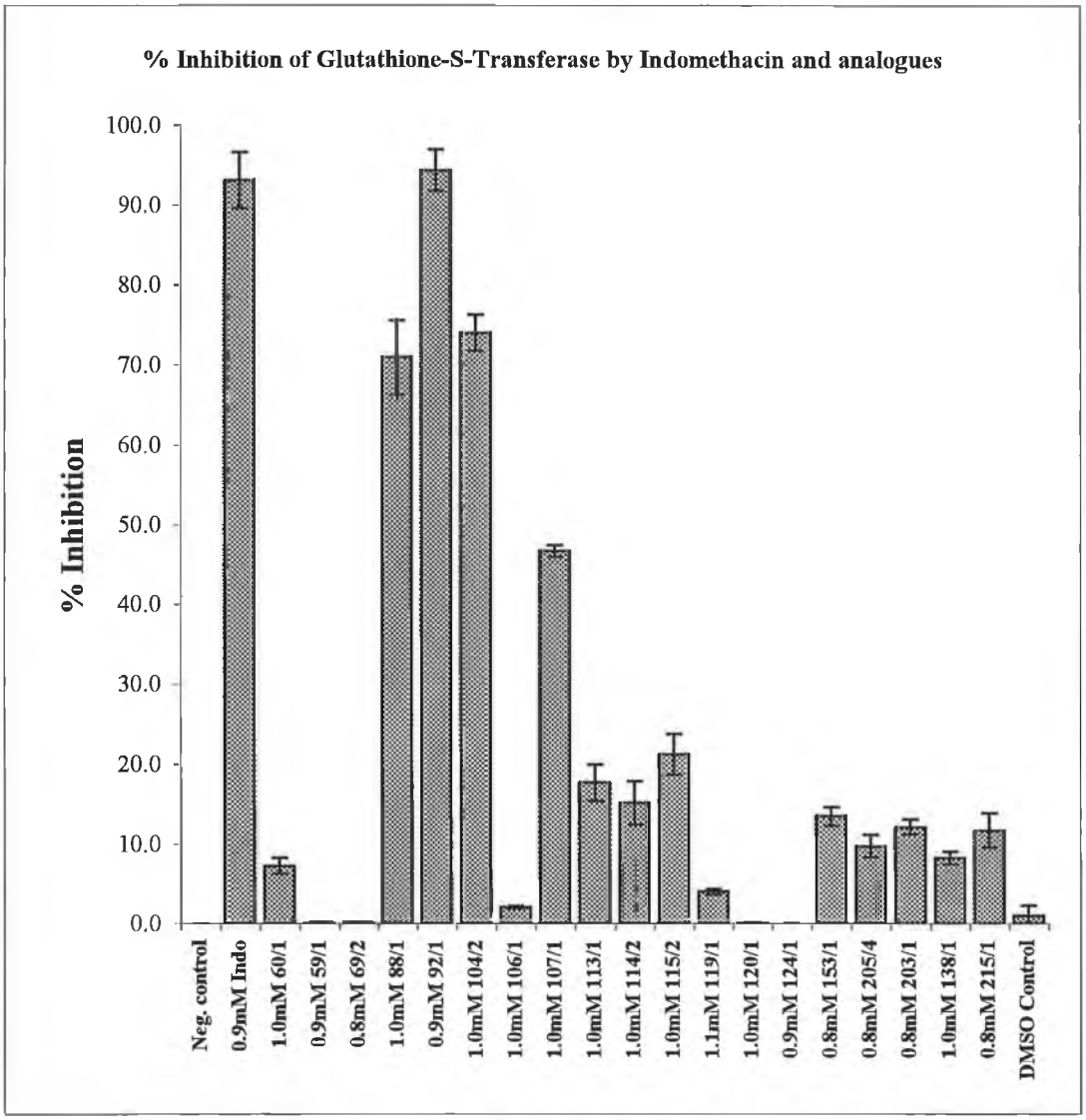


Figure 3.2.1: GST assay results showing the % inhibition of production of glutathione conjugates by indomethacin and indomethacin analogues.
The results are the average of two readings for each compound from a minimum of three assay repeats.

3.3 Inside Out Vesicle Assay

Protein analysis studies failed to show the existence of MRP protein in whole cell extracts of DLKP. It had been demonstrated that all other cell lines in which the toxicity enhancement effect had been found to occur expressed MRP (Duffy *et al.,* 1998). The drug profile for which the NSAID-mediated toxicity enhancement effect was found to occur was exactly similar to the range of drugs believed to be transported by MRP. This suggested that co-treatment of MRP-expressing cells with an MRP-substrate drug and an NSAID with the ability to interfere with the drug pumping ability of MRP, may have resulted in increased retention of drug within the cell. This would ultimately cause an enhancement of cytotoxic drug-induced cell kill. Although the toxicity enhancement effect was found to occur in DLKP, MRP expression had not been detected in this cell line. It was suggested that DLKP expressed MRP at levels undetectable by Western blotting of whole cell extracts. In order to isolate the plasma membrane from these cells and specifically target Western blotting analysis to the area in which the MRP molecule may have localised, Inside Out Vesicles were prepared from DLKP cells. It was found that MRP was detectable by Western blotting, in IOVs isolated from DLKP. The level of MRP protein in DLKP was significantly lower than the levels found in IOVs isolated from HL60/ADR cells but this level appears to be functionally effective for drug transport out of the cell (Duffy *et al.,* 1998; Elliott, 1997).

The glutathione conjugate, LTC_4, was found to be transported into vesicles in an ATP-dependent manner (Jedlitschly *et al.,* 1994 and Leier *et al.,* 1994). Duffy *et al.,* (1998), demonstrated the influence of various NSAIDs on MRP activity in HL60/ADR IOVs by measuring the ability of the compounds to inhibit the transport of LTC_4. The authors reported that that the positive NSAIDs, especially sulindac and indomethacin, have MRP pump inhibitory activity, whereas, inactive NSAIDs, naproxen and piroxicam, do not.

To further determine if indomethacin and the indomethacin analogues were acting on MRP, an assay was devised in which a pure preparation of MRP (an active enzyme preparation) was used along with a radiolabelled MRP substrate, LTC_4. This pure preparation of MRP was contained within vesicles, prepared from HL60/ADR cells, which were subsequently turned inside out. (To allow measurement of the transport of LTC_4 - instead of MRP pumping

the substrate out of the vesicle it now pumped it in to the vesicle using ATP as an energy source). The ability of the indomethacin analogues to inhibit MRP was determined by measuring their ability to inhibit the uptake of Leukotriene C_4 into the vesicle.

The results of the Inside Out Vesicle assay show that all but one of the compounds, positive in the combination toxicity assay, were effective inhibitors of MRP. From the data, the strongest MRP inhibitors appeared to be BRI 92/1 and 153/1, followed closely by 88/1, 115/2 and 114/2. Of the positive group, BRI 60/1 and 104/2 were the weakest inhibitors of the uptake of LTC_4 in to the vesicle. Compounds, which were negative in the combination toxicity assay, were used as negative controls and the results (Figure 3.4.12) indicate that these compounds are not inhibitors of MRP activity in the IOVs.

Therefore, there appears to be a good correlation between the combination toxicity assay and the IOV assay in that it seems likely that indomethacin and its analogues potentiate the toxicity of adriamycin on the cancer cells by inhibiting the expulsion of the chemotherapeutic drug from the cell by MRP.

However, the one exception was compound BRI 138/1, the *meta-* form of compound 115/2. Though this compound was very positive in the combination toxicity assay in the DLKP cells with adriamycin, it was only weakly positive in the IOV assay. This result would suggest that this compound is not exerting its effect through an interaction with MRP1 or perhaps its *meta*-structure is effecting the affinity of this compound for MRP1. It may be possible that this analogue of indomethacin is potentiating the toxicity of adriamycin through an interaction with another form of MRP1 or possibly by interacting with Pgp (MDR1) which is also expressed in DLKP cells.

Comp.	Molarity of compound in test solution	Average % Inhibition	S.D.
Indomethacin	46.0μM	83.1	10.4
BRI 60/1	47.6μM	57.7	4.7
BRI 88/1	51.0μM	64.4	7.8
BRI 92/1	41.0μM	85.0	2.8
BRI 104/2	50.2μM	60.4	8.0
BRI 115/2	50.1μM	67.1	13.5
BRI 153/1	41.8μM	85.0	9.0
BRI 205/4	39.8μM	0.0	0.8
BRI 114/2	47.2μM	66.9	7.7
BRI 203/1*	13.3μM	65.1	19.5
BRI 138/1	50.1μM	26.8	5.2
BRI 215/1	38.2μM	22.5	5.6

Table 3.3.1: Effect of indomethacin and selected analogues on transport of [^{3}H]-LTC_4 in to inside-out vesicles from HL60/ADR cells.

The relative ATP-dependent rates are expressed as a percentage of untreated control, taken as 100%, by subtracting the rate in the presence of AMP, which was used as the blank.

% inhibition was calculated using the following formula:

$$\left(\frac{LTC_4 \text{ uptake @ T180 seconds (untreated) - Levels of } LTC_4 \text{ @ T180 seconds (+ compound)}}{LTC_4 \text{ uptake @ T180 seconds (untreated)}}\right) \times 100$$

Data given are from a minimum of three assay repeats.

* Indomethacin analogue 203/1 proved very difficult to dissolve in the reaction mixture -- results shown may be a reflection of solubility problems and not a true reflection of the inhibiting ability of the compound.

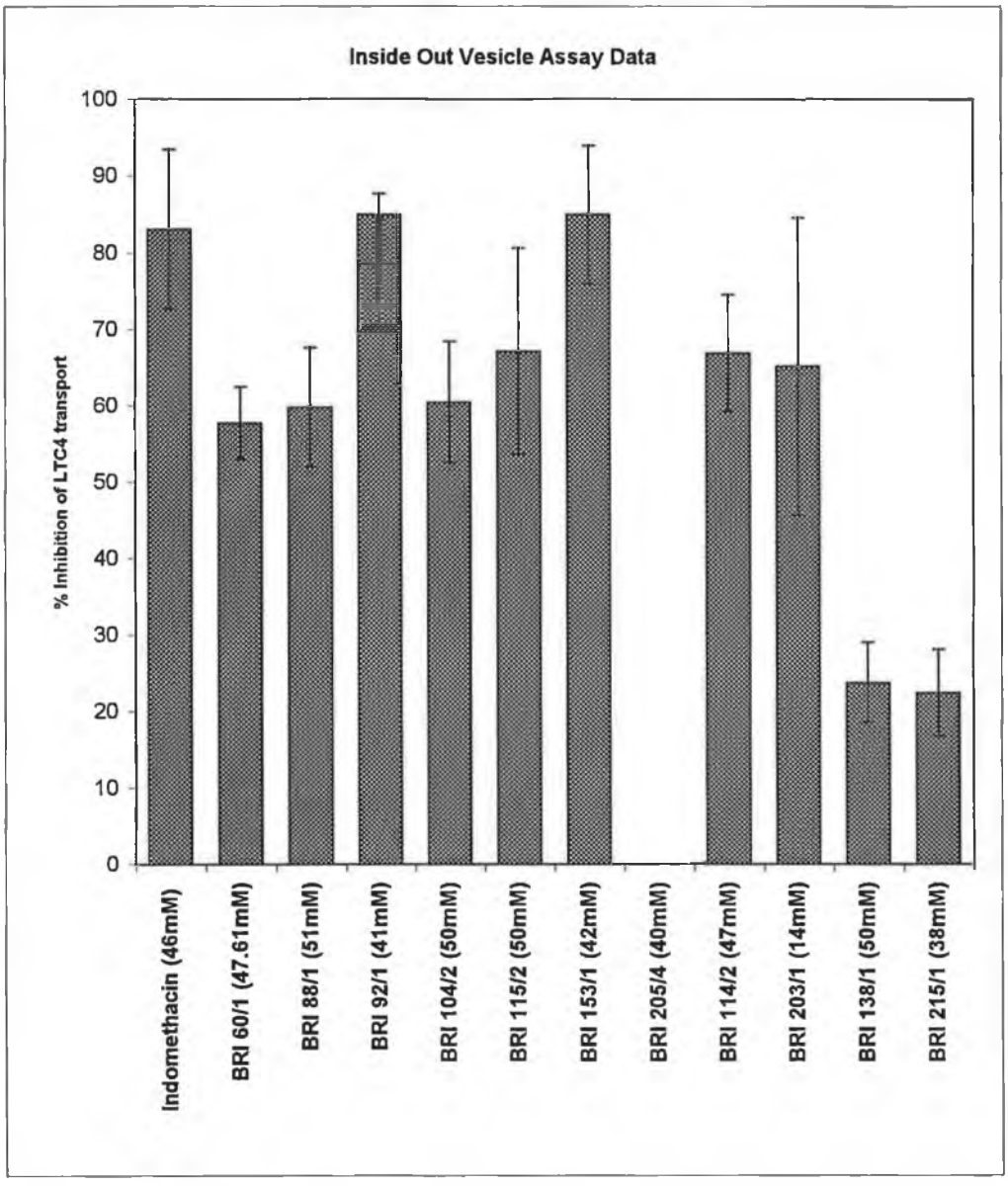


Figure 3.3.1: Graph showing the effect of indomethacin and indomethacin analogues on the transport of [^{3}H]-LTC4 in to inside-out vesicles from HL60/ADR cells. Data are the average of at least three minimum repeats.

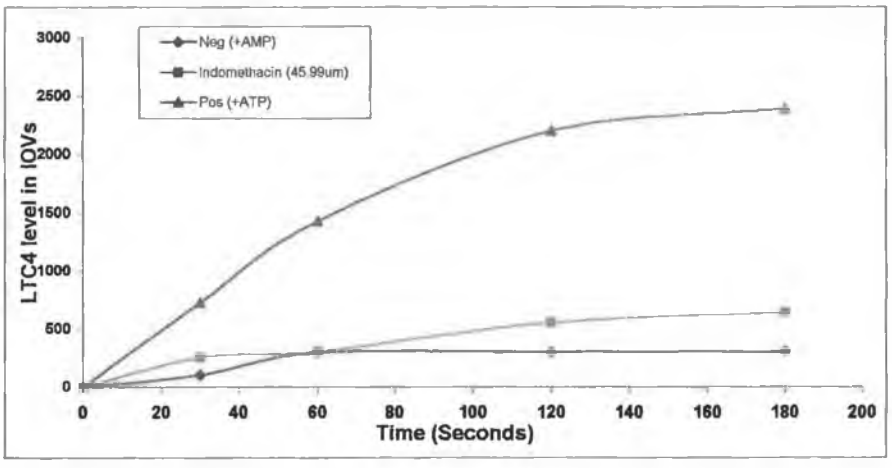


Figure: 3.3.2: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and indomethacin Similar results were obtained in at least one additional experiment.

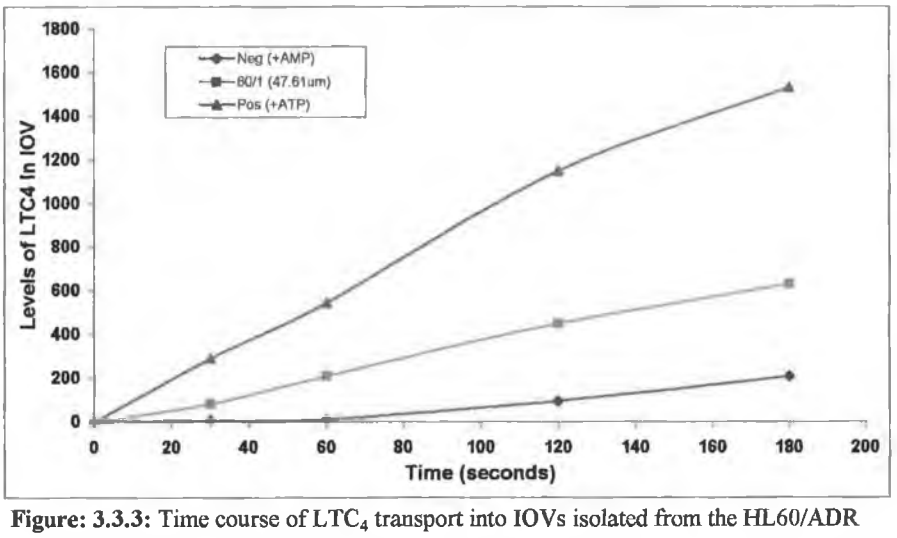


Figure: 3.3.3: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 60/1 Similar results were obtained in at least one additional experiment.

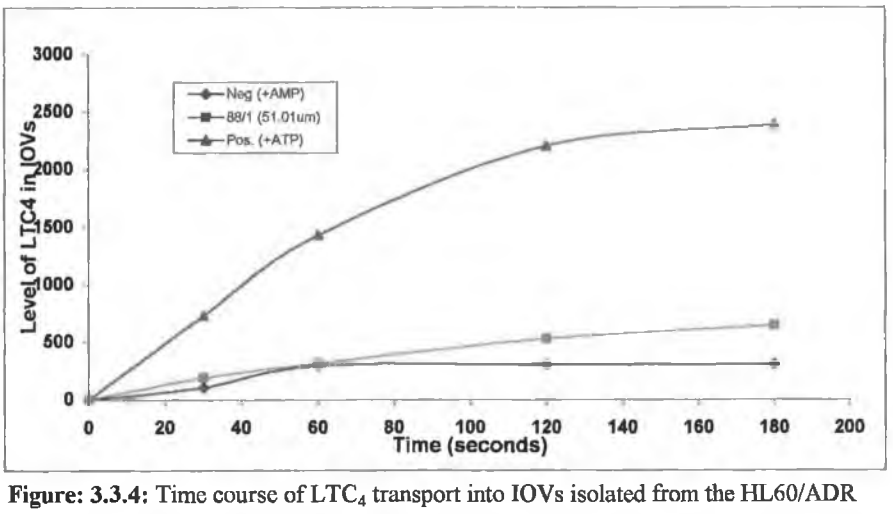


Figure: 3.3.4: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 88/1 Similar results were obtained in at least one additional experiment.

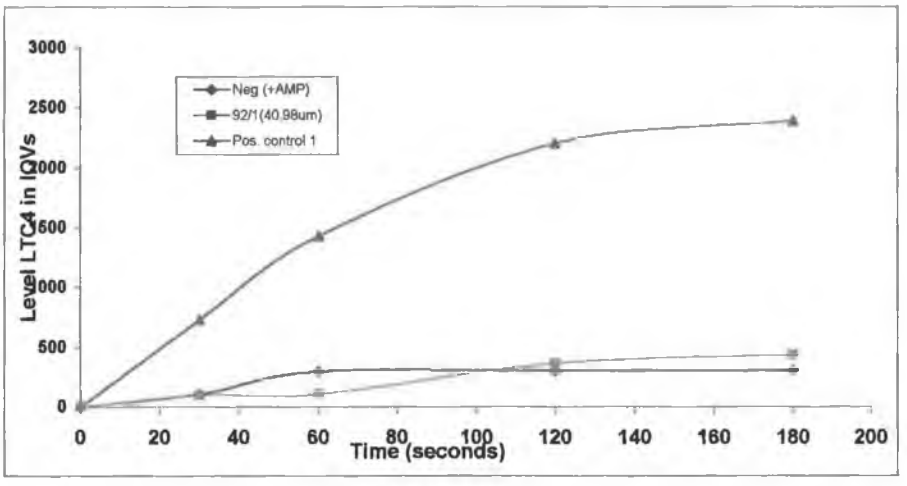


Figure: 3.3.5: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 92/1
Similar results were obtained in at least one additional experiment.

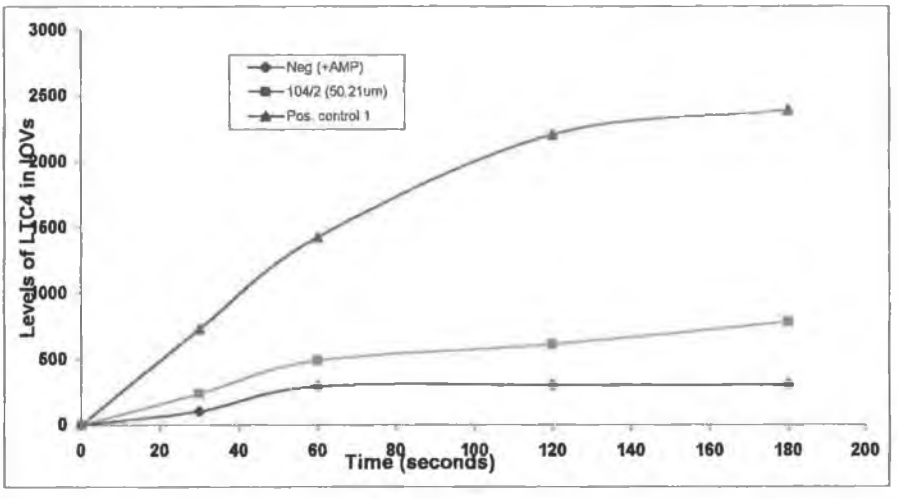


Figure: 3.3.6: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 104/2
Similar results were obtained in at least one additional experiment.

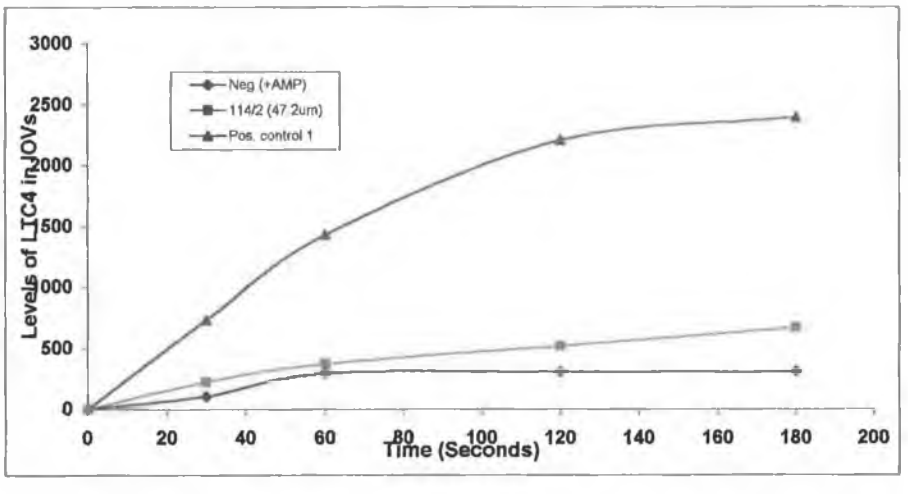


Figure: 3.3.7: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 114/2
Similar results were obtained in at least one additional experiment.

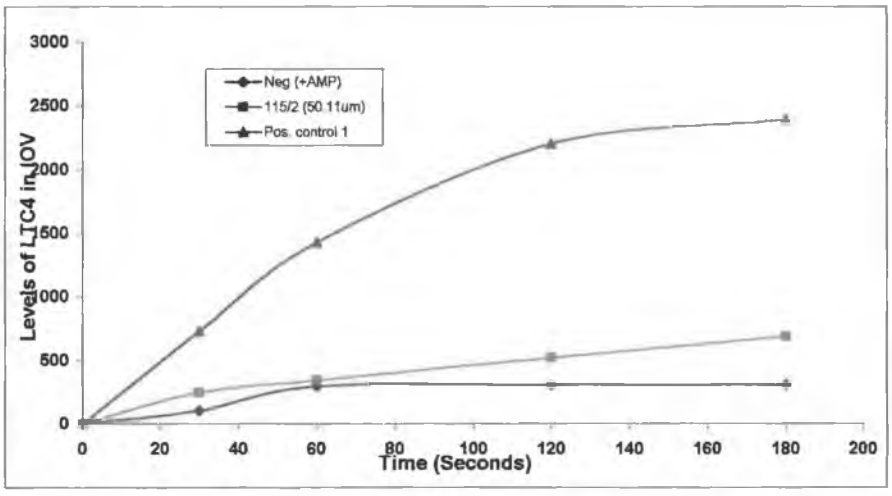


Figure: 3.3.8: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 115/2
Similar results were obtained in at least one additional experiment.

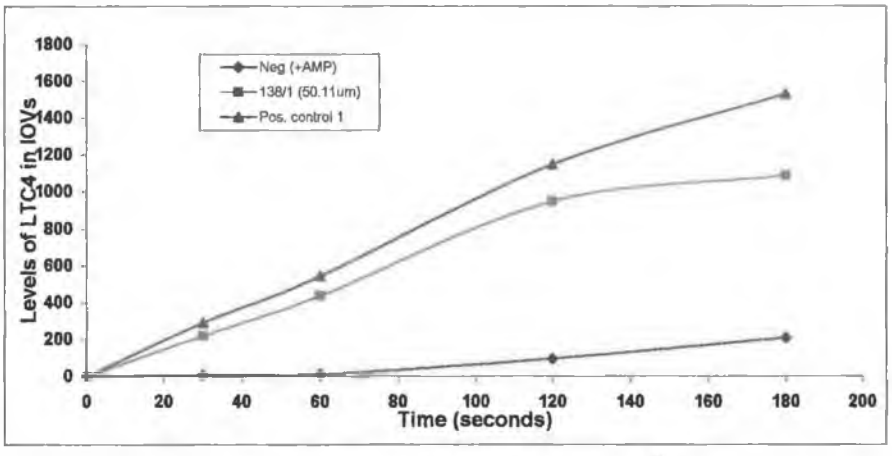


Figure: 3.3.9: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 138/1
Similar results were obtained in at least one additional experiment.

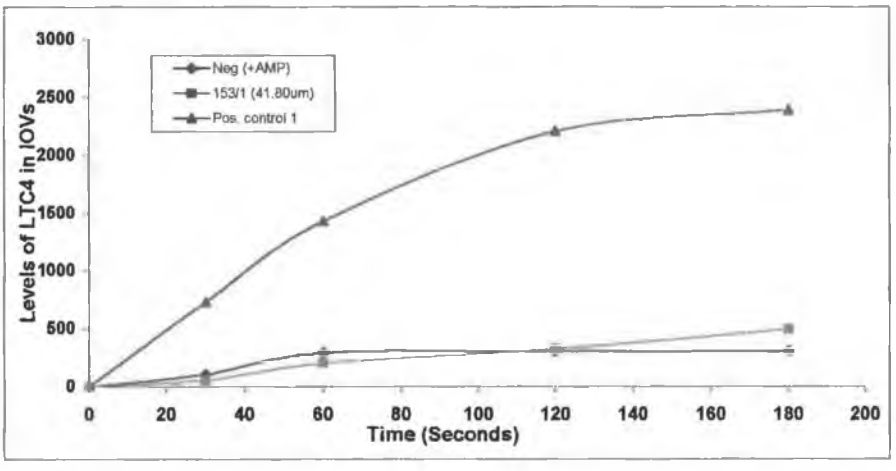


Figure: 3.3.10: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 153/1
Similar results were obtained in at least one additional experiment.

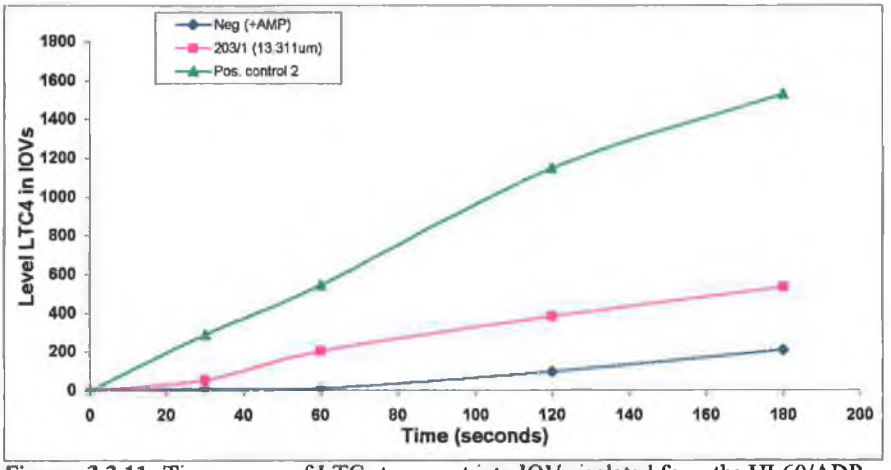


Figure: 3.3.11: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 203/1
Similar results were obtained in at least one additional experiment.

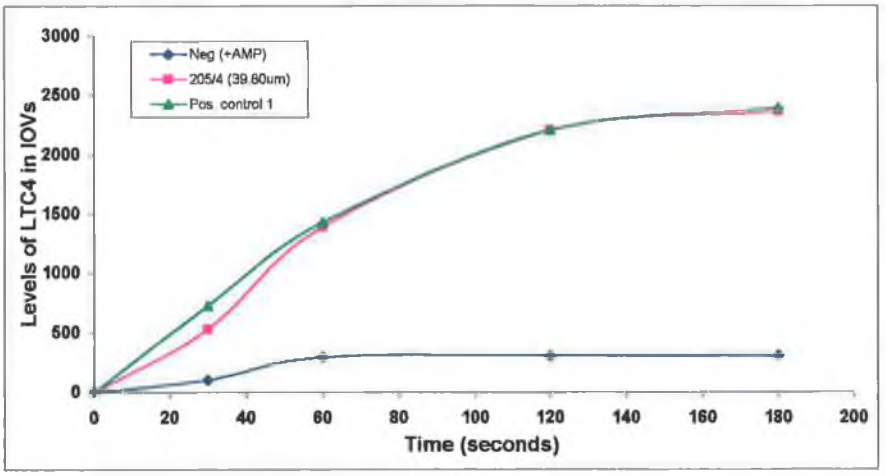


Figure: 3.3.12: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 205/4
Similar results were obtained in at least one additional experiment.

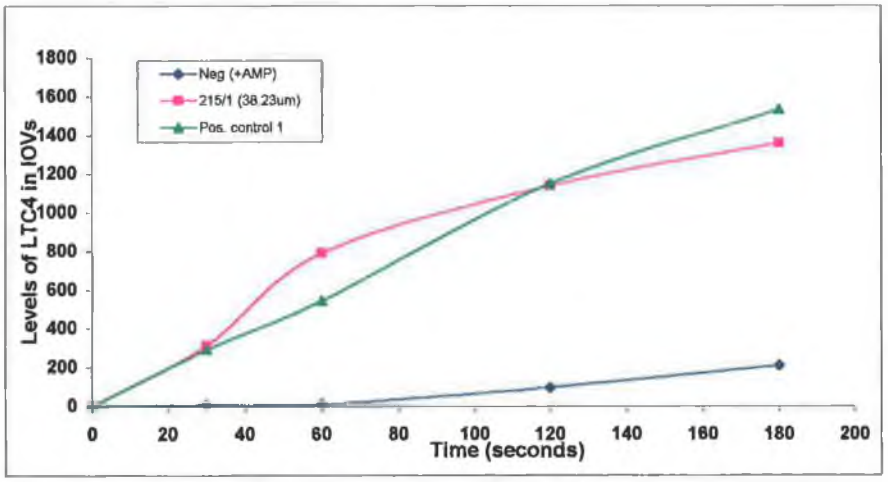


Figure: 3.3.13: Time course of LTC_4 transport into IOVs isolated from the HL60/ADR cell line in the presence of ATP, AMP or a combination of ATP and BRI 215/1
Similar results were obtained in at least one additional experiment.

3.4.1 Cyclooxygenase 1 assays

Further *in vitro* testing was carried out on the indomethacin analogues, particularly on the positive compounds, to determine if they had any inhibitory activity on either Cyclooxygenase 1 (COX-1) or Cyclooxygenase 2 (COX-2). The anti-inflammatory properties of NSAIDs are known to be mediated by COX inhibition and many have attributed their antineoplastic properties to reduction of prostaglandin levels in the target tissue (Vane *et al.,* 1991; Vainio *et al.,* 1997; Levy *et al.,* 1997; Vane *et al.,* 1996 and Abramson *et al.,* 1989). It is possible that COX inhibition does not mediate the anti-neoplastic properties of NSAIDs and this is of considerable clinical significance because a reduction in prostaglandin levels, through inhibition of COX-1, is known to be responsible for the gastrointestinal and renal toxicity that accompanies chronic NSAID administration. If COX inhibition is not necessary or sufficient for the anti-neoplastic properties of NSAIDs it should be feasible to develop less toxic NSAID-like drugs to aid in the treatment of cancer (Piazza *et al.,* 1997). COX-1 is the constitutive form of the Cox enzymes whereas COX-2 is the induced form. Inhibition of Cox explains both the therapeutic effects (inhibition of COX-2) and side effects (inhibition of COX-1) of NSAIDs. An NSAID, which selectively inhibits COX-2, is likely to retain maximal anti-inflammatory efficacy combined with less toxicity.

The Cyclooxygenase 1 assay is a spectrophotometric assay, based on the assay used by Boopathy *et al.,* (1986) and Piazza *et al.,* (1997), to measure inhibition of COX-1 by various compounds. The experimental method is as per section 2.8. In brief, COX-1 was incubated with 100μm arachidonic acid and cofactors (0.5mM glutathione, 0.5mM hydroquinone, 0.625μm haemoglobin and 1.25mM $CaCl_2$ in 100mM TRIS-HCL, pH 7.4-8.0) at 37°C for 20 min in the presence of various NSAIDs or their solvent (1% DMSO final concentration). The reaction was terminated by the addition of trichloroacetic acid. Enzyme activity was measured by the thiobarbituric acid colour reaction of malonaldehyde formed in the reaction and determined by a spectrophotometer at 530nm.

Results obtained from the COX-1 assay (Table 3.4.1.1 and Figure 3.4.1.1) indicated that, of the positive indomethacin analogues, BRI 60/1, 114/2, 115/2 153/1 and 138/1 were not good inhibitors of COX-1. Analogues BRI 88/1 and BRI 104/2 compared favourably with the COX-1 inhibitory ability of indomethacin. The results for BRI 92/1 indicated that it might be a stronger COX-1 inhibitor than indomethacin. Overall, the strongest COX-1 inhibitor appeared to be BRI 92/1 and the weakest inhibitors were BRI 153/1 and 138/1. The remainder of the compounds tested were poor inhibitors of COX-1. The compounds can be listed in order of decreasing ability to inhibit COX-1 as follows: BRI 92/1 > BRI 88/1 ≥ indomethacin ≥ BRI 104/2 > BRI 114/2 > BRI 115/2 > BRI 60/1 > BRI 153/1 ≥ BRI 138/1.

Compound (12μg/ml)	Molar conc. of compound in assay (μM)	Average % Inhibition of COX-1	% S.D.
Indomethacin	0.033	60.9	12.2
BRI 60/1	0.035	16.3	12.4
BRI 88/1	0.038	59.9	13.2
BRI 92/1	0.030	79.0	11.3
BRI 114/2	0.030	24.5	5.8
BRI 104/2	0.037	57.3	12.9
BRI 115/2	0.037	26.6	9.9
BRI 153/1	0.031	-0.1	7.4
BRI 138/1	0.037	0.8	3.6
BRI 203/1	0.032	22.1	8.2
BRI 215/1	0.030	1.7	8.1
BRI 205/4	0.037	-7.4	2.8
BRI 113/1	0.038	-1.0	7.4
No compound (Control)	N/A	0.0	0.0
With DMSO (no compound)	N/A	-1.3	9.0

Table 3.4.1.1: Cyclooxygenase-1 (COX-1) assay results showing the % inhibition of COX-1 activity by indomethacin and indomethacin analogues.
All of the indomethacin analogues analysed, except BRI 215/1, 205/4 and 113/1, were positive in the combination toxicity assays.
The results are the average of a minimum of three assay repeats. Data is expressed as % inhibition relative to an untreated control.

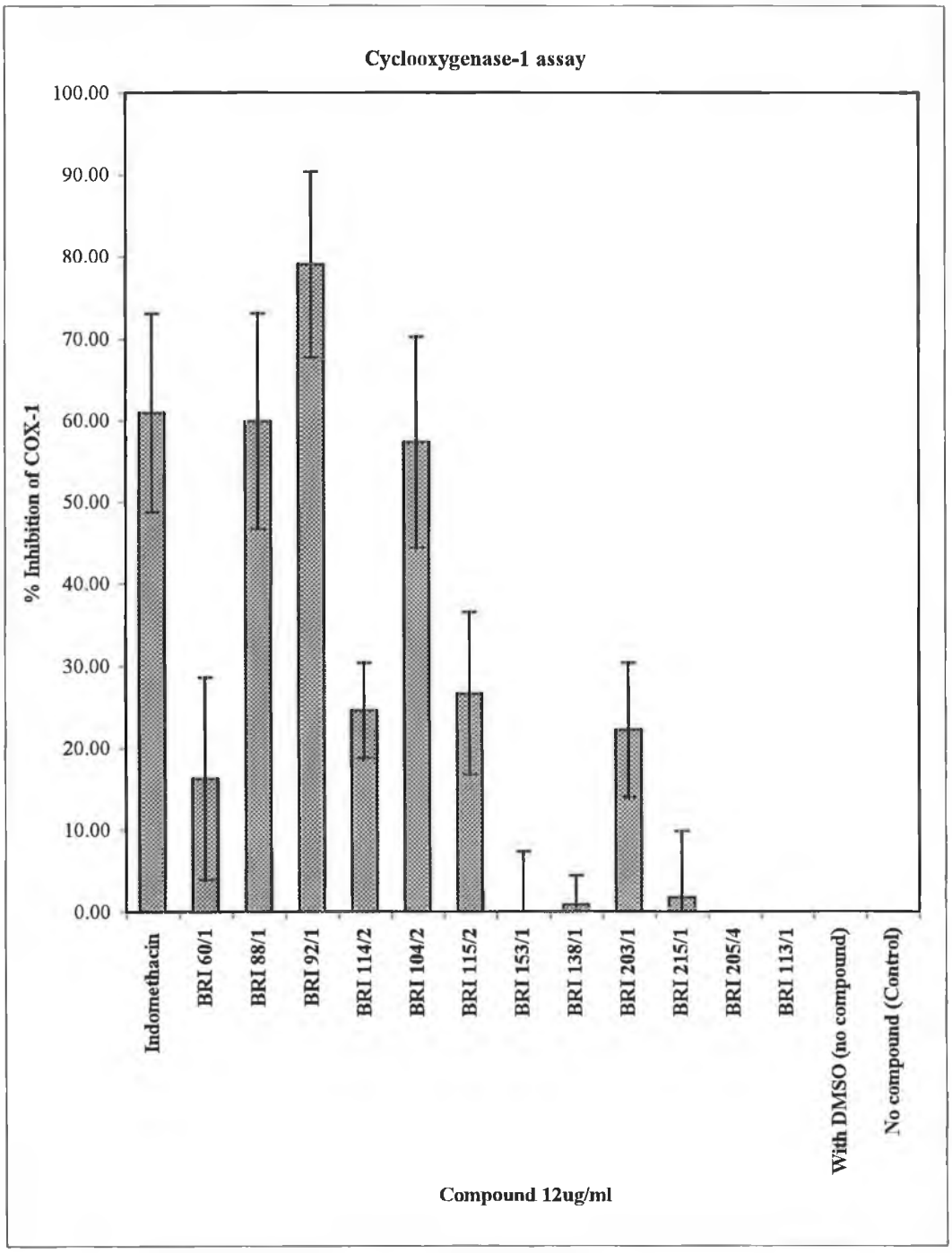


Figure 3.4.1: Graph showing the % inhibition of COX-1 activity by indomethacin and indomethacin analogues.
The results are the average of a minimum of three assay repeats.
Data is expressed as % inhibition relative to an untreated control.

3.4.2. Cyclooxygenase 2 assays

Cytokines such as IL-1 are produced by many cells and appear to bring about the effects of disease or damage such as fever and inflammation. IL-1β can act directly and/or stimulate the release of other pro-inflammatory cytokines and growth factors, which then act upon target cells. These actions include the up-regulation of certain key enzymes involved in the generation of eicosanoids such as PLA_2 and COX-2 (Croxtall *et al.*, 1996; Endo *et al.*, 1995). Typically the release of arachidonic acid and PgE_2 is increased following IL-1β treatment of many cells (Croxtall *et al.*, 1996).

The cell line, A549, a human lung adenocarcinoma, was chosen for the experiment as it was demonstrated by Asano *et al.*, (1996), that COX-2 is the constitutive and dominant isoform in unstimulated and stimulated cultured human lung epithelial cells. A549 cells express COX-2 mRNA and protein when they are stimulated with epidermal growth factor or pro-inflammatory cytokines such as IL-1β (Asano *et al.*, 1996).

A preliminary ELISA for PgE_2 was carried out on A549 cells, treated with varying concentrations of IL-1β (0ng/mL -20ng/mL), and it was found that the optimum level of PgE_2 production in the A549 cells was obtained with 10ng/mL IL-1β. Western blotting was carried out on protein extracted from A549 cells treated with 0ng/mL and 10ng/mL IL-1β to assess the expression of COX-2. Expression of COX-2 could not be detected in A549 cells treated with 0ng/mL IL-1β. However, low expession of COX-2 was observed in the cells treated with 5ng/ml IL-1β and expression of COX-2 was increased in those A549 cells treated with 10ng/mL IL-1β.

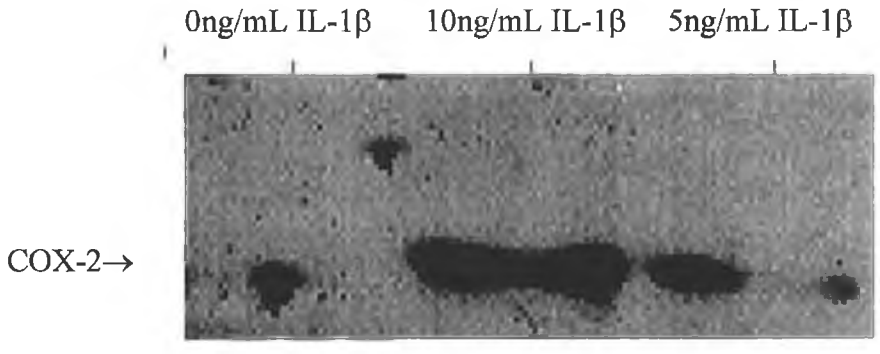


Figure 3.4.2.1: Western blot analysis of COX-2 protein from A549 cells treated with varying concentrations of IL-1β

A study was carried out to investigate if co-treatment of the A549 cell line with IL-1β and indomethacin or indomethacin analogues, resulted in inhibition of the production of PgE_2 by COX-2. A PgE_2 ELISA assay was used to quantify the PgE_2 production by A549 cells during treatment with IL-1β, indomethacin and BRI compounds. The experimental protocol used in these experiments is detailed in section 2.8. In summary, cells were seeded at a density of 1.5 x 10^5 cells per well in 6 well plates and allowed to attach overnight. The waste media was then removed and replaced with freshly prepared media containing the compounds of interest. Indomethacin and indomethacin analogues (those compounds positive in the combination toxicity assays) were added with and without IL-1β to ensure that the addition of indomethacin and the indomethacin analogues alone did not induce the production of COX-2. After a further 24 hours, aliquots of media were then removed from each of the treated wells and added to the ELISA plate for analysis. The results of this study are analysed in table 3.5.2.1. Analysis of the results demonstrated that when the unstimulated PgE_2 production is taken as zero, IL-1β- induced A549 cells produced approximately 112 ρg PgE_2 per 10^5 cells. 10nM of indomethacin and indomethacin analogue were added to the A549 cells and it was found that indomethacin, BRI 60/1, 88/1, 92/1 and 104/2 were most capable of inhibiting PgE_2 production (90.2 - 73.6 % inhibition of PgE_2). BRI 92/1 was the most potent inhibitor of PgE_2 production (90.2% inhibition of PgE_2). BRI 114/2, 115/2 were only capable of inhibiting COX-2 by 39.7 and 33.6% respectively whereas indomethacin analogues 153/1, 203/1, and 138/1 were not capable of inhibiting PgE_2 production by COX-2. BRI 215/1, which was negative in the combination toxicity assay, was also negative in the COX-2 assay.

Treatment	% Inhibition of production of PgE_2 by Cox-2	S.D.
Cell control	0.0	0.0
IL-1B (10mg/ml)	0.0	0.0
Indomethacin (10nM) + IL-1B	86.1	5.9
60/1 (10nM) + IL-1B	78.1	11.1
88/1(10nM) + IL-1B	80.4	12.7
92/1(10nM) + IL-1B	88.1	4.4
104/2 (10nM) + IL-1B	79.6	12.2
114/2 (10nM) + IL-1B	44.1	12.2
115/2 (10nM) + IL-1B	40.1	18.5
153/1(10nM) + IL-1B	14.7	15.0
138/1 (10nM) + IL-1B	13.6	10.6
203/1(10nM) + IL-1B	24.2	8.1
215/1 (10nM) + IL-1B	0.2	0.3
205/4 (10nM) + IL-1B	48.0	0.0

Table 3.4.2.1: Effect of indomethacin and analogues on production of PgE_2 by COX-2. Results are represented as means ± S.D. for duplicate determinations carried out on three separate occasions. Inhibition is expressed as a percentage of untreated control (IL-1β (10mg/ml)), taken as 100%.

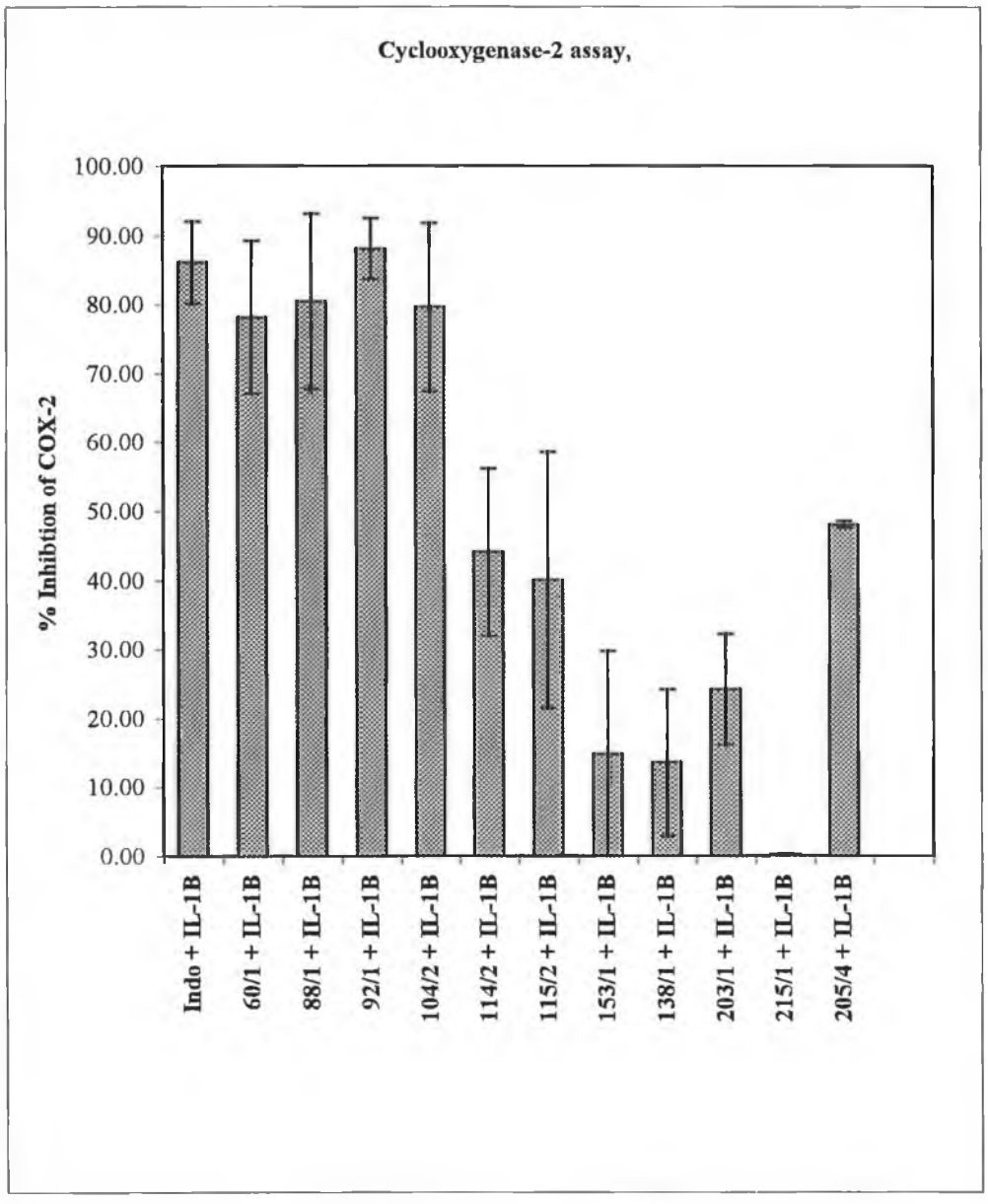


Figure: 3.4.2: Graph showing % inhibition of COX-2 enzyme by indomethaci and indomethacin analogues. Results are represented as means +/- S.D. for triplicate determinations carried out on three separate occasions.

	Combination Toxicity Assay	GST	IOV	Cox-1	Cox-2
Indomethacin	+++	+++	+++	++	+++
BRI 60/1	+++	-	++	+	+++
BRI 59/1	-	-	-		
BRI 69/2	-	-	-		
BRI 88/1	+++	++	++(+)	++	+++
BRI 92/1	+++	+++	+++	+++	+++
BRI 104/2	+++	++	++	++	+++
BRI 106/1	-	-			
BRI 107/1	-	+/-			
BRI 114/2	+++	-	++(+)	+	++
BRI 115/2	+++	+/-	+++	+	++
BRI 113/1	-	+/-		-	
BRI 124/1	-	-			
BRI 119/1	-	-	-		
BRI 120/1	-	-	-		
BRI 153/1	+++	-	+++	-	+/-
BRI 203/1	+++	-	++	+	+
BRI 205/4	-	-	-	-	++
BRI 215/1	-	-	+/-	+/-	-
BRI 138/1	+++	-	+	-	+/-

Table 3.4.3: Summary table of all results from investigations of the SAR (Structure Activity Relationship) of indomethacin (section 3.1 to section 3.4).

+++ Very strong positive; ++ Strong positive; + positive; +/- weakly positive; - negative.

3.5 Indomethacin and adriamycin efflux studies using HPLC analysis.

3.5.1 Adriamycin efflux studies

There is strong evidence to suggest that indomethacin and analogues potentiate the toxicity of adriamycin by interacting with MRP. It is postulated that these compounds have a strong affinity for MRP and when added to an MRP-expressing cell line, in combination with a chemotherapeutic drug, the compounds inhibit or interfere with the activity of the pump and reduce the rate at which the chemotherapeutic drug is effluxed. It is possible that the active compounds may also be very good substrates for MRP and compete with the chemotherapeutic drug for this protein. Adriamycin quantitation was carried out on DLKP cells treated with a combination of adriamycin and indomethacin or indomethacin analogues. The cells, which were approximately 80% confluent in $75cm^2$ flasks, were 'loaded' for two hours at 37°C with 10μM adriamycin alone, or, adriamycin and 28μM indomethacin combined. After this initial loading period, the media was removed from the cells (T0 hr) the flasks were washed twice with fresh media and media containing the test compound (indomethacin/indomethacin analogue), minus adriamycin, was added to the appropriate flasks. The cells were incubated for a further 5 hours (T5 hr). At required time points, the cells were rapidly trypsinised, counted, pelleted and frozen at -20°C until analysed by HPLC as described in section 2.13. An analogue of indomethacin, which was positive in the combination toxicity assay (138/1) and a negative analogue (205/4) were also used in this assay.

The results of this HPLC analysis showed a notable difference in the cellular adriamycin content in DLKP cells incubated with 10μm adriamycin alone versus DLKP cells incubated with adriamycin and 28μm indomethacin or 138/1 combined. After the initial 2 hour loading period, there was similar accumulation of adriamycin in the cells treated with adriamycin alone (2.4μM adriamycin/million cells) and the cells treated with a combination of adriamycin + indomethacin or 138/1 (2.2μM adriamycin /million cells). When the levels of indomethacin and 138/1 were maintained in the cells for a further five hours the level of adriamycin were only slightly decreased in these cells

(1.8 – 1.9μM adriamycin/million cells). However, there was decreased accumulation of adriamycin in those cells treated with adriamycin alone (1.0μM adriamycin/million cells). The negative indomethacin analogue, BRI 205/4, had no effect on the accumulation of adriamycin in the DLKP cells. Maintaining the levels of BRI 205/4 in the medium, during the five hour incubation period, did not reduce the efflux of adriamycin from the cells. The results suggest that the export of adriamycin from MRP-expressing cells can be inhibited by indomethacin and it's active analogues.

Time	Treatment	Average μM adriamycin per million cells	S.D.
T0	Adriamycin alone	2.4	0.05
T5	Adriamycin alone	1.0	0.09
T0	Adriamycin + Indomethacin	2.3	0.15
T5	(+) Indomethacin	1.8	0.17
T5	(-) Indomethacin	1.2	0.17
T0	Adriamycin + 138/1	2.3	0.12
T5	(+) 138/1	1.9	0.07
T5	(-) 138/1	1.0	0.22
T0	Adriamycin + 205/4	2.0	0.17
T5	(+) 205/4	0.9	0.09
T5	(-) 205/4	0.7	0.09

Table 3.5.1.1: Adriamycin levels in DLKP cells treated with adriamycin alone versus DLKP cells treated with adriamycin and indomethacin / indomethacin analogues combined. Data shown are the average of three separate determinations.

T0: Time point immediately after initial 2 hour loading period

T5: Time point 5 hours after initial 2 hour loading period

(+) Indomethacin/analogue: Flasks re-fed with either indomethacin or analogue after initial 2 hour loading period.

(-) Indomethacin/analogue: Flasks re-fed with media only after initial 2 hour loading period.

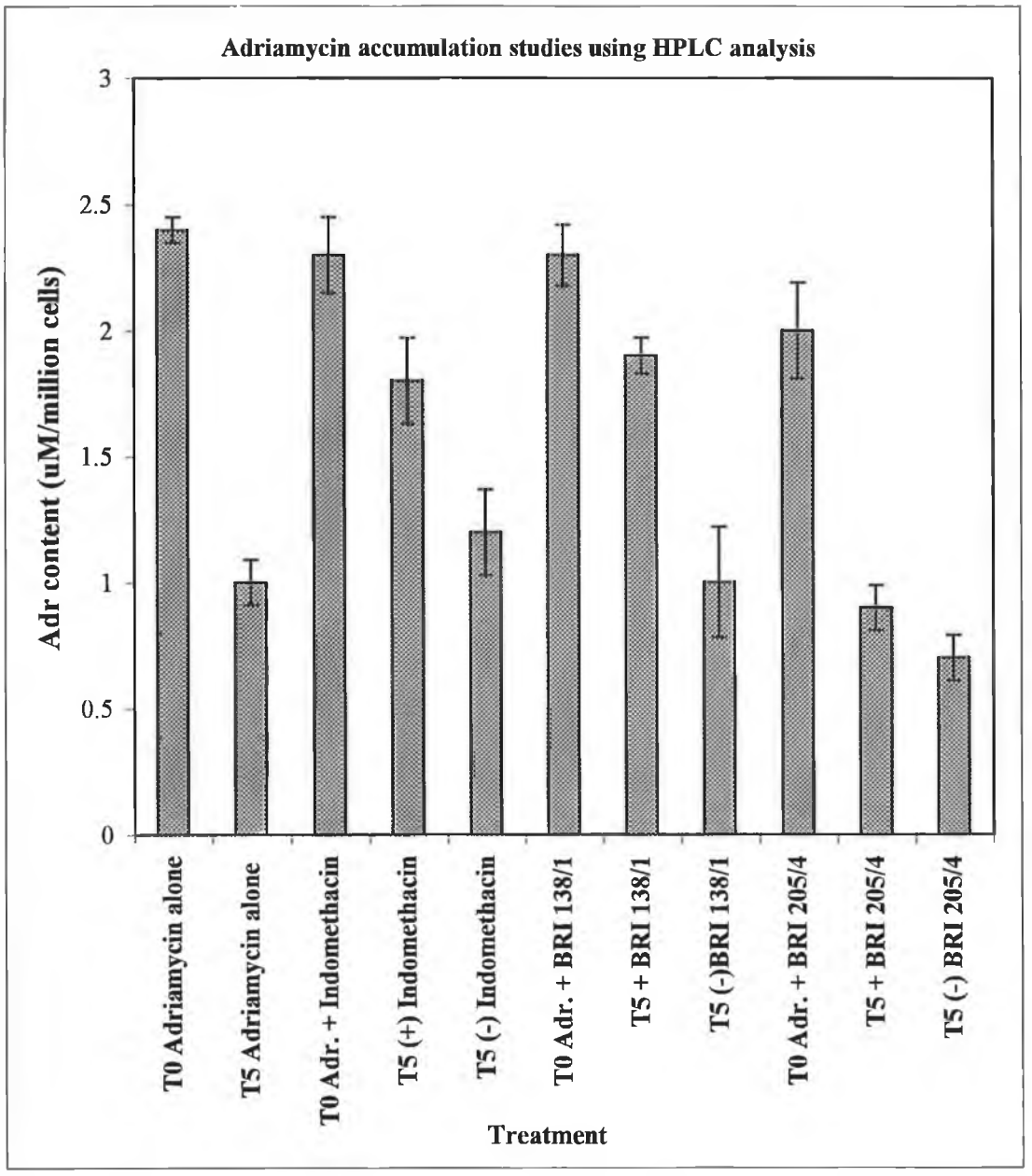


Figure 3.5.1: HPLC analysis of the effect of indomethacin and indomethacin analogues on adriamycin content in DLKP cells. Results are represented as means +/- S.D. for triplicate determinations.

3.5.2 Indomethacin efflux studies

Indomethacin efflux was also analysed, by HPLC, in cells treated with indomethacin alone or adriamycin and indomethacin combined. The cells, which were approximately 80% confluent, were 'loaded' for two hours at 37°C with 28μm indomethacin alone, or indomethacin and 10μm adriamycin combined. After the initial loading period samples were taken at T0 and the remaining flasks were re-fed with either fresh media or fresh media containing adriamycin to assess if maintaining the level of the anticancer drug in the cells would have an effect on the accumulation of indomethacin in the cells. The cells were incubated for a further 90 minutes. (This time point was chosen as preliminary HPLC experiments indicated that following two hours incubation, after the initial loading period, the levels of indomethacin in the cells had decreased to almost undetectable levels).

The results indicate that after the initial loading period the level of indomethacin in the cells was higher in those cells treated with a combination of indomethacin and adriamycin (0.044μM indomethacin/million cells) than the level of indomethacin in those cells treated with indomethacin alone (0.03μM indomethacin/million cells). In those cells incubated with both indomethacin and adriamycin during the initial loading period, and then refed with adriamycin alone for a further 90 minutes, there was almost no efflux of indomethacin from the cells at the end of this period (0.03μM indomethacin/million cells) as compared to those cells incubated with fresh media alone (0.006μM indomethacin/million cells). This suggests that in the presence of adriamycin the efflux of indomethacin from the cell is reduced.

An experiment was also carried out to investigate if co-incubation of indomethacin with adriamycin during the initial loading period was required for maintanance of the cellular indomethacin content. The cells were loaded for two hours with indomethacin and after this initial period the media was removed from the cells and the cells were refed with adriamycin alone for 90 minutes incubation. After this incubation the level of indomethacin in the cells was measured and was found to have decreased to 0.007μM indomethacin/million cells (as compared to 0.3μM indomethacin per million cells in those cells co-incubated with indomethacin and adriamycin). These

results indicate that co-incubation of indomethacin with adriamycin is required for the maintenance of cellular indomethacin content. This suggests that adriamycin and indomethacin compete for the same binding site on the MRP molecule and the results indicate that adding adriamycin to the cells after the initial loading period only was not as effective as indomethacin had already bound to the MRP binding site.

Time	Treatment	Average μM indomethacin per million cells	S.D.
T0	Indomethacin alone	0.03	0.002
T90min	Indomethacin alone	0.01	0.002
T0	Indomethacin + Adriamycin	0.04	0.008
T90min	(+) Adriamycin	0.03	0.007
T90min	(-) Adriamycin	0.01	0.001
T90min	Indomethacin (+ Adr @T0)*	0.01	0.002

Table 3.5.2.1: Indomethacin content in DLKP cells treated with indomethacin alone versus DLKP cells treated with adriamycin and indomethacin combined. Data shown are the average of three separate determinations.

T0: Time point immediately after initial 2 hour loading period

T90min: Time point 90 minutes after initial 2 hour loading period

(+) adriamycin: Flasks re-fed with adriamycin after initial 2 hour loading period.

(-) adriamycin: Flasks re-fed with media only after initial 2 hour loading period.

* Flasks were incubated with indomethacin alone for the initial 2 hour loading period and were then washed and refed with media containing adriamycin. These flasks were subsequently incubated for a further 90 minutes.

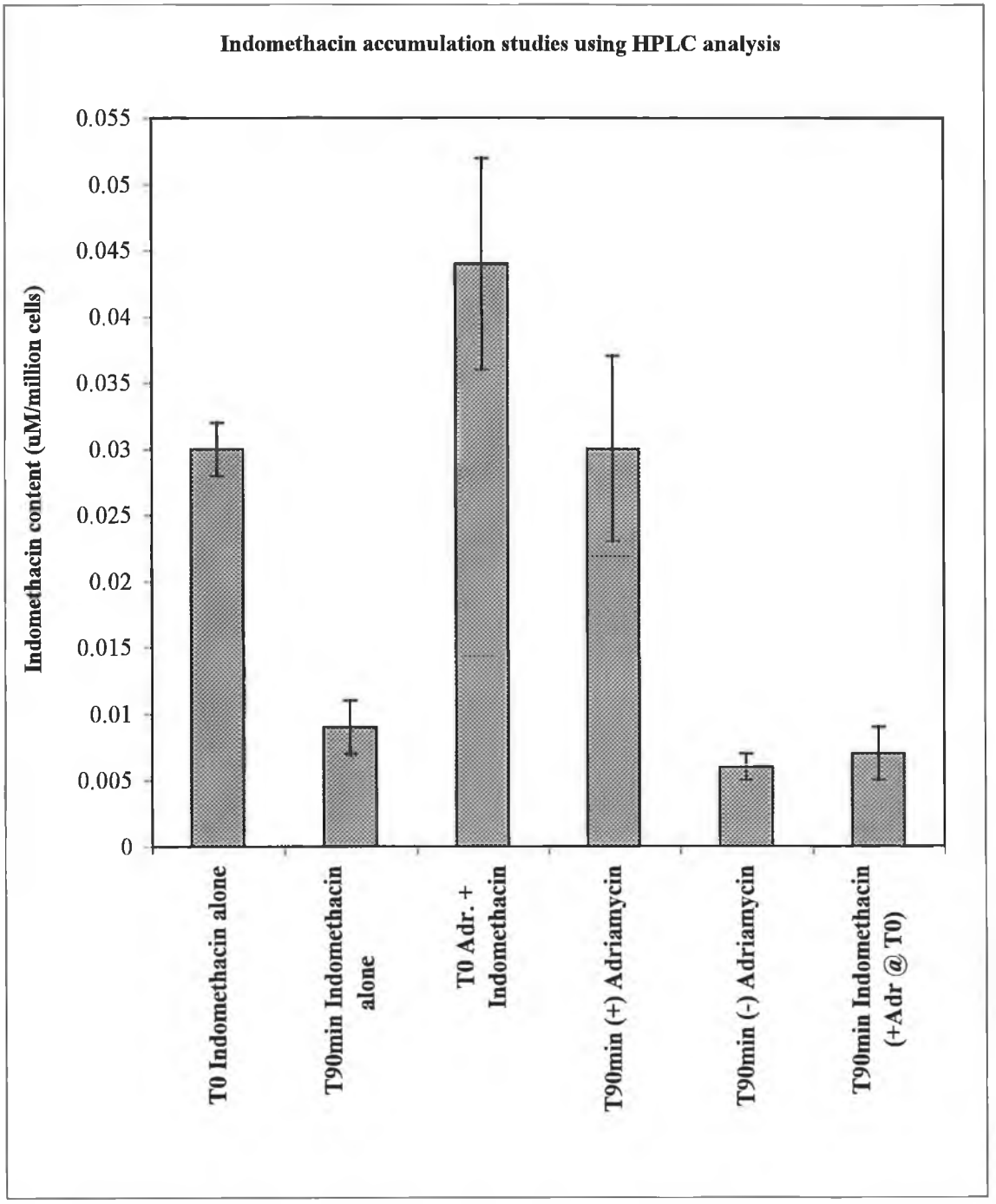


Figure 3.5.2: HPLC analysis of the effect of adriamycin on indomethacin content in DLKP cells.
Results are represented as means +/- S.D. for triplicate determinations.

3.6 *In vitro* combination toxicity assays – CORL23 cell line

The resistant variant of the cell line CORL23, CORL23(R), overexpresses MRP1 which is not overexpressed by the parental form, CORL23(P) (Kool *et al.*, 1998). A Western blot carried out on both cell lines demonstrates the difference in the expression of MRP protein in the cell lines. There is a very low level of expression of MRP in the CORL23(P) cell line as opposed to a very strong expression in the CORL23(R) cell line (figure 3.6.1). As it was postulated that the NSAIDs and indomethacin analogues potentiated the toxicity of chemotherapeutic drugs by inhibiting MRP (Duffy et al., 1998), this difference in expression of MRP in the CORL23 cells was exploited to further assess the mode of action of the compounds. A number of the BRI compounds, which had been positive in the combination toxicity assay in DLKP cells (section 3.1), were added at non-toxic concentrations to both the CORL23(R) and CORL23(P) cells, in combination with a range of chemotherapeutic drugs. The expected result was that the active analogues would potentiate toxicity only in the resistant cell line.

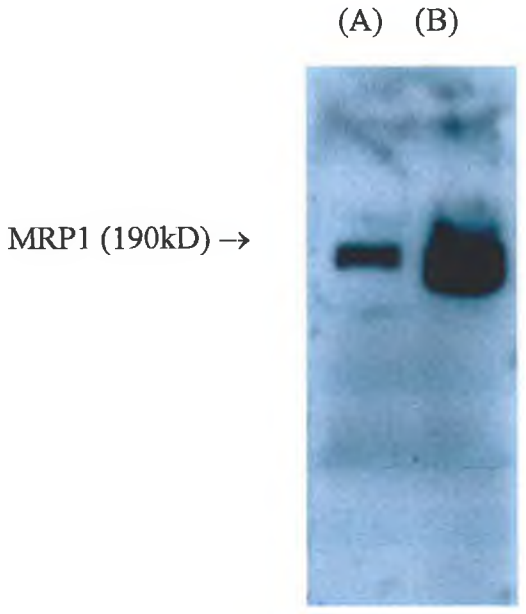


Figure 3.2a: Western blot analysis of CORL23 variants for MRP1 expression using an MRP1 specific monoclonal antibody, MRPr1: (A) CORL23(S); (B) CORL23(R).

Indomethacin, BRI 153/1, BRI 88/1 and BRI 92/1 in combination with the chemotherapeutic drugs, vincristine, adriamycin and VP-16, resulted in an increase in the toxicity of the chemotherapeutic drugs in the CORL23(R) cell line (Table 3.6.6b, 3.6.9b and 3.6.15b). There was evidence of an increase in the toxicity of the same drugs in the CORL23(P) cell line (Table 3.6.6a, 3.6.9a and 3.6.15a). However, there is low expression of MRP1 in this parental cell line (Kool *et al.,* 1997) (and as shown in figure 3.2a) which indicates that this potentiation of toxicity by the indomethacin analogues is evident even in the presence of a very low expression of MRP1. The hypothesis that the compounds interact with MRP was further strengthened by the results obtained in the combination toxicity assays on the CORL23 cell lines using the indomethacin analogues and the MRP-negative chemotherapeutic drugs 5'Fluorouracil and cisplatin. There was no evident increase in toxicity of these drugs in any of the toxicity assays when used in combination with BRI 88/1, 92/1, 153/1, sulindac and indomethacin (Table 3.6.12b).

Interestingly, sulindac, indomethacin and the positive indomethacin analogues, BRI 88/1, 92/1 and 153/1 also potentiated the toxicity of the taxanes, taxol and taxotere, in both the resistant and the parental CORL23 cell lines. There was only slightly greater enhancement in the resistant cell line than in the sensitive cell line. The combination index values for the short-term combination toxicity assays were lower than those obtained with the long-term assays using the same drug combinations suggesting that sulindac is not as effective at potentiating the toxicity of these anticancer drugs in the short-term assay and requires longer incubation times to exert the optimum effect (see section 3.8). Taxol and taxotere toxicity was also analysed in these cell lines in combination with the negative indomethacin analogue 205/4. The results indicated that there was also no enhancement of the toxicity of taxol or taxotere by this negative analogue. These results would suggest that the enhancement of the toxicity of taxol and taxotere, by the active compounds, is through the same mechanism by which the toxicity of adriamycin, vincristine and VP-16 are potentiated. However, the taxanes had previously been reported by a number of authors including Cole *et al.,* (1992), and Twentyman *et al.,* (1994), to be poor MRP1 substrates.

The results from the toxicity assays on the parental CORL23 cell line, using a combination of vincristine and indomethacin, 88/1 or 92/1 demonstrated a significant enhancement of the toxicity of the chemotherapeutic drug. This enhancement was comparable to the magnitude of the enhancement effect in the resistant CORL23 cell line. The CORL23(R) cells were more resistant to the range of anti-cancer drugs analysed in this set of experiments which seems to correlate with the overexpression of MRP1 in the CORL23(R) cell line. There was slightly greater enhancement of adriamycin, vincristine, VP16, taxol and taxotere in the CORL23(R) cell line than in the CORL23(P) cell line. However, the combination index values obtained for both of the cell lines suggest that the level of the expression of MRP does not appear to strictly correlate to the rate of potentiation of the anticancer drug by the positive NSAIDs. Duffy *et al.,* (1998), also demonstrated higher levels of MRP protein expression in the A549, a human lung adenocarcinoma cell line, than in the DLKP cell line. Yet, the authors reported comparable synergistic combination of selected NSAIDs with certain chemotherapeutic drugs in both cell lines.

Note:

- All toxicity assays in this section, except for those specifically referred to as "Short-term" were carried according to section 2.7.3a i.e. 7 day toxicity assays whereby drug and/or compound were added to the cells on Day 2 and remain in the cells until Day 7 when the cells were analysed.
- The Short-term toxicity assays referred to in this section were performed using the method described in section 2.7.3b i.e. 6 day toxicity assays whereby the cytotoxic drugs and test inhibitors are added to the cells on Day 2 and were incubated in the assay for a period of four hours only. The cells were then re-fed with fresh media and incubated until Day 6 when the cells were analysed.

CORL23(P), Adriamycin + BRI 153/1 and Indomethacin.		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 20 ng/ml	52.8	4.7
Indo (2.5μg/ml)	101.6	5.6
Indo + Adr	**31.9**	**6.4**
Indo (1.25μg/ml)	104.5	5.7
Indo + Adr	**32.9**	**4.3**
Indo (0.625μg/ml)	98.7	5.0
Indo + Adr	**38.5**	**5.7**
153/1 (50μg/ml)	97.5	5.9
153/1 + Adr	**34.8**	**5.0**
153/1 (25μg/ml)	105.0	4.3
153/1 + Adr	**38.1**	**9.0**
153/1 (12.5μg/ml)	104.2	4.4
153/1 + Adr	**41.4**	**5.1**
Adr. 10 ng/ml	95.45	4.7
Indo (2.5μg/ml)	101.3	4.1
Indo + Adr	**87.1**	**4.4**
Indo (1.25μg/ml)	101.4	2.5
Indo + Adr	**91.0**	**1.9**
Indo (0.625μg/ml)	100.7	1.9
Indo + Adr	**95.2**	**1.6**
153/1 (50μg/ml)	100.3	4.7
153/1 + Adr	**81.1**	**6.0**
153/1 (25μg/ml)	103.7	3.0
153/1 + Adr	**86.0**	**1.6**
153/1 (12.5μg/ml)	103.8	3.8
153/1 + Adr	**90.1**	**6.2**

Table 3.6.1: % Survival of CORL23 (parental) cells in the presence of various concentrations of adriamycin, indomethacin and BRI 153/1 as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

CORL23(R), Adriamycin + BRI 153/1 and Indomethacin.		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 250 ng/ml	46.7	3.1
Indo (2.5μg/ml)	98.1	3.8
Indo + Adr	**16.1**	**5.7**
Indo (1.25μg/ml)	101.0	4.3
Indo + Adr	**21.4**	**3.6**
Indo (0.625μg/ml)	100.6	201
Indo + Adr	**28.7**	**4.1**
153/1 (50μg/ml)	103.4	5.8
153/1 + Adr	**13.0**	**1.4**
153/1 (25μg/ml)	103.1	3.0
153/1 + Adr	**25.7**	**3.8**
153/1 (12.5μg/ml)	106.5	5.1
153/1 + Adr	**36.3**	**3.1**
Adr. 125 ng/ml	74.6	2.8
Indo (2.5μg/ml)	102.3	3.7
Indo + Adr	**31.2**	**7.5**
Indo (1.25μg/ml)	102.0	3.6
Indo + Adr	**47.0**	**5.1**
Indo (0.625μg/ml)	99.1	7.6
Indo + Adr	**60.2**	**6.5**
153/1 (50μg/ml)	102.2	4.4
153/1 + Adr	**18.5**	**5.2**
153/1 (25μg/ml)	98.3	7.6
153/1 + Adr	**48.8**	**2.9**
153/1 (12.5μg/ml)	105.0	4.6
153/1 + Adr	**63.7**	**4.7**

Table 3.6.2: % Survival of CORL23 (resistant) cells in the presence of various concentrations of adriamycin, indomethacin and BRI 153/1 as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

CORL23(P), Adriamycin + BRI 88/1, 92/1 and Indomethacin.		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 20 ng/ml	38.0	7.2
Indo (2.5μg/ml)	98.9	1.2
Indo + Adr	**18.5**	**4.6**
Indo (1.25μg/ml)	101.7	2.8
Indo + Adr	**21.04**	**11.1**
Indo (0.625μg/ml)	100.7	2.9
Indo + Adr	**24.3**	**13.7**
88/1 (5μg/ml)	103.0	5.6
88/1 + Adr	**17.2**	**1.3**
88/1 (2.5μg/ml)	97.8	4.8
88/1 + Adr	**19.1**	**1.6**
88/1 (1.25μg/ml)	97.1	6.8
88/1 + Adr	**18.4**	**2.1**
92/1 (5μg/ml)	102.0	11.7
92/1 + Adr	**24.6**	**8.6**
92/1 (2.5μg/ml)	107.7	4.1
92/1 + Adr	**25.2**	**9.8**
92/1 (1.25μg/ml)	108.4	9.5
92/1 + Adr	**26.5**	**8.3**

Table 3.6.3: % Survival of CORL23 (parental) cells in the presence of 20ng/ml adriamycin + various concentrations of indomethacin, BRI 88/1 or BRI 92/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

CORL23(R), Adriamycin + BRI 88/1, 92/1 and Indomethacin.		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 250 ng/ml	29.2	11.4
Indo (2.5μg/ml)	99.3	1.4
Indo + Adr	**13.0**	**2.9**
Indo (1.25μg/ml)	99.8	2.1
Indo + Adr	**20.4**	**4.8**
Indo (0.625μg/ml)	101.0	4.8
Indo + Adr	**31.9**	**12.8**
88/1 (5μg/ml)	101.9	2.5
88/1 + Adr	**5.8**	**1.0**
88/1 (2.5μg/ml)	100.9	5.2
88/1 + Adr	**7.8**	**0.7**
88/1 (1.25μg/ml)	102.4	5.9
88/1 (3) + Adr	**11.1**	**1.8**
92/1 (5μg/ml)	94.0	7.6
92/1 + Adr	**9.7**	**0.2**
92/1 (2.5μg/ml)	100.7	6.5
92/1 + Adr	**14.9**	**1.0**
92/1 (1.25μg/ml)	103.4	9.1
92/1 + Adr	**19.6**	**2.5**

Table 3.6.4: % Survival of CORL23 (resistant) cells in the presence of 250ng/ml adriamycin + various concentrations of Indomethacin, BRI 88/1 or BRI 92/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

CORL23(P) Adriamycin and BRI 205/4		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 20ng/ml	32.7	4.6
205/4 (25μg/ml)	98.1	6.0
205/4 + Adr	**28.6**	**2.6**
205/4 (12.5μg/ml)	97.7	4.7
205/4 + Adr	**29.9**	**3.5**
205/4 (6.25μg/ml)	98.2	3.1
205/4 + Adr	**31.3**	**3.1**

CORL23(R) Adriamycin and BRI 205/4		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 250ng/ml	50.8	2.9
205/4 (25μg/ml)	103.2	6.4
205/4 + Adr	**46.9**	**2.6**
205/4 (12.5μg/ml)	96.4	3.1
205/4 + Adr	**47.9**	**2.4**
205/4 (6.25μg/ml)	103.3	6.4
205/4 + Adr	**51.1**	**4.3**

Table 3.6.5: % Survival of CORL23(P) and CORL23(R) cells in the presence of adriamycin, in combination with 205/4 as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

	No Anticancer agent	Adriamycin (20ng/ml)	CI values
No NSAID	100 ± 0.0	52.8 ± 4.7	
Indomethacin 2.5μg/ml	101.6 ± 5.6	31.9 ± 6.4	0.779
BRI 153/1 50μg/ml	97.5 ± 5.9	34.8 ± 5.0	0.813
No NSAID	100 ± 0.0	38.0 ± 7.2	
Indomethacin 2.5μg/ml	98.9 ± 1.2	18.5 ± 4.6	0.672
BRI 88/1 5μg/ml	103.0 ± 5.6	17.2 ± 1.3	0.670
BRI 92/1 5μg/ml	102.0 ± 11.7	24.6 ± 8.6	0.749
No NSAID	100 ± 0.0	38.9 ± 5.5	
BRI 205/4 2.5μg/ml	98.1 ± 6.0	36.3 ± 4.2	1.0

Table: 3.6.6a: CORL23(P), Adriamycin + highest non-toxic concentrations of indomethacin and indomethacin analogues.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

Statistical weight of the results is indicated by CI values (see section 2.16)

CI: Combination index values.

CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.

	No Anticancer agent	Adriamycin (20ng/ml)	CI values
No NSAID	100 ± 0.0	46.7 ± 3.1	
Indomethacin 2.5μg/ml	98.1 ± 3.8	16.1 ± 5.7	0.542
BRI 153/1 50μg/ml	103.4 ± 5.9	13.0 ± 1.4	0.382
No NSAID	100 ± 0.0	29.2 ± 11.4	
Indomethacin 2.5μg/ml	99.3 ± 1.4	13.0 ± 2.9	0.704
BRI 88/1 5μg/ml	101.9 ± 2.5	5.8 ± 1.0	0.432
BRI 92/1 5μg/ml	94.0 ± 7.6	9.7 ± 0.2	0.552
No NSAID	100 ± 0.0	50.8 ± 2.9	
BRI 205/4 2.5μg/ml	103.2 ± 6.4	47.0 ± 2.6	1.0

Table: 3.6.6b: CORL23(R), Adriamycin + highest non-toxic concentrations of indomethacin and indomethacin analogues.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

Statistical weight of the results is indicated by CI values (see section 2.16)

CI : Combination index.

CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.

CORL23(P), Vincristine + BRI 88/1, 92/1 and Indomethacin.		
Test Sample	**% Cell Survival**	**S.D.**
Vin. 1 ng/ml	42.0	2.4
Indo (2.5μg/ml)	99.3	0.9
Indo + Vin	**11.3**	**3.2**
Indo (1.25μg/ml)	99.5	0.7
Indo + Vin	**12.6**	**0.5**
Indo (0.625μg/ml)	98.8	0.7
Indo + Vin	**16.4**	**3.4**
88/1 (5μg/ml)	90.5	5.1
88/1 + Vin	**12.9**	**1.2**
88/1 (2.5μg/ml)	97.1	8.5
88/1 + Vin	**18.6**	**2.3**
88/1 (1.25μg/ml)	101.3	6.2
88/1 + Vin	**31.9**	**3.4**
92/1 (5μg/ml)	106.8	7.7
92/1 + Vin	**19.0**	**2.4**
92/1 (2.5μg/ml)	109.1	7.8
92/1 + Vin	**20.6**	**0.9**
92/1 (1.25μg/ml)	108.2	1.9
92/1 + Vin	**22.8**	**1.6**

Table 3.6.7: % Survival of CORL23 (parental) cells in the presence of 1ng/ml Vincristine + various concentrations of indomethacin BRI 88/1, 92/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D.

Results are the average of triplicate determinations in three separate experiments.

CORL23(R), Vincristine + BRI 88/1, 92/1 and Indomethacin.

Test Sample	% Cell Survival	S.D.
Vin. 12 ng/ml	57.5	15.1
Indo (2.5μg/ml)	98.9	1.7
Indo + Vin	**16.9**	**3.0**
Indo (1.25μg/ml)	105.0	4.4
Indo + Vin	**27.7**	**3.6**
Indo (0.625μg/ml)	105.3	4.5
Indo + Vin	**38.7**	**3.2**
88/1 (5μg/ml)	90.4	4.8
88/1 + Vin	**15.2**	**1.5**
88/1 (2.5μg/ml)	92.0	8.0
88/1 + Vin	**18.9**	**2.1**
88/1 (1.25μg/ml)	92.5	2.8
88/1 + Vin	**44.4**	**1.43**
92/1 (5μg/ml)	93.3	7.3
92/1 + Vin	**11.5**	**3.7**
92/1 (2.5μg/ml)	96.0	9.4
92/1 + Vin	**17.7**	**6.9**
92/1 (1.25μg/ml)	100.3	6.0
92/1 + Vin	**25.0**	**10.4**

Table 3.6.8: % Survival of CORL23 (resistant) cells in the presence of 12 ng/ml Vincristine + various concentrations of indomethacin BRI 88/1, 92/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D.

Results are the average of triplicate determinations in three separate experiments.

	No Anticancer agent	Vincristine (1ng/ml)	CI values
No NSAID	100 ± 0.0	42.0 ± 2.4	
Indomethacin 2.5μg/ml	99.3 ± 0.9	11.3 ± 3.2	0.333
BRI 88/1 5μg/ml	90.5 ± 5.1	12.9 ± 1.2	0.554
BRI 92/1 5μg/ml	106.8 ± 7.7	19.0 ± 2.36	0.532

Table: 3.6.9a: CORL23(P), Vincristine + highest non-toxic concentrations of indomethacin and indomethacin analogues.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

	No Anticancer agent	Vincristine (12ng/ml)	CI values
No NSAID	100 ± 0.0	57.5 ± 15.1	
Indomethacin 2.5μg/ml	98.9 ± 1.7	16.9 ± 3.0	0.417
BRI 88/1 5μg/ml	90.4 ± 1.5	15.2 ± 1.5	0.329
BRI 92/1 5μg/ml	93.3 ± 7.3	11.5 ± 3.7	0.284

Table: 3.6.9b: CORL23(R), Vincristine + highest non-toxic concentrations of indomethacin and indomethacin analogues.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

Statistical weight of the results is indicated by CI values (see section 2.16)

CI: Combination index values.

CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.

CORL23(P), 5-FU + BRI 92/1, 88/1 and Indomethacin.		
Test Sample	**% Cell Survival**	**S.D.**
5-FU. 0.4 μg/ml	28.9	1.1
Indo (2.5μg/ml)	97.4	2.8
Indo + 5-FU	**27.7**	**7.0**
Indo (1.25μg/ml)	99.6	1.9
Indo + 5-FU	**27.0**	**7.3**
Indo (0.625μg/ml)	98.8	1.6
Indo + 5-FU	**27.2**	**6.5**
92/1 (5μg/ml)	103.5	11.6
92/1 + 5-FU	**28.2**	**9.3**
92/1 (2.5μg/ml)	103.3	7.8
92/1 + 5-FU	**27.7**	**8.8**
92/1 (1.25μg/ml)	105.2	8.2
92/1 (3) + 5-FU	**28.7**	**9.2**
88/1 (5μg/ml)	97.8	4.7
88/1 + 5-FU	**28.4**	**3.1**
88/1 (2.5μg/ml)	99.1	2.0
88/1 + 5-FU	**28.9**	**4.0**
88/1 (1.25μg/ml)	99.0	4.3
88/1 + 5-FU	**28.2**	**7.1**

Table 3.6.10: % Survival of CORL23 (parental) cells in the presence of 0.4 μg/ml 5'Fluorouracil + various concentrations of indomethacin, BRI 88/1 and BRI 92/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

CORL23(R), 5-FU + BRI 92/1, 88/1 and Indomethacin.		
Test Sample	**% Cell Survival**	**S.D.**
5-FU. 0.4 μg/ml	15.5	1.2
Indo (2.5μg/ml)	99.0	4.6
Indo + 5-FU	**14.0**	**5.0**
Indo (1.25μg/ml)	99.9	6.5
Indo + 5-FU	**14.0**	**4.0**
Indo (0.625μg/ml)	101.9	6.3
Indo (4) + 5-FU	**14.2**	**3.5**
92/1 (5μg/ml)	94.6	0.9
92/1 + 5-FU	**15.7**	**3.5**
92/1 (2.5μg/ml)	92.9	1.8
92/1 + 5-FU	**15.3**	**3.0**
92/1 (1.25μg/ml)	97.1	8.2
92/1 (3) + 5-FU	**15.4**	**3.5**
88/1 (5μg/ml)	98.8	5.8
88/1 + 5-FU	**14.9**	**1.4**
88/1 (2.5μg/ml)	99.1	2.0
88/1 + 5-FU	**15.1**	**1.8**
88/1 (1.25μg/ml)	99.0	4.3
88/1 + 5-FU	**15.6**	**1.8**

Table 3.6.11: % Survival of CORL23 (resistant) cells in the presence of 2.0 μg/ml 5'Fluorouracil + various concentrations of indomethacin, BRI 88/1 and BRI 92/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

	No Anticancer agent	5-FU (0.4μg/ml)	CI values
No NSAID	100 ± 0.0	28.9 ± 1.1	
Indomethacin 2.5μg/ml	97.4 ± 2.8	27.7 ± 7.0	1.000
BRI 88/1 5μg/ml	97.8 ± 4.7	28.4 ± 3.1	1.000
BRI 92/1 5μg/ml	103.5 ± 11.6	28.2 ± 9.3	1.146

Table: 3.6.12a: CORL23(P), 5-FU + highest non-toxic concentrations of indomethacin and indomethacin analogues.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

	No Anticancer agent	5-FU (2.0μg/ml)	CI values
No NSAID	100 ± 0.0	15.45 ± 1.2	
Indomethacin 2.5μg/ml	99.0 ± 4.6	14.0 ± 5.2	1.114
BRI 88/1 5μg/ml	98.8 ± 5.8	14.9 ± 1.4	1.151
BRI 92/1 5μg/ml	94.6 ± 0.9	15.7 ± 3.5	1.127

Table: 3.6.12b: CORL23(R), 5-FU + highest non-toxic concentrations of indomethacin and indomethacin analogues*.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

Statistical weight of the results is indicated by CI values (see section 2.16)

CI: Combination index values.

CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.

CORL23(P), VP-16 + BRI 88/1, BRI 92/1 and Indomethacin		
Test Sample	**% Cell Survival**	**S.D.**
VP-16. 0.4 μg/ml	23.4	2.8
Indo (2.5μg/ml)	99.0	4.6
Indo + VP-16	**15.5**	**6.7**
Indo (1.25μg/ml)	99.9	6.5
Indo + VP-16	**18.5**	**2.1**
Indo (0.625μg/ml)	101.9	6.3
Indo + VP-16	**21.7**	**1.7**
88/1 (5μg/ml)	90.8	3.6
88/1 + VP-16	**16.2**	**1.3**
88/1 (2.5μg/ml)	102.6	8.0
88/1 + VP-16	**20.7**	**3.7**
88/1 (1.25μg/ml)	101.2	3.9
88/1 + VP-16	**22.1**	**7.2**
92/1 (5μg/ml)	96.0	7.1
92/1 + VP-16	**16.8**	**4.4**
92/1 (2.5μg/ml)	98.7	7.8
92/1 + VP-16	**19.2**	**3.6**
92/1 (1.25μg/ml)	101.4	8.5
92/1 + VP-16	**20.4**	**3.0**

Table 3.6.13: % Survival of CORL23 (parental) cells in the presence of 0.1μg/ml VP-16 + various concentrations of indomethacin, BRI 88/1 and BRI 92/1 as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

CORL23(R), VP-16 + BRI 88/1, BRI 92/1 and Indomethacin		
Test Sample	**% Cell Survival**	**S.D.**
VP-16. 2.5 μg/ml	34.5	5.3
Indo (2.5μg/ml)	99.0	4.6
Indo + VP-16	**9.9**	**3.4**
Indo (1.25μg/ml)	99.9	6.5
Indo + VP-16	**15.1**	**7.2**
Indo (0.625μg/ml)	101.9	6.3
Indo + VP-16	**19.0**	**4.4**
88/1 (5μg/ml)	93.8	3.6
88/1 + VP-16	**9.8**	**1.1**
88/1 (2.5μg/ml)	94.5	3.6
88/1 + VP-16	**13.1**	**2.3**
88/1 (1.25μg/ml)	98.1	5.1
88/1 + VP-16	**19.8**	**3.5**
92/1 (5μg/ml)	96.0	7.1
92/1 + VP-16	**10.1**	**5.1**
92/1 (2.5μg/ml)	98.7	7.8
92/1 + VP-16	**14.6**	**1.5**
92/1 (1.25μg/ml)	101.4	8.5
92/1 + VP-16	**21.2**	**3.3**

Table 3.6.14: % Survival of CORL23 (resistant) cells in the presence of 2.5μg/ml VP16 + various concentrations of BRI 88/1, BRI 92/1 and indomethacin as found using the protocol detailed in section 2.7.3a.

Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

	No Anticancer agent	VP-16 (0.1μg/ml)	CI values
No NSAID	100 ± 0.0	28.9 ± 1.1	
Indomethacin 2.5μg/ml	99.0 ± 4.6	15.5 ± 6.7	0.893
BRI 88/1 5μg/ml	90.8 ± 3.6	16.2 ± 1.3	0.922
BRI 92/1 5μg/ml	96.0 ± 7.1	16.8 ± 4.4	0.895

Table: 3.6.15a: CORL23(P), VP16 + highest non-toxic concentrations of indomethacin and indomethacin analogues.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

	No Anticancer agent	VP-16 (2.5μg/ml)	CI values
No NSAID	100 ± 0.0	34.5 ± 5.3	
Indomethacin 2.5μg/ml	99.0 ± 4.6	9.9 ± 3.4	0.572
BRI 88/1 5μg/ml	93.8 ± 3.6	9.8 ± 1.1	0.569
BRI 92/1 5μg/ml	96.0 ± 7.1	10.1 ± 5.1	0.541

Table: 3.6.15b: CORL23(R), VP16 + highest non-toxic concentrations of indomethacin and indomethacin analogues.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

Statistical weight of the results is indicated by CI values (see section 2.16)

CI: Combination index values.

CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.

CORL23, Taxol, Taxotere & Sulindac		
Test Sample	**% Cell Survival**	**S.D.**
CORL23(P) Taxotere and Sulindac		
Taxotere. 1.0ng/ml	45.7	4.9
Sul (8μg/ml)	92.1	0.4
Sul + Taxotere	**23.9**	**2.1**
Sul (4μg/ml)	98.7	2.1
Sul + Taxotere	**32.5**	**3.9**
Sul (2μg/ml)	101.1	2.0
Sul + Taxotere	**40.4**	**11.0**
CORL23(R) Taxotere and Sulindac		
Taxotere. 2.0ng/ml	50.7	6.6
Sul (8μg/ml)	91.0	0.1
Sul + Taxotere	**27.7**	**1.7**
Sul (4μg/ml)	98.9	2.5
Sul + Taxotere	**31.5**	**3.5**
Sul (2μg/ml)	102.5	3.5
Sul + Taxotere	**33.6**	**0.1**
CORL23(P) Taxol and Sulindac		
Taxol. 1.0ng/ml	34.5	4.2
Sul (8μg/ml)	93.8	1.9
Sul + Taxol	**16.8**	**0.5**
Sul (4μg/ml)	99.1	4.5
Sul + Taxol	**23.4**	**5.5**
Sul (2μg/ml)	104.9	1.9
Sul + Taxol	**25.1**	**7.4**
CORL23(R) Taxol and Sulindac		
Taxol. 7.0ng/ml	39.5	3.8
Sul (8μg/ml)	94.0	2.8
Sul + Taxol	**22.1**	**0.9**
Sul (4μg/ml)	98.4	1.8
Sul + Taxol	**28.5**	**2.4**
Sul (2μg/ml)	102.1	2.6
Sul + Taxol	**33.6**	**0.6**

Table 3.6.16: % Survival of CORL23(P) and CORL23(R) cells in the presence of Taxotere and Taxol, in combination with sulindac, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

SHORT TERM ASSAY: CORL23, Taxol, Taxotere & Sulindac		
Test Sample	**% Cell Survival**	**S.D.**
CORL23(P) Taxotere and Sulindac		
Taxotere. 4.0ng/ml	46.4	1.9
Sul (8μg/ml)	92.4	0.3
Sul + Taxotere	**22.4**	**2.1**
Sul (4μg/ml)	98.6	5.3
Sul + Taxotere	**29.0**	**0.4**
Sul (2μg/ml)	99.6	0.6
Sul + Taxotere	**31.9**	**1.8**
CORL23(R) Taxotere and Sulindac		
Taxotere. 8.0ng/ml	48.2	1.9
Sul (8μg/ml)	99.6	0.6
Sul + Taxotere	**28.4**	**4.8**
Sul (4μg/ml)	100.5	1.4
Sul + Taxotere	**33.3**	**1.7**
Sul (2μg/ml)	99.6	0.6
Sul (3) + Taxotere	**36.0**	**4.4**
CORL23(P) Taxol and Sulindac		
Taxol. 90ng/ml	43.4	0.2
Sul (8μg/ml)	101.3	0.7
Sul + Taxol	**29.7**	**4.4**
Sul (4μg/ml)	99.9	0.5
Sul + Taxol	**36.4**	**0.1**
Sul (2μg/ml)	98.9	0.5
Sul + Taxol	**38.0**	**1.0**
CORL23(R) Taxol and Sulindac		
Taxol. 180ng/ml	55.7	4.6
Sul (8μg/ml)	96.3	7.4
Sul + Taxol	**39.9**	**4.0**
Sul (4μg/ml)	97.5	3.6
Sul + Taxol	**43.3**	**1.9**
Sul (2μg/ml)	101.9	2.0
Sul + Taxol	**48.4**	**3.8**

Table 3.6.17: % Survival of CORL23(P) and CORL23(R) cells in the presence of Taxotere and Taxol, in combination with sulindac, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

Long-term assay

	No NSAID	Sulindac (8 μg/ml)	CI values
CORL23(P)			
No Anticancer agent	100 ± 0.0	92.1 ± 0.4	
Taxol 1.0ng/ml	34.5 ± 4.2	16.8 ± 0.57	0.652
Taxotere 1.0ng/ml	45.7 ± 4.9	23.9 ± 2.1	0.679
CORL23(R)			
No Anticancer agent	100 ± 0.0	94.0 ± 2.8	
Taxol 7.0ng/ml	39.5 ± 3.8	22.1 ± 0.9	0.559
Taxotere 2.0ng/ml	50.7 ± 6.6	27.7 ± 1.7	0.474

Table: 3.6.18a: Long-term assay: CORL23 cell lines + Taxol/Taxotere + Highest non-toxic concentrations of Sulindac.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

Short-term assay

	No NSAID	Sulindac (8 μg/ml)	CI values
CORL23(P)			
No Anticancer agent	100 ± 0.0	96.3 ± 7.4	
Taxol 90.0ng/ml	43.4 ± 0.2	29.7 ± 4.4	0.905
Taxotere 4.0ng/ml	46.4 ± 1.9	22.4 ± 2.1	0.779
CORL23(R)			
No Anticancer agent	100 ± 0.0	96.3 ± 7.4	
Taxol 180.0ng/ml	55.7 ± 4.6	39.9 ± 4.0	0.852
Taxotere 8.0ng/ml	48.2 ± 1.9	28.4 ± 4.8	0.729

Table: 3.6.18b: Short-term assay: CORL23 cell lines + Taxol/Taxotere + Highest non-toxic concentrations of Sulindac.*

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

CI: Combination index values.

CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.

CORL23 cell lines + Taxol and Indomethacin		
Test Sample	**% Cell Survival**	**S.D.**
CORL23(P) Taxol and Indomethacin		
Taxol. 1.0ng/ml	33.1	2.7
Indo (2.5μg/ml)	100.5	0.8
Indo + Taxol	**17.0**	**2.1**
Indo (1.25μg/ml)	100.6	1.5
Indo + Taxol	**23.2**	**2.4**
Indo (0.625μg/ml)	97.7	0.8
Indo + Taxol	**25.8**	**2.4**
CORL23(R) Taxol and Indomethacin		
Taxol. 7.0ng/ml	41.2	0.1
Indo (2.5μg/ml)	96.5	3.5
Indo + Taxol	**26.3**	**0.8**
Indo (1.25μg/ml)	100.0	0.1
Indo + Taxol	**28.8**	**1.6**
Indo (0.625μg/ml)	100.9	2.3
Indo + Taxol	**31.2**	**0.3**
CORL23(P) Taxol and BRI 153/1		
Taxol. 1.0ng/ml	30.8	3.2
153/1 (50μg/ml)	94.5	0.9
153/1 + Taxol	**9.8**	**2.6**
153/1 (25μg/ml)	96.7	0.4
153/1 + Taxol	**17.9**	**1.7**
153/1 (12.5μg/ml)	98.9	0.0
153/1 + Taxol	**21.2**	**1.0**
CORL23(R) Taxol and BRI 153/1		
Taxol. 7.0ng/ml	42.1	1.7
153/1 (50μg/ml)	94.6	0.2
153/1 + Taxol	**17.6**	**0.2**
153/1 (25μg/ml)	98.5	2.7
153/1 + Taxol	**23.7**	**1.3**
153/1 (12.5μg/ml)	100.4	0.6
153/1 + Taxol	**23.4**	**0.2**

Table 3.6.19: % Survival of CORL23(P) and CORL23(R) cells in the presence of taxol, in combination with indomethacin and BRI 153/1 as found using the protocol detailed in section 2.7.3. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

Test Sample	% Cell Survival	S.D.
CORL23(P) Taxol and BRI 205/4		
Taxol. 1.0ng/ml	38.9	5.5
205/4 (25μg/ml)	96.8	3.0
205/4 + Taxol	**36.3**	**4.2**
205/4 (12.5μg/ml)	100.9	0.6
205/4 + Taxol	**37.2**	**4.5**
205/4 (6.25μg/ml)	100.7	0.6
205/4 + Taxol	**38.1**	**5.6**
CORL23(R) Taxol and BRI 205/4		
Taxol. 7.0ng/ml	39.9	4.9
205/4 (25μg/ml)	99.2	5.1
205/4 + Taxol	**34.4**	**5.4**
205/4 (12.5μg/ml)	100.7	2.4
205/4 + Taxol	**35.7**	**5.6**
205/4 (6.25μg/ml)	101.1	1.9
205/4 + Taxol	**39.7**	**2.8**

Table 3.6.20: % Survival of CORL23(P) and CORL23(R) cells in the presence of Taxol, in combination with 205/4 as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

	No Anticancer agent	Taxol (1.0μg/ml)	CI values
No NSAID	100 ± 0.0	33.1 ± 2.7	
Indomethacin 2.5μg/ml	100.5 ± 0.8	17.0 ± 2.1	0.659
BRI 153/1 50μg/ml	94.5 ± 0.2	9.8 ± 2.6	0.352
BRI 205/4 25μg/ml	96.8 ± 3.0	36.3 ± 4.2	1.365

Table: 3.6.21a: CORL23(P), Taxol + Highest non-toxic concentrations of Indomethacin and Indomethacin analogues.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

	No Anticancer agent	Taxol (7.0μg/ml)	CI values
No NSAID	100 ± 0.0	42.1 ± 1.7	
Indomethacin 2.5μg/ml	96.5 ± 3.5	23.3 ± 0.8	0.688
BRI 153/1 50μg/ml	94.6 ± 0.2	17.6 ± 0.2	0.337
BRI 205/4 25μg/ml	103.2 ± 6.4	46.9 ± 2.6	1.045

Table: 3.6.21b: CORL23(R), VP16 + Highest non-toxic concentrations of Indomethacin and Indomethacin analogues.

Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

CI: Combination index values.

CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.

CORL23 (S)	Adr.	Vin.	VP-16	5-FU	Taxol	Taxotere
Indo.	**0.725**	**0.333**	**0.893**	**1.000**	**0.659**	
Sulindac					**0.652**	**0.679**
BRI 88/1	**0.670**	**0.554**	**0.922**	**1.000**		
BRI 92/1	**0.749**	**0.532**	**0.895**	**1.146**		
BRI 153/1	**0.813**				**0.352**	
BRI 205/4	**1.000**				**1.365**	

Table 3.6.22a: Summary table of CI values from combination toxicity assays in CORL23(P) cells in section 3.6.

CI: Combination index values.

CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.

CORL23(R)	Adr.	Vin.	VP-16	5-FU	Taxol	Taxotere
Indo.	**0.626**	**0.417**	**0.572**	**1.114**	**0.688**	
Sulindac					**0.559**	**0.474**
BRI 88/1	**0.432**	**0.329**	**0.569**	**1.151**		
BRI 92/1	**0.552**	**0.284**	**0.541**	**1.127**		
BRI 153/1	**0.382**				**0.337**	
BRI 205/4	**1.000**				**1.045**	

Table 3.6.22b: Summary table of CI values from combination toxicity assays in CORL23(R) cells in section 3.6.

CI: Combination index values.

CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.

3.7 cMOAT experiments in HepG2, lung carcinoma cell line.

A human hepatic cancer cell line, HepG2, was reported by a number of authors to express a high level of cMOAT (MRP2), both at protein (Koike *et al.*, 1997), and mRNA levels (Narasaki *et al.*, 1997). Kool *et al.*, (1997), Tanaguchi *et al.*, (1996) and Koike *et al* (1997), provide indirect evidence that an organic pump, notably cMOAT, could contribute to cisplatin resistance by exporting the cisplatin -GSH complex. But they also agree that other mechanisms may contribute to resistance. In a more recent paper Oguri *et al.*, (1998), observed no association between ante-mortem platinum drug exposure and steady state cMOAT mRNA and suggests that cMOAT does not play a major role in platinum drug resistance or transport. They also suggest that the increased expression of cMOAT mRNA, after exposure to platinum drugs, is part of the normal stress response to xenobiotics.

3.7.1 Combination toxicity assays in the HepG2 cell line

Duffy *et al.*, (1998), reported that the positive NSAIDs and indomethacin analogues were unable to potentiate the toxicity of cisplatin.

Combination toxicity assays were carried out on the HepG2 cell line using a combination of adriamycin and indomethacin, or cisplatin and indomethacin to assess if indomethacin was capable of potentiating the toxicity of the cytotoxic drug in cMOAT-overexpressing HepG2 cells. Previously the combination toxicity assays were carried out on cell lines, which overexpress MRP1 (DLKP and CORL23). Cisplatin is not a substrate for MRP1 (Duffy *et al.*, 1998). The mechanism by which cells develop resistance to cisplatin is presently unclear but it is postulated that this resistance may be a result of the activity of the MRP analogue, cMOAT in certain cell lines. The combination toxicity assay was carried out on this cell line using a combination of cisplatin and indomethacin to try to assess if MRP analogues, other than MRP1, could be inhibited in the same way as MRP1.

The results demonstrated that indomethacin was capable of potentiating the toxicity of adriamycin in HepG2 and DLKP cells (Table 3.7.1). The highest non-toxic concentration of indomethacin used in the HepG2 cells was 10μg/ml as compared to 2.5μg/ml for DLKP cells. However, results from the

combination toxicity assays with cisplatin and indomethacin in HepG2 and DLKP cells revealed that indomethacin was not capable of potentiating the toxicity of cisplatin in these cells (3.7.2). Interestingly the toxicity of cisplatin seemed to decrease when combined with indomethacin in the HepG2 and DLKP cell lines. The results indicate that either indomethacin is not a good substrate for cMOAT (MRP2) and is unable to inhibit the activity of this pump in the HepG2 cells. Or they may also indicate that cisplatin is not a substrate for cMOAT and inhibition of the activity of cMOAT in the HepG2 cells by indomethacin did not affect the transport of cisplatin out of the cell. Koike *et al.* (1997) did not detect MRP1 protein expression in the HepG2 cell line. However, Narasaki *et al.,* (1997), reported that HepG2 cells expressed MRP1 at mRNA levels. It is possible that the enhancement of adriamycin toxicity by indomethacin in the HepG2 cells is entirely due to the expression of MRP1 and may not be attributed to the expression of MRP2.

DLKP, Adriamycin and Indomethacin

Test Sample	% Survival	S.D.
Adr. 10ng/ml	46.5	1.7
Indo. (2.5μg/ml)	96..9	2.7
Indo. + Adr	**11.5**	**6.5**
Indo. (1.25μg/ml)	102.7	1.5
Indo. + Adr	**14.1**	**0.9**
Indo. (0.625μg/ml)	102.5	1.4
Indo. + Adr	**19.6**	**4.35**

HepG2, Adriamycin and Indomethacin

Test Sample	% Survival	S.D.
Adr. 20ng/ml	58.3	1.3
Indo. (10μg/ml)	82.7	2.7
Indo. + Adr	**20.6**	**6.2**
Indo. (5μg/ml)	91.4	2.5
Indo. + Adr	**20.5**	**0.5**
Indo. (2.5μg/ml)	102.7	1.4
Indo. + Adr	**23.6**	**3.2**

Table 3.7.1: % Survival of DLKP and HepG2 cells in the presence of various concentrations of adriamycin and indomethacin as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

DLKP, Cisplatin and Indomethacin

Test Sample	% Survival	S.D.
Cis. 250ng/ml	33.6	2.9
Indo. (2.5μg/ml)	95.5	1.3
Indo. + Cis	**44.1**	**4.2**
Indo. (1.25μg/ml)	100.7	0.9
Indo. + Cis	**38.1**	**2.4**
Indo. (0.625μg/ml)	104.5	5.6
Indo. + Cis	**35.9**	**3.5**

HepG2, Cisplatin and indomethacin

Test Sample	% Survival	S.D.
Cis. 250ng/ml	32.8	0.9
Indo. (10μg/ml)	96.1	1.8
Indo. + Cis	**46.7**	**4.5**
Indo. (5μg/ml)	106.3	7.4
Indo. + Cis	**49.4**	**0.5**
Indo. (2.5μg/ml)	100.1	1.4
Indo. + Cis	**40.4**	**3.2**

Table 3.7.2: Survival of DLKP and HepG2 cells in the presence of various concentrations of cisplatin and indomethacin as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

3.8: In Vitro Combination Toxicity Assays: 2008 cell line.

Certain NSAIDs can potentiate the toxicity of a number of anticancer drugs in cell lines which overexpress MRP1, e.g. CORL23(R), DLKP, HL60/ADR and A549 cell lines (Duffy *et al.,* 1998). Western Blot analysis has indicated that the over-expression of MRP1 is the main mode of resistance in these cell lines (Duffy *et al.,* 1998; Kool *et al.,* 1997 and Connolly, 1999). It is now known that there are at least 6 members of the human MRP family, MRP 1-6 (Borst *et al.,* 1999). Analysis for the presence of a selection of MRP homologues was undertaken to assess if the potentiation of the toxicity of the anticancer drugs was associated with expression of one member of the MRP family or if this effect was also evident in cell lines expressing different members of this transport family.

Three cell lines were generated by transfection of an ovarian carcinoma cell line, 2008, with the cDNA for MRP1, MRP2 or MRP3 and were used in a number of experiments to assess the effect of the expression of these proteins on the toxicity of a number of anticancer drugs. These cell lines were received as a kind gift from Dr. Marcel Kool and Prof. Piet Borst from the Department of Molecular Biology, Netherlands Cancer Institute, Amsterdam.

The Long-term toxicity assays referred to in this section were carried out using the same procedure used for the toxicity assays carried out in section 3.1 and according to section 2.7.1a and 2.7.3a (i.e. 7 day toxicity assays whereby drug and/or compound were added to the cells on Day 2 and remain in the cells until Day 7 when the cells were analysed).

The Short-term toxicity assays referred to in this section were performed using the method described in section 2.7.1b and 2.7.3b (i.e. 6 day toxicity assays whereby the drug/compound were added to the cells on Day 2 and were incubated in the cells for a period of four hours only. The cells were then re-fed with fresh media and were incubated until Day 6 when the cells were analysed) (Kool *et al.,* 1999). Reasons for using Short-term toxicity assays versus Long-term toxicity assays to analyse the effects of chemotherapeutic drug on these cell lines are described in detail in section 4.11.

The drug resistance profile of the 2008 parental and transfected cell lines is provided in table 3.8.1a (Long-term toxicity assays) and 3.8.1b (Short-term toxicity assays).

Long-term	Adr. ng/ml	MTX μg/ml	Taxol ng/ml	Taxotere ng/ml	Cisplatin ng/ml
2008 P	21.0 ± 1.2	5.0 ± 0.6	7.18 ± 1.1	0.98 ± 0.11	202.9 ± 15.4
2008 MRP1	117.5 ± 4.2	4.8 ± 0.2	3.0 ± 0.5	1.02 ± 0.09	52.1 ± 3.7
2008 MRP2	10.0 ± 0.5	5.1 ± 0.3	2.94 ± 0.7	0.95 ± 0.08	15.6 ± 1.3
2008 MRP3	19.2 ± 1.8	5.1 ± 0.2	3.1 ± 1.5	1.08 ± 0.10	31.1 ± 0.8

Table 3.8.1a: IC_{50} values of chemotherapeutic drugs in 2008 parental and transfected cell lines determined using the long-term toxicity assay. These values are identified as the drug concentrations giving rise to 50% cell kill relative to untreated control cells according to protocol detailed in section 2.7.1a.

Short-term	Adr. ng/ml	MTX μg/ml	Taxol ng/ml	Taxotere ng/ml
2008 P	82.3 ± 5.7	10.4 ± 1.1	984 ± 41.0	50.6 ± 4.4
2008 MRP1	246.6 ± 9.8	903.2 ± 25.2	2011 ± 27.4	4.0 ± 0.5
2008 MRP2	129.0 ± 6.5	312.7 ± 19.6	968 ± 31.8	49.8 ± 5.9
2008 MRP3	250.4 ± 11.2	888.1 ± 30.4	1992 ± 20.7	26.4 ± 2 .1

Table 3.8.2a: IC_{50} values of chemotherapeutic drugs in 2008 parental and transfected cell lines determined using the short-term toxicity assay. These values are identified as the drug concentrations giving rise to 50% cell kill relative to untreated control cells according to protocol detailed in section 2.7.1b.

3.8.1 Analysis of 2008 cells for enhancement of adriamycin, methotrexate and cisplatin toxicities by indomethacin.

Toxicity assays with adriamycin and methotrexate were carried on the four cell lines, 2008 P (parental), 2008 MRP1, 2008 MRP2 and 2008 MRP3, to assess their toxicity profile. The results obtained from the long-term toxicity assays with adriamycin (table 3.8.1a) indicated that 2008 MRP1 was approximately

6-10 fold more resistant to adriamycin than the other three cell lines. 2008 MRP3 and 2008 P cells were two-fold more resistant to adriamycin than 2008 MRP2. These results suggested that overexpression of MRP1 resulted in the greatest level of resistance to adriamycin. Short-term toxicity assays on the 2008 cells using adriamycin (table 3.8.1b) indicated that, in short-term exposure experiments, where the cells were exposed to the anticancer drug for four hours only (as described in section 2.7.1b), there was equal resistance to adriamycin in both the MRP1 and MRP3 transfected cells. These cell lines were only two-fold more resistant to adriamycin than 2008 MRP2 and three-fold more resistant than the 2008 parental cells. Initial long-term toxicity assays on these cell lines using methotrexate (MTX) (table 3.8.1a) failed to demonstrate any difference in resistance levels between the parental and the MRP-transfected cells. However, short-term MTX toxicity assays on the 2008 cells showed 2008 MRP1 and 2008 MRP3 to be almost 100-fold more resistant to MTX than the parental cell line. 2008 MRP2 cells were 30-fold more resistant to MTX than the parental cell line (table 3.8.1b).

Because of the differences in the toxicity profiles between the short-term and the long-term toxicity assays, both long-term and short-term combination toxicity assays, with indomethacin in combination with adriamycin or MTX, were carried out on these cell lines. These assays were carried out to assess if potentiation of the toxicity of either anti-cancer drug was possible using either method. The results for adriamycin in combination with indomethacin (Tables 3.8.2 and 3.8.6) indicate that the toxicity of adriamycin was potentiated by indomethacin in the long term assay, in all four cell lines, particularly in the 2008 MRP1 cells. In the short term assay there was potentiation of the toxicity of adriamycin by indomethacin in the MRP1-transfected cells and to a lesser extent in the MRP3-transfected cells. There was no potentiation of adriamycin toxicity by indomethacin in either the parental or 2008 MRP2 cells. Indomethacin has been shown, using Inside Out Vesicles prepared from MRP1 overexpressing HL60/ADR cells, to be a good substrate for MRP1 (see section 3.3). The results from the combination toxicity assays in 2008 cells indicate that indomethacin may also be a substrate for MRP2 and MRP3. Western Blot analysis of the 2008 cell lines indicated a strong expression of the transfected MRP1 in the 2008 MRP1 transfected cell line. However, basal expression of MRP1 was detected in the 2008 P, 2008 MRP2 and 2008 MRP3 cells

(Connolly, 1999). Results from previous experiments in the CORL23 cell lines (Section 3.6) indicated that MRP1 protein levels did not correlate with the magnitude of enhancement by indomethacin and related active compounds in these cell lines. RT-PCR analysis showed that MRP1 was expressed at mRNA levels in all the 2008 cell lines. The expression of MRP1 mRNA was only slightly stronger in the MRP1 transfected cell line (section 3.16). Together these results may indicate that indomethacin is a better substrate for MRP1 than MRP2 and MRP3 and the potentiation of adriamycin by indomethacin may simply be due to the expression of MRP1 in all the cell lines. The poorest combination index values for adriamycin in combination with indomethacin were obtained in the 2008 MRP2 cells (Table 3.8.5). In the short-term assays the low potentiation of adriamycin by indomethacin may suggest that a four hour exposure period in the cells is insufficient for indomethacin to exert its potentiation effect on 2008 cells. It appears that indomethacin is required in the cells for a longer period of time for sufficient potentiation of adriamycin toxicity to occur.

Indomethacin potentiated the toxicity of MTX in 2008 MRP1 and to a lesser extent in the 2008 MRP3 cells only, in the long-term toxicity assay (Table 3.8.4). However, in the short-term combination toxicity assays there was increased potentiation of MTX in both the 2008 P and 2008 MRP1 cells. There was poorer potentiation of MTX toxicity in the 2008 MRP3 cells in the short-term assay than in the long-term assay. The poorest combination index values for methotrexate in combination with indomethacin were obtained in the 2008 MRP2 cells (Table 3.8.5) where there was no evidence of potentiation of MTX toxicity by indomethacin in the 2008 MRP2 cell line in either the long-term or the short-term assays.

Potential indomethacin interactions with cisplatin were investigated using long-term toxicity assays. The results clearly show that there was no potentiation of the toxicity of cisplatin by indomethacin in any of the 2008 cell lines (Table 3.8.4 and 3.8.5). Interestingly, co-incubating cisplatin with indomethacin appeared to increase the resistance of the cells to the anti-cancer drug instead of increasing the toxicity of the drug as might be expected. The results indicate that cisplatin is not a good substrate for any of the MRP

homologues expressed in the 2008 cells. Interestingly, comparing the IC_{50} results for cisplatin in the 2008 cell lines indicate that the parental cell line is more resistant to cisplatin (200ng/ml) than the transfected 2008 cells (50, 15 and 30ng/ml for MRP1, MRP2 and MRP3 transfected cells respectively).

LONG TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Adriamycin and Indomethacin		
Adriamycin 20ng/ml	56.4	8.8
Indo (1)	97.6	3.7
Indo (1) + Adriamycin	**33.3**	**7.9**
Indo (2)	99.1	1.5
Indo (2) + Adriamycin	**39.8**	**7.6**
Indo (3)	100.0	1.0
Indo (3) + Adriamycin	**41.6**	**8.7**
2008 MRP1 Adriamycin and Indomethacin		
Adriamycin 120ng/ml	55.6	6.6
Indo (1)	93.5	3.0
Indo (1) + Adriamycin	**15.7**	**1.2**
Indo (2)	99.1	3.4
Indo (2) + Adriamycin	**20.7**	**5.1**
Indo (3)	99.0	3.5
Indo (3) + Adriamycin	**25.0**	**3.4**
2008 MRP2 Adriamycin and Indomethacin		
Adriamycin 10ng/ml	47.8	1.3
Indo (1)	98.9	2.3
Indo (1) + Adriamycin	**28.9**	**0.6**
Indo (2)	100.2	2.6
Indo (2) + Adriamycin	**31.0**	**2.1**
Indo (3)	99.2	1.2
Indo (3) + Adriamycin	**32.0**	**0.8**
2008 MRP3 Adriamycin and Indomethacin		
Adriamycin 20ng/ml	70.7	2.4
Indo (1)	95.0	0.8
Indo (1) + Adriamycin	**45.9**	**4.0**
Indo (2)	98.0	1.8
Indo (2) + Adriamycin	**52.4**	**2.1**
Indo (3)	99.0	2.3
Indo (3) + Adriamycin	**53.8**	**1.7**

Table 3.8.2a: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of adriamycin, in combination with indomethacin, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of indomethacin were: **(1)** 10μg/ml; **(2)** 5μg/ml; **(3)** 2.5μg/ml.

SHORT TERM ASSAY (-Indomethacin @T4 hours)		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Adriamycin and Indomethacin		
Adriamycin 80ng/ml	57.4	2.5
Indo (1)	92.2	0.8
Indo (1) + Adriamycin	**49.5**	**1.9**
Indo (2)	93.0	2.1
Indo (2) + Adriamycin	**50.2**	**1.4**
Indo (3)	95.7	1.8
Indo (3) + Adriamycin	**52.2**	**0.2**
2008 MRP1 Adriamycin and Indomethacin		
Adriamycin 250ng/ml	74.2	2.9
Indo (1)	92.6	1.7
Indo (1) + Adriamycin	**43.8**	**5.0**
Indo (2)	94.6	1.7
Indo (2) + Adriamycin	**52.4**	**8.0**
Indo (3)	96.1	4.1
Indo (3) + Adriamycin	**59.0**	**5.8**
2008 MRP2 Adriamycin and Indomethacin		
Adriamycin 125ng/ml	72.0	4.4
Indo (1)	90.1	2.5
Indo (1) + Adriamycin	**58.6**	**4.0**
Indo (2)	98.0	7.3
Indo (2) + Adriamycin	**63.8**	**4.9**
Indo (3)	101.1	9.7
Indo (3) + Adriamycin	**67.6**	**5.6**
2008 MRP3 Adriamycin and Indomethacin		
Adriamycin 250ng/ml	54.6	3.3
Indo (1)	97.4	2.9
Indo (1) + Adriamycin	**41.3**	**1.0**
Indo (2)	96.5	3.2
Indo (2) + Adriamycin	**42.7**	**0.9**
Indo (3)	96.0	3.2
Indo (3) + Adriamycin	**47.3**	**4.4**

Table 3.8.2b: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of adriamycin, in combination with indomethacin, as found using the protocol detailed in section 27.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of indomethacin were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

LONG TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Methotrexate and Indomethacin		
Methotrexate 5μg/ml	25.5	7.6
Indo (1)	96.9	1.2
Indo (1) + Methotrexate	**22.5**	**4.2**
Indo (2)	99.4	0.9
Indo (2) + Methotrexate	**23.8**	**1.8**
Indo (3)	102.3	2.3
Indo (3) + Methotrexate	**24.8**	**2.6**
2008 MRP1 Methotrexate and Indomethacin		
Methotrexate 5μg/ml	28.5	3.6
Indo (1)	93.5	0.6
Indo (1) + Methotrexate	**14.1**	**5.2**
Indo (2)	93.9	2.6
Indo (2) + Methotrexate	**22.4**	**4.8**
Indo (3)	96.7	1.0
Indo (3) + Methotrexate	**25.6**	**3.6**
2008 MRP2 Methotrexate and Indomethacin		
Methotrexate 5μg/ml	30.1	9.6
Indo (1)	90.2	4.2
Indo (1) + Methotrexate	**27.0**	**4.1**
Indo (2)	97.1	2.7
Indo (2) + Methotrexate	**28.1**	**5.4**
Indo (3)	98.2	1.4
Indo (3) + Methotrexate	**29.7**	**9.0**
2008 MRP3 Methotrexate and Indomethacin		
Methotrexate 5μg/ml	50.3	0.8
Indo (1)	95.5	3.8
Indo (1) + Methotrexate	**36.6**	**5.9**
Indo (2)	97.9	2.2
Indo (2) + Methotrexate	**46.5**	**1.5**
Indo (3)	99.9	0.7
Indo (3) + Methotrexate	**46.4**	**4.0**

Table 3.8.3a: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of methotrexate, in combination with indomethacin, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of indomethacin were: **(1)** 10μg/ml; **(2)** 5μg/ml; **(3)** 2.5μg/ml.

SHORT TERM ASSAY (-Indomethacin @T4 hours)		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Methotrexate and Indomethacin		
Methotrexate 10ng/ml	53.3	6.9
Indo (1)	93.4	1.7
Indo (1) + Methotrexate	**21.0**	**5.5**
Indo (2)	96.4	2.0
Indo (2) + Methotrexate	**25.8**	**7.9**
Indo (3)	95.3	1.4
Indo (3) + Methotrexate	**35.3**	**9.3**
2008 MRP1 Methotrexate and Indomethacin		
Methotrexate 900ng/ml	42.5	1.7
Indo (1)	98.5	2.0
Indo (1) + Methotrexate	**22.8**	**3.0**
Indo (2)	96.1	4.1
Indo (2) + Methotrexate	**34.2**	**5.0**
Indo (3)	100.7	4.3
Indo (3) + Methotrexate	**36.4**	**3.3**
2008 MRP2 Methotrexate and Indomethacin		
Methotrexate 300ng/ml	30.0	4.7
Indo (1)	90.0	2.9
Indo (1) + Methotrexate	**24.7**	**2.9**
Indo (2)	92.8	0.6
Indo (2) + Methotrexate	**26.6**	**2.6**
Indo (3)	95.8	1.8
Indo (3) + Methotrexate	**27.6**	**2.8**
2008 MRP3 Methotrexate and Indomethacin		
Methotrexate 900ng/ml	37.4	3.9
Indo (1)	96.4	1.2
Indo (1) + Methotrexate	**28.2**	**7.5**
Indo (2)	97.6	3.2
Indo (2) + Methotrexate	**29.3**	**7.5**
Indo (3)	100.4	2.0
Indo (3) + Methotrexate	**30.3**	**4.8**

Table 3.8.3b: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of methotrexate, in combination with indomethacin, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of indomethacin were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

LONG TERM ASSAY		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Cisplatin and Indomethacin		
Cisplatin 200ng/ml	55.5	7.6
Indo (1)	98.2	5.8
Indo (1) + Cisplatin	**76.8**	**6.5**
Indo (2)	102.6	5.0
Indo (2) + Cisplatin	**69.1**	**8.5**
Indo (3)	102.5	4.2
Indo (3) + Cisplatin	**60.2**	**6.9**
2008 MRP1 Cisplatin and Indomethacin		
Cisplatin 50ng/ml	59.5	3.1
Indo (1)	96.4	0.9
Indo (1) + Cisplatin	**59.2**	**4.7**
Indo (2)	95.9	4.8
Indo (2) + Cisplatin	**59.1**	**10.0**
Indo (3)	99.7	1.9
Indo (3) + Cisplatin	**58.0**	**7.9**
2008 MRP2 Cisplatin and Indomethacin		
Cisplatin 15ng/ml	61.7	9.2
Indo (1)	103.5	4.2
Indo (1) + Cisplatin	**75.0**	**4.4**
Indo (2)	103.4	2.6
Indo (2) + Cisplatin	**68.7**	**7.6**
Indo (3)	103.6	4.5
Indo (3) + Cisplatin	**61.9**	**8.1**
2008 MRP3 Cisplatin and Indomethacin		
Cisplatin 30ng/ml	75.1	3.5
Indo (1)	92.3	3.2
Indo (1) + Cisplatin	**84.4**	**6.4**
Indo (2)	104.8	1.2
Indo (2) + Cisplatin	**79.8**	**6.1**
Indo (3)	104.4	1.5
Indo (3) + Cisplatin	**76.9**	**5.7**

Table 3.8.4: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of cisplatin, in combination with indomethacin, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of indomethacin were: **(1)** 10μg/ml; **(2)** 5μg/ml; **(3)** 2.5μg/ml.

	Adr + Indo		MTX + Indo		Cis + Indo
	L.T	S.T	L.T	S.T	L.T
2008P	0.488	1.048	1.056	0.372	2.422
MRP1	0.186	0.683	0.653	0.581	1.345
MRP2	0.493	0.958	1.064	1.000	2.030
MRP3	0.433	0.860	0.738	0.879	2.198

Table 3.8.5: Summary of combination index (CI) values for adriamycin (Adr), methotrexate (MTX) or cisplatin (Cis) in combination with indomethacin, obtained using the method of Chou and Talalay as described in section 2.16

(CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.)

L.T: Long-term assay as described in section 2.7.3a

S.T (1): Short-term assay. Drug and compound removed from the cells after 4 hours incubation and replaced with fresh medium only as described in section 2.7.3b

S.T (2): Short-term assay. Drug and NSAID removed from the cells after 4 hours incubation and replaced with NSAID only as described in section 2.7.3b

3.8.2 Analysis of 2008 cells for enhancement of adriamycin or methotrexate toxicities by sulindac.

To further investigate the effects of drug combinations in the transfected 2008cell lines, long-term and short-term combination toxicity assays were carried out using a number of anti-cancer drugs in combination with the active NSAID, sulindac. Duffy *et al.,* (1998), previously reported the ability of sulindac to enhance the anti-cancer effect of a number of chemotherapeutic drugs in MRP-overexpressing cell lines. The results for the long-term toxicity assays with adriamycin in combination with sulindac were similar to those obtained with adriamycin in combination with indomethacin (Table 3.8.6). Sulindac potentiated the toxicity of adriamycin in all of the 2008 cell lines, particularly in the 2008 P and 2008 MRP1 cell lines. In the long term assays

the concentration of sulindac used in the assays (35μg/ml (98.3μM)) was 3-fold higher than the indomethacin concentrations used in these cells (10μg/ml (28μM)) as sulindac was less toxic to the cells than indomethacin. This could account for the greater potentiation of adriamycin by sulindac in the 2008 cells in the long-term assay. The results suggest that sulindac is a better substrate for MRP1 than MRP2 and MRP3 in the long-term combination assays. The short-term combination toxicity assays with adriamycin and sulindac (Table 3.8.7) also produced similar results to those obtained with adriamycin and indomethacin. There was no potentiation of adriamycin toxicity in the 2008 P and 2008 MRP2 cell lines. There was reduced potentiation of adriamycin toxicity by sulindac in the 2008 MRP1 and MRP3 cells as compared to that observed in the long-term assays. As the short-term combination assays with indomethacin indicated that short-term exposure to indomethacin is insufficient to significantly enhance the toxicity of adriamycin, an experiment was carried out to assess if re-addition of the NSAID, after removing the anti-cancer drug and NSAID from the medium at the end of the four hour exposure time, would increase the potentiation of the drug toxicity by the NSAID. There was increased potentiation of adriamycin in the short term assay after re-addition of sulindac (Table 3.8.6a) compared to the short-term assay where sulindac was completely removed from the medium (Table 3.8.6b). However, the combination effect was less than that obtained in the long-term toxicity assay. This may be due to adriamycin being effluxed during the washing stage of the short-term assay as described in section 2.7.1b. The procedure requires four washes to ensure complete removal of the drug from the medium (Kool *et al.,* 1999), so by the time sulindac-containing medium was added to the cells it is possible that there was less adriamycin in the cells on which to exert its effect.

Identical experiments were carried out using methotrexate in combination with sulindac and the results indicated that there was poorer potentiation of MTX toxicity in the long-term combination toxicity assays (Table 3.8.7a) than in the short-term assays for 2008 P and 2008 MRP1 cells (Table 3.8.7b). The re-addition of sulindac in the short-term assay further enhanced the toxicity of methotrexate in these cells which indicates that long–term maintenance of sulindac levels is required for optimal potentiation of the drug to occur (Table 3.8.7c). There was poor potentiation of MTX toxicity by sulindac in both the

2008 MRP2 and MRP3 cells in both the long term and the short term assays. Re-addition of sulindac to the medium in the short-term assay did not significantly increase the potentiation of methotrexate toxicity. The results suggest that MRP2 and MRP3 are also poorer transporters of sulindac than MRP1.

3.8.3 Analysis of 2008 cells for enhancement of taxol and taxotere toxicities by sulindac.

The effect of sulindac on taxotere and taxol was subsequently analysed to try to assess other possible substrates for MRP1-3 and also to assess if synergism between the anticancer drug and the NSAID would occur. Initial long-term and short-term toxicity assays were carried out to assess the toxicity profile of these drugs in the 2008 cell lines. In the long-term assay the IC_{50} values for taxotere were the same for all the 2008 cells (1ng/ml). In the short-term assay the IC_{50} value increased to 50ng/ml for both the parental and MRP2-transfected cells and 25ng/ml for 2008 MRP3. However, a most unexpected result was obtained for 2008 MRP1. The IC_{50} value for 2008 MRP1 increased only to 4ng/ml, 12-fold less resistant than the parental and 2008 MRP2 cells. The result indicate that overexpression of MRP1 resulted in decreased resistance to taxotere in the short-term assays. The toxicity results for taxol show the parental 2008 cells to be approximately 2-fold more resistant to taxol than the transfected cell lines (7ng/ml versus 3ng/ml). In the short-term toxicity assay the IC_{50} concentration for the parental and MRP2-transfected cell lines increased to 1000ng/ml and the IC_{50} concentration for 2008 MRP1 and MRP3 increased to 2000ng/ml.

The combination toxicity assay results indicate that greater synergy between taxotere/taxol and sulindac was evident in the long-term combination toxicity assays than in the short-term toxicity assays (Tables 3.8.8a and 3.8.8b). Again, re-addition of sulindac in the short-term assay demonstrated that long term exposure to sulindac was more effective in enhancing the anti-cancer effect of the chemotherapeutic drugs. The combination index values indicate that there was a good combination effect between taxotere and sulindac in all of the cell lines (Table 3.8.10a). This effect was strongest in the MRP1 and parental 2008

cell lines despite the reduced resistance associated with MRP1. In the case of taxol, there was excellent synergism in all cell lines between taxol and sulindac (Table 3.8.9a). The strongest synergism was observed in the 2008 MRP1-transfected cells and the lowest combination index value for this particular combination was obtained for the 2008 MRP2 cells (Table 3.8.10a). The results for taxotere followed the trend observed with methotrexate and adriamycin with the strongest combination effect observed in the MRP1 and parental cells and poorer effects observed in the 2008 MRP2 and MRP3 cells. However, the results for taxol are very surprising. The combination index results indicate that sulindac can potentiate the toxicity of taxol to a greater extent than any of the other chemotherapeutic drugs. It is possible that the effect of sulindac on taxol may not be entirely due to sulindac interacting with MRP1 and could be due to an additional mechanism of action (see section 4.11.2).

LONG TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Adriamycin and Sulindac		
Adriamycin 20ng/ml	65.4	5.1
Sul (1)	96.2	3.9
Sul (1) + Adriamycin	**14.3**	**8.5**
Sul (2)	99.4	2.6
Sul (2) + Adriamycin	**24.4**	**4.5**
Sul (3)	101.2	2.2
Sul (3) + Adriamycin	**27.6**	**9.0**
2008 MRP1 Adriamycin and Sulindac		
Adriamycin 120ng/ml	61.9	7.0
Sul (1)	92.1	4.4
Sul (1) + Adriamycin	**4.4**	**1.5**
Sul (2)	97.6	1.0
Sul (2) + Adriamycin	**26.9**	**6.0**
Sul (3)	96.8	1.1
Sul (3) + Adriamycin	**52.2**	**5.4**
2008 MRP2 Adriamycin and Sulindac		
Adriamycin 10ng/ml	60.7	0.4
Sul (1)	95.2	3.1
Sul (1) + Adriamycin	**34.3**	**6.6**
Sul (2)	96.9	3.4
Sul (2) + Adriamycin	**43.4**	**5.7**
Sul (3)	101.7	3.6
Sul (3) + Adriamycin	**44.9**	**4.6**
2008 MRP3 Adriamycin and Sulindac		
Adriamycin 20ng/ml	55.0	3.8
Sul (1)	93.9	7.2
Sul (1) + Adriamycin	**30.9**	**2.1**
Sul (2)	101.9	0.2
Sul (2) + Adriamycin	**34.4**	**1.4**
Sul (3)	97.8	3.8
Sul (3) + Adriamycin	**35.1**	**4.1**

Table 3.8.6a: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of adriamycin, in combination with sulindac, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **(1)** 35μg/ml; **(2)** 17.5μg/ml; **(3)** 8.75μg/ml.

SHORT TERM ASSAY (-Sulindac @T4 hours)		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Adriamycin and Sulindac		
Adriamycin 80ng/ml	59.1	2.7
Sul (1)	97.3	3.6
Sul (1) + Adriamycin	**59.3**	**6.4**
Sul (2)	98.2	3.4
Sul (2) + Adriamycin	**59.8**	**6.1**
Sul (3)	99.4	1.4
Sul (3) + Adriamycin	**60.5**	**6.4**
2008 MRP1 Adriamycin and Sulindac		
Adriamycin 250ng/ml	76.4	3.6
Sul (1)	92.8	2.8
Sul (1) + Adriamycin	**45.0**	**10.8**
Sul (2)	99.2	2.0
Sul (2) + Adriamycin	**52.9**	**9.8**
Sul (3)	98.0	1.4
Sul (3) + Adriamycin	**61.6**	**9.5**
2008 MRP2 Adriamycin and Sulindac		
Adriamycin 125ng/ml	76.0	3.4
Sul (1)	92.8	2.3
Sul (1) + Adriamycin	**71.9**	**1.8**
Sul (2)	96.7	1.8
Sul (2) + Adriamycin	**72.4**	**2.0**
Sul (3)	101.4	2.9
Sul (3) + Adriamycin	**74.4**	**2.9**
2008 MRP3 Adriamycin and Sulindac		
Adriamycin 250ng/ml	61.9	1.9
Sul (1)	96.1	1.9
Sul (1) + Adriamycin	**47.5**	**4.2**
Sul (2)	102.3	4.5
Sul (2) + Adriamycin	**48.6**	**2.7**
Sul (3)	97.7	2.4
Sul (3) + Adriamycin	**50.1**	**5.6**

Table 3.8.6b: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of adriamycin, in combination with sulindac, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

SHORT TERM ASSAY (+Sulindac @T4 hour)		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Adriamycin and Sulindac		
Adriamycin 80ng/ml	59.5	2.7
Sul (1)	96.0	6.8
Sul (1) + Adriamycin	**33.9**	**9.8**
Sul (2)	99.7	3.2
Sul (2) + Adriamycin	**47.3**	**4.1**
Sul (3)	95.9	4.2
Sul (3) + Adriamycin	**48.8**	**4.5**
2008 MRP1 Adriamycin and Sulindac		
Adriamycin 250ng/ml	76.7	2.1
Sul (1)	92.9	1.4
Sul (1) + Adriamycin	**23.8**	**1.5**
Sul (2)	93.9	1.6
Sul (2) + Adriamycin	**48.2**	**6.7**
Sul (3)	98.7	3.1
Sul (3) + Adriamycin	**64.5**	**0.4**
2008 MRP2 Adriamycin and Sulindac		
Adriamycin 125ng/ml	57.7	2.7
Sul (1)	92.8	2.9
Sul (1) + Adriamycin	**51.7**	**3.0**
Sul (2)	100.3	0.3
Sul (2) + Adriamycin	**54.4**	**6.2**
Sul (3)	96.6	0.2
Sul (3) + Adriamycin	**54.9**	**5.9**
2008 MRP3 Adriamycin and Sulindac		
Adriamycin 250ng/ml	51.5	10.7
Sul (1)	94.4	0.5
Sul (1) + Adriamycin	**40.2**	**9.6**
Sul (2)	100.4	7.2
Sul (2) + Adriamycin	**40.4**	**11.2**
Sul (3)	95.7	0.4
Sul (3) + Adriamycin	**42.3**	**6.7**

Table 3.8.6c: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of adriamycin, in combination with sulindac, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **@T0: (1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml; **@T4hours: (1)** 35 μg/ml; **(2)** 17.5 μg/ml; **(3)** 8.75 μg/ml

LONG TERM ASSAY		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Methotrexate and Sulindac		
Methotrexate 5μg/ml	21.2	5.3
Sul (1)	98.4	3.5
Sul (1) + Methotrexate	**12.8**	**3.1**
Sul (2)	101.8	2.1
Sul (2) + Methotrexate	**14.4**	**3.3**
Sul (3)	102.9	2.4
Sul (3) + Methotrexate	**16.2**	**4.4**
2008 MRP1 Methotrexate and Sulindac		
Methotrexate 5μg/ml	24.2	2.2
Sul (1)	93.5	2.4
Sul (1) + Methotrexate	**11.8**	**1.4**
Sul (2)	96.5	4.8
Sul (2) + Methotrexate	**14.5**	**2.8**
Sul (3)	99.1	5.4
Sul (3) + Methotrexate	**19.1**	**3.6**
2008 MRP2 Methotrexate and Sulindac		
Methotrexate 5μg/ml	21.6	1.4
Sul (1)	98.1	2.2
Sul (1) + Methotrexate	**17.6**	**0.7**
Sul (2)	99.5	1.4
Sul (2) + Methotrexate	**18.7**	**1.0**
Sul (3)	98.9	1.8
Sul (3) + Methotrexate	**18.7**	**0.2**
2008 MRP3 Methotrexate and Sulindac		
Methotrexate 5μg/ml	30.6	1.5
Sul (1)	91.9	3.9
Sul (1) + Methotrexate	**20.5**	**2.0**
Sul (2)	101.1	2.1
Sul (2) + Methotrexate	**26.8**	**3.8**
Sul (3)	105.2	3.9
Sul (3) + Methotrexate	**28.0**	**2.4**

Table 3.8.7a: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of methotrexate, in combination with sulindac, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **(1)** 35μg/ml; **(2)** 17.5μg/ml; **(3)** 8.75μg/ml.

SHORT TERM ASSAY (-Sulindac @T4 hours)		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Methotrexate and Sulindac		
Methotrexate 10μg/ml	59.2	4.8
Sul (1)	93.5	5.8
Sul (1) + Methotrexate	**38.2**	**7.9**
Sul (2)	98.2	2.1
Sul (2) + Methotrexate	**38.3**	**9.4**
Sul (3)	99.6	2.4
Sul (3) + Methotrexate	**41.5**	**9.9**
2008 MRP1 Methotrexate and Sulindac		
Methotrexate 900μg/ml	32.1	1.9
Sul (1)	97.7	2.0
Sul (1) + Methotrexate	**18.3**	**1.6**
Sul (2)	98.3	2.0
Sul (2) + Methotrexate	**22.7**	**2.8**
Sul (3)	96.7	3.6
Sul (3) + Methotrexate	**28.6**	**2.4**
2008 MRP2 Methotrexate and Sulindac		
Methotrexate 300μg/ml	20.2	6.9
Sul (1)	93.2	3.1
Sul (1) + Methotrexate	**14.3**	**5.8**
Sul (2)	103.3	8.4
Sul (2) + Methotrexate	**15.4**	**6.7**
Sul (3)	97.8	4.3
Sul (3) + Methotrexate	**17.2**	**6.2**
2008 MRP3 Methotrexate and Sulindac		
Methotrexate 900μg/ml	28.1	3.7
Sul (1)	95.8	6.4
Sul (1) + Methotrexate	**22.9**	**4.3**
Sul (2)	97.2	4.6
Sul (2) + Methotrexate	**24.6**	**5.9**
Sul (3)	101.5	4.6
Sul (3) + Methotrexate	**26.6**	**6.5**

Table 3.8.7b: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of methotrexate, in combination with sulindac, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

SHORT TERM ASSAY (+Sulindac @T4 hours)		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Methotrexate and Sulindac		
Methotrexate 10μg/ml	50.9	6.2
Sul (1)	92.7	1.0
Sul (1) + Methotrexate	**16.7**	**7.7**
Sul (2)	98.6	1.0
Sul (2) + Methotrexate	**20.8**	**7.4**
Sul (3)	102.6	7.8
Sul (3) + Methotrexate	**30.9**	**7.3**
2008 MRP1 Methotrexate and Sulindac		
Methotrexate 900μg/ml	45.0	5.2
Sul (1)	92.0	3.3
Sul (1) + Methotrexate	**16.4**	**2.0**
Sul (2)	96.0	3.6
Sul (2) + Methotrexate	**27.8**	**6.7**
Sul (3)	102.5	6.3
Sul (3) + Methotrexate	**35.0**	**5.4**
2008 MRP2 Methotrexate and Sulindac		
Methotrexate 300μg/ml	25.5	1.4
Sul (1)	95.7	4.0
Sul (1) + Methotrexate	**19.2**	**1.5**
Sul (2)	100.1	2.0
Sul (2) + Methotrexate	**20.4**	**3.2**
Sul (3)	101.0	2.2
Sul (3) + Methotrexate	**22.2**	**3.1**
2008 MRP3 Methotrexate and Sulindac		
Methotrexate 900μg/ml	31.2	6.7
Sul (1)	92.0	0.2
Sul (1) + Methotrexate	**17.8**	**2.3**
Sul (2)	99.7	3.8
Sul (2) + Methotrexate	**21.9**	**1.0**
Sul (3)	100.9	3.4
Sul (3) + Methotrexate	**25.5**	**2.4**

Table 3.8.7c: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of methotrexate, in combination with sulindac, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **@T0: (1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml; **@T4hours: (1)** 35 μg/ml; **(2)** 17.5 μg/ml; **(3)** 8.75 μg/ml.

LONG TERM ASSAY		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Taxotere and Sulindac		
Taxotere 1.0ng/ml	60.0	1.6
Sul (1)	95.1	2.7
Sul (1) + Taxotere	**26.5**	**2.7**
Sul (2)	96.7	1.5
Sul (2) + Taxotere	**37.8**	**2.3**
Sul (3)	94.5	3.2
Sul (3) + Taxotere	**43.4**	**4.8**
2008 MRP1 Taxotere and Sulindac		
Taxotere 1.0ng/ml	34.4	5.8
Sul (1)	89.5	2.3
Sul (1) + Taxotere	**10.6**	**3.2**
Sul (2)	94.8	2.8
Sul (2) + Taxotere	**14.3**	**3.9**
Sul (3)	99.5	2.5
Sul (3) + Taxotere	**22.1**	**3.1**
2008 MRP2 Taxotere and Sulindac		
Taxotere 1.0ng/ml	34.2	6.9
Sul (1)	95.9	2.9
Sul (1) + Taxotere	**15.3**	**2.3**
Sul (2)	99.2	0.5
Sul (2) + Taxotere	**26.5**	**0.7**
Sul (3)	97.7	0.8
Sul (3) + Taxotere	**31.0**	**3.5**
2008 MRP3 Taxotere and Sulindac		
Taxotere 1.0ng/ml	54.1	9.2
Sul (1)	93.5	3.8
Sul (1) + Taxotere	**32.1**	**6.9**
Sul (2)	101.5	4.8
Sul (2) + Taxotere	**45.1**	**8.4**
Sul (3)	102.5	0.6
Sul (3) + Taxotere	**47.0**	**10.6**

Table 3.8.8a: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxotere, in combination with sulindac, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **(1)** 35μg/ml; **(2)** 17.5μg/ml; **(3)** 8.75μg/ml.

SHORT TERM ASSAY (-Sulindac @T4 hours)		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Taxotere and Sulindac		
Taxotere 50.0ng/ml	47.5	1.4
Sul (1)	96.5	2.3
Sul (1) + Taxotere	**43.9**	**4.5**
Sul (2)	102.5	6.1
Sul (2) + Taxotere	**43.1**	**5.6**
Sul (3)	100.5	4.0
Sul (3) + Taxotere	**44.4**	**4.7**
2008 MRP1 Taxotere and Sulindac		
Taxotere 4.0ng/ml	56.5	4.5
Sul (1)	95.3	1.2
Sul (1) + Taxotere	**46.6**	**7.1**
Sul (2)	102.5	4.5
Sul (2) + Taxotere	**50.4**	**4.9**
Sul (3)	97.9	4.1
Sul (3) + Taxotere	**51.6**	**6.8**
2008 MRP2 Taxotere and Sulindac		
Taxotere 50ng/ml	47.7	6.3
Sul (1)	94.8	1.5
Sul (1) + Taxotere	**35.7**	**9.8**
Sul (2)	98.6	2.3
Sul (2) + Taxotere	**36.6**	**9.2**
Sul (3)	99.4	1.0
Sul (3) + Taxotere	**40.3**	**8.7**
2008 MRP3 Taxotere and Sulindac		
Taxotere 25ng/ml	48.1	4.9
Sul (1)	92.9	3.5
Sul (1) + Taxotere	**38.2**	**4.7**
Sul (2)	100.6	4.4
Sul (2) + Taxotere	**47.0**	**4.7**
Sul (3)	97.9	0.7
Sul (3) + Taxotere	**47.0**	**3.4**

Table 3.8.8b: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxotere, in combination with sulindac, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of Sulindac were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

SHORT TERM ASSAY (+Sulindac @T4 hours)		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Taxotere and Sulindac		
Taxotere 50.0ng/ml	49.3	4.2
Sul (1)	95.0	2.7
Sul (1) + Taxotere	**12.5**	**3.2**
Sul (2)	98.8	2.4
Sul (2) + Taxotere	**31.5**	**3.0**
Sul (3)	100.9	4.8
Sul (3) + Taxotere	**38.6**	**8.5**
2008 MRP1 Taxotere and Sulindac		
Taxotere 4.0ng/ml	68.6	1.9
Sul (1)	91.01	1.2
Sul (1) + Taxotere	**26.0**	**4.0**
Sul (2)	93.6	1.7
Sul (2) + Taxotere	**42.9**	**1.9**
Sul (3)	98.7	2.0
Sul (3) + Taxotere	**52.5**	**9.4**
2008 MRP2 Taxotere and Sulindac		
Taxotere 50ng/ml	37.1	6.7
Sul (1)	95.6	3.1
Sul (1) + Taxotere	**21.9**	**4.2**
Sul (2)	99.6	5.5
Sul (2) + Taxotere	**30.0**	**8.3**
Sul (3)	101.9	4.6
Sul (3) + Taxotere	**32.9**	**6.9**
2008 MRP3 Taxotere and Sulindac		
Taxotere 25ng/ml	51.5	8.1
Sul (1)	92.6	4.2
Sul (1) + Taxotere	**24.6**	**6.0**
Sul (2)	98.9	1.4
Sul (2) + Taxotere	**39.1**	**1.8**
Sul (3)	97.7	2.1
Sul (3) + Taxotere	**42.3**	**3.1**

Table 3.8.8c: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxotere, in combination with sulindac, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **@T0: (1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml; **@T4hours: (1)** 35 μg/ml; **(2)** 17.5 μg/ml; **(3)** 8.75 μg/ml

LONG TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Taxol and Sulindac		
Taxol 7.0ng/ml	35.2	3.6
Sul (1)	98.0	2.1
Sul (1) + Taxol	**7.8**	**4.7**
Sul (2)	97.7	1.2
Sul (2) + Taxol	**14.3**	**2.4**
Sul (3)	99.7	1.9
Sul (3) + Taxol	**19.7**	**4.0**
2008 MRP1 Taxol and Sulindac		
Taxol 3.0ng/ml	46.3	1.1
Sul (1)	96.1	1.0
Sul (1) + Taxol	**5.0**	**0.9**
Sul (2)	101.1	4.8
Sul (2) + Taxol	**11.4**	**1.3**
Sul (3)	100.9	2.9
Sul (3) + Taxol	**30.3**	**1.8**
2008 MRP2 Taxol and Sulindac		
Taxol 3.0ng/ml	35.9	1.1
Sul (1)	99.6	2.0
Sul (1) + Taxol	**13.3**	**1.7**
Sul (2)	100.8	0.4
Sul (2) + Taxol	**18.9**	**1.5**
Sul (3)	100.1	0.4
Sul (3) + Taxol	**24.3**	**5.2**
2008 MRP3 Taxol and Sulindac		
Taxol 3.0ng/ml	40.3	5.7
Sul (1)	95.4	3.8
Sul (1) + Taxol	**8.4**	**5.5**
Sul (2)	103.2	4.3
Sul (2) + Taxol	**13.0**	**5.3**
Sul (3)	102.5	4.0
Sul (3) + Taxol	**15.8**	**4.6**

Table 3.8.9a: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxol, in combination with sulindac, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **(1)** 35μg/ml; **(2)** 17.5μg/ml; **(3)** 8.75μg/ml.

SHORT TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Taxol and Sulindac		
Taxol 1000ng/ml	50.0	7.4
Sul (1)	98.3	2.3
Sul (1) + Taxol	**33.9**	**10.5**
Sul (2)	94.7	2.0
Sul (2) + Taxol	**35.7**	**11.2**
Sul (3)	99.3	3.0
Sul (3) + Taxol	**41.1**	**10.6**
2008 MRP1 Taxol and Sulindac		
Taxol 2000ng/ml	48.9	2.8
Sul (1)	94.0	4.6
Sul (1) + Taxol	**29.3**	**8.7**
Sul (2)	97.5	0.4
Sul (2) + Taxol	**40.3**	**12.5**
Sul (3)	100.7	0.4
Sul (3) + Taxol	**42.3**	**5.6**
2008 MRP2 Taxol and Sulindac		
Taxol 1000ng/ml	59.8	0.3
Sul (1)	97.1	0.7
Sul (1) + Taxol	**34.2**	**0.6**
Sul (2)	100.5	4.2
Sul (2) + Taxol	**54.7**	**8.9**
Sul (3)	99.9	3.1
Sul (3) + Taxol	**54.5**	**3.3**
2008 MRP3 Taxol and Sulindac		
Taxol 2000ng/ml	62.6	1.5
Sul (1)	96.1	3.4
Sul (1) + Taxol	**50.5**	**0.8**
Sul (2)	97.3	1.0
Sul (2) + Taxol	**59.0**	**4.2**
Sul (3)	96.9	4.5
Sul (3) + Taxol	**59.6**	**0.6**

Table 3.8.9b: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxol, in combination with sulindac, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

	Taxotere + Sulindac			Taxol + Sulindac	
	L.T	S.T(1)	S.T(2)	L.T	S.T(1)
2008P	0.371	1.365	0.324	0.172	0.631
MRP1	0. 465	0.963	0.279	0.046	0.652
MRP2	0.612	0.977	0.851	0.283	0.590
MRP3	0.516	0.996	0.537	0.199	0.780

Table 3.8.10a: Combination index (CI) values for taxotere or taxol in combination with sulindac obtained using the method of Chou and Talalay as described in section 2.16.

	Methotrexate + Sulindac			Adriamycin + Sulindac		
	L.T	S.T(1)	S.T(2)	L.T	S.T(1)	S.T(2)
2008P	0.729	0.550	0.220	0.218	1.082	0.534
MRP1	0. 591	0.357	0.343	0.091	0.528	0.357
MRP2	0.883	0.823	0.792	0.463	1.009	0.918
MRP3	0.709	0.790	0.640	0.509	0.786	0.658

Table 3.8.10b: Combination index (CI) values for taxotere or taxol in combination with sulindac obtained using the method of Chou and Talalay as described in section 2.16.

(CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.)

L.T: Long-term assay as described in section 2.7.3a

S.T (1): Short-term assay. Drug and compound removed from the cells after 4 hours incubation and replaced with fresh medium only as described in section 2.7.3b

S.T (2): Short-term assay. Drug and NSAID removed from the cells after 4 hours incubation and replaced with NSAID only as described in section 2.7.3b.

3.8.4 Analysis of 2008 cells for enhancement of adriamycin, taxol, taxotere and cisplatin toxicities by BRI 138/1 and piroxicam.

Long-term and short-term combination toxicity assays were carried out on the 2008 cells using taxol and taxotere in combination with piroxicam. Duffy *et al.*, (1998), reported that piroxicam was not an MRP substrate and did not potentiate the toxicity of any of the chemotherapeutic drugs analysed. Hence, this NSAID was used in the combination toxicity assays as a negative control. The results for taxotere in combination with piroxicam in both the long-term and the short-term assays indicate that there is no significant synergy between piroxicam and taxotere in the 2008 cell lines (Table 3.8.11 and 3.8.17a). **However, the results for taxol and piroxicam are interesting. There appears to be some potentiation of the toxicity of taxol by the NSAID piroxicam in the long-term toxicity assay (Table 3.8.12).** The combination index values for taxol and piroxicam in the 2008 cell lines range from 0.795 for 2008 MRP1 to 0.820 for 2008 P (Table 3.8.17a). These results indicate significant potentiation of the toxicity of taxol by piroxicam in the long-term toxicity assay. Considering these results, it is possible that there is an alternative or additional mechanism of taxol toxicity potentiation by NSAIDs.
BRI 138/1, an indomethacin analogue was found to be positive in the combination toxicity assays in both DLKP and CORL23 cells. BRI 138/1 was analysed in the 2008 cell lines in combination with taxol and taxotere to assess if this compound was also active in an ovarian carcinoma cell line. The results obtained from these assays indicate that BRI 138/1 potentiated the toxicity of taxol, taxotere and adriamycin in the long-term combination toxicity assays (Tables 3.8.13, 3.8.14 and 3.8.15). Again, the toxicity of taxol was potentiated to a greater extent by BRI 138/1 as compared to the potentiation of adriamycin and taxotere by this indomethacin analogue. There was no significant potentiation of the toxicity of the chemotherapeutic drugs in the short-term assays suggesting that BRI 138/1 required a longer period of time in the cells to exert its enhancement effect. This indomethacin analogue, like indomethacin, was not able to potentiate the toxicity of cisplatin in the 2008 cells (Table 3.8.16). This result suggests that cisplatin is not an MRP substrate.

LONG TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Taxotere and Piroxicam		
Taxotere 1.0ng/ml	58.9	4.8
Pirox (1)	92.4	2.1
Pirox (1) + Taxotere	**48.8**	**2.4**
Pirox (2)	99.7	0.9
Pirox (2) + Taxotere	**48.8**	**3.5**
Pirox (3)	100.6	1.6
Pirox (3) + Taxotere	**51.2**	**1.8**
2008 MRP1 Taxotere and Piroxicam		
Taxotere 1.0ng/ml	50.7	6.2
Pirox (1)	93.3	1.7
Pirox (1) + Taxotere	**45.7**	**5.2**
Pirox (2)	95.5	3.5
Pirox (2) + Taxotere	**47.6**	**4.0**
Pirox (3)	94.2	6.2
Pirox (3) + Taxotere	**49.8**	**4.4**
2008 MRP2 Taxotere and Piroxicam		
Taxotere 1.0ng/ml	44.0	10.9
Pirox (1)	97.6	1.1
Pirox (1) + Taxotere	**39.4**	**6.6**
Pirox (2)	100.2	2.8
Pirox (2) + Taxotere	**40.0**	**3.9**
Pirox (3)	96.8	0.5
Pirox (3) + Taxotere	**41.8**	**4.1**
2008 MRP3 Taxotere and Piroxicam		
Taxotere 1.0ng/ml	47.4	9.1
Pirox (1)	98.1	1.1
Pirox (1) + Taxotere	**43.4**	**0.9**
Pirox (2)	99.1	0.5
Pirox (2) + Taxotere	**45.5**	**3.7**
Pirox (3)	98.3	2.8
Pirox (3) + Taxotere	**46.8**	**5.4**

Table 3.8.11a: Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxotere, in combination with piroxicam, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate± S.D. The untreated cell survival % is taken as 100%. Results are the average of triplicate determinations in three separate experiments. The concentrations of piroxicam were: **(1)** 40μg/ml; **(2)** 20μg/ml; **(3)** 10μg/ml.

SHORT TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Taxotere and Piroxicam		
Taxotere 50.0ng/ml	47.2	1.8
Pirox (1)	98.8	1.8
Pirox (1) + Taxotere	**45.0**	**2.4**
Pirox (2)	98.7	1.0
Pirox (2) + Taxotere	**47.0**	**2.1**
Pirox (3)	99.1	1.0
Pirox (3) + Taxotere	**46.6**	**1.7**
2008 MRP1 Taxotere and Piroxicam		
Taxotere 4.0ng/ml	41.6	7.1
Pirox (1)	99.6	3.1
Pirox (1) + Taxotere	**40.9**	**6.4**
Pirox (2)	100.3	2.7
Pirox (2) + Taxotere	**41.3**	**7.1**
Pirox (3)	100.6	0.9
Pirox (3) + Taxotere	**41.6**	**7.1**
2008 MRP2 Taxotere and Piroxicam		
Taxotere 50ng/ml	50.6	6.6
Pirox (1)	99.9	1.0
Pirox (1) + Taxotere	**47.5**	**5.8**
Pirox (2)	99.2	1.1
Pirox (2) + Taxotere	**49.4**	**6.6**
Pirox (3)	94.8	0.6
Pirox (3) + Taxotere	**49.4**	**8.2**
2008 MRP3 Taxotere and Piroxicam		
Taxotere 25ng/ml	57.8	1.8
Pirox (1)	95.2	1.1
Pirox (1) + Taxotere	**56.9**	**0.9**
Pirox (2)	95.9	1.3
Pirox (2) + Taxotere	**57.6**	**1.9**
Pirox (3)	98.2	2.6
Pirox (3) + Taxotere	**58.1**	**0.3**

Table 3.8.11b: Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxotere, in combination with piroxicam, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of piroxicam were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

LONG TERM ASSAY		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Taxol and Piroxicam		
Taxol 7.0ng/ml	48.9	5.0
Pirox (1)	98.4	1.5
Pirox (1) + Taxol	**36.1**	**5.5**
Pirox (2)	96.5	2.6
Pirox (2) + Taxol	**37.7**	**7.8**
Pirox (3)	99.6	1.7
Pirox (3) + Taxol	**39.9**	**3.2**
2008 MRP1 Taxol and Piroxicam		
Taxol 3.0ng/ml	51.2	3.5
Pirox (1)	97.7	1.6
Pirox (1) + Taxol	**38.3**	**5.7**
Pirox (2)	101.4	0.3
Pirox (2) + Taxol	**42.4**	**3.0**
Pirox (3)	102.6	4.1
Pirox (3) + Taxol	**49.1**	**6.2**
2008 MRP2 Taxol and Piroxicam		
Taxol 3.0ng/ml	50.2	2.9
Pirox (1)	96.9	0.5
Pirox (1) + Taxol	**39.8**	**6.1**
Pirox (2)	97.8	0.3
Pirox (2) + Taxol	**42.6**	**4.8**
Pirox (3)	99.1	0.2
Pirox (3) + Taxol	**44.1**	**5.1**
2008 MRP3 Taxol and Piroxicam		
Taxol 3.0ng/ml	55.5	5.2
Pirox (1)	97.4	2.0
Pirox (1) + Taxol	**42.3**	**2.5**
Pirox (2)	99.1	0.2
Pirox (2) + Taxol	**45.9**	**5.0**
Pirox (3)	100.3	2.3
Pirox (3) + Taxol	**51.9**	**8.0**

Table 3.8.12a: Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxol, in combination with piroxicam, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of piroxicam were: **(1)** 40μg/ml; **(2)** 20μg/ml; **(3)** 10μg/ml.

SHORT TERM ASSAY		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Taxol and Piroxicam		
Taxol 1000ng/ml	57.6	0.8
Pirox (1)	99.0	0.4
Pirox (1) + Taxol	**51.5**	**4.8**
Pirox (2)	95.1	1.6
Pirox (2) + Taxol	**52.8**	**1.9**
Pirox (3)	97.9	3.6
Pirox (3) + Taxol	**57.2**	**1.0**
2008 MRP1 Taxol and Piroxicam		
Taxol 2000ng/ml	53.7	3.0
Pirox (1)	98.9	0.6
Pirox (1) + Taxol	**50.6**	**1.2**
Pirox (2)	101.2	4.1
Pirox (2) + Taxol	**51.2**	**0.7**
Pirox (3)	99.7	0.9
Pirox (3) + Taxol	**53.4**	**1.0**
2008 MRP2 Taxol and Piroxicam		
Taxol 1000ng/ml	52.3	0.8
Pirox (1)	96.5	1.9
Pirox (1) + Taxol	**50.5**	**0.8**
Pirox (2)	97.7	0.4
Pirox (2) + Taxol	**50.9**	**1.0**
Pirox (3)	99.0	1.0
Pirox (3) + Taxol	**51.2**	**0.6**
2008 MRP3 Taxol and Piroxicam		
Taxol 2000ng/ml	59.4	6.2
Pirox (1)	98.8	1.1
Pirox (1) + Taxol	**55.9**	**3.0**
Pirox (2)	99.3	0.7
Pirox (2) + Taxol	**57.2**	**1.3**
Pirox (3)	101.9	4.5
Pirox (3) + Taxol	**57.5**	**0.9**

Table 3.8.12b: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxol, in combination with piroxicam, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of piroxicam were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

LONG TERM ASSAY		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Taxotere and BRI 138/1		
Taxotere 1.0ng/ml	42.8	9.2
138/1 (1)	95.6	1.8
138/1 (1) + Taxotere	**20.6**	**0.5**
138/1 (2)	100.0	2.5
138/1 (2) + Taxotere	**27.9**	**3.0**
138/1 (3)	100	7.2
138/1 (3) + Taxotere	**33.8**	**1.3**
2008 MRP1 Taxotere and 138/1		
Taxotere 1.0ng/ml	43.6	5.4
138/1 (1)	96.7	3.2
138/1 (1) + Taxotere	**9.2**	**1.9**
138/1 (2)	100.0	0.8
138/1 (2) + Taxotere	**13.2**	**0.9**
138/1 (3)	100.2	0.8
138/1 (3) + Taxotere	**21.8**	**1.3**
2008 MRP2 Taxotere and 138/1		
Taxotere 1.0ng/ml	38.1	3.9
138/1 (1)	91.4	5.4
138/1 (1) + Taxotere	**16.0**	**3.0**
138/1 (2)	101.9	4.4
138/1 (2) + Taxotere	**17.0**	**0.8**
138/1 (3)	100.9	3.1
138/1 (3) + Taxotere	**19.1**	**2.0**
2008 MRP3 Taxotere and 138/1		
Taxotere 1.0ng/ml	40.0	7.7
138/1 (1)	93.3	2.5
138/1 (1) + Taxotere	**9.5**	**0.9**
138/1 (2)	95.5	1.5
138/1 (2) + Taxotere	**10.5**	**3.7**
138/1 (3)	99.9	2.8
138/1 (3) + Taxotere	**12.2**	**5.4**

Table 3.8.13a: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxotere, in combination with BRI 138/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of 138/1 were: **(1)** 35μg/ml; **(2)** 17.5μg/ml; **(3)** 8.75μg/ml.

SHORT TERM ASSAY		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Taxotere and 138/1		
Taxotere 50.0ng/ml	47.3	3.2
138/1 (1)	93.6	5.7
138/1 (1) + Taxotere	**35.2**	**4.5**
138/1 (2)	98.5	4.3
138/1 (2) + Taxotere	**40.5**	**3.7**
138/1 (3)	100.1	0.6
138/1 (3) + Taxotere	**43.2**	**2.6**
2008 MRP1 Taxotere and 138/1		
Taxotere 4.0ng/ml	60.6	2.7
138/1 (1)	94.8	2.8
138/1 (1) + Taxotere	**51.4**	**6.6**
138/1 (2)	96.8	0.5
138/1 (2) + Taxotere	**56.1**	**8.3**
138/1 (3)	101.5	1.1
138/1 (3) + Taxotere	**59.4**	**4.9**
2008 MRP2 Taxotere and 138/1		
Taxotere 50ng/ml	44.5	0.6
138/1 (1)	91.3	0.1
138/1 (1) + Taxotere	**32.9**	**2.8**
138/1 (2)	95.9	8.9
138/1 (2) + Taxotere	**40.3**	**3.4**
138/1 (3)	100.0	2.6
138/1 (3) + Taxotere	**40.5**	**3.2**
2008 MRP3 Taxotere and 138/1		
Taxotere 25ng/ml	43.6	7.1
138/1 (1)	94.3	3.0
138/1 (1) + Taxotere	**31.2**	**7.5**
138/1 (2)	95.6	2.3
138/1 (2) + Taxotere	**34.9**	**6.7**
138/1 (3)	100.7	6.9
138/1 (3) + Taxotere	**37.0**	**5.0**

Table 3.8.13b: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxotere, in combination with BRI 138/1, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of 138/1 were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

LONG TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Taxol and BRI 138/1		
Taxol 7.0ng/ml	39.6	0.5
138/1 (1)	96.6	3.1
138/1 (1) + Taxol	**10.4**	**1.0**
138/1 (2)	98.9	2.7
138/1 (2) + Taxol	**15.6**	**1.6**
138/1 (3)	100.3	0.8
138/1 (3) + Taxol	**24.0**	**1.0**
2008 MRP1 Taxol and BRI 138/1		
Taxol 3.0ng/ml	35.4	1.2
138/1 (1)	96.5	3.9
138/1 (1) + Taxol	**5.6**	**2.9**
138/1 (2)	97.2	1.3
138/1 (2) + Taxol	**15.3**	**0.7**
138/1 (3)	99.3	1.3
138/1 (3) + Taxol	**23.1**	**2.4**
2008 MRP2 Taxol and BRI 138/1		
Taxol 3.0ng/ml	35.3	7.2
138/1 (1)	93.9	2.2
138/1 (1) + Taxol	**7.6**	**5.1**
138/1 (2)	98.0	1.5
138/1 (2) + Taxol	**15.6**	**8.2**
138/1 (3)	100.3	1.9
138/1 (3) + Taxol	**20.5**	**8.3**
2008 MRP3 Taxol and BRI 138/1		
Taxol 3.0ng/ml	32.6	7.1
138/1 (1)	90.5	10.3
138/1 (1) + Taxol	**3.4**	**0.6**
138/1 (2)	99.8	0.3
138/1 (2) + Taxol	**11.0**	**1.9**
138/1 (3)	101.5	2.5
138/1 (3) + Taxol	**21.8**	**6.5**

Table 3.8.14a: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxol, in combination with BRI 138/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of BRI 138/1 were: **(1)** 35µg/ml; **(2)** 17.5µg/ml; **(3)** 8.75µg/ml.

SHORT TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Taxol and BRI 138/1		
Taxol 1000ng/ml	52.9	2.6
138/1 (1)	93.0	3.2
138/1 (1) + Taxol	**43.5**	**1.8**
138/1 (2)	97.6	0.6
138/1 (2) + Taxol	**47.8**	**0.5**
138/1 (3)	98.6	1.6
138/1 (3) + Taxol	**50.3**	**2.3**
2008 MRP1 Taxol and BRI 138/1		
Taxol 2000ng/ml	49.6	1.0
138/1 (1)	92.5	0.6
138/1 (1) + Taxol	**44.5**	**1.3**
138/1 (2)	97.7	2.6
138/1 (2) + Taxol	**46.5**	**0.1**
138/1 (3)	99.3	1.9
138/1 (3) + Taxol	**48.5**	**1.0**
2008 MRP2 Taxol and BRI 138/1		
Taxol 1000ng/ml	54.6	2.1
138/1 (1)	95.6	2.7
138/1 (1) + Taxol	**51.6**	**4.3**
138/1 (2)	96.5	2.4
138/1 (2) + Taxol	**51.6**	**4.1**
138/1 (3)	94.1	1.7
138/1 (3) + Taxol	**56.8**	**0.9**
2008 MRP3 Taxol and BRI 138/1		
Taxol 2000ng/ml	46.1	1.0
138/1 (1)	93.8	1.7
138/1 (1) + Taxol	**35.3**	**3.0**
138/1 (2)	94.3	2.9
138/1 (2) + Taxol	**40.9**	**2.3**
138/1 (3)	101.7	2.4
138/1 (3) + Taxol	**39.2**	**0.6**

Table 3.8.14b: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of taxol, in combination with BRI 138/1, as found using the protocol detailed in section 2.7.3b (Short term assay). Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of BRI 138/1 were: **(1)** 100 μg/ml; **(2)** 50 μg/ml; **(3)** 25 μg/ml.

LONG TERM ASSAY

Test Sample	% Cell Survival	S.D.
2008 P Adriamycin and BRI 138/1		
Adriamycin 20ng/ml	54.1	2.4
138/1 (1)	93.9	4.0
138/1 (1) + Adriamycin	**25.7**	**2.4**
138/1 (2)	100.4	2.6
138/1 (2) + Adriamycin	**38.3**	**1.2**
138/1 (3)	103.7	4.0
138/1 (3) + Adriamycin	**48.1**	**1.1**
2008 MRP1 Adriamycin and BRI 138/1		
Adriamycin 120ng/ml	49.3	3.7
138/1 (1)	94.5	4.1
138/1 (1) + Adriamycin	**12.6**	**3.2**
138/1 (2)	98.7	4.9
138/1 (2) + Adriamycin	**25.1**	**3.3**
138/1 (3)	101.5	3.1
138/1 (3) + Adriamycin	**32.8**	**1.3**
2008 MRP2 Adriamycin and BRI 138/1		
Adriamycin 10ng/ml	46.5	5.5
138/1 (1)	92.2	1.3
138/1 (1) + Adriamycin	**22.6**	**3.5**
138/1 (2)	94.5	1.6
138/1 (2) + Adriamycin	**33.2**	**2.2**
138/1 (3)	100.2	4.4
138/1 (3) + Adriamycin	**36.7**	**4.8**
2008 MRP3 Adriamycin and BRI 138/1		
Adriamycin 20ng/ml	75.9	3.2
138/1 (1)	93.1	2.1
138/1 (1) + Adriamycin	**49.2**	**4.8**
138/1 (2)	95.7	1.7
138/1 (2) + Adriamycin	**54.4**	**5.8**
138/1 (3)	98.0	2.0
138/1 (3) + Adriamycin	**63.4**	**4.0**

Table 3.8.15: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of adriamycin, in combination with BRI 138/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of BRI 138/1 were: **(1)** 35μg/ml; **(2)** 17.5μg/ml; **(3)** 8.75μg/ml.

LONG TERM ASSAY		
Test Sample	**% Cell Survival**	**S.D.**
2008 P Cisplatin and BRI 138/1		
Cisplatin 200ng/ml	56.7	8.3
138/1 (1)	93.9	5.9
138/1 (1) + Cisplatin	**74.9**	**9.7**
138/1 (2)	97.6	1.6
138/1 (2) + Cisplatin	**70.4**	**7.9**
138/1 (3)	98.9	5.5
138/1 (3) + Cisplatin	**64.7**	**7.4**
2008 MRP1 Cisplatin and BRI 138/1		
Cisplatin 50ng/ml	58.0	4.9
138/1 (1)	93.0	2.9
138/1 (1) + Cisplatin	**59.1**	**4.0**
138/1 (2)	99.2	8.2
138/1 (2) + Cisplatin	**58.6**	**6.6**
138/1 (3)	101.2	7.0
138/1 (3) + Cisplatin	**58.0**	**5.8**
2008 MRP2 Cisplatin and BRI 138/1		
Cisplatin 15ng/ml	58.0	3.8
138/1 (1)	94.0	8.9
138/1 (1) + Cisplatin	**64.2**	**3.5**
138/1 (2)	96.1	2.1
138/1 (2) + Cisplatin	**62.2**	**5.0**
138/1 (3)	100.7	7.3
138/1 (3) + Cisplatin	**59.5**	**7.0**
2008 MRP3 Cisplatin and BRI 138/1		
Cisplatin 30ng/ml	78.4	0.8
138/1 (1)	92.0	3.4
138/1 (1) + Cisplatin	**90.1**	**6.8**
138/1 (2)	108.0	0.1
138/1 (2) + Cisplatin	**83.0**	**9.1**
138/1 (3)	110.4	11.5
138/1 (3) + Cisplatin	**81.5**	**0.3**

Table 3.8.16: % Survival of 2008 P, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells in the presence of cisplatin, in combination with BRI 138/1, as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of BRI 138/1 were: **(1)** 35μg/ml; **(2)** 17.5μg/ml; **(3)** 8.75μg/ml.

	Taxotere+ Piroxicam		Taxol + Piroxicam	
	L.T	S.T	L.T	S.T
2008P	0.978	0.960	0.820	1.007
MRP1	0.942	1.004	0.795	1.042
MRP2	0.938	0.978	0.817	1.000
MRP3	0.954	1.026	0.801	0.930

Table 3.8.17a: Combination index (CI) values for taxotere or taxol in combination with piroxicam, obtained using the method of Chou and Talalay as described in section 2.16.

	Taxotere +138/1		Taxol +138/1		Adr.+138/1	Cis. +138/1
	L.T	S.T	L.T	S.T	L.T	LT
2008P	0.576	0.940	0.580	1.212	0.476	1.007
MRP1	0.340	1.000	0.230	1.336	0.222	1.042
MRP2	0.649	0.927	0.235	1.503	0.454	1.000
MRP3	0.438	0.930	0.227	1.188	0.724	0.930

Table 3.8.17b: Combination index (CI) values for taxotere, taxol, adriamycin (Adr) or cisplatin (Cis) in combination with BRI 138/1, obtained using the method of Chou and Talalay as described in section 2.16.

(CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.)

L.T: Long-term assay as described in section 2.7.3a

S.T (1): Short-term assay. Drug and compound removed from the cells after 4 hours incubation and replaced with fresh medium only as described in section 2.7.3b

	Adriamycin			Methotrexate			Cisplatin
Indomethacin	LT	ST(1)	ST(2)	LT	ST(1)	ST(2)	LT
2008 P	0.488	1.048		1.056	0.372		2.422
2008 MRP1	0.186	0.683		0.653	0.581		1.345
2008 MRP2	0.493	0.958		1.064	1.000		2.030
2008 MRP3	0.433	0.860		0.738	0.879		2.198
Sulindac							
2008 P	0.218	1.082	0.534	0.729	0.550	0.220	
2008 MRP1	0.091	0.528	0.357	0.591	0.357	0.343	
2008 MRP2	0.463	1.009	0.918	0.883	0.823	0.792	
2008 MRP3	0.509	0.786	0.658	0.709	0.790	0.640	
BRI 138/1							
2008 P	0.476						1.007
2008 MRP1	0.222						1.042
2008 MRP2	0.454						1.000
2008 MRP3	0.724						0.930

Table 3.8.18a: Summary table with Combination index (CI) values for the highest concentrations of adriamycin, methotrexate and cisplatin in combination with indomethacin, sulindac, BRI 138/1 and piroxicam.

(CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.)

L.T: Long-term assay as described in section 2.7.3a.

S.T (1): Short-term assay. Drug and compound removed from the cells after 4 hours incubation and replaced with fresh medium only as described in section 2.7.3b.

S.T (2): Short-term assay. Drug and NSAID removed from the cells after 4 hours incubation and replaced with NSAID only as described in section 2.7.3b.

	Taxol		Taxotere		
	LT	ST(1)	LT	ST(1)	ST(2)
Sulindac					
2008 P	0.172	0.631	0.371	1.365	0.324
2008 MRP1	0.046	0.652	0.465	0.963	0.279
2008 MRP2	0.283	0.590	0.612	0.977	0.851
2008 MRP3	0.199	0.780	0.516	0.996	0.537
BRI 138/1					
2008 P	0.580	1.212	0.576	0.940	
2008 MRP1	0.230	1.336	0.340	1.000	
2008 MRP2	0.235	1.503	0.649	0.927	
2008 MRP3	0.227	1.188	0.438	0.930	
Piroxicam					
2008 P	0.820	1.007	0.978	0.960	
2008 MRP1	0.795	1.042	0.942	1.004	
2008 MRP2	0.817	1.000	0.938	0.978	
2008 MRP3	0.801	0.930	0.954	1.026	

Table 3.8.18b: Summary table with Combination index (CI) values for the highest concentrations of taxol and taxotere in combination with indomethacin, sulindac, BRI 138/1 and piroxicam.

(CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.)

L.T: Long-term assay as described in section 2.7.3a.

S.T (1): Short-term assay. Drug and compound removed from the cells after 4 hours incubation and replaced with fresh medium only as described in section 2.7.3b.

S.T (2): Short-term assay. Drug and NSAID removed from the cells after 4 hours incubation and replaced with NSAID only as described in section 2.7.3b.

3.9 RT-PCR analysis of 2008(P), 2008 MRP1, 2008 MRP2 and 2008 MRP3 cell lines.

RT-PCR analysis was carried out on all the 2008 cell lines to characterise the MRP mRNA expression in these cells and to compare the levels of MRP mRNA to protein expression in the analysed cells. Photographs of the RT-PCR gels and densitometric analysis of the bands are in section 3.16. The sequences for the primers used for MRP1-5 PCR were identical to those used by Kool *et al.,* (1997). The sequence for the primers for MRP6 were obtained from O'Neill *et al.,* 1998.

3.9.1 MRP1 mRNA expression

MRP1 RT-PCR analysis of the 2008 P, 2008 MRP1, MRP2 and MRP3 cells indicated that there was very little difference in expression of MRP1 in all of the 2008 cell lines. There appeared to be an up-regulation of MRP1 expression in the transfected 2008 MRP1 cell line and slight down regulation of MRP1 mRNA in the 2008 MRP3 cells (Figure 3.16.1a). Western blot analysis of these cell lines using the MRP specific rat monoclonal antibody (MAb), MRPr1, demonstrated a very strong expression of MRP1 in the MRP1 transfected cells. However, there was also a low level of MRP expression in the 2008 P, 2008 MRP2 and 2008 MRP3 cells (Connolly, 1999). The results indicate that regulation of MRP1 expression may be post-transcriptional and that MRP1 mRNA expression does not correlate with MRP1 protein levels in these 2008 cell lines.

3.9.2 MRP2 mRNA expression

MRP2 RT-PCR analysis of the 2008 P, 2008 MRP1, MRP2 and MRP3 cells indicated that there was a very low expression of MRP2 in the parental 2008 cell line. MRP2 was upregulated in the 2008 MRP1 and MRP3 transfected cell lines as compared to the parental cell line. However, the expression of MRP2 mRNA was strongest in the 2008 cell line transfected with MRP2 cDNA (Figure 3.16.2a). Western blot analysis of these cell lines using the MRP2 specific, mouse MAb, M2III-6, demonstrated a very strong expression of

MRP2 in the MRP2-transfected cells only (Connolly, 1999). There appeared to be no expression of MRP2 in the parental and MRP1 and MRP3 transfected 2008 cell lines.

3.9.3 MRP3 mRNA expression

MRP3 RT-PCR analysis of the 2008 P, 2008 MRP1, MRP2 and MRP3 cells indicated that there was highest expression of MRP3 mRNA in the 2008 MRP3- transfected cell line. There were similarly low levels of MRP3 mRNA expression in the 2008 parental and 2008 MRP1 cell lines. The expression of MRP3 mRNA was down-regulated in the 2008 MRP2-transfected cell line as compared to expression of MRP3 mRNA in the parental 2008 cell line (Figure 3.16.3). Western blot analysis of these cell lines using the MRP3 specific mouse MAb, M3II-21, demonstrated a very strong expression of MRP3 in the MRP3 transfected cells only (Connolly, 1999). There seemed to be no expression of MRP3 in the parental and MRP1 and MRP2-transfected 2008 cell lines.

3.9.4 MRP4 mRNA expression

MRP4 RT-PCR analysis of the 2008 P, 2008 MRP1, MRP2 and MRP3 cells indicated that there was a low expression of MRP4 mRNA in the 2008 parental cell line. A similar level of expression of MRP4 mRNA was observed in the 2008 MRP3 transfected cell line. There was an up-regulation of MRP4 mRNA in the MRP2 2008 transfected cells as compared to the 2008 parental cell line. The expression of MRP4 mRNA was strongest in the 2008 MRP1 transfected cell line (Figure 3.16.4a). Western Blot analysis for MRP4 was not carried out on these cell lines.

3.9.5 MRP5 mRNA expression

MRP5 RT-PCR analysis of the 2008 P, 2008 MRP1, MRP2 and MRP3 cells indicated that MRP5 mRNA was expressed in all four cells lines. There appeared to be down-regulation of MRP5 mRNA in the 2008 MRP1, MRP2 and MRP3 transfected cell lines as compared to the level of MRP5 mRNA in

the parental cell line (Figure 3.16.5a). Western Blot analysis for MRP5 was not carried out on these cell lines.

3.9.6 MRP6 mRNA expression

MRP6 RT-PCR analysis of the 2008 P, 2008 MRP1, MRP2 and MRP3 cells indicated that MRP6 mRNA was expressed in all four cells lines. There appeared to an up-regulation of MRP6 mRNA in the 2008 MRP1, MRP2 and MRP3 transfected cell lines as compared to the level of MRP6 mRNA in the parental cell line (Figure 3.16.6). The highest expression of MRP6 mRNA was evident in the 2008 MRP2 cell line. Western Blot analysis for MRP6 was not carried out on these cell lines.

3.9.7 MDR1 mRNA expression

MDR1 RT-PCR analysis of the 2008 P, 2008 MRP1, MRP2 and MRP3 cells indicated that MDR1 mRNA was expressed in all four cells lines. There appeared to an up-regulation of MDR1 mRNA in the 2008 MRP1 and MRP2 transfected cell lines as compared to the level of MDR1 mRNA in the parental 2008 and MRP3 transfected cell lines (Figure 3.16.7). The highest expression of MDR1 mRNA was evident in the 2008 MRP2 cell line. Western Blot analysis for MDR1 was not carried out on these cell lines.

3.10 Investigation of enhancement of chemotherapeutic drugs by sulindac and indomethacin in a range of cancer cell lines.

To investigate if the potentiation of taxol and taxotere by sulindac and indomethacin was limited to particular cell lines, combination toxicity assays using taxol, taxotere and adriamycin in combination with indomethacin or sulindac were carried out on four more cell lines. The cell lines analysed were a breast cancer cell line, MCF-7, a poorly differentiated human lung cancer cell line, DLKP, and a human lung adenocarcinoma cell line, A549, from two different sources, the NCTCC cell culture collection (originally from the ATCC) and Quintiles. Long-term combination toxicity assays were used to analyse these cell lines as it had been demonstrated in section 3.8 that both indomethacin and sulindac required longer incubation periods in the cells to exert their enhancement effect. As it was already known that indomethacin enhances the toxicity of adriamycin in the DLKP cell line (section 3.1), initial combination toxicity assays were carried out to assess if this effect was similar in the other cell lines. The results indicate that indomethacin also potentiated the toxicity of adriamycin in the MCF-7 and A549 cell lines, although the enhancement effect was not as good as that observed in the DLKP cells (Table 3.10.1).

The toxicity of taxol or taxotere in combination with indomethacin was subsequently analysed in the four cell lines. The results indicated that indomethacin was unable to significantly potentiate the toxicity of taxol in the DLKP, MCF-7 and A549 cell lines (Table 3.10.2). There is a small enhancement of the toxicity of taxotere by indomethacin in the DLKP and MCF-7 cell lines (Table 3.10.3) but no enhancement of the toxicity of this drug by indomethacin in the A549 cell lines.

Taxol and taxotere were then analysed in these cell lines in combination with sulindac and the results showed that there is very good synergism between taxol + sulindac and taxotere + sulindac in the DLKP cell line. There was average potentiation of taxol by sulindac in the MCF-7 cell line and only a very weak potentiation of taxotere by sulindac in the same cell line. Sulindac was unable to potentiate the toxicity of taxol or taxotere in either the A549 cells (Tables 3.10.5, 3.10.6 and 3.10.7).

Test Sample	% Cell Survival	S.D.
DLKP, Adriamycin and Indomethacin		
Adr. 10ng/ml	46.6	5.1
Indo (1)	96.0	2.4
Indo (1) + Adr	**12.9**	**1.1**
Indo (2)	95.7	2.4
Indo (2) + Adr	**28.1**	**3.9**
Indo (3)	99.3	0.4
Indo (3) + Adr	**40.1**	**1.1**
MCF-7 (Quintiles) Adriamycin and Indomethacin		
Adr. 30ng/ml	54.0	1.8
Indo (1)	96.0	2.7
Indo (1) + Adr	**29.1**	**5.3**
Indo (2)	97.0	2.5
Indo (2) + Adr	**37.0**	**5.4**
Indo (3)	98.3	3.9
Indo (3) + Adr	**41.8**	**7.4**
A549 (NCTCC) Adriamycin and Indomethacin		
Adr. 50ng/ml	47.2	5.0
Indo (1)	94.6	6.5
Indo (1) + Adr	**24.2**	**5.2**
Indo (2)	100.3	0.4
Indo (2) + Adr	**36.4**	**5.2**
Indo (3)	99.3	1.3
Indo (3) + Adr	**41.7**	**7.8**
A549 (Quintiles) Adriamycin and Indomethacin		
Adr. 50ng/ml	48.2	3.6
Indo (1)	93.6	2.6
Indo (1) + Adr	**28.9**	**3.6**
Indo (2)	98.4	1.9
Indo (2) + Adr	**34.1**	**0.5**
Indo (3)	98.6	1.3
Indo (3) + Adr	**39.4**	**2.7**

Table 3.10.1: % Survival of DLKP, MCF-7 (Quintiles), A549 (Quintiles) and A549 (NCTCC) cells in the presence of various concentrations of adriamycin and indomethacin as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of indomethacin were: **(1)** 2.5 μg/ml; **(2)** 1.25 μg/ml; **(3)** 0.625 μg/ml.

Test Sample	% Cell Survival	S.D.
DLKP, Taxol and Indomethacin		
Taxol. 1.0ng/ml	59.0	6.2
Indo (1)	97.8	4.7
Indo (1) + Taxol	**50.7**	**3.9**
Indo (2)	100.7	1.1
Indo (2) + Taxol	**56.1**	**3.5**
Indo (3)	93.9	0.7
Indo (3) + Taxol	**57.5**	**4.4**
MCF-7 (Quintiles) Taxol and Indomethacin		
Taxol. 2.0ng/ml	33.6	7.9
Indo (1)	96.0	2.3
Indo (1) + Taxol	**27.7**	**8.5**
Indo (2)	102.3	1.5
Indo (2) + Taxol	**31.9**	**5.2**
Indo (3)	108.5	7.5
Indo (3) + Taxol	**30.2**	**1.9**
A549 (NCTCC) Taxol and Indomethacin		
Taxol. 1.2ng/ml	78.4	9.4
Indo (1)	92.6	3.5
Indo (1) + Taxol	**65.8**	**6.9**
Indo (2)	91.7	4.0
Indo (2) + Taxol	**67.7**	**8.0**
Indo (3)	99.2	3.3
Indo (3) + Taxol	**72.4**	**3.9**
A549 (Quintiles) Taxol and Indomethacin		
Taxol. 1.2ng/ml	50.0	4.7
Indo (1)	95.6	1.5
Indo (1) + Taxol	**42.7**	**0.6**
Indo (2)	99.8	2.2
Indo (2) + Taxol	**50.9**	**1.9**
Indo (3)	95.8	5.1
Indo (3) + Taxol	**46.7**	**3.2**

Table 3.10.2: % Survival of DLKP, MCF-7 (Quintiles), A549 (Quintiles) and A549 (NCTCC) cells in the presence of various concentrations of taxol and indomethacin as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of indomethacin were: **(1)** 2.5 μg/ml; **(2)** 1.25 μg/ml; **(3)** 0.625 μg/ml.

Test Sample	% Cell Survival	S.D.
DLKP, Taxotere and Indomethacin		
Taxotere. 1.0ng/ml	46.0	4.8
Indo (1)	96.8	0.9
Indo (1) + Taxotere	**38.0**	**2.9**
Indo (2)	98.2	1.7
Indo (2) + Taxotere	**39.4**	**1.6**
Indo (3)	96.7	5.5
Indo (3) + Taxotere	**41.4**	**5.1**
MCF-7 (Quintiles) Taxotere and Indomethacin		
Taxotere. 2.0ng/ml	26.5	5.9
Indo (1)	102.7	7.3
Indo (1) + Taxotere	**20.4**	**2.2**
Indo (2)	97.3	1.2
Indo (2) + Taxotere	**21.3**	**1.9**
Indo (3)	98.3	2.8
Indo (3) + Taxotere	**22.2**	**2.0**
A549 (NCTCC) Taxotere and Indomethacin		
Taxotere. 1.2ng/ml	42.1	6.5
Indo (1)	98.3	1.7
Indo (1) + Taxotere	**33.1**	**7.7**
Indo (2)	102.1	5.3
Indo (2) + Taxotere	**36.2**	**6.1**
Indo (3)	102.7	2.6
Indo (3) + Taxotere	**39.2**	**5.4**
A549 (Quintiles) Taxotere and Indomethacin		
Taxotere. 1.2ng/ml	42.0	5.9
Indo (1)	93.9	1.2
Indo (1) + Taxotere	**36.2**	**3.9**
Indo (2)	97.3	3.2
Indo (2) + Taxotere	**39.6**	**2.5**
Indo (3)	96.6	2.0
Indo (3) + Taxotere	**40.0**	**3.2**

Table 3.10.3: % Survival of DLKP, MCF-7 (Quintiles), A549 (Quintiles) and A549 (NCTCC) cells in the presence of various concentrations of taxotere and indomethacin as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of indomethacin were: **(1)** 2.5 μg/ml; **(2)** 1.25 μg/ml; **(3)** 0.625 μg/ml.

	Adr + Indo L.T	Taxol + Indo L.T	Taxotere + Indo L.T
DLKP	0.319	0.930	0.851
MCF7	0.507	1.213	0.856
A549	0.564	1.066	0.952
A549Q	0.607	1.166	1.464

Table 3.10.4: Combination index (CI) values for Adriamycin (Adr), taxol or taxotere in combination with indomethacin, obtained using the method of Chou and Talalay as described in section 2.16.

(CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.)

L.T: Long-term assay as described in section 2.7.3a

Test Sample	% Cell Survival	S.D.
DLKP, Taxol and Sulindac		
Taxol. 1.0ng/ml	51.6	3.1
Sul (1)	94.5	2.6
Sul (1) + Taxol	**18.8**	**5.4**
Sul (2)	98.7	0.9
Sul (2) + Taxol	**41.8**	**2.4**
Sul (3)	98.9	3.3
Sul (3) + Taxol	**48.2**	**2.4**
MCF-7 (Quintiles) Taxol and Sulindac		
Taxol. 2.0ng/ml	43.7	5.1
Sul (1)	95.6	4.4
Sul (1) + Taxol	**26.5**	**6.6**
Sul (2)	101.7	1.1
Sul (2) + Taxol	**35.7**	**8.0**
Sul (3)	100.8	3.7
Sul (3) + Taxol	**36.5**	**4.9**
A549 (NCTCC) Taxol and Sulindac		
Taxol. 1.2ng/ml	77.2	8.5
Sul (1)	90.1	2.7
Sul (1) + Taxol	**59.1**	**6.1**
Sul (2)	92.0	3.0
Sul (2) + Taxol	**68.3**	**3.5**
Sul (3)	95.8	5.4
Sul (3) + Taxol	**71.2**	**1.5**
A549 (Quintiles) Taxol and Sulindac		
Taxol. 1.2ng/ml	54.4	3.8
Sul (1)	94.2	2.2
Sul (1) + Taxol	**40.5**	**2.4**
Sul (2)	98.0	0.9
Sul (2) + Taxol	**41.6**	**1.7**
Sul (3)	102.2	5.9
Sul (3) + Taxol	**48.9**	**4.1**

Table 3.10.5: % Survival of DLKP, MCF-7 (Quintiles), A549 (Quintiles) and A549 (NCTCC) cells in the presence of various concentrations of taxol and sulindac as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **(1)** 8μg/ml; **(2)** 4μg/ml; **(3)** 2μg/ml.

Test Sample	% Cell Survival	S.D.
DLKP, Taxotere and Sulindac		
Taxotere. 0.5ng/ml	46.3	2.3
Sul (1)	97.7	2.6
Sul (1) + Taxotere	**11.5**	**0.4**
Sul (2)	99.7	2.2
Sul (2) + Taxotere	**21.3**	**4.3**
Sul (3)	100.4	2.4
Sul (3) + Taxotere	**24.4**	**7.8**
MCF-7 (Quintiles) Taxotere and Sulindac		
Taxotere. 1.5ng/ml	34.2	5.0
Sul (1)	98.7	1.6
Sul (1) + Taxotere	**24.7**	**4.0**
Sul (2)	94.9	2.4
Sul (2) + Taxotere	**26.1**	**4.8**
Sul (3)	96.5	6.1
Sul (3) + Taxotere	**27.2**	**5.0**
A549 (NCTCC) Taxotere and Sulindac		
Taxotere. 1.2ng/ml	28.9	7.7
Sul (1)	94.6	2.7
Sul (1) + Taxotere	**23.7**	**6.1**
Sul (2)	98.3	1.1
Sul (2) + Taxotere	**25.9**	**7.1**
Sul (3)	98.4	3.2
Sul (3) + Taxotere	**28.6**	**7.9**
A549 (Quintiles) Taxotere and Sulindac		
Taxotere 1.2ng/ml	33.6	7.3
Sul (1)	97.5	1.3
Sul (1) + Taxotere	**27.4**	**6.1**
Sul (2)	98.8	1.0
Sul (2) + Taxotere	**31.1**	**6.3**
Sul (3)	98.8	3.6
Sul (3) + Taxotere	**31.5**	**7.6**

Table 3.10.6: % Survival of DLKP, MCF-7 (Quintiles), A549 (Quintiles) and A549 (NCTCC) cells in the presence of various concentrations of taxotere and sulindac as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments. The concentrations of sulindac were: **(1)** 8μg/ml; **(2)** 4μg/ml; **(3)** 2μg/ml.

	Taxol + Sulindac L.T	Taxotere + Sulindac L.T
DLKP	0.323	0.261
MCF7	0.686	0.915
A549	1.062	1.338
A549Q	1.094	1.122

Table 3.10.7: Combination index (CI) values for Taxol or Taxotere in combination with Sulindac, obtained using the method of Chou and Talalay as described in section 2.16

L.T: Long-term assay as described in section 2.7.3a

3.11 Pulse selection of DLKP cells with indomethacin.

Results obtained from the IOV assays (section 3.3), drug efflux experiments (section 3.5) and combination toxicity assays carried out in DLKP, CORL23 and 2008 cell lines (section 3.1, 3.6, and 3.8 respectively) strongly indicated that indomethacin is a good substrate for MRP. Pulse selection of DLKP cells with indomethacin for a period of 6 weeks was carried out (i.e. 4 hour pulse with indomethacin once each week after which the cells were re-fed in fresh media) to assess the effect of this NSAID on the resistance profile of the DLKP cells. The DLKP cells were grown up in 25 cm^2 flasks until approximately 80% confluent and then pulsed with 300μg/ml indomethacin. This concentration of indomethacin was found to kill approx. 80-90% of the DLKP cells in the $25cm^2$ flasks.

3.11.1 IC_{50} values

The IC_{50} values for a range of chemotherapeutic drugs and indomethacin were obtained after pulsing the DLKP cells for four weeks (DLKP Indo4) and six weeks (DLKP Indo6) (Table 3.11.1).

	DLKP	DLKP Indo4	DLKP Indo6
Adriamycin (ng/ml)	12.7 ± 0.38	16.1 ± 0.35	19.0 ± 0.14
Vincristine (ng/ml)	1.41 ± 0.23	1.80 ± 0.28	2.05 ± 0.28
VP-16 (ng/ml)	96.7 ± 16.0	160.5 ± 12.02	190.0 ± 7.1
5-FU (ng/ml)	483.3 ± 30.6	1410 ± 147.3	1860 ± 410.1
Cisplatin (ng/ml)	310.0 ± 20.1	500.0 ± 28.3	610.7 ± 15.2
Indomethacin (μg/ml)	15.4 ± 1.24	31.4 ± 3.9	34.1 ± 4.7

Table 3.11.1: IC_{50} values for adriamycin, vincristine, VP-16, 5-FU, cisplatin and indomethacin on parental DLKP cells and DLKP cells pulsed with 300μg/ml indomethacin for four weeks (DLKP Indo4) and six weeks (DLKP Indo6). Data are the average of results from three separate experiments.

The results indicate an increase in IC_{50} values for all drugs analysed. A particularly significant increase in the IC_{50} value was evident for the non-MRP substrate, 5-FU, which was increased more than 4-fold after pulsing for 6 weeks. Resistance to cisplatin, a non-MRP1 substrate was also increased two-fold in the pulsed cell lines as compared to the parental DLKP cells. Approximately, a 2-fold increase in resistance was evident with the MRP substrates adriamycin, vincristine, VP16 and indomethacin after pulsing the DLKP cells with indomethacin for 6 weeks.

3.11.2 RT-PCR analysis of DKLP and DLKP pulsed cells.

RT-PCR analysis of DLKP, DLKP Indo4 and DLKP Indo6 was carried out to assess if pulse selecting DLKP cells for a number of weeks with indomethacin resulted in a change in the expression of the multidrug resistance associated proteins expressed in DLKP cells. The results indicated an up-regulation of MRP1 mRNA in the both DLKP Indo4 and DLKP Indo6 cells. The highest expression of MRP1 was observed in the DLKP Indo-6 cells (Figure 3.16.1a). There also appeared to be slight up-regulation of MRP2 in both the pulsed cell lines as compared to the parental DLKP cells (Figure 3.16.2a). Again the highest expression of MRP2 was in the cells pulsed for six weeks with indomethacin. There was no detectable expression of MRP3 mRNA in the parental DLKP and pulsed DLKP cells indicating that the increased drug resistance in the DLKP cells, pulsed with indomethacin, was not due to expression of MRP3 (Figure 3.16.3). MRP4 RT-PCR analysis of the DLKP cell lines, indicated that there is an increased expression of MRP4 mRNA in the DLKP Indo4. This expression was further increased in the DLKP Indo6 cells (3.16.4a). These results suggest that pulsing the DLKP cells with indomethacin resulted in an up-regulation of MRP4 (Figure 3.16.4) and MRP6 mRNA (Figure 3.16.6). The results for RT-PCR analysis of the DLKP cell lines indicated that there was a very slight down-regulation of MRP5 in both the DLKP Indo4 and DLKP Indo6 cells as compared to the parental DLKP cells (Figure 3.16.5a).. MDR1 RT-PCR analysis of the DLKP cells indicate that there was almost undetectable levels of MDR1 in the parental DLKP cells. There was no increase in MDR1 expression after pulsing these cells with indomethacin for 6 weeks (Figure 3.16.7). These results indicate that pulsing

DLKP cells with indomethacin for 4-6 weeks resulted in an up-regulation of MRP1, 2, 4 and 6 mRNA expression and a down-regulation of MRP5 mRNA expression. MRP3 and MDR1 do not appear to be associated with the drug resistance mechanisms in the DLKP and DLKP pulsed cells.

3.11.2 Combination toxicity assays in DLKP and DLKP pulsed cells

Combination toxicity assays were then carried out on the DLKP and DLKP Indo4 and DLKP Indo6 cell lines, using indomethacin in combination with adriamycin, to assess if an increase in the resistance to indomethacin in the pulsed cells resulted in a loss of the combination effect already shown to be present in DLKP cells (section 3.1). The results indicate that although there was an increase in resistance to both indomethacin and adriamycin, both the pulsed cell lines demonstrated a combination effect equal to that achieved in the parental cell line (Table 3.11.2).

DLKP, Adriamycin and Indomethacin		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	46.6	5.1
Indo (2.5μg/ml)	96.0	2.4
Indo + Adr	**12.9**	**1.1**
Indo (1.25μg/ml)	95.7	2.4
Indo + Adr	**28.1**	**3.9**
Indo (0.625μg/ml)	99.3	0.4
Indo + Adr	**40.1**	**1.1**
DLKP Indo4, Adriamycin and Indomethacin		
Adr. 20ng/ml	44.8	1.8
Indo (2.5μg/ml)	99.9	1.2
Indo + Adr	**11.6**	**4.3**
Indo (1.25μg/ml)	99.9	1.2
Indo + Adr	**16.6**	**4.7**
Indo (0.625μg/ml)	99.3	1.1
Indo + Adr	**27.5**	**0.5**
DLKP Indo6, Adriamycin and Indomethacin		
Adr. 20ng/ml	47.3	0.3
Indo (2.5μg/ml)	99.3	3.4
Indo + Adr	**10.0**	**4.5**
Indo (1.25μg/ml)	101.6	3.2
Indo + Adr	**16.9**	**2.6**
Indo (0.625μg/ml)	98.9	2.3
Indo + Adr	**27.8**	**3.1**

Table 3.11.2: % Survival of DLKP (parental), DLKP Indo4 and DLKP Indo6 cells in the presence of various concentrations of adriamycin and Indomethacin as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

3.12 Effect of pulsing HepG2 cells with Cisplatin

HepG2 cells were pulse selected with cisplatin for four weeks i.e. 4 hour pulse once each week after which the cells were re-fed in fresh media. The HepG2 cells were grown up in 25 cm^2 flasks until approximately 80% confluent and then pulsed with 4000ng/mL cisplatin. This concentration of cisplatin was found to kill approx. 80-90% of the HepG2 cells in the 25cm^2 flasks.

3.12.1. Toxicity profile of cytotoxic drugs in HepG2 cell line

Toxicity assays were carried out on the HepG2 cell line, prior to pulsing and after pulsing for 10 weeks with cisplatin, to assess the toxicity profile of the chemotherapeutic drugs on this cell line and to assess if there was a difference in these toxicity profiles after pulsing. The IC_{50} of these drugs (the concentration of the drugs at which 50 % cell kill is obtained) in HepG2 parental and pulsed cells were calculated after pulsing for 4 weeks and 10 weeks (Table 3.12.1).

Drug	HepG2 (parental)	HepG2 4P	HepG2 10P
Adriamycin	20.1 ± 4.7 ng/ml	13.0 ± 4.2 ng/ml	8.5 ± 3.5 ng/ml
Vincristine	4.2 ± 1.1 ng/ml	3.5 ± 0.9 ng/ml	1.5 ± 0.6 ng/ml
5'fluorouracil	164.5 ± 36.3 ng/ml	380 ± 32.1 ng/ml	400 ± 35.1 ng/ml
VP-16	250.0 ± 25.1ng/ml	189 ± 15.1ng/ml	100 ± 7.5ng/ml
Cisplatin	215.0 ± 35.4 ng/ml	2065 ± 194 ng/ml	2518 ± 202 ng/ml

Table 3.12.1: IC_{50} of Cytotoxic drugs in the HepG2 cell line pulsed with cisplatin. Results are the average of triplicate determinations in three separate experiments.

The results indicate that pulsing the HepG2 cells with 4000ng/ml cisplatin for 4 weeks, resulted in a decrease in the resistance to adriamycin, vincristine and VP-16. These anti-cancer drugs are good substrates for MRP1, 2 and 3 substrates (Duffy *et al.,* 1998; Cole *et al.,* 1994 and Hipfner *et al.,* 1999, Borst *et al.,* 1999 and Konig *et al.,* 1999). However, the results indicate that the

resistance to both 5-FU and cisplatin increased after pulsing HepG2 cells with 4000ng/ml cisplatin for four weeks. This resistance was further increased after pulsing these cells for a period of ten weeks. A number of authors including Duffy *et al.*, (1998) and Nishiyama *et al.*, (1999), reported that MRP did not seem to play an important role in 5-FU resistance. The mechanism of cisplatin resistance is uncertain and reports linking cisplatin resistance to MRP are inconclusive and conflicting. (See section 1.6.8).

3.12.2 RT-PCR analysis of HepG2 parental cells.

RT-PCR analysis was carried out on the HepG2 cell line to assess if MRP was expressed at the RNA level and also to assess which form of MRP was expressed in the cell line. RT-PCR analysis was carried out on the HepG2 cells, using primers for MRP1, cMOAT, MRP3 and MRP4, prior to pulsing. The results show that HepG2 cells express mRNA for MRP1 (3.12.1), MRP2 (cMOAT) (3.12.2), MRP3 (3.12.2) and MRP4 (3.12.3). Narasaki *et al.*, (1997), have demonstrated the presence of cMOAT and MRP1 but as of yet the presence of MRP3 and MRP4 in HepG2 cells had not previously been reported. RT-PCR was not carried out using primers for MRP5 and 6 but these primers will be included in future RT-PCR experiments on HepG2 parental and pulsed cells so that complete MRP expression in these cells can be assessed.

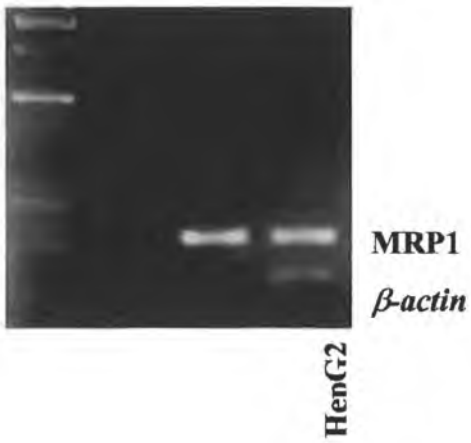


Figure 3.12.1: RT-PCR analysis of MRP1 expression in HepG2 cells carried out according to the method described in section 2.15.

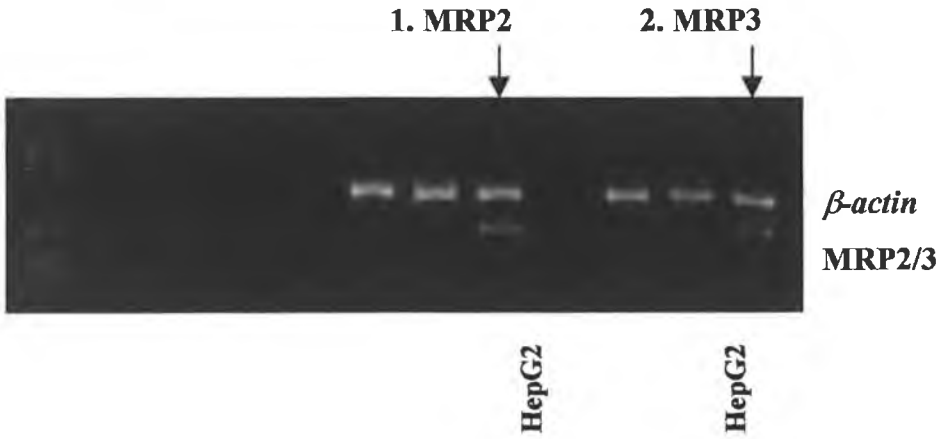


Figure 3.12.2: RT-PCR analysis of MRP2 and MRP3 expression in HepG2 cells carried out according to the method described in section 2.15.

Figure 3.12.3: RT-PCR analysis of MRP4 expression in HepG2 cells carried out according to the method described in section 2.15.

3.13 Investigation of the mechanism of resistance in DLKP C14 cells

3.13.1 Toxicity profile of cytotoxic drugs in DLKPC 14 cell line

The parental cell line, DLKP, was exposed to varying concentrations of carboplatin to develop a resistant variant called DLKPC 14 (Cleary, 1995). The parental DLKP cells were initially exposed to 1μg/mL carboplatin and adjusted to growth in 6.2 μg/mL and after approximately four months, to 14μg/mL. The resistant cell line, selected to and maintained in 14μg/mL carboplatin were designated DLKPC 14.

Toxicity assays were carried out on the parental DLKP and the DLKPC 14 cell lines to assess the toxicity profile of the chemotherapeutic drugs on this cell line. The IC_{50} of these drugs (the concentration of the drugs at which 50 % cell kill is obtained) in DLKPC 14 are as follows:

Cytotoxic drug	IC_{50} concentrations DLKP	IC_{50} concentrations DLKPC 14
Adriamycin	14.13 ± 2.7 ng/ml	15.1 ± 2.5 ng/ml
Vincristine	2.25 ± 0.2 ng/ml	3.2 ± 0.22ng/ml
5-FU	590 ± 52.3 ng/ml	720 ± 61.3 ng/ml
VP-16	112.5 ± 17.6ng/ml	210 ± 14.1ng/ml
Cisplatin	480.0 ± 35.4 ng/ml	3010.0 ± 156.4 ng/ml

Table 3.13.1: IC_{50} of Cytotoxic drugs in the DLKPC 14 cell line.
Results are the average of triplicate determinations in three separate experiments.

Immunocytochemical studies on the expression of the 190kDa protein, MRP, revealed no alteration in the level of MRP in the DLKPC 14 cell line when compared to the parental DLKP cell line (Cleary, 1995). These results suggest that MRP was not involved in cisplatin resistance in these cell lines. Further

evidence to support this theory was obtained from RT-PCR studies, where no significant difference was observed in MRP1 mRNA levels (Lorraine O'Driscoll, personal communication). Cleary (1995), also reported no detectable levels of Pgp in the parental cells of the resistant variants.

3.13.2 Combination toxicity assays in DLKPC 14 cells

Combination toxicity assays were carried out on the DLKPC 14 cell line using a combination of adriamycin and indomethacin, or cisplatin and indomethacin. Previous experiments demonstrated that the cytotoxic drug was rendered more toxic in a number of cancer cell lines including DLKP (section 3.1), CORL23 (section 3.6), HepG2 (section 3.7) and 2008 cells (section 3.8) when combined with indomethacin by a process involving inhibition of the activity of MRP1. The aims of carrying out the combination toxicity assays on the DLKPC 14 cell line were to assess if indomethacin (and analogues) were capable of potentiating the toxicity of the cytotoxic drugs, cisplatin and adriamycin, in a carboplatin and cisplatin resistant cell line.

The results of the combination toxicity assay are reported in Tables 3.13.2 and 3.13.3. The results demonstrated that indomethacin was capable of potentiating the toxicity of adriamycin in DLKPC 14 and DLKP cells. The highest non-toxic concentration of indomethacin used in both the DLKPC 14 and DLKP cells was 2.5μg/ml. However, results from the combination toxicity assays with cisplatin and indomethacin in DLKPC 14 and DLKP cells revealed that indomethacin was not capable of potentiating the toxicity of cisplatin in these cells. The toxicity of cisplatin seemed to decrease when combined with indomethacin in the DLKPC 14 and DLKP cell lines. This interesting effect was also evident in the HepG2 cells (section 3.7) treated with a combination of cisplatin and indomethacin and the in 2008 cell lines (section 3.8) treated with a combination of cisplatin with either indomethacin and sulindac. Combining an active NSAID, indomethacin or sulindac, with cisplatin, appears to render the cells more resistant to cisplatin.

DLKP, Adriamycin and Indomethacin

Test Sample	% Survival	S.D.
Adr. 10ng/ml	46.5	1.7
Indo. (2.5μg/ml)	96.9	2.7
Indo. + Adr	**11.5**	**6.5**
Indo. (1.25μg/ml)	102.7	1.5
Indo. + Adr	**14.1**	**0.9**
Indo. (0.625μg/ml)	102.5	1.4
Indo. + Adr	**19.6**	**4.35**

DLKP C14, Adriamycin and indomethacin

Test Sample	% Survival	S.D.
Adr. 10ng/ml	43.2	7.5
Indo. (2.5μg/ml)	89.1	4.4
Indo. + Adr	**6.5**	**3.6**
Indo. (1.25μg/ml)	90.4	2.5
Indo. + Adr	**8.4**	**1.5**
Indo. (0.625)	103.0	3.2
Indo. + Adr	**10.6**	**6.2**

Table 3.13.2: % Survival of DLKP and DLKP C14 cells in the presence of various concentrations of adriamycin and indomethacin as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate± S.D. The untreated cell survival % is taken as 100%.

Results are the average of triplicate determinations in three separate experiments. The concentrations of the compounds were: **(1)** 2.5 μg/ml; **(2)** 1.25 μg/ml **(3)** 0.625 μg/ml

DLKP, Cisplatin and Indomethacin

Test Sample	% Survival	S.D.
Cis. 250ng/ml	33.6	2.9
Indo. (2.5μg/ml)	95.5	1.3
Indo. + Cis	**44.1**	**4.2**
Indo. (1.25μg/ml)	100.7	0.9
Indo. + Cis	**38.1**	**2.4**
Indo. (0.625μg/ml)	104.5	5.6
Indo. + Cis	**35.9**	**3.5**

DLKP C14, Cisplatin and Indomethacin

Test Sample	% Survival	S.D.
Cis. 3000ng/ml	19.1	2.9
Indo. (2.5μg/ml)	96.1	2.5
Indo. + Cis	**20.5**	**2.2**
Indo. (1.25μg/ml)	98.6	6.8
Indo. + Cis	**19.4**	**2.6**
Indo. (0.625μg/ml)	99.4	0.9
Indo. + Cis	**19.2**	**5.1**

Table 3.13.3: % Survival of DLKP and DLKP C14 cells in the presence of various concentrations of cisplatin and indomethacin as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

3.14 Analysis of Adriamycin Metabolite

One of the problems associated with using adriamycin as a chemotherapeutic drug in human cancers is the cardiotoxicity associated with long-term adriamycin use *in vivo*. This toxicity is primarily associated with the primary metabolite of adriamycin, adriamycinol. Adriamycinol was analysed in toxicity assays and combination toxicity assays to assess if this metabolite of adriamycin was as toxic as adriamycin in DLKP cells and if the toxicity of this drug was also enhanced by indomethacin. adriamycinol was found to have an IC_{50} value of 40.3 ± 2.5ng/ml in DLKP cells as compared to an IC_{50} value of 12.7 ± 0.38 for adriamycin in these cells.

Combination toxicity assays were carried out using both adriamycin and the adriamycinol in combination with indomethacin to compare the potentiation of toxicity of both these drugs when combined with indomethacin. The results indicate that there is a poorer combination effect in the cells treated with indomethacin and adriamycin metabolite. The toxicity of the metabolite is potentiated by indomethacin but not to the same extent as adriamycin (Table 3.14.1). Combination toxicity assays were also carried out on A549 cells treated with epirubicin in combination with sulindac. Epirubicin has been reported by Minotti *et al.,* (1999), and Stewart *et al.,* (1993), to be an analogue of adriamycin which causes less cardiotoxicity. These combination toxicity assays were carried out to assess if sulindac was capable of enhancing the toxicity of epirubicin as effectively as it enhances the toxicity of adriamycin. The results indicate that this is the case (3.14.2). There was greater potentiation of epirubicin by sulindac in the A549 cell line than was observed when adriamycin was combined with sulindac. It is also interesting to note that the concentration of epirubicin used in the A549 cells was 4-fold less than the concentration of adriamycin. These results indicate that epirubicin is more effective than adriamycin in this cell line and greater potentiation of this chemotherapeutic drug can be achieved when combined with sulindac.

DLKP, Adriamycin and Indomethacin		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	45.2	4.9
Indo (2.5μg/ml)	95.5	2.2
Indo + Adr	**10.3**	**5.2**
Indo (1.25μg/ml)	97.3	2.4
Indo + Adr	**28.1**	**3.6**
Indo (0.625μg/ml)	98.0	2.6
Indo + Adr	**37.2**	**5.7**
DLKP, Adriamycinol and Indomethacin		
Adr. 40ng/ml	53.5	1.5
Indo (2.5μg/ml)	96.9	3.3
Indo + Adrol	**33.4**	**2.9**
Indo (1.25μg/ml)	99.9	3.6
Indo + Adrol	**44.0**	**3.5**
Indo (0.625)	100.8	1.4
Indo + Adrol	**50.4**	**1.0**

Table 3.14.1: % Survival of DLKP (parental) cells in the presence of various concentrations of adriamycin /adriamycinol and indomethacin. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

A549, Adriamycin and Sulindac		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 50ng/ml	52.3	4.7
Sul (8.0μg/ml)	96.0	5.4
Sul + Adr	**25.1**	**3.6**
Sul (4.0μg/ml)	100.1	2.4
Sul + Adr	**31.5**	**6.2**
Sul (2.0μg/ml)	101.0	0.9
Sul + Adr	**42.5**	**3.3**
A549, Epirubicin and Sulindac		
Epi. 12ng/ml	72.7	2.1
Sul (8.0μg/ml)	97.5	3.3
Sul + Epi	**26.0**	**1.4**
Sul (4.0μg/ml)	99.9	1.0
Sul + Epi	**40.9**	**5.0**
Sul (2.0μg/ml)	98.7	1.7
Sul + Epi	**57.1**	**1.9**

Table 3.14.2: % Survival of A549 cells in the presence of various concentrations of adriamycin/epirubicin and sulindac as found using the protocol detailed in section 2.7.3. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in two separate experiments.

3.15 Investigation of other drugs as possible MRP1 substrates

Studies were carried out on a number of TB/AIDS related drugs to determine if there was a correlation between acquisition of chemotherapeutic resistance mechanisms and resistance to TB and /or AIDS drugs.

Hollo *et al.* (1996), reported that the antimalarial compound quinine is a substrate of MRP. Rifampicin, an anti-TB agent, had previously been reported to down-modulate Pgp mediated drug efflux (Fardel *et al.,* 1995 and Furusara *et al.,* 1997). It is now reported that rifampicin can increase the accumulation of two MRP substrates, vincristine and calcein, in GLC4/ADR, an adriamycin resistant human lung cancer cell line (Courtois *et al.,* 1999). There is no evidence to date that AZT (Zidovudine), an anti-AIDs drug or tetracycline, an antibiotic, are substrates for MRP but it is possible that one of the mechanisms by which cells develop resistance to these compounds is through the activity of a cellular pump with actions similar to MRP.

Combination toxicity assays were carried out in DLKP cells using indomethacin and varying concentrations of the compounds, AZT, rifampicin, quinine and tetracycline. These assays were carried out to assess if these compounds, were substrates for MRP and to determine if indomethacin could potentiate the toxicity of compounds other than the chemotherapeutic drugs already known to be substrates of MRP1. The compounds were analysed in the DLKP cells at non-toxic concentrations.

Of the four compounds analysed, both rifampicin and quinine were found to potentiate the toxicity of adriamycin in the DLKP cells (CI values of 0.833 and 0.769 respectively). AZT and tetracycline had no effect on the toxicity of adriamycin in the DLKP cells (Figures 3.15.1 and 3.15.2). The results indicate that quinine and rifampicin may be substrates for MRP and are effective in reducing the efflux of adriamycin from the cells

DLKP, Adriamycin and AZT, Rifampicin, Quinine and Tetracycline		
Test Sample	**% Cell Survival**	**S.D.**
Adr. 10ng/ml	56.9	3.1
AZT (50μg/ml)	96.2	1.9
AZT + Adr	**52.6**	**2.2**
AZT (25μg/ml)	97.9	1.6
AZT + Adr	**54.2**	**2.7**
AZT (12.5μg/ml)	102.5	1.6
AZT + Adr	**57.3**	**1.9**
Adr. 10ng/ml	59.8	7.7
Rif (15μg/ml)	94.8	0.6
Rif + Adr	**44.1**	**3.9**
Rif (7.5μg/ml)	98.3	1.7
Rif + Adr	**50.6**	**5.9**
Rif (3.75μg/ml)	100.4	1.3
Rif + Adr	**54.1**	**4.7**
Adr. 10ng/ml	61.4	9.4
Quin (15μg/ml)	95.0	1.2
Quin + Adr	**42.2**	**6.1**
Quin (7.5μg/ml)	96.7	2.9
Quin + Adr	**54.4**	**4.3**
Quin (3.75μg/ml)	99.4	0.6
Quin + Adr	**61.1**	**7.0**
Adr. 10ng/ml	59.3	5.7
Tetra (5μg/ml)	90.3	1.5
Tetra + Adr	**56.4**	**4.1**
Tetra (2.5μg/ml)	100.5	1.3
Tetra + Adr	**55.6**	**3.7**
Tetra (1.25μg/ml)	100.2	3.6
Tetra + Adr	**56.9**	**5.2**

Table 3.15.1: % Survival of DLKP cells in the presence of various concentrations of adriamycin and AZT, Rifampicin (Rif), Quinine (Quin) and Tetracycline (Tetra) as found using the protocol detailed in section 2.7.3a. Survival is represented as a % of the growth of untreated cells in the same plate ± S.D. Results are the average of triplicate determinations in three separate experiments.

	No Anticancer agent	Adr.* (10ng/ml)	CI values
No compound	100 ± 0.0	59.3 ± 5.7	
AZT 50μg/ml	96.2 ± 1.9	52.6 ± 2.2	1.578
Rifampicin 15μg/ml	94.8 ± 0.6	44.1 ± 3.9	0.833
Quinine 15μg/ml	95.0. ± 1.2	42.2 ± 6.1	0.769
Tetracycline 5μg/ml	90.3 ± 1.5	56.4 ± 4.1	1.701

Table: 3.15.2: DLKP and Adriamycin + highest non-toxic concentrations of AZT, Rifampicin, Quinine and Tetracycline.

*Data are expressed as % Cell Survival ± standard deviation for a minimum of three assay repeats.

CI : Combination index.

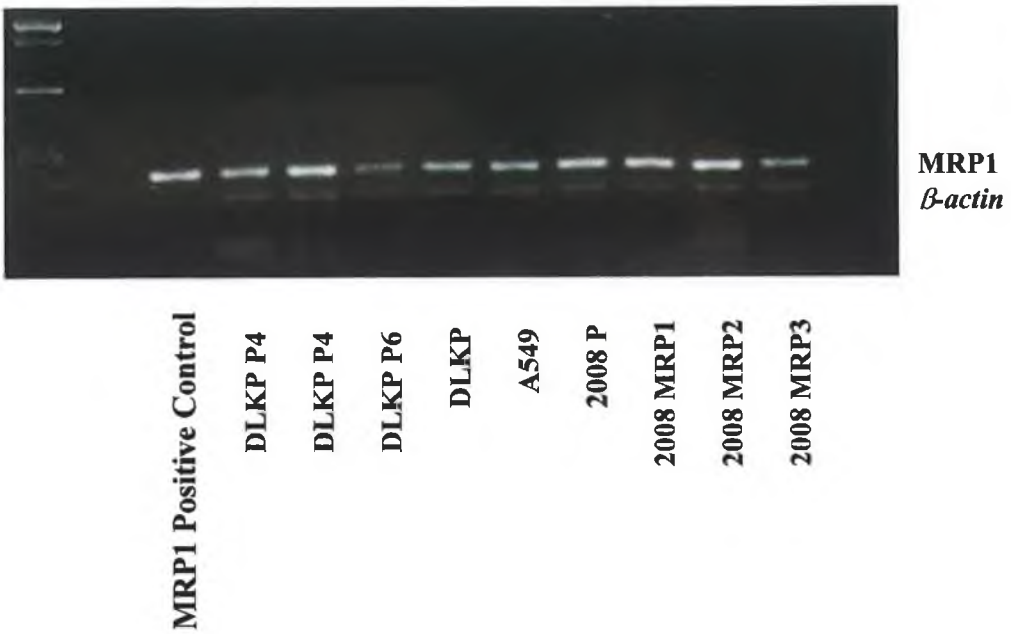
(CI < 1: synergism; CI > 1: antagonism; CI = 1: additive.)

3.16 RT-PCR analysis of MRP1, MRP2, MRP3, MRP4, MRP5, MRP6 and MDR1 mRNA expression in cancer cell lines.

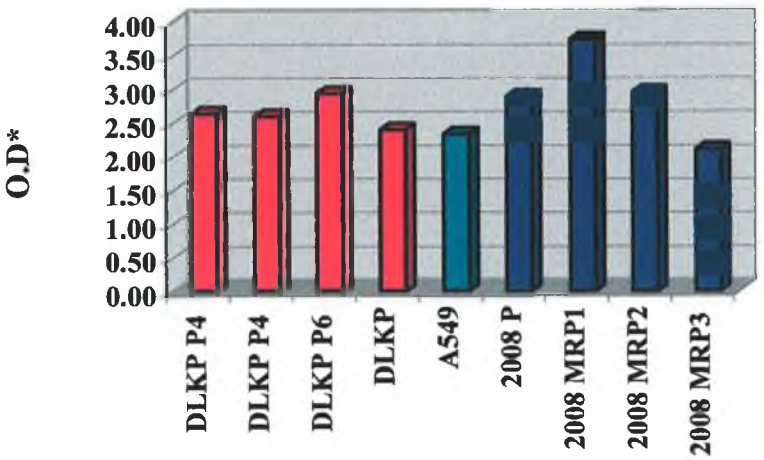
RT-PCR analysis was carried out on RNA extracted from DLKP, DLKP Indo4, DLKP Indo6, A549, 2008 P, 2008 MRP1, 2008 MRP2, 2008 MRP3, HL60 (S) and HL60/ADR cells to determine the expression of MRP1-6 and MDR1 mRNA these cell lines. The RT-PCR procedure was carried out according to section 2.15. The primers used in the detection if the MRP and MDR genes are described in Appendix A.

3.16 RT-PCR analysis of MRP1, MRP2, MRP3, MRP4, MRP5, MRP6 and MDR1 in cancer cell lines.

Figure 3.16.1a RT-PCR analysis of MRP1 expression



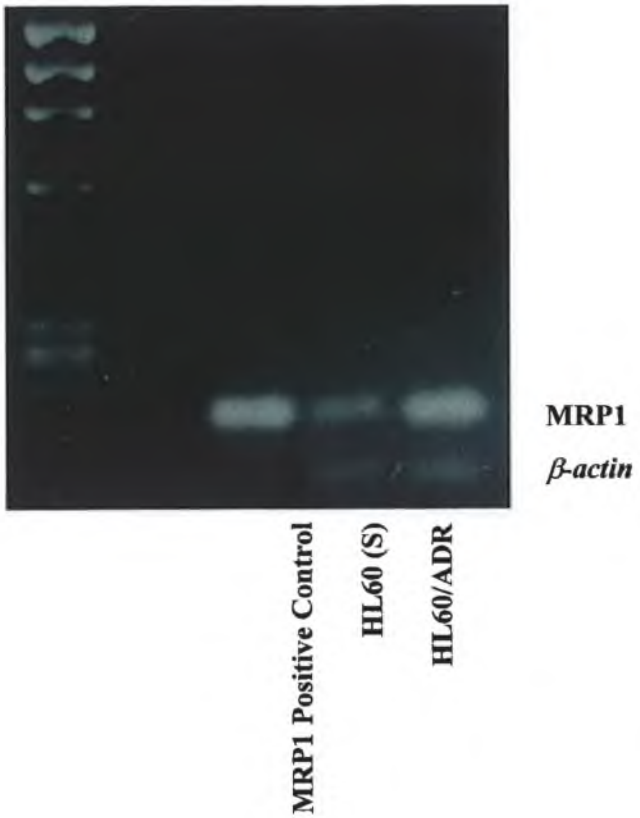
MRP1 - desitometric analysis

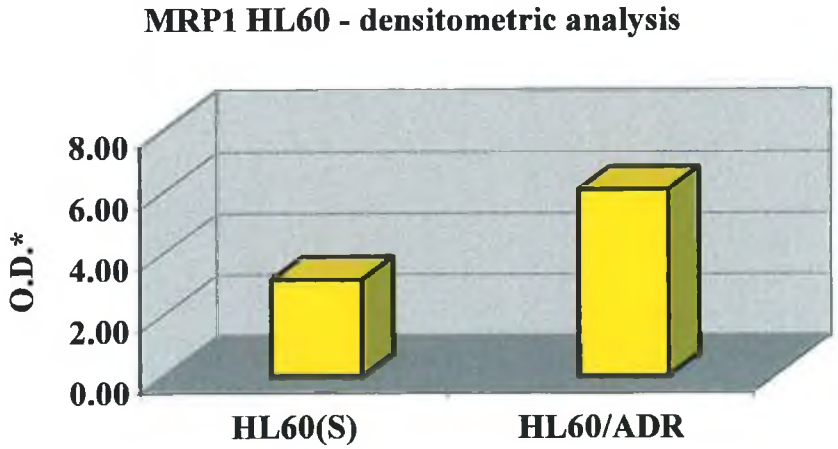


(**Note:** DLKP P4 = DLKP Indo4; DLKPP6 = DLKP Indo6)

* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

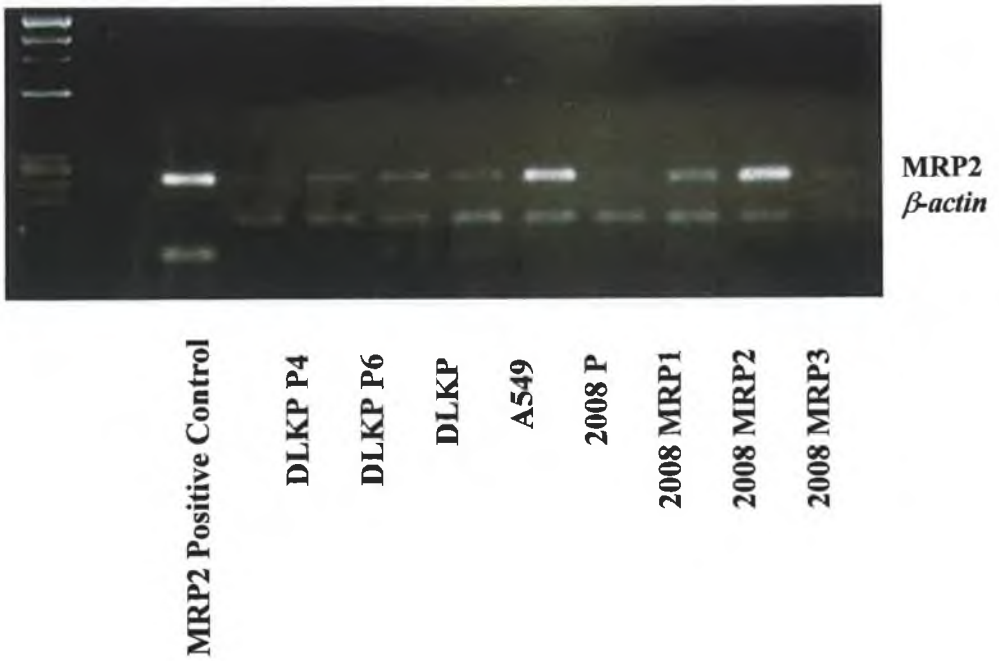
Figure 3.16.1b RT-PCR analysis of MRP1 expression in HL60(S) and HL60/ADR cell lines



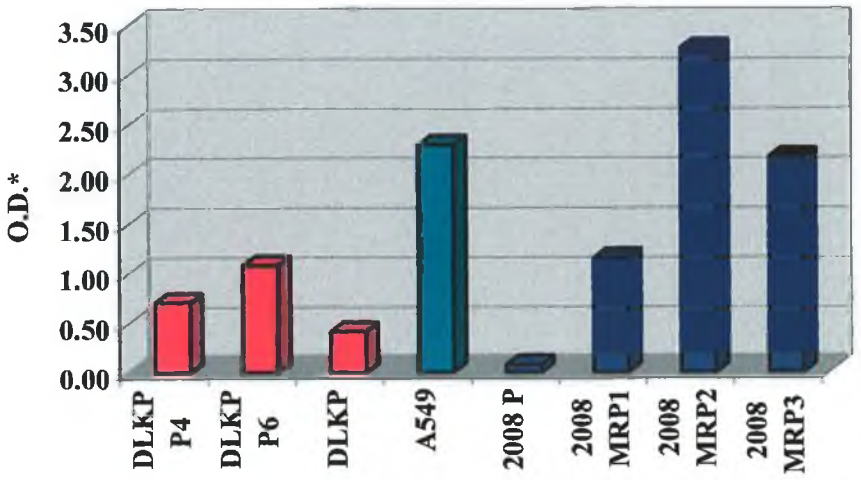


* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

Figure 3.16.2a RT-PCR analysis of MRP2 expression



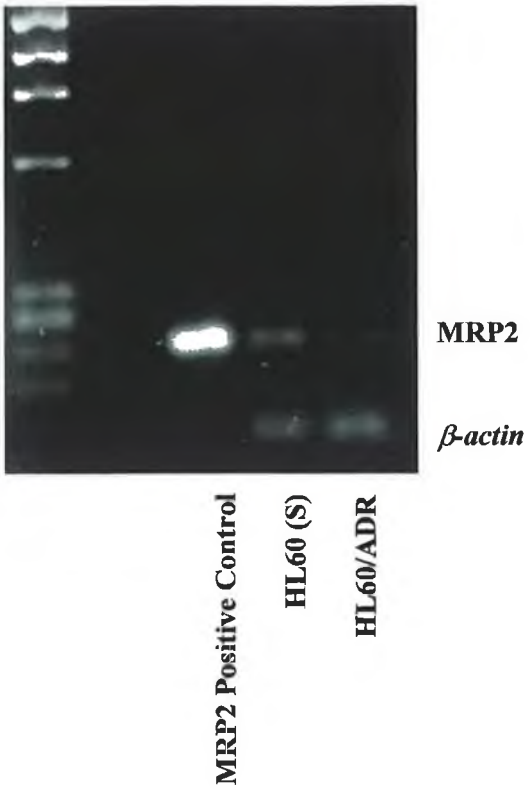
MRP2 - densitometric analysis

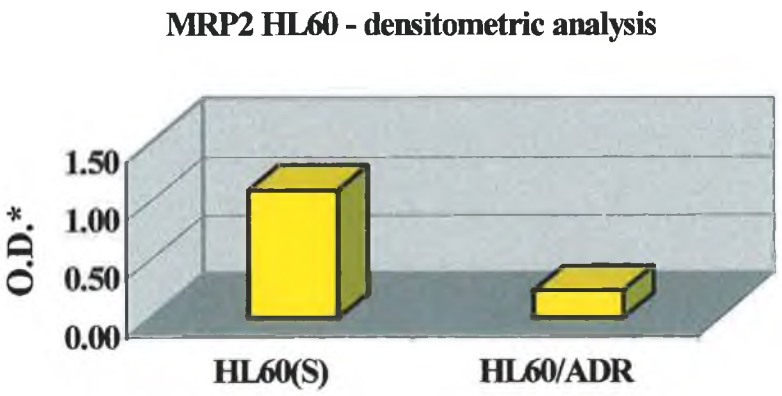


(**Note:**DLKP P4 = DLKP Indo4; DLKPP6 = DLKP Indo6)

* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

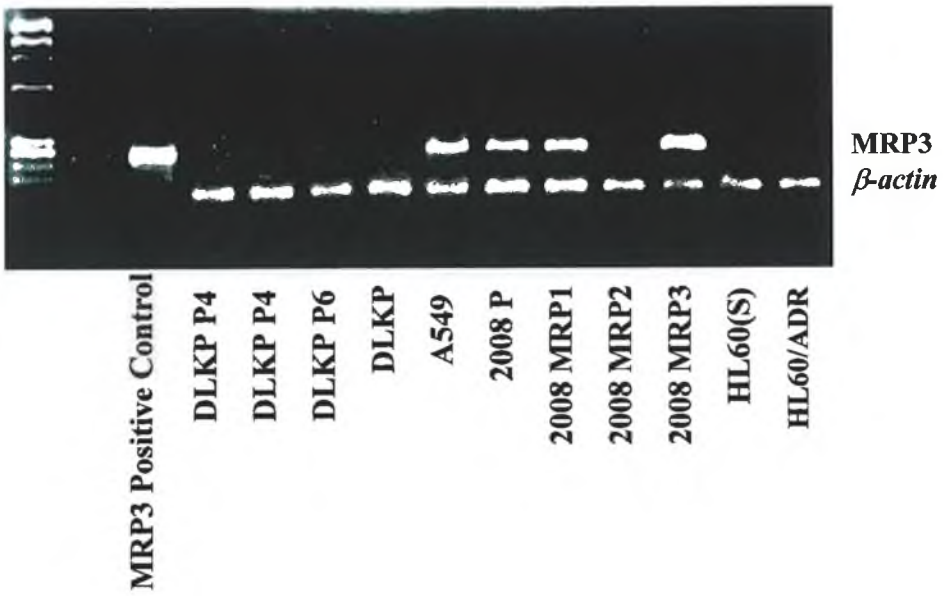
Figure 3.16.2b RT-PCR analysis of MRP2 expression in HL60(S) and HL60/ADR



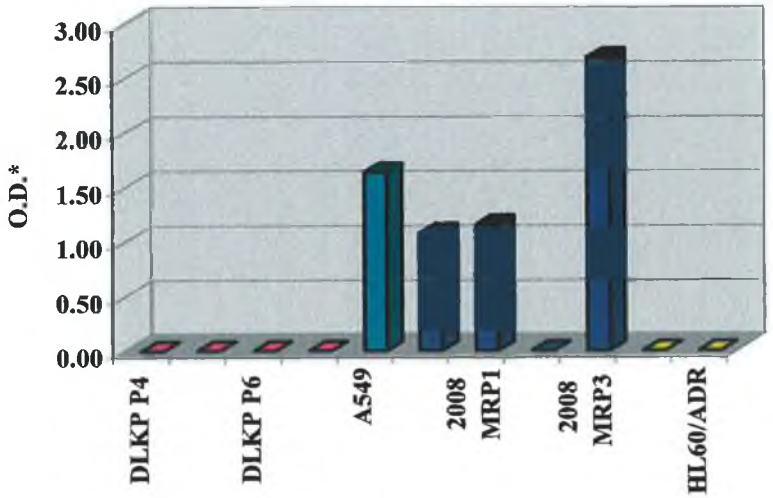


* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

Figure 3.16.3 RT-PCR analysis of MRP3 expression



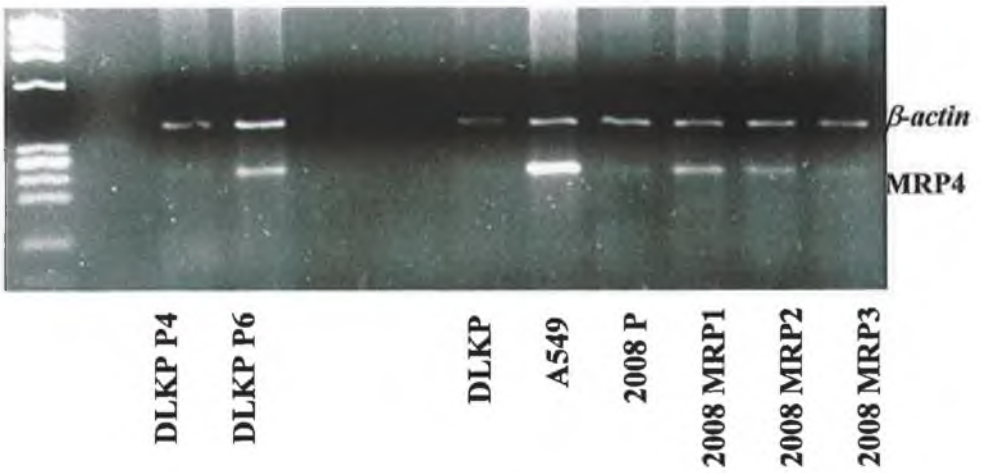
MRP3 - densitometric analysis

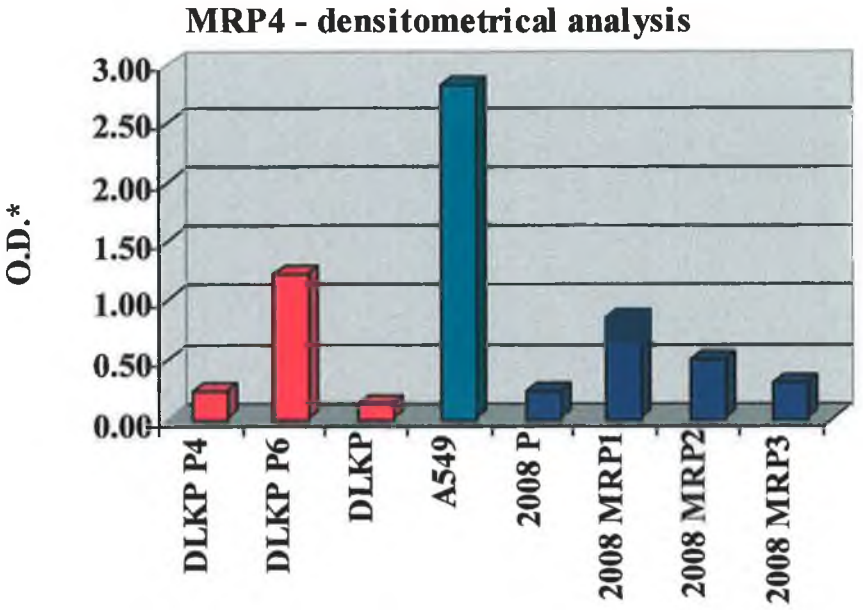


(**Note:** DLKP P4 = DLKP Indo4; DLKPP6 = DLKP Indo6)

* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

Figure 3.16.4a RT-PCR analysis of MRP4 expression

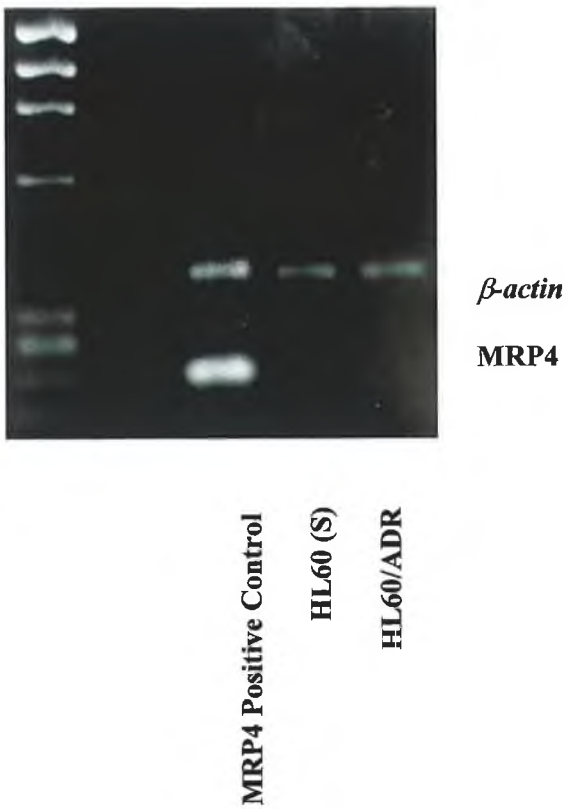




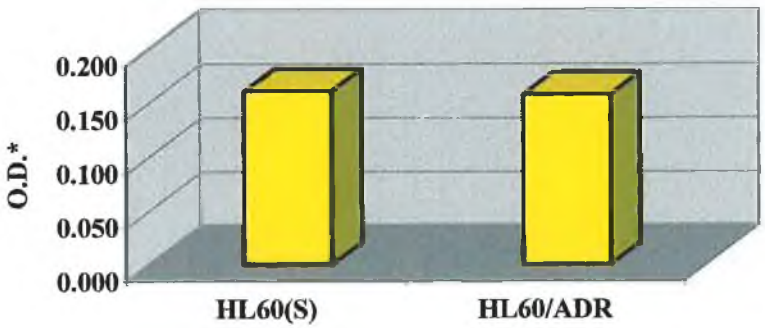
(**Note:** DLKP P4 = DLKP Indo4; DLKPP6 = DLKP Indo6)

* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

Figure 3.16.4b RT-PCR analysis of MRP4 expression in HL60(S) and HL60/ADR

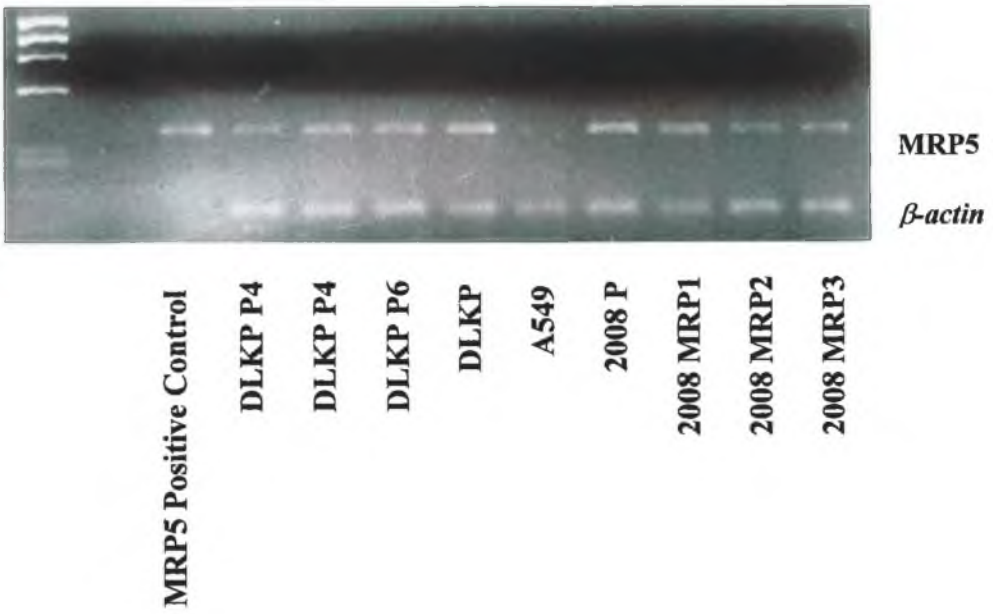


MRP4 HL60 - densitometric analysis

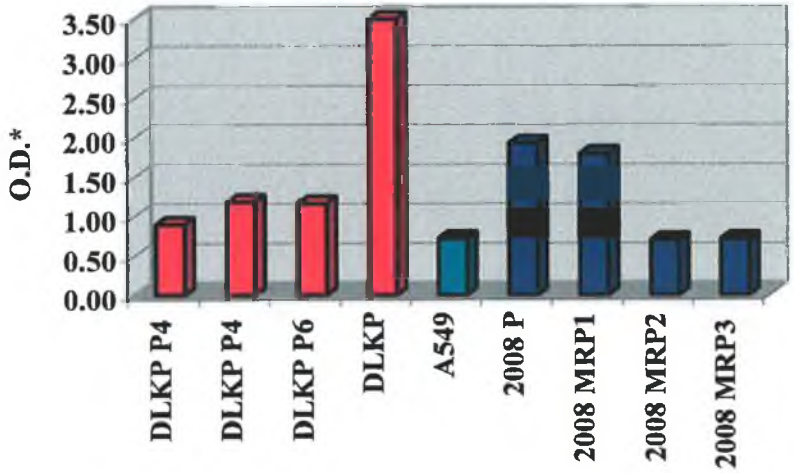


* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

Figure 3.16.5a RT-PCR analysis of MRP5 expression



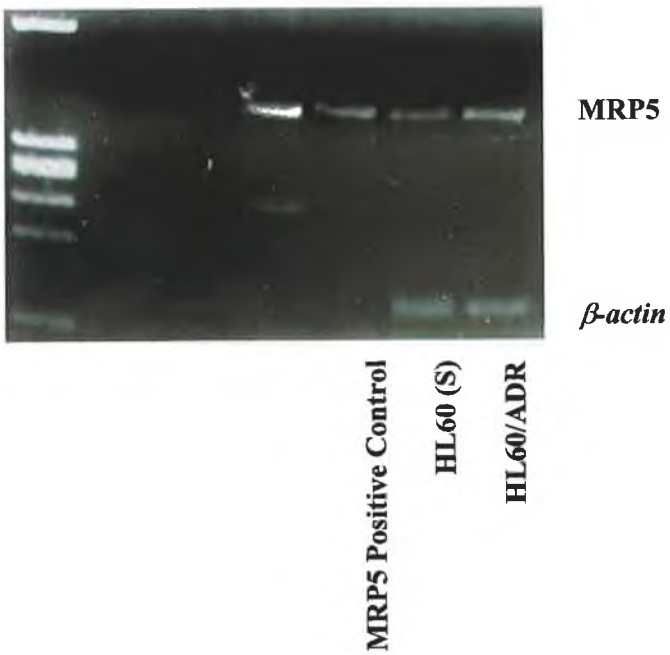
MRP5 - densitometric analysis

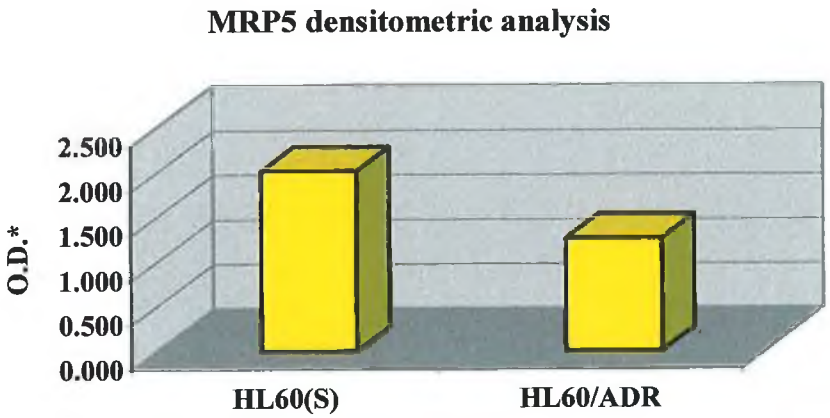


(**Note:** DLKP P4 = DLKP Indo4; DLKPP6 = DLKP Indo6)

* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

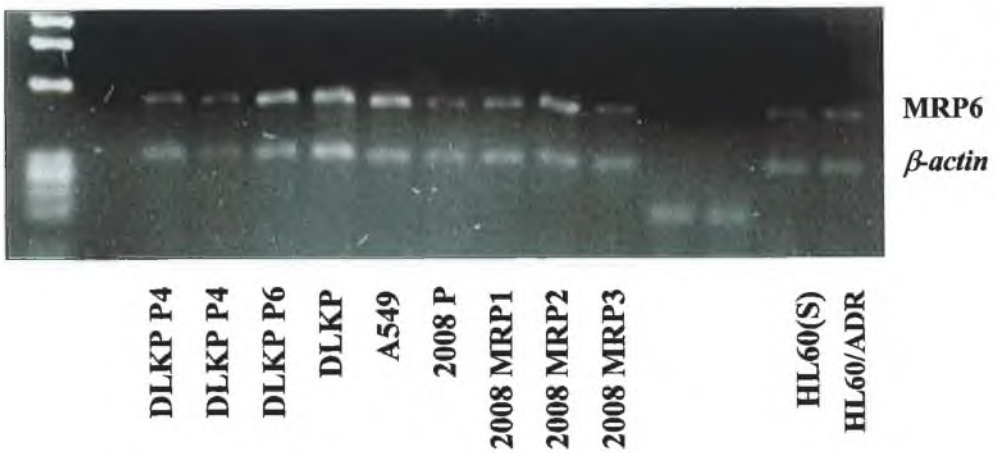
Figure 3.16.5b RT-PCR analysis of MRP5 expression in HL60(S) and HL60/ADR



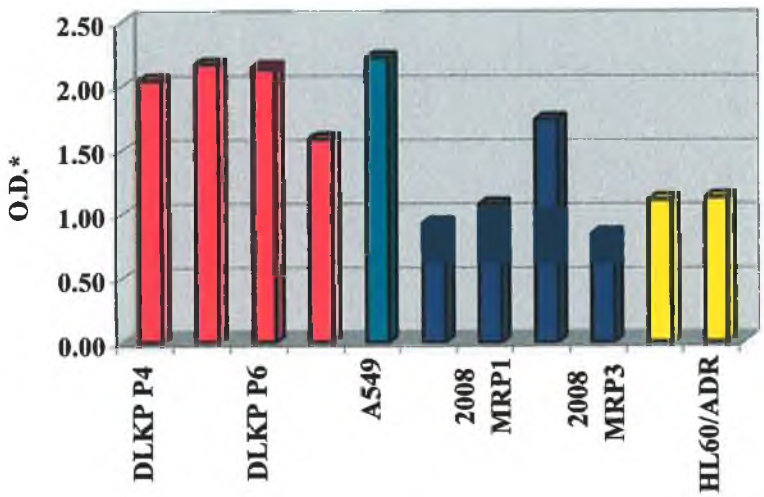


* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

Figure 3.16.6 RT-PCR analysis of MRP6 expression



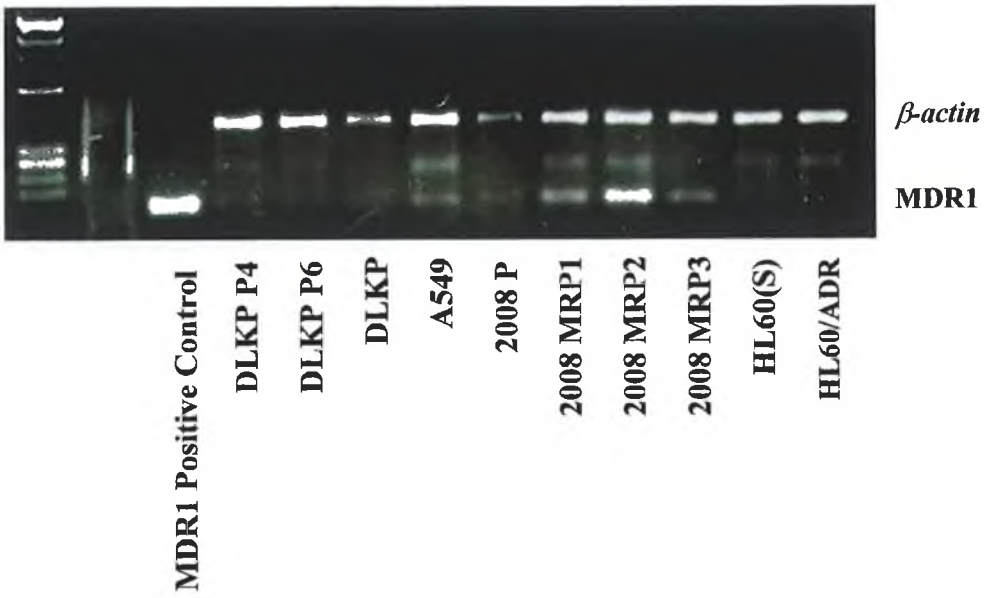
MRP6 - densitometric analysis

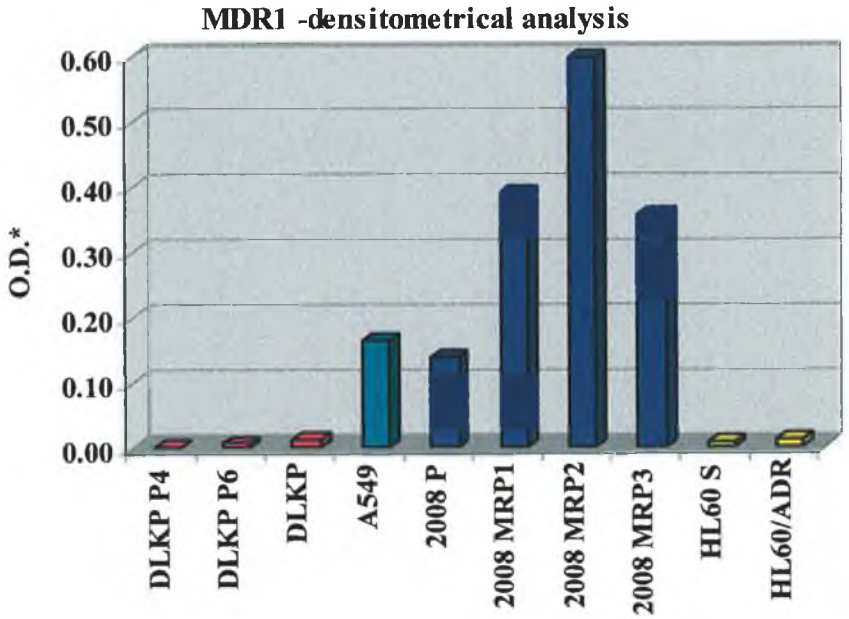


(**Note:** DLKP P4 = DLKP Indo4; DLKPP6 = DLKP Indo6)

* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

Figure 3.16.7 RT-PCR analysis of MDR1 expression





(**Note:** DLKP P4 = DLKP Indo4; DLKPP6 = DLKP Indo6)

* O.D. is presented as the ratio of the levels of each specific gene product to the internal β-actin.

4.0 Discussion

4.1 General discussion

Overexpression of the 170kDa drug efflux pump, P-glycoprotein, has been observed in many multidrug resistant cell lines and some human tumours (Gottesman *et al.,* 1993; Arceci, 1993; Aszalos *et al.,* 1998). However, it has been clear for some time that alternate mechanisms of multidrug resistance must exist both *in vivo* and *in vitro.* A number of multidrug resistant cell lines have been described that do not overexpress P-glycoprotein but display drug resistance profiles very similar to those that do (Mirski *et al.,* 1987; McGrath *et al.,* 1987; Hill *et al.,* 1993).

Until the early 1990s, non-Pgp-mediated multidrug resistance was generally attributed to a variety of mechanisms, including enhanced expression of drug detoxification enzymes and alterations in topoisomerase II (Cole *et al.,* 1990; 1992a and 1992b). In addition, elevated levels of a number of uncharacteristic proteins had been reported in some non-Pgp multidrug-resistant cell lines. In 1992, Cole *et al.,* cloned MRP and hence permitted the identification of one of these uncharacteristic proteins as a member of the ATP-binding cassette transporter superfamily as described in section 1.6. Grant *et al.,* (1994), subsequently demonstrated that MRP-overexpression was sufficient to confer multidrug resistance to previously sensitive cells. Since the cloning of MRP cDNA from the multidrug resistant H60Ar cell line, overexpression of MRP has been reported in many other previously described non-P-glycoprotein multidrug resistant cell lines (Cole *et al.,* 1992; Krishnamachary *et al.,* 1993; Zaman *et al.,* 1993; Barrand *et al.,* 1994; Kruh *et al.,* 1994). These observations suggest that MRP may be a relatively common mediator of resistance, at least *in vitro.*

As described in section 1.6, other homologues of MRP have now been identified, MRP2 (cMOAT), MRP3, MRP4, MRP5 and MRP6 (Kool *et al.,* 1997 and Borst *et al.,* 1999). The mechanisms of these transport proteins have yet to be fully elucidated but it is thought that these proteins may also have some role in drug transport and chemo-resistance.

During the last decade, there has been much research in the field of multidrug resistance circumvention. Fischer and Sikic (1995), carried out clinical trials using a range of Pgp modulators. The result of these clinical trials were

relatively disappointing as it had been found that currently available Pgp modulators lack the potency to completely reverse the MDR phenotype at clinically achievable concentrations without significant side-effects or toxicity. A number of MRP-modulating agents have been described in section 1.6.9. Although some of these compounds seem promising *in vitro* it is not known if the MRP-modulating agents, listed in section 1.6.9, could be used to modulate MRP-mediated resistance *in vivo*. It is possible that these compounds could cause serious toxic side effects when added at concentrations required for modulation of MRP.

The NSAID, indomethacin, is a well-known inhibitor of prostaglandin synthesis (Vane *et al.,* 1996) and has also been shown to be a potent inhibitor of glutathione-S-transferase (Primiano *et al.,* 1993). It has previously been shown to enhance the anti-cancer activity of chlorambucil (Hall *et al.,* 1989), methotrexate (Henderson *et al.,* 1994), vincristine (Draper *et al.,* 1997 and Kobayashi *et al.,* 1997), VP-16, methotrexate (Maca, 1991), and adriamycin (Kobayashi *et al.,* 1997).

The concentrations at which indomethacin was effective in enhancing the cytotoxicity of the cancer cells was also reported by Kobayashi *et al.,* (1997), to be clinically safe (approx. 2μg/ml).

Duffy *et al.*, (1998), examined the effect on cytotoxicity of combining a range of clinically important non-steroidal anti-inflammatory drugs (NSAIDs) with a variety of chemotherapeutic drugs in the human lung cancer cell lines DLKP, A549, CORL23R and in the human leukemia cell line HL60/ADR. They found that a specific group of NSAIDs (indomethacin, sulindac, tolmetin, acemetacin, zomepirac and mefenamic acid) all at non-toxic levels, significantly increased the cytotoxicity of the anthracyclines (doxorubicin, daunorubicin and epirubicin), as well as tenopside, VP-16 and vincristine, but not the other vinca alkaloids, vinblastine and vinorelbine. Other anti-cancer drugs, including methotrexate, 5-fluorouracil, cytarabine, hydroxyurea, chlorambucil, cyclo-phosphamide, cisplatin, carboplatin, mitoxantrone, actinomycin D, bleomycin, paclitaxel and camptothecin, were also tested but displayed no synergy in combination with the NSAIDs. The enhancement of cytotoxicity was observed in a range of tumour cell lines which over-expressed MRP but did not occur in multidrug resistant cell lines which overexpress P-170 (Pgp). As it appeared that the NSAIDs exerted their positive effect only in combination with MRP

substrates and in MRP positive cell lines, it seemed logical to postulate that the NSAIDs were affecting the activity of MRP and reducing the expulsion of the chemotherapeutic drugs from the cell. This resulted in a reduction in the resistance of the cell to the chemotherapeutic drug.

The mechanism behind indomethacin's ability to chemosensitise MRP-overexpressing cells remains uncertain. It is unlikely that the enhancement effect is due to the inhibition of prostaglandin synthesis as explained in section 1.8. Therefore, it is most probable that indomethacin exerts its effect on the chemotherapeutic drugs through interacting with the functioning of MRP. Zaman *et al.,* (1995), reported that MRP is an extremely effective transporter of glutathione conjugates. Drug transport in MRP- but not Pgp- overexpressing MDR tumour cell lines can be regulated by intracellular GSH levels (Versantvoort *et al.,* 1995). Duffy *et al.,* (1998), discovered that glutathione-S-transferase activity was inhibited by the NSAIDs found to have the ability to potentiate the toxicity of chemotherapeutic drugs, but not the inactive NSAIDs. Our group also found that the efflux of adriamycin from cells treated with indomethacin was significantly retarded relative to untreated cells or cells treated with the inactive NSAIDs. Therefore, it would appear that the positive NSAIDs might function by directly inhibiting the MRP pump and as a result of this binding, inhibit efflux of the drugs. Alternatively, the NSAIDs may act as a competitive substrate for MRP resulting in the NSAIDs being preferentially pumped out of the cells, therefore, allowing the cytotoxic drugs to remain in the cell for a longer period exerting their chemotherapeutic effect.

This thesis describes an investigation of the modulation of MRP by indomethacin and a number of indomethacin analogues. These experiments were carried out to:

i. Determine the structure activity relationship of MRP inhibition by indomethacin analogues
ii. Discover an analogue of MRP with greater MRP modulating activity than indomethacin at less toxic concentrations with less side effects;
iii. Determine the mechanism by which indomethacin and similar compounds potentiate the toxicity of a range of chemotherapeutic drugs.

4.2 Investigation of Structure Activity Relationship (SAR) of indomethacin-mediated toxicity enhancement

As a result of the results reported by Duffy *et al.*, (1998), a study was undertaken by our group, in collaboration with the chemistry department in University College, Cork, to develop a series of compounds based on the basic structure of indomethacin. These indomethacin analogues were then analysed in a number of assays to determine the Structure Activity Relationship (SAR) of the compounds. The aim of such experiments was to find the structure with optimum ability to enhance the cytotoxicity of the chemotherapeutic drugs and to determine the function of the various substituents on the indomethacin structure.

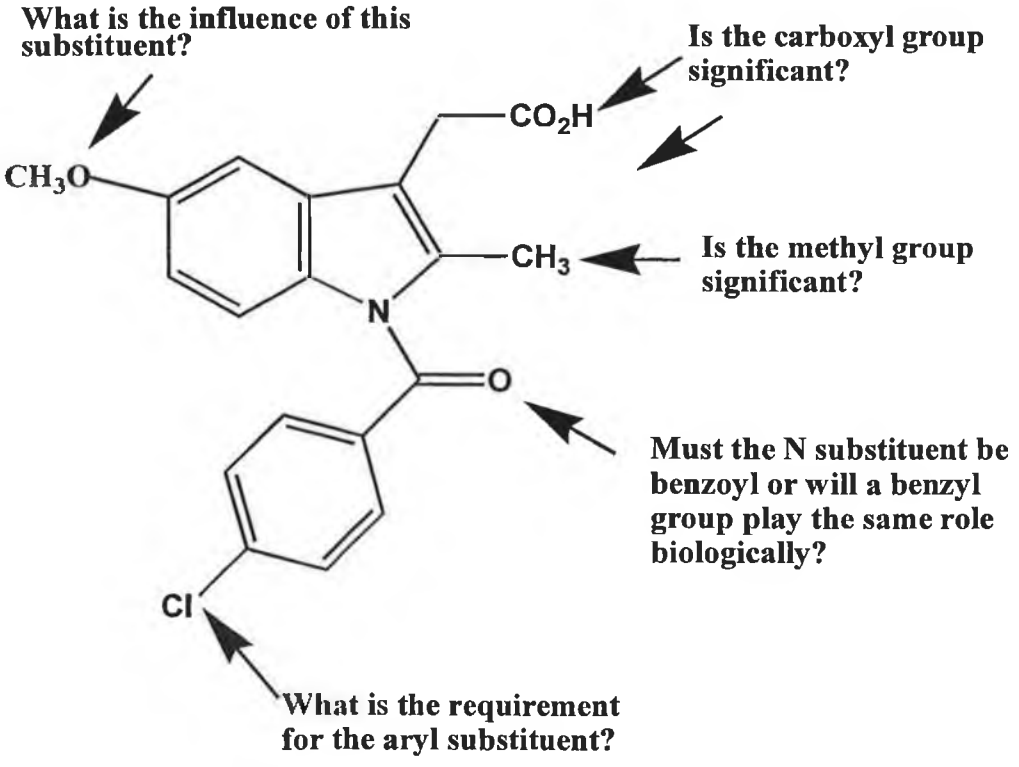


Figure 4.1: Indomethacin structure indicating which substituents were varied to generate the indomethacin analogues.

23 of these indomethacin analogues were generated (section 3.1). 19 of these compounds were directly related to indomethacin with substituent variation to determine the SAR of the compounds. The remaining four compounds were based on the structure of a known PLA_2 inhibitor with a basic structure similar to that of indomethacin.

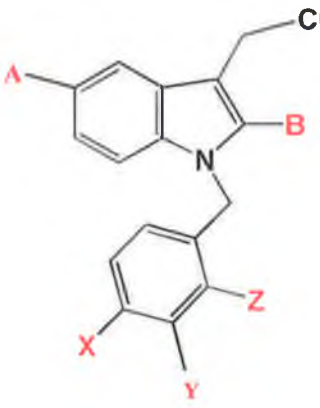
4.2.1 Investigation of ability of indomethacin analogues to enhance the toxicity of adriamycin in DLKP cells *in vitro*.

The compounds were initially assayed in combination toxicity assays in DLKP cells to assess their ability to enhance the cytotoxicity of the anthracycline, adriamycin, as compared to indomethacin. The highest non-toxic concentration of indomethacin, which exerted its positive effect, was 2.5μg/ml. The concentrations of adriamycin used in the combination toxicity assays were 10ng/ml and 5ng/ml. The IC_{50} of adriamycin in DLKP cells is approximately 10ng/ml. The procedure for the combination toxicity assay was carried out as per section 2.7.3a. The structures of the compounds assayed are described in section 3.1.

The results obtained from the combination toxicity assays indicated that:

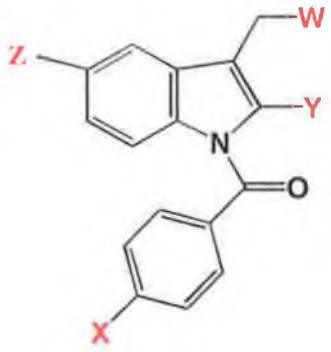
- The activity of the compounds was concentration dependent.
- A number of the substituents on the indomethacin structure were critical in the functioning of the compounds.

Figure 4.1a: Analogues of N-benzyl-indomethacin



BRI 60/1:	A=MeO;	B=CH_3;	X=Cl;	Y=H;	Z=H
BRI 114/2:	A=MeO;	B=CH_3;	X=Br;	Y=H;	Z=H
BRI 115/2:	A=MeO;	B=CH_3;	X=F;	Y=H;	Z=H
BRI 113/1:	A=MeO;	B=CH_3;	X=H;	Y=H;	Z=H
BRI 124/1:	A=MeO;	B=CH_3;	X=MeS;	Y=H;	Z=H
BRI 106/1:	A=MeO;	B=CH_3;	X=H;	Y=Cl;	Z=H
BRI 107/1:	A=MeO;	B=CH_3;	X=H;	Y=H;	Z=Cl
BRI 138/1:	A=MeO;	B=CH_3;	X=H;	Y=F;	Z=H
BRI 119/1:	A=H;	B=CH_3;	X=Cl;	Y=H;	Z=H
BRI 120/1:	A=MeO;	B=H;	X=Cl;	Y=H;	Z=H

Figure 4.1b: Analogues of N-benzoyl-indomethacin



Indo:	W=CO_2H;	X=Cl;	Y=CH_3;	Z=CH_3O
BRI 59/1:	W=COOMe;	X=Cl;	Y=CH_3;	Z=CH_3O
BRI 69/2:	W=CONHTol;	X=Cl:	Y=CH_3;	Z=CH_3O
BRI 88/1:	W=CO_2H;	X=H;	Y=CH_3;	Z=CH_3O
BRI 92/1:	W=CO_2H;	X=Br;	Y=CH_3;	Z=CH_3O
BRI 104/2:	W=CO_2H;	X=Cl;	Y=CH_3;	Z=H

The first significant results from the combination toxicity assay demonstrated that the benzoyl substituent on the indole nitrogen atom was not required for the potentiation ability of indomethacin. This N-benzyl-indomethacin analogue (BRI 60/1) was found to be active in the combination toxicity assay and moreover, its activity was found to be comparable to that of indomethacin. Removing the benzoyl substituent also rendered this indomethacin analogue less toxic (2-fold) than N-benzoyl-indomethacin to the DLKP cells.

Subsequent assessment of a number of benzyl derivatives of indomethacin, with various substituent variations, demonstrated the importance of a number of these substituents for the enhancement of toxicity of adriamycin by indomethacin. Changing the position of the chlorine from the *para*-position on the benzyl ring to the *meta*-position (BRI 106/1) or the *ortho*-position (BRI 107/1) rendered N-benzyl-indomethacin incapable of potentiating the toxicity of adriamycin. Removing the halogen from the benzene ring (BRI 113/1) also

resulted in loss of ability to enhance the toxicity of adriamycin. These results indicated that in the benzyl series, not only was the presence of a halogen necessary for the potentiating activity of N-benzyl indomethacin, but the *para*-position of the halogen was also critical for a positive effect in the combination toxicity assays. Further investigations indicated that the chlorine substituent in the *para*-position on the benzene ring of N-benzyl-indomethacin could be replaced with either bromine (BRI 114/2) or fluorine (BRI 115/2) without loss of activity in the combination toxicity assays. Replacing chlorine with fluorine very slightly reduced the potentiating activity of indomethacin. Replacing chlorine with bromine did not result in any notable decrease in activity and when analysed in the combination toxicity assays, this analogue of indomethacin was found to compare very favorably to indomethacin. In addition, replacing chlorine with bromine or fluorine resulted in a 2-fold decrease in toxicity to DLKP cells as compared to N-benzyl indomethacin and a 4-fold decrease in toxicity as compared to N-benzoyl indomethacin.

An additional benzyl-derivative was generated in which the fluorine, attached to the benzene ring in N-benzyl-indomethacin, was moved from the *para*- to the *meta*-position. Moving the chlorine from the *para*- to the *meta* and *ortho*-positions on the benzene ring in N-benzyl-indomethacin had already been shown to result in a loss of activity of these indomethacin analogues in the combination toxicity assay. However, moving the fluorine to the *meta*-position on the benzene ring resulted in the generation of an analogue of indomethacin that still demonstrated an ability to potentiate adriamycin in the combination toxicity assays (BRI 138/1). Although this analogue required concentrations 3-fold higher than N-benzyl-indomethacin to obtain a comparable potentiation effect, it was found to be 1.5-fold less toxic than the *para*-form of this indomethacin analogue and 3-fold less toxic than N-benzyl-indomethacin to the DLKP cells. The effect of moving fluorine to the *ortho*-position was not examined. In addition, the effect of moving bromine to either the *meta*- or the *ortho*-position was not investigated. The development and subsequent analysis of additional indomethacin analogues are required to fully elucidate the roles of these halogens on the N-benzyl-indomethacin structure.

Further analysis of N-benzyl-indomethacin analogues demonstrated that replacing chlorine on the benzene ring with a non-halogen substituent, a methylthio substituent (BRI 124/1), rendered this N-benzyl-indomethacin

analogue inactive in the combination toxicity assays. This result indicated that a halogen was the desired substituent at this position on the benzyl-indomethacin structure.

Variations of the halogen group on the benzene ring in the N-benzoyl-indomethacin series were also investigated with the development of two benzoyl derivatives where the chlorine was completely removed from the benzene ring (BRI 88/1) or was replaced with bromine in the *para*-position (BRI 92/1). Results from the combination toxicity assay showed these two compounds to be positive with comparable activity to indomethacin. However, both of these compounds were 2-fold less toxic to the DLKP cells than N-benzoyl-indomethacin. This result indicates that the presence of the halogen in the N-benzoyl-indomethacin series is not critical and when removed does not render the compound inactive in the combination toxicity assay. However, no compounds were developed in which the halogen was placed in the *ortho*- and *meta*- position in the N-benzoyl derivatives of indomethacin so one cannot affirm for certain that the positioning of the halogen is not critical.

In subsequent analysis of N-benzyl derivatives of indomethacin, two compounds were developed in which the methoxy (BRI 119/1) or the methyl substituents (BRI 120/1) were removed. Both analogues were inactive in the combination toxicity assay indicating that both these substituents were required for the benzyl compounds to be active. Another interesting result was obtained with the development of an analogue of N-benzoyl indomethacin in which the methoxy substituent was removed (BRI 104/2). Removal of this substituent was not tolerated in the N-benzyl series, but in the N-benzoyl derivative the removal of this substituent did not render the compound inactive. This analogue was comparable in activity to indomethacin in the combination toxicity assay when used at similar concentrations and was also found to be 2-fold less toxic than N-benzoyl-indomethacin in the DLKP cells.

The acetic acid side chain was manipulated in two of the N-benzoyl-indomethacin analogues in which this substituent was converted to a methyl ester or a tolyl amide. This structural change rendered both these compounds inactive in the combination toxicity assay. These compounds were relatively insoluble in DMSO and media and this was most likely due to the changes made to the acetic acid side chain as the rest of the indomethacin structure remained unchanged.

Therefore, results obtained from analysis of the N-benzyl derivatives of indomethacin indicate that the nature and the position of the functional groups are critical when the benzoyl substituent is removed from the indomethacin structure. It appears that the presence of the benzoyl group on the indole nitrogen atom confers greater structure rigidity to N-benzoyl-indomethacin. There is a large barrier to free rotation associated with groups joined by a double bond (Solomon, 1996). Therefore, upon removal of this benzoyl group, the rigidity of the structure of indomethacin is affected which may affect the binding of the structure to MRP. As it is postulated that indomethacin potentiates the activity of adriamycin by binding to MRP and subsequently inhibiting the efflux of adriamycin (Duffy *et al.,* 1998), it is very likely that variations to the indomethacin structure can affect its binding to the MRP protein.

Seelig *et al.,* (2000), suggested that MRP1 binds its substrates via hydrogen bond formation and that the transport of anionic substrates by MRP1 is facilitated by cationic amino acid residues present in the transmembrane helices of MRP1. The authors demonstrated that the compounds analysed in the study and found to interact with MRP1, carried units consisting of two electron donor groups with a spatial separation of 2.5 ± 0.3 Å (defined as type I units) or, less frequently, units consisting of three electron donor groups with the two outer groups exhibiting a spatial separation of 4.6 ± 0.6 Å (defined as type II units). They also reported that negatively charged electron donor units e.g. the carboxylic acid group, COO^-, are required for efficient transport by MRP1.

It appears that MRP transports amphiphilic anionic and some cationic substrates (section 1.6.6). The carboxyl (COOH) substituent is required on the indomethacin structure to render the indomethacin molecule more water-soluble. When the carboxyl group was substituted with either COOMe (BRI 59/1) or CONHTol (BRI 69/2), in two N-benzoyl-indomethacin analogues, the potentiation ability of the compound was lost. Therefore, it is possible that altering the carboxyl side chain in N-benzoyl-indomethacin rendered these analogues too lipid soluble making them poor substrates for MRP. Substituting the carboxyl group may also have had the effect of reducing the ability of the

analogues to bind to MRP due to the loss of this negatively charged electron donor group which appears to be necessary for substrate binding to MRP1.

It is postulated that when the benzoyl group is removed from the indomethacin structure, the chlorine substituent on the benzene ring functions to maintain the rigid structure of indomethacin, which may be most suited to binding with MRP. If the halogen is required in the *para*-position on the benzene ring to maintain the rigid structure of indomethacin, it is difficult to explain why moving the fluorine group to the *meta*-position did not render the compound inactive. Fluorine is the least dense of the halogens and due to its smaller size (atomic mass of fluorine = 18.99) (chlorine = 35.45, bromine = 79.9) it is possible that moving its position to the *meta*-position did not have a large effect on the structure of indomethacin. But moving the larger chlorine structure resulted in a greater alteration to the shape of the indomethacin structure. Fluorine is more electronegative (4.1) than chlorine (2.8) or bromine (2.7), and has a greater dipole moment (1.91μ (D)) than chlorine (1.08μ (D)) or bromine (0.80μ (D)) when bonded to hydrogen. These properties may result in stronger binding of the *meta*-fluoro-benzyl indomethacin structure to the MRP molecule than if chlorine or bromine were in the *meta*-position.

Another hypothesis concerns the actual binding site on the MRP molecule. Perhaps one side of the indomethacin structure binds to a particular binding site on the MRP. Presumably the side that binds to the MRP binding site is that with the carboxylic acid group (Seelig *et al.,* 2000). If this is so, it is possible that the position of the chlorine substituent, either in the *ortho*- or the *meta*-position of the benzene ring, but on the same side of the indomethacin structure as the carboxylic acid group, might result in an indomethacin structure which is too large to bind to the MRP binding site. The smaller size of fluorine, when it is in the *meta*-position, may not render the indomethacin structure too large for the binding site on MRP.

It is also possible that the lipophilicity of the indomethacin structure is affected by the position of the halogen on the benzene ring and in particular in N-benzyl-indomethacin analogues. MRP transports amphiphilic anionic compounds and the chlorine substituent helps to maintain the lipophilic properties of certain compounds (O'Connor, 1995). Moving chlorine to the *meta*- or the *ortho*-position on the benzene ring may reduce the lipophilicity of N-benzyl-indomethacin rendering it a poorer substrate for MRP. However, this

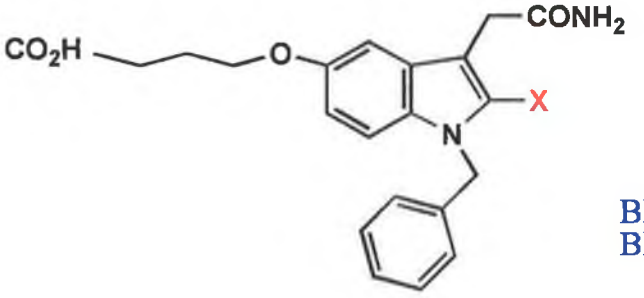
hypothesis is unlikely, as removing the halogen group completely from the benzene ring in the N-benzoyl-indomethacin analogues did not render the indomethacin analogues inactive. Therefore, it is most likely that the position and the nature of the halogen in the N-benzyl-indomethacin analogues affect the overall conformation (i.e. size and electron distribution) of the compound and as a result, its ability to bind to MRP.

The fact that the removal of the methoxy substituent on the N-benzoyl indomethacin structure did not result in a loss of activity in the combination toxicity assays, but removing the same substituent in the N-benzyl analogue of indomethacin resulted in loss of activity, strongly suggests that the nature and the function of the substituents on the benzene ring are not as crucial for N-benzoyl-indomethacin due to the greater structural rigidity of the N-benzoyl substituted indole nucleus.

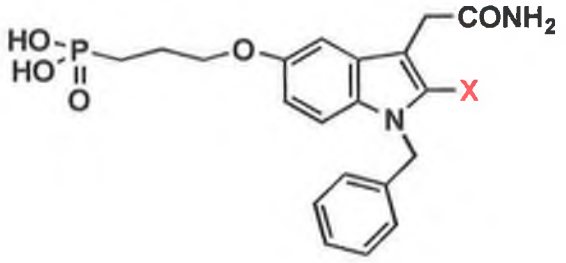
As indomethacin is a known PLA_2 inhibitor (Kaplan *et al.,* 1978; Lobo *et al.,* 1994), four compounds were developed which are PLA_2 inhibitors, adapted from studies of Fleisch *et al.,* (1996) and Mihelich *et al.,* (1997), of Lilly research laboratories. Another suggested explanation for the ability of the NSAIDs to potentiate the toxicity of the chemotherapeutic drug, was through inhibition of the enzyme PLA_2 by these compounds. It was postulated that through the inhibition of the production of arachidonic acid the NSAIDs might exert their cytotoxic enhancing effects. Arachidonic acid is released from phopholipid stores in the cell membrane by the action of phopholipase A_2 (Lehr *et al.,* 1997; Fleisch *et al.,* 1996 and Fox *et al.,* 1996). This polyunsaturated fatty acid is a substrate for the lipoxygenase and cyclooxygenase enzymes and leads to a family of bioactive lipids that include the leukotrienes, prostaglandins, prostacyclins and thromboxanes (Mann *et al.,* 1994). Despite a great deal of investigation, the roles that these arachidonic acid metabolites play in cancer have yet to be fully elucidated. However, as described in section 1.7, a number of studies, including those carried out by Marnett *et al.,* (1992) and Ara *et al.,* (1996), have discussed the lipoxygenase and cyclo-oxygenase inhibitory properties of NSAIDs and their effects on tumour growth and promotion.

The structures of the four PLA_2 inhibitors developed for analysis, BRI 153/1, 203/1, 205/4 and 215/1, are described in figures 3.1.20, 3.1.23 and 4.2 (below). BRI 153/1 and BRI 203/1 were N-benzyl-1-(m)ethylindole-3-acetamides

functionalised at the 5-position with a short chain alkoxy unit terminated by a carboxylic acid unit. Both compounds were structurally identical except for an ethyl substituent in BRI 153/1 was replaced with a methyl substituent in BRI 203/1. When analysed in the combination toxicity assay, BRI 153/1 was non-toxic to the DLKP cells at concentrations up to 50μg/ml (0.130mM). It was only at this concentration that BRI 153/1 enhanced the toxicity of adriamycin in the DLKP cells at a level comparable that obtained by indomethacin at 2.5μg/ml (0.007mM). BRI 203/1 was more toxic to the cells than 153/1. The highest non-toxic concentration of BRI 203/1 was 20μg/ml (0.054mM), indicating that the methyl substituent was involved in increasing the toxicity of the compound to the DLKP cells. At this highest non-toxic concentration BRI 203/1 was positive in the combination toxicity assay but at its highest non-toxic concentration was not as effective in enhancing the toxicity of adriamycin as indomethacin at 2.5μg/ml.



BRI 203/1: $X=CH_3$
BRI 153/1/1: $X=CH_2CH_3$



BRI 205/4: $X=CH_3$
BRI 215/1: $X=CH_2CH_3$

BRI 205/4 and BRI 215/1 were also developed with structures similar to that of 153/1. These compounds were N-benzyl-2-(m)ethylindole-3-acetamindes, functionalised at the 5-position. However, the short-chain alkoxy unit was

terminated by a phosphonic acid residue instead of a carboxylic acid residue. Again, BRI 205/4 and BRI 215/1 were structurally identical except for an ethyl substituent in BRI 215/1 was replaced with a methyl substituent in BRI 205/4. In the combination toxicity assay BRI 205/4 was negative. This compound had no cytotoxic enhancing ability when used at its highest non-toxic concentration of 25μg/ml (0.062mM). BRI 215/1 could also be used at concentrations up to 50μg/ml (0.119mM), again indicating that the replacing of the ethyl substituent for a methyl substituent increases the toxicity of the compound in the cells. Although BRI 215/1 was at such high concentrations it was only weakly positive as compared to indomethacin. The results obtained in the combination toxicity assay for the four PLA_2 inhibitors, indicate that BRI 153/1 and BRI 203/1 were most positive. This indicates that the phosphonic acid residue, terminating the short chain alkoxy unit in BRI 215/1 and 205/4 had a negative effect on the ability of these compounds to enhance the toxicity of adriamycin. As explained previously, Seelig *et al.,* (2000), reported that the carboxylic acid residue is a typical, negatively charged type I unit, (unit consisting of two electron donor groups with a spacial separation 2.5 ± 0.3 Å) suitable for substrate binding to MRP. Therefore, it is possible that when the carboxylic acid residue on the short chain alkoxy unit of BRI 153/1 and 203/1 is removed, and replaced with a phosphonic acid residue on BRI 205/4 and 215/1, these compounds lose their ability to bind to MRP. As a result BRI 205/4 and 215/1 cannot potentiate the toxicity of adriamycin. The results also suggested that the ethyl substituent was the most favourable in terms of lower toxicity in the cell. At its highest non-toxic concentration, BRI 153/1 was the most positive of the PLA2 compounds analysed. Yet it could only achieve enhancement of the toxicity of adriamycin, comparable to that obtained by indomethacin at 2.5μg/ml, when used at 50μg/ml. These results suggest that the potentiating ability of indomethacin and positive indomethacin analogues is not through PLA_2 inhibition.

	Combination Toxicity Assay		Combination Toxicity Assay
Indomethacin	+	BRI 115/2	+
BRI 60/1	+	BRI 113/1	-
BRI 59/1	-	BRI 124/1	-
BRI 69/2	-	BRI 119/1	-
BRI 88/1	+	BRI 120/1	-
BRI 92/1	+	BRI 153/1	+
BRI 104/2	+	BRI 203/1	+
BRI 106/1	-	BRI 205/4	-
BRI 107/1	-	BRI 215/1	-
BRI 114/2	+	BRI 138/1	+

Table 4.2.1: Summary table of results from combination toxicity assays.

+ Positive; - Negative.

4.2.2 Investigation of the ability of indomethacin and indomethacin analogues to inhibit Glutathione S-transferase activity.

One postulated mode by which the chemotherapeutic drugs are transported by MRP is through the formation of glutathione conjugates, which are then effluxed from the cell by MRP, resulting in the development of multidrug resistance (Paul *et al.,* 1996). The findings that MRP can transport cysteinyl leukotrienes (e.g. LTC_4) as well as other GSH conjugates suggest that this protein may be a GSH conjugate/organic anion transporter (Leier *et al.,* 1994; Jedlitschly *et al.,* 1994; Loe *et al.,* 1996 and Cole *et al.,* 1994). However, this theory is questionable as glutathione conjugates of the natural drug products for which MRP confers resistance have not been isolated (Tew *et al.,* 1994) and MRP transfectants do not exhibit increased resistance to alkylating agents, a class of drugs for which glutathione conjugation is known to occur (Grant *et al.,* 1994). Loe *et al.,* (1996), demonstrated the direct uptake of unmodified vincristine by MRP-enriched vesicles in an ATP and GSH-dependent manner and that the tripeptide structure of GSH is a requirement for stimulation of VCR transport to occur. However, co-transport mechanisms still cannot explain all MRP-mediated resistance mechanisms, in particular with respect to the anthracycline antibiotics since GSH displays little or no ability to enhance either the transport directly or their ability to inhibit ATP-dependent, MRP-mediated LTC_4 transport (Loe *et al.,* 1996b).

Our group postulated that the mode of action of indomethacin and positive indomethacin analogues, in circumventing resistance in MRP positive cell lines, was through some form of inhibition of the activity of MRP. An investigation was carried out to assess if these positive compounds were active against MRP through inhibition of the enzyme glutathione S-transferase (GST) which is the enzyme involved in the formation of glutathione conjugates (Yang *et al.,* 1992). Hall *et al.,* (1989), showed a partial reversal of chlorambucil resistance in Chinese hamster ovary cells by preincubation of the cells with indomethacin. These CHO cells were found to exhibit resistance to bifunctional nitrogen mustards while maintaining sensitivity to a range of other alkylating agents. This enhanced drug resistance was associated with a greater than 40-fold increase in the level of GST as compared to the parental, CHO-K1 cell line (Robson *et al.,* 1986). Hall *et al.,* (1989), found that indomethacin was

an inhibitor of GST enzyme activity and also demonstrated that following exposure of the CHO cells to 500μm of indomethacin, the cytotoxicity of chlorambucil was potentiated. In contrast, the anti-inflammatory agent acetylsalicylic acid (aspirin) failed to inhibit the activity of GST and caused no potentiation of chlorambucil activity, suggesting that the potentiation by indomethacin is not due to the effects of this drug on prostaglandin synthesis. These studies led the authors to believe that GSTs may be involved in the development of resistance to bifunctional alkylating agents and suggest that indomethacin, or agents with similar activities may be of value as an adjunct to chemotherapy in some patients with tumours resistant to treatment with alkylating agents.

Results from the GST assays demonstrated varying ability among indomethacin and analogues to inhibit GST. Indomethacin was used as the positive compound control as its GST inhibiting ability was known and DMSO was used as the negative control (no compound). At approx. 500μm, indomethacin was found to inhibit GST by approx. 50% and 1,000μm inhibited GST activity by approx. 100%. The indomethacin analogues were analysed at similar concentrations. The results obtained from these assays were interesting. BRI 60/1, 88/1, 92/1, 104/2, 114/2, 115/2, 153/1, 138/1 and 203/1 were positive in the combination toxicity assays in DLKP cells. However, only three of those compounds, BRI 88/1, 92/1, 104/2, positive in the combination toxicity assay, were strongly positive in the GST assay. GST inhibition by BRI 92/1 was comparable to that of indomethacin whereas the other two compounds were slightly less inhibitory. It was also interesting to note that indomethacin and BRI 88/1, 92/1, 104/2, are N-benzoyl compounds and vary only with indomethacin by the removal/replacement of one substituent in each of the compounds (Figures 1.10.5 - 1.10.7). The remaining indomethacin analogues, positive in the combination assay, are N-benzyl derivatives of indomethacin, and demonstrate insignificant or no GST inhibitory ability. This suggests that the GST active site is more selective for substrate binding than the MRP active site and the rigid structure of N-benzoyl-indomethacin is required for binding to this GST active site. The results indicate that inhibition of GST is not important for the enhancement of the toxicity of the chemotherapeutic by indomethacin and the active indomethacin analogues.

	GST Assay Results		GST Assay Results
Indomethacin	+++	BRI 115/2	+/-
BRI 60/1	-	BRI 113/1	+/-
BRI 59/1	-	BRI 124/1	-
BRI 69/2	-	BRI 119/1	-
BRI 88/1	++	BRI 120/1	-
BRI 92/1	+++	BRI 153/1	-
BRI 104/2	++	BRI 203/1	-
BRI 106/1	-	BRI 205/4	-
BRI 107/1	+/-	BRI 215/1	-
BRI 114/2	-	BRI 138/1	-

Table 4.2.2: Summary table of results from GST assay

+++ Very strong positive; ++ Strong positive; + positive; +/- poor positive; - negative.

4.2.3 Investigation of MRP inhibiting ability of indomethacin and indomethacin analogues using a membrane-enriched preparation of MRP.

Western blotting analysis failed to demonstrate expression of MRP protein in whole cell extracts of DLKP (Duffy *et al.,* 1998). This contradicted the suggestion that NSAIDs interfered with MRP-mediated drug efflux resulting in chemotherapeutic drug toxicity enhancement. It was found, however, that MRP was detectable by Western blotting of inside-out vesicles (IOVs) isolated from DLKP. The levels were relatively low in comparison to the levels present in IOVs isolated from the MRP- overexpressing cell line HL60-ADR, but these levels were sufficient to render the cells less sensitive to chemotherapeutic drug and to allow circumvention of the resistance by the active NSAIDs (Elliott, 1997 and Duffy *et al.,* 1998).

The precise mechanism by which MRP renders the cells resistant to cytotoxic insults has not been definitely established but it is believed (at least as one possible mechanism) to act as an efflux pump for GSH conjugates of

xenobiotics (Ishikawa *et al.,* 1994). Active NSAIDS, indomethacin, sulindac and tolmetin have previously been demonstrated by our group to inhibit the uptake of leukotriene C_4, an MRP substrate, using inside-out vesicles prepared from the plasma membrane of HL60/ADR cells (Duffy *et al.,* 1998).

To gain insight into the mechanism of action of indomethacin and its analogues, the *in vitro* substrate specificity of MRP was examined by analysing the uptake of LTC_4 into membrane vesicles prepared from MRP-overexpressing HL60/ADR cells in the presence or absence of indomethacin or its analogues. Membrane vesicles were prepared as described in section 2.10, and according to the methods described by Paul *et al.,* (1996), and Leier *et al.,* (1994b). Uptake of LTC_4 was measured by the rapid filtration technique described by Horio *et al.,* (1988), and Paul *et al.,* (1996).

The results obtained from these assays demonstrated that approximately 50mM indomethacin inhibited the transport of LTC_4 by approx. 80%. Similar molar concentrations of each of the active analogues were assayed and the results demonstrated that, of the nine indomethacin analogues active in the combination toxicity assay, eight of these compounds were very positive in the IOV assay. Analogues of indomethacin that were found to be negative in the combination toxicity assay were also found to be negative in the IOV assay in that they failed to inhibit the uptake of LTC_4. These results strengthen the theory that the enhancement of the toxicity of the chemotherapeutic drug is through the interaction of the positive analogues with MRP. It is suggested that indomethacin and its analogues exerted their positive effect through inhibition of the pumping ability of MRP either by:

i. Binding to MRP and inhibiting the activity of the pump;

ii. Competing with LTC_4 for a particular binding site on the MRP molecule, inhibiting the binding of LTC_4 to MRP and curtailing the pumping of this substrate;

iii. Competitively inhibiting the pumping of LTC_4 by MRP, causing the MRP pump to preferentially transport indomethacin and its analogues from the cell (i.e. indomethacin and analogues may be competitive substrates for MRP).

The glutathione conjugate transporter is also believed to be involved in the transport of cytotoxic drugs (Paul *et al.,* 1996; Ishikawa *et al.,* 1995; Shen *et al.,* 1996) (Section 1.6.6). This may indicate that the NSAIDs and the indomethacin analogues potentiate the toxicity of the chemotherapeutic drugs through direct interaction with the MRP pump, causing a reduction in the amount of drug being pumped out of the cell and as a result increasing the toxicity of the drug to the cell.

However, one of the indomethacin analogues, BRI 138/1 (meta-fluorobenzyl-indomethacin), which was active in the combination toxicity assay in DLKP cells, was only weakly positive in the IOV assay. Approximately 50μM of BRI 138/1 was only capable of inhibiting the uptake of LTC_4 by approx. 20% as compared to approx. 80% inhibition by equivalent concentrations of indomethacin. This would suggest that BRI 138/1 is a very poor MRP inhibitor/substrate even though it potentiated very strongly the toxicity of adriamycin in the combination toxicity assay, (although a potentiation ability comparable to indomethacin was only evident when BRI 138/1 was added to the combination toxicity assay at 6-times the concentration of indomethacin).
It may be possible that BRI 138/1 is exerting its effect on a different analogue of MRP which might have different substrate specificities than MRP1. RT-PCR analysis of DLKP cells (Section 3.11.2) has demonstrated a strong expression of MRP1 in these cells. Low expressions of MRP2, -4, -5 and –6 mRNA have also been detected in DLKP cells (Section 3.16). mRNA expression of the various homologues of MRP was also investigated in the HL60/ADR cell line, the cell line from which the IOVs were prepared. Again, a strong expression of MRP1 mRNA was detected in these cells with lower levels of MRP2, -5 and –6 mRNA expression also detected. There are no detectable levels of MRP4 or MRP3 in the HL60/ADR cells (Section 3.16). Perhaps BRI 138/1 potentiated the toxicity of adriamycin by interacting with MRP4 which was detectable in DLKP cells but not in HL60/ADR cells. However, there is no evidence to date to suggest that adriamycin or indomethacin/indomethacin analogues are substrates for MRP4.
BRI 138/1 was negative in the GST assays so its activity is most likely not through inhibition of the formation of glutathione conjugates. BRI 115/2, the *para*-form of BRI 138/1, was positive in the IOV assay. It may be possible that

because of its particular *meta*-structure (other *meta*-analogues of benzyl indomethacin were inactive in the combination toxicity assay), BRI 138/1 has a lower affinity for the binding site on the MRP molecule than LTC_4 and the other active analogues. As a result of this, if the actual mode of action by which the positive analogues work is via competitive inhibition of MRP it may be unable to compete with LTC_4 for preferential pumping by MRP. In contrast, in the combination toxicity assay, the levels of BRI 138/1 added to the cells is quite high, (15μg/ml (46μM)), when compared to the concentration of adriamycin added (10ng/ml (17.2nM)) to the same cells. As a result BRI 138/1 may be capable of competing with adriamycin for preferential pumping by the MRP pump due to the greater concentration of this compound in the cell. Hence, affinities of the compounds for the MRP binding site may not be so critical in the combination toxicity assay.

The comparison between the concentrations of indomethacin and indomethacin analogues used in both the combination toxicity assay and the IOV assay is also of interest. In the combination toxicity assay, BRI 138/1 was required at 6-times the concentration of indomethacin to produce a similar potentiation effect whereas, BRI 153/1 was required at 50-times the concentration of indomethacin. In comparison, in the IOV assay, the concentration of BRI 153/1 (42μM) was similar to that of indomethacin (46μM), with similar abilities to inhibit LTC_4 transport. However, BRI 138/1 at a concentration of 50μM was 3-fold less effective than indomethacin in the IOV assay. The fact that indomethacin and BRI 153/1 could be used at similar concentrations in the IOV assay, but not in the combination toxicity assay suggests differences in substrate requirements for binding to MRP between both assays. It appears that, although the fluorine in the *meta*-position on BRI 138/1 did not affect binding to the MRP binding site in the combination toxicity assay, the position of this halogen rendered BRI 138/1 a poor substrate in the IOV assay. It is possible that the procedure of turning vesicles inside-out, and thus, the direction of transport by MRP, affected, in some way, the conformation of the MRP active site which resulted in less effective binding by BRI 138/1.

Alternatively, this discrepancy with the results obtained with BRI 138/1 may be simply due to the solubility of the compound in the IOV assay. Moving the fluorine from the *para*-to the *meta*-position in fluorobenzyl indomethacin may effect the solubility and electronegativity of the compound. MRP seems to

have a preference for amphiphilic xenobiotics (Cole *et al.*, 1994 and Loe *et al.*, 1996a). Fluorine is a smaller molecule (MW 18.998) than chlorine (MW 35.453) and bromine (MW 79.904) and is also more electronegative which may be associated with reduced lipophilicity. In the combination toxicity assay, BRI 138/1 was dissolved in DMSO and added to medium prior to adding to the cells. However, in the IOV assay, BRI 138/1 was dissolved in DMSO and then added to an incubation buffer, which consisted of a high concentration (8% w/v) of sucrose in 10mM TRIS-HCL. Therefore, the solubility of BRI 138/1 in this buffer and, thus, in the IOV assay, may have been affected by the fluorine in the *meta*-position.

Further experiments are required to determine if BRI 138/1 is exerting its effect through an interaction with MRP and if its mode of action is similar to that of the other active indomethacin analogues. Efflux studies (section 3.5) suggest that the DLKP cells efflux chemotherapeutic drug, in combination with BRI 138/1, in a manner similar to the efflux of drug with indomethacin. These efflux experiments may also be expanded to determine preferential pumping of one indomethacin analogue over another. The affinity of BRI 138/1 versus other indomethacin analogues for MRP may be analysed here.

	IOV Assay Results		IOV Assay Results
Indomethacin	+++	BRI 115/2	+++
BRI 60/1	++	BRI 113/1	N/D
BRI 59/1	-	BRI 124/1	N/D
BRI 69/2	-	BRI 119/1	-
BRI 88/1	++(+)	BRI 120/1	-
BRI 92/1	+++	BRI 153/1	+++
BRI 104/2	++	BRI 203/1	++
BRI 106/1	N/D	BRI 205/4	-
BRI 107/1	N/D	BRI 215/1	+/-
BRI 114/2	++(+)	BRI 138/1	+

Table 4.2.3: Summary table of results from IOV assay

+++ Very strong positive; ++ Strong positive; + positive; +/- poor positive; - negative.

4.2.4 Investigation of the ability of indomethacin and indomethacin analogues to inhibit the cycloxygenase enzymes, COX-1 and COX-2.

Indomethacin has been shown by a number of researchers (Vane *et al.,* 1996; Engelhardt *et al.,* 1996; Mitchell *et al.,* 1993) to be an inhibitor of both cyclooxygenase-1 and -2 (COX-1 and COX-2). This NSAID is slightly more potent against COX-1 than COX-2 but is generally considered as a non-selective NSAID (Reindeau *et al.,* 1997). Indomethacin is also known for its propensity to cause gastric damage (Bateman, 1994).

It is unlikely that the cyclooxygenase inhibitory activity of the NSAIDs is involved in the enhancement of the cytotoxicity of chemotherapeutic drugs because:

- Many NSAIDs known to be cycloxygenase inhibitors have been shown in our laboratories not to have the ability to potentiate the toxicity of chemotherapeutic drugs. These negative NSAIDs include aspirin, diclofenac, fenoprofen, flufenamic acid, naproxen and piroxicam (Duffy *et al.,* 1998).
- Sulindac sulfone, a metabolite of sulindac, is an active toxicity enhancer (Duffy *et al.,* 1998), although it does not possess cyclo-oxygenase inhibitory activity (Piazza *et al.,* 1995; 1997a; 1997b and Levy *et al.,* 1997).
- The addition of PgD_2 or PgE_2 does not reverse the enhancement effect (Duffy *et al.,* 1998; De Mello *et al.,* 1980; Elliot 1997).

NSAIDs are reported to inhibit the synthesis of cytoprotective prostaglandins by COX-1 in the gastrointestinal tract leading to the accumulation of surplus arachidonic acids. These can enhance the generation of leukotrienes via the lipoxygenase pathway inducing neutrophil adhesion to endothelium and vasoconstriction. The NSAIDs containing a carboxyl group also inhibit oxidative phosphorylation (OXPHOS) lowering adenosine-triphosphate (ATP) generation, leading to loss of mucosal cell tight junctions and increased mucosal permeability (Fosslien, 1998). Inhibition of COX-2 by NSAIDs reduces synthesis of pro-inflammatory prostaglandins and produces analgesia (Fosslien, 1998). Evidence, therefore, suggests that the GI toxicity associated with NSAID use is primarily

the result of inhibition of COX-1 and anti-inflammatory effects are largely due to the inhibition of COX-2. An NSAID, which selectively inhibits COX-2, and has little effect on COX-1, would be ideal for the purpose of our work especially since COX-2 inhibitors may have anti-tumour activity (section 1.7). Therefore, the indomethacin analogues were analysed to investigate their COX-1 and COX-2 inhibitory abilities and to assess if one of the analogues of indomethacin, positive in the combination toxicity assay, might be capable of exerting its effect on the chemotherapeutic drug without causing the gastric damage customarily associated with NSAIDs, including indomethacin.

Indomethacin was used as the positive control in both the COX-1 and COX-2 assays as its ability to inhibit both enzymes was known. BRI 215/1 and 205/4 were also analysed in the COX assays to assess if analogues of indomethacin, which were negative in the combination toxicity assay, would also be devoid of COX-inhibitory activity. Separate assays were utilised to analyse the inhibitory effect of the compounds on the enzymes. The COX-1 assay was a spectrophotometric assay based on the assays utilised by Boopathy *et al.*, (1986), and Piazza *et al.*, (1997). The assay used to measure the inhibition of COX-2 was an ELISA- based assay and the experiment was based on the fact that COX-2 is the constitutive and dominant isoform in stimulated and unstimulated cultured human lung epithelial cells (Asano *et al.*, 1996). The cell line A549, a human lung adenocarcinoma, was shown to express COX-2 mRNA and protein when it is stimulated with epidermal growth factor or pro-inflammatory cytokines such as IL-1β.

The compounds analysed in both the COX-1 and COX-2 assays were those compounds that were positive in the combination toxicity assay in DLKP cells. Of the agents analysed, the results indicate that only four of the compounds positive in the combination toxicity assay, including indomethacin were good inhibitors of both COX-1 and COX-2. The indomethacin analogues most effective at inhibiting COX-1 and COX-2 were BRI 88/1, 92/1 and 104/2. Of these compounds BRI 92/1, bromo-indomethacin, was most effective at inhibiting both enzymes and seemed to be slightly more effective than indomethacin. 12μg/ml (33nM) indomethacin inhibited COX-1 by 60.9 ± 12.2% and at slightly lower molar concentrations BRI 92/1 at 30nM inhibited COX-1 by 79.0 ± 11.3%. Indomethacin at 10nM inhibited the activity of COX-2 by 86.1 ± 5.9%. BRI 92/1 at 10nM inhibited the activity of COX-2 by 88.1 ±

4.4%. Indomethacin, BRI 88/1 and BRI 104/2 (N-benzoyl-indomethacin analogues) had similar COX-inhibiting ability. Perhaps the replacement of the chlorine substituent with bromine in N-benzoyl indomethacin increased the COX- inhibitory ability of the compound.

BRI 60/1, BRI 114/2, BRI 115/2, BRI 203/1 and BRI 215/1 (negative control compound) inhibited COX-1 by approximately 20-30% (Table 3.4.1.1), so were relatively weak COX-1 inhibitors as they were approximately 50% less effective than indomethacin at inhibiting COX-1. These compounds are all N-benzyl derivatives of indomethacin and this indicates that the N-benzoyl unit in indomethacin is critical in COX-1 inhibition and removal of this unit renders the compound too dissimilar to indomethacin to be an effective COX-1 inhibitor. Zoete *et al.,* (1999), reported that electron–donating properties are essential in the action of NSAIDs as inhibitors of prostaglandin cyclooxygenase activity. Perhaps removal of the benzoyl group diminishes the electron-donor ability of indomethacin rendering it unable to inhibit COX-1.

Of interest here are the compounds BRI 153/1 and BRI 138/1. These compounds demonstrated extremely low ability to inhibit the activity of COX-1. BRI 153/1 and BRI 138/1 had no inhibitory activity against COX-1. This suggests that as well as the N-benzoyl unit being critical for COX-1 inhibition, the presence of the halogen in the *para*-position also seems to be critical for inhibition of COX-1. Both these compounds were positive in the combination toxicity assays, BRI 153/1 was very positive in the IOV assay but BRI 138/1 was only weakly positive. These compounds would be of clinical interest as they seem to both have a good enhancement effect on the chemotherapeutic drugs *in vitro* and have very weak COX-1 inhibitory ability. These properties would make them attractive as active compounds, with adriamycin potentiation ability, without the characteristic adverse side effects of NSAIDs which result from inhibition of COX-1.

BRI 60/1 was the only indomethacin analogue which exhibited a weak COX-1 inhibitory activity but a strong COX-2 inhibitory activity comparable to that of indomethacin (≈ 85% inhibition of COX-2). BRI 114/2 and BRI 115/2 inhibited COX-2 by approximately 40% which is only 50% the COX-2 inhibitory ability of indomethacin. BRI 153/1, BRI 138/1 and BRI 203/1 only inhibited COX-2 by approx. 20%. BRI 215/1 exhibited no COX-2 inhibitory activity. BRI 60/1 was the only benzyl analogue of indomethacin to exhibit

strong COX-2 inhibitory activity. The results seem to indicate that there is a relationship between the benzoyl/benzyl unit and the halogen attached to the benzene ring. In the benzoyl series, removal of the halogen (BRI 88/1) or replacing it with another halogen (BRI 92/1) did not seem to effect the compounds ability to inhibit COX-2 activity once the benzoyl unit was present. However, in the benzyl series, removing the chlorine from the benzene ring, changing its position from *para*-, or replacing it with another halogen, affected the ability of the compound to inhibit COX-2. The COX active site is a long hydrophobic channel (Vane *et al.,* 1996) and it is possible that changing the structure of indomethacin, in particular altering the halogen substituent, might result in a number of analogues becoming less lipophilic and incapable of binding to the COX active site as effectively as indomethacin. Meade *et al.,* (1993), reported that the active site of COX-2 is larger than that of COX-1 and can accept a wider range of structures as substrates. Perhaps this explains why removal of the benzyl group in BRI 60/1 did not diminish its COX-2 inhibitory activity. The structure was too dissimilar to indomethacin for significant COX-1 inhibition but due to the wider substrate specificity of the COX-2 active site, BRI 60/1 was able to bind to the active site of enzyme.

	Combination Toxicity Assay	GST	IOV	COX-1	COX-2
Indomethacin	+++	+++	+++	++	+++
BRI 60/1	+++	-	++	+	+++
BRI 59/1	-	-	-		
BRI 69/2	-	-	-		
BRI 88/1	+++	++	++(+)	++	+++
BRI 92/1	+++	+++	+++	+++	+++
BRI 104/2	+++	++	++	++	+++
BRI 106/1	-	-			
BRI 107/1	-	+/-			
BRI 114/2	+++	-	++(+)	+	++
BRI 115/2	+++	+/-	+++	+	++
BRI 113/1	-	+/-		-	
BRI 124/1	-	-			
BRI 119/1	-	-	-		
BRI 120/1	-	-	-		
BRI 153/1	+++	-	+++	-	+/-
BRI 203/1	+++	-	++	+	+
BRI 205/4	-	-	-	-	++
BRI 215/1	-	-	+/-	+/-	-
BRI 138/1	+++	-	+	-	+/-

Table 4.2.4: Summary table of results from investigation of SAR of indomethacin

+++ Very strong positive; ++ Strong positive; + positive; +/- poor positive;

- negative.

In summary, investigations of the structure activity relationship of indomethacin have indicated that the N-benzoyl substituent on the indomethacin structure is crucial for GST and COX-1 inhibitory activity. This substituent does not seem to be as critical in N-benzyl-indomethacin analogues, for inhibition of COX-2. But, this is only so when the halogen group is present and in the *para*-position which suggests, in the absence of the N-benzoyl substituent, that the *para*-halogen bonds to a particular part of the active site of COX-2, locking the compound in to position. Analysis of the indomethacin analogues in the SAR assays has yielded a number of interesting indomethacin analogues. Of primary interest are those compounds which were found to be active in the combination toxicity assay. Six of these compounds, N-benzyl indomethacin analogues, BRI 60/1, 114/2 and 115/2, and N-benzoyl indomethacin analogues, BRI 88/1 92/1 and 104/2, demonstrated potentiating ability similar to indomethacin at similar concentrations but they also demonstrated reduced toxicity in the DLKP cells as compared to indomethacin. These indomethacin analogues were 2-fold (BRI 60/1, 88/1 and 92/1), and 4-fold (BRI 114/2 and BRI 115/2) less toxic than indomethacin in the DLKP cells. The required concentrations of the remaining active indomethacin analogues, BRI 138/1, 153/1 and 203/1, are higher than the required concentration of indomethacin, for enhancement of adriamycin toxicity. However, although they are less potent than indomethacin, these analogues are also less toxic to the DLKP cells than indomethacin. As a result, these indomethacin analogues may prove to be more clinically beneficial than indomethacin for enhancement of chemotherapeutic drug activity due to their reduced toxicity.

Of particular interest also, are those indomethacin analogues with reduced COX-1 inhibitory ability. The N-benzyl-indomethacin analogues demonstrated reduced ability to inhibit COX-1 as compared to the indomethacin and the N-benzoyl indomethacin analogues. This reduced COX-1 inhibition would be of great benefit, as the toxicity of the anticancer drugs could be enhanced by the active indomethacin analogues without the gastrointestinal toxic side effects associated with indomethacin. In particular, BRI 138/1 and 153/1, N-benzyl indomethacin analogues, were active in the combination toxicity assay, were less toxic than indomethacin and demonstrated no COX-1 and some COX-2 inhibitory activity. BRI 60/1 also demonstrated very low COX-1inhibitory

ability but, in contrast to BRI 138/1 and 153/1, this indomethacin analogue was also a very strong COX-2 inhibitor. The results for BRI 60/1 suggest a potential clinical application due to possible lower toxic side effects than indomethacin and in addition, increased ability as a tumour suppresser due to inhibition of COX-2.

Duffy *et al.,* (1998), reported that indomethacin was unable to potentiate the toxicity of chemotherapeutic drugs in Pgp-overexpressing cell lines. Therefore, indomethacin and indomethacin analogues function as selective inhibitors of MRP-mediated efflux of the chemotherapeutic drugs from MRP-overexpressing cell lines and are effective at biologically relevant concentrations. There is enough evidence to suggest that these compounds interact with MRP1. The implications of using these compounds in cell lines which express MRP homologues other than MRP1 are discussed in section 4.11 of this thesis.

The active indomethacin analogues are promising candidates for future clinical applications, in particular those with reduced toxicity and COX-1 inhibitory activity. Two of the active indomethacin analogues with reduced COX-1 inhibitory activity, BRI 115/2 and 138/1, are completely novel (Dr. Anita Maguire, personal communication) and so may also be promising candidates for patent and commercial applications.

However, extensive biological analysis of these compounds in animal experiments, clinical trials etc. are required to fully elucidate the effectiveness of these compounds before they can be used in the treatment of human cancers.

4.3 Adriamycin and indomethacin accumulation studies using drug efflux studies.

4.3.1 Investigation of Adriamycin efflux

Drug efflux experiments were carried out to determine the accumulation of adriamycin in DLKP cells in the presence of indomethacin/indomethacin analogues and the accumulation of indomethacin in the cells in the presence of adriamycin.

The results of the adriamycin efflux studies (Table 3.5.1) showed a notable difference in the levels of cellular adriamycin content in DLKP cells incubated with 10μm (5.44μg/ml) adriamycin alone versus co-incubation of adriamycin and 28μm (10μg/ml) indomethacin. After the initial 2 hour loading period, there was similar accumulation of adriamycin in the cells treated with adriamycin alone and adriamycin + indomethacin. When the levels of indomethacin were maintained in the cells for a further five hours the level of adriamycin remained almost constant with only a slight decrease evident after this time. When indomethacin was removed from the medium after the initial loading period, the concentration of adriamycin in the cells greatly decreased. However, there was a decrease in the levels of adriamycin in those cells treated with adriamycin alone.

4.3.2 Effect of indomethacin analogues on adriamycin efflux

Similar results to indomethacin were obtained with BRI 138/1. BRI 138/1 was positive in the combination toxicity assay (section 3.1), in that it could potentiate the toxicity of adriamycin in DLKP cells. The efflux of adriamycin from the DLKP cells was also reduced in the presence of BRI 138/1, also at 28μm, as compared to cells incubated with adriamycin alone. Removing BRI 138/1 from the DLKP cells also resulted in a decrease in adriamycin accumulation. However, when this experiment was carried out using an analogue of indomethacin, BRI 205/4, which was negative in the combination toxicity assay (section 3.1), the efflux of adriamycin from the cells was similar to that observed in cells treated with adriamycin alone i.e. this negative

indomethacin analogue had no effect on adriamycin accumulation in the DLKP cells. The results suggest that the efflux of adriamycin from MRP-expressing cells can be significantly reduced by indomethacin and BRI 138/1 and as a result can lead to greater adriamycin toxicity to the cell. This supports results obtained in the combination toxicity assay (section 3.1) in which in the presence of indomethacin and BRI 138/1, the toxicity of adriamycin was potentiated in the DLKP cells. It appears that the active compounds have direct inhibitory potential against the activity of MRP. It is possible that indomethacin and indomethacin analogues exert their positive effect through inhibition of the pumping ability of MRP either by:

i. Binding to MRP and inhibiting the activity of the pump.
ii. Competing with an MRP substrate, such as LTC_4 or chemotherapeutic drug, for a particular binding site on the MRP molecule, inhibiting the binding of the substrate.
iii. Competitively inhibiting the pumping of the substrate by MRP, causing the MRP pump to preferentially transport active NSAIDs such as indomethacin (competitive substrate).

4.3.3 Investigation of Indomethacin efflux

An investigation was then carried out to assess the effect of adriamycin on the efflux of indomethacin from the DLKP cell.

The results indicate that after the initial loading period, the level of indomethacin in the cells was higher in those cells treated with a combination of indomethacin and adriamycin, than in those cells treated with indomethacin alone. After the cells were refed with fresh media alone, subsequent to loading with both adriamycin and indomethacin, the concentration of indomethacin in the cells decreased considerably. However, there was almost no efflux of indomethacin from the cells refed with adriamycin and incubated for a further 90 minutes. The results indicate that in the presence of adriamycin the efflux of indomethacin from the DLKP cells is notably reduced. These experiments demonstrate that indomethacin as well as adriamycin is effluxed from the DLKP cells. This suggests that both compounds are competitive substrates for

MRP1 and reduce the efflux of one another from the DLKP cells by competing for the same binding site on the MRP protein and competing for preferential pumping from the cell by MRP1.

An experiment was also carried out to investigate if co-incubation of indomethacin with adriamycin during the initial loading period was required for maintenance of the cellular indomethacin content. The cells were loaded for two hours with indomethacin and after this initial period the medium was removed from the flask and the cells were refed with adriamycin containing medium alone for 90 minutes incubation. The level of indomethacin in the cells was found to have decreased to similar levels observed in the cells treated with indomethacin alone, indicating that co-incubation of indomethacin with adriamycin is required for the maintenance of cellular indomethacin content.

Adriamycin also seems to have exerted an effect on the initial uptake of indomethacin by the DLKP cells. The results indicate that in the presence of adriamycin there was a greater uptake of indomethacin in the cell than when the cells were incubated with indomethacin alone (23.3ng/million cells and 12.8 ng/million cells respectively). It is possible that indomethacin is such a good substrate for MRP due to its amphiphilic anionic properties and the presence of the carboxylic acid group which seems to be required for binding to MRP, that it is effluxed from the cell almost immediately, resulting in the low levels of indomethacin being present in the cells after the initial 'loading' period. In the presence of adriamycin, the efflux of indomethacin seems to be reduced, resulting in a greater net uptake of indomethacin in the cell after the initial 'loading' period.

The concentrations at which indomethacin and adriamycin are added to the DLKP cells must also be taken in to consideration. A concentration of 27.95μM indomethacin was added to the DLKP cells in comparison to 10μM adriamycin. Perhaps indomethacin is only capable of competing with MRP when present in the cell at such high concentrations. However, preliminary drug efflux experiments which were carried out to determine the length of time adriamycin or indomethacin were detectable in the DLKP cells demonstrated that five hours after the initial loading period, low levels of adriamycin was

still detected in the DLKP cells. In contrast, very low levels of indomethacin could only be detected up to 2 hours after the initial loading period.

These preliminary results thus indicate that indomethacin is pumped out of the cells at a much faster rate than adriamycin, suggesting that indomethacin /indomethacin analogues are pumped preferentially by MRP1 when combined with adriamycin, resulting in increased toxicity of the chemotherapeutic drug to the cells. As mentioned previously, this preferential binding is possibly due to the amphiphilic anionic properties of indomethacin in contrast to the more hydrophilic, cationic properties of adriamycin. Although MRP is reported to transport both of these compounds (section 1.6) it seems to have a preference for amphiphilic anionic substrates with negatively charged substituents (e.g. the carboxylic acid substituent on indomethacin) (Seelig *et al.*, 2000).

Thus, it appears that these active compounds compete with the chemotherapeutic drugs, which are MRP1 substrates, for a binding site on the MRP pump and are preferentially pumped out of the cell. As a result of this, while MRP is effluxing the active NSAIDs out of the cell, there is less efflux of the chemotherapeutic drug resulting in greater accumulation of the chemotherapeutic drug in the cell and greater cell kill.

Interesting future experiments could include similar drug efflux studies with DLKP cells using combinations of adriamycin and a number of the active and inactive indomethacin analogues. The concentrations of adriamycin and indomethacin/indomethacin analogues used in these efflux studies were chosen as initial experiments carried out in our laboratory indicated that these concentrations were the minimum concentrations required for detection by the HPLC apparatus (Dr. Robert O' Connor, personal communication). It would be interesting to add both the compound and the drug to the cell at the same concentrations to analyse the effect.

These drug efflux studies have been carried out with adriamycin only, in combination with indomethacin and indomethacin analogues. Preliminary work has been carried out on a number of other chemotherapeutic drugs to validate protocols for measuring these drugs using HPLC analysis. To further elucidate the mechanisms by which the active NSAIDs potentiate the anticancer drugs a large number of drug efflux experiments are required using indomethacin/indomethacin analogues and other NSAIDs such as sulindac and

piroxicam in combination with a range of anti-cancer drugs which were shown to be potentiated or unpotentiated in the combination toxicity assays. These experiments would determine if the toxicity of the anticancer drugs were potentiated by the active NSAIDs by a reduction of efflux from the cells or if there are different mechanisms of toxicity enhancement by the active NSAIDs other than modulation of efflux. Efflux studies using other cell lines such as MRP+ and MRP- cell lines and cell lines which over-express Pgp, could also be carried out to compare the rate of efflux of indomethacin, indomethacin analogues and other active NSAIDs in these cell lines.

4.4 Investigation of potentiation of adriamycin, vincristine, VP-16 and 5-FU by indomethacin and indomethacin analogues in the CORL23 parental and resistant cell lines.

From the human lung cancer cell line CORL23, a resistant variant was developed by growth of the parental cells in increasing concentrations of doxorubicin over a period of eight weeks (Mirski *et al.,* 1987 and Twentyman *et al.,* 1994). This CORL23(R) subline does not overexpress Pgp but has high levels of a 190-kDa protein we now know to be MRP1. Borst *et al.,* (1997), and Kool *et al.,* (1997), characterised the CORL23 cell line and determined the levels of MRP present in the parental and resistant variants. Both variants do not express Pgp. The parental CORL23 cell line expresses only very low levels of MRP1 and MRP4, and a very low level of MRP5 was detected. After continuous selection of the cell line with adriamycin, the CORL23(R) cell line was found to highly overexpress MRP1. This overexpression of MRP1 in the resistant cell line resulted in a ten-fold increase in resistance to vincristine and adriamycin, and a 6-fold increase in resistance to VP-16. As the resistant variant does not express Pgp, even after continuous exposure to adriamycin, this resistance can be attributed to the over expression of MRP1.

This characteristic of the CORL23 cell line was exploited to further analyse a number of the indomethacin analogues. combination toxicity assays were carried out on both the CORL23(P) and CORL23(R) to determine if those compounds, which enhanced the toxicity of chemotherapeutic drugs in DLKP

cells, were active in another MRP positive cell line (CORL23(R)). Experiments had previously been carried out on the CORL23(R) cell line, by Duffy *et al.*, (1998), and it was found that the positive NSAIDs, including indomethacin, potentiated the toxicity of a range of chemotherapeutic drugs in this cell line. Therefore, indomethacin was used as the positive control NSAID in these combination toxicity assays. Duffy *et al.*, (1998), also reported that 5'fluorouracil displayed no synergy in combination with NSAIDs in the MRP1 overexpressing cell lines so 5'fluorouracil was used as the negative control chemotherapeutic drug in these assays. BRI 205/4, an indomethacin analogue, negative in the combination toxicity assay in DLKP cells, was used as a negative control compound in these assays. The results show that BRI 205/4 was also found to be unable to potentiate the toxicity of the anti-cancer drugs in the CORL23 cell lines. These results indicate that indomethacin and indomethacin analogues are potentiating the toxicity of the chemotherapeutic drugs using a mechanism similar to that observed in the DLKP cells.

A number of the active indomethacin analogues were found to potentiate the toxicity of MRP1 substrates, adriamycin, VP-16 and vincristine, in the MRP overexpressing cell line, CORL23(R). There was also a potentiation of the same drugs in the parental CORL23 cell line when combined with the active analogues. MRP1 is expressed in both the CORL23 variants (Borst *et al.*, 1997 and Kool *et al.*, 1997), albeit very little expressed in the CORL23 parental cells. However, the fact that MRP1 is expressed at such a low level in DLKP cells (i.e. it was only visible on Western blots when a concentrated plasma membrane preparation was used (Duffy *et al.*, 1998)), indicates that even the presence of a small amount of functional MRP1 may be sufficient to render cells less sensitive to chemotherapeutic drugs. The results obtained in the CORL23(R) and parental cell lines suggest that the level of expression of MRP does not appear to strictly correlate to the rate of the potentiation of the anticancer drug by the positive NSAIDs. Duffy *et al.*, (1998), also demonstrated higher levels of MRP1 protein expression in A549, a human lung adenocarcinoma cell line, than in the DLKP cell line. Yet the authors reported comparable synergistic combination of selected NSAIDs with certain chemotherapeutic drugs in both cell lines. Therefore, it seems that the enhancement of toxicity of the chemotherapeutic drugs by the active compound

is not dependent on the level of expression of MRP1 protein or perhaps a plateau of MRP activity is reached at low protein level.

MRP is understood to not play an important role in 5-FU resistance (Nishiyama *et al.,* 1999). Kirihara *et al.,* (1999), also report that the mechanisms of 5-FU resistance appear to be different from drug resistance associated with the multidrug resistance phenotype related to Pgp and MRP. Indomethacin and the active analogues were unable to potentiate the toxicity of 5-FU in both the CORL23 parental and resistant cell lines. These results concur with the hypothesis that the mode of action of the active NSAIDs is through an interaction with MRP.

Kobayashi *et al.,* (1997), reported that vincristine is a most effective chemotherapeutic drug in combination with indomethacin in two pulmonary adenocarcinoma cell lines. In our experiments the active indomethacin analogues did not demonstrate as strong an ability to potentiate the toxicity of adriamycin and VP-16, in the parental cell line as they did in the resistant variant. However, the magnitude of the enhancement of the toxicity of vincristine by indomethacin, BRI 88/1 and BRI 92/1 was almost equivalent in the parental CORL23 and in the resistant cells. Vincristine is an extremely good substrate for MRP and Loe *et al.,* (1996), demonstrated the direct uptake of unmodified vincristine by MRP in an ATP and GSH-dependent manner, this unmodified uptake has not been demonstrated for adriamycin or VP-16. It may be possible, due to the fact vincristine is a better substrate for MRP than adriamycin and VP-16, that inhibition of even low levels of MRP results in a dramatic potentiation of the toxicity of vincristine, as it appears that low levels of MRP would be capable of effluxing vincristine very effectively.

Alternatively, the greater increase in vincristine toxicity, in both the CORL23 sensitive and resistant cell lines, by indomethacin and indomethacin analogues, may simply be as a result of the lower concentrations of this chemotherapeutic drug in the cells. In the parental CORL23 cells the IC_{50} for vincristine was 1ng/ml (1.21μM). 20ng/ml (34.4μM) of adriamycin was required to achieve the same level of cell kill in these cells. Similarly, in the resistant CORL23 cells 50% cell kill was achieved using vincristine at 12ng/ml (14.52μM) as compared to 250ng/ml (430μM) for adriamycin. Therefore, this data indicates that indomethacin may potentiate the toxicity of vincristine better than

adriamycin and VP-16 due to the lower concentrations of vincristine with which it has to compete for a binding site on the MRP protein.

The mechanisms of action of the drugs used in this combination assay may also contribute to an explanation for the strong potentiation of the toxicity of vincristine in the parental CORL23 cell as opposed to a lower potentiation of adriamycin and VP-16. Both VP-16 and adriamycin interact with the DNA of the cell. The major actions of adriamycin include high affinity binding to DNA through intercalation, with consequent blockade of the synthesis of DNA and RNA, and DNA strand scission through effects on topoisomerase II. The mode of action of VP-16 (etoposide) involves inhibition of topoisomerase II which, results in DNA damage through strand breakage induced by the formation of a ternary complex of drug, DNA and enzyme. However, the mode of action of vincristine does not involve binding to DNA. Its mechanism of action involves depolymerisation of microtubules, which are an important part of the cytoskeleton and the mitotic spindle. The drug binds specifically to the microtubular protein tubulin in dimeric form; the drug-tubulin complex adds to the forming end of the microtubules to terminate assembly, and depolymerisation of the microtubules then occurs. This results in mitotic arrest at the metaphase, dissolution of the mitotic spindle, and interference with chromosome segregation (Pratt *et al.,* 1994; Katzung, 1998). Hence, due to the fact that vincristine does not bind to DNA in the nucleus, it is possible that there is more vincristine freely available in the outer part of the cells, for effluxing from the cell by MRP. As a result any inhibition of the MRP pump may result in a large increase in the amount of vincristine within in the cell, resulting in a greater level of toxicity to the cells.

If the proposed working model for MRP, described in section 1.6.8.1a, which involves the presence of two co-operative binding sites, is correct, it may support an additional hypothesis for the superior enhancement of vincristine toxicity in the CORL23 cell lines. As vincristine transport is reported by Loe *et al.,* (1996) to require co-transport with GSH, it is postulated that vincristine binds to the D-site while GSH binds to the G-site. Indomethacin is reported by Borst *et al.,* (1999), to stimulate GSH transport in MDCKII-MRP2 cells. Therefore, it appears that indomethacin preferentially binds to the D-site of the MRP protein, reducing the amount of vincristine bound to this site, resulting in reduced efflux of this chemotherapeutic drug from the cell. Whereas, it is

postulated that adriamycin and VP-16, chemotherapeutic drugs which do not appear to form glutathione conjugates or require co-transport with GSH (Tew *et al.,* 1994 and Loe *et al.,* 1996b), can bind to both the G- and the D-sites (Borst *et al.,* 1999). Therefore, modification of the efflux of these chemotherapeutic drugs requires binding to both the G- and the D- site by indomethacin also. Or if indomethacin only binds to the D-site there still may be some transport of adriamycin and VP-16 attached to the G-site.

4.5 Investigation of potentiation of taxol and taxotere in the CORL23 parental and resistant cell lines.

Taxanes are a new class of anti-mitotic anticancer drugs with a unique mechanism of action (Van Ark-Otte *et al.,* 1998). Unlike vinca alkaloids, a group of antimicrotubule drugs which act mainly as spindle poisons and inhibit the polymerisation of tubulin, taxanes promote the polymerisation of tubulin (Jordan *et al.,* 1993). Microtubules formed in the presence of taxanes are stable and non-functional, leading to cell death by disruption of the normal microtubule dynamics required for cell division and vital interphase processes (Van Ark-Otte *et al.,* 1998). Taxol (paclitaxel), a compound derived from the bark of *Taxus brevifolia*, and taxotere (docetaxol), a semi-synthetic taxane extracted from the needles of *Taxus baccata* have both been shown to have significant activity in ovarian breast and non-small cell lung cancer (Rowinsky *et al.,* 1995).

Previously, our group reported that the toxicity of taxol (paclitaxel) was not potentiated in A549 cells when in combination with the active NSAIDs (Duffy *et al.,* 1998). However, when taxol was analysed in the CORL23 cells in combination with sulindac, indomethacin and BRI 153/1, the toxicity of the drug was increased in both the resistant and the sensitive variants. Taxol has been reported to be a substrate for Pgp (Germann, 1993). Cole *et al.,* (1994), reported that when two different eukaryotic expression vectors containing MRP complementary DNA, were transfected into HeLa cells, the drug resistance patterns of the two MRP-transfected cell populations were similar and demonstrated a low (≤3-fold) level of resistance to taxol. More recently

there have been reports suggesting that these taxanes may be better MRP substrates than first believed (Vanhoefer *et al.,* 1996 and 1997). Vanhoefer *et al.,* (1996), demonstrated the ability of PAK-104P (see section 1.6.9) to completely reverse taxol resistance in HL60/ADR cells and concluded that this effect was due to an interaction with MRP1. PAK-104P was also shown to restore sensitivity to both taxol and taxotere (docetaxel) in MRP-expressing HT1080 (human sarcoma) tumour xenografts (Vanhoefer *et al.,* 1997). The authors also reported that taxotere (docetaxol) at its Maximum Tolerated Dose (MTD) was more active against MRP-expressing tumour xenografts than taxol and that the observed resistance to taxol and taxotere appears to be related to MRP.

Combination toxicity assays were carried out in the CORL23 cells with taxotere in combination with sulindac. The results also demonstrated potentiation of taxotere by sulindac in both the resistant and parental CORL23 cell lines. Slightly greater potentiation of this chemotherapeutic drug was observed in the resistant cell line than in the parental CORL23 cells which is most likely due to the higher expression of MRP1 in the resistant CORL23 cells. However, there was no significant difference between the potentiation of taxol by indomethacin, sulindac and BRI 153/1 in the resistant and the sensitive CORL23 cell lines.

Indomethacin and sulindac were unable to potentiate the toxicity of a range of chemotherapeutic drugs in Pgp over-expressing cell lines, such as DLKPA (Duffy *et al.,* 1998). Kool *et al.,* (1997), did not detect mRNA expression for Pgp, MRP2, MRP3 in the CORL23 parental or resistant cells and there was only a very low expression of MRP4 and MRP5. Therefore, it appears that the enhancement of taxol and taxotere by indomethacin and the other active NSAIDs is MRP1-related.

These results were extremely interesting as it was the first time the potentiation of taxol and taxotere by the active NSAIDs, indomethacin and sulindac (at non-toxic concentrations), had been demonstrated. Soriano *et al.,* (December 1999) reported the ability of sulindac sulfone (exsulind), a metabolite of sulindac which does not inhibit COX enzymes, to potentiate the toxicity of taxotere, VP-16 and cisplatin in a number of non-small cell lung cancer cell lines. However, the concentrations of sulindac sulfone used by the authors to obtain potentiation of cisplatin and taxotere toxicities were quite toxic

concentrations (100-400μM) as compared to concentrations of sulindac (< 25μM) used in this thesis.

Van Ark-Otte *et al.,* (1998), reported that taxol and taxotere achieved greater cell kill in a non-small cell lung cancer (NSCLC) cell line, SW-1573, a human breast cancer cell line, MCF-7, and an adenosquamous NSCLC cell line NCI-H322, than adriamycin and cisplatin at similar concentrations. Au *et al.,* (1998), have reported that taxol is one of the most important anticancer drugs developed in the past two decades as it has shown impressive activity against human solid tumours i.e. ovarian, head and neck, bladder, breast and lung cancers. Taxotere had been reported by a number of authors including Vanhoefer *et al.,* (1997), and Van Ark-Otte *et al.,* (1998), to be even more potent than taxol. Therefore, the taxanes appear to be the more promising choice of treatment for a wide range of cancer types. The implications of potentiating the toxicity of taxol and taxotere are very exciting for the future treatment of cancer.

However, to assess that the potentiation of taxol and taxotere was not specific to the CORL23 cell lines, the ability of indomethacin and sulindac to potentiate the toxicity of taxol and taxotere was assessed in a range of cancer cell lines as described in sections 4.6 and 4.7.

4.6 Investigation of chemotherapeutic drug enhancement by sulindac and indomethacin in DLKP, A549 and MCF-7 cell lines.

Duffy *et al.,* (1998) and Elliot (1997), both in our laboratories, had reported that the active NSAIDs were not able to potentiate the toxicity of taxol in A549 cells. However, results from sections 3.6 and 3.8 demonstrated the ability of indomethacin, indomethacin analogue BRI 138/1 and sulindac to potentiate the toxicity of both taxol and taxotere in the CORL23 and the 2008 cell lines. A549 overexpresses MRP1 and combination assays carried out by Duffy *et al.,* (1998), and Elliot (1997), in A549 cells, using other MRP1 substrate, chemotherapeutic drugs, in combination with the active NSAIDs, demonstrated potentiation of these drugs by the NSAIDs. To investigate the non-potentiation

of taxol by NSAIDs in the A549 cells a range of cancer cell lines were investigated to determine if perhaps the effect with taxol and taxotere was cell line specific.

The cell lines investigated included the poorly differentiated human lung cancer cell line, DLKP, a breast cancer cell line, MCF7, and two A549 cell lines. One of the A549 cell lines was obtained from the culture collection in the NCTCC (originally from the ATCC) and the other was obtained from an external source to investigate if different drug resistance profiles were observed in the same cell line from different sources. Initial toxicity assays were carried out on the cell lines and the IC_{50} values obtained for adriamycin, taxol and taxotere were identical for both the A549 cell lines.

Combination toxicity assays were carried out on the four cell lines with adriamycin, taxol or taxotere in combination with indomethacin, and taxol or taxotere in combination with sulindac. The results demonstrated that indomethacin was able to potentiate adriamycin in all four cell lines with the greatest potentiation observed in the DLKP cells. MRP1 mRNA expression was demonstrated in all four cell lines (Duffy *et al.,* (1998); Lorraine O'Driscoll, personal communication, and section 3.16) and this expression correlated with MRP1 protein expression in these cell lines (Dr. Lisa Connolly, personal communication). Therefore, these results, and evidence presented in previous sections of this thesis, strongly support the hypothesis that the potentiation of adriamycin by indomethacin in these cell lines was as a result of an interaction with MRP1.

There was poorer potentiation of taxol and taxotere by indomethacin in the four cell lines as compared to the magnitude of the potentiation of adriamycin by indomethacin in the same cell lines. Low, but significant enhancement of taxol and taxotere toxicity by indomethacin was observed in the DLKP cells. Low enhancement of taxotere, but not of taxol toxicity was observed in the MCF7 cell line. There was no potentiation of taxol or taxotere toxicity by indomethacin in either of the A549 cell lines.

In contrast there was very strong potentiation of taxol and taxotere by sulindac in the DLKP cell line. There was lower but significant potentiation of these two chemotherapeutic drugs by sulindac in the MCF7 cell line. These results indicate that in these cell lines sulindac was a better potentiator of taxol and

taxotere toxicity than indomethacin. This increased potentiation by sulindac was also observed in the CORL23(R) cell line (section 3.6) and in the 2008 cell lines (section 3.10).

However, there was no potentiation of taxol or taxotere by sulindac in either the A549 cell lines. This supports findings by Duffy *et al.*, (1998) and Elliot (1997). Therefore, it appears that the potentiation of taxol and taxotere by the active NSAIDs, including indomethacin and sulindac, is cell line specific. All cell lines had previously been shown to express MRP1. RT-PCR analysis also demonstrated higher levels of MRP2, MRP3, MRP4 and MRP6 in the A549 cells as compared to the DLKP cell line (section 3.16). A549 (NCTCC) cells have been shown by RT-PCR (section 3.16) to express very high levels of MRP4 mRNA as compared to MRP4 levels expressed in DLKP cells (11-fold higher expression) and the 2008 cell lines (at least 4-fold higher expression). Expression of MRP4 protein levels in this cell line has not been determined, as there was no commercially available MRP4 antibody.

MRP4 has been reported to be directly linked to the efflux of nucleoside monophosphate analogs from mammalian cells (Schuetz *et al.*, 1999) (see section 1.6.9.4). It is hypothesized that taxol and taxotere may act as GTP mimics due to the following facts:

i. Taxol stabilizes microtubules, and microtubule function is GTP-dependent (Caudron *et al.*, 2000; Phelps *et al.*, 2000 and Martin *et al.*, 2000). It is possible that taxol competes with GTP at a specific binding site.
ii. Taxol and taxotere have been shown to alter the phosphorylation status of proteins in the apoptotic pathway (e.g. bcl-2) (Wang *et al.*, 2000 and Kalechman *et al.*, 2000) suggesting that the taxanes may interfere in the phosphorylation mechanisms (mainly involving ATP and GTP) in cells.

Therefore, it is possible that taxol/taxotere are 'seen' by the MRP binding site as nucleoside analogues, transported by MRP4. Perhaps when the NSAIDs are combined with taxol and taxotere in the A549 cells the ability of MRP1 to pump taxol and taxotere is reduced but this is overcome by the activity of MRP4. In addition, the active NSAIDs may not be effective inhibitors of MRP4 activity. However, it has not yet been determined if indomethacin is a

substrate for MRP4. The complete range of substrates for MRP4 has yet to be elucidated (section 1.6.9.4).

Interestingly, Theodossiou *et al.,* (1998), reported that a nucleoside-based drug, gemcitabine, when combined with taxol, could antagonise the effect of the chemotherapeutic drug. The authors reported that taxol imposed a G2/M block, while gemcitabine blocks entry into S-phase. Cells that were delayed during S-phase by gemcitabine could not proceed through the cell cycle and, therefore, the cytotoxic effects of taxol, which required entry into mitosis, were diminished. If taxol is 'seen' by MRP4 as a nucleoside analogue, it might be expected that both gemcitabine and taxol would be transported by MRP4, and would therefore, act as competitive substrates for this transport pump, resulting in synergism between both agents. However, antagonism of taxol cytotoxicity was observed when combined with gemcitabine. It is possible that gemcitabine and taxol compete for some, as yet uncharacterised, uptake mechanism.

Further investigations in to the range of substrates for MRP4 and the mechanism of transport is required to fully understand the contribution (if any) by MRP4 to taxol and taxotere resistance. Interestingly, there are higher levels of MRP5 mRNA expressed in the DLKP cells and the 2008 parental and MRP1-transfected cell lines than in the A549 cell line (section 3.16). The best potentiation of taxol and taxotere by sulindac were observed in these cell lines (section 3.8 and 3.10). Again, the substrates for MRP5 have yet to be determined (section 1.6.9.5) but it is possible that there may be a connection between this expression of MRP5 and taxol/taxotere potentiation.

Soriano *et al.,* (1999), reported the ability of sulindac sulfone to enhance the toxicity of taxotere in A549 cells. However, as stated previously in section 4.5, the concentrations of sulindac sulfone which were used to potentiate the toxicity of taxotere (100μM-400μM) were greater than the highest non-toxic concentrations of sulindac used in the combination toxicity assays outlined in this thesis (< 23μM).

4.7 Investigation of chemotherapeutic drug enhancement in 2008, an ovarian carcinoma cell line, transfected with MRP1, -2 and -3.

There is much evidence to support the hypothesis that indomethacin, positive indomethacin analogues and other positive NSAIDs, such as sulindac, enhance the toxicity of a number of chemotherapeutic drugs through an interaction with MRP1. It is not known, however, if these NSAIDs are capable of this potentiation in cell lines expressing MRP2 - 6.

The results from the experiments described in sections 4.5 and 4.6 demonstrate an enhancement of toxicity of taxol and taxotere in a number of MRP1-expressing cell lines. However, in A549 cells, where MRP1 was also expressed sulindac and indomethacin were unable to potentiate the toxicity of taxol and taxotere, even though there was strong potentiation of adriamycin by indomethacin in the A549 cell line. As RT-PCR analysis demonstrated that there was a very high expression of MRP4 in the A549 cells (section 3.16) it was postulated that perhaps the expression of other homologues of MRP might modify in some way the potentiation of the chemotherapeutic drug by the NSAIDs. These findings raised the question as to whether or not the potentiation of taxol and taxotere was due to an interaction with MRP1 only or if it was possible to potentiate the toxicity of these drugs in cell lines expressing homologues of MRP other than MRP1.

4.7.1 MRP homologues and chemotherapeutic drug resistance

To determine if indomethacin, sulindac and indomethacin analogues could potentiate the toxicity of a range of anticancer drugs in cell lines which overexpressed MRP2 or MRP3, an ovarian carcinoma cell line, transfected with cDNA for MRP1, MRP2 or MRP3 and kindly donated to us by Dr. Marcel Kool from the Netherlands Cancer Institute, Amsterdam, was used.

ong-term	Adr.	MTX	Taxol	Taxotere	Cisplatin
2008 P	1.0	1.0	1.0	1.0	1.0
008 MRP1	5.6	1.0	0.4	1.0	0.3
008 MRP2	0.5	1.0	0.4	1.0	0.1
008 MRP3	0.9	1.0	0.4	1.0	0.2

Table 4.7.1a: Fold resistance values for chemotherapeutic drug in 2008 transfected cell lines relative to the parental 2008 cell line in the Long-term toxicity assay (section 2.7.1a).

Short-term	Adr.	MTX	Taxol	Taxotere
2008 P	1.0	1.0	1.0	1.0
2008 MRP1	3.0	86.8	2.0	0.1
2008 MRP2	1.6	30.1	1.0	1.0
2008 MRP3	3.0	85.4	2.0	0.5

Table 4.7.1b: Fold resistance values for chemotherapeutic drug in 2008 transfected cell lines relative to the parental 2008 cell line in the Short-term toxicity assay (section 2.7.1b).

Toxicity assays and combination toxicity assays were carried out on the cells using a range of both NSAIDs and chemotherapeutic drugs. Initial long-term toxicity assays (6-day exposure to drug) on these cell lines using methotrexate (MTX) failed to demonstrate any difference in resistance levels between the parental and the MRP transfected cells. However, short-term MTX toxicity assays (4-hour exposure to drug) on the 2008 cells showed 2008 MRP1 and 2008 MRP3 to be almost 100-fold more resistant to MTX than the parental cell line. 2008 MRP2 cells were 30-fold more resistant to MTX than the parental cell line. Kool *et al.,* (1999), and Hooijberg *et al.,* (1999), reported that the transfected 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells showed a marked level of resistance to the polyglutamatable antifolate, methotrexate. These results were obtained by analysing the cells using a short-term toxicity assay as described in section 2.7.1b, in which the cells are exposed to the anti-cancer drug for 4-hours only, as opposed to a 6-day exposure in the long-term toxicity

assay (2.7.1a). The authors suggest that the difference in toxicity of MTX between the two assays was due to the fact that MTX is polyglutamylated after entering the cell. The polyglutamylated form is an effective inhibitor of dihydrofolate reductase. Polyglutamylation of MTX is slow and after short-term exposures to MTX, cells with a good MTX efflux pump can pump out the monoglutamate MTX. These cells will accumulate much less long-chain polyglutamates of MTX. In continuous exposures to MTX, a good efflux pump for MTX makes no difference, because these cells will eventually accumulate enough polyglutamylated MTX to block the dihydrofolate reductase efficiently, resulting in cell death.

As a result of these findings, both long-term and short-term toxicity assays were carried out with a number of chemotherapeutic drugs in the 2008 parental (2008 P) and transfected cells. These cells showed greater resistance to adriamycin, methotrexate, taxol and taxotere in short-term assays versus long-term assays (table 4.11.2).

Resistance to adriamycin was greatest in the 2008 MRP1 cell line in the long-term assay but in the short-term assay both 2008 MRP1 and MRP3 were equally resistant to adriamycin. MRP1 has been associated with adriamycin resistance (Cole *et al.,* 1992; Cole *et al.,* 1994; Zaman *et al.,* 1993; Loe *et al.,* 1996a). Protein analysis of the 2008 cell lines indicated that although there was basal expression of MRP1 in all the 2008 cell lines (Dr. Lisa Connolly, personal communication) there was a much higher level of MRP1 protein expression evident in the 2008 MRP1 cell line which accounts for the higher resistance to adriamycin in this cell line over the other 2008 cells in the long-term assay. Perhaps, the equal resistance to adriamycin in the short-term assay is due to the expression of MRP1 in both 2008 MRP1 and 2008 MRP3 cells which, although the expression is higher in 2008 MRP1 cells, functions at a similar level in both 2008 MRP1 and MRP3 cells in short-term exposure assays. However, the results may also indicate that MRP3 is involved in adriamycin resistance and MRP3 involvement in drug resistance has also been suggested by Young *et al.,* (1999).

2008 MRP2 is less resistant to adriamycin than the parental 2008 cells in the long-term assays. RT-PCR analysis of these cells has indicated a decrease in MRP3 and MRP5 mRNA expression in these cells as compared to the parental cell line. The substrate specificities for these transporters remain uncertain but

it is possible that MRP3 and MRP5 contribute to adriamycin resistance in the 2008 cell lines.

	Adr.	MTX	Taxol	Taxotere
2008 P	4.0	2.1	137.0	51.6
2008 MRP1	2.1	188.1	670.3	3.92
2008 MRP2	13.0	61.3	329.3	52.4
2008 MRP3	13.0	174.1	642.6	24.4

Table 4.7.2: Fold resistance values for chemotherapeutic drug in 2008 parental and transfected cell lines in the Short-term toxicity assay relative to resistance of these chemotherapeutic drugs in the parental and transfected 2008 cell lines in the Long-term toxicity assay (section 2.7.1a).

There was only a 4-fold increase in resistance to taxotere in the 2008 MRP1 cells in the short term assay relative to the long-term assay and these cells were at least 6-fold less resistant to taxotere than the other 2008 cell lines. In contrast there was highest resistance to adriamycin, taxol and methotrexate in the 2008 MRP1 cells. Vanhoefer *et al.,* (1997), demonstrated that both taxol and taxotere were substrates for MRP1 (section 4.6) in MRP1-expressing HT1080/DR4 cells. The authors also reported that taxotere was not as readily transported by MRP1 as taxol leading to an increased therapeutic ratio in MRP1-expressing tumours *in vivo*. They suggested that taxotere may have therapeutic advantages in the clinical treatment of MRP-expressing tumours. Therefore, the lower resistance to taxotere in the 2008 MRP1 cells may be due to the fact that taxotere is a poorer MRP1 substrate than taxol.

4.7.2 Problems associated with determining resistance profiles of transfected cell lines

Kool *et al.,* (1999) and Borst *et al.,* (1999), have reported a number of problems observed when studying cells transfected with MRP. The authors reported that it has been difficult to get MRP transfectants giving high expression and product routed to the cell membrane. Transfected cells produce a transporter that is trapped in an endosomal compartment and does not reach

the plasma membrane (Borst *et al.,* 1999). The problem was illustrated by data for MRP2. Some cell lines transfected with a retroviral MRP2 construct did not express the protein at all, or in a patchy fashion with only 10% of the cells positive. This 10% was maintained after cloning and was thought to reflect a deleterious effect of the transporter on cell viability Some of the initial transfection constructs produced low levels of transporter (Borst *et al.,* 1999). Evers *et al.,* (1998), reported that intra-cellular routing of MRP2 is also sensitive to growth conditions and in kidney cells or hepatocytes, MRP2 only goes to the plasma membrane if cells are in contact with one another.

In the case of 2008 MRP3, Kool *et al.,* (1999), reported that they were not successful in isolating non-polarized cells with high MRP3 levels. As a result it was difficult for the author to fully determine the range of compounds transported by MRP3. The transfection of 2008 cells with MRP1 cDNA was the most successful of the three transfectants (Kool *et al.,* 1999 and Marcel Kool, personal communication). It is possible that the large increase in resistance to adriamycin, methotrexate, taxol and taxotere in the 2008 MRP2 and MRP3 cells is due to the fact that even though there may not be high expression of MRP2 and MRP3 in the 2008 transfected cells, there is sufficient protein expressed, to effectively pump the anticancer drugs from the cell in the short exposure time (4hrs), in the short-term toxicity assay. Whereas in the long-term assays the low levels of MRP2 or MRP3 in the cells are insufficient to effectively efflux the chemotherapeutic drug from the cells over a long period of time.

In addition, most cells contain endogenous, organic anion transporters, resulting in background transport activity. As the endogenous transporters are often uncharacterised and, hence, undetectable by antibody it is difficult to find a solution to this problem (Borst *et al.,* 1999). The 2008 parental cells express basal levels of MRP1 protein and significant levels of MRP1 mRNA. Although it is possible that the drug resistance observed in the 2008 cells may be as a result of the transfected MRP2 or MRP3, due to the basal levels of MRP1 in the parental cells, attempts to relate the resistance profile of such cells to a single transporter is risky.

Borst *et al.,* (1999), also report that cells transfected with MRP1, MRP2 or MRP5 cDNAs secrete GSH in to the medium, suggesting that they must be

producing GSH at a high rate to counteract this drain. This suggests that, if the mechanism by which the MRP substrates are transported is through conjugation or in conjunction with GSH, there may not be sufficient GSH present in the cells to support transport of MRP substrates from the cells. Therefore, it may be difficult to assess the complete range of substrates for cells transfected with MRP1 or MRP2. Cells with high levels of MRP3 do not secrete GSH (Kool *et al.,* 1999) which may limit the ability of MRP3 to transport non-anionic organic molecules.

4.7.3 Investigation of the potentiation of adriamycin and methotrexate toxicity in the 2008 cell lines.

In the combination toxicity assays, indomethacin was shown to potentiate the toxicity of adriamycin in all 2008 cell lines and in particular in 2008 MRP1, in the long-term assay. This potentiation was not so notable in the short-term assay with the best potentiation also observed in 2008 MRP1 cell line. Results obtained from HPLC experiments on DLKP cells (section 2.5) indicated that when indomethacin and adriamycin were removed from the medium after a two-hour loading period, indomethacin was effluxed from the MRP expressing cells very rapidly (< 2 hours). This was followed by a rapid efflux of adriamycin from the cells. However, when the level of indomethacin was maintained in the medium there was much reduced efflux of adriamycin from the cells. Hence, it appears that maintaining indomethacin in the cells and in the medium surrounding the cells was required for the enhancement of the toxicity of the anti-cancer drug. In the long-term combination toxicity assays the concentration of indomethacin in the medium and cells remained constant throughout the total incubation period (6 days). In the short-term combination toxicity assays, indomethacin was removed from the medium after four hours. It is possible that as there was no indomethacin in the medium to maintain the required levels of indomethacin in the cell there was an increased efflux of adriamycin from the cells. The positive indomethacin analogue, BRI 138/1 was also shown to potentiate adriamycin in the 2008 cells, in the long-term assay at a similar rate to indomethacin, with greatest potentiation in the 2008

MRP1 cells. These results indicate that this indomethacin analogue functions similarly to indomethacin.

Both Kool *et al.,* (1999), and Hooijberg *et al.,* (1999), reported that MRP1, MRP2 and MRP3-overexpression is associated with resistance to short-term exposure of polyglutamatable antifolate drugs such as MTX. Due to the pharmacological characteristics of methotrexate, as discussed previously, it was not surprising to observe very little enhancement of MTX toxicity by indomethacin in the 2008 cells in the long-term assays. However, there was potentiation of methotrexate by indomethacin in the MRP1-transfected cell line. Perhaps this indicates that indomethacin is a better substrate for MRP1, and to a lesser extent MRP3, than MRP2 in long-term assays. In the short-term assays the potentiation of methotrexate was greatest in the parental 2008 cell line. This may be due to the fact that the IC_{50} of methotrexate in the parental cell line was only 10μg/ml compared to 900μg/ml for both 2008 MRP1 and MRP3 and 300μg/ml for 2008 MRP2. Work outlined earlier indicates that indomethacin enhances the toxicity of MRP substrates by acting as a competitive substrate for MRP, i.e. indomethacin and other such NSAIDs compete with the anticancer drug for preferential pumping from the cell by the MRP pump. In our previous combination toxicity experiments indomethacin was always used at much higher concentrations in the cells than the anti-cancer drugs (μg quantities versus ng quantities). Perhaps the potentiation effect is greater in the parental cell line in the short-term assay due to the lower concentrations of methotrexate used in these cells and the concentrations of indomethacin present in the parental cell line were sufficient to compete with methotrexate for MRP-efflux from the cell. There was very little potentiation of the toxicity of methotrexate in the 2008 MRP3 and no potentiation in the 2008 MRP2 cells (Table 3.7.2b). In the transfected cell lines the concentration of methotrexate used in the cells was 100-fold greater than the concentration of indomethacin in the cells. This indicates that indomethacin was not capable of competing successfully for preferential pumping by MRP and as a result methotrexate was pumped out of the cells at a greater rate. In addition, as mentioned previously, a four-hour exposure of the cells to indomethacin may not be sufficient to sufficiently reduce the efflux of methotrexate from the cells. The results for 2008 MRP1, MRP2 and MRP3 cells may also suggest that

indomethacin is a better substrate for MRP1 than MRP2 and MRP3 and hence is better capable to preferentially pump indomethacin before methotrexate.

A number of authors, including Hooijberg *et al.,* (1999), and Bakos *et al.,* (2000), have reported that methotrexate is a particularly good substrate for MRP2. However, Bakos *et al.,* (2000), reported that low concentrations of indomethacin (< 100μM) enhanced the efflux of N-ethylmaleimide glutathione (NEM-GS), an MRP1 and MRP2 substrate, in S*f*9 cells transfected with a recombinant baculoviruse containing MRP2 cDNA. Indomethacin was unable to potentiate the toxicity of methotrexate in the 2008 MRP2-transfected cell line. Therefore, as a result of the above findings by Bakos *et al.,* (2000), it is possible that indomethacin was interacting with MRP2 to increase the efflux of methotrexate from the 2008 MRP2 cell line. These results suggest that, in the case of methotrexate, there are differences in transport properties for this drug in each of the 2008 cell lines which may indicate that the expression of MRP1 does not account for all of the potentiation effects observed in the 2008 cell lines.

Results from the combination toxicity assays in the 2008 cell lines using adriamycin or methotrexate in combination with sulindac, yielded similar results to those obtained when these anti-cancer drugs were used in combination with indomethacin. The best potentiation of both chemotherapeutic drugs was observed in the 2008 MRP1 transfected cells. However, there was also a small but significant potentiation of methotrexate by sulindac in the 2008 MRP2 cell line which indicates that sulindac does not behave similarly to indomethacin in this particular cell line. It appears that sulindac does not function to enhance the efflux of methotrexate in cells lines overexpressing MRP2 and that this effect is specific to indomethacin. It was also observed that sulindac, like indomethacin, required a longer incubation period in the cells than the 4hrs incubation period of the short-term assay, for optimum potentiation of the chemotherapeutic drugs. The results also suggest that MRP2 and MRP3 are poorer transporters of sulindac than MRP1.

4.7.4 Investigation of potentiation of taxol and taxotere in the 2008 cell lines.

The combination toxicity assay results for taxol and taxotere in combination with sulindac indicated that long-term exposure to sulindac resulted in better potentiation of these chemotherapeutic drugs by this NSAID. The strongest combination effect was, as before, observed in the MRP1-transfected cells. However, the results obtained with taxol were surprising as sulindac was able to potentiate the toxicity of taxol to a greater extent than the other three chemotherapeutic drugs analysed. In addition, when taxotere was combined with the NSAID piroxicam, in the 2008 cells, no potentiation of this chemotherapeutic drug was observed. Duffy *et al.,* (1998), had found that piroxicam was not effective in potentiating the toxicity of chemotherapeutic drugs in any of the cell lines analysed. The authors postulated that this NSAID was not a substrate for MRP. However, when piroxicam was used in combination with taxol there was a statistically significant potentiation of the anti-cancer drug in the 2008 cell line. These results suggest the presence of an additional mechanism of enhancement of taxol toxicity or that taxol/taxotere use a different or modified active site on MRP. Moos *et al.,* (1999), indicated that taxol (but not taxotere) increased COX-2 (but not COX-1) protein and mRNA expression in RAW 264.7 murine macrophages. The authors also report that taxol also induced COX-2 in human and murine monocytes via a p38 mitogen-associated protein kinase pathway. Taxotere was reported to induce COX-2 protein expression in human monocytes only. A number of studies have reported that truncation mutations of the APC gene occur somatically in a large percentage of colorectal cancers (Sheng *et al.,* 1998; Oshima *et al.,* 1996). This mutation in the APC gene has been associated with increased levels of COX-2 and a decrease in apoptosis in a number of cancer cell lines (section 1.7.9) (Sheng *et al.,* 1998; Kargman *et al.,* 1995; Kutchera *et al.,* 1996 and Watson *et al.,* 1997). Indomethacin, sulindac and piroxicam are recognised as being non-selective NSAIDs, inhibiting both COX-1 and COX-2 (Riendeau *et al.,* 1997 and Hinz *et al.,* 1999). It is suggested that the inhibition of COX-2 by the active NSAIDs, in addition to an interaction with MRP, may explain the increased enhancement of taxol toxicity in the 2008 cell lines. Piroxicam has no MRP inhibitory ability so the slight potentiation of taxol toxicity by

piroxicam may be due to inhibition of COX-2, resulting in an increase in apoptosis in the 2008 cell lines. The possibility that this effect is cell line specific cannot be ruled out as the combination of taxol/taxotere and piroxicam was only analysed in the 2008 cell lines. It is necessary to carry out this combination in a number of different cell lines to assess if this potentiation of taxol toxicity by piroxicam can be demonstrated. In addition, combination toxicity assays using COX-2 specific inhibitors in combination with taxol and taxotere would also be interesting. A recent publication by Hida *et al.,* (2000), reported the ability of a COX-2 inhibitor, nimesulide, to enhance the toxicity of taxotere in four NSCLC cell lines. The potentiation of taxol toxicity was not assessed. Although the results in this thesis only demonstrated the potentiation of taxol by piroxicam, this result was observed in one cell line only so it is possible that assessment of a range of cell lines may show potentiation of both taxol and taxotere toxicities by COX-2 inhibitors. The results serve to demonstrate the presence of a complex mechanism by which taxol and taxotere may be potentiated by NSAIDs.

4.8 Investigation of the effect on cisplatin toxicity of indomethacin and possible correlation between MRP2 (cMOAT) expression and cisplatin resistance.

A number of authors including Cole *et al.,* (1992), and Loe *et al.,* (1996), reported that MRP1 did not confer resistance to platinum-containing compounds such as cisplatin and carboplatin. Cisplatin is an anticancer drug used widely for the treatment of various cancers. However the development of resistance to its cytotoxic effect is a major problem in its clinical use. Cisplatin binds to the N7 position of guanosine and forms intrastrand and interstrand cross-links. In addition to its direct binding to DNA, the other cellular effects of cisplatin that have been reported include disruption of the mitochondrial membrane potential, depolymerisation of the microtubules and collapse of the intermediate filament network (Parekh *et al.,* 1995).

cMOAT, a 190kDa membrane glycoprotein and a GS-X pump was first recognised by Tanaguchi *et al.,* (1996), as a member of the MRP family which shows 46% similarity to human MRP1. cMOAT mediates the ATP-dependent

transport of various hydrophobic anionic compounds in liver canalicular membranes and other tissues (Koike *et al.,* 1997). The spectrum of hydrophilic anionic compounds transported by cMOAT resembles that of MRP (Roelofsen *et al.,* 1999). The function of cMOAT in drug transport and cancer is at present unclear.

Tanaguchi *et al.,* (1996), postulated that human cMOAT may function as a cellular cisplatin transporter, as expression of human cMOAT was enhanced in these cisplatin resistant cell lines with decreased cellular accumulation of cisplatin (section 1.6.8). Kool *et al.,* (1996), also demonstrated a correlation between cMOAT transcript levels and sensitivity to cisplatin. However, there are conflicting theories concerning the function of cMOAT in clinical resistance and, in particular, its relationship with cisplatin resistance. Tanaguchi *et al.,* (1996) and Koike *et al.,* (1996), acknowledge that their results are not conclusive and agree with authors such as Oguri *et al.,* (1998), who observed no association between ante-mortem platinum drug exposure and steady state cMOAT mRNA, and Kauffman *et al.,* (1997), who reported that the increased expression of cMOAT mRNA, after exposure to platinum drugs, may be part of the normal stress response to xenobiotics.

4.8.1 Investigation of the potentiation of cisplatin toxicity in 2008 cells transfected with MRP1, -2, -3 and the HepG2 cell line which constitutively expresses MRP1, -2, -3.

Duffy *et al.,* (1998), reported that cisplatin toxicity was not potentiated in a number of MRP1 overexpressing cell lines, A549 and DLKP. The results from the long-term toxicity assays in the 2008 cell lines with cisplatin were interesting. Transfecting MRP1, MRP2 or MRP3 cDNA in to the 2008 parental cell line resulted in a decrease in resistance to cisplatin (table 3.8.1a). The greatest decrease in resistance was evident in the 2008 MRP2 transfected cell line. The reasons for the decrease in resistance to cisplatin in the 2008 transfected cell lines are uncertain. RT-PCR analysis (section 3.16) of the 2008 cell lines indicated a decrease in the expression of MRP5 mRNA in the transfected cell lines relative to the parental cell line. The expression of MRP5 was increased slightly in three cell lines selected for cisplatin resistance

(T24/DDP10, HCT8DDP and KCP-4) (Kool *et al.,* 1997). Therefore, MRP5 may be involved in cisplatin resistance but there is little evidence in the literature to support this hypothesis.

Combination toxicity assays were carried out in the 2008 cell lines, with cisplatin in combination with indomethacin and the positive indomethacin analogue 138/1, to assess the ability of these compounds to enhance the toxicity of cisplatin in cell lines expressing MRP1, -2, or –3. The results (section 3.8) demonstrated that there was no potentiation of cisplatin in any of the 2008 cell lines except for a very low level of synergism in the 2008 MRP3 cell line (CI = 0.930) when used in combination with BRI 138/1. This may indicate that cisplatin is a weak MRP3 substrate. Young *et al.,* (1999), reported highly significant correlation between MRP3 and resistance to vincristine, etoposide and cisplatin. However, to date, there have been no other publications to support these findings and the substrate specificities for MRP3 remain uncertain. The accurate range of substrates for each transport protein cannot be elucidated until cell lines are generated which express single transporters, without background expression of other transporters as discussed in section 4.7.

The human hepatic cancer cell line, HepG2, was found to express high levels of cMOAT mRNA and protein (Narasaki *et al.,* 1997; Koike *et al.,* 1997), and antisense transfection work with cMOAT in this cell line, resulted in the cell line becoming more sensitive to a range of drugs including cisplatin (section 1.6.9.1). There was also an increased level of glutathione in the antisense transfectants indicating that the levels of cMOAT were inversely correlated to levels of glutathione. RT-PCR analysis carried out on this cell line (section 3.16) demonstrated the expression of MRP1, MRP2, MRP3 and MRP4 but not MRP5 mRNA. The expression of MRP6 mRNA in this cell line was not investigated.

Combination toxicity assays were carried out on HepG2 cells to assess if the potentiation of adriamycin by indomethacin was also observed in a cell line which constitutively over-expresses MRP2. These assays were also carried out to investigate if cisplatin could be potentiated by indomethacin in this cell line which constitutively over-expresses MRP2 and has also been shown by RT-PCR (section 3.16) to express MRP3 and MRP4 mRNA. In addition to

combination toxicity assays in the HepG2 cell line, concurrent combination toxicity assays were carried out in DLKP cells as a control.

Indomethacin significantly enhanced the toxicity of adriamycin in both the HepG2 and DLKP cell lines which may be attributed to the expression of MRP1 in these cells. However, indomethacin was unable to potentiate cisplatin in both of these cell lines. In fact, indomethacin appears to antagonise cisplatin toxicity in both the DLKP and HepG2 cells, i.e. the cells appeared to become more resistant to cisplatin when combined with indomethacin resulting in an increase in cell survival. This effect was also observed when cisplatin was combined with indomethacin in the 2008 cell lines. Significant differences were observed between the combination index (CI) values obtained for cisplatin in combination with indomethacin and the values obtained for the combination with the indomethacin analogue BRI 138/1 in the 2008 cell lines. Significant antagonism of the toxicity of cisplatin was observed when combined with indomethacin in the 2008 cells. The CI values ranged from 1.345 for 2008 MRP1 to 2.422 for the 2008 parental cell line. In contrast the CI values were approximately 1.0 for the 2008 parental, 2008 MRP1 and 2008 MRP2 cell lines when cisplatin was co-incubated with BRI 138/1. As discussed above, the CI value for cisplatin and BRI 138/1 in 2008 MRP3 was 0.930, which suggested synergism between these two compounds in the 2008 MRP3 cell line. BRI 138/1 is an N-benzyl analogue of indomethacin, as described in section 3.1, with the chlorine in the *para*-position on the benzene ring replaced with fluorine in the *meta*-position. These results suggest that although the structures of indomethacin and BRI 138/1 are similar, changing the indomethacin structure to generate BRI 138/1 also resulted in functional differences between the two compounds. BRI 138/1 is less toxic than indomethacin in the DLKP cells (section 3.1) and could be used in the 2008 cells at higher concentrations than indomethacin, 35μg/ml versus 10μg/ml. The higher concentrations of BRI 138/1 in the 2008 cells might partially explain the lower CI values.

It is also possible that the increase in the resistance to cisplatin is associated with indomethacin alone. Apart from the set of combination toxicity assays carried out in the 2008 cell lines with BRI 138/1 in combination with cisplatin, combination toxicity assays were only carried out in the 2008, HepG2 and DLKPC14 (sections 3.15 and 4.9) cell lines with indomethacin in combination

with cisplatin. Cisplatin reacts readily with compounds containing SH groups (Pratt *et al.,* 1994). Thus, resistance to cisplatin has been found to correlate with increased glutathione content (Behrens *et al.,* 1987 and Parekh *et al.,* 1995). It has been suggested that quenching of the platinum compounds by reaction with nucleophilic SH groups in glutathione may be a major determinant of tumour response to initial therapy. It is interesting that the antagonism to cisplatin toxicity is observed with indomethacin but not with BRI 138/1. Results from the GST assays (section 3.2) demonstrated that indomethacin was a very good GST inhibitor whereas BRI 138/1 was unable to inhibit the activity of this enzyme. However, the relevance of this to the observed antagonism of cisplatin toxicity is unclear.

A study was carried out by Cosolo *et al.,* (1991), on the pharmacokinetics of cisplatin in five cancer patients receiving cisplatin (50-100mg/m^2) in combination with indomethacin (12.5µg/ml). These patients were receiving cisplatin for carcinoma of the ovary, testicular carcinoma or transitional carcinoma of the bladder. The authors reported that there was an increase in free cisplatin in the plasma of these patients when co-treated with indomethacin at 12.5µg/ml. The authors did not offer any explanations for this interaction. They did not investigate the expression of MRP in these cancers and did not investigate the effects of indomethacin on cisplatin toxicity. The results of this study suggest that indomethacin might be capable of increasing the efflux of cisplatin from the cancer cells resulting in increased cisplatin in the plasma. If MRP is present in these cancer types, the results suggest that indomethacin might enhance the ability of the MRP pump to export cisplatin from the cells more rapidly than MRP alone. It remains to be elucidated if cisplatin is a substrate for any of the MRP homologues and what part they play in cisplatin resistance. It is also possible that indomethacin inhibits in some way the uptake of cisplatin into the cells.

Future experiments will include drug efflux experiments to measure both the uptake and the efflux of cisplatin in a number of cell lines, in the presence or absence of active NSAIDs such as indomethacin.

In addition, further experiments are required using cisplatin in combination with a range of active NSAIDs such as sulindac and a number of the active indomethacin analogues (± GST inhibitory activity), to assess if the effect is

indomethacin specific and also to investigate if any of these compounds are capable of potentiating the toxicity of cisplatin.

4.8.2 Correlation between cisplatin resistance and MRP2

Both DLKP and HepG2 cells express MRP1 (section 3.16 and Narasaki *et al.*, 1997). The findings from this thesis seem to suggest that the active NSAIDs may be only capable of potentiating the toxicity of those drugs which are substrates for MRP1. However, it was not possible to determine from these results if indomethacin was capable of potentiating the toxicity of adriamycin by interacting with MRP2. MRP2 is reported to have a similar substrate specificity to MRP1 (Oude Elferink *et al.*, 1995; Keppler *et al.*, 1997 and Roelofsen *et al.*, 1999), and Koike *et al.*, (1997), reported that adriamycin resistance is also conferred by MRP2. Indomethacin is an amphiphilic organic anion so it is possible that indomethacin can interact with MRP2 as well as with MRP1.

However, it is also possible that indomethacin may not have an affinity for the binding site on cMOAT and may not be able to compete with the natural substrates of cMOAT. LTC_4, the GSH conjugate, which is a substrate for MRP1, is also a substrate for cMOAT (Mayer *et al.*, 1995; Koike *et al.*, 1997). However, Keppler *et al.*, (1998, 1999a and 1999b) and Konig *et al.*, (1999), reported that the LTC_4 has greater affinity for MRP1 than cMOAT Similarities in the substrate specificity between MRP1 and cMOAT (Roelofsen *et al.*, 1999), indicate that the binding between protein and substrate in both MRP and cMOAT requires similar conditions. However, the sensitivity of HepG2 cells to etoposide (VP-16), which is a substrate for MRP1, was not increased when transfected with antisense MRP2 cDNA (Koike *et al.*, 1997). In contrast, these transfected cells displayed an enhanced sensitivity to cisplatin, CPT-11, ((4*S*)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino)carbonyloxy]dione-hydrochloride triethydrate), and SN-38, (7-ethyl-10-hydroxycamptothecin) which are reported not to be MRP1 substrates (Koike *et al.*, 1997). This seems to indicate that there are some differences in substrate specificity between the two MRP homologues. If the mode of action of the active NSAIDs is via competitive

inhibition with the chemotherapeutic drugs for an active site on the MRP protein, this difference in substrate specificity may account for the inability of the active compounds to bind to the MRP2 protein and exert their effect.

Versantvoort *et al.,* (1995), demonstrated that glutathione metabolism is a critical element in the drug efflux capabilities of MRP. It was shown that buthionine sulphoximine (BSO), an inhibitor of GSH (reduced glutathione) synthesis caused an inhibition of the drug efflux activity of MRP in MRP-overexpressing cell lines. Loe *et al.,* (1996b) and (1998), have demonstrated the ability of MRP to co-transport vincristine with GSH in membrane vesicles. Perhaps the active NSAIDs and analogues also require co-transport with glutathione to be good MRP substrates. Koike *et al.,* (1997), demonstrated that the stable transfection of HepG2 cells with cMOAT antisense construct led to a marked increase in cellular GSH. Narasaki *et al.,* (1997), have demonstrated, using quantitative RT-PCR, that the highest expression of cMOAT mRNA is found in HepG2 cells. It appears that as a result of the high expression of cMOAT in HepG2 cells there is a low level of GSH in the cells. This low level of GSH may reduce the ability of the active compounds to be effluxed from the cell by cMOAT in preference to cisplatin. However, as discussed previously, resistance to cisplatin has also been associated with increased GSH. Therefore, it appears that cisplatin resistance may not be due to the expression of cMOAT in the cells.

There have been a number of publications suggesting that MRP3 does not secrete GSH (Zeng *et al.,* 1999 and Borst *et al.,* 1999). Therefore, the high GSH content in cells expressing MRP3 may contribute to cisplatin resistance in MRP3 expressing cell lines. Chen *et al.,* (1999), reported that an unknown transporter, distinct from MRP1, MRP2 and Pgp, was expressed in the cisplatin-resistant human epidermoid carcinoma cell line, KCP-4, and was involved in cisplatin transport in these cells. The authors also report the ability of this pump to transport LTC_4, which is an excellent substrate for MRP1 (Leier *et al.,* 1994 and Jedlitschy *et al.,* 1994). It is possible that a member of the MRP family of transporters, other than MRP1 and MRP2, contributes to cisplatin resistance.

4.9 Investigations in to the mechanisms involved in increased cisplatin resistance in DLKP C 14 cells

The DLKPC 14 cell line was developed by Cleary (1995), by continuous exposure of the parental cell line DLKP in varying concentrations of the platinum drug, carboplatin. This drug selection rendered the cells more resistant to a number of chemotherapeutic drugs, in particular, carboplatin (Cleary, 1995) and cisplatin. The toxicity assays carried out on the DLKPC 14 and parental DLKP cells indicate that there was a significant increase in resistance to cisplatin in the DLKPC 14 cells (IC_{50} 3010 ± 156.4 ng/ml) as compared to the resistance of DLKP to the same drug (IC_{50} 480.0 ± 35.4 ng/ml). There was no significant cross-resistance to the chemotherapeutic agents adriamycin and vincristine. The resistant variant showed slight increase in resistance to 5'fluorouracil (approx. 1.2-fold) and VP-16 (approx. 2-fold).

It has been postulated by a number of authors that cisplatin resistance is as a result of the overexpression of cMOAT in the cancer cells (section 1.6.9.1 and section 4.8 and 4.13). Immunocytochemical studies and RT-PCR analysis demonstrated that there was no increase in the expression of MRP1 or Pgp in the DLKPC 14 cells as compared to the parental DLKP cells (Cleary, 1995). These results suggest that resistance to cisplatin in this particular cell line, DLKPC 14, is not by overexpression of Pgp or MRP1. Future RT-PCR experiments will investigate the roles of MRP2, MRP3, MRP4, MRP5 and MRP6 in cisplatin and carboplatin resistance in this carboplatin-selected DLKP cell line.

Cleary (1995), reported that the cisplatin resistant cells display a unique cross-resistance profile to multiple agents including antimetabolites, such as 5'fluorouracil and methotrexate, topoisomerase inhibitors such as camptothecin and etoposide, and DNA polymerase inhibitors such as azidothymidine. The cell lines resistant to cisplatin and mentioned in the literature have been shown to have multifactorial mechanisms of resistance (see section 4.8). Four biological alterations capable of producing cisplatin resistance have been reported and include:

- Decreased cellular accumulation of cisplatin,
- Increased levels of glutathione or increased glutathione-S-transferase activity,
- Increased levels of intracellular metallothionein,
- Enhanced DNA repair,

(Cleary, 1995 and Parekh *et al.*, 1995).

Cleary (1995), carried out a number of experiments to try to determine the mechanism of resistance in the DLKPC 14 cells and found that:

- Glutathione did not appear to play a major role in mediating drug resistance in the cisplatin resistant DLKP cell lines. The addition of BSO, the glutathione biosynthesis inhibitor, to the DLKPC 14 cells did not significantly alter the toxicity profile of carboplatin in these cells.
- GST activity did not play a major role in drug resistance in the DLKPC variants. Cleary (1995), demonstrated a slight decrease in the activity of GST in the DLKPC 14 cell line. Furthermore, the addition of ethacrynic acid, which has been reported by Tew *et al.,* (1988), to cause a reduction in the levels of GST activity and an increase in the cytotoxicity of a number of alkylating agents in resistance cell lines, caused no significant alteration in the cytotoxicity of carboplatin in the DLKPC 14 cells (Cleary, 1995).
- An overexpression of metallothionein in the DLKPC 14 cell line may be involved in mediating drug resistance in these cells
- Topoisomerases did not seem to play a major role in the drug resistance of the DLKPC variants as Western blot and RT-PCR analysis indicated that there was no significant difference in the levels of topoisomerase I and II in the DLKPC 14 cells relative to the parental DLKP cells.

A number of interesting observations were reported by Parekh *et al.,*(1995). The authors reported that a cisplatin resistant human ovarian carcinoma cell line, 2008/C13, contained markedly lower levels (6-fold) of cytokeratin 18,

when compared to the cisplatin sensitive 2008 cell line. They also demonstrated that the transfection of a full length cytokeratin 18 cDNA in to the cisplatin resistant 2008/C13 cell line, resulted in clones with increased levels of cytokeratin 18, which was accompanied in the majority of the clones by a marked increase in their sensitivity to cisplatin. These authors also report that a decreased expression of cytokeratin 14 was reported in cisplatin-resistant variant of a human lung squamous cell carcinoma line and since intermediate filament proteins bind to cisplatin-damaged DNA it is possible that they may play a role in the cytotoxic action of cisplatin.

Combination toxicity assays carried out on the DLKPC 14 cell line using a combination of adriamycin or cisplatin with indomethacin, gave similar results to those obtained from combination toxicity assays carried out on HepG2 cells (section 3.6) and 2008 transfected and parental cells (section 3.8). The results indicate that an enhancement effect was only observed when indomethacin was combined with adriamycin. An antagonistic effect was observed in DLKPC 14 cells treated with cisplatin and indomethacin. These results support the hypothesis that the enhancement of the toxicity of chemotherapeutic drugs by the active NSAIDs, is only observed for those drugs which are substrates for MRP1. An increase in resistance to cisplatin was also observed in the DLKPC 14 cells when this chemotherapeutic drug was combined with indomethacin. Results presented in this thesis have shown that this effect is not cell line specific. The mechanism of this increase in resistance is unclear (see section 4.8) but data presented in this thesis suggest an interesting interaction between indomethacin and cisplatin.

4.10 Investigation of the effect of pulsing HepG2 cells with cisplatin.

HepG2 cells were pulsed with cisplatin for a period of ten weeks to assess the effects on chemoresistance in HepG2 cells due to pulsing with this chemotherapeutic drug. As described in section 1.6.9.1, HepG2 cells were found to highly express both MRP2 mRNA and protein (Narasaki *et al.,* 1997

and Koike *et al.*, 1997). There has been a great deal of conflicting evidence concerning cisplatin as a substrate for MRP (section 1.6.9.1 and section 4.12) and, as in the case of the 2008 cells (section 4.9), it has so far been impossible to obtain a resistant cell line with resistance attributed to one single transport pump to elucidate the substrate specificity of each individual transporter.
Combination toxicity assays carried out in the HepG2 cells, with cisplatin in combination with indomethacin, demonstrated the inability of indomethacin to enhance the toxicity of cisplatin (section 3.7) and in fact the cells seemed to develop greater resistance to cisplatin in the presence of indomethacin as described in section 4.8.

RT-PCR analysis on RNA extracted from the parental HepG2 cell line demonstrated the expression of MRP1, MRP2, MRP3 and MRP4 mRNA in the cells (section 3.16). No expression of MRP5 mRNA was detected and MRP6 expression was not investigated. Narasaki *et al.*, (1997) demonstrated the presence of MRP1 and a low level of Pgp mRNA expression. The results obtained to date support the findings of Narasaki *et al.*, (1997) and Koike *et al.*, (1997). In addition the results presented in this thesis demonstrate the presence of at least two more MRP homologues in the HepG2 cell line, MRP3 and MRP4.

Toxicity assays were carried out on the HepG2 cells after pulsing for four weeks and ten weeks, to assess if resistance to adriamycin, vincristine, VP-16, 5-FU and cisplatin increased or decreased in the pulsed cell lines relative to the parental HepG2 cells. The results were surprising in that the resistance to adriamycin, vincristine and VP-16 was decreased approximately 2.5-fold after pulsing for ten weeks with cisplatin. This reduction in resistance was also evident, but not quite so notable, after pulsing for four weeks. However, in contrast, the resistance in the pulsed HepG2 cells increased 10-fold to cisplatin and 2.5 fold to 5-FU. Due to time constraints, RT-PCR analysis was not carried out on the pulsed HepG2 cell lines. This analysis will be carried out in the immediate future to assess if pulsing the HepG2 cells with cisplatin resulted in changes in the expression of any of the MRP homologues. These results would help to explain the changes in resistance to the chemotherapeutic drugs after pulsing with cisplatin.

Adriamycin, vincristine and VP-16 are substrates for MRP1 (Cole *et al.*, 1994, Duffy *et al.*, 1998), and MRP2 (Koike *et al.*, 1997 and Konig *et al.*, 1999). These results suggest that pulsing with cisplatin resulted in a decrease in a transport mechanism shared by adriamycin, vincristine and VP-16, possibly MRP1. It is possible that 5-FU is a substrate for MRP4 (section 4.11). As there is MRP4 present in the parental HepG2 cells prior to pulsing it is possible that pulsing these cells with cisplatin resulted in an increase in the expression of MRP4 and hence, an increase in 5-FU resistance. Nishiyama *et al.*, (1999), reported that selecting gastrointestinal cell lines with cisplatin (0.1μg/ml), resulted in an increase in GSTπ and dihydropyrimidine dehydrogenase (DPD) expression, which have been associated with 5-FU resistance (section 4.11). It is possible that these genes are also being overexpressed in the HepG2 cells after pulsing with cisplatin for a period of time.

It is not clear what resistance mechanism(s) caused the large increase in resistance to cisplatin after pulsing the HepG2 cells with cisplatin. Again RT-PCR analysis will hopefully help to answer some of the questions. Possibly, pulsing with cisplatin resulted in an increase in MRP2 (cMOAT) expression. If this is the case it could explain the increase in sensitivity to adriamycin, vincristine and VP-16. It is postulated that these anticancer drugs are transported from the cells by MRP1 in a GSH dependent manner. Koike *et al.*, (1997), observed reduced levels of glutathione when cMOAT was expressed in the HepG2 cell line. If cMOAT is overexpressed in these pulsed cells, it is possible that the level of GSH in the cells is also reduced which may result in a reduction of the GSH dependent transport of adriamycin, vincristine and VP-16 from the cells. Further detailed analysis of the pulsed cells is required to determine the resistant mechanisms present in these pulsed cells.

4.11 Investigation of effect of pulsing DLKP cells with indomethacin.

It has yet to be determined which of the MRP homologues transport indomethacin. It is clear that this NSAID is a substrate for MRP1 as determined by experiments carried out in this thesis and previous work carried

out by Duffy *et al.*, (1998) and Elliot (1997). In the 2008 transfected cell lines the true substrates of MRP2 and MRP3 could not be determined as there was background expression of MRP1 mRNA (section 3.16) and protein (Connolly, 1999), in these cell lines. Therefore, DLKP cells were pulse selected with indomethacin to assess if pulsing with this NSAID would result in an increase in expression of MRP1 or the other MRP homologues.

Pulsing DLKP cells with 300μg/ml indomethacin resulted in an increase in resistance to all chemotherapeutic drugs analysed (section 3.11.1 and table 4.11.1) after 4 weeks and a greater increase in resistance after pulsing for 6 weeks. There was also a small but significant increase in resistance to indomethacin in the pulsed cell lines relative to the parental DLKP cells (table 4.11.1). It must be noted, however, that the levels of indomethacin used in this assay are approximately 100-fold greater than the normal pharmacological relevant concentrations (2.5μg/ml) used for indomethacin.

Drug	DLKP	DLKP Indo6
Adriamycin	1.0	1.5
Vincristine	1.0	1.5
VP-16	1.0	2.0
5-FU	1.0	3.8
Cisplatin	1.0	2.0
Indomethacin	1.0	2.0

Table 4.11.1: Fold resistance values for chemotherapeutic drug and indomethacin in DLKP Indo6 cells relative to the parental DLKP cells.

The mRNA expression was analysed in the DLKP cells after pulsing with indomethacin for 4 weeks and 10 weeks to compare the expression of MRP1-6 in these cells with the MRP expression in the parental DLKP cells. The results indicated that there was only a slight increase in expression of MRP1 in the pulsed cells as compared to the parental cells. There was also an increase in MRP2, MRP4 and MRP6 mRNA expression. There were undetectable levels of MRP3 in the DLKP parental and the DLKP pulsed cells. Interestingly, pulsing the DLKP cells for 4-6 weeks resulted in a notable decrease in MRP5

mRNA expression. There was a very low expression of Pgp in the parental DLKP cells which was not increased or decreased in the pulsed cells. Therefore, it is possible that indomethacin is a substrate for MRP1, MRP2, MRP4 and MRP6 or that these MRP homologues all contribute to a resistance mechanism, in DLKP cells, to indomethacin. It is possible also that the increased expression of these MRP homologues are part of a very generalised response, associated with exposure to high toxic doses of indomethacin (300μg/ml) making it difficult to assess which (or any) of the MRP homologues actually transport indomethacin. In the DLKP cell line, indomethacin does not appear to be a substrate for MRP3 or Pgp. It is possible also, that, as in the case of the bcl and bax family of proteins, the upregulation of one of the MRP proteins may result in an upregulation or down regulation of another of the MRP proteins. However, there is no evidence to support this hypothesis. The increase in resistance to adriamycin, vincristine, VP-16 and indomethacin may be attributed to an increase in expression of MRP1 or MRP2, for which these compounds are known substrates (section 1.6). However, the increase in resistance to cisplatin and 5-FU is more difficult to explain as the mechanisms of resistance to these chemotherapeutic drugs are not clear. There has been no conclusive evidence to suggest that expression of MRP is important in conveying resistance to cisplatin and 5-FU in any particular cell line. However, a number of authors have suggested that cisplatin is a substrate for MRP2 (cMOAT) (section 1.6.9). It is possible that an increase in the expression of MRP2 contributed to an increase in cisplatin resistance but it is also possible that pulsing DLKP cells with indomethacin results in an increase in other mechanisms of cisplatin resistance and other transport proteins. No clear-cut simple mechanism for cisplatin resistance has been identified as discussed in sections 4.8 - 4.10.

Mechanisms of 5-FU resistance have also been much researched over the last number of years. MRP is understood not to play an important role in 5-FU resistance (Nishiyama *et al.,* 1999). Kirihara *et al.,* (1999), also reports that the mechanisms of 5-FU resistance appear to be different from drug resistance associated with the multidrug resistance phenotype related to Pgp and MRP. However, both authors have reported an increase in MRP1 expression in 5-FU-selected gastrointestinal cell lines. They also reported a very high increase of thymidylate synthase (TS) gene expression in these cells as well as increased

levels of glutathione S-transferase π (GSTπ) and dihydropyrimidine dehydrogenase (DPD). It is possible that the expression of MRP is an effect due to or associated with an increase in the expression of the other 5-FU resistance mechanisms but does not have direct involvement in 5-FU drug resistance. However, there is no evidence to support this hypothesis. 5-FU can be metabolised to 5'fluorodeoxyuridine monophosphate (FdUMP), which inhibits thymidylate synthase and consequently DNA synthesis. 5-FU can also be metabolised to the ribonucleoside triphosphate FUTP, and incorporated into various types of RNA, inhibiting their metabolism and function. A very low % of this chemotherapeutic drug can also be converted to the deoxyribonucleoside triphosphate, FdUTP, which is incorporated in to DNA (Pratt *et al.,* 1994). As described in section 1.6.9.4, MRP4 has been linked with efflux of nucleoside monophosphate analogues (Schuetz *et al.,* 1999). RT-PCR analysis demonstrated an increase in MRP4 mRNA expression in the pulsed DLKP cells as compared to the parental DLKP cells. Therefore, it is possible that 5-Fu resistance is as a result of MRP4 expression in resistant cells.

Pulsing DLKP cells with indomethacin did not diminish the ability of this NSAID to potentiate the toxicity of adriamycin in these cells. However, pulsing the DLKP cells with indomethacin resulted in greater resistance to the anti-cancer drugs. This could potentially be a worrying factor if indomethacin is to be used in cancer chemotherapy to overcome resistance to a range of anti-cancer drugs. Although, it must be considered that the concentrations used in the pulse selection are more than 100-fold greater than the concentration of indomethacin used in the combination toxicity assays (2.5μg/ml). The plasma relevant concentrations of indomethacin are also approximately 2.5μg/ml. Therefore the high concentration of indomethacin, which was used in the pulse selection, would not be administered to patients in a clinical setting.

4.12 Investigation of other possible MRP substrates

Studies were carried out on a number of TB/AIDS related drugs to determine if there was a correlation between acquisition of chemotherapeutic resistance

mechanisms and resistance to TB and /or AIDS drugs. Resistance to TB and AIDS related drugs is not generally considered a 'cellular' phenomenon. Acquired resistance is usually associated with the microorganism acquiring the resistance and not as a result of the intracellular availability of the drug to the microbe, such as by a membrane pump extruding the drug from the cell (Larder *et al.,* 1991). However, there have been a number of publications, which consider the possibility that resistance to certain compounds, in particular resistance of HIV-1 to AZT might also involve the cellular expression of multidrug resistance Pgp (Antonelli *et al.,* 1992 and Cinatl *et al.,* 1994). In 1994, Dianzani *et al.,* reported that exposure of the drug sensitive CEM cell line to continuous increasing concentrations of AZT, a nucleoside analogue, resulted in a cell line resistant to both the anti-proliferative and anti-viral action of the drug. However, Pgp levels, sensitivity to chemotherapeutic drugs and sensitivity to other anti-HIV drugs (even within the same class) remained unaltered. The author suggests that there may be other alternative possibilities to explain the AZT-induced cellular resistance:

i. other transmembrane transporter proteins could be overexpressed in the CEM resistant cell line similar to those proteins that are responsible for the expulsion of anticancer agents

ii. the mechanism underlying AZT resistance resides in a defect in thymidine kinase activity. This latter suggestion is supported by the findings of Turrizianni *et al.,* (1996).

Sawchuk *et al.,* (1999), investigated the distribution, transport and uptake of anti-HIV drugs, including AZT, to the central nervous system (CNS) and reported that the active efflux transport of AZT out of the CNS appears to be a predominant mechanism limiting nucleoside access to the CNS. The authors have speculated that this efflux transport is due to the activity of an MRP-like transporter in blood-brain and blood-cerebrospinal fluid barriers. Wijnholds *et al.,* (2000), has also reported that MRP1 helped to limit the tissue distribution of certain drugs, including AZT and contributed to the blood-cerebrospinal fluid permeability barrier.

There have been no publications associating TB resistance to cellular resistance mechanisms such as transmembrane pumps and altered accumulation of the

drug in the cell. However, Takiff *et al.* (1996) reported the appearance of Fluoroquinolone (FQ) resistant strains of tuberculosis. The authors selected a gene that confers low-level FQ resistance when present on a multicopy plasmid. This gene IfrA, encodes a putative membrane efflux pump of the major facilitator family, which appears to recognise the hydrophilic FQ, ethidium bromide, acridine and some quaternary ammonium compounds. It is homologous to qacA from *Staphylococcus aureus.*

MRP transports a range of compounds that include glutathione-S-conjugates, amphiphilic anionic drugs, and natural product toxins. Vezmar *et al.,* (1998), demonstrated that the lysosomotropic or antimalarial drug chloroquine was a substrate for MRP and that MRP modulated the transport of chloroquine by direct binding. Hollo *et al.* (1996), has also reported that the antimalarial compound quinine is also a substrate of MRP. Recently, Vezmar *et al.,* (2000), reported that a number of quinoline drugs, chloroquine, quinine, quinidine and primaquine potentiated the toxicity of adriamycin in a concentration-dependent manner in two MRP overexpressing cell lines, HL60/ADR and H69/AR. Moreover, the mechanism of reversal appeared to be mediated through direct binding to MRP. These findings suggest that resistance to quinine could possibly be due to overexpression of MRP.

Courtois *et al.,* (1999), also reported the ability of rifampicin, the anti-tuberculosis drug, to inhibit MRP. The authors demonstrated that rifampicin was capable of increasing intracellular accumulation of calcein, a fluorescent dye substrate for MRP and PgP, in MRP-overexpressing GLC4/ADR, human lung cancer cells, through inhibition of its export out of cells. In addition, rifampicin also enhanced levels of accumulation of the anticancer drug vincristine, another known substrate of MRP, in GLC4/ADR cells. Zaman *et al.,* (1993), demonstrated that GLC4/ADR cells do not express Pgp which indicates that the inhibition of efflux by rifampicin is through an interaction with MRP. The exact mechanism by which rifampicin modulates MRP-mediated drug transport remains to be determined. Courtois *et al.,* (1999), hypothesised that rifampicin interferes with substrate-binding sites on MRP. This conclusion is supported by the fact that rifampicin and other rifamycins may be considered substrates for the MRP efflux pump since GLC4/ADR cells

were shown to display low, but significant levels of cross-resistance to these compounds when compared to parental drug-sensitive GLC4 cells. However, both quinine and rifampicin were used in experimental procedures carried out by Courtois *et al.,* (1999) and Vezmar *et al.,* (2000), at quite toxic concentrations (20μm and 100-200μm respectively). So while they may be promising as inhibitors/modulators of MRP further studies are required to develop analogues of these compounds which are effective against MRP at less toxic concentrations.

Tetracycline resistance has been attributed to the afflux activities of secondary multidrug transporters such as the chromosomally encoded TetA (L) protein of *Bacillus subtilis* (Wang *et al.,* 2000), the TetA (K) protein of *Staphylococcus aureus* (Ginn *et al.,* 2000 and Gibbons *et al.,* 2000), the TetQ protein in *Prevotella intermedia* (Okamoto *et al.,* 2000), Tet (W) in bacteria from human gut (Scott *et al.,* 2000), EnvD and TetA in *E. coli,* Tel (L) in *Bacillus subtilis* and MexB in *Pseudomonas areuginosa* (Nikaido *et al.,* 1994). However, to date, there is no evidence that the antibiotic tetracycline, is a substrate for MRP or that one of the mechanisms by which cells develop resistance to these compounds is through activity of MRP.

The results from the combination toxicity assays (section 3.13) indicate that only rifampicin and quinine were found to potentiate the toxicity of adriamycin in the DLKP cells. The enhancement of adriamycin toxicity was slightly better in the presence of quinine than rifampicin. These results support the experimental findings of Courtois *et al.,* (1999), and Vezmar *et al.,* (2000). As both quinine and rifampicin are reported by these authors to bind directly with MRP it is possible that they are competitive substrates for the MRP pump, competing with adriamycin for pumping from the cell by MRP. It appears that these compounds potentiate adriamycin in a manner similar to indomethacin. If rifampicin and quinine are substrates for MRP it is also possible that MRP may contribute to resistance to these compounds. Further studies are required to determine the mechanism by which rifampicin and quinine potentiate the toxicity of adriamycin and to elucidate if these compounds are substrates for MRP1 alone or for other homologues of MRP also. Future experiments should include inside-out vesicle assays to determine if these compounds inhibit the transport of LTC_4 by a pure MRP preparation and would provide evidence that

these compounds are substrates for MRP. Future work should also include GST assays to assess if these compounds inhibit GS-X conjugation. Their ability to inhibit the activity of GSTγ, the rate limiting step in the production of GSH, should also be investigated as it is possible rifampicin and quinine potentiate the toxicity of adriamycin by inhibiting GSH which may be required for the transport of adriamycin by MRP. Drug efflux studies would also aid in determining the potentiation mechanism of these two compounds.

Tetracycline and AZT were unable to potentiate the toxicity of adriamycin in the DLKP cells. MRP1 is overexpressed in DLKP cells so the results indicate that these two compounds are not substrates for MRP1. However, DLKP cells express very low levels of MRP2 and almost undetectable levels of MRP3 and MRP4 (section 3.16). It is possible that tetracycline and AZT are substrates for other MRP homologues but this would be difficult to ascertain until cell lines, with one single transport protein and no background expression of other pumps, are generated to elucidate the range of substrates for each transport protein. Of particular interest is the fact that AZT is a nucleoside analogue, 3'-azido-3'-deoxythymidine (Sawchuck *et al.,* 1999; Batrakova *et al.,* 1999 and Arion *et al.,* 2000). MRP4 has been reported by Schuetz *et al.,* (1999), to be involved in nucleoside monophosphate analogue transport from mammalian cells (section 1.6.9) and, thus, in resistance to these drugs. It is possible that AZT is also transported by MRP4. There are almost undetectable levels of MRP4 in DLKP cells so it is possible that transport of AZT would be evident in cell lines such as A549 which overexpresses MRP4.

4.13 Investigation of potentiation of chemotherapeutic drugs with reduced cardiotoxicity.

Adriamycin is an anthracycline antineoplastic agent used to treat a wide variety of solid and hematogenous tumours and differs from daunorubicin by the presence of an additional hydroxyl group on the adriamycin structure. Adriamycin and other anthracyclines induce cytotoxicity through several different mechanisms. Adriamycin complexes with DNA by intercalating between DNA base pairs causing the helix to change shape. This simple act of

changing the conformation of DNA can interfere with strand elongation by inhibiting DNA polymerase and can inhibit protein synthesis due to affects on RNA polymerase. (Pratt *et al.,* 1994).

Unfortunately, the risk of congestive cardiomyopathy increases rapidly once the total cumulative lifetime dose of adriamycin exceeds 450-500mg/m^2 (Minow *et al.,* 1977). The authors report that adriamycin induced toxicity can cause substantial morbidity and may prove fatal. Moreover, there is a concern that use of adriamycin as part of adjuvent or curative chemotherapy may result in decreased cardiac reserve that will lead to the development of congestive heart failure at some time following discontinuation of adriamycin treatment (Stewart *et al.,* 1993). Consequently adriamycin analogues have been developed, such as epirubicin (Yeung *et al.,* 1989), and idarubicin (Ganzina *et al.,* 1986), that in preclinical studies retain doxorubicin's antineoplastic activity but with a lesser degree of cardiotoxicity.

Stewart *et al.,* (1993), suggested that there is a gradual conversion of adriamycin to adriamycinol within cardiac tissue. Adriamycin may be converted to adriamycinol by the activity of the cytoplasmic enzyme aldo-keto reductase, which is present in many tissues including the heart. Adriamycinol appears to be taken up in to the cells at a slower rate than adriamycin but adriamycinol produced from adriamycin within the cell is potentially cytotoxic and may cause even more cardio-damage than adriamycin (Ferrazzi *et al.,* 1991). Minotti *et al.,* (1995), have reported that the cardiotoxicity of adriamycin and other quinone-containing antitumour anthracyclines has been tentatively attributed to the formation of drug semiquinones which generate superoxide anion and reduce ferritin-bound Fe(III), favouring the release of Fe(II) and its subsequent involvement in free radical reactions. This cardiotoxicity has also been reported by Minotti *et al.,* (1999), to also be as a result of the formation of a secondary alcohol metabolite of adriamycin, adriamycinol, which is involved in a non-enzymatic and superoxide anion-independent redox coupling with Fe(III)-binding proteins distinct from ferritin. This coupling results in the regeneration of stoichiometric amounts of adriamycin, mobilising a two-fold excess of Fe(II) ions. It has previously been postulated that the reduced cardiac toxicity of epirubicin relative to doxorubicin could be due to the relatively low conversion of epirubicin to epirubicinol (Sweatman *et al.,* 1987). Epirubicin, daunorubicin and idarubicin

are reported to be significantly less effective than adriamycin in mobilising Fe(II) (Minotti *et al.*, 1999).

Combination toxicity assays were carried out in the DLKP cell line using doxorubicin, adriamycinol or epirubicin in combination with indomethacin to investigate if the toxicity of adriamycinol and the less cardiotoxic analogue, epirubicin, can be potentiated by indomethacin to the same extent as adriamycin. The results (section 3.12) indicate that to achieve 50% cell kill in the DLKP cell line, a 4-fold greater concentration of adriamycinol than adriamycin was required to obtain this level of cell kill. Indomethacin was capable of potentiating the toxicity of adriamycinol but the enhancement was not as notable as the magnitude of the enhancement of adriamycin by indomethacin. This is of interest as this metabolite of adriamycin causes greater cardiotoxicity and if both adriamycin and adriamycinol are present in the cell the toxicity of adriamycin will be preferentially potentiated by indomethacin.

However, more promising results were obtained from the combination toxicity assays carried out in the A549 cell line with adriamycin/epirubicin in combination with sulindac. Greater potentiation of epirubicin than adriamycin was observed when these chemotherapeutic drugs were combined with sulindac. In addition, to obtain similar cell kill to adriamycin, a 4-fold lower concentration of epirubicin was sufficient. These results indicate that epirubicin has greater chemotherapeutic ability in A549 cells than adriamycin, at lower concentrations. Epirubicin is also more effective than adriamycin when combined with sulindac in this cell line. This would suggest that epirubicin could effectively replace adriamycin *in vitro* resulting in reduced cardiotoxicity with greater cell kill.

As the combination toxicity assays with epirubicin were carried out in one cell line only, with one NSAID, it is not clear if this effect is cell line specific or if this chemotherapeutic drug would be more effective than adriamycin in a range of cell lines. Therefore, future experiments will include analysis of epirubicin in a range of cell lines from different tumour types. Combination toxicity assays using epirubicin in combination with a range of active NSAIDs and indomethacin analogues to assess if better potentiation of this drug can be obtained. It would also be of interest to combine epirubicin with an active NSAID, which causes reduced gastrointestinal toxicity, which would result in a

combination of NSAID and chemotherapeutic drug which is effective against cancer with much reduced toxic side effects.

5.0 Conclusions

5.1 Investigation of SAR of indomethacin analogues

The results obtained from the *in vitro* combination toxicity assay using an MRP expressing cell line, DLKP, indicated that the activity of indomethacin and analogues was concentration-dependent and that a number of the substituents on the indomethacin structure were critical for the toxicity potentiating ability of the compounds. Experimental findings are summarised as follows:

i. Changing indomethacin from a benzoyl (indomethacin, figure 1.10.1) to a benzyl compound (BRI 60/1, figure 1.10.3) (i.e. removing the carbonyl oxygen, thus allowing potential free rotation) did not affect the potentiating activity of the structure. However, in the benzyl and the benzoyl series of indomethacin analogues the structural requirements for drug toxicity enhancement differ.

ii. Alterations to the benzyl structure indicated that the presence of the halogen on the benzene ring was necessary for the potentiating activity of N-benzyl-indomethacin (BRI 113/1, figure 1.10.9) and could not be replaced with non-halogen substituents such as a methylthio substituent (BRI 124/1, figure 1.10.9).

iii. The activity of N-benzyl-indomethacin was retained when the chlorine, in the *para*-position, was replaced with bromine (BRI 114/2, figure 1.10.8) and fluorine (BRI 115/2, figure 1.10.8). The compound was inactive when chlorine was in the *meta*-position (BRI 106/1, figure 1.10.8) and in the *ortho*-position (BRI 107/1, figure 1.10.8). However, when fluorine was moved to the *meta*-position the compound was still active (BRI 138/1, figure 1.10.10). Perhaps this is due to the smaller size of the fluorine molecule, which may allow a different type of interaction with the active site.

iv. Removal of the halogen in N-benzoyl-indomethacin (BRI 88/1, figure 1.10.5) or replacing the chlorine molecule with bromine (BRI 92/1, figure 1.10.6) indicated that this substituent was not required for the potentiating activity of the compound. Perhaps the lack of free rotation in the benzoyl compound equates to "locking" into a particular conformation in the benzyl series via interactions between a *para*-halogen and a group in the active site of MRP.

v. Further investigations of N-benzyl-indomethacin compounds determined that both the methoxy (on the benzene ring) and methyl (on the indole ring) groups were necessary for the activity (BRI 119/1 and BRI 120/1, figure 1.10.11) of N-benzyl-indomethacin.

vi. In the benzoyl series, changing the acetic acid side chain to a methyl ester (BRI 59/1, figure 1.10.4) or a tolyl amide (BRI 69/2, figure 1.10.4) reduced the solubility of the compound and rendered the compound inactive.

vii. Removal of the methoxy substituent from the N-benzoyl-indomethacin structure did not render the compound inactive (BRI 104/2, figure 1.10.7).

Four compounds were produced with chemical structures similar to known PLA_2 inhibitors and of these, two compounds, both of which were N-benzyl-2-(m)ethylindole-3-acetamides functionalised at the 5-position with a short chain alkoxy unit terminated by a carboxylic acid, were capable of potentiating the toxicity of adriamycin, albeit at higher molar concentrations than that required for indomethacin. The two remaining compounds from this group of four were N-benzyl-2-(m)ethylindole-3-acetamides with a phosphonic acid residue terminating the short chain alkoxy unit. Neither of these compounds had significant toxicity-enhancing ability. These results suggest that the potentiating ability of the NSAIDs was not through PLA_2 inhibition. However, the specific activity of these compounds and the indomethacin analogues on PLA_2 activity need to be fully investigated before definite conclusions re PLA_2 activity can be drawn.

The nine analogues of indomethacin which exhibited toxicity enhancing ability were all less toxic to DLKP cells than indomethacin but only three of these compounds had similar potentiation ability to indomethacin. The results from the *in vitro* toxicity assays indicate that in terms of potentiation ability, the structure of indomethacin is the optimum structure and the substituent variation reported in this thesis did not significantly enhance the potentiation ability of this NSAID; some of the compounds, however, have properties which may make them preferable to indomethacin for *in vivo* use.

A number of experiments were carried out to determine the mechanism by which indomethacin and positive indomethacin analogues potentiate the toxicity of

chemotherapeutic drugs. Results which were crucial in elucidation of the mechanism involved are summarised below:

i. A number of the indomethacin analogues, which were shown to be positive in the combination toxicity assays, did not inhibit the enzyme Glutathione S-transferase (GST) which is active in the formation of glutathione conjugates. In particular, the N-benzyl-indomethacin analogues (e.g. BRI 60/1, BRI 114/2 and BRI 115/2) were poor GST inhibitors indicating that the carbonyl substituent is important in the inhibition of GST. The results indicate that indomethacin and the active indomethacin analogues do not potentiate the toxicity of the chemotherapeutic drugs by inhibiting the formation of glutathione conjugates.

ii. Experiments were carried out to assess if these compounds had a direct effect on the MRP pump. Using inside-out vesicles prepared from the plasma membrane of HL60/ADR cells, which overexpress MRP, it was demonstrated previously that the active NSAIDs, indomethacin, sulindac and tolmetin, inhibited uptake of LTC_4, an MRP1 substrate (Duffy *et al.*, 1998). This method was used to assess if the indomethacin analogues had similar ability to inhibit MRP-mediated pumping of this substrate. All of the active indomethacin analogues were shown to have the ability to inhibit the uptake of LTC_4 into the vesicles. However, one of the indomethacin analogues, BRI 138/1, where the chlorine in the *para*-position on the benzene ring was replaced with a fluorine in the *meta*-position, was a very strong potentiator in the combination toxicity assay but, although it modulated the transport of LTC_4 in the IOV assay, this modulation was relatively weak as compared to indomethacin and the other active indomethacin analogues. Overall, however, the results indicate that these compounds are inhibiting the activity of the MRP1 pump, in some way, and preventing the pumping of the MRP1 substrate LTC_4.

iii. Indomethacin and the indomethacin analogues were assayed in another MRP1-overexpressing lung carcinoma cell line, CORL23(R) to assess that the potentiation effect was not cell line specific. The results showed that the active indomethacin analogues were capable of potentiating the toxicity of a range of chemotherapeutic drugs in the CORL23(R) cell line. Interestingly potentiation of these drugs by indomethacin and active

analogues was also observed to a slightly lesser extent in the parental cell line, CORL23(S). Western blotting indicated that there is a basal level of expression of MRP1 in the parental cell line (Borst *et al.*,1997, Duffy *et al.*,1998 and Kool *et al.*,1997), which is increased after the stepwise selection with adriamycin used to generate the CORL23(R) cell line. The combination index values obtained for both of the cell lines (section 3.6) suggest that the level of expression of MRP does not appear to strictly correlate with the rate of potentiation of the anticancer drugs by the NSAIDs. It appears that even the presence of a small amount of functional MRP can be sufficient to render cells less sensitive to chemotherapeutic drugs.

iv. Indomethacin and the indomethacin analogues were capable of potentiating the toxicity of adriamycin, vincristine and VP-16 in the CORL23 cell lines. These chemotherapeutic drugs have been reported to be MRP1 substrates (Cole *et al.*, 1998 and Hipfner *et al.*, 1999). The positive indomethacin analogues were unable to enhance the toxicity of non-MRP1 substrates 5-FU and cisplatin which indicates that the enhancement of the toxicity of chemotherapeutic drugs in an MRP1-overexpressing cell lines is specific for those drugs which are MRP1 substrates. These results further indicate an interaction with MRP as the mechanism of action for the potentiation of toxicity by indomethacin and the positive analogues.

v. The efflux of adriamycin from DLKP cells treated with indomethacin or active indomethacin analogues was significantly retarded relative to untreated cells or cells treated with an inactive indomethacin analogue. These results strongly indicate that the active compounds have direct inhibitory potential against the activity of MRP. It is postulated that indomethacin and indomethacin analogues exert their positive effect either by binding to MRP and inhibiting the activity of the pump or by competing with an MRP substrate, such as LTC_4 or chemotherapeutic drug, for preferential pumping from the cell by the MRP molecule, inhibiting the binding and subsequent pumping of other substrates. Further efflux studies demonstrated that adriamycin was also capable of reducing the efflux of indomethacin from the DLKP cells, suggesting that indomethacin and adriamycin may be competitive substrates for MRP.

vi. Duffy *et al.*, (1998), had previously reported that the enhancement effect was not due to the cyclooxygenase inhibitory activity of NSAIDs. However, the indomethacin analogues were analysed for COX-1 and COX-2 activity to assess if any of the active analogues were capable of enhancing the toxicity of the chemotherapeutic drug without COX-1 inhibition, and therefore possibly without causing the gastric damage customarily associated with NSAIDs including indomethacin which is believed to be associated with COX-1 inhibition. Again, the N-benzoyl-indomethacin analogues were most effective at inhibiting COX-1 and COX-2, which indicates that the benzoyl unit in indomethacin may be critical for COX-1 and COX-2 inhibition and removal of this unit renders the analogues too dissimilar to indomethacin to be effective cyclooxygenase inhibitors. It appears that removing the chlorine from the benzene ring, changing its position from *para-*, or replacing it with another halogen, also reduces the ability of the N-benzyl-indomethacin analogues to inhibit COX-2.

Two of the active indomethacin analogues, BRI 138/1 and 153/1, were found to have almost no COX-1 inhibitory activity and were weak inhibitors of COX-2 which renders these compounds quite interesting in terms of high chemosensitising ability with likely minimal gastrointestinal toxicity. Of great interest is the positive indomethacin analogue BRI 60/1 which is a very poor COX-1 inhibitor but, in contrast to BRI 138/1 and 153/1, this analogue is a very good COX-2 inhibitor. Therefore, BRI 60/1 would potentially be very useful in a clinical situation for its high chemosensitising ability, minimal gastrointestinal toxicity and inhibition of tumour enhancing properties associated with COX-2 (as discussed in section 1.7).

5.2 Enhancement of chemotherapeutic drug toxicity by NSAIDs in cell lines with varying expression of MRP1-6.

To assess the ability of the NSAIDs to potentiate the toxicity of a range of chemotherapeutic drugs in cell lines which overexpress homologues of MRP other than MRP1 an ovarian carcinoma cell line, 2008, transfected with MRP1, MRP2 or MRP3, was used in a number of combination toxicity assays.

5.2.1 Enhancement of adriamycin toxicity

i. Indomethacin and sulindac were both shown to have the ability to potentiate the toxicity of the MRP1 substrate, adriamycin, in the 2008 parental and transfected cell lines. The results indicate that although the toxicity of the chemotherapeutic drug was potentiated in all the 2008 transfected cell lines, the effect was greatest in the MRP1 transfected cells. The results also suggest that the enhancement effect, overall, was slightly better in the 2008 MRP3 cell line than in the 2008 MRP2 cell line. However, MRP1 mRNA and protein expression was detected in the 2008 parental and transfected cell lines. Therefore, it cannot be ruled out that the enhancement effect observed in the 2008 cell lines is as a result of MRP1 expression in all these cell lines.

ii. Adriamycin has also been reported to be a substrate for MRP1 (Cole *et al.*, 1992), MRP2 (Koike *et al.*, 1997), and MRP3 (Young *et al.*, (1999). However, Kool *et al.*, (1999), have shown that transfecting MRP3 cDNA into the 2008 cell line did not confer resistance to adriamycin, indicating that adriamycin was not a substrate for MRP3 expressed in the 2008 cell line. Therefore, if this is the case, potentiation of adriamycin in the 2008 MRP3 cell line is most likely due to the NSAIDs interacting with MRP1. However, in the short-term assays, similar resistance to adriamycin was demonstrated in the 2008 MRP3 and 2008 MRP1 cells. There is lower protein expression of MRP1 in the 2008 MRP3 cells than in the 2008 MRP1 cells (Dr. Lisa Connolly, personal communication), and the 2008 MRP3 cells overexpress MRP3 protein, therefore, it is possible that resistance to adriamycin in the short-term assays is due to the expression of both MRP3 and MRP1. mRNA and protein analysis of the 2008 MRP3 cell line have shown undetectable levels of MRP2 (section 3.8 and 3.16). From the results detailed in section 3.8 it is difficult to determine if indomethacin or sulindac potentiate the toxicity of adriamycin in the 2008 MRP2 cells. Adriamycin is poorly potentiated in the short-term assays by either sulindac or indomethacin in the 2008 MRP2 cells which suggests that either these NSAIDs are poor substrates for MRP2 or that the small observed potentiation of adriamycin toxicity is due to the low protein expression of MRP1 in the 2008 MRP2 cells. No MRP2 protein expression

was observed in any of the 2008 cell lines except 2008 MRP2 as expected. Therefore, the potentiation of adriamycin in the other 2008 cell lines is most likely not due to the NSAIDs interacting with MRP2 To fully elucidate the roles of the active NSAIDs and indomethacin analogues in MRP2 and MRP3 expressing cell lines it will be necessary to obtain cell lines transfected with MRP1, 2 or 3 but with no basal expression of MRP1 or other MRP transporters in the parental cell.

5.2.2 Enhancement of methotrexate toxicity

Methotrexate has been shown to be a substrate for MRP1, MRP2 (Hooijberg *et al.*, 1999) and MRP3 (Kool *et al.*, 1999). However, in our experiments there was potentiation of methotrexate in 2008 P, 2008 MRP1 and 2008 MRP3 only, by indomethacin, with greatest potentiation in the parental and 2008 MRP1 cell lines. There was no observed potentiation of methotrexate in the 2008 MRP2 cell line, by indomethacin, but there was some potentiation by sulindac in the same cell line. Sulindac was capable of potentiating methotrexate in all the 2008 cell lines with greatest potentiation in the parental and 2008 MRP1 cell lines. A number of authors, including Hooijberg *et al.*, (1999), and Bakos *et al.*, (2000), have reported that methotrexate is a particularly good substrate for MRP2. However, Bakos *et al.*, (2000), reported that low concentrations of indomethacin (< 100μM) enhanced the efflux of N-ethylmaleimide glutathione (NEM-GS), an MRP1 and MRP2 substrate, in *Sf*9 cells transfected with a recombinant baculoviruses containing MRP2 cDNA.

Indomethacin was unable to potentiate the toxicity of methotrexate in the 2008 MRP2 transfected cell line. Therefore, as a result of the above findings by Bakos *et al.*, (2000), it is possible that indomethacin was interacting with MRP2 to increase the efflux of methotrexate from the 2008 MRP2 cell line. These results suggest that, in the case of methotrexate, there are differences in transport properties for this drug in each of the 2008 cell lines which may indicate that the expression of MRP1 does not account for all of the potentiation effects observed in the 2008 cell lines. It is also possible that the transporter, MRP2, is trapped in an endosomal compartment within the 2008 cells and cannot reach the plasma membrane so the problems with the intracellular routing of MRP2 may make it difficult to determine the exact substrate specificities for MRP2.

5.2.3 Enhancement of taxol and taxotere toxicity

i. Initial experiments were carried out in the CORL23 parental and resistant cell lines, using indomethacin and an indomethacin analogue, BRI 153/1, in combination with taxol, a poor MRP substrate (Cole *et al.,* 1994 and Breuninger *et al.,* 1995), as a negative control. Previous experiments by Duffy *et al.,* (1998), demonstrated that the toxicity of taxol was not potentiated by the active NSAIDs in A549 cells. However, indomethacin and BRI 153/1 enhanced the toxicity of taxol in the CORL23 parental and resistant cells. The effect was not specific for indomethacin as sulindac could also potentiate the toxicity of taxol in the CORL23 cell lines.

Another member of the taxanes, taxotere, was also found to be potentiated by sulindac in the CORL23 cells.

ii. Both these taxanes were analysed in the 2008 cell lines and their toxicity was potentiated by sulindac, indomethacin and one of the indomethacin analogues, BRI 138/1. The potentiation effect was most evident in the MRP1 transfected cell line suggesting that these drugs are better MRP1 substrates. Recent reports have suggested that both taxol and taxotere are substrates for MRP1 (Vanhoefer *et al.,* 1997). Therefore, it is possible that the enhancement of taxol and taxotere toxicity is due to an interaction between the NSAID and MRP1. It also appears that the effect is cell line specific as the enhancement of taxol and taxotere toxicity by indomethacin or sulindac was evident in DLKP cells, very slightly in the MCF-7 cells, but not in the A549 cell lines (from two different external sources). All cell lines had previously been shown to express MRP1. A549 cells have been shown by RT-PCR to express very high levels of MRP4 mRNA. Expression of MRP4 protein levels in this cell line has not been determined as there was no commercially available MRP4 antibody. MRP4 has been reported to be directly linked to the efflux of nucleoside monophosphate analogs from mammalian cells (Schuetz *et al.,* 1999 and section 1.6.8.4). The range of substrates for MRP4 has not been elucidated yet, so it is possible that taxol and taxotere are substrates for MRP4. Perhaps when the NSAIDs are combined with taxol and taxotere in the A549 cells, the ability of MRP1 to pump taxol and taxotere is reduced but this is overcome by the activity of MRP4.

This theory is also dependent on the active NSAIDs not being substrates for MRP4. We have not been able to determine this yet. Taxol and taxotere toxicities were potentiated in the 2008 cell lines, which have been shown to express MRP4, but the levels are much lower than that observed in the A549 cell line.

iii. Piroxicam was unable to potentiate the toxicity of any of the chemotherapeutic drugs analysed in the 2008 cells, except for taxol where the combination index values indicated a slight potentiation of taxol toxicity by piroxicam. Moos *et al.,* (1999), indicated that taxol (but not taxotere) increased COX-2 (but not COX-1) protein and mRNA expression in RAW 264.7 murine macrophages. It is suggested that the inhibition of COX-2 by the active NSAIDs, in addition to an interaction with MRP, may explain the increased enhancement of taxol toxicity in the 2008 cell lines. Piroxicam has no MRP-inhibitory ability so the slight potentiation of taxol toxicity by piroxicam may be due to inhibition of COX-2, resulting in an increase in apoptosis in the 2008 cell lines. The possibility that this effect is cell line specific cannot be ruled out as the combination of taxol/taxotere and piroxicam was analysed only in the 2008 cell lines. This combination needs to be tested in a number of different cell lines.

5.2.4 Enhancement of cisplatin toxicity

i. The mechanism of cisplatin resistance has proven difficult to elucidate but a number of reports have linked cisplatin resistance to MRP2 (cMOAT) expression (Narasaki *et al.,* 1997; Koike *et al.,* 1997; Taniguchi *et al.,* 1996 and Kool *et al.,* 1996). The toxicity of cisplatin, a non-MRP1 substrate, could not be potentiated by indomethacin in any of the cell lines assayed in this thesis including the 2008 MRP1-3 cell lines, HepG2, a MRP2-overexpressing cell line, and DLKPC 14, a carboplatin selected cell line resistant to cisplatin. This indicates that either cisplatin is not a substrate for any of the MRP homologues analysed in this thesis or that indomethacin is not capable of competing with this chemotherapeutic drug for the cisplatin resistance mechanism. In fact, resistance to cisplatin was increased in all of the above cell lines in the presence of indomethacin.

Shen *et al.,* (2000), reported that a decrease in cisplatin uptake may account for some resistance to this chemotherapeutic drug in a number of cancer cell lines, and that this reduction of influx was independent of MRP1 and MRP2.

ii. However, BRI 138/1 potentiated very slightly the toxicity of cisplatin in 2008 MRP3 suggesting that both BRI 138/1 and cisplatin are weak substrates for MRP3. In addition resistance to cisplatin was not increased in the presence of BRI 138/1 in the other 2008 parental and transfected cell lines which suggests that this enhancement of resistance to cisplatin may be an indomethacin specific mechanism.

5.3 Effect of pulsing DLKP cells with indomethacin

i Pulsing DLKP cells with indomethacin resulted in an increase in resistance to adriamycin (≈ 1.5-fold), vincristine (≈ 1.5-fold), VP-16 (≈ 2-fold), cisplatin (≈ 2-fold), 5-FU (≈ 4-fold) and indomethacin (≈ 2.2-fold). RT-PCR analysis indicated that pulsing DLKP cells with indomethacin resulted in an increase in the expression of MRP1, 2, 4 and 6, no change in MRP3 or MDR1 expression and a down-regulation of MRP 5 mRNA. Adriamycin, vincristine and VP-16 are reported to be substrates for MRP1 and 2 and the overexpression of these proteins could account for the increase in resistance to these three chemotherapeutic drugs. However, there is no evidence that MRP4 is involved in the transport of chemotherapeutic drugs. Although experiments in this thesis have not produced evidence that cisplatin is a substrate for MRP2 it is possible that MRP2 is involved in some way in a complicated mechanism of cisplatin resistance. Pulsing the DLKP cells with indomethacin may also have had a negative effect on the uptake of chemotherapeutic drug in to the cell.

ii. The mechanisms of 5-FU resistance are known to be different from drug resistance associated with the multidrug resistance phenotype related to Pgp and MRP (Kirihara *et al.,* 1999). However, a number of authors have reported that although it appears that 5-FU resistance is not due to MRP or Pgp, MRP was identified as a predictor of primary 5-FU resistance (Kirihara *et al.,* 1999 and Nishiyama *et al.,* 1999). It is possible that

pulsing the DLKP cell line with indomethacin resulted in an increased activity of thymidylate synthase (TS) or dihydropyrimidine dehydrogenase (DPD), a rate limiting enzyme of 5-FU catabolism (Fischel *et al.,* 1995), in the cells, resulting in resistance to 5-FU. Future experiments should determine the activity of both enzymes in the DLKP cells prior to and after pulsing the cells with indomethacin to determine if they are involved in 5-FU resistance in DLKP cells. 5-FU can be metabolised to 5'fluorodeoxyuridine monophosphate, which inhibits thymidylate synthase and consequently DNA synthesis. Therefore, it is also possible that 5-FU is a substrate for the MRP4 which has been linked to the transport of nucleoside monophosphate analogs (Schuetz *et al.,* 1999).

Indomethacin, positive indomethacin analogues, sulindac and other active NSAIDs are capable of potentiating the toxicity of a class of chemotherapeutic drugs known to be MRP1 substrates, in cell lines which have been shown to overexpress MRP1. The results indicate that these active NSAIDs may be substrates for MRP2 and MRP3 also but cell lines with expression of these MRP homologues only, without expression of MRP1, are required to elucidate the activity of the active NSAIDs on MRP2 and 3 and this has proven difficult. The potentiation of taxol and taxotere by the active NSAIDs and positive indomethacin analogues is very interesting as it was believed that the taxanes were poor MRP substrates and that the potentiation effect would not be evident with these drugs. However, there also appears to be an alternative or additional mechanism of enhancement of taxol toxicity in certain cell lines which may involve the inhibition of taxol induced COX-2 by the NSAIDs. The lack of potentiation of these taxanes in the A549 cells may be due to the high expression of MRP4 (or another taxane efflux pump) in these cells. Further research is required in this area to fully elucidate the mechanism of taxol/taxotere potentiation in any of the cell lines analysed.

Overall, the results have demonstrated the ability of indomethacin, a number of indomethacin analogues and other active NSAIDs, which appear to be relatively selective MRP inhibitors, to have the ability to potentiate the toxicity of a number of clinically important chemotherapeutic drugs at non-toxic concentrations. For certain cancers, where drug resistance is particularly as a result of MRP over-

expression, these active NSAIDs and analogues are promising as potentiators of the toxicity of chemotherapeutic drug in future cancer treatments and therefore, enhanced treatment for cancer patients.

6.0 Future Experiments

6.0 Future experiments

There are a number of future experiments areas that could follow from work in this thesis:

i. The generation and analysis of cell lines, which express a single MRP transport protein only, without background expression of other MRP homologues or Pgp, to elucidate the range of substrates for each transport protein. Ideally this would involve transfection of cell lines which have no background expression of any MRP family members.

ii. Toxicity assays will be carried out in the 2008 cells using a wider range of chemotherapeutic drugs, including VP-16 and 5-FU, to assess the resistance profiles for a wider range of drugs in the 2008 cells than those used in section 3.8 of this thesis.

iii. Investigations of the ability of NSAIDs to potentiate the toxicity of anticancer drugs where drug resistance is due to the expression of MRP homologues other than MRP1. Again, cell lines expressing single transport proteins, as described above, would be ideal for some experiments.

iv. Analysis of the potentiation of taxol and taxotere in a wide range of cell lines, in combination with the active NSAIDs, and in particular with piroxicam, to further asses if the potentiation of taxol and taxotere is cell line-specific and also to determine if the potentiation of taxol by piroxicam is observed in cell lines other than the 2008 cell line.

v. Western blot analysis of a number of cell lines, exposed to taxol or taxotere, to assess if the expression of COX-2 is increased or induced in these cell lines. This experiment would help to clarify if potentiation of taxol by piroxicam is through inhibition of COX-2.

vi. Pulse selecting DLKP cells with indomethacin resulted in an increase in the expression of MRP4. Toxicity assays should be carried out in these pulsed cells using taxol and taxotere to assess if there is increased resistance to taxol and taxotere in the pulsed cells versus the parental cells. Also, combination assays should be carried out on the parental and pulse selected DLKP cell lines to elucidate if taxol and taxotere are potentiated in the pulsed cell line where there is a higher expression of MRP4 as compared to the parent cell

line. This work would aid in determining the role of MRP4 in taxol and taxotere resistance.

vii. It would also be of interest to pulse select DLKP cells with a combination of both indomethacin and chemotherapeutic drug to assess the difference in toxicity profiles between the cells pulsed with indomethacin alone and those pulsed with a combination if indomethacin and chemotherapeutic drug.

viii. Pulse selecting DLKP cells (which express very low levels of MRP4, with taxol or taxotere to assess if an increase of any of the MRP transport proteins, in particular MRP4, is observed.

ix. RT-PCR analysis of the HepG2 cells which were pulsed with cisplatin, to assess if the expression of MRP1 or its homologues have increased or decreased due to the pulse selection of these cells with cisplatin.

x. To assess if the changes in the expression of the MRP1 and MRP homologues are also observed at protein level as well as mRNA level, in the indomethacin pulsed DLKP cell line and the cisplatin pulsed HepG2 cell line, Western blotting should be carried out on both cell lines to further elucidate the effects of pulsing these cell lines with indomethacin or cisplatin.

xi. As described in section 4.6, further drug efflux studies would also be beneficial in defining the effect of indomethacin, indomethacin analogues or other active NSAIDs, such as sulindac, on the efflux of a range of chemotherapeutic drugs, especially taxol, taxotere and cisplatin. These efflux studies would also aid in determining mechanisms of resistance in cancer cells to these chemotherapeutic drugs.

xii. It would also be interesting to carry out efflux studies on cells treated with two active NSAIDs or two chemotherapeutic drugs, to assess if there is preferential pumping of one NSAID over the other, or one chemotherapeutic drug over another drug in MRP expressing cell lines. Cell lines expressing single transport mechanisms would also be useful so that the efflux of drugs or compound can be attributed to specific transport mechanisms.

xiii. To aid in determining the mode of action of the NSAIDs in potentiating the toxicity of certain chemotherapeutic drugs, the ability of the NSAIDs to inhibit GSH synthesis should be examined. It is possible that the active NSAIDs potentiate the toxicity of the chemotherapeutic drugs through inhibition of the synthesis of GSH which may be required for their transport

by MRP. This experiment would also help to determine if the transport of the chemotherapeutic drugs, which are substrates for MRP, is GSH dependent.

xiv. It would also be interesting to investigate further how cytokeratins influence cisplatin resistance in the HepG2 and the DLKPC 14 cells. Possible future experiments include the transfection of cytokeratin 18 or cytokeratin 14 in to DLKPC 14 cells, to determine if increased levels of either cytokeratin in the cells results in increased sensitivity of the cells to cisplatin. Perhaps also, Northern blot analysis could be carried out to investigate if DLKPC 14 cells express cytokeratin 18 or cytokeratin 14 and assess if the levels in the DLKPC 14 cells compare to the levels of the cytokeratins in the parental cell line. Additional future experiments to be performed in this area include RT-PCR and Western blot analysis of the parental and resistant cell lines to establish if pulsing the DLKP cells with carboplatin resulted in an increase in MRP2, -3, -4, -5, -6. These experiments would help to elucidate the contribution (if any) of these transporters to platinum drug resistance.

7.0 References

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Appendix A PCR primers used for the detection of MDR genes.

Gene	Primer length	A+T:G+C	Tm*	Amplified cDNA length (bases)
[1]MRP1	21	13:8	55	203
[2]MRP2	20	11:8	53	241
[2]MRP3	19	7:12	63	262
[2]MRP4	19	10:9	42	239
[2]MRP5	18	9:9	49	381
[3]MRP6	20	9:11	68	221
[1]MDR1	21	10:11	56	324

*Tm: Annealing temperature

[1] NicAmhlaoibh, (1997); [2] Kool *et al.*, (1997); [3] O'Neill *et al.*, (1998).

Appendix B Abbreviations

AA	Arachidonic Acid
ABC	ATP Binding cassette
ADR	Adriamycin
AMP	Adenosine 5'-monophosphate
ATCC	American Tissue Culture Centre
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSO	DL-Buthionine-[S,R]-sulfoximine
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
COX	Cyclooxygenase or Prostaglandin H synthase
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbeccos modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNDP	Deoxynucleotide Diphosphate (where N= A,C,G,T,U)
dNMP	Deoxynucleotide Monophosphate (where N= A,C,G,T,U)
dNTP	Deoxynucleotide Triphosphate (where N= A,C,G,T,U)
ECACC	European Collection of animal cell culture
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminineetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
FCS	Foetal calf serum
5-FU	5-fluorouracil
GSH	Reduced glutathione
GST	Glutathion S-transferase
HCl	Hydrochloric acid
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
HIV	Human Immunodeficiency Virus
HPLC	High pressure liquid chromatography
IC_{50}	Inhibitory Concentration 50%
IL-1β	Interleukin 1β
IMS	Industrial Methylated Spirits
Indo	Indomethacin
IOV	Inside Out Vesicles
Kcl	Potassium chloride
K_m	Substrate concentration which allows reaction to proceed at one half of maximum rate

LRP	Lung related-resistance protein
Lt	Leukotriene
LT	Long-term
MDR	Multidrug resistance
Mef	Mefenamic acid
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
MMLV-RT	Molomey Murine Leukemia Virus-Reverse Transcriptase
mRNA	Messenger ribonucleic acid
MRP	Multidrug Resistance Protein
MTX	Methotrexate
NaCl	Sodium chloride
NADPH	Nicotine adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCTCC	National Cell and Tissue Culture Centre
NEAA	Non-essential amino acids
NSAID	Nonsteroidal antiinflammatory drugs
NSCLC	Non-small cell lung carcinoma
NTP	Nucleotide Triphosphate (where N=ACGTU)
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pg	Prostaglandin
PGHS	Cyclooxygenase or Prostaglandin H synthase
PgI_2	Prostacycline
Pgp	P-glycoprotein
PKC	Protein Kinase C
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribonucleic acid
r.p.m.	Revolutions per minute
RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCLC	Small cell lung carcinoma
SD	Standard deviation
SDS	Sodium dodedecyl sulphate
ST	Short-Term
Sul	Sulindac
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
TGF-β	Transforming growth factor β
Topo II	Topoisomerase II

Tris	Tris(hydroxymethyl)aminomethane
TS	Thymidylate Synthase
UV	Ultraviolet
Vcr	Vincristine
V_{max}	Maximum rate of reaction when substrate present at saturation levels
VP-16	Etoposide

Appendix C Molecular Weights

Compound	Molecular Weight
Adriamycin	580
ATP	509
Cisplatin	300
Daunorubicin	564
5-FU	130
GSH	307
Indomethacin	358
BRI 60/1	344
BRI 88/1	322
BRI 92/1	402
BRI 104/2	328
BRI 59/1	372
BRI 69/2	447
BRI 106/1	344
BRI 107/1	344
BRI 114/2	388
BRI 115/2	327
BRI 113/1	308
BRI 124/1	391
BRI 138/1	327
BRI 119/1	314
BRI 120/1	329
BRI 153/1	384
BRI 203/1	370
BRI 205/4	406
BRI 215/1	420
Leukotriene C_4	626
Methotrexate	454
Piroxicam	331
Sulindac	356
Taxol	854
Taxotere	808
Vincristine	825
VP-16	589

Appendix D

Chemical analysis of indomethacin and indomethacin analogues

The indomethacin analogues described in this thesis were synthesised by Dr. Anita Maguire and Dr. Stephen Plunkett in the Department of Chemistry, University College Cork (UCC). To identify, characterise and assess purity of each of the analogues a number of analytical procedures were carried out on the indomethacin analogues in UCC prior to being sent to our group for biological analysis. These analytical procedures consisted of the following (Dr. Anita Maguire, personal communication):

- Nuclear Magnetic Resonance spectroscopy (NMR) was recorded on a Joel PMX60SI (60 MHZ) spectrometer;
- Mass spectra were recorded on a Kratos Profile HV-4 double focussing high resolution mass spectrometer;
- IR spectra were recorded on a Perkin Elmer Paragon 1000 FT-IR spectrometer as liquid films, chloroform ($CHCl_3$) solutions or potassium bromide (KBr) disks;
- Elemental analyses were recorded on a Perkin Elmer 240 elemental analyser;
- Thin layer chromatography was performed on DC-Alufoilen Kieselgel $60F_{254}$ 0.2mm plates (Merck) and visualised under UV light with a vanillin stain;