Investigations on the role of efflux pumps in drug resistance of human cancer cells.

A thesis submitted for the degree of Ph.D.

by

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

Signed: Cathal Elliott **I.D. No.:** 94970262

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Abstract

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The toxicity of a specific group of chemotherapeutic drugs was demonstrated to be enhanced in the presence of non-toxic concentrations of a number of non-steroidal anti-inflammatory drugs (NSAIDs). This was found to occur in the drug sensitive A549 human lung adenocarcinoma and DLKP human lung squamous carcinoma cell lines.

The chemotherapeutic drugs for which the toxicity enhancement effect was found to occur (adriamycin, daunorubicin, epirubicin, vincristine and VP-16) share the property of being potential substrates for the multidrug resistance associated protein (MRP). The toxicity of drugs which were not MRP-substrates, was unaffected by NSAID treatment. The toxicity of non-chemotherapeutic drug substrates for MRP, such as the heavy metal sodium arsenate was also found to be enhanced in the presence of a suitable NSAID.

NSAIDs are believed to exert their anti-inflammatory, analgesic and antipyretic effects *in vivo* as a result of their ability to inhibit the enzyme prostaglandin H_2 synthase (PGHS), which is also known as cyclooxygenase (COX). The only NSAIDs found to possess chemotherapeutic drug toxicity enhancing abilities were: indomethacin, sulindac, sulindac sulfide, sulindac sulfone, tolmetin, acemetacin, mefenamic acid and zomepirac. The mechanism by which these NSAIDs enhanced the toxicity of a suitable chemotherapeutic drug was demonstrated to be independent of any effects of NSAIDs on PGHS. This was conclusively demonstrated by the ability of sulindac sulfone (a metabolite of sulindac which is devoid of inhibitory effects on PGHS) to enhance chemotherapeutic drug toxicity to the same extent as achieved using sulindac sulfide (a metabolite of sulindac with potent inhibitory effects on PGHS).

MRP protein was demonstrated by Western blotting to be present in low levels in DLKP plasma membrane-derived inside-out vesicles (IOVs). The MRP in DLKP IOVs was demonstrated to actively transport the MRP substrate leukotriene C_4 (LtC_4). This transport was shown to be inhibited by NSAIDs with drug toxicity enhancing abilities such as indomethacin, but was unaffected by NSAIDs which were without influence on chemotherapeutic drug toxicity. These results suggested that NSAID-mediated enhancement of chemotherapeutic drug toxicity was dependent on the ability of an NSAID to interfere with the drug efflux activity of MRP.

The presence of MRP protein in IOVs facilitated investigation into the cytotoxic drug transporting characteristics of MRP. The LtC_4 transport activity of MRP in IOVs from the MRP-overexpressing cell line HL60-ADR was found to be significantly inhibited by vincristine (an MRP substrate) but not by Taxol or cisplatin (non-MRP substrates). This inhibition in LtC_4 transport with vincristine was synergistically enhanced in the presence of non-inhibitory concentrations of reduced glutathione (GSH). These results suggested that MRP substrate drugs such as vincristine may be actively transported by MRP in the presence of GSH.

Demonstration of the presence of active MRP protein in DLKP suggested that MRP may also have been present in resistant variants of DLKP. Active MRP protein was shown to be present in a variety of DLKP-related drug resistant cell lines namely DLKP-A10, DLKPA2B, DLKPA11B, DLKPA6B and DLKPA5F. Preliminary evidence suggested that MRP may be involved in certain aspects of the cytotoxic drug accumulation characteristics of these drug resistant variants of DLKP.

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1.0 Introduction

1.1 General introduction

The failure of cancer cells to respond to cytotoxic chemotherapy in the laboratory as well as in the clinic continues to be a significant problem for cancer researchers and clinical oncologists. It is estimated that cancer is responsible for approximately 22% of all deaths in the United States, with 1 million cases diagnosed each year (Cooper, 1992). Cancer may be treated by surgery, radiotherapy or chemotherapy. Approximately half of all cancer patients can be cured by surgery and radiation therapy if tumors have not spread to other sites in the body. (Davey and Davey, 1997). Significant developments have been made in the field of cancer chemotherapy over the years but in many cases it is still found that although a tumor may be initially sensitive to chemotherapy, a relapse after treatment can occur. Often clinical drug resistance may involve the development of resistance to several mechanistically and structurally unrelated cytotoxic drugs simultaneously, which can cause treatment failure when the cancer recurs. In addition to this form of resistance, some tumors may be intrinsically resistant to chemotherapy (Lehnert, 1996). Multidrug resistance implies that structurally and functionally unrelated lipophilic drugs are recognised and processed by the molecular system which protects multidrug resistant cells against lipophilic cytotoxic drugs (Lehnert, 1996; Clynes , 1993; Kartner and Ling, 1989).

A number of mechanisms of drug resistance have been characterised in multidrug resistant cancer cell lines. These mechanisms include expression of energy dependent transmembrane drug efflux pumps such as P-glycoprotein (Pgp) and multidrug resistance associated protein (MRP), reduced expression of topoisomerase II, and overexpression of the drug detoxifying enzyme family, the glutathione S-transferases (Bellamy, 1996; Clynes, 1993). The knowledge developed from studying the resistance mechanisms in cellular models of multidrug resistance (MDR), has resulted in trials using novel chemotherapeutic strategies which aim to overcome the drug resistance possessed by some tumors (Ferry *et al.*, 1996). A number of these trials involve the use of drug efflux pump modulators such as verapamil in combination with cytotoxic drugs such as adriamycin. The results of these trials have been disappointing as these

compounds have been found to lack the potency to completely reverse the MDR phenotype at clinically relevant concentrations. Many of the modulators currently being investigated in clinical trials have unacceptable toxic side effects and this seriously limits their potential for use as modulators of clinical drug resistance (Fisher and Sikic, 1995).

1.2 Cancer chemotherapy

The origins of modern cancer chemotherapy date from 1942 when Gilman, Goodman, Lindskog and Dougherty used nitrogen mustard in a patient with lymphosarcoma (Pratt *et al.,* 1994). Since that first treatment a large number of chemotherapeutic drugs have been developed for clinical use. These drugs may be classified into a number of specific drug classes primarily based on their mode of activity. These classes include the anthracyclines, epipodophyllotoxins, the vinca alkaloids, the antimetabolites, as well as the covalent and non-covalent inhibitors of DNA (Pratt *et al.*, 1994).

Tumours with a high rate of growth such as in human lymphomas and childhood leukaemias, can generally be effectively treated by chemotherapy (Cooper, 1992). The majority of chemotherapeutic drugs either inhibit DNA synthesis, cause DNA damage or in some way interfere with cell division. For this reason, one of the limiting factors in chemotherapy is the relative drug sensitivity of the cancer cells compared to the patient's normal cells which possess a high growth rate such as in the bone marrow. Colorectal, lung and breast tumors account for almost half of all cancer deaths in the United States (Cooper, 1992). These tumors possess a low fraction of dividing cells and this contributes to them being relatively resistant to treatment by chemotherapy alone. Cancers of the colon and rectum are among the most common cancers in developed countries, accounting for 13% of all cancers diagnosed and 11% of all cancer deaths (Weiss and Forman, 1996), with the incidence rate remaining relatively constant in recent years. The morbidity from lung cancer has increased considerably over the past 50 years. The prognosis is very poor with nearly 80% of lung cancer patients dying within 1 year of diagnosis. Despite advances in

chemotherapy, long term survival is obtained in only 5-10% of the patients (Mattern and Volm, 1995). Breast cancer is one of the most frequent cancers in women, accounting for approximately 30% of all cancers in females with the mortality rate second only to lung cancer in American women (Cooper, 1992). The relative inability of current chemotherapy regimes to significantly reduce the mortalities resulting from colorectal, lung, breast and other cancers has stimulated the development of novel strategies to overcome the resistance of these cancers to chemotherapy.

1.2.1 The anthracyclines

The anthracyclines are among the most commonly used cancer chemotherapeutic drugs. This group describes a class of antitumor antibiotics isolated from various *Streptomyces* species and includes adriamycin (also known as doxorubicin), daunorubicin and epirubicin. Adriamycin is used in the treatment of a variety of carcinomas including lung, bladder, breast, endometrium, stomach, ovarian and thyroid, soft tissue sarcomas, paediatric solid tumours as well as lymphoid and myelogenous tumours (Pratt *et al.*, 1994). It is administered intravenously at a dose of 60 mg/m^2 which is repeated after a period of 21 days. This is associated with a rapid uptake of the drugs in the lungs, heart, kidney, liver and spleen. Adriamycin is metabolised in the liver and excreted in the bile resulting in the elimination of adriamycin from the body. This elimination follows a 2 phase pharmokinetic profile with a initial half life (α t½) of approximately 10 minutes and a second half life (β t½) of approximately 30 hours (Pratt *et al.*, 1994). As with all cancer chemotherapeutic drugs, adriamycin has serious side effects. This drug is a carcinogen and a mutagen, but in addition it may also cause immunosuppression (Pratt *et al.*, 1994). Adriamycin also possesses significant cardiotoxic effects and this cardiotoxicity is shared by other anthracyclines (Gaudin *et al.*, 1993; Unverferth *et al.*, 1982) and is believed to be due to the generation of active oxygen species from oxygen (Pratt *et al.*, 1994).

The therapeutic advantage in the use of anthacyclines in cancer chemotherapy is a result of the ability of these drugs to bind to DNA and RNA. These drugs bind tightly to DNA and during their intercalation, their amino sugar moiety becomes located within the minor groove of DNA. This causes extensive interaction between the hydrophobic faces of the base pairs and the drug. Intercalation of the aglycone moiety between adjacent DNA base pairs can cause an inhibition of DNA replication as well as RNA synthesis and transcription. A stabilisation of the topoisomerase II-DNA complex, which causes protein associated DNA strand breaks has also been reported to occur in the presence of anthracyclines (Pratt *et al.*, 1994). Anthracyclines have been shown to undergo metabolism to form a free radical molecule with the ability to form superoxides which can cause single strand DNA breaks, lipid peroxidation and disruption of cell membrane function and integrity (Skladanowski and Konopa, 1994; Skladanowski and Konopa, 1993; Bachur *et al.*, 1977). The cytotoxicity induced by adriamycin has been reported as occurring even in the absence of significant DNA damage (Triton and Lee, 1982). This does not appear to be the dominant mechanism of action of anthracyclines, as when adriamycin is altered so that the ability of the drug to bind DNA and RNA is decreased, the level of cytotoxicity achieved by the drug is significantly reduced (Pratt *et al.*, 1994).

1.2.2 Vinca alkaloids

The vinca alkaloids are derived from extracts isolated from the periwinkle plant *Catharanthus roseus* and include chemotherapeutic drugs such as vincristine and vinblastine. Vincristine is often used in the treatment of childhood leukemia and vinblastine is regularly used in a variety of combination chemotherapy regimes often involving cisplatin or bleomycin (Pratt *et al.*, 1994). Vincristine can also be used to treat acute leukaemia, aggressive non-Hodgkin's lymphoma, Hodgkin's lymphoma, small cell lung cancer, Wilm's tumour and neuroblastoma (Pratt *et al.*, 1994). Vincristine is administered intravenously and is eliminated from the body with characteristic triexponential kinetics, with α and ß half lives of 3 minutes and 100 minutes respectively, and a terminal half life of over 85 hours (Pratt *et al.*, 1994).

Vincristine is eliminated from the body after metabolism in the liver and by biliary excretion. Only a fraction of vinca alkaloid end products are found to be present in the urine (Owellan *et al.*, 1977).

Vinca alkaloids can cause cell cycle metaphase arrest and a resultant block in mitosis. Vincristine disrupts the mitotic spindle which results in the cells becoming blocked in the mitotic phase of the cell cycle. The disruption of the mitotic spindle is caused by the binding of vincristine to free tubulin dimers. This results in the formation of paracrystalline aggregates which interfere with microtubule polymerisation and ultimately cause dissolution of the microtubules (Pratt *et al.*, 1994). In the absence of an intact mitotic spindle, the chromosomes become unable to segregate correctly and this eventually results in cell death. Mutations involving alterations in tubulin proteins have been shown to effect the ability of vinca alkaloids to achieve their cytotoxic effects (Cabral *et al.*, 1980; Ling *et al.*, 1979). The vinca alkaloids have also been shown to indirectly affect DNA, RNA and lipid biosynthesis (Jordan *et al.*, 1992; Jordan *et al.*, 1991).

1.2.3 Epipodophyllotoxins

The plant *Podophyllum peltatum* (American mandrake) produces podophyllotoxin, a mitotic inhibitor that acts by binding to tubulin and that prevents the formation of microtubules by blocking polymerisation of tubulin (Long, 1992). Podophyllotoxin was evaluated in the clinic in the early 1950s but had poor antitumor activity and excessive toxicity. Semi-synthetic derivatives of podophyllotoxin are now available and these include etoposide (VP-16) and teniposide (VM-26). These semisynthetic derivatives have been used in the treatment of a number of different types of cancers. VP-16 is used in the treatment of small cell bronchogenic carcinoma, testicular carcinoma, lymphoma, leukaemia and Kaposi's sarcoma (Van Maanen *et al.*, 1988a; Van Maanen *et al.*, 1988b).

VP-16 and VM-26 are normally administered intravenously with at a dose of 60-120 mg/m^2 daily for 5 days (Carter *et al.*, 1987). Metabolism follows biphasic kinetics with a half lives of 3 and 15 hours. Approximately 50% of the drug can be recovered unchanged in urine with a significant fraction recovered in the bile as hydroxy acid, glucuronide and sulphate conjugate metabolites (Pratt *et al.*, 1994). Haematologic toxicity is usually the dose limiting factor in the treatment of cancer patients with epipodophyllotoxins. The use of these drugs can result in leukopenia and thrombocytopenia. Gastrointestinal toxicity results in nausea and mild vomiting, but in addition alopecia can occur in of 8-20% of patients (Carter *et al.*, 1987).

The primary target of VP-16 and VM-26 is cellular DNA. Topoisomerase II binds to double stranded DNA making a transient double strand break. Topoisomerase II is activated by sites on chromosomes where two double helices become crossed over one another. When the topoisomerase binds, it causes a reversible breakage in one double helix to create a DNA gate. This allows the second double helix to pass through this break. Topoisomerase II then reseals the breakage in DNA and finally dissociates from the DNA, (Alberts *et al.*, 1994). The epiodophyllotoxins achieve their cytotoxic effects by stabilising the complex formed between topoisomerase II and the newly cleaved DNA. This causes an interference in the activity of topoisomerase II which ultimately results in double strand breaks and cell death.

1.3 The arachidonic acid Cascade

The arachidonic acid cascade is a cascade of events that results in the formation of prostaglandins (Pgs), leukotrienes (Lts), thromboxanes (Txs) and various hydroxy (HETEs) and hydroperoxy fatty acids (HPETEs). The end products of the arachidonic acid cascade are collectively referred to as eicosanoids and the biochemical pathway required for their formation is shown in simplified schematic form in Figure 1.3.1. In humans the most important precursor for eicosanoid biosynthesis is arachidonic acid (AA), a C_{20} polyunsaturated fatty acid with four non-conjugated double bonds which is derived directly from the diet or via modification of linoleic acid (Burgoyne and Morgan, 1990). The rate limiting step in the arachidonic acid cascade is the initial step involving the calcium dependent release of free AA from the cell phospholipid pool by phospholipases such as phospholipase A_2 (Lapetina, 1982). This cytosolic enzyme hydrolyses acyl groups at C_2 of phopholipids such as phosphatidylcholine, phophatidylethanolamine and phosphatidylinositol, (Lauritsen *et al.*, 1994). An increase in free AA levels within the cell can also be generated directly or indirectly by phospholipid breakdown by other phospholipases such as phospholipases A_1, C or D (Burgoyne and Morgan, 1990). Activation of phospholipase A_2 can occur upon stimulation of specific cell surface receptors. These stimuli include bradykinin, thrombin, ADP, chemotactic peptides and histamine, (Lapetina, 1982). Enzyme studies have shown that maximal phospholipase A_2 activation is dependent on the synergistic action of two factors (Berlin and Preston, 1995). The first of these factors involves an increase in intracellular free calcium levels which is necessary for enzyme catalysis and translocation to the membrane. The second factor involves specific phosphorylation of the enzyme by a mitogen activated protein (MAP) kinase (Berlin and Preston, 1995).

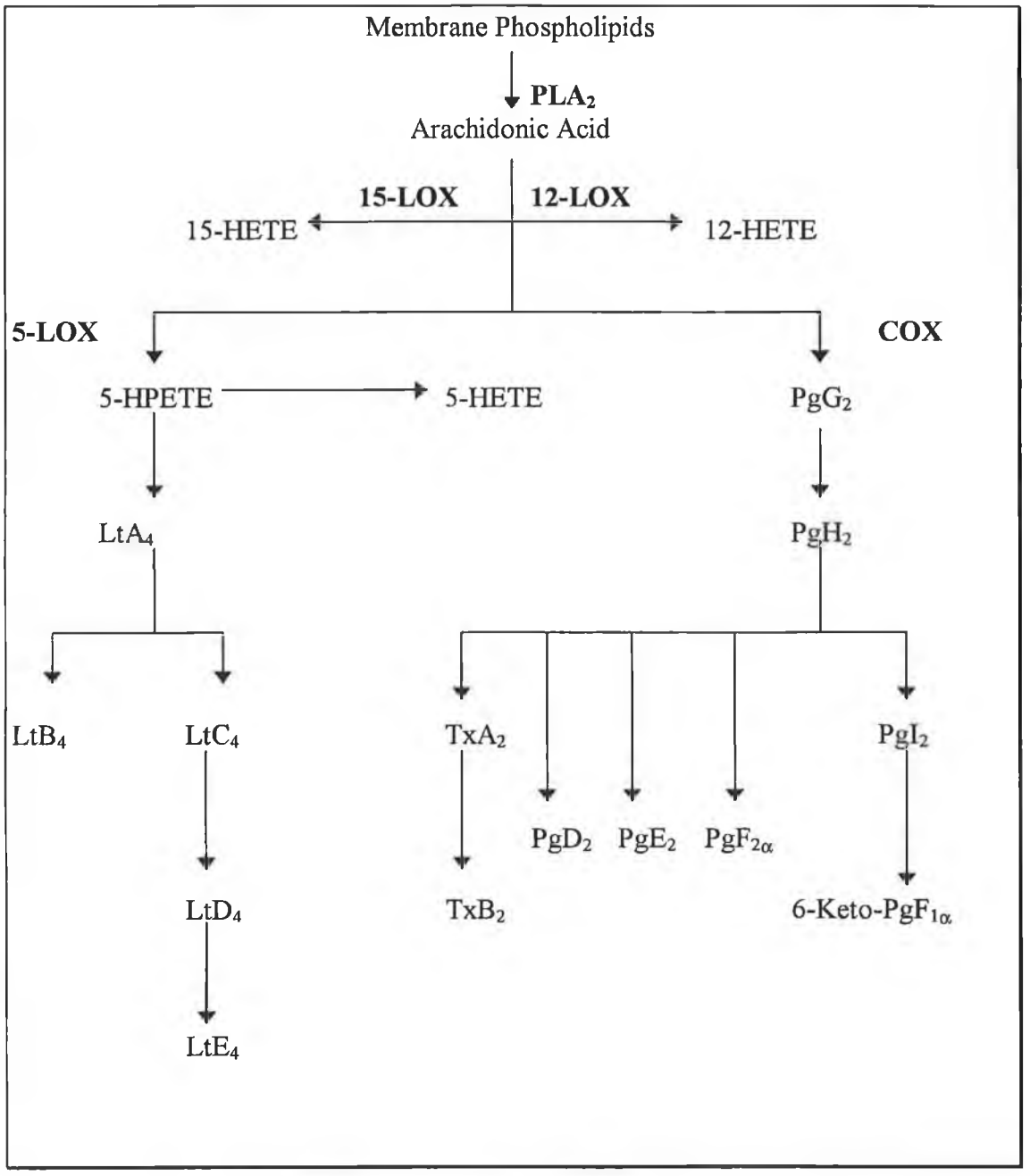

Figure 1.3.1: The arachidonic acid cascade. All enzymes are signified by bold type. PLA_2 indicates phospholipase A_2, COX indicates prostaglandin H_2 synthase and LOX indicates lipoxygenase. Details of other abbreviations are provided in Section 1.3

After liberation from cell membrane phospholipids, AA than becomes available for enzymatic modification to eicosanoids (Needleman *et al.*, 1986). The most extensively characterised branches of the arachidonic acid cascade are the prostaglandin endoperoxide synthase (also known as PGHS, cyclooxygenase or COX) and the lipoxygenase (LOX) catalysed branches of the arachidonic acid cascade, (Needleman *et al.*, 1986). The COX or PGHS catalysed branch of the AA cascade results in the formation of prostaglandins (such as PgE_2), thromboxanes (TxA_2 and TxB_2) and prostacyclin (PgI_2), which are collectively known as the prostanoids (Needleman *et al.*, 1986). The lipoxygenase catalysed branch of the arachidonic acid cascade may result in the formation of leukotrienes (such as LtC_4), as well as hydroxy and hydroperoxy fatty acids (HETEs and HPETEs), (Henderson, 1994).

Prostaglandins possess a cyclopentane ring and two side chains with a carboxyl group in one side chain (Needleman *et al.*, 1986). The first prostaglandins to be discovered were designated PgE and PgF respectively because of their preferential solubility in ether (E) and phosphate buffer (F for *fosfat*, or phosphate in Swedish). A subscript number such as in PgE_2 denotes the number of double bonds in the two chains, with two double bonds being most common for prostaglandins derived from arachidonic acid (Mathews and van Holde, 1990). A subscript α such as in the case of $PgF_{2\alpha}$ indicates that the hydroxyl group at C_9 (carbons are numbered from the carboxyl group at the end of the side chain) is on the same side of the ring as the adjacent C_8 to C_7 bond, (Mathews and van Holde, 1990). Similar nomenclature is used for thromboxanes and prostacyclin.

The labile intermediate PgH_2 is the immediate precursor of all series-2 prostaglandins (such as PgE_2), thromboxanes and prostacyclin. The fate of PgH_2 is dependent on the relative activities of the enzymes catalysing the specific inter-conversions to either of the series-2 prostaglandins, thromboxanes and prostacyclin, (Hubbard *et al.*, 1988). The primary prostaglandins (PgE_2, D_2 and $F_{2\alpha}$) can be formed by non-enzymatic degradation of the endoperoxides (PgG_2 and PgH_2), but formation of PgE_2 and PgD_2 can also be catalysed by an isomerase (Vane, 1987). The intermediate PgH_2 can also be metabolised to TxA_2 by thromboxane synthase and to PgI_2 by PgI_2 synthase

(Needleman *et al.*, 1986). These labile compounds are subsequently and rapidly converted to TxB_2 and 6-keto-$PgF_{1\alpha}$ which are stable but usually biologically inactive.

The relative amounts of individual prostanoids formed from PgH_2 depend on the cell type in question (Lau *et al.*, 1987). Prostanoids exert a wide variety of effects on diverse tissues and cells through specific cell surface receptors (Negishi *et al.*, 1995). Prostanoid receptors are classified into five basic types on the basis of sensitivity to the five primary prostanoids PgE_2, D_2, $F_{2\alpha}$, I_2 and TxA_2. These receptors are termed EP, DP, FP, IP and TP respectively. The EP family of receptors is composed of four subtypes namely EP_1 to EP_4 which are produced through alternative RNA splicing from a single gene and which differ only in their carboxy terminal tails (Negishi *et al.*, 1995). The prostanoid receptors are G-protein linked and so prostanoid receptor binding can have a wide variety of signal transduction effects, (Di Marzo, 1995).

Leukotrienes are products of the AA cascade formed through the action of the 5-lipoxygenase (5-LOX) enzyme pathway (Ford-Hutchinson, 1994). To date, three animal lipoxygenases have been identified namely 5, 12- and 15-lipoxygenase, which are named after the position where oxygen is inserted into AA (Honn *et al.*, 1994). The 5-LOX enzyme resides in the cytosol until cell activation induces a calcium dependent translocation to a putative cell membrane receptor. This enzyme and a 18 kDa membrane protein named 5-LOX activating protein, (FLAP), are required for leukotriene biosynthesis in intact cells, (Brooks and Day, 1991). The activity of 5-LOX initially results in the formation of 5-HPETE and then in the formation of the unstable epoxide LtA_4. This may then serve as the substrate for two enzymes namely LtA_4 hydrolase or LtC_4 synthase (Needleman *et al.*, 1986). The LtA_4 hydrolase enzyme catalyses the production of LtB_4. LtC_4 synthase catalyses conjugation of glutathione to LtA_4, resulting in LtC_4 production (Nelson and Seitz, 1994). The leukotriene LtC_4 can be further metabolised to LtD_4 by a glutamyl transferase enzyme and this can subsequently be converted to LtE_4 by a dipeptidase enzyme (Eberhart and DuBois, 1995). The leukotrienes C_4, D_4 and E_4 are collectively known as the cysteinyl leukotrienes or the slow reacting substances of anaphylaxis (SRS-A), (Needleman *et al.*, 1986). The precise number as well as the specificity of receptors for cysteinyl

leukotrienes is currently unclear. It is known that LtD_4 binds to a specific receptor that differs from LtB_4 receptors and LtC_4 receptors (Snyder and Fleisch, 1989). There have been no reports of a specific receptor for LtE_4. These receptors are similar to prostanoid receptors as they are G protein linked and so receptor activation can give rise to a variety of cellular effects (Henderson, 1994).

1.3.1 Prostaglandin endoperoxide H synthases -1 and -2

Prostaglandin endoperoxide H synthase (PGHS) or cyclooxygenase (COX) is one of the most important enzymes involved in the production of prostanoids from arachidonic acid. This enzyme possesses two enzymatic activities namely a cyclooxygenase activity, which inserts two molecules of O_2 into arachidonic acid to yield PgG_2, and a peroxidase activity which reduces PgG_2 to the corresponding 15-hydroxy analogue PgH_2, (Needleman *et al.*, 1986). This enzyme is now known to exist in two separate isoforms namely PGHS-1 and PGHS-2, also known as COX-1 and COX-2. The PGHS-1 isozyme is produced constitutively in many cell types but synthesis of the PGHS-2 isozyme in inducible by a wide range of extracellular and intracellular stimuli including interleukins (Croxtall *et al.*, 1996), lipopolysaccharide (Geisel *et al.*, 1994), tumor necrosis factor (Geng *et al.*, 1995) and other factors such as serum, retinoic acid, epidermal growth factor, transforming growth factor-α and platelet activating factor (Williams and DuBois, 1996). PGHS-1 is believed to be a ubiquitous constitutive enzyme whereas PGHS-2 seems to be present in only a select number of tissues such as fibroblasts, endothelial cells, monocytes and ovarian follicles in response to an appropriate stimulus (Smith *et al.*, 1996). The PGHS-2 enzyme is expressed constitutively in a number of locations including tracheal epithelia, brain and testes (Smith *et al.*, 1996).

The human PGHS-1 gene is 22.5 kb in length and composed of 11 exons and 10 introns and is located on chromosome 9 at locus 9q32-33.3, (Kosaka *et al.*, 1994). The human PGHS-2 gene has been mapped to chromosome 1q25.2-q25.3, and is approximately 8.3 kB in size with 10 exons, (Kosaka *et al.*, 1994). The PGHS-1 gene lacks a TATA box but little is known of the regulation of expression of this gene. Expression of the PGHS-2 gene can be induced through multiple signalling pathways involving protein kinase C, protein kinase A, tyrosine kinases and *src,* (Smith *et al.*, 1996). The transcription of PGHS-1 and PGHS-2 gives rise to mRNAs that are 2.7 and 4.5 kb in size (O'Banion *et al.*, 1991). The PGHS-1 mRNA codes for the production of homodimer of 70 kDa subunits with 565 amino acid residues as well as a short signal peptide and four N-linked glycosylation sites. The PGHS-2 mRNA

codes for a homodimer protein composed of 70 kDa subunits, which is approximately 75% homologous to PGHS-1 at the protein level (Williams and DuBois, 1996).

The crystallographic structure of ovine PGHS-1 has been characterised by Picot *et al.*, (1994). This group showed that the enzyme possesses three independent folding units namely an epidermal growth factor domain, a membrane binding motif and an enzymatic domain. It was found that the cyclooxygenase active site was created by a long hydrophobic channel which was the site of NSAID (non-steroidal anti-inflammatory drug) binding. The peroxidase active site was located within a wide cleft on the exterior surface of the protein, (Picot *et al.*, 1994). The enzyme was also shown to be located in a single leaflet of the lipid bilayer (Picot *et al.*, 1994). The crystal structure of PGHS-2 has been recently been reported and has been shown to be extremely similar to that of PGHS-1 (Smith *et al.*, 1996). The PGHS-1 and PGHS-2 isozymes are integrated into the lumenal surface of the endoplasmic reticulum and into the contiguous outer membrane of the nuclear envelope (Otto and Smith, 1994).

1.3.2 Eicosanoids in cancer

A wide range of eicosanoids have been shown to be involved in the regulation, initiation and promotion steps in tumor development (Marnett, 1992). These potent biological mediators have also been shown to have important roles in the rate of tumor growth and dissemination in many tumor types (Young *et al.*, 1985). A wide variety of human and animal tumor types have been shown to produce abnormally high levels of eicosanoids, (Bennett, 1986). It is estimated that PGHS-2 protein levels are increased in 90% of human colon cancers and 40% of pre-malignant colorectal adenomas, but PGHS-2 protein is not expressed in non-tumor colon tissue (Boolbol *et al.*, 1996). There is no specific relationship between type of tumor and the range or quantities of eicosanoids produced (Milas and Hanson, 1995). Hubbard *et al.*, (1988) characterised the profile of prostanoids formed from endogenous arachidonic acid in 16 cell lines from different histological classes of carcinomas (colorectal adenocarcinomas, ovarian adenocarcinomas, prostate adenocarcinoma, non-small cell lung carcinoma and small cell lung carcinoma). It was found that PgE_2 and $PgF_{2\alpha}$ were the only prostanoids synthesised in detectable quantities and that only one of three colorectal adenocarcinoma derived cell lines, one of three ovarian adenocarcinomas derived cell lines and four of five non-small cell lung carcinoma cell lines produced significant levels of eicosanoids, (Hubbard *et al.*, 1988). None of the other cell lines tested produced significant levels of prostanoids.

Eicosanoids have been shown to exert both stimulatory (Qiao *et al.*, 1995; Zhang and Fulton, 1994; De Asua *et al.*, 1975) and inhibitory effects on tumor cell growth (Ellis *et al.*, 1990; Santoro *et al.*, 1976). Studies carried out into the effects of eicosanoids on tumor cell growth *in vivo* are complicated by the fact that it is often unclear if the tumor cell is responsible for eicosanoid production, or if the eicosanoids in question originate from other cell types in the vicinity of the tumor. Marnett, (1992) has reviewed studies involving human colon tumors which appeared to produce higher levels of PgE_2 than the surrounding mucosa. A number of these studies found that enhanced prostaglandin production was due to host peripheral mononuclear cell activity rather than due to enhanced PgE_2

production by the tumor cells. For this reason, assessments of the direct eicosanoid producing abilities of tumor cells are most effectively carried out using tumor cell lines (Lau *et al.*, 1987).

Eicosanoids have been shown to be involved in tumor promotion, initiation and regulation as well as mutagenesis, immune suppression and metastasis (Marnett, 1992). The peroxidase activity of PGHS is primarily responsible for the involvement of eicosanoids in mutagenesis (Marnett, 1994). This activity catalyses the oxidation of a wide range of xenobiotics including several classes of chemical carcinogens (Eling and Curtis, 1992). This oxidation can result in the formation of mutagenic derivatives (Eling *et al.*, 1990). The PGHS catalysed branch of the arachidonic acid cascade is also responsible for the production of a direct acting mutagen, malondialdehyde which has been shown to be mutagenic in bacterial and mammalian models as well as being carcinogenic in rats (Marnett, 1994). This mutagen is produced as a result of both enzymatic and non-enzymatic breakdown of PgH_2 (Needleman *et al.*, 1986). Malondialdehyde has been shown to cause frame-shift and base-pair substitution mutations in Salmonella typhimurium (Mukai and Goldstein, 1976) and is known to form adducts with deoxyguanosine, deoxycytosine and deoxyadenosine residues (Marnett, 1994). In addition to generating the direct acting mutagen malondialdehyde, PGHS can generate peroxy radicals as reactive intermediates during the catalysis process, (Marnett, 1994). These radicals can oxidise the double bonds of pre-carcinogens such as benzopyrene-7,8-diol to the carcinogenic form (Levy, 1997).

Tumor growth is often associated with immune suppression and it has been shown that eicosanoids can have critical roles in this immune suppression process (Plescia *et al.*, 1975). Tumors can directly suppress host immune defences (directly effecting tumor infiltrating lymphocytes, circulating T-cells, natural killer cells and macrophages) through production of soluble immune suppressive factors such as PgE_2. Alternatively tumors can indirectly cause immune suppression by stimulating host immune suppressor cells such as $CD8^+$ suppressor T-cells, (Young, 1994). Prostanoids such as PgE_2 have the ability when present in high levels in areas surrounding tumors, to diminish immune surveillance. Inhibition of this

synthesis due to the presence of an NSAID, can cause this immune suppression to be reversed, (Taffet and Russell, 1981).

The influence of eicosanoids on the immune response is intricately linked to the role of eicosanoids in metastasis. A wide range of eicosanoids have been shown to be involved in metastasis (Honn *et al.*, 1994) but the involvement if PgI_2 and TxA_2 illustrate the complexity of the relationship between eicosanoids and metastasis (Honn *et al.*, 1981a). Formation of aggregates of tumor cells and platelets has been suggested as one possible activation mechanism for tumor cell attachment to vascular endothelium, providing an immunological shield for tumor cells and aiding in the installation of tumor cells in small capillary beds, (Marnett, 1992). Any of these events could partially facilitate tumor cell metastasis. Prostacyclin, PgI_2 is a product of arachidonic acid metabolism in vascular endothelial cells and TxA_2 is produced by platelets. Prostacyclin is a potent inhibitor of platelet aggregation whereas TxA_2 promotes platelet aggregation (Marnett, 1992). For this reason the balance between TxA_2 and PgI_2 levels becomes a critical factor in determining if platelets aggregate. Studies carried out with the potent inhibitor of platelet aggregation, PgI_2 have shown that this eicosanoid can inhibit metastasis in colon, lung, breast, pancreas and prostate carcinomas (Honn *et al.*, 1981b; Marnett, 1992). A significant amount of interest has recently arisen in the use of aspirin in the treatment of digestive tract cancers, (Thun, 1994). There are a number of possible explanations for the relative successes achieved in epidemiological studies. One of the possible explanations for the anti-tumor activity of aspirin involves the influence of aspirin on TxA_2 and PgI_2 production in vascular endothelial cell and in platelets. The acetylation of PGHS by aspirin is irreversible and so cells such as platelets with a low protein synthetic ability have their PGHS activity irreversibly inhibited. This results in an absence of platelet derived TxA_2 until new platelets are synthesised (which can take a number of days). The vascular endothelial cells have a superior level of protein synthetic ability and so PgI_2 returns to normal levels a short period after aspirin treatment. This results in PgI_2 levels predominating over TxA_2 levels which ultimately results in an effective inhibition of platelet aggregation. The eicosanoid 12-HETE is derived from 12-lipoxygenase catalysed insertion of oxygen into the

AA moiety and has also been shown to have some important involvement in cancer metastasis. Honn *et al.*, (1994) have shown that the ability of certain tumor cell types to generate 12-HETE is directly related to their metastatic potential. This eicosanoid has been shown to have involvement in tumor cell motility, angiogenesis and vasculature-tumor cell interactions (Honn *et al.*, 1994). In addition, Tang *et al.*, (1996) have shown that 12-HETE is a critical regulator of cell survival and apoptosis in rat Walker 256 carcinosarcoma cells.

1.4 Nonsteroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are a group of compounds prescribed for use as anti-inflammatories, antipyretics or analgesics and are believed to account for 3% to 9% of the total numbers of prescriptions issued world-wide (Alhava, 1994). In the United Kingdom over 20 million prescriptions for NSAIDs are issued each year for a variety of conditions including management of cancer pain (Pace, 1995). NSAIDs may be classified by chemical structure and so may be subdivided into five main groups, namely the salicylates (such as aspirin and diflunisal), oxicams (such as piroxicam and tenoxicam), 5-pyrazolone derivatives (such as phenylbutazone), arylacetic acid derivatives (such as indomethacin, acemetacin, sulindac, tolmetin and zomepirac) and the fenamates and isoteres (such as mefenamic and flufenamic acid), (Korolkovas, 1988).

NSAIDs are believed to achieve their therapeutic effects by inhibition of PGHS which ultimately results in a reduction in prostanoid production (Vane, 1994). NSAIDs vary in the mechanism by which they inhibit the cyclooxygenase activity of PGHS. Aspirin transfers an acetyl moiety to a single serine (Ser^{530} in the case of PGHS-1 and Ser^{516} in the case of PGHS-2) hyroxyl group of PGHS. This inhibits PGHS activity by denying arachidonic acid entry to the cyclooxygenase active site of PGHS (De Witt *et al.*, 1990). Aspirin totally inhibits prostanoid synthesis by PGHS-1 but causes the activity of PGHS-2 to be altered so that an overproduction of 15-HETE results (Meade *et al.*, 1993). The majority of NSAIDs such as ibuprofen compete competitively with AA for binding to the cyclooxygenase site of PGHS (Mitchell *et al.*, 1994). NSAIDs such as

indomethacin bind to the cyclooxygenase active site and induce a conformational change which makes the active site inaccessible to AA (Levy, 1997).

NSAIDs show significant variation in their respective abilities to inhibit either the PGHS-1 or PGHS-2 isozymes (Frolich, 1997). Aspirin preferentially inhibits PGHS-1 but the majority of NSAIDs such as ibuprofen, indomethacin, diclofenac and piroxicam show both intermediate levels of PGHS-1 inhibitory activity and low levels of PGHS-2 inhibitory activity (Vane and Botting, 1996). Few NSAIDs possess highly significant PGHS-2 inhibitory activity but Meloxicam has recently been shown by Engelhardt *et al.*, (1996a) to possess a high preference in intact cells for inhibition of PGHS-2 rather than for the PGHS-1 isozyme. Meloxicam has also been shown to preferentially inhibit PGHS-2 *in vivo* when tested in the rat pleurisy model of the inflammatory process (Engelhardt *et al.*, 1996b). The PGHS isozyme preference of NSAIDs is important in terms of the ability of an NSAID to achieve their anti-inflammatory, antipyretic and analgesic effects. In achieving the desired therapeutic effect, a given NSAID must not cause serious side effects such as ulceration. This can be caused by the inhibition of constitutive prostanoid production in healthy tissue (Fries, 1996). The side effects associated with NSAIDs are unfortunately very common and are responsible for the largest number of adverse effect reports to the FDA in the United States (Pace, 1995). Eicosanoids have a variety of roles in conditions such as inflammation where eicosanoid production is increased as a result of some form of inflammatory stimulus (Samuelsson, 1983). This increased eicosanoid production can often be the result of PGHS-2 induction whereas PGHS-1 levels remain unchanged (Munroe and Lau, 1995). Ideally one would aim to inhibit PGHS-2 by NSAID treatment and so eliminate the abnormally high levels of prostanoid production. PGHS-1 activity should not be affected so that the normal physiological activities of prostanoids such as cytoprotection of the gastric mucosa would remain unaffected (Whittle *et al.*, 1980).

1.4.1 The effects of NSAIDs on tumor cell growth

There have been numerous reports over the last 10 years describing the ability of NSAIDs to significantly reduce or in some cases eliminate the occurrence and development of various types of cancer. Three related areas of research have provided evidence of the anti-tumor activity of NSAIDs. Firstly, in animal models of colon carcinogenesis, NSAIDs such as sulindac, indomethacin and piroxicam, when administered at high concentrations (often up to 80% of maximum tolerated dose), have been repeatedly shown to cause a reduction in the number of tumors per animal as well as an overall reduction in the number of tumor bearing animals (Reddy *et al.*, 1990; Moorghen *et al.*, 1988; Narisawa *et al.*, 1981). The second line of evidence comes from studies of familial adenomatous polyposis (FAP), an autosomal dominant disorder characterised by the formation of hundreds of colorectal adenomas and eventually development of colon cancer. Numerous studies such as performed by Giardiello *et al.*, (1993) have shown that sulindac can prevent the formation of colorectal polyps in FAP patients. In addition there is some evidence that NSAIDs such as sulindac can also cause the regression of sporadic colorectal adenomatous polyps (Matsuhashi *et al.*, 1997). A mouse model of FAP was used by Boolbol *et al.*, (1996) where a strain containing a dominant mutation in the APC (adenomatous polyposis coli) tumor suppressor gene was developed. This strain (known as Min mice) developed gastrointestinal adenomas by 110 days of age. It was found that the Min animals produced increased levels of PGHS-2 and PgE_2 compared to wild type mice. Treatment with sulindac inhibited tumor 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 in wild-type mice). The third indication of the ability of NSAIDs to influence tumor growth comes from epidemiological studies (Weiss and Forman, 1996). Results of 15 colorectal cancer studies are now available and the results illustrate that patient treatment with NSAIDs, particularly aspirin results in a significant reduction in colorectal cancer risk (Berkel *et al.*, 1996).

The mechanism by which a number of individual NSAIDs can in some cases influence tumor growth is presently unclear. In addition it is has not yet been fully proven if the effects of NSAIDs in the three types of studies listed above are a result of only one or even a number of aspects of a given NSAID's activities. There are a number of possible explanations for the anti-tumor activity of NSAID and these include inhibition of prostanoid biosynthesis, interference with tumor cell cycle and induction of apoptosis (Krishnan and Brenner, 1997) as well as effects of NSAIDs on the immune system (Gilman and Chang, 1987).

The influence of NSAIDs on eicosanoid production from arachidonic acid was examined in Section 1.4, but basically it can be generalised that NSAIDs can reduce prostanoid biosynthesis by inhibiting the cyclooxygenase activity of PGHS. Many tumor types overproduce prostanoids and these prostanoids have been shown to increase the proliferation rate of tumor cell lines and related cells *in vivo* (Qiao *et al.*, 1995). Inhibition of this growth stimulatory activity due to the presence of an NSAID such as indomethacin, obviously can cause a resultant reduction in tumor cell growth (Tripathi *et al.*, 1996; Tanaka *et al.*, 1989). It is known that the influence of NSAIDs on tumor cell growth is not entirely dependent on PGHS inhibition because of a number of factors. The first of these factors stems from studies which have shown no correlation between the ability of an NSAID to inhibit tumor cell growth and the potency of that NSAID as an inhibitor of PGHS (DeMello *et al.*, 1980). Secondly, sulindac sulfone, which is devoid of any PGHS inhibitory activity has been shown to inhibit HT-29 colon carcinoma cell growth to an extent comparable to that achieved with sulindac sulfide, a potent inhibitor of PGHS (Piazza *et al.*, 1995). In addition, a number of studies such as performed by Planchon *et al.*, (1995) with the MCF-7 breast carcinoma cell line have shown that exogenous addition of prostanoids such as PgE_2 was ineffective for achieving a reversal of NSAID mediated growth inhibition. Hanif *et al.*, (1996) carried out a detailed investigation into the effects of sulindac sulfide and piroxicam on the growth of the HT-29 and HCT-15 human colon adenocarcinoma cell lines. The HT-29 cell line produces PgE_2, $PgF_{2\alpha}$ and PgI_2, but HCT-15 does not possess PGHS and hence is devoid of prostanoid synthesising ability (Hanif *et al.*, 1996). It was found that treatment of these cell lines with sulindac sulfide

and piroxicam had comparable growth inhibitory effects and that these effects could not be reversed by exogenous addition of PgE_2, $PgF_{2\alpha}$ and PgI_2. For this reason, it was obvious that both sulindac sulfide and piroxicam exerted their growth inhibitory effects independently of any inhibition of PGHS (Hanif *et al.*, 1996).

NSAIDs have recently been shown to have effects on the cell cycle progression and apoptosis of tumor cells (Piazza *et al.*, 1997a; Goldberg, 1996; Pasricha *et al.*, 1995; Piazza *et al.*, 1995). Shiff *et al.*, (1996) investigated the effects of aspirin, indomethacin, naproxen and piroxicam on proliferation, cell cycle phase distribution and apoptosis in HT-29 cells. It was found that these NSAIDs reduced the proliferation and altered the morphology of HT-29 in a time dependent and concentration dependent fashion. Only indomethacin and aspirin increased the fraction of cells in the G_0/G_1 phase and accordingly reduced the fraction of cells in the S and G_2/M phases of the cell cycle. In addition, these NSAIDs were shown to reduce the levels of the cdks (cyclin dependent kinases), $p34^{cdc2}$ and $p33^{cdk2}$ which have critical roles in the G_1/S and G_2/M transitions respectively (Shiff *et al.*, 1996). Indomethacin, piroxicam and naproxen but not aspirin were shown in this study to induce apoptosis in the HT-29 colon adenocarcinoma cell line. The HT-29 cell line expresses mutant p53 protein only (Rodrigues *et al.*, 1990) and so this apoptosis occurred independently of wild-type p53 protein.

The tumor suppressor gene p53 is critical in the regulation of the G_1/S checkpoint of the cell cycle (Kastan *et al.*, 1991). The levels of the p53 protein have been shown to increase in response to DNA damage, which ultimately results in a G_1 cell cycle arrest (allowing for DNA repair to occur) or apoptosis (Shaw *et al.*, 1992). It has been reported that over 50% of human lung, breast and colon tumors possess missense p53 mutations (Hollstein *et al.*, 1991; Nigro *et al.*, 1989) with mutant p53 unable to cause G_1 arrest or apoptosis. The p21 tumor suppressor gene is induced during activation of the p53 mediated G_1/S checkpoint but it can also be regulated by p53 independent mechanisms. The p21 protein has the ability to inhibit the kinase activity of a variety of cdks which can prevent phosphorylation of a variety of substrates including pRb (retinoblastoma protein) (Harper *et al.*, 1993). The pRb tumor suppressor gene product causes cell cycle arrest in late G_1 by regulating the activity of the E_2F family of

transcription factors (Weinberg, 1995). The pRb protein also possesses the ability to inhibit apoptosis (Haas-Kogan *et al.*, 1995) but the role of pRb in carcinogenesis has not been fully elucidated. Goldberg *et al.*, (1996) investigated the influence of sulindac and sulindac sulfide on p53, p21 and pRb protein levels in HT-29 cell line. It was found that these compounds caused a G_0/G_1 cell cycle block and exit from the cell division cycle. This was associated with an initial increase followed by an abrupt decrease in the levels of $p34^{cdc2}$, a reduction in the levels of the mitotic cyclins A and B_1, an increase in p21 levels, a reduction in total pRb and an increase in hypophosphorylated pRb as well as a reduction in the levels of p53 (Goldberg *et al.*, 1996). Piazza *et al.*, (1995) showed that sulindac sulfide and sulindac sulfone inhibited the growth of a variety of cell lines but these compounds also were growth inhibitory for normal fibroblasts and normal epithelial cells. Both of these sulindac derivatives induced apoptosis in the HT-29 cell line, and this was shown to be independent of their inhibitory effect on cell cycle progression (Piazza *et al.*, 1997a; Piazza *et al.*, 1995). It was recently reported by Thompson *et al.*, (1997) that sulindac sulfone inhibited the occurrence of mammary carcinoma which were classified as having either the wild type or mutant Ha-*ras* gene, but with significantly more potent inhibition in 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*, (Thompson *et al.*, 1997).

The relationship between NSAID treatment and apoptosis in rat intestinal epithelial (RIE) cells was examined by Tsujii and DuBois, (1995). These RIE cells were permanently transfected with a PGHS-2 expression vector. The transfected cells showed high levels of PGHS-2 protein and were resistant to butyrate-induced apoptosis. In addition, these transfected cells possessed elevated bcl-2 protein and reduced transforming growth factor (TGF-β) levels, all of which are indications of increased tumorigenic potential. It was found that the resistance to apoptosis was overcome by sulindac sulfide treatment. In a related study, Tsujii *et al.*, (1997) reported that PGHS-2 transfected human colon cancer cells (CaCo-2) possessed significantly increased RNA levels for activated metalloproteinase-2, and that this resulted in increased metastatic potential. This increased invasiveness and the

increased prostanoid production due to overexpression of PGHS-2 was shown to be eliminated by sulindac sulfide.

1.4.2 NSAIDs in cancer chemotherapy

NSAIDs have been extensively shown to inhibit the growth of some tumors *in vivo* and *in vitro*. NSAIDs have also been shown to potentiate tumor radioresponse, (Furata *et al.*, 1988), but only a select number of studies have investigated the possibility of NSAID-mediated enhancement of chemotherapeutic drug toxicity.

Powles *et al.*, (1978) reported that indomethacin and flurbiprofen rendered a chemo-resistant variant of the Walker tumor more resistant to chlorambucil treatment *in vivo*. Bennett *et al.*, (1982) showed that flurbiprofen and indomethacin decreased cancer development and spread in mice bearing a transplanted mammary adenocarcinoma when treated in combination with methotrexate and melphalan. This toxicity enhancement effect was also shown *in vitro* using indomethacin in combination with methotrexate, (Gaffen *et al.*, 1985). Flurbiprofen, unlike indomethacin, was unable to enhance methotrexate cytotoxicity *in vitro* (Bennett *et al.*, 1987). Indomethacin was also shown to increase methotrexate accumulation *in vitro* (Bennett *et al.*, 1987).

The toxicity of methotrexate in LoVo human colon cancer cells was unaffected by co-treatment with indomethacin, (Gaffen *et al.*, 1989). Indomethacin increased the cytotoxicity of methotrexate to the human breast cell lines DU4475 and T47D, (Bennett *et al.*, 1989). The mechanism of enhancement of methotrexate toxicity by indomethacin has not been conclusively proven. A number of interesting observations have been made namely that the toxicity enhancement effect is unlikely to be due to displacement of methotrexate from binding sites on serum proteins, inhibition of prostaglandin synthesis, inhibition of cAMP phosphodiesterase or calcium transport, (Yazici *et al.*, 1992). Indomethacin does not have a beneficial interaction with all cytotoxic drugs. Sato *et al.*, (1983)

reported that indomethacin in combination with 5-fluorouracil was no more effective at killing a mouse colon adenocarcinoma *in vivo* than 5-fluorouracil treatment alone. A select number of other drugs have been successfully used in combination with indomethacin. Hall *et al.*, (1989) showed that the toxicity of chlorambucil was potentiated 5.5 fold in a resistant Chinese Hamster Ovary cell line following exposure to indomethacin.

A study by Maca, (1991) showed that non-toxic concentrations of indomethacin enhanced the sensitivity to VP-16 and Methotrexate in a variety of cell lines. Cultured Lewis lung carcinoma (LLC) cells, mouse lymphoma cells, a human acute lymphoblastic leukaemia cell line and human chronic myelogenous leukaemia cells were tested. These cell lines are relatively insensitive to VP-16 alone but indomethacin was found to enhance VP-16 cytotoxicity in all cases. Indomethacin also increased the response of LLC cells to methotrexate but not to bleomycin. It was concluded that indomethacin enhances VP-16 cytotoxicity by increasing the cellular concentration of VP-16. 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. This report suggested that indomethacin acted as a specific inhibitor of MRP and so modulated drug resistance as a result of this activity. Hollo *et al.*, (1996) characterised the transport properties of MRP in MRP-transfected and drug selected MRP overexpressing cells and found that indomethacin at concentrations of 10-20 μM inhibited MRP activity by approximately 50%. Kobayashi *et al.*, (1997) examined the drug sensitivity of two pulmonary adenocarcinoma cell lines to a variety of chemotherapeutic drugs in the presence of various NSAIDs. It was found that indomethacin, mefenamic acid, sulindac and tolmetin enhanced the toxicity of both adriamycin and vincristine. 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 (Kobayashi *et al.*, 1997).

A number of additional NSAIDs have been used in combination with a select number of cytotoxic drugs. The use of piroxicam in a cytotoxic drug treatment regimen was reported by Braun *et al,* (1987). Patients with lung cancer receiving mitomycin C, vinblastine and cisplatin treatment were treated with piroxicam for 31 days. This group found that the immuno-modulatory effects of cytotoxic chemotherapy were ameliorated by adding piroxicam to the cytotoxic drug treatment regimen.

Teicher *et al.*, (1994) reported on the use of mefenamic acid, diflunisal, sulindac and indomethacin as modulators of cytotoxic drug therapies in the treatment of the Lewis lung carcinoma in mice. A mixture consisting of indomethacin or sulindac combined with phenidone (a lipoxygenase inhibitor) and cisplatin was 2.5-fold more effective in causing tumor growth delay than was cisplatin treatment alone. Sulindac in combination with either minocycline (a collagenase inhibitor) or phenidone increased the tumor growth delay achieved by cyclophosphamide by 2.0-fold. Sulindac in combination with minocycline or phenidone and mefenamic acid in combination with minocycline caused a 4.5-fold increase in tumor growth delay achieved by melphalan. Sulindac or diflunisal in combination with minocycline caused a 3.0-fold increase in the tumor growth delay caused by carmustine.

1.5 Multiple drug resistance (MDR) in cancer

1.5.1 P-glycoprotein in MDR

Kessel *et al.*, (1968) reported that anthracycline selected drug resistant cells exhibited a drug accumulation deficit relative to the drug sensitive parental cell line. Since that initial report, a wide variety of multidrug resistant (MDR) cell lines have been isolated as a result of exposure to chemotherapeutic drugs such as anthracyclines, epipodophyllotoxins, vinca alkaloids, actinomycin D and colchicine (Germann, 1996; Clynes, 1993; Beck and Danks, 1991). These resistant cell lines may be obtained in three ways namely by adaptation to gradually increasing concentrations of an appropriate drug, transfection with an appropriate drug resistance cDNA or alternatively by establishment of the cell line from an inherently resistant tumor (Clynes, 1993). These cell lines generally exhibit cross resistance to a range of structurally unrelated drugs but are relatively sensitive to 5-fluorouracil and platinum compounds (Hill, 1993; Beck and Danks, 1991). Overexpression of a plasma membrane efflux pump, known as P-glycoprotein or Pgp has been shown to be involved in the resistance of many of these MDR cell lines (Germann, 1996). The physiological role of Pgp in normal tissues is presently unclear, but it is known to be highly expressed in the adrenal cortex, proximal tubules of the kidney, bile canaliculi of the liver, colon and jejunum (Izquierdo, 1996a; Favrot *et al.*, 1991; Sugawara *et al.*, 1988). The Pgp protein may have a role in normal tissues as a detoxifying transporter of xenobiotics (foreign organic compounds not produced by metabolic cellular activity), (Garberoglio *et al.*, 1992; Cordon-Cardo *et al.*, 1989) and as a steroid transporter in the adrenal gland (Van Kalken *et al.*, 1993; Van Kalken *et al.*, 1992). The overexpression of Pgp is often found in the case of tumors derived from tissues which normally express Pgp such as carcinomas of the adrenal gland, lung, colon, kidney, liver and pancreas (Izquierdo, 1996a). A study involving over 400 human cancers showed that elevated expression of the mdr1 gene responsible for Pgp synthesis, occurred in the case of colon cancer, renal cell carcinoma, and hepatoma (Goldstein *et al.*, 1989). In many cases, Pgp expressing tumors have been shown to have a poor prognosis (Fisher and Sikic, 1995).

1.5.1.1 Structure of the gene encoding P-glycoprotein

The Pgp protein in humans is encoded by the mdr1 gene, which is present on the long arm of chromosome 7, band q21.1 (Chen *et al.*, 1986). Transfection experiments have shown that a full length cDNA clone coding for mdr1 conferred drug resistance when transfected into drug sensitive cell lines (Gros *et al.*, 1986). This gene possesses 27 exons in the protein coding region of the gene and 14 of these exons code for the left and 13 for the right half of the Pgp molecule (Roninson, 1991). The nomenclature used to describe mdr1 related genes (the MDR gene family) in different species, according to a scheme involving sequence similarity of the 3' untranslated regions, is summarised in Table 1.5.1.1, (Heenan, 1994). This illustrates that humans only have two, while mice and hamsters possess three classes of members of the MDR gene family. In hamsters and mice only class I and II are believed to be involved in drug resistance but in humans only class I is believed to convey drug resistance to cells. As mentioned above, transfection of full length cDNA for mdr1 conferred drug resistance when transfected into drug sensitive cell lines (Gros *et al.*, 1986). In addition transfection of human mdr3 or mouse mdr2 cDNA did not result in drug resistance (Van der Bliek *et al.*, 1988). The mdr1 locus is 230 kb in length and is approximately 300 bp upstream of the mdr3 gene (Lincke *et al.*, 1991; van der Bliek *et al.*, 1988). The mdr1 and mdr3 genes encode 4.1 to 4.5 kb mRNA transcripts which share a significant level of homology (Roninson, 1991).

Class	Human	Hamster	Mouse -Scheme A	Mouse -Scheme B
I	mdr1	pgp1	mdr3	mdr1a
II		pgp1	mdr1	mdr1b
III	mdr 3/2	pgp1	mdr2	mdr2

Table 1.5.1.1: Classification and nomenclature of mammalian P-glycoproteins

Increases in mdr1 mRNA have been shown to occur in the presence and absence of gene amplification (Bradley *et al.*, 1989; Shen *et al.*, 1986; Roninson *et al.*, 1986). Two promoter regions for this gene have been identified (Ueda *et al.*, 1987a, b) one of which is upstream and the other which is downstream. This downstream promoter is preferentially used by the majority of resistant cells *in vitro* (Ueda *et al.*, 1987b). A wide variety of agents have been shown to influence transcription of the mdr1 gene. The treatment of a number of colon carcinoma cell lines with dimethyl sulfoxide, sodium butyrate or dimethyl formamide have been shown to increase mRNA and protein levels of Pgp (Mazzanti *et al.*, 1996; Mickley *et al.*, 1989). Treatment of human cell lines with chemotherapeutic drugs or UV radiation has also been shown to increase mdr1 expression (Chaudhary and Roninson, 1993; Uchiumi *et al.*, 1993). Phorbol ester response element and a number of heat shock consensus elements have also been shown to be involved in the transcription of mdr1 under appropriate conditions (Angel *et al.*, 1997; Chin *et al.*, 1990; Angel *et al.*, 1987).

1.5.1.2 P-glycoprotein protein structure

The mdr1 gene product is a 170 kDa membrane associated protein of 1280 amino acids which contains 12 transmembrane domains with 6 extracellular loops and 2 cytoplasmic ATP binding domains. (Gros *et al.*, 1986). A model predicted by computer assisted hydropathy analysis and amino acid sequence comparisons with bacterial transport proteins (Chen *et al.*, 1986; Gros *et al.*, 1986) suggests that both the N- and C-terminal membrane associated domains possess 6 transmembrane regions. The N- and C-termini and nucleotide binding folds are predicted to be present intracellularly with the overall structure composed of two halves of similar structure bridged by a linker region (Van der Bliek *et al.*, 1987). In addition, the first extracellular loop is predicted to be glycosylated (Chen *et al.*, 1986; Gros *et al.*, 1986). The Pgp protein is predominately localised in the plasma membrane of MDR cells (Izquierdo, 1996a) but low levels of expression have been shown to be present in association with the endoplasmic reticulum and Golgi apparatus (Germann, 1996).

The human Pgp protein is produced as a 120-140 kDa non-glycosylated precursor which can be processed to the 170 kDa form within 1-2 hours (Richert *et al.*, 1988). The primary structure of mammalian Pgp suggests the presence of several potential N-linked glycosylation sites within the first extracellular loop with Asn^{91}, Asn^{94} and Asn^{99} the relevant sites in human Pgp (Schinkel *et al.*, 1993). The precise role of glycosylation of Pgp is unclear but it has been suggested that it is not critical for Pgp mediated drug efflux (Germann, 1996). For example, multidrug resistant sublines have been established from a glycosylation defective hamster mutant cell line (Ling *et al.*, 1983). In addition, tunicamycin, an inhibitor of glycosylation did not affect the drug resistance characteristics of the human T-cell leukemia resistant cell line CCRF-CEM (Beck and Cirtain, 1982). It has been suggested that glycosylation is required for translocation of the Pgp protein to the plasma membrane and this suggestion has developed from studies such as performed by Kramer *et al.*, (1995) where a tunicamycin treated human colon carcinoma cell clone was shown to possess reduced levels of plasma membrane associated Pgp.

P-glycoprotein is a phosphorylated glycoprotein but a number of the details related to the phosphorylation mechanisms involving this protein require further investigation. Pgp has the ability to undergo rapid cycles of phosphorylation and dephosphorylation (Ma *et al.*, 1991). Studies carried out by Chambers *et al.*, (1990) using the human MDR KB-V1 carcinoma cell line showed that protein kinase C (PKC) and type 1 or 2A phosphatases were responsible for this activity. Increased phosphorylation of Pgp was shown to cause an increase in Pgp-mediated drug efflux activity and inhibition of phosphorylation was shown to decrease this activity (Chambers *et al.*, 1990). The phosphorylation of Pgp by PKC was believed to occur on serine residues only, namely Ser^{661} and Ser^{671} and one or more of Ser^{667}, Ser^{675} and Ser^{683} which occur in the linker domain of Pgp (Chambers *et al.*, 1993). The Ser^{667} residue is now known to be the third site for PKC phosphorylation of Pgp (Chambers *et al.*, 1994). The cAMP dependent protein kinase (PKA) has also been shown to phosphorylate Pgp *in vitro* (Mellado and Horwitz, 1987). Treatment of Pgp overexpressing cells with protein kinase inhibitors such as staurosporine and calphostin C, causes a reduction in the levels of phosphorylation of Pgp and in some cases a resultant inhibition of the drug efflux activity of Pgp (Bates *et al.*, 1993; Ma *et al.*, 1991). These kinase inhibitors are

relatively non-specific, exerting a variety of effects on cells and so results such as these should be interpreted with caution. Mutational analysis performed with Ser^{661}, Ser^{671}, Ser^{667}, Ser^{675} and Ser^{683} residues on Pgp, where these residues were substituted with alanine residues (which cannot be phosphorylated), showed that non-phosphorylated Pgp possessed the same ability to confer MDR as the wild-type phosphorylated form (Germann, 1996). It is now believed that phosphorylation 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.

1.5.1.3 P-glycoprotein mediated transport

The role of Pgp in normal tissues is currently unclear but it is believed to have a role in protection against natural xenobiotics (Cordon-Cardo *et al.*, 1989). It has been shown to be induced with cytochrome P-450 genes in rat liver after xenobiotic administration (Burt and Thoreirsson, 1988), and is proposed as being a modulator of an endogenous chloride channel (Hardy *et al.*, 1995). The overexpression of Pgp in tumor cells can confer cross-resistance to a broad range of chemically and mechanistically unrelated drugs including anthracyclines, vinca alkaloids, epipodophyllotoxins, actinomycin D and Taxol (Clynes, 1993). 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 electric membrane potential (Roepe *et al.*, 1994; Roepe *et al.*, 1993). The potential drug binding sites of Pgp have been identified using azidopine in photoaffinity labelling experiments with proteolytic fragments of Pgp (Skach *et al.*, 1993). The binding was shown to occur primarily in the cytoplasmic domain in the region adjacent to transmembrane region number 6 and in the cytoplasmic region immediately following transmembrane region number 12. This suggests that one binding site is formed by the two homologous halves of Pgp. A number of point mutations have been shown to influence the drug transporting abilities of Pgp. Dhir *et al.*, (1993) showed that alterations in Ser^{939} of the mouse mdr3 gene product conferred a significant reduction in the levels of vinblastine resistance. Replacing this residue with alanine or cysteine had no effects on adriamycin and colchicine cross

resistance. Replacement of the serine residue with aspartic acid practically caused a complete loss of adriamycin and cholchicine resistance, (Dhir *et al.*, 1993). The suggestions of direct involvement of Pgp in drug transport have been supported by the results of a number of related studies as discussed by Germann, (1996). As described earlier transfection of full length cDNA for mdr1 conferred drug resistance when transfected into drug sensitive cell lines (Gros *et al.*, 1986). In addition, cellular Pgp levels often correlate with drug resistance levels in MDR cell lines (Fojo *et al.*, 1985). Pgp has been shown to directly bind to cytotoxic drugs, as demonstrated by photoaffinity labelling experiments with a photoactive analogue of vinblastine and 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).

1.5.1.4 Modulation of Pgp mediated drug resistance

Verapamil was the first agent to be shown successfully to reverse the MDR phenotype in Pgp expressing cells *in vitro* (Tsuruo *et al.*, 1981). Since this initial study a large number of compounds have been shown to be potential modulators of Pgp activity. Clinical studies have shown that in general, the maximum tolerated dose of the clinically available modulators yielded serum levels significantly below those required for Pgp inhibition (Fisher *et al.*, 1996). MDR modulators may interfere with cytotoxic drug efflux by acting as competitive or non-competitive inhibitors or by binding to chemosensitiser binding sites that cause allosteric changes. These allosteric changes can result in the inhibition of cytotoxic drug binding to the relevant efflux pump (Ford, 1995). The majority of chemosensitisers described to date may be grouped into six categories (Ford, 1995) namely immunosuppressive drugs (cyclosporin A and SDZ PSC833), calcium channel blockers (verapamil), calmodulin antagonists (trifluoroperazine), steroidal agents (tamoxifen), vinca alkaloid analogues (vindoline), and miscellaneous compounds (quinidine).

The MDR modulator verapamil has been shown to inhibit Pgp mediated drug efflux. This modulating effect is independent of this compound's ability to directly affect calcium channels (Robert *et al.*, 1987). Verapamil inhibits the binding of various chemotherapeutic drugs to Pgp (Ford, 1995). This has been confirmed by photoaffinity labelling experiments which showed that this calcium channel blocker binds directly to the Pgp protein (Safa, 1988). Cyclosporin A is a cyclic hydrophobic peptide of 11 amino acids and it has been found to reverse drug resistance in MDR cells. Cyclosporin A may act as a substrate for P-glycoprotein and antagonises Pgp activity through competitive inhibition of cytotoxic drug efflux (Twentyman, 1988). The direct binding of cyclosporin A to Pgp has been shown by photoaffinity labeling experiments (Foxwell *et al.*, 1989). A number of less immunosuppressive cyclosporin analogues such as PSC 833 and PSC 280-446 have also been developed and are being examined as possible modulators of Pgp *in vivo* (Ford, 1995; Gaveriaux *et al.*, 1991).

Research into the area of MDR modulators is continuing, but the compounds used to date *in vivo* have been shown to be required in very high doses. These high doses often exceed the maximum tolerated doses of these compounds, causing unacceptable toxic side effects (Erlichman *et al.*, 1993). Ferry *et al.*, (1996) reported on the results of a randomised trial of verapamil in a chemotherapeutic regime for the treatment of small cell lung cancer. Patients were treated with cyclophosphamide, adriamycin, vincristine and VP-16. Verapamil at a dose of 480 mg/day (the maximum tolerated dose) was also included in the treatment, and at this dose verapamil reached a serum level of 0.8 μM. This level is approximately 8-fold lower than the verapamil concentration needed to reverse Pgp mediated resistance *in vitro*, (Ferry *et al.*, 1996). Twentyman and Versantvoort, (1996), stated that any strategy designed to suppress efflux of cytotoxic drugs by a drug efflux pump was highly probable to increase normal tissue toxicity. Even if this was the case, the development of novel circumventing and drug modulating strategies would permit the accumulation of information which would ultimately lead to a successful therapeutic treatment for drug resistant cancer.

1.5.1.5 Pgp as a member of the ATP-binding cassette superfamily

The MDR gene family in mammals is related to the ATP-binding cassette (ABC) superfamily with can exist in non-mammalian species. The (ABC) superfamily are structurally diverse with over 50 members, but all contain at least one hydrophobic transmembrane region and a cytoplasmic nucleotide binding domain (NBD), (Higgins, 1992). These proteins are involved in energy dependent transport of a variety of substrates across membranes. In eukaryotic cells, ABC genes often encode four domains composed of two NBDs and two transmembrane regions, (Leveille-Webster and Arias, 1995). The ATP binding NBDs contain conserved residues (termed Walker A and B motifs) spaced by 90-120 amino acids (Walker C motif). There is wide variation in the chemical nature of the molecules transported by ABC proteins, which vary from chloride ions to antigenic peptides associated with the endoplasmic reticulum of immune cells, (Bellamy, 1996). The Pgp protein in humans shows a significant degree of homology with the Hly B protein of *Escherichia coli* with 50% amino acid sequence identity between the two proteins, (Juranka *et al.*, 1989). P-glycoprotein is also homologous with the leukotoxin secretion protein lkt in *Pasteurella Haemolytica,* (Strathdee and Lo, 1989). The human Pgp protein exhibits significant homology to the ndvA protein in *Rhizobium Meliloti* which possesses glycan transporting abilities, (Stansfield *et al.*, 1988). The STE 6 gene product in *Saccharomyces cerevisiae* shares homologous regions with Pgp in their nucleotide binding regions and transmembrane domains (McGrath and Varshavsky, 1989). Several mammalian homologues of Pgp have been identified including the TAP1 and TAP2 proteins (Section 1.5.3), CFTR (cystic fibrosis transmembrane conductance regulator protein) which is an ATP dependent cAMP regulated chloride channel and PMP 70, which when mutated results in a fatal cerebro-hepato-renal dysfunction known as Zellweger syndrome, (Leveille-Webster and Arias, 1995).

1.5.2 Multidrug Resistance-associated Protein

The drug transporter Pgp has been shown to be present in a variety of human tumors and multidrug resistant cell lines. Numerous examples now exist of multidrug resistant cell lines and tumors which have Pgp-independent drug resistance mechanisms (Broxterman *et al.*, 1995). One of these mechanisms involves the multidrug resistance-associated protein, MRP. Since the initial discovery of MRP in the drug resistant, small cell lung cancer cell line, H69AR, (Cole *et al.*, 1992), this transporter molecule has been shown to be present in non-Pgp multidrug resistant cell lines from a variety of tumor types. These tumors include leukaemias, fibrosarcoma, small cell and non-small cell lung carcinomas, breast, cervix, prostate and bladder carcinomas (Izquierdo *et al.*, 1996c; Loe *et al.*, 1996a). There have also been reports of co-expression of Pgp and MRP in a number of drug selected cell lines (Brock *et al.*, 1995). MRP 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).

Cell lines overexpressing MRP are typically cross-resistant to anthracyclines, epipodophyllotoxins and vinca alkaloids (Loe *et al.*, 1996a). Although the resistance profiles of drug-selected MRP or Pgp overexpressing cell lines are similar, considerable differences exist particularly with regard to Taxol resistance (Loe *et al.*, 1996a). The explanation for a cell developing MRP rather than Pgp-mediated resistance during drug exposure remains unclear, but it is believed that overexpression of MRP may confer initial levels of resistance, while Pgp overexpression develops as higher levels of resistance are required for survival. A study by Brock *et al.*, (1995) showed that in the small cell lung cancer cell line, H69, MRP is over-expressed during selection in low concentrations of VP-16. Following further selection in higher concentrations of drug, MRP expression remained relatively constant, but Pgp overexpression developed. The expression of MRP protein and mRNA is not limited to drug selected MRP-overexpressing

cells. Berger *et al.*, (1997) reported that a significant number of drug sensitive non-small cell lung cancer cell lines expressed MRP mRNA and protein.

1.5.2.1 MRP gene structure

The MRP gene is distantly related to the mdr1 gene, and has been localised to chromosome 16p13.1 (Cole *et al.* 1992). Cloning and sequence analysis performed by Zhu and Center, (1994), of a 2.2 kb 5'-flanking sequence of genomic MRP, (a region of strong promoter activity) of HL60-ADR cells indicated the presence of a number of regulatory elements. These included AP-1, AP-2, SP-1, ERE (estrogen response element), GRE (glucocorticoid response element) and CRE (cyclic AMP response element). The MRP gene has been shown to be amplified in a number of drug selected cell lines (Cole *et al.*, 1992). In other cases MRP mRNA levels have been shown to increase in the absence of gene amplification (Slapak *et al.*, 1994) and this is believed to be due to alterations in transcriptional control of the MRP gene. It is suggested that the major promoter activity of MRP resides in a highly GC-rich region of 194 nucleotides (Zhu and Center, 1994). This region does not contain a TATA box for directing site-specific transcriptional initiation, but contains multiple start sites for transcription. This is similar to the situation found in the case of Pgp (Ueda *et al.*, 1987b). The MRP gene codes for a message of 6.5 kb which is capable of encoding the 1531 amino acid protein. Transfection studies using full length MRP cDNA have shown that MRP can confer resistance to drug sensitive cells (Grant *et al.*, 1994).

1.5.2.2 MRP protein structure

After MRP mRNA had been isolated from the H69AR cell line (Cole *et al.* 1992), analysis showed that this mRNA coded for a member of the ABC superfamily of transmembrane transporters. The MRP protein was predicted to be 171 kDa, containing two nucleotide binding domains, each preceded by a multi-spanning transmembrane region. A number of models have been proposed (reviewed by Loe *et al.*, 1996a) for the secondary structure of MRP, but it is now believed that the transporter contains 12 transmembrane segment in the NH_2-proximal half and six transmembrane segments in the COOH-proximal half. Three other ABC transporters may also have topologies similar to MRP, namely the β cell sulfonylurea receptor (SUR) which is involved in regulating insulin secretion, the MOAT (multispecific organic anion transporter) molecule and the yeast cadmium resistance factor YCF1 (Lautier *et al.*, 1996).

MRP was initially believed to be predominately located in the endoplasmic reticulum of resistant cells but significant levels are now known to be present in the plasma membrane (Krishnamachary and Center, 1993). The phenotypes of MDR cell lines with high levels of MRP vary significantly with regards to their drug accumulation characteristics. Some drug-selected MRP-overexpressing cell lines can accumulate less drug or have higher rates of efflux than the sensitive cells from which they were derived, (Coley *et al.*, 1991). A number of MRP expressing cell lines also appear to sequester drug into cytoplasmic vesicles (Marquardt and Center 1992). If this vesicular sequesterisation occurs, it may divert drug from the relevant cellular target without affecting cellular accumulation levels. This could possibly explain some of the unusual drug accumulation characteristics of MRP-expressing cell lines. This also complicates any potential attempt to correlate MRP expression with total drug accumulation characteristics of MRP expressing cells. Analysis of subcellular distribution of MRP, as performed by Almquist *et al.*, (1995), using isopyknic density gradient centrifugation, showed that 80% of MRP in HeLa transfectants was associated with a low density plasma membrane fraction. The comparable fraction in drug-

selected MRP-overexpressing H69AR cells contained 50% of the protein. The remaining MRP and plasma membrane markers were co-distributed in higher density fractions which indicated the presence of MRP in endocytotic vesicles in H69AR cells. The MRP-transfected HeLa cells accumulated less drug over time than the parental HeLa cell line, but H69AR drug resistant cells and H69 parental cells accumulated comparable levels of drug over time. The presence of MRP-containing endocytotic vesicles in H69AR was believed to contribute to drug resistance in this cell line. Encapsulation of drug in these vesicles was suggested as having prevented drugs from reaching their target destination in the cell. This would explain both the drug resistance of MRP-overexpressing H69AR cells and the fact that both H69AR and H69 cells accumulated comparable levels of drug over time.

The amino acid sequence of MRP contains various sites known to be relevant for ATP binding and post-translational modification (Loe *et al.*, 1996a). MRP 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 (Almquist *et al.*, 1995). It is known to take approximately 90 minutes to produce the 190 kDa MRP molecule from the 170 kDa polypeptide, with the mature protein possessing a half life of 20 hours (Almquist *et al.*, 1995). Human MRP contains twelve potential sites for N-linked glycosylation, but it is believed that only three of these sites are external to the plasma membrane and so ultimately glycosylated (Almquist *et al.*, 1995). The effect of glycosylation on MRP activity is not fully known. It has been demonstrated that tunicamycin-induced inhibition of glycosylation has little effect on the cellular drug accumulation characteristics of MRP expressing cells (Almquist *et al.*, 1995).

The precise location and functional role of the phosphorylated residues on MRP are also unclear. In Pgp and CFTR (cystic fibrosis transmembrane conductance regulator), phosphorylation occurs predominately in the linker region (Chambers *et al.*, 1993) and it is believed that this is also the case for phosphorylation of

MRP. Serine is the single amino acid phosphorylated in MRP and the phosphate groups are contained in nine tryptic peptides (Ma *et al.*, 1995). The phosphorylation of these amino acids was shown to be blocked by compounds such as staurosporine. This resulted in an increase in drug accumulation in MRP overexpressing HL60-ADR cells (Ma *et al.*, 1995). The protein kinases involved in this phosphorylation have not yet been clearly identified, partly because of the fact that agents such as staurosporine simultaneously inhibit a variety of protein kinases with varying levels of potency. There are potential sites for phosphorylation of MRP by cyclic AMP/cyclic GMP-dependent protein kinases, protein kinase C, casein kinase II and tyrosine kinases (Almquist *et al.*, 1995). The primary sequence requirements for many of these sites are not strict and their presence is not necessarily indicative that they are functional. In addition, comparison of the linker region amino acid sequences of human and murine MRP have shown that there is only a low level of conservation of potential PKC (believed to be particularly important for MRP phosphorylation) phosphorylation sites between these two homologs, which otherwise share a very high proportion of conserved sequences (Lautier *et al.*, 1996).

1.5.2.3 MRP Transport Activity

The MRP transporter has the ability to transport cysteinyl leukotrienes such as leukotriene C_4 (LtC_4) and glutathione conjugates such as glutathione disulphide (GSSG), (Loe *et al.*, 1996a). A wide variety of organic anions are believed to serve as possible physiological substrates of MRP including anionic conjugates of bile salts and steroid hormones (Loe *et al.*, 1996a). Transport of heavy metal oxyanions by MRP has also been demonstrated (Cole *et al.*, 1994).

The mechanism of MRP mediated transport of chemotherapeutic drugs is presently unclear. It was known that MRP had the ability to transport glutathione conjugates and so it was suggested that cytotoxic drugs became conjugated to glutathione and in this way became potential substrates for MRP transport. This suggestion was complicated by the fact that glutathione conjugation is not known to occur for natural product drugs. In addition, MRP transfectants do not exhibit increased resistance to alkylating agents, the class of drugs for which glutathione conjugation is known to occur (Loe *et al.*, 1996a). Although MRP has been indirectly shown to reduce cellular accumulation of drugs such as adriamycin, only a limited number of reports have described the direct transport of these drugs by MRP. Studies carried out by Paul *et al.*, (1996c) showed that MRP isolated from MRP-transfected NIH 3T3 fibroblasts, could serve as an ATP dependent pump for a spectrum of natural product, unconjugated cytotoxic drugs including anthracyclines, vincristine and etoposide. Paul *et al.*, (1996b) demonstrated that MRP isolated from the HL60-ADR human leukaemia drug resistant cell line actively transported daunorubicin, VP-16 and vincristine but not vinblastine or Taxol. Shen *et al.*, (1996) reported that the activity of MRP isolated from the HL60-ADR cell line was potently inhibited by daunorubicin, vincristine and VP-16. Heijn, *et al.*, (1997) reported that MRP activity in the GLC_4/ADR small cell lung carcinoma drug resistant cell line was inhibited by daunorubicin and VP-16.

Loe *et al.*, (1996b) demonstrated that transport of vincristine by MRP occurred only in the presence of glutathione. It was suggested that either MRP co-transports glutathione and cytotoxic drugs, or that glutathione causes some form of conformational change in MRP which enables cytotoxic drugs to bind to the transporter (Loe *et al.*, 1996b). Stride *et al.*, (1997) also provided evidence that MRP-transfected cells only possess the ability to transport vincristine when glutathione is present.

The analysis of drug resistance profiles of MRP-overexpressing drug selected cell lines has generated information on the potential drug transporting abilities of MRP, (Versantvoort *et al.*, 1992; Coley *et al.*, 1991). This information is complicated by the fact that these cell lines were obtained by a stepwise selection in drug, which facilitates the development of multiple collateral resistance mechanisms. This may result in the simultaneous expression of a variety of drug resistance mechanisms which ultimately results in difficulties in interpreting the contribution of any individual mechanism to drug resistance levels, (Brock *et al.*, 1995, Hasegawa *et al.*, 1995). Studies carried out with MRP-transfected cell lines serve as more useful models for definition of 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.

A study carried out by Breuninger *et al.*, (1995) using NIH 3T3 fibroblasts, which had been transfected with MRP cDNA, showed that these transfectants displayed increased resistance to several lipophilic drugs. These included adriamycin (9.4-fold more resistant than negative control transfectant), daunorubicin (7.8-fold resistance), VP-16 (11.4-fold resistance), actinomycin D (2.3-fold resistance) and vincristine (3.8-fold resistance). These tranfectants showed very low to negligible levels of resistance to vinblastine (1.2-fold resistance) and Taxol (1.3-fold resistance), but were more sensitive to cisplatin (0.9-fold resistance). The absence of high level resistance to Taxol in MRP-transfected cells is significant as this drug is a high affinity substrate for transport by Pgp. In a similar study carried out by Cole *et al.*, (1994) it was found that MRP-transfected cell populations

possessed a moderate level of resistance to adriamycin (6.9-fold resistance), daunorubicin (6.3-fold resistance), epirubicin (8.6-fold resistance), vincristine (11.6-fold resistance) and VP-16 (10.3-fold resistance). These cells showed only low level of resistance to Taxol (1.7-fold resistance), vinblastine (3-fold resistance) and colchicine (2.1-fold resistance). In addition, these transfectants were not resistant to 9-alkyl anthracyclines (1.0-fold resistance), mitoxantrone (1.0-fold resistance) or cisplatin (0.9-fold resistance). The MRP transfected cells were also resistant to a selection of heavy metal anions including arsenite, arsenate and trivalent and pentavalent antimonials, but were not resistant to cadmium chloride, (Cole *et al.*, 1994).

1.5.2.4 Modulation of MRP mediated drug resistance

As was the case for defining the drug substrate menu of MRP, identification of specific modulators of MRP is complicated by the fact that this transporter exhibits various similarities to other drug transporter molecules such as Pgp. In drug selected cell lines, co-expression of various drug resistance mechanisms makes it extremely difficult to conclusively deduce if a given modulator influences drug accumulation due to interference with MRP activity only. In many cases, potential modulators of MRP can have significant effects on drug transporters such as Pgp. For this reason, ideally all screening of potential modulators of MRP should be performed in MRP-transfected cell lines. 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. This chemosensitisation was believed to be independent of interference with MRP activity as the degree of chemosensitisation by these agents was similar in MRP-transfected cells and in cells transfected with the vector alone. Both verapamil and cyclosporin A caused a slight increase in vincristine accumulation in resistant cells, but did not return the level of accumulation to that achieved by the drug sensitive cells. A further study by Breuninger *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.

The chemosensitisers verapamil and cyclosporin A are known to be much less effective at reversing drug resistance in MRP overexpressing drug selected cell lines, rather than in Pgp overexpressing drug selected cell lines, (Coley *et al.*, 1991). In addition, MRP could not be labelled with a photoaffinity analogue of verapamil in the MRP-overexpressing drug selected HL60-ADR cell line, (McGrath *et al.*, 1989). This suggests that verapamil does not directly interact with MRP, whereas in the case of Pgp, verapamil is known to bind directly to the protein and interfere with transport in this manner, (Safa, 1988).

A significant number of compounds have now been suggested as having the ability to modulate MRP activity. Practically all of these studies have been carried out using drug selected MRP-expressing cells and so the selectivity of inhibition may be questioned as many of these compounds can have effects on a variety of cellular drug resistance mechanisms. The use of these compounds as modulators of MRP has been reviewed by Twentyman and Versantvoort, (1996). The two most interesting compounds to emerge as potentially selective MRP modulators include the anionic quinoline LtD_4 receptor antagonist MK571 and the bisindolylmaleimide protein kinase C inhibitor GF109203X. The leukotriene D_4 receptor antagonist MK571 has been extensively characterised by Gekeler *et al.*, (1995a) in drug selected Pgp and MRP expressing cell lines. A complete reversal of vincristine resistance was achieved by co-incubation with a non-toxic level (40 μM) of MK571 in an MRP-expressing cell line. This compound was found to have no effect on the level of toxicity achieved in a Pgp-overexpressing cell line. 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 GF109203X 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. The effect of this

compound on drug resistance of a Pgp-overexpressing cell line was not examined in this study (Gekeler *et al.*, 1995b). The bisindolylmaleimide GF109203X is reported as being a highly selective inhibitor of PKC isozymes, with inhibitory effects on other kinases only at very high concentrations. It was assumed by Gekeler *et al.*, (1995b) that this compound exerted an inhibitory influence on MRP activity due to interference with phosphorylation and so activity of the transporter, or that it interacted directly with the transporter. No explanation was offered for the inability of this compound to reverse adriamycin resistance.

Although the majority of studies into modulation of MRP involve the use of compounds such as verapamil, the use of antisense oligonucleotides to reduce MRP protein expression has also been reported, (Stewart *et al.*, 1996). A sequence was identified, ISIS 7597, complementary to nucleotides 2107-2126 of MRP that reduced levels of MRP mRNA and protein in transfected HeLa cells by 90% and 70% respectively. Cells treated with ISIS 7597 have been shown to enhance sensitivity to adriamycin (Stewart *et al.*, 1996), but the decreases in MRP mRNA and protein levels were short lived and only a partial sensitisation to adriamycin was achieved.

1.5.2.5 MRP as a member of the ATP-binding cassette superfamily

The ATP-binding cassette (ABC) superfamily are structurally diverse but all contain at least one hydrophobic transmembrane region and a cytoplasmic nucleotide binding domain (NBD) as described earlier. These proteins are involved in energy dependent transport of a variety of substrates across membranes. In eukaryotic cells, ABC genes often encode four domains composed of two NBDs and two transmembrane regions. The ATP binding NBDs contain conserved residues (termed Walker A and B motifs) spaced by 90-120 amino acids (Walker C motif). There is wide variation in the chemical nature of the molecules transported by ABC proteins, which vary from chloride ions to antigenic peptides associated with the endoplasmic reticulum of immune cells, (Loe *et al.*, 1996a).

A total of 21 new genes from the human ABC superfamily have recently been identified, bringing the total number of characterised human ABC genes to 33, (Allikmets *et al.*, 1996). A number of the previously characterised human ABC proteins have been well characterised and these include proteins involved in drug transport (Pgp and MRP), peptide transporters involved in antigen presentation (TAP), and those involved in inherited human diseases (CFTR (cystic fibrosis transmembrane conductance regulator), ALD (adrenoleukodystrophy), SUR (sulfonylurea receptor) and PMP70 (human peroxisomal membrane protein)). Members of the ABC superfamily with which MRP has greatest degree of sequence homology include CFTR (19% amino acid identity) which mediates chloride conductance in airway epithelia and other tissues, rat SUR (29% amino acid identity), ItpgpA (32% amino acid identity), a *Leishmania* protein which confers resistance to arsenic and antimony centred oxyanions, YORI (33% amino acid identity) a yeast protein that mediates oligomycin resistance and YCF1 (43% amino acid identity), which is a yeast protein that mediates cadmium resistance (Loe *et al.*, 1996a). It has been suggested that MRP may be involved in the regulation of endogenous ion channels. As described above, MRP possesses significant sequence homology with CFTR (outwardly rectifying Cl^- channel), SUR (inwardly rectifying K^+ channel) and Pgp (volume regulated Cl^- channel), but there have been only a limited number of reports describing MRP's influence on ion channel activity. Jirsch *et al.*, (1994) reported that Cl^- and K^+ channel activity was increased in MRP expressing H69AR cells relative to the drug sensitive parent.

The MRP transporter has the ability to transport a wide variety of hydrophobic anionic compounds such as oxidised glutathione and a number of different steroid glucuronides and bile salt derivatives, (Leier *et al.*, 1996). The multispecific organic anion transporter (MOAT) in liver canicular membranes and other tissues (Mayer *et al.*, 1995) also has the ability to transport molecules of this nature, and it was suggested that the MRP-1 gene was responsible for cMOAT synthesis. The extremely low expression of MRP-1 protein in the liver and the localisation of MRP-1 in the basolateral hepatocyte membrane indicated that this suggestion was

incorrect. Recent studies by Paulusma *et al.*, (1996) provided evidence that MRP-1 and cMOAT are encoded by two different genes namely MRP-1 and MRP-2.

The role of glutathione and glutathione S-transferases will be described briefly in Section 1.5.5.2. It has been shown relatively conclusively that increased levels of GST are involved in resistance to alkylating agents (Puchalski and Fahl, 1990) but it has proven more difficult to conclusively prove involvement in other forms of resistance such as to cisplatin and anthracyclines. Ishikawa and Ali-Osman, (1993) showed that cisplatin resistance is complex as it involves two steps, namely the initial formation of a glutathione (GSH) S-conjugate and the subsequent removal of the toxic conjugate from the cell by a glutathione conjugate pump, which this group describes as the GS-X pump. It is now also known as a MOAT protein or the leukotriene C_4 transporter (Leier *et al.*, 1994). Conjugation of cisplatin and GSH can occur non-enzymatically but export from the cell requires the GS-X pump. The activity of the GS-X pump has been shown to be enhanced in one cisplatin resistant mutant (Ishikawa *et al.*, 1994). Muller *et al.*, (1994) found no increased activity of the GS-X pump in a cisplatin resistant cell line, but found a substantial increase in GS-X pump activity in the MRP-overexpressing GLC_4/ADR cell line, relative to the sensitive parental cells. The GS-X pump is described as being an ATP-dependent export pump for organic anions such as cysteinyl leukotrienes and certain organic anions such as methotrexate. The studies by Muller *et al.*, (1994) and Leier *et al.*, (1994) showed that overexpression of the MRP gene in cells resulted in increased ATP dependent GS-conjugate transport. Ishikawa *et al.*, (1996) assumes from this evidence that the MRP gene product is a GS-X pump and that the MRP and GS-X pumps possess similar if not identical substrate specificity. The sensitivity of the vast majority of MRP overexpressing cell lines to cisplatin was explained by Ishikawa *et al.*, (1996) as being a result of insufficient intracellular levels of GSH in these cells. This group concludes that simultaneously elevated expression of the GS-X pump and increased GSH biosynthesis, are the critical factors in determining resistance levels to cisplatin, alkylating agents and heavy metals.

Database screening has permitted the identification of three new homologues of the MRP-1 gene, which is responsible for the MRP-1 gene product, and the MRP2 gene, which is responsible for cMOAT production. These newly discovered genes have been designated MRP-3, MRP-4 and MRP-5. The MRP-3 protein, like cMOAT is mainly expressed in the liver, with MRP-4 expressed at very low levels in a number of different tissues, and MRP-5 expressed in almost all tissues analysed (Kool *et al.*, 1997). Preliminary investigations have also been carried out to investigate the role of these MRP homologues in drug resistance. It was found that MRP-4 was not over-expressed in any of the cell lines tested. The MRP-3 and MRP-5 transporters were only over-expressed in a limited number of cell lines but mRNA levels in these cell lines did not correlate with levels of adriamycin or cisplatin resistance. In addition, it was found that cMOAT was over-expressed in a number of cell lines and that mRNA levels correlated with cisplatin but not adriamycin resistance (Kool *et al.*, 1997). Further investigations are necessary in order to investigate the exact contribution of these homologues of MRP to drug resistance in MRP expressing cells.

1.5.3 Transporter associated with Antigen Presentation

The transporter associated with antigen presentation (TAP) is a recently discovered member of the ABC superfamily which has been found to be over-expressed in a number of MDR cell lines (Izquierdo *et al.*, 1996d). TAP is a heterodimer formed by the products of the TAP1 and TAP2 genes. This protein is involved in MHC (major histocompatibility complex) class I antigen presentation where it facilitates peptide translocation over the endoplasmic reticulum membrane (Neefjes *et al.*, 1993). Pgp has also been reported as having the ability to transport peptides (Sarkadi *et al.*, 1994). The TAP1 and Pgp proteins share highly homologous regions among their transmembrane sections (Manavalan *et al.*, 1993). The transfection of the TAP genes into mutant lymphoblastoid cells lacking TAP caused the levels of resistance of these cells to VP-16, adriamycin and vincristine to increase 2-fold. It was also demonstrated that high VP-16 or vincristine concentrations inhibited the transport of a model peptide by TAP into the endoplasmic reticulum, (Izquierdo *et al.*, 1996d). It has been suggested that TAP may have a role in low levels of MDR but further investigations will be necessary in order to obtain more definitive information on the possible involvement of TAP in MDR, (Izquierdo *et al.*, 1996d).

1.5.4 Lung Resistance-related Protein

The lung resistance related protein (LRP) was identified by Scheper *et al.*, (1993) as a 110 kDa vesicular protein over-expressed in a non-Pgp expressing MDR lung cell line. LRP protein expression has been demonstrated in the epithelia of the bronchus, digestive tract, as well as in keratinocytes, macrophages and the adrenal cortex (Izquierdo *et al.*, 1996b). The LRP gene is localised on chromosome 16p11.2 which is close to the locus for the MRP gene (16p13.1), (Izquierdo, 1996a). LRP has been shown to be over-expressed in a large number of Pgp negative drug selected MDR cell lines including small cell lung cancer cell lines such as GLC_4-ADR, breast cancer lines such as MCF7-MR, fibrosarcoma derived lines such as HT1080-DR4 and myeloma cell lines such as 8226-MR40 (Scheper *et al.*, 1993). When the LRP gene

was cloned and sequenced it was found to share over 87% amino acid identity with the major vault protein in *Rattus norvigecus* (Scheffer *et al.,* 1995).

Vaults have been isolated from a variety of species and are highly conserved among these different species, (Kedersha *et al.,* 1990). Vaults in other species are most abundant in epithelial cells and macrophages, as is found in the case of LRP in normal human tissues, (Kedersha *et al.,* 1990). Vaults are ribonucleoprotein particles that in the rat are composed of a major vault protein of 104 kDa (accounting for over 70% of the mass of the particle), three minor proteins of 210, 192 and 54 kDa and a small RNA molecule (Izquierdo, 1996a). These vault constituents are constructed into a barrel-like structure of approximately 57 x 32 nm. The vault particle has 2-fold symmetry and each half of the vault particle can be opened into a flower-like structure which contains eight petals surrounding a central ring (Kedersha *et al.,* 1991). Most vaults are present in the cytoplasm and it is believed that the majority of cell types possess thousands of vaults (Chugani *et al.,* 1993). Vault structure and localisation support a transport function for this particle which could involve a variety of substances, but the precise function of vaults is currently unknown (Izquierdo, 1996a).

In a study conducted by Izquierdo *et al.,* (1996b), relatively low levels of LRP protein were shown to be present in tissue samples from acute myeloid neuroblastoma, leukaemia, and testicular cancer. Intermediate levels of LRP were present in ovarian cancer and high levels present in colon, renal and pancreatic carcinomas. The simultaneous overexpression of MRP and LRP was reported by Izquierdo *et al.,* (1996c) as being a frequent event in non-Pgp MDR cell lines. In this study, MRP and LRP were found to be widely expressed in a panel of 61 human cancer cell lines. These cell lines possessed a low level of expression of P-glycoprotein. LRP was found to be a more accurate indicator of *in vitro* drug resistance of these cell lines, compared to P-glycoprotein and MRP (Izquierdo *et al.,* 1996c). Expression of LRP in primary ovarian cancer was significantly associated with a poor response to chemotherapy and shorter overall survival (Izquierdo *et al.,* 1995). It has been suggested that LRP may be a potentially useful marker of clinical drug resistance particularly in the case of acute lyphoblastic leukaemia (ALL), acute myeloid leukaemia (AML) and in some cases of ovarian carcinoma (Izquierdo *et al.,* 1996e). The extent of the involvement of

LRP in the manifestation of MDR requires further investigations but it has been suggested by Izquierdo *et al.*, (1996e) that vaults regulate the cytoplasmic redistribution and nucleocytoplasmic transport of drugs. Scheffer *et al.*, (1995) showed that transfection of the LRP gene did not confer the MDR phenotype on the transfected cells.

1.5.5 Additional mechanisms of resistance in MDR

In addition to Pgp, MRP, TAP and LRP expression in multidrug resistant cells, a number of alternative mechanisms of resistance have been identified as potentially being involved in MDR. These mechanisms can include alterations in the levels of topoisomerase II, glutathione S-transferases or metallothionein expression. Metallothioneins are proteins of 6-7 kDa molecular weight which have the ability to bind heavy metal ions (Mattern and Volm, 1995). The production of metallothioneins by tumor cells has been suggested as a possible mechanism for the intracellular inactivation of metal containing chemotherapeutic agents such as cisplatin (Mattern and Volm, 1995). Kasahara *et al.*, (1991) showed that metallothionein content correlated with the sensitivity of human small cell lung cancer cell lines to cisplatin. Kelley *et al.*, (1988) showed that overexpression of metallothioneins in a cell line contributed to resistance to cisplatin, chlorambucil, melphalan and adriamycin.

1.5.5.1 Topoisomerase II

The type I and type II DNA topoisomerases are highly conserved enzymes that catalyse the critical steps in DNA replication, transcription, chromosome segregation and DNA recombination. All cells possess the type I topoisomerase which makes single stranded cuts in DNA and the type II topoisomerase which cut and pass double stranded DNA (Wang, 1985). Inhibitors of DNA topoisomerases can often interfere with enzyme catalysis at a variety of stages of the process (Nitiss and Beck, 1996). The activity of the topoisomerase II enzyme is affected by certain categories of chemotherapeutic drugs (De Isabella *et al.*, 1990). These drugs stabilise the cleavable

complex formed between topoisomerase II and DNA, resulting in increased DNA excision. It has been shown that alterations in topoisomerase II levels can lead to resistance to anthracyclines and the epipodophyllotoxins (De Isabella *et al.*, 1990) but not to the vinca alkaloids. A correlation between topoisomerase II gene expression and sensitivity to adriamycin, VP-16 and cisplatin has been shown in non-drug selected lung cancer cell lines (Giaccone *et al.*, 1992). A number of cell lines selected for resistance to VP-16 have decreased topoisomerase II content (Heenan, 1994) and this may contribute significantly to the levels of resistance to specific drugs. This decrease in topoisomerase II content can result in a decreased number of drug induced DNA strands breaks. This ultimately can result in the conferral of resistance to topoisomerase II specific drugs. The decrease in topoisomerase II content can be caused by gene inactivation (Tan *et al.*, 1989) or may also be a post-translational event (Deffie *et al.*, 1992). Additional factors such as topoisomerase II activity, isoform expression and degree of phosphorylation can also contribute to the development of the MDR phenotype (De Isabella *et al.*, 1990).

1.5.5.2 Glutathione S-transferases

Glutathione is a tripeptide that can protect the cell against two forms of metabolic stress (Mathews and van Holde, 1990). The first form involves free radicals or peroxides that can accumulate in cells under oxidising conditions. Glutathione has the ability to non-enzymatically reduce these potentially damaging free radicals. Glutathione can also be conjugated to xenobiotics or electrophiles (which can be produced by the activity of cytochrome P-450 enzymes) and this conjugation can be catalysed by the glutathione-S-transferase enzymes (GSTs). This conjugation can result in a potentially toxic compound becoming less toxic against cellular targets. In addition the compound becomes more hydrophilic which aids the excretion and elimination of the toxin in question. The GSTs are ubiquitously present in many normal tissues as well as in tumors (O'Brien and Tew, 1996). GSTs are the products of a multigene family and consist of different isozymes namely α, π, μ and θ which have the ability to conjugate reduced glutathione to a wide variety of electrophilic compounds (O'Brien and Tew, 1996). The ultimate result of GST metabolism as

stated above is the conversion of lipophilic compounds to more polar derivatives which enables elimination from the body. These enzymes catalyse the conjugation of electrophiles to the sulphur atom of the cysteine residue of glutathione (which is composed of cysteine, glutamate and glycine), (Alberts *et al.*, 1994). A number of the common cancer chemotherapeutic agents are electrophiles and can be conjugated with molecules such as glutathione and glucuronic acid to facilitate transport, metabolism and ultimately excretion. Known chemotherapeutic drug substrates of GST include chlorambucil, melphalan and cyclophosphamide (Cheng *et al.*, 1997; O'Brien and Tew, 1996). Eastman, (1987) reported on the direct conjugation of cisplatin to glutathione.

Many of the various forms of human cancer exhibit overexpression of GSTs, although a significant proportion of liver and breast carcinomas may show no expression despite being resistant to drugs metabolised by these enzymes (Harrison, 1995). Overexpression of GST could be potentially detrimental to a patient undergoing chemotherapeutic drug treatment as it may result in a more rapid detoxification and so a reduction in the efficacy of treatment of any drug that is potentially a substrate for GST. The GST-π isozyme has been shown to be present in abnormally high levels in a number of solid tumors including colon, ovary, oesophagus, pancreas and bladder, (Hayes *et al.*, 1991; Singh *et al.*, 1990). The cellular levels of glutathione are also believed to be important factors in GST mediated drug resistance because GST can only act as a detoxification mechanism if there are adequate levels of glutathione for conjugation to the appropriate drug. An inhibitor of the γ-glutamylcysteine synthase enzyme which is critical for glutathione synthesis, (buthionine-S-sulphoximine or BSO), has been shown reduce cellular GSH levels and in some cases a reversal in the resistance of MDR cell lines (Kunzmich *et al.*, 1992; Mistry *et al.*, 1991; Green *et al.*, 1984). Significant increases in glutathione have also been shown to be present in some ovarian, breast, small cell and non-small cell lung carcinomas (Singh *et al.*, 1994; Buser *et al.*, 1991). Agents such as ethacrynic acid (a diuretic drug with the ability to conjugate with GSH) have been shown to reverse chlorambucil resistance in a CLL (chronic lymphocytic leukaemia) patient (O'Dwyer *et al.*, 1991).

The role of GSTs in MDR requires further investigation, but a number of transfection studies have shown that GSTs may be involved in drug resistance, (O'Brien and Tew, 1996). Greenbaum *et al.*, (1994) showed that transfection of GSTα cDNA into NIH 3T3 fibroblasts conferred increased levels of resistance to chlorambucil. In a similar study, Nakagawa *et al.*, (1990) showed that when NIH 3T3 fibroblasts were transfected with GSTπ, the cells exhibited resistance to adriamycin and ethacrynic acid (an inhibitor of GST isozymes) but did not develop resistance to cisplatin, melphalan and chlorambucil. The results of these transfection studies are encouraging but have generated some unexpected results such as found by Nakagawa *et al.*, (1990). This group found that resistance to known GST substrates did not develop and that resistance to non-GST substrates such as adriamycin did develop.

The conjugation of electrophilic chemotherapeutic drugs to glutathione is only an efficient method of detoxification if these conjugates can be efficiently eliminated from the cell. The GS-X pump is an ATP dependent glutathione-conjugate export pump for organic anions such as cysteinyl leukotrienes and certain organic anions such as methotrexate, as has already been described in Section 1.5.2.5. This pump is closely associated with the MRP protein which is believed to have the ability to pump a variety of natural product drugs such as anthracyclines, epipodophyllotoxins and vinca alkaloids. MRP mediated resistance is not normally associated with resistance to alkylating agents, the category of drugs which are known to undergo glutathione conjugation. This has been explained for the case of cisplatin sensitivity by Ishikawa *et al.*, (1996) as being a result of a cell being deficient in some aspect of the glutathione conjugation system such as low GST or GSH levels. This can explain the sensitivity to agents such as cisplatin, but the simultaneous MRP mediated resistance to other drugs such as anthracyclines, epipodophyllotoxins or vinca alkaloids in these cells requires further explanation if MRP is assumed to be acting as a glutathione conjugate pump.

1.6 Aims of thesis

The initial aim of the research described in this thesis was the identification of chemotherapeutic drugs whose toxicity was enhanced in the drug sensitive A549 human lung adenocarcinoma cell line, in the presence of non-toxic levels of the NSAIDs indomethacin and sulindac. This was performed in order to identify a characteristic which was shared by all chemotherapeutic drugs which had their toxicity enhanced in the presence of indomethacin or sulindac but which was not possessed by drugs from other categories. Once this had been performed, further *in vitro* toxicity assay screening was performed with a wide range of NSAIDs in order to identify the specific NSAIDs which possessed the ability to enhance the toxicity of adriamycin and vincristine in the A549 cell line. The therapeutic effects of NSAIDs are generally attributed to the ability of these compounds to inhibit the activity of the prostaglandin endoperoxide H synthase (PGHS) enzyme. Studies were conducted in order to investigate if this activity was involved in the ability of an NSAID such as indomethacin to enhance the toxicity of a chemotherapeutic drug such as adriamycin.

The profile of NSAIDs with the ability to enhance the toxicity of adriamycin and vincristine was shown to be exactly similar in both the A549 human lung adenocarcinoma and DLKP human lung squamous carcinoma cell lines. This indicated that these compounds affected a drug resistance mechanism common to both of these cell lines. The specific group of chemotherapeutic drugs with which the NSAID-mediated toxicity enhancement effect could be demonstrated suggested the involvement of the multidrug resistance associated protein (MRP). The demonstration of NSAID-mediated chemotherapeutic drug toxicity enhancement in DLKP appeared to contradict this suggestion, as MRP protein had not been detected in whole cell extracts of DLKP. For this reason, plasma membrane derived inside-out vesicles were isolated from DLKP in order to demonstrate that active MRP protein was present in DLKP and to investigate the mechanisms by which NSAIDs interfered with MRP activity in this cell line.

The presence of MRP in DLKP suggested that some of the unusual drug accumulation characteristics of DLKP-related drug resistant cell lines may have been explainable by the presence of MRP in these cell lines. The DLKP-A10 drug resistant cell line was known to possess non-P-glycoprotein related drug accumulation characteristics. Experiments were conducted in order to determine if active MRP protein was present in this cell line and if this drug transporter was involved in the unusual drug accumulation characteristics possessed by DLKP-A10.

A number of clonal subpopulations of the DLKP-derived DLKP-A drug resistant cell line exhibited drug resistance characteristics that were not fully explainable by the levels of P-glycoprotein or topoisomerase II present in the cells. Inside-out vesicles were isolated from these clones and their MRP protein levels and activities characterised in an attempt to explain if this drug resistance mechanism contributed to the drug resistance characteristics of these clones.

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 by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP) to a standard of 12-18 MΩ / cm resistance. The quality of UHP was continuously monitored by a conductivity meter in the system.

2.2 Treatment of glassware

Solutions used in all stages of cell culture were stored in sterile glass bottles. Bottles and all other glassware used for cell culture related applications were prepared using the following protocol: 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. Materials were then washed in an industrial dishwasher, using Neodisher detergent and rinsed twice with ultrapure water, (UHP). Materials were finally sterilised by autoclaving as described in Section 2.3.

2.3 Sterilisation

Water, glassware and all thermostable solutions were sterilised by autoclaving at 121°C for 20 min. at 15 p.s.i.. Thermolabile solutions were filtered through 0.22 μm 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 according to the formulations illustrated in Table 2.4. 10X media were added to sterile UHP water, buffered with HEPES and $NaHCO_3$ as required and adjusted to pH 7.5 using sterile 1.5 N NaOH and 1.5 N HCl. The media were then filtered through sterile 0.22 μm bell filters (Gelman, 12158) and stored in sterile 500 mL bottles at 4°C. Sterility checks were performed on each bottle of media using the protocol described in Section 2.5.5. Basal media were stored at 4°C for up to 3 months. The HEPES buffer was prepared by dissolving 23.8 g HEPES in 80 mL UHP water followed by autoclaving. Following autoclaving, 5 mL sterile 5 N NaOH was added to give a final volume of 100 mL. $NaHCO_3$ was prepared by dissolving 7.5 g in 100 mL of UHP water followed by autoclaving. Volumes of 100 mL of basal media were in the majority of cases, supplemented with 2 mM L-glutamine (Gibco, 043-05030) and 5% foetal calf serum (Sigma, F7524). Volumes of 1 mL 100X non-essential amino acids (Gibco, 11140-035) and 100 mM sodium pyruvate (Gibco, 11360-035) were also added to MEM. Complete media was maintained at 4°C for up to a maximum of 1 week

	DMEM (Gibco 042-02501M)	**Hams F12** (Gibco 042-01430M)	**MEM** (Gibco 074-01700N)
10X Medium	500 mL	Powder	500 mL
Ultrapure water	4300 mL	4700 mL	4300 mL
1M HEPES* (Sigma H9136)	100 mL	100 mL	100 mL
7.5% $NaHCO_3$ (BDH 30151)	45 mL	45 mL	45 mL

* HEPES = N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)

Table 2.4: Preparation of basal media for cell culture

2.5 Cells and cell culture

All cell culture manipulations were performed in a class II laminar air-flow cabinet (Nuaire Biological Laminar Air-Flow Cabinet). Any experiments involving cytotoxic compounds were conducted in a cytogard laminar air-flow cabinet (Gelman Sciences, CG Series). The laminar air-flow cabinet was cleaned with 70% industrial methylated spirits (IMS) before and after use. This was also performed for any all items brought into the laminar air-flow cabinet. Only one cell line was used in the laminar air-flow cabinet at any given time. After using a given cell line, the laminar air-flow cabinet was allowed to clear for 20 minutes in order to eliminate any possibilities of cross contamination. The cabinet was cleaned weekly with industrial detergents. The detergents used were alternated every month (Virkon, Antec or TEGO, Goldschmidt). Details pertaining to the cell lines used for the experiments detailed in this thesis are provided in Table 2.5.1. For experiments conducted with DLKP in serum free media, the media components were insulin (10 μmol/L), transferrin (5 μmol/L), L-glutamine (1×10^{-2} μmol/L), selenium (3×10^{-2} μmol/L) and linoleic acid (3×10^{-1} μmol/L). All cell lines were incubated at 37°C in an atmosphere of 5% CO_2 where required. Cells were fed with fresh media every 2-3 days in order to maintain active cell growth. The cell lines listed in Table 2.5.1 are anchorage dependent cell lines with the exception of the HL60-ADR cell line which was grown in suspension in vented 75 cm^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 with 1 mL of a trypsin/EDTA solution (0.25% trypsin (Gibco, 043-05090), 0.01% EDTA (Sigma, E9884) solution in PBS (Oxoid, BR14a)).

2. A volume of 4 mL trypsin was added to the flask, which was then incubated at 37°C until the cells were seen to have detached from the surface of the flask (approximately 5 minutes).
3. Trypsin was inactivated by addition of an equal volume of complete medium to the flask.
4. This cell suspension was then added to a sterile universal container (Sterilin, 128a) and centrifuged at 1,000 r.p.m. for 5 minutes.
5. Supernatant was discarded and the cell pellet resuspended in growth medium. A cell count was performed and an aliquot of cells used to reseed a flask at the desired cell density.

Cell line	Basal medium[1,4]	Cell type	Source[2,3]
A549	DME	Human lung adenocarcinoma	ATCC
DLKP	ATCC	Human lung squamous carcinoma	NCTCC
DLKP-A	ATCC	Adriamycin resistant variant of DLKP	NCTCC
DLKP-VP3	ATCC	VP-16 selected variant of DLKP	NCTCC
DLKP-VP8	ATCC	VP-16 selected variant of DLKP	NCTCC
DLKP-A10	ATCC	Adriamycin selected variant of DLKP-A	NCTCC
DLKPA2B	ATCC	Clonal subpopulation of DLKP-A	NCTCC
DLKPA6B	ATCC	Clonal subpopulation of DLKP-A	NCTCC
DLKPA11B	ATCC	Clonal subpopulation of DLKP-A	NCTCC
DLKPA5F	ATCC	Clonal subpopulation of DLKP-A	NCTCC
SKMES-1	MEM	Human lung squamous carcinoma	ATCC
SKMES-1/ADR	MEM	Adriamycin resistant variant of SKMES-1	NCTCC
HL60-ADR	RPMI 1640	Adriamycin resistant variant of HL60 human leukaemia cell line	Dr. M. Center Univ. Kansas USA

[1]: ATCC basal media consists of a 1:1 mixture of DMEM and Hams F12.
[2]: ATCC = American Tissue Culture Collection.
[3]: NCTCC = National Cell and Tissue Culture Centre.
[4]: RPMI 1640 media supplied as a 1X stock (Gibco, 52400-025).

Table 2.5.1 Source, description and media requirements of cell lines.

2.5.2 Assessment of cell number

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 10 μL 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 1 mL 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 containers which was equivalent to a temperature of -80°C.

4 After a period of 3 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 9 mL of fresh growth medium was added to a sterile universal. The cryopreserved cells were removed from the liquid nitrogen and thawed at 37°C. The cells were removed from the vials and transferred to the aliquoted media.
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 cell 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 into Columbia blood agar plates (Oxoid, CM331), Sabauraud dextrose (Oxoid, CM217) and Thioglycollate broths (Oxoid, CM173). These facilitated the detection of bacteria, fungus and yeast contamination. Complete cell culture media were sterility tested at least 4 days prior to use using Columbia blood agar.

2.6 *Mycloplasma* 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 *Mycloplasma* analysis

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

2.6.2 Direct Staining procedure for *Mycloplasma* analysis

Direct staining for *Mycoplasma* analysis involved inoculating samples onto a *Mycoplasma* culture broth (Oxoid, CM403). This was supplemented with 16% serum, 0.002% DNA (BDH, 42026), 2 μg/mL fungizone (Gibco, 042 05920), $2x10^3$ units penicillin (Sigma, Pen-3) and 10 mL 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 3 weeks at 37°C while exposed to CO_2. The plates were examined microscopically every 7 days. The appearance of small oval shaped colonies indicated the presence of *Mycoplasma* infection.

2.7 Minitiaturised *in vitro* toxicity assays

2.7.1 *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 100 µL of these cell suspensions 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 were prepared at 2X their final concentration in cell culture medium. Volumes of the drug dilutions (100 µL) were then added to each well using a multichannel pipette. Plates were then mixed gently as above.
4. Cells were incubated for 6 days at 37°C and 5% CO_2. At this point the control wells would have reached approximately 80% confluency.
5. Assessment of cell survival in the presence of drug was determined by acid phosphatase assay (Section 2.7.2). The concentration of drug which caused 50% cell kill, (the IC_{50} of the drug) was determined from a plot of % survival (relative to 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 100 µL PBS. This was then removed and 100 µL of freshly prepared phosphatase substrate (10 mM *p*-nitrophenol phosphate (Sigma, 104-0) in 0.1 M 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 50 µL of 1 N NaOH.
5. The plate was read in a dual beam plate reader at 405 nm with a reference wavelength of 620 nm.

2.7.3 NSAID mediated drug toxicity enhancement 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 100 µL 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 50 µL of the drug dilution and 50 µL of the NSAID dilution were then added to each relevant well so that a total final volume of 200 µL was present in each well. All potential toxicity enhancing agents were dissolved in DMSO, ethanol or media as illustrated in Table 2.7.3.1. Stock solutions were prepared at approximately 15 mg/10 mL media, filter sterilised with a 0.22 µm 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. The sulindac sulfide and sulfone analogues were synthesised by Dr. Robert O' Connor at the NCTCC as described by Hucker *et al.*, (1973). In assays where more than two compounds were used in combination (such as adriamycin, indomethacin and PgE_2), 8X concentrations of the relevant agents were individually prepared in media and 25 µL of these compounds added to the appropriate wells. The final volume in each well was always maintained at 200 µL.

4. Cells were incubated for 6 days at 37°C and 5% CO_2. At this point the control wells would have reached approximately 80% 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 2.13.

NSAID	Stock solution solubility	Sigma product number	Working concentrations (µg/mL)
Acetaminophen	1 mL DMSO + 9 mL DME	A5000	60, 30, 15
Acemetacin	1 mL DMSO + 9 mL DME	A1674	40, 20, 10
Aspirin	10 mL DME	A3160	150, 75, 30
Aurothioglucose	10 mL DME	A0632	3.0, 1.5, 0.8
Chloroquine	10 mL DME	C8138	10, 5, 2.5
Diclofenac	1 mL EtOH + 9 mL DME	D6899	12, 6, 3
Fenoprofen	1 mL DMSO + 9 mL DME	F1517	50, 25, 13
Flufenamic acid	1 mL DMSO + 9 mL DME	F9005	6, 3, 2
Flurbirprofen	1 mL DMSO + 9 mL DME	F8514	6, 3, 2
Ibuprofen	1 mL EtOH + 9 mL DME	I4883	16, 8, 4
Indomethacin	1 mL DMSO + 9 mL DME	I7378	2.50, 1.25, 0.60
Ketoprofen	1 mL DMSO + 9 mL DME	K1751	50, 25, 13
Levamisole	10 mL DME	L9756	30, 15, 8
Meclofenamic acid	1 mL DMSO + 9 mL DME	M4531	8, 4, 2
Mefenamic acid	1 mL DMSO + 9 mL DME	M4267	6, 3, 2
Naproxen	10 mL DME	M1275	100,50,30
Phenylbutazone	1 mL DMSO + 9 mL DME	P8386	12,6,3
Piroxicam	1 mL DMSO + 9 mL DME	P5654	20, 10, 5
Sulindac	1 mL DMSO + 9 mL DME	S8139	6, 3, 1.5
Sulindac sulfide	1 mL DMSO + 9 mL DME	N/A	6, 3, 1.5
Sulindac sulfone	1 mL DMSO + 9 mL DME	N/A	20, 10, 5
Tolmetin	10 mL DME	T6779	20, 10, 5
Zomepirac	1 mL DMSO + 9 mL DME	Z2625	40, 20, 10

Table 2.7.3.1: Solubility data for NSAIDs and related compounds

2.8 ELISA for assessment of PgE_2 production

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

1. Cells were seeded at high density (2.5×10^5 cells per well) in 6 well plates (Falcon, 3046).
2. Plates were then incubated overnight in serum containing media.
3. Media was removed and cells washed twice with DME. The compounds of interest (such as NSAIDs) were then added to the cells, at a concentration appropriate for the cell density present. Control wells were treated with media only.
4. At appropriate timepoints, 150 µL aliquots were removed and then 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 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 PgE_2 present. For analysis of PgE_2 levels released by cells into the media during incubation in 175 cm^2 flasks, a 1 mL media aliquot was taken at the appropriate timepoint and 50 µL of this volume subjected to analysis as described above.

2.9 Western blotting

2.9.1 Sample preparation

1. Cells were seeded at a density of $3x10^6$ cells per 175 cm^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 (aliquot taken for PgE_2 ELISA if necessary) and cells were trypsinised as described in 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 1 mL of NP-40 lysis buffer. Table 2.9.1 below provides the details of lysis buffer. Immediately before use, 10 µL of the 100X stocks listed in Table 2.9.2 were added to 1 mL of lysis buffer.

Addition required per 500 mL stock	Final concentration
425 mL UHP water	-
25 mL 1 M Tris-HCl (pH 7.5)	50 mM Tris-HCl (pH 7.5)
15 mL 5 M NaCl	150 mM NaCl
2.5 mL NP-40	0.5% NP-40

Table 2.9.1: NP-40 lysis buffer

100X Stock	Preparation instructions
100 mM Na_3VO_4	1.83 g Na_3VO_4 in 100 mL UHP
100 mM DTT	154 mg in 10 mL UHP
100 mM PMSF	174 mg in 10 mL 100 % ethanol
100X Protease inhibitors	2.5 mg/mL leupeptin, 2.5 mg/mL aprotinin, 15 mg/mL benzamidine and 1 mg/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 2 mg/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 20 µL volume of protein standard dilution or sample (1:2 dilution) was added to 1 mL of diluted dye reagent and the mixture vortexed.
5. After 5 minutes incubation, absorbance was assessed at 570 nm.
6. The concentration of the protein samples was determined from the plot of the absorbance at 570 nm 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 2.9.3.1. The gels were set in clean 10 cm x 8 cm gel cassettes which consisted of a glass plate and an aluminium plate. These plates were separated by 0.75 cm 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 15-20 µL).

	7.5% Resolving Gel	15% Resolving Gel	5% Stacking Gel
Acrylamide stock	3.8 mL	5.0 mL	0.8 mL
UHP Water	8.0 mL	6.8 mL	3.6 mL
1.875 M Tris-HCl pH 8.8	3.0 mL	3.0 mL	-
1.25 M Tris-HCl pH 6.8	-	-	0.5 mL
10 % SDS	150 µL	150 µL	50 µL
10 % NH_4 - Persulfate	60 µL	50 µL	17 µL
TEMED	9 µL	10 µL	8 µL

Table 2.9.3.1: Preparation protocol for SDS-PAGE gels (2 x 0.75 mm gels).

The acrylamide stock in Table 2.9.3.1 was composed of 29.1 g Acrylamide (Sigma, A8887) and 0.9 g NN'-methylene bis-acrylamide (Sigma, N7256) made up to 100 mL with UHP water. In advance of samples being loaded into the relevant sample wells, 15 µg of protein was diluted in 5X loading buffer (2.5 mL 1.25 M Tris-HCl, 1.0 g SDS, 2.5 mL mercaptoethanol (Sigma, M6250), 5.8 mL glycerol (BDH, 44305) and 0.1% bromophenol blue (Sigma, B8026) made up to 10 mL with distilled water). The molecular weight markers (Sigma, P1677) and protein samples (for PGHS-2 analysis) were heated to 95°C for 2 minutes. Following heating, equal amounts (15 µg in 5X loading buffer in a total volume of 10 µL) of protein were loaded into each well. The gels were run at 250 V and 45 mA 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 (25 mM Tris (Sigma, T8404), 192 mM 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 3 mm filter paper were soaked in freshly prepared transfer buffer. These were then placed on the cathode plate of a semi-dry blotting apparatus (Bio-rad). Air pockets were then removed from between the filter paper. Nitrocellulose was also soaked in 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 34 mA at 15 V 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 (125 mM NaCl, 20 mM Tris pH 7.5) at 4°C.
4. Membranes were treated with primary antibody overnight at 4°C (with prostaglandin H synthase 1 monoclonal antibody (Cayman Chemicals, 160110) or prostaglandin H synthase 2 (human) polyclonal antibody (Cayman Chemicals, 160107) diluted 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; A6782) 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 20 (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 final washings 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 3 mL 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 cling film. All excess air bubbles were removed.
5. The nitrocellulose was then exposed to autoradiographic film (Kodak; X-OMAT S) 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; LX24).
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 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 (IOVs)

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 20 μg IOV 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 for 40°C for 1 minute (Bio-Rad, 161-0309).
3. Membranes were blocked using 5% milk (Cadburys; Marvel skimmed milk) in TBS (125 mM NaCl, 20 mM 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, P0450) 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 1.5 mL SuperSignal Reagent 1 with 1.5 mL SuperSignal 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. Autoradiograph film was processed as described in Section 2.9.5.

2.10 Inside-out vesicle (IOV) preparation

2.10.1 Spinner flask preparation

Spinner flasks were soaked in a warm solution of 2% RBS for one hour and then scrubbed vigorously with a scrubbing brush. They were then rinsed three times in tap water and then three times in UHP water. Spinner flasks were then allowed to completely dry at 37°C, and after this point were treated with 10 mL 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 a 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 75 cm^2 flasks at a density of $2x10^5$ cells per flask.
2. Once cells reached 70% to 80% confluency, the cells were trypsinised and used to seed a 175 cm^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 500 mL spinner flask (Techne, TR174-30) containing 250 mL of 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 100 mL of media after three days of growth in the spinner flasks.
7. Cells were harvested after seven days at which time cell number was approximately 1.5×10^8 cells per flask.

8. When IOVs were isolated from HL60-ADR, cells (in RPMI media supplemented with 10% serum) were cultured in five vented 75 cm^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 1,000 r.p.m. for 5 minutes. The pellets were then resuspended in 5 mL media and used to seed a roller bottle (Falcon, 3027) containing 500 mL of media. Cells were cultured in the roller bottle until 60% confluent. Once this had been achieved, cells were split equally into an additional four roller bottles and the final volume in each of the five roller bottles made up to 500 mL 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 5,000 r.p.m (1,200 g) for 10 minutes at 4°C in a Sorvall refrigerated centrifuge.
2. The supernatant was decanted and the pellets resuspended in 50 mL ice cold PBS. The combined pellets were then transferred to a 50 mL tube and spun at 4,000 r.p.m for 5 minutes.
3. The resulting cell pellet was then resuspended in 230 mL hypotonic buffer, (Table 2.10.3.1). The PMSF was added to the buffer immediately before use.

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

Table 2.10.3.1: Hypotonic buffer for IOV isolation

4. Cells were lysed by gentle agitation at 4°C for 1.5 hours.
5. The cell lysate was then centrifuged at 28,000 r.p.m (100,000 g) 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 10 mL 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 20 mL with incubation buffer which was prepared as shown in Table 2.10.3.2.

Buffer constituent	Preparation instructions
10 mM Tris-HCl (pH 7.4)	1.211 g Tris in 1 L UHP water
250 mM Sucrose	42.79 g Sucrose in 500 mL 10 mM Tris-HCl (pH 7.4)

Table 2.10.3.2: Incubation buffer for IOV isolation

8. A 100 µL aliquot of this crude membrane fraction was then retained for marker enzyme assays.
9. The crude membrane fraction was layered over 38% sucrose/10 mM Tris-HCl pH 7.4, (38 g sucrose in 100 mL 10 mM Tris-HCl pH 7.4) and centrifuged at 28,000 r.p.m (100,000 g) for 35 minutes at 4 °C with a SW28 rotor. A volume of 10 mL crude membrane fraction was layered over 28.5 mL 38% sucrose/10 mM 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 total volume of 20 mL with incubation buffer.
11. The suspension was then centrifuged at 38,200 r.p.m (100,000 g) for 35 minutes at 4°C using a Beckman 70.1 rotor.
12. The pellets were then resuspended in 0.2 mL incubation buffer. Vesicles were formed by passing resuspended pellets through a 27-gauge needle 20 times using a 1 mL syringe.
13. A protein assay was then performed (Section 2.10.4) and the IOV preparation was then diluted to a concentration of 5 mg protein/mL with incubation buffer. Volumes of 50 µL IOVs were then frozen at -80°C.
14. Before freezing, a 50 µL aliquot was taken for the marker enzyme assays. These assays had to be performed on the day of the IOV preparation in order to ensure that enzyme activity remained intact.

2.10.4 Protein assay

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

2.10.5 Marker enzyme assays - Plasma membrane

1. The solutions listed in Table 2.10.5.1 were prepared freshly in advance of the assay.

Buffer constituent	Preparation instructions
90 mM 2-amino-2-methyl-1-propanol (pH 10.5)	0.34 g in 30 mL UHP water.
22.0 mM $MgCl_2$	10.5 mg in 5 mL of 90 mM 2-amino-2-methyl-1-propanol (pH 10.5).

Table 2.10.5.1: Plasma membrane assay buffers

2. A solution of 8.0 mM *p*-nitrophenylphosphate was prepared by dissolving 26.3 mg *p*-nitrophenylphosphate in 9.55 mL 90 mM 2-amino-2-methyl-1-propanol (pH 10.5) and 0.45 mL 22.0 mM $MgCl_2$.
3. A volume of 5 mL of 8.0 mM *p*-nitrophenylphosphate solution was removed and a volume of 5 µL Triton X-100 (Sigma, T-9284), added to the reaction buffer. This was used to disrupt the IOVs and so allow assessment of the % IOVs in the preparation.
4. To perform the assay, 100 µL of the reaction buffer (one volume without Triton X-100 and a second volume containing Triton X-100) was added to a 96-well plate and 10 µL of the IOV preparation or crude membrane extract added to each relevant well. A reagent blank was also included on the plate.
5. The plates were incubated in the dark at 37°C for 35 minutes.

6. Absorbance was then analysed at 405 nm on a dual beam ELISA plate reader.

2.10.6 Marker enzyme assays - Lysosome

1. This assay was performed in exactly the same manner as the acid phosphatase assay detailed in Section 2.7.2. In this case, 0.0263 g of *p*-nitrophenylphosphate was added to 10 mL of acid phosphatase buffer.
2. Volumes of 100 µL of this reagent were added to a 96-well plate and 10 µL of IOV or crude membrane was then added. Reagent blanks were also included on the plate.
3. The plates were incubated in the dark at 37°C for 35 minutes and then analysed at 405 nm on the ELISA plate reader.

2.11 Transport assays with IOVs

Transport assays with IOVs were performed as described by Ishikawa *et al.*, (1994). The protocol used in these assays was 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 2.10.3.2. An ATP/creatine phosphate/$MgCl_2$/10 mM Tris-HCl (pH 7.4) solution is prepared as detailed in Table 2.11.1. Volumes of 200 µL were then frozen at -80°C.

Buffer constituent	Preparation instructions
$MgCl_2$ $6H_2O$	203.3 mg in 30 mL Incubation buffer
ATP (Disodium salt)	6.05 mg ATP in 3 mL $MgCl_2$. $6H_2O$ solution
Creatine phosphate	32.7 mg in 3 mL ATP solution

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

2. For the AMP solution, 4.99 mg AMP (Sigma, A1752) was substituted for the ATP (Sigma, A7699). Once prepared as in Table 2.11.1 above, 100 µL volumes were frozen at -80°C.
3. A creatine kinase solution (2 mg/mL), (Sigma, C3755) was prepared in incubation buffer and 50 µL aliquots frozen at -80°C.
4. Prior to performing the assay, filters (Millipore, GSWP-02500) were soaked in the incubation buffer for one hour at 4°C. Once soaked, the filters were applied to the filter apparatus (Millipore, 12-25 Sampling Manifold) and vacuum was applied to the system.
5. An Eppendorf thermomixer (Eppendorf, 5436) was allowed to equilibrate at 37°C and once at this 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: 60 µL incubation buffer, 30 µL ATP, 5 µL creatine kinase, 5 µL [^{3}H]-LtC_4 (DuPont NEN, NET-1018, 0.01 mCi/mL) and 10 µL 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 20 µL were removed at appropriate timepoints and added into 1 mL volumes of ice cold incubation buffer.
8. These were then washed through the filter apparatus. The eppendorf was washed out with 1 mL 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 8 mL 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 scintillation 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 30 µL incubation buffer.
11. For assessment of a compounds ability to inhibit LtC_4 transport ability, the compound of interest was dissolved in incubation buffer at 1.83X the final desired concentration. An aliquot of 60 µL of this volume was then added to an eppendorf. The standard volumes of ATP, AMP, creatine kinase, LtC_4 and IOV were then added to a total final volume of 110 µL. The final concentration of solvent used for dissolution of the compound of interest was maintained below 0.5% in all cases possible. Indomethacin (Sigma, I7378), Taxol (Sigma, T7402), verapamil (Sigma, V4629), MK571 (Cayman Chemicals, 70720-M010) and antimycin A (Sigma, A8674) were prepared as 10 mM stocks in ethanol before final dissolution to the desired concentration in incubation buffer. Naproxen (Sigma, M1275), glutathione (Sigma, 6529) and sodium azide (S8032) were prepared as 10 mM stocks in incubation buffer.

Water based formulations of vincristine (David Bull Laboratories), adriamycin (Farmitalia Carlo Erba Ltd.), cyclosporin A (Sandoz) and cisplatin (David Bull Laboratories) were used in all LtC_4 transport inhibition experiments. These compounds were dissolved in incubation buffer to the desired final concentration. In control experiments it was demonstrated that at the concentrations used in these studies, the solvent used to dissolve these compounds did not have any significant effects on transport of LtC_4 into IOVs.

2.11.1 Determination of kinetic parameters of [^{3}H]-LtC_4 transport.

1. An eppendorf was placed in the thermomixer and the following added sequentially: 60 µL incubation buffer, 30 µL ATP and 5 µL creatine kinase. A volume of 1 µL unlabelled LtC_4 (Sigma, L4886), at the desired final concentration was then added to the reaction mixture. The unlabelled LtC_4 was supplied at a stock concentration of 54 µg/mL (86 µM) in 70% ethanol. A volume of 4 µL [^{3}H]-LtC_4 (DuPont NEN, NET-1018, 0.01 mCi/mL, 165 Ci/mmol) was then added to the reaction mixture. Final LtC_4 concentration (labelled and unlabelled LtC_4) in the reaction mixture was varied from 3 nM to 750 nM by preparation of various concentrations of unlabelled LtC_4 in 70% ethanol and using 1 µL of this unlabelled LtC_4 in the mixture as described above. A volume of 10 µL IOVs was then added to the reaction mixture. After every sequential addition the thermomixer was adjusted to half speed mixing to allow agitation of the various components of the mixture.

2. Aliquots of the mixture were then removed at appropriate timepoints and added to 1 mL ice cold incubation buffer. For determination of kinetic parameters, only initial rates of LtC_4 transport were monitored. A timepoint of 1 minute was used in the case of DLKP and 20 seconds in the case of HL60-ADR for all LtC_4 transport rate determinations.
3. The removed aliquot was then washed through the filter apparatus. The eppendorf was subsequently washed with 1 mL of ice cold incubation buffer. The filter was finally washed with 2 mL of ice cold incubation buffer.
4. Filters were then removed and placed in 8 mL 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 scintillation counter.
5. Total LtC_4 uptake (labelled plus unlabelled) was calculated by dividing the amount of accumulated [^{3}H]-labelled LtC_4 (in pmol/mg) as found from scintillation counting, by the quantity of [^{3}H]-labelled LtC_4 initially added. This was then multiplied by the total quantity of LtC_4 (labelled and unlabelled) initially added in order to approximate the total uptake of LtC_4.
6. Data was plotted as initial rate (V_0) of LtC_4 transport (pmol/mg/min) versus total final LtC_4 concentration (nM). Kinetic parameters were determined from regression analysis of the Lineweaver-Burk transformation of the data. This involved plotting $(V_0)^{-1}$ versus $[LtC_4]^{-1}$ with the values of V_{max} and K_m determined from the Y- and X-axes intercepts (Y-axis intercept of $(V_{max})^{-1}$ and X-axis intercept of $(-K_m)^{-1}$).

2.11.2 Assessment of [^{3}H]-vincristine transport into IOVs

1. Assessment of [^{3}H]-vincristine transport into IOVs was performed as described for [^{3}H]-LtC_4. Modifications to the procedure detailed in Section 2.11 were made in order to minimise non-specific binding of [^{3}H]-vincristine to the filters. This involved the use of glass fiber (type A/E) filters (Gelman sciences, 61630) which had been soaked in incubation buffer for 2 hours in advance of the assay.

2. [^{3}H]-Vincristine (Amersham, TRK478, $3.7x10^{-11}$ mol/μL, 6.7 Ci/mmol) was added to the reaction mixture at a final concentration of 200 nM. The addition of ATP, AMP, creatine kinase and IOV was performed as described in Section 2.11. Filters were washed with 5 x 1 mL volumes of ice cold incubation buffer in order to minimise any non-specific binding to the glass fiber filters. Quantification of [^{3}H]-vincristine transport into the IOVs was performed by scintillation counting. Results were corrected for non-specific binding of [^{3}H]-vincristine to the filters.

2.12 Assessment of [^{14}C]-Indomethacin accumulation in DLKP and DLKP-A.

1. Cells in the exponential phase of growth were trypsinised and set up at a density of $1x10^5$ cells per well on 6-well plates. These plates were then incubated at 37°C, 5% CO_2 for 48 hours.
2. Labelled indomethacin (DuPont NEN, NEC-786, 22.3 mCi/mmol) at a concentration of 7 μg/mL, ([^{14}C]-labelled) and unlabelled indomethacin (94 μg/mL) were added to the cells for an appropriate time period.
3. At various time points, media was removed from the cells. The cells were then washed twice with ice cold PBS and the plates blotted dry.
4. Cells were solubilised by overnight treatment with 0.2 N NaOH at room temperature. The cell lysates were then neutralised with 0.2 N HCl.

5. The levels of [^{14}C]-indomethacin present in the cell lysates was then determined by scintillation counting following mixing of cell lysates with Ecolite scintillation cocktail (ICN, 882475). A standard curve of c.p.m due to [^{14}C]-indomethacin present, versus [^{14}C]-indomethacin concentration was used to convert c.p.m values obtained for the cell lysates into the equivalent [^{14}C]-indomethacin concentration accumulated in the cells.

2.13 Fractional product method

Analysis of experiments investigating possible synergy between cytotoxic drugs and compounds such as NSAIDs was performed initially using the fractional product method. This method involves using the following equation:

Equation 2.13.1: 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.

If 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 drugs, 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 product method were confirmed using a computer package for multiple drug effect analysis, "Dose - Effect Analysis with Microcomputers", (Chou and Chou, 1987).

2.14 Bioluminescent ATP assay

The concentration of ATP in the incubation mixture used in the LtC_4 transport assays was quantified using a bioluminescent ATP assay (Labsystems, 5080200). The assay is based on the quantitative measurement of light produced as a result of firefly luciferase activity in the presence of ATP. The conditions used in the ATP assay kit are designed so that the light emitted is directly proportional to the ATP concentration present.

1. Unlabelled LtC_4 (Sigma, L4886) was diluted in incubation buffer so that a final concentration of 2.75 nM LtC_4 was achieved in the 110 μL volume of the LtC_4 transport assay incubation mixture.
2. The incubation mixture was composed of 60 μL incubation buffer, 30 μL ATP, 5 μL creatine kinase (in the case of the negative control, this was replaced by 5 μL incubation buffer), 5 μL of the above LtC_4 dilution and 10 μL IOVs. The reaction mixture was incubated at 37°C as described in Section 2.11.
3. At the relevant timepoints, 9 μL of the reaction mixture was removed and dissolved in 4 mL ice cold incubation buffer. A total of 100 μL was subsequently removed from this volume and added to 900 μL of ice cold incubation buffer.

4. A 50 μL aliquot of the final dilution of the sample was added to 110 μL of 0.1 M tris-acetate buffer in a well of 96-well assay plate (Labsystems, 95029510). A volume of 40 μL ATP monitoring reagent (containing firefly luciferase) was then added and the assay plate loaded into a luminometer (Luminoskan RS).
5. The luminescent signal was then quantified using a 2 second lagtime and 10 second integration time.
6. The concentration of ATP in the IOV incubation mixture was calculated from a standard curve constructed using an ATP standard (Labsystems, 1620140).

2.15 Quantification of adriamycin accumulation in DLKP-A10

1. DLKP or DLKP-A10 cells were seeded into 75 cm^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 (10 μM) added. In some cases, DLKP-A10 cells were treated with adriamycin and verapamil (60 μM) or adriamycin and cyclosporin A (8 μM).
   At relevant timepoints, medium was removed and the flasks washed twice with PBS. Cells were then trypsinised as described in Section 2.5.1. Pellets were then washed with PBS and frozen at -20°C. SKMES-1 and SKMES-1/ADR cells were treated in the manner described above for DLKP and DLKP-A10.
2. For adriamycin accumulation experiments involving antimycin A (Sigma, A8674) and sodium azide (Sigma, S8032), DLKP and DLKP-A10 cells were treated with glucose free medium. Cells were seeded at $0.5x10^6$ cells per flask and incubated at 37°C for 48 hours as described above. Cells were then washed twice in PBS and then pre-incubated for 2 hours in glucose free medium only (Gibco, 11963-022).

Cells were then treated with adriamycin (10 μM), adriamycin and antimycin A (10 μM) or adriamycin and sodium azide (10 mM) in glucose free medium. At relevant timepoints, cells were trypsinised and pellets washed and frozen as described above.

3. The frozen pellets were thawed and resuspended in 100 μL water. A volume of 100 μL 33% silver nitrate (Sigma, S6506) was then added to the pellets, followed by mixing for 5 minutes. A quantity of 1.5 μg daunorubicin (Farmitalia Carlo Erba Ltd.), dissolved in 300 μL of methanol (Labscan) was then added to the mixture as an internal standard. Mixing was then continued for a further 5 minutes.
4. A volume of 1.3 mL acetonitrile (Labscan) was added to the mixture, which was then maintained at 4°C for 1 hour. This was followed by centrifugation at 4000 r.p.m. for 40 minutes.
5. The supernatant (1.1 mL) was removed and added to HPLC autosampler vials. All solvent was then removed under a stream of nitrogen gas.
6. The remaining solids were resuspended in 50 μL of HPLC mobile phase.
7. The HPLC mobile phase was prepared as follows: 32 mL of 0.1 M phosphoric acid (Sigma, P6560) was added to 244 mL UHP water. The pH was then adjusted to 2.3 with 1 N potassium hydroxide (Sigma, P6310). A volume of 124 mL acetonitrile was added finally and the completed mobile phase allowed to degas at 4°C overnight.
8. The 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 1 mL per minute with a total run time of 15 minutes. The column used for HPLC analysis of adriamycin in DLKP and DLKP-A10 was a C_{18} reversed phase Prodigy 5 μm particle size ODS-3 column (Phenomenex, U.K.). Absorbance was monitored at 253 nm.
9. 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.

2.16 Materials

The product numbers of materials used, but which have not already been provided, are as follows: sodium fluoride (Sigma, S1504), sodium orthovanadate (Sigma, S6508), DTT (Sigma, D5545), PMSF (Sigma, P7626), leupeptin (Sigma, L2023), aprotinin (Sigma, A6279), benzamidine (Sigma, B6506), trypsin inhibitor (Sigma, T9003), SDS (Sigma, L4509), ammonium persulfate (Sigma, A6761), TEMED (Sigma, T9281), sodium phosphate (Sigma, P5379), EGTA (Sigma, E4378), sucrose (Sigma, S7903), amino-methyl-propanol (Sigma, A9199), $MgCl_2$ (Sigma, M8266), $MgCl_2$. $6H_2O$ (Sigma, M2670), PgE_2 (Sigma, P5640), PgD_2 (Sigma, P5172), GF109203X (Calbiochem, 203290).

3.0 Results

3.1 Cytotoxic drug resistance profiles of cell lines used in NSAID screening experiments.

The A549 human lung adenocarcinoma and DLKP human lung squamous carcinoma cell lines were used for the identification of NSAIDs with chemotherapeutic drug toxicity-enhancing abilities. The drug resistance profile of the A549 cell line is provided in Table 3.1.1. The DLKP and A549 cell lines are regarded as drug sensitive as they do not exhibit the high level of drug resistance found for multidrug resistant cell lines such as DLKP-A, (Clynes *et al.*, 1992).

Anticancer drug category	Drug	A549 IC_{50},
Antimetabolites	5-Fluorouracil	0.13 ± 0.01 μg/mL
Covalent DNA binding drugs	Carboplatin	3.50 ± 0.20 μg/mL
Noncovalent DNA binding drugs	Adriamycin	55.00 ± 3.00 ng/mL
	Daunorubicin	15.00 ± 0.60 ng/mL
	Epirubicin	12.00 ± 0.40 ng/mL
	Mitoxantrone	2.00 ± 0.30 ng/mL
Inhibitors of chromatin function	Etoposide (VP-16)	0.28 ± 0.05 μg/mL
	Vincristine	9.00 ± 0.50 ng/mL
	Taxol	1.20 ± 0.10 ng/mL

Table 3.1.1: IC_{50} values of chemotherapeutic drugs in A549. These values were identified as the drug concentrations giving rise to 50% cell kill relative to untreated control cells according to the protocol detailed in Section 2.7.

3.2 Chemotherapeutic drug *in vitro* toxicity assay screening with indomethacin and sulindac in the A549 cell line

Non-toxic levels of the NSAIDs, indomethacin and sulindac have been shown to enhance the toxicity of adriamycin in a number of drug sensitive cell lines, (Duffy *et al.*, 1997). An enhancement in 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 toxicities of the drug and NSAID (as described in Section 2.13). Drug screening experiments illustrated the ability of indomethacin and sulindac to significantly enhance the toxicity of adriamycin in the A549 cell line (Tables 3.2.2 and 3.2.3). The toxicity enhancing abilities of these NSAIDs occurred when the NSAIDs were added at relatively non-toxic levels (causing only 0-5% cell kill). A certain degree of toxicity appeared to be necessary for achievement of the toxicity enhancement effect. The data in Table 3.2.6 illustrates this fact as it can be seen that minor toxicity was caused due to the presence of 3.20 μg/mL indomethacin. This concentration of indomethacin was found to enhance epirubicin induced cell kill. At a concentration of 0.90 μg/mL indomethacin, cell kill is negligible and no significant synergistic cell kill is observed in the presence of a variety of epirubicin concentrations (11, 6 or 4 ng/mL).

Further drug screening experiments with indomethacin and sulindac showed that in addition to adriamycin, the toxicity of vincristine, VP-16, daunorubicin and epirubicin could be enhanced in the presence of non-toxic levels of these NSAIDs. It was also discovered that indomethacin and sulindac did not enhance the toxicity of carboplatin, 5-fluorouracil or Taxol. The results are shown in summary in Table 3.2.1 with the relevant screening results in Tables 3.2.2 to 3.2.13 inclusive. As can be seen from Table 3.2.1, no differences were found between indomethacin and sulindac as regards their ability to enhance the toxicity of a given chemotherapeutic drug. Drug and NSAID combinations where the toxicity enhancement effect was observed have been included in Tables 3.2.2 to 3.2.11. In Tables 3.2.12 and 3.2.13 sample data is provided where a drug (Taxol) and

NSAID combination give rise to an additive rather than a synergistic level of cell kill. All other drug and NSAID combinations where the toxicity enhancement effect was not observed generated similar results

Drug	NSAID	Toxicity enhancement *	Drug	NSAID	Toxicity enhancement*
Adriamycin	Indomethacin	(+)	Adriamycin	Sulindac	(+)
Carboplatin	Indomethacin	(-)	Carboplatin	Sulindac	(-)
Daunorubicin	Indomethacin	(+)	Daunorubicin	Sulindac	(+)
Epirubicin	Indomethacin	(+)	Epirubicin	Sulindac	(+)
5-Fluorouracil	Indomethacin	(-)	5-Fluorouracil	Sulindac	(-)
Taxol	Indomethacin	(-)	Taxol	Sulindac	(-)
Vincristine	Indomethacin	(+)	Vincristine	Sulindac	(+)
VP-16	Indomethacin	(+)	VP-16	Sulindac	(+)

*: Toxicity enhancement is scored with a (+) if a level of toxicity is achieved with a drug plus NSAID combination, which is significantly greater than the level of toxicity predicted by the fractional product method and as described in Section 2.13.

Table 3.2.1: Chemotherapeutic drug toxicity enhancement abilities of indomethacin and sulindac as found in the A549 cell line.

A549 (P 98), Adriamycin and Indomethacin

Adriamycin conc. 1 :- 0.04 μg/mL
Adriamycin conc. 2 :- 0.02 μg/mL
Adriamycin conc. 3 :- 0.01 μg/mL

Indomethacin conc. 1 :- 2.40 μg/mL
Indomethacin conc. 2 :- 1.20 μg/mL
Indomethacin conc. 3 :- 0.60 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	60.60	7.96
Indo 1 control	92.18	7.09
Adriamycin 1 + Indo	21.00*	3.56
Adriamycin 2 control	81.54	7.26
Adriamycin 2 + Indo	30.54*	3.74
Adriamycin 3 control	84.89	10.98
Adriamycin 3 + Indo	56.48*	5.58
Adriamycin 1 control	53.40	10.37
Indo 2 control	87.54	13.07
Adriamycin 1 + Indo	23.20*	4.25
Adriamycin 2 control	70.18	11.09
Adriamycin 2 + Indo	30.28*	5.51
Adriamycin 3 control	85.40	13.11
Adriamycin 3 + Indo	55.07	10.72
Adriamycin 1 control	49.71	4.99
Indo 3 control	92.38	13.30
Adriamycin 1 + Indo	25.48*	2.99
Adriamycin 2 control	78.90	13.92
Adriamycin 2 + Indo	40.43	8.96
Adriamycin 3 control	79.92	11.95
Adriamycin 3 + Indo	60.89	8.26

Table 3.2.2: Survival of A549 cells in the presence of various concentrations of adriamycin and indomethacin (Indo) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 98), Adriamycin and Sulindac

Adriamycin conc. 1 :- 0.04 μg/mL
Adriamycin conc. 2 :- 0.02 μg/mL
Adriamycin conc. 3 :- 0.01 μg/mL

Sulindac conc. 1 :- 6.30 μg/mL
Sulindac conc. 2 :- 3.20 μg/mL
Sulindac conc. 3 :- 1.50 μg/mL

Treatment	% Cell Survival	S.D.
Cell Control	100	
Adriamycin 1 control	49.87	4.74
Sul 1 control	96.01	5.36
Adriamycin 1 + Sul	27.14*	3.64
Adriamycin 2 control	72.41	6.65
Adriamycin 2 + Sul	35.27*	6.44
Adriamycin 3 control	83.21	6.14
Adriamycin 3 + Sul	59.72*	7.58
Adriamycin 1 control	47.56	5.57
Sul 2 control	101.0	12.14
Adriamycin 1 + Sul	27.72*	4.57
Adriamycin 2 control	69.75	8.57
Adriamycin 2 + Sul	50.50	7.70
Adriamycin 3 control	83.95	9.17
Adriamycin 3 + Sul	70.02	7.29
Adriamycin 1 control	42.11	6.89
Sul 3 control	100.0	13.20
Adriamycin 1 + Sul	28.51	5.17
Adriamycin 2 control	65.99	10.27
Adriamycin 2 + Sul	55.94	6.18
Adriamycin 3 control	84.61	11.86
Adriamycin 3 + Sul	79.29	9.37

Table 3.2.3: Survival of A549 cells in the presence of various concentrations of adriamycin and sulindac (Sul) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 95), Daunorubicin and Indomethacin

Daunorubicin conc. 1 :- 0.012 μg/mL
Daunorubicin conc. 2 :- 0.006 μg/mL
Daunorubicin conc. 3 :- 0.003 μg/mL

Indomethacin conc. 1 :- 3.20 μg/mL
Indomethacin conc. 2 :- 1.60 μg/mL
Indomethacin conc. 3 :- 0.90 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Daunorubicin 1 control	51.31	3.78
Indomethacin 1 control	95.75	5.64
Dauno 1 + Indo	23.80*	1.92
Dauno 2 control	83.44	8.04
Dauno 2 + Indo	31.77*	3.57
Dauno 3 control	94.12	4.00
Dauno 3 + Indo	73.40	5.69
Daunorubicin 1 control	47.90	3.40
Indomethacin 2 control	106.21	14.75
Dauno 1 + Indo	21.03*	1.52
Dauno 2 control	78.30	5.07
Dauno 2 + Indo	39.79*	5.78
Dauno 3 control	91.48	8.08
Dauno 3 + Indo	78.87	7.59
Daunorubicin 1 control	46.64	3.47
Indomethacin 3 control	100.87	7.51
Dauno 1 + Indo	20.69*	3.15
Dauno 2 control	77.04	4.61
Dauno 2 + Indo	53.77*	3.75
Dauno 3 control	95.70	7.31
Dauno 3 + Indo	89.27	6.83

Table 3.2.4: Survival of A549 cells in the presence of various concentrations of daunorubicin (Dauno) and indomethacin (Indo) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 95), Daunorubicin and Sulindac

Daunorubicin conc. 1 :- 0.012 μg/mL
Daunorubicin conc. 2 :- 0.006 μg/mL
Daunorubicin conc. 3 :- 0.003 μg/mL

Sulindac conc. 1 :- 8.40 μg/mL
Sulindac conc. 2 :- 4.40 μg/mL
Sulindac conc. 3 :- 2.20 μg/mL

Treatment	**% Cell Survival**	**S.D.**
Cell control	100	
Daunorubicin 1 control	53.05	19.03
Sulindac 1 control	87.27	25.79
Dauno 1 + Sul	25.65*	16.80
Daunorubicin 2 control	61.36	15.72
Dauno 2 + Sul	44.42	15.94
Daunorubicin 3 control	65.15	16.54
Dauno 3 + Sul	56.17	18.46
Daunorubicin 1 control	58.79	20.16
Sulindac 2 control	94.09	20.57
Dauno 1 + Sul	38.83*	8.90
Daunorubicin 2 control	65.09	10.51
Dauno 2 + Sul	53.34	9.28
Daunorubicin 3 control	69.72	9.64
Dauno 3 + Sul	66.82	6.14
Daunorubicin 1 control	55.35	17.43
Sulindac 3 control	92.91	23.80
Dauno 1 + Sul	46.20	16.33
Daunorubicin 2 control	70.61	22.63
Dauno 2 + Sul	61.38	17.04
Daunorubicin 3 control	72.34	20.39
Dauno 3 + Sul	70.81	24.09

Table 3.2.5: Survival of A549 cells in the presence of various concentrations of daunorubicin (Dauno) and sulindac (Sul) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 95), Epirubicin and Indomethacin

Epirubicin conc. 1 :- 0.011 μg/mL
Epirubicin conc. 1 :- 0.006 μg/mL
Epirubicin conc. 1 :- 0.004 μg/mL

Indomethacin conc. 1 :- 3.20 μg/mL
Indomethacin conc. 2 :- 1.60 μg/mL
Indomethacin conc. 3 :- 0.90 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Epirubicin 1 control	44.62	4.20
Indomethacin 1 control	89.01	6.47
Epi 1 + Indo	17.15*	1.46
Epi 2 control	68.62	5.38
Epi 2 + Indo	20.42*	3.32
Epi 3 control	80.30	8.17
Epi 3 + Indo	26.72*	3.04
Epirubicin 1 control	49.70	4.39
Indomethacin 2 control	90.55	12.05
Epi 1 + Indo	22.88*	1.74
Epi 2 control	70.37	6.17
Epi 2 + Indo	30.01*	2.94
Epi 3 control	78.16	6.38
Epi 3 + Indo	48.22	5.14
Epirubicin 1 control	53.11	4.86
Indomethacin 3 control	99.77	18.14
Epi 1 + Indo	26.76	2.16
Epi 2 control	73.71	10.55
Epi 2 + Indo	42.70	4.20
Epi 3 control	82.78	6.02
Epi 3 + Indo	66.40	6.48

Table 3.2.6: Survival of A549 cells in the presence of various concentrations of epirubicin (Epi) and indomethacin (Indo) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 95), Epirubicin and Sulindac

Epirubicin conc. 1 :- 0.011 μg/mL
Epirubicin conc. 2 :- 0.006 μg/mL
Epirubicin conc. 3 :- 0.004 μg/mL

Sulindac conc. 1 :- 8.40 μg/mL
Sulindac conc. 2 :- 4.40 μg/mL
Sulindac conc. 3 :- 2.20 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Epirubicin 1 control	56.24	1.33
Sulindac 1 control	92.03	4.08
Epi 1 + Sul	25.69*	5.67
Epirubicin 2 control	67.80	8.84
Epi 2 + Sul	44.64*	8.52
Epirubicin 3 control	75.57	12.17
Epi 3 + Sul	52.07	9.60
Epirubicin 1 control	59.20	13.93
Sulindac 2 control	103.88	19.55
Epi 1 + Sul	45.92	8.67
Epirubicin 2 control	88.95	13.67
Epi 2 + Sul	71.09	13.90
Epirubicin 3 control	91.31	12.98
Epi 3 + Sul	81.39	13.24
Epirubicin 1 control	53.42	7.84
Sulindac 3 control	96.14	14.94
Epi 1 + Sul	46.02	9.04
Epirubicin 2 control	64.87	11.63
Epi 2 + Sul	61.46	11.25
Epirubicin 3 control	71.68	11.61
Epi 3 + Sul	70.61	10.68

Table 3.2.7: Survival of A549 cells in the presence of various concentrations of epirubicin (Epi) and sulindac (Sul) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 100), Vincristine and Indomethacin

Vincristine conc. 1 :- 0.008 μg/mL
Vincristine conc. 2 :- 0.004 μg/mL
Vincristine conc. 3 :- 0.002 μg/mL

Indomethacin conc. 1 :- 2.60 μg/mL
Indomethacin conc. 2 :- 1.30 μg/mL
Indomethacin conc. 3 :- 0.60 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	67.33	6.07
Indo 1 control	92.89	6.74
Vincristine 1 + Indo	16.78*	2.17
Vincristine 2 control	89.05	7.22
Vincristine 2 + Indo	31.20*	3.86
Vincristine 3 control	90.99	7.97
Vincristine 3 + Indo	57.08*	8.19
Vincristine 1 control	59.71	7.05
Indo 2 control	92.23	6.37
Vincristine 1 + Indo	21.52*	3.84
Vincristine 2 control	87.36	7.50
Vincristine 2 + Indo	39.61*	5.27
Vincristine 3 control	88.28	6.50
Vincristine 3 + Indo	72.28	7.88
Vincristine 1 control	60.88	11.00
Indo 3 control	98.50	7.03
Vincristine 1 + Indo	27.75*	4.03
Vincristine 2 control	89.43	6.55
Vincristine 2 + Indo	60.53*	9.38
Vincristine 3 control	98.54	10.82
Vincristine 3 + Indo	90.46	8.83

Table 3.2.8: Survival of A549 cells in the presence of various concentrations of vincristine and indomethacin (Indo) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 101), Vincristine and Sulindac

Vincristine conc. 1 :- 0.008 μg/mL
Vincristine conc. 2 :- 0.004 μg/mL
Vincristine conc. 3 :- 0.002 μg/mL

Sulindac conc. 1 :- 6.20 μg/mL
Sulindac conc. 2 :- 3.10 μg/mL
Sulindac conc. 3 :- 1.60 μg/mL

Treatment	**% Cell Survival**	**S.D.**
Cell control	100	
Vincristine 1 control	74.07	6.99
Sul 1 control	89.56	9.73
Vincristine 1 + Sul	28.47*	3.57
Vincristine 2 control	90.18	11.10
Vincristine 2 + Sul	45.32*	9.71
Vincristine 3 control	90.60	10.38
Vincristine 3 + Sul	67.17	13.82
Vincristine 1 control	57.95	4.00
Sul 2 control	95.01	8.25
Vincristine 1 + Sul	31.61*	7.28
Vincristine 2 control	84.20	11.02
Vincristine 2 + Sul	61.47	8.62
Vincristine 3 control	92.93	11.45
Vincristine 3 + Sul	75.72	11.43
Vincristine 1 control	47.20	2.95
Sul 3 control	97.28	5.90
Vincristine 1 + Sul	32.92*	5.59
Vincristine 2 control	78.42	9.01
Vincristine 2 + Sul	64.36	4.66
Vincristine 3 control	75.85	8.70
Vincristine 3 + Sul	68.32	6.83

Table 3.2.9: Survival of A549 cells in the presence of various concentrations of vincristine and sulindac (Sul) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 102), VP-16 and Indomethacin

VP-16 conc. 1 :- 0.080 μg/mL
VP-16 conc. 2 :- 0.040 μg/mL
VP-16 conc. 3 :- 0.020 μg/mL

Indomethacin conc. 1 :- 4.50 μg/mL
Indomethacin conc. 2 :- 2.10 μg/mL
Indomethacin conc. 3 :- 1.20 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
VP-16 1 control	97.14	3.50
Indomethacin 1 control	99.46	10.20
VP-16 1 + Indo	53.45*	7.95
VP-16 2 control	105.01	12.39
VP-16 2 + Indo	77.87*	8.23
VP-16 3 control	100.73	11.98
VP-16 3 + Indo	82.60	34.65
VP-16 1 control	96.24	8.20
Indomethacin 2 control	100.30	8.05
VP-16 1 + Indo	60.92*	3.86
VP-16 2 control	100.73	12.96
VP-16 2 + Indo	86.84	8.51
VP-16 3 control	99.80	13.68
VP-16 3 + Indo	93.46	11.07
VP-16 1 control	93.03	10.07
Indomethacin 3 control	98.92	14.64
VP-16 1 + Indo	76.90	5.17
VP-16 2 control	98.23	10.60
VP-16 2 + Indo	92.91	9.31
VP-16 3 control	95.04	8.75
VP-16 3 + Indo	96.91	12.46

Table 3.2.10: Survival of A549 cells in the presence of various concentrations of VP-16 and indomethacin (Indo) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 106), VP-16 and Sulindac

VP-16 conc. 1 :- 0.10 μg/mL
VP-16 conc. 2 :- 0.05 μg/mL
VP-16 conc. 3 :- 0.03 μg/mL

Sulindac conc. 1 :- 5.80 μg/mL
Sulindac conc. 2 :- 2.90 μg/mL
Sulindac conc. 3 :- 1.50 μg/mL

Treatment	**% Cell Survival**	**S.D.**
Cell Control	100	
VP-16 1 control	72.26	6.28
Sul 1 control	99.24	3.45
VP-16 1 + Sul	46.59*	4.56
VP-16 2 control	94.30	14.27
VP-16 2 + Sul	77.36	6.57
VP-16 3 control	96.39	12.84
VP-16 3 + Sul	90.40	8.61
VP-16 1 control	64.80	3.10
Sul 2 control	98.36	2.40
VP-16 1 + Sul	49.11*	5.80
VP-16 2 control	86.22	5.50
VP-16 2 + Sul	76.86	5.46
VP-16 3 control	92.54	7.36
VP-16 3 + Sul	89.13	10.94
VP-16 1 control	59.62	5.55
Sul 3 control	102.7	9.82
VP-16 1 + Sul	56.95	8.75
VP-16 2 control	82.99	11.84
VP-16 2 + Sul	82.91	7.61
VP-16 3 control	95.01	9.39
VP-16 3 + Sul	86.92	9.33

Table 3.2.11: Survival of A549 cells in the presence of various concentrations of VP-16 and sulindac (Sul) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 100), Taxol and Indomethacin

Taxol conc. 1 :- 1.80 ng/mL
Taxol conc. 2 :- 0.90 ng/mL
Taxol conc. 3 :- 0.50 ng/mL

Indomethacin conc. 1 :- 2.60 μg/mL
Indomethacin conc. 2 :- 1.30 μg/mL
Indomethacin conc. 3 :- 0.65 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Taxol 1 control	17.25	1.79
Indo 1 control	96.43	10.79
Taxol 1 + Indo	16.48	2.05
Taxol 2 control	39.24	4.22
Taxol 2 + Indo	41.46	6.00
Taxol 3 control	69.84	8.05
Taxol 3 + Indo	76.83	7.01
Taxol 1 control	16.45	2.44
Indo 2 control	99.05	10.83
Taxol 1 + Indo	14.83	2.13
Taxol 2 control	36.49	3.60
Taxol 2 + Indo	38.73	6.18
Taxol 3 control	72.62	6.79
Taxol 3 + Indo	69.43	7.31
Taxol 1 control	16.25	2.20
Indo 3 control	100.03	5.82
Taxol 1 + Indo	16.22	2.41
Taxol 2 control	35.51	5.65
Taxol 2 + Indo	35.71	3.84
Taxol 3 control	73.05	6.10
Taxol 3 + Indo	74.15	6.85

Table 3.2.12: Survival of A549 cells in the presence of various concentrations of Taxol and indomethacin (Indo) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 101), Taxol and Sulindac

Taxol conc. 1 :- 2.25 ng/mL
Taxol conc. 2 :- 1.10 ng/mL
Taxol conc. 3 :- 0.60 ng/mL

Sulindac conc. 1 :- 6.39 μg/mL
Sulindac conc. 2 :- 3.10 μg/mL
Sulindac conc. 3 :- 1.64 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Taxol 1 control	10.90	1.37
Sul 1 control	101.36	9.86
Taxol 1 + Sul	9.89	0.83
Taxol 2 control	53.85	7.46
Taxol 2 + Sul	46.43	5.66
Taxol 3 control	69.56	7.26
Taxol 3 + Sul	70.37	8.14
Taxol 1 control	11.60	1.37
Sul 2 control	102.05	8.32
Taxol 1 + Sul	10.39	1.15
Taxol 2 control	60.08	7.33
Taxol 2 + Sul	54.80	8.18
Taxol 3 control	75.00	7.33
Taxol 3 + Sul	77.04	7.54
Taxol 1 control	11.05	1.80
Sul 3 control	101.95	10.64
Taxol 1 + Sul	10.89	1.17
Taxol 2 control	52.84	6.26
Taxol 2 + Sul	51.75	7.24
Taxol 3 control	70.24	8.59
Taxol 3 + Sul	62.41	9.40

Table 3.2.13: Survival of A549 cells in the presence of various concentrations of Taxol and sulindac (Sul) as found using the protocol detailed in Section 2.7.3. Survival is represented as a % relative to the control untreated cells ± S.D. Statistically significant results are indicated by an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

3.3 Identification of NSAIDs with an ability to enhance the toxicity of adriamycin and vincristine in the A549 cell line.

The data provided in Tables 3.2.1 to 3.2.13 illustrated that indomethacin and sulindac had the ability to enhance the toxicity of a specific group of chemotherapeutic drugs. Further studies were conducted to investigate if the toxicity enhancement abilities of indomethacin and sulindac in the A549 cell line were shared by other NSAIDs. The results of these studies are summarised in Table 3.3.1 with relevant individual data in Tables 3.3.2. to 3.3.14. It was found that acemetacin, indomethacin, mefenamic acid, sulindac, sulindac sulfide, sulindac sulfone, tolmetin and zomepirac enhanced the toxicity of both adriamycin and vincristine in the A549 cell line. None of these NSAIDs enhanced the toxicity of only one of these drugs. This indicated that these NSAIDs interfered with a cellular resistance mechanism which is common for both adriamycin and vincristine. As found in the case of indomethacin and sulindac, the toxicity enhancement effect appeared to be concentration dependent as the effect was highly significant at higher concentrations of drug and NSAID. In some cases, the effect was absent at lower concentrations of drugs and NSAIDs. For example 5.80 μg/mL mefenamic acid significantly enhanced the toxicity of 15 ng/mL adriamycin from approximately 22% to 66%. This dose of mefenamic acid is insufficient to enhance the level of toxicity achieved using 4 ng/mL adriamycin (Table 3.3.3). Sample data has been included in Tables 3.2.13 and 3.2.14 to illustrate the level of cell survival achieved in the presence of an NSAID such as acetaminophen which is ineffective for enhancement of the toxicity of drugs such as adriamycin. In the presence of this NSAID an additive rather than a synergistic level of cell kill, as determined from the fractional product method (Section 2.13), was achieved. The NSAIDs tested in combination with adriamycin and vincristine may be classified into a number of structural categories as described in Section 1.4. It was found that with the exception of mefenamic acid, all NSAIDs with the ability to enhance the toxicity of adriamycin and vincristine were members of the heteroarylacetic acid NSAIDs. This suggests that a characteristic structural feature may be required for an NSAID to enhance the toxicity of a given drug.

This situation is complicated by the fact that mefenamic acid but not other fenamic acid NSAIDs (such as meclofenamic acid) had the ability to enhance adriamycin and vincristine toxicity in the drug sensitive A549 cell line.

Drug	NSAID	Toxicity enhancement *,**	Drug	NSAID	Toxicity enhancement *,**
Adriamycin	Acemetacin	(+)	Vincristine	Acemetacin	(+)
Adriamycin	Acetamino-phen	(-)	Vincristine	Acetamino-phen	(-)
Adriamycin	Aspirin	(-)	Vincristine	Aspirin	N.D.
Adriamycin	Aurothio-glucose	(-)	Vincristine	Aurothio-glucose	(-)
Adriamycin	Chloroquine	(-)	Vincristine	Chloroquine	N.D.
Adriamycin	Diclofenac	(-)	Vincristine	Diclofenac	N.D.
Adriamycin	Etodolac	(-)	Vincristine	Etodolac	(-)
Adriamycin	Flufenamic acid	(-)	Vincristine	Flufenamic acid	(-)
Adriamycin	Flurbiprofen	(-)	Vincristine	Flurbiprofen	(-)
Adriamycin	Ibuprofen	(-)	Vincristine	Ibuprofen	(-)
Adriamycin	Indomethacin	(+)	Vincristine	Indomethacin	(+)
Adriamycin	Levamisole	(-)	Vincristine	Levamisole	N.D.
Adriamycin	Meclofenamic acid	(-)	Vincristine	Meclo-fenamic acid	(-)
Adriamycin	Mefenamic acid	(+)	Vincristine	Mefenamic acid	(+)
Adriamycin	Naproxen	(-)	Vincristine	Naproxen	N.D.
Adriamycin	Pheylbutazone	(-)	Vincristine	Pheyl-butazone	(-)
Adriamycin	Sulindac	(+)	Vincristine	Sulindac	(+)
Adriamycin	Sulindac sulfide	(+)	Vincristine	Sulindac sulfide	(+)
Adriamycin	Sulindac sulfone	(+)	Vincristine	Sulindac sulfone	(+)
Adriamycin	Tolmetin	(+)	Vincristine	Tolmetin	N.D.
Adriamycin	Zomepirac	(+)	Vincristine	Zomepirac	(+)

* Toxicity enhancement is scored with a (+) if a level of toxicity is achieved with a drug plus NSAID combination, which is significantly greater than the level of toxicity predicted by the fractional product method as described in Section 2.13.
** N.D. = Not determined.

Table 3.3.1: Summary table of ability of various NSAIDs to enhance adriamycin or vincristine toxicity in the drug sensitive A549 cell line.

A549 (P109), Adriamycin and Acemetacin

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.007 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

Acemetacin conc. 1 :- 37.5 μg/mL
Acemetacin conc. 2 :- 19.2 μg/mL
Acemetacin conc. 3 :- 11.5 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	68.99	6.69
Acemetacin 1 control	85.04	10.13
Adriamycin 1 + Ace	19.25*	1.80
Adriamycin 2 control	89.24	16.14
Adriamycin 2 + Ace	29.33*	2.66
Adriamycin 3 control	93.16	11.91
Adriamycin 3 + Ace	54.82*	3.17
Adriamycin 1 control	61.43	4.73
Acemetacin 1 control	97.30	8.46
Adriamycin 1 + Ace	25.73*	2.65
Adriamycin 2 control	82.14	6.55
Adriamycin 2 + Ace	51.07*	3.95
Adriamycin 3 control	94.61	6.79
Adriamycin 3 + Ace	83.58	6.65
Adriamycin 1 control	53.14	4.07
Acemetacin 1 control	102.08	8.76
Adriamycin 1 + Ace	30.66*	3.82
Adriamycin 2 control	81.10	6.27
Adriamycin 2 + Ace	68.55	6.22
Adriamycin 3 control	96.68	9.29
Adriamycin 3 + Ace	92.20	6.72

Table 3.3.2: Survival of A549 cells in the presence of various concentrations of adriamycin and acemetacin (Ace). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P106), Adriamycin and Mefenamic Acid

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.007 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

Mefenamic Acid conc. 1 :- 5.80 μg/mL
Mefenamic Acid conc. 2 :- 2.90 μg/mL
Mefenamic Acid conc. 3 :- 1.50 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	78.19	7.23
Mefenamic Acid 1 control	99.95	8.40
Adriamycin 1 + Mef	34.77*	4.05
Adriamycin 2 control	97.87	13.41
Adriamycin 2 + Mef	70.55	7.33
Adriamycin 3 control	103.34	17.39
Adriamycin 3 + Mef	95.41	16.32
Adriamycin 1 control	65.52	5.52
Mefenamic Acid 2 control	100.07	10.12
Adriamycin 1 + Mef	36.65*	4.59
Adriamycin 2 control	87.56	5.38
Adriamycin 2 + Mef	73.73	6.99
Adriamycin 3 control	93.49	8.76
Adriamycin 3 + Mef	91.86	7.63
Adriamycin 1 control	72.99	4.08
Mefenamic Acid 3 control	103.47	10.70
Adriamycin 1 + Mef	59.19*	4.16
Adriamycin 2 control	93.84	6.44
Adriamycin 2 + Mef	90.50	6.85
Adriamycin 3 control	98.79	7.28
Adriamycin 3 + Mef	97.82	7.27

Table 3.3.3: Survival of A549 cells in the presence of various concentrations of adriamycin and mefenamic acid (Mef). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 96), Adriamycin and Sulindac Sulfide

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.008 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

Sulindac sulfide conc. 1 :- 6.00 μg/mL
Sulindac sulfide conc. 2 :- 2.70 μg/mL
Sulindac sulfide conc. 3 :- 1.40 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	74.21	4.29
Sulindac sulfide 1 control	90.19	9.63
Adriamycin 1 + Sul sulfide	26.79*	1.99
Adriamycin 2 control	85.46	5.95
Adriamycin 2 + Sul sulfide	34.12*	2.86
Adriamycin 3 control	88.23	7.89
Adriamycin 3 + Sul sulfide	48.86*	5.63
Adriamycin 1 control	69.66	3.43
Sulindac sulfide 2 control	104.91	5.57
Adriamycin 1 + Sul sulfide	34.80*	2.39
Adriamycin 2 control	92.93	4.90
Adriamycin 2 + Sul sulfide	39.02*	2.94
Adriamycin 3 control	101.55	6.81
Adriamycin 3 + Sul sulfide	62.56	4.32
Adriamycin 1 control	68.49	6.73
Sulindac sulfide 3 control	105.83	6.50
Adriamycin 1 + Sul sulfide	26.74*	1.76
Adriamycin 2 control	83.99	5.89
Adriamycin 2 + Sul sulfide	39.34*	3.79
Adriamycin 3 control	90.77	6.44
Adriamycin 3 + Sul sulfide	65.59	4.23

Table 3.3.4: Survival of A549 cells in the presence of various concentrations of adriamycin and sulindac (Sul) sulfide. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 96), Adriamycin and Sulindac Sulfone

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.008 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

Sulindac sulfone conc. 1 :- 19.50 μg/mL
Sulindac sulfone conc. 2 :- 8.90 μg/mL
Sulindac sulfone conc. 3 :- 4.50 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	66.68	6.90
Sulindac sulfone 1 control	76.77	6.49
Adriamycin 1 + Sul sulfone	22.16*	1.78
Adriamycin 2 control	81.15	5.90
Adriamycin 2 + Sul sulfone	30.93*	2.90
Adriamycin 3 control	99.80	9.25
Adriamycin 3 + Sul sulfone	49.45	4.51
Adriamycin 1 control	70.50	5.85
Sulindac sulfone 2 control	102.21	8.88
Adriamycin 1 + Sul sulfone	26.60*	2.87
Adriamycin 2 control	86.69	7.74
Adriamycin 2 + Sul sulfone	41.88*	4.10
Adriamycin 3 control	96.38	8.67
Adriamycin 3 + Sul sulfone	64.71	8.28
Adriamycin 1 control	66.18	4.98
Sulindac sulfone 3 control	101.87	10.52
Adriamycin 1 + Sul sulfone	36.67*	2.68
Adriamycin 2 control	81.24	5.35
Adriamycin 2 + Sul sulfone	60.77*	5.30
Adriamycin 3 control	97.91	10.23
Adriamycin 3 + Sul sulfone	79.48	12.34

Table 3.3.5: Survival of A549 cells in the presence of various concentrations of adriamycin and sulindac (Sul) sulfone. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 104), Adriamycin and Tolmetin

Adriamycin conc. 1 :- 0.02 μg/mL
Adriamycin conc. 2 :- 0.01 μg/mL
Adriamycin conc. 3 :- 0.005 μg/mL

Tolmetin conc. 1 :- 24.20 μg/mL
Tolmetin conc. 2 :- 14.10 μg/mL
Tolmetin conc. 3 :- 8.50 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	79.64	3.95
Tol 1 control	99.24	10.35
Adr 1 + Tol	31.82*	2.73
Adr 2 control	98.73	14.08
Adr 2 + Tol	52.83	4.33
Adr 3 control	101.32	11.33
Adr 3 + Tol	86.36	5.82
Adriamycin 1 control	81.10	5.17
Tol 2 control	97.59	9.85
Adr 1 + Tol	38.83*	3.07
Adr 2 control	94.37	8.70
Adr 2 + Tol	73.74	3.55
Adr 3 control	96.94	10.01
Adr 3 + Tol	92.23	9.41
Adriamycin 1 control	73.38	5.58
Tol 3 control	91.84	4.30
Adr 1 + Tol	44.66*	3.13
Adr 2 control	94.40	11.86
Adr 2 + Tol	76.54	6.69
Adr 3 control	95.87	11.00
Adr 3 + Tol	92.04	9.55

Table 3.3.6: Survival of A549 cells in the presence of various concentrations of adriamycin and tolmetin (Tol). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P108), Adriamycin and Zomepirac

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.007 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

Zomepirac conc. 1 :- 39.40 μg/mL
Zomepirac conc. 2 :- 19.80 μg/mL
Zomepirac conc. 3 :- 9.30 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	78.82	7.65
Zomepirac 1 control	88.82	8.25
Adriamycin 1 + Zom	21.40*	2.67
Adriamycin 2 control	90.80	9.53
Adriamycin 2 + Zom	30.94*	2.23
Adriamycin 3 control	94.42	7.58
Adriamycin 3 + Zom	54.56	4.32
Adriamycin 1 control	72.39	5.45
Zomepirac 2 control	95.26	11.73
Adriamycin 1 + Zom	20.88*	1.90
Adriamycin 2 control	90.59	10.84
Adriamycin 2 + Zom	31.94*	2.89
Adriamycin 3 control	98.00	10.70
Adriamycin 3 + Zom	58.20	5.17
Adriamycin 1 control	73.62	8.47
Zomepirac 3 control	98.25	13.72
Adriamycin 1 + Zom	26.25*	2.15
Adriamycin 2 control	90.48	11.79
Adriamycin 2 + Zom	40.52*	3.71
Adriamycin 3 control	97.98	12.86
Adriamycin 3 + Zom	71.45	4.93

Table 3.3.7: Survival of A549 cells in the presence of various concentrations of adriamycin and zomepirac. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P106), Vincristine and Acemetacin

Vincristine conc. 1 :- 0.010 µg/mL
Vincristine conc. 2 :- 0.005 µg/mL
Vincristine conc. 3 :- 0.002 µg/mL

Acemetacin conc. 1 :- 37.5 µg/mL
Acemetacin conc. 2 :- 19.2 µg/mL
Acemetacin conc. 3 :- 11.5 µg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	71.57	5.80
Acemetacin 1 control	76.27	5.85
Vincristine 1 + Ace	13.48*	1.02
Vincristine 2 control	95.41	7.55
Vincristine 2 + Ace	24.77*	2.34
Vincristine 3 control	97.31	11.40
Vincristine 3 + Ace	50.00	5.17
Vincristine 1 control	71.99	7.28
Acemetacin 2 control	99.74	7.10
Vincristine 1 + Ace	28.38*	3.97
Vincristine 2 control	99.67	6.79
Vincristine 2 + Ace	64.09	6.85
Vincristine 3 control	100.65	6.59
Vincristine 3 + Ace	96.49	10.42
Vincristine 1 control	80.08	4.30
Acemetacin 3 control	96.96	6.26
Vincristine 1 + Ace	46.51*	3.02
Vincristine 2 control	95.55	7.63
Vincristine 2 + Ace	85.91	3.56
Vincristine 3 control	101.52	10.10
Vincristine 3 + Ace	97.84	6.32

Table 3.3.8: Survival of A549 cells in the presence of various concentrations of vincristine and acemetacin (Ace). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 98), Vincristine and Mefenamic acid

Vincristine conc. 1 :- 0.010 μg/mL
Vincristine conc. 2 :- 0.005 μg/mL
Vincristine conc. 3 :- 0.002 μg/mL

Mefenamic acid conc. 1 :- 7.90 μg/mL
Mefenamic acid conc. 2 :- 4.00 μg/mL
Mefenamic acid conc. 3 :- 2.30 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	35.78	2.83
Mefenamic acid 1 control	85.57	5.03
Vincristine 1 + Mef	14.01*	1.06
Vincristine 2 control	79.18	4.24
Vincristine 2 + Mef	28.83*	1.95
Vincristine 3 control	91.24	4.99
Vincristine 3 + Mef	72.98	5.44
Vincristine 1 control	29.57	2.87
Mefenamic acid 2 control	94.19	5.08
Vincristine 1 + Mef	14.83*	1.32
Vincristine 2 control	73.21	4.53
Vincristine 2 + Mef	38.57*	3.16
Vincristine 3 control	94.20	5.83
Vincristine 3 + Mef	88.26	6.91
Vincristine 1 control	32.50	3.76
Mefenamic acid 3 control	97.35	13.49
Vincristine 1 + Mef	21.25	1.90
Vincristine 2 control	73.76	6.54
Vincristine 2 + Mef	54.49	5.03
Vincristine 3 control	90.78	4.68
Vincristine 3 + Mef	91.68	4.00

Table 3.3.9: Survival of A549 cells in the presence of various concentrations of vincristine and mefenamic acid (Mef). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 99), Vincristine and Sulindac Sulfide

Vincristine conc. 1 :- 0.010 μg/mL
Vincristine conc. 2 :- 0.006 μg/mL
Vincristine conc. 3 :- 0.003 μg/mL

Sulindac sulfide conc. 1 :- 9.50 μg/mL
Sulindac sulfide conc. 2 :- 4.70 μg/mL
Sulindac sulfide conc. 3 :- 2.40 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	44.71	3.02
Sulindac sulfide 1 control	99.01	13.58
Vincristine 1 + Sul sulfide	18.34*	1.08
Vincristine 2 control	91.79	10.05
Vincristine 2 + Sul sulfide	24.09*	1.30
Vincristine 3 control	94.70	8.89
Vincristine 3 + Sul sulfide	51.80*	3.31
Vincristine 1 control	44.34	2.99
Sulindac sulfide 2 control	92.11	10.93
Vincristine 1 + Sul sulfide	19.16*	1.60
Vincristine 2 control	91.97	21.04
Vincristine 2 + Sul sulfide	25.83*	2.33
Vincristine 3 control	99.47	14.68
Vincristine 3 + Sul sulfide	59.35*	3.78
Vincristine 1 control	49.16	3.92
Sulindac sulfide 3 control	107.65	12.50
Vincristine 1 + Sul sulfide	22.09*	1.76
Vincristine 2 control	99.08	10.57
Vincristine 2 + Sul sulfide	31.62*	2.23
Vincristine 3 control	101.53	11.17
Vincristine 3 + Sul sulfide	74.32	3.72

Table 3.3.10: Survival of A549 cells in the presence of various concentrations of vincristine and sulindac (Sul) sulfide. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 99), Vincristine and Sulindac Sulfone

Vincristine conc. 1 :- 0.010 μg/mL
Vincristine conc. 2 :- 0.006 μg/mL
Vincristine conc. 3 :- 0.003 μg/mL

Sulindac sulfone conc. 1 :- 20.70 μg/mL
Sulindac sulfone conc. 2 :- 10.00 μg/mL
Sulindac sulfone conc. 3 :- 6.10 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	43.76	3.77
Sulindac sulfone 1 control	102.91	8.50
Vincristine 1 + Sul sulfone	18.42*	1.83
Vincristine 2 control	92.44	12.62
Vincristine 2 + Sul sulfone	24.29*	2.50
Vincristine 3 control	97.29	5.64
Vincristine 3 + Sul sulfone	46.27*	4.35
Vincristine 1 control	46.13	6.09
Sulindac sulfone 2 control	98.99	17.53
Vincristine 1 + Sul sulfone	23.34*	2.51
Vincristine 2 control	90.80	17.36
Vincristine 2 + Sul sulfone	34.81*	3.20
Vincristine 3 control	100.53	13.49
Vincristine 3 + Sul sulfone	85.34	13.78
Vincristine 1 control	40.43	4.62
Sulindac sulfone 3 control	104.95	14.02
Vincristine 1 + Sul sulfone	27.91*	2.61
Vincristine 2 control	87.06	6.43
Vincristine 2 + Sul sulfone	43.91*	3.59
Vincristine 3 control	103.86	13.06
Vincristine 3 + Sul sulfone	99.81	13.10

Table 3.3.11: Survival of A549 cells in the presence of various concentrations of vincristine and sulindac (Sul) sulfone . Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 97), Vincristine and Zomepirac

Vincristine conc. 1 :- 0.010 μg/mL
Vincristine conc. 2 :- 0.005 μg/mL
Vincristine conc. 3 :- 0.002 μg/mL

Zomepirac conc. 1 :- 38.80 μg/mL
Zomepirac conc. 2 :- 20.70 μg/mL
Zomepirac conc. 3 :- 11.40 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	80.11	6.68
Zomepirac 1 control	93.49	9.05
Vincristine 1 + Zom	20.86*	2.39
Vincristine 2 control	101.32	15.14
Vincristine 2 + Zom	39.96*	2.50
Vincristine 3 control	99.59	11.23
Vincristine 3 + Zom	82.22	9.76
Vincristine 1 control	86.43	12.92
Zomepirac 2 control	102.60	11.90
Vincristine 1 + Zom	23.96*	2.12
Vincristine 2 control	96.12	12.19
Vincristine 2 + Zom	51.04*	3.65
Vincristine 3 control	100.62	12.52
Vincristine 3 + Zom	95.93	9.50
Vincristine 1 control	87.91	10.55
Zomepirac 3 control	103.38	14.77
Vincristine 1 + Zom	26.66*	2.32
Vincristine 2 control	107.94	13.98
Vincristine 2 + Zom	63.71*	5.15
Vincristine 3 control	102.73	16.10
Vincristine 3 + Zom	98.55	17.98

Table 3.3.12: Survival of A549 cells in the presence of various concentrations of vincristine and zomepirac (Zom). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 98), Adriamycin and Acetaminophen

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.007 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

Acetaminophen conc. 1 :- 0.01 μg/mL
Acetaminophen conc. 2 :- 0.005 μg/mL
Acetaminophen conc. 3 :- 0.002 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	68.53	6.42
Acetaminophen 1 control	82.91	7.30
Adriamycin 1 + Acet	55.04	3.86
Adriamycin 2 control	91.74	13.68
Adriamycin 2 + Acet	60.83	10.39
Adriamycin 3 control	97.57	12.03
Adriamycin 3 + Acet	77.13	8.16
Adriamycin 1 control	59.52	8.42
Acetaminophen 2 control	89.44	19.78
Adriamycin 1 + Acet	40.70	5.21
Adriamycin 2 control	88.23	13.34
Adriamycin 2 + Acet	66.76	10.90
Adriamycin 3 control	93.17	10.30
Adriamycin 3 + Acet	76.33	15.25
Adriamycin 1 control	79.09	4.63
Acetaminophen 3 control	99.39	8.85
Adriamycin 1 + Acet	69.76	7.38
Adriamycin 2 control	93.84	13.38
Adriamycin 2 + Acet	90.72	8.05
Adriamycin 3 control	97.46	6.30
Adriamycin 3 + Acet	92.48	9.57

Table 3.3.13: Survival of A549 cells in the presence of various concentrations of adriamycin and acetaminophen (Acet). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 98), Vincristine and Acetaminophen

Vincristine conc. 1 :- 0.010 μg/mL
Vincristine conc. 2 :- 0.005 μg/mL
Vincristine conc. 3 :- 0.002 μg/mL

Acetaminophen conc. 1 :- 0.01 μg/mL
Acetaminophen conc. 2 :- 0.005 μg/mL
Acetaminophen conc. 3 :- 0.002 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	60.05	6.31
Acetaminophen 1 control	94.01	8.43
Vincristine 1 + Acet	55.56	5.74
Vincristine 2 control	93.96	10.50
Vincristine 2 + Acet	84.35	9.56
Vincristine 3 control	104.54	12.37
Vincristine 3 + Acet	95.32	12.28
Vincristine 1 control	63.89	7.62
Acetaminophen 2 control	94.29	9.08
Vincristine 1 + Acet	61.62	9.55
Vincristine 2 control	96.22	12.24
Vincristine 2 + Acet	91.61	10.50
Vincristine 3 control	99.34	9.02
Vincristine 3 + Acet	102.39	11.04
Vincristine 1 control	68.77	9.80
Acetaminophen 3 control	104.21	11.28
Vincristine 1 + Acet	72.52	12.15
Vincristine 2 control	101.64	9.24
Vincristine 2 + Acet	100.09	10.87
Vincristine 3 control	105.08	9.00
Vincristine 3 + Acet	101.11	8.81

Table 3.3.14: Survival of A549 cells in the presence of various concentrations of vincristine and acetaminophen (Acet). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

3.4 Ability of NSAIDs to enhance the toxicity of heavy metals in the A549 cell line

It was known that the toxicity of a suitable chemotherapeutic drug could be enhanced in the presence of various NSAIDs, but it was unclear if this effect was a result of the NSAID interfering with a resistance mechanism unique for chemotherapeutic drugs or if the NSAID affected an intrinsic cellular resistance mechanism common to toxins other than chemotherapeutic drugs. Further *in vitro* toxicity assay screening was carried out with the A549 cell line in order to investigate the ability of NSAIDs such as indomethacin and tolmetin to enhance the toxicity of heavy metals such as sodium arsenate or potassium antimony tartrate. Heavy metals such as these compounds have been shown to be substrates for the drug transporter molecule MRP, (Cole *et al.*, 1994). Interference with their transport out of MRP-expressing cells should result in an increase in cellular accumulation and an augmentation of heavy metal-induced cell kill. As can be seen from Tables 3.4.1 and 3.4.2, indomethacin and tolmetin, when present in high concentrations (3 and 24 μg/mL respectively) enhanced the toxicity of sodium arsenate in A549 cells. This result suggested that indomethacin and tolmetin may have in some way interfered with MRP-mediated transport of sodium arsenate out of the cell and so caused the enhancement of heavy metal toxicity. This suggestion was complicated by the fact that the cytotoxicity of potassium antimony tartrate was unaffected by the presence of indomethacin or tolmetin (Tables 3.4.3 and 3.4.4). The influence of an NSAID such as acetaminophen which did not possess chemotherapeutic drug toxicity enhancing abilities, to influence heavy metal toxicity was not investigated.

A549 (P 99), Sodium Arsenate and Indomethacin

Sodium arsenate conc. 1 :- 15.20 μg/mL
Sodium arsenate conc. 2 :- 7.70 μg/mL
Sodium arsenate conc. 3 :- 4.00 μg/mL

Indomethacin conc. 1 :- 3.00 μg/mL
Indomethacin conc. 2 :- 1.50 μg/mL
Indomethacin conc. 3 :- 1.00 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Na Arsenate 1 control	68.88	5.20
Indomethacin 1 control	98.95	3.99
Na Arsenate 1 + Indo	35.10*	2.20
Na Arsenate 2 control	67.58	8.13
Na Arsenate 2 + Indo	54.81	5.22
Na Arsenate 3 control	78.17	9.32
Na Arsenate 3 + Indo	71.41	6.18
Na Arsenate 1 control	69.85	2.65
Indomethacin 2 control	100.36	3.89
Na Arsenate 1 + Indo	40.33*	5.99
Na Arsenate 2 control	64.98	6.54
Na Arsenate 2 + Indo	66.86	5.41
Na Arsenate 3 control	79.50	6.31
Na Arsenate 3 + Indo	80.23	6.29
Na Arsenate 1 control	63.29	7.97
Indomethacin 3 control	95.74	5.35
Na Arsenate 1 + Indo	54.31	3.51
Na Arsenate 2 control	64.74	5.35
Na Arsenate 2 + Indo	65.70	4.81
Na Arsenate 3 control	79.15	5.23
Na Arsenate 3 + Indo	80.83	5.10

Table 3.4.1: Survival of A549 cells in the presence of various concentrations of sodium arsenate and indomethacin (Indo). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 98), Sodium Arsenate and Tolmetin

Sodium arsenate conc. 1 :- 14.50 μg/mL
Sodium arsenate conc. 2 :- 7.50 μg/mL
Sodium arsenate conc. 3 :- 4.20 μg/mL

Tolmetin conc. 1 :- 24.30 μg/mL
Tolmetin conc. 2 :- 11.90 μg/mL
Tolmetin conc. 3 :- 6.10 μg/mL

Treatment	**% Cell Survival**	**S.D.**
Cell control	100	
Na Arsenate 1 control	85.97	7.01
Tolmetin 1 control	96.58	9.92
Na Arsenate 1 + Tol	48.77*	4.26
Na Arsenate 2 control	93.22	8.21
Na Arsenate 2 + Tol	73.23	3.99
Na Arsenate 3 control	99.30	11.49
Na Arsenate 3 + Tol	89.72	9.08
Na Arsenate 1 control	91.02	9.10
Tolmetin 2 control	97.29	8.61
Na Arsenate 1 + Tol	58.29*	7.10
Na Arsenate 2 control	94.82	13.96
Na Arsenate 2 + Tol	87.34	7.84
Na Arsenate 3 control	92.39	12.89
Na Arsenate 3 + Tol	91.41	9.61
Na Arsenate 1 control	93.30	7.81
Tolmetin 3 control	100.99	11.04
Na Arsenate 1 + Tol	86.88	8.59
Na Arsenate 2 control	99.51	13.90
Na Arsenate 2 + Tol	97.88	11.90
Na Arsenate 3 control	100.92	9.25
Na Arsenate 3 + Tol	101.92	15.25

Table 3.4.2: Survival of A549 cells in the presence of various concentrations of sodium arsenate and tolmetin (Tol). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 95), Potassium Antimony Tartrate and Indomethacin

Potassium Antimony Tartrate conc. 1:- 3.00 μg/mL
Potassium Antimony Tartrate conc. 2:- 1.50 μg/mL
Potassium Antimony Tartrate conc. 3:- 0.75 μg/mL

Indomethacin conc. 1 :- 2.60 μg/mL
Indomethacin conc. 2 :- 1.30 μg/mL
Indomethacin conc. 3 :- 0.80 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Pott antimony tartrate 1 control	38.27	7.61
Indomethacin 1 control	90.49	6.43
Pott antimony tartrate 1 + Indo	66.12	5.59
Pott antimony tartrate 2 control	53.02	9.09
Pott antimony tartrate 2 + Indo	75.25	7.60
Pott antimony tartrate 3 control	76.12	7.83
Pott antimony tartrate 3 + Indo	83.48	6.05
Pott antimony tartrate 1 control	34.39	9.33
Indomethacin 2 control	96.58	10.07
Pott antimony tartrate 1 + Indo	66.34	7.10
Pott antimony tartrate 2 control	46.86	10.41
Pott antimony tartrate 2 + Indo	67.42	7.30
Pott antimony tartrate 3 control	76.09	6.01
Pott antimony tartrate 3 + Indo	86.40	6.88
Pott antimony tartrate 1 control	45.21	5.71
Indomethacin 3 control	96.73	4.55
Pott antimony tartrate 1 + Indo	76.27	6.27
Pott antimony tartrate 2 control	64.24	7.76
Pott antimony tartrate 2 + Indo	80.29	7.55
Pott antimony tartrate 3 control	83.72	7.89
Pott antimony tartrate 3 + Indo	87.12	5.63

Table 3.4.3: Survival of A549 cells in the presence of various concentrations of Potassium antimony tartrate and indomethacin (Indo). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 98), Potassium Antimony Tartrate and Tolmetin

Potassium Antimony Tartrate conc. 1:- 3.50 μg/mL
Potassium Antimony Tartrate conc. 2:- 1.70 μg/mL
Potassium Antimony Tartrate conc. 3:- 0.90 μg/mL

Tolmetin conc. 1 :- 24.30 μg/mL
Tolmetin conc. 2 :- 11.90 μg/mL
Tolmetin conc. 3 :- 6.10 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Pott antimony tartrate 1 control	99.19	14.30
Tolmetin 1 control	101.32	14.63
Pott antimony tartrate 1 + Tol	87.40	6.37
Pott antimony tartrate 2 control	97.49	14.87
Pott antimony tartrate 2 + Tol	94.44	12.27
Pott antimony tartrate 3 control	98.02	12.76
Pott antimony tartrate 3 + Tol	92.73	10.58
Pott antimony tartrate 1 control	90.24	9.17
Tolmetin 2 control	98.51	11.78
Pott antimony tartrate 1 + Tol	89.72	8.57
Pott antimony tartrate 2 control	93.03	10.21
Pott antimony tartrate 2 + Tol	93.14	9.48
Pott antimony tartrate 3 control	94.21	8.61
Pott antimony tartrate 3 + Tol	93.23	8.97
Pott antimony tartrate 1 control	92.52	9.81
Tolmetin 3 control	99.02	12.23
Pott antimony tartrate 1 + Tol	89.38	10.53
Pott antimony tartrate 2 control	94.42	10.22
Pott antimony tartrate 2 + Tol	94.71	12.81
Pott antimony tartrate 3 control	92.81	9.41
Pott antimony tartrate 3 + Tol	94.26	6.98

Table 3.4.4: Survival of A549 cells in the presence of various concentrations of Potassium antimony tartrate and tolmetin (Tol). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

3.5 Identification of NSAIDs with an ability to enhance the toxicity of adriamycin and vincristine in the DLKP cell line.

The NSAID screening performed with the A549 cell line identified a select number of NSAIDs with an ability to enhance the toxicity of adriamycin and vincristine. In order to investigate if this profile of toxicity enhancing NSAIDs was unique to the A549 cell line, further *in vitro* toxicity assay screening was performed using a second drug sensitive cell line, the human lung squamous carcinoma cell line DLKP. It was found that in DLKP, adriamycin and vincristine toxicity could be enhanced in the presence of acemetacin, indomethacin, sulindac, tolmetin, mefenamic acid and zomepirac. The toxicity of these cytotoxic drugs was unaffected by the presence of an NSAID such as etodolac (Table 3.5.9). As described in Section 3.3, these NSAIDs also enhance adriamycin and vincristine toxicity in the A549 cell line. This suggested that the NSAIDs in question directly affected a drug resistance mechanism which is common to the DLKP and A549 cell lines. The NSAID screening results for the DLKP cell line are summarised in Table 3.5.1 with the specific screening results presented in Tables 3.5.3 to 3.5.16. From the data it can be seen that the toxicity enhancement effect occurs to the greatest extent at the highest concentrations of drug and NSAID tested. For example in Table 3.5.4, a concentration of 2.5 μg/mL indomethacin (a non-toxic dose) significantly enhances the toxicity of 10 ng/mL adriamycin from approximately 26% to 85%. This concentration of indomethacin has no effect on the level of toxicity achieved by a dose of 3 ng/mL adriamycin. The magnitude of the toxicity enhancement effect in the DLKP cell line is comparable to the effect in the A549 cell line. For example in the A549 cell line (Table 3.2.3), a non-toxic dose of sulindac (6.3 μg/mL) enhanced the toxicity of 20 ng/mL adriamycin from approximately 28% to 65%, In DLKP (Table 3.5.6) a non-toxic dose of sulindac (7.6 μg/mL) enhanced the toxicity of 20 ng/mL adriamycin from approximately 35% to 75%.

A serum free medium has been developed for the DLKP cell line, (Meleady, 1997). NSAID *in vitro* toxicity assay screening was carried out using serum free conditions (media composition detailed in Section 2.5) in order to investigate the possible dependence of the toxicity enhancement effect on the various growth factors present in serum. It was found that chemotherapeutic drug toxicity was enhanced under serum free conditions to an extent comparable to that found under standard serum containing conditions. The results are provided in summary in Table 3.5.2. This illustrated that the ability of a suitable NSAID to enhance the toxicity of a drug such as adriamycin was independent of any potential activities of the various growth factors present in serum.

Drug	NSAID	Toxicity enhancement *	Drug	NSAID	Toxicity enhancement *
Adriamycin	Acemetacin	(+)	Vincristine	Acemetacin	(+)
Adriamycin	Etodolac	(-)	Vincristine	Etodolac	(-)
Adriamycin	Indomethacin	(+)	Vincristine	Indomethacin	(+)
Adriamycin	Mefenamic acid	(+)	Vincristine	Mefenamic acid	(+)
Adriamycin	Sulindac	(+)	Vincristine	Sulindac	(+)
Adriamycin	Tolmetin	(+)	Vincristine	Tolmetin	(+)
Adriamycin	Zomepirac	(+)	Vincristine	Zomepirac	(+)

* Toxicity enhancement is scored with a (+) if a level of toxicity is achieved with a drug plus NSAID combination, which is significantly greater than the level of toxicity predicted by the fractional product method as described in Section 2.3. A (-) indicates no significant toxicity enhancement effects.

Table 3.5.1: Ability of various NSAIDs to enhance the toxicity of adriamycin and vincristine in the DLKP cell line.

Drug	NSAID	Toxicity enhancement*
Adriamycin	Indomethacin	(+)
Adriamycin	Sulindac	(+)
Adriamycin	Tolmetin	(+)
Adriamycin	Naproxen	(-)

* Toxicity enhancement is scored with a (+) if a level of toxicity is achieved with a drug + NSAID combination, which is significantly greater than the level of toxicity predicted by the fractional product method. A (-) indicates no significant toxicity enhancement effects.

Table 3.5.2: Ability of various NSAIDs to enhance the toxicity of adriamycin and vincristine in the DLKP cell line under serum free medium growth conditions as described in Section 2.5.

DLKP (P 16), Adriamycin and Acemetacin

Adriamycin conc. 1 :- 0.010 µg/mL
Adriamycin conc. 2 :- 0.005 µg/mL
Adriamycin conc. 3 :- 0.003 µg/mL

Acemetacin conc. 1 :- 37.50 µg/mL
Acemetacin conc. 2 :- 18.30 µg/mL
Acemetacin conc. 3 :- 7.50 µg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	63.00	4.93
Acemetacin 1 control	76.82	9.90
Adriamycin 1 + Ace	14.93*	1.92
Adriamycin 2 control	88.64	5.34
Adriamycin 2 + Ace	28.91*	2.98
Adriamycin 3 control	95.17	6.23
Adriamycin 3 + Ace	32.72*	3.39
Adriamycin 1 control	59.58	4.96
Acemetacin 2 control	89.33	8.45
Adriamycin 1 + Ace	11.38*	1.72
Adriamycin 2 control	83.68	4.56
Adriamycin 2 + Ace	60.50	5.35
Adriamycin 3 control	93.76	7.93
Adriamycin 3 + Ace	83.67	7.21
Adriamycin 1 control	56.38	5.30
Acemetacin 3 control	96.41	6.73
Adriamycin 1 + Ace	10.77*	2.00
Adriamycin 2 control	86.80	8.51
Adriamycin 2 + Ace	62.22	3.73
Adriamycin 3 control	92.29	5.77
Adriamycin 3 + Ace	91.65	8.79

Table 3.5.3: Survival of DLKP cell in the presence of various concentrations of adriamycin and acemetacin (Ace). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 17), Adriamycin and Indomethacin

Adriamycin conc. 1 :- 0.010 μg/mL
Adriamycin conc. 2 :- 0.005 μg/mL
Adriamycin conc. 3 :- 0.003 μg/mL

Indomethacin conc. 1 :- 2.50 μg/mL
Indomethacin conc. 2 :- 1.40 μg/mL
Indomethacin conc. 3 :- 0.70 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	73.52	5.41
Indomethacin 1 control	96.70	4.93
Adriamycin 1 + Indo	14.63*	4.58
Adriamycin 2 control	94.85	5.72
Adriamycin 2 + Indo	69.83*	4.02
Adriamycin 3 control	102.02	5.57
Adriamycin 3 + Indo	96.70	8.61
Adriamycin 1 control	70.44	5.26
Indomethacin 2 control	98.05	6.00
Adriamycin 1 + Indo	23.47*	3.35
Adriamycin 2 control	90.46	6.37
Adriamycin 2 + Indo	76.92	6.39
Adriamycin 3 control	96.47	9.13
Adriamycin 3 + Indo	93.46	7.40
Adriamycin 1 control	70.69	11.75
Indomethacin 3 control	102.18	15.08
Adriamycin 1 + Indo	36.24*	8.12
Adriamycin 2 control	90.98	12.42
Adriamycin 2 + Indo	85.74	11.35
Adriamycin 3 control	97.68	9.03
Adriamycin 3 + Indo	89.50	8.53

Table 3.5.4: Survival of DLKP cells in the presence of various concentrations of adriamycin and indomethacin (Indo). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 21), Adriamycin and Mefenamic acid

Adriamycin conc. 1 :- 0.010 μg/mL
Adriamycin conc. 2 :- 0.005 μg/mL
Adriamycin conc. 3 :- 0.003 μg/mL

Mefemamic acid conc. 1 :- 7.60 μg/mL
Mefemamic acid conc. 2 :- 4.00 μg/mL
Mefemamic acid conc. 3 :- 2.00 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	91.08	13.76
Mefenamic acid 1 control	96.12	11.58
Adriamycin 1 + Mef	30.46*	3.82
Adriamycin 2 control	99.59	13.55
Adriamycin 2 + Mef	85.69	6.16
Adriamycin 3 control	97.48	9.08
Adriamycin 3 + Mef	95.58	12.72
Adriamycin 1 control	91.67	8.66
Mefenamic acid 2 control	100.67	12.04
Adriamycin 1 + Mef	46.77*	4.08
Adriamycin 2 control	99.33	13.46
Adriamycin 2 + Mef	100.90	12.58
Adriamycin 3 control	98.58	8.48
Adriamycin 3 + Mef	99.56	12.98
Adriamycin 1 control	86.49	8.60
Mefenamic acid 3 control	95.24	13.15
Adriamycin 1 + Mef	67.64	4.61
Adriamycin 2 control	96.80	11.01
Adriamycin 2 + Mef	94.02	10.67
Adriamycin 3 control	92.65	8.92
Adriamycin 3 + Mef	93.69	11.42

Table 3.5.5: Survival of DLKP cells in the presence of various concentrations of adriamycin and mefenamic acid (Mef). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 18), Adriamycin and Sulindac

Adriamycin conc. 1 :- 0.020 μg/mL
Adriamycin conc. 2 :- 0.010 μg/mL
Adriamycin conc. 3 :- 0.005 μg/mL

Sulindac conc. 1 :- 7.60 μg/mL
Sulindac conc. 2 :- 3.90 μg/mL
Sulindac conc. 3 :- 2.00 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	64.84	3.40
Sulindac 1 control	103.04	8.41
Adriamycin 1 + Sul	24.35*	1.90
Adriamycin 2 control	87.06	15.18
Adriamycin 2 + Sul	58.68*	3.66
Adriamycin 3 control	95.37	16.29
Adriamycin 3 + Sul	88.74	12.41
Adriamycin 1 control	56.52	5.15
Sulindac 2 control	102.10	8.37
Adriamycin 1 + Sul	22.26*	2.23
Adriamycin 2 control	94.15	11.20
Adriamycin 2 + Sul	64.17*	4.36
Adriamycin 3 control	95.24	10.55
Adriamycin 3 + Sul	94.28	13.69
Adriamycin 1 control	64.68	4.93
Sulindac 3 control	101.25	8.40
Adriamycin 1 + Sul	37.55*	2.72
Adriamycin 2 control	98.71	8.29
Adriamycin 2 + Sul	83.16	4.01
Adriamycin 3 control	104.01	13.87
Adriamycin 3 + Sul	104.26	13.77

Table 3.5.6: Survival of DLKP cells in the presence of various concentrations of adriamycin and sulindac (Sul). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 19), Adriamycin and Tolmetin

Adriamycin conc. 1 :- 0.010 μg/mL
Adriamycin conc. 2 :- 0.005 μg/mL
Adriamycin conc. 3 :- 0.003 μg/mL

Tolmetin conc. 1 :- 26.80 μg/mL
Tolmetin conc. 2 :- 13.40 μg/mL
Tolmetin conc. 3 :- 6.50 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	54.72	4.82
Tolmetin 1 control	94.72	6.00
Adriamycin 1 + Tol	18.44*	1.44
Adriamycin 2 control	81.61	6.51
Adriamycin 2 + Tol	65.51*	4.64
Adriamycin 3 control	92.37	7.01
Adriamycin 3 + Tol	87.33	6.64
Adriamycin 1 control	51.03	3.42
Tolmetin 2 control	99.00	5.15
Adriamycin 1 + Tol	22.90*	2.56
Adriamycin 2 control	82.79	5.00
Adriamycin 2 + Tol	71.86	4.48
Adriamycin 3 control	95.35	5.97
Adriamycin 3 + Tol	93.85	4.36
Adriamycin 1 control	48.64	3.73
Tolmetin 3 control	97.48	5.71
Adriamycin 1 + Tol	30.83*	3.43
Adriamycin 2 control	80.32	6.17
Adriamycin 2 + Tol	75.45	4.17
Adriamycin 3 control	91.76	6.04
Adriamycin 3 + Tol	90.76	6.01

Table 3.5.7: Survival of DLKP cells in the presence of various concentrations of adriamycin and tolmetin (Tol). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 19), Adriamycin and Zomepirac

Adriamycin conc. 1 :- 0.010 μg/mL
Adriamycin conc. 2 :- 0.005 μg/mL
Adriamycin conc. 3 :- 0.003 μg/mL

Zomepirac conc. 1 :- 37.20 μg/mL
Zomepirac conc. 2 :- 17.20 μg/mL
Zomepirac conc. 3 :- 8.40 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	61.11	5.52
Zomepirac 1 control	89.20	8.20
Adriamycin 1 + Zom	12.05*	1.89
Adriamycin 2 control	92.03	21.41
Adriamycin 2 + Zom	54.14*	3.76
Adriamycin 3 control	99.93	20.02
Adriamycin 3 + Zom	76.62	8.86
Adriamycin 1 control	55.73	6.25
Zomepirac 2 control	90.71	17.22
Adriamycin 1 + Zom	9.06*	1.40
Adriamycin 2 control	87.95	14.65
Adriamycin 2 + Zom	53.93*	8.82
Adriamycin 3 control	94.32	13.73
Adriamycin 3 + Zom	80.18	13.40
Adriamycin 1 control	53.87	10.08
Zomepirac 3 control	87.08	6.64
Adriamycin 1 + Zom	10.27*	2.35
Adriamycin 2 control	84.63	6.55
Adriamycin 2 + Zom	51.78	7.64
Adriamycin 3 control	94.62	14.30
Adriamycin 3 + Zom	76.03	12.33

Table 3.5.8: Survival of DLKP cells in the presence of various concentrations of adriamycin and zomepirac (Zom). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 21), Adriamycin and Etodolac

Adriamycin conc. 1 :- 0.010 µg/mL
Adriamycin conc. 2 :- 0.005 µg/mL
Adriamycin conc. 3 :- 0.003 µg/mL

Etodolac conc. 1 :- 15.20 µg/mL
Etodolac conc. 2 :- 7.60 µg/mL
Etodolac conc. 3 :- 3.70 µg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	72.88	10.50
Etodolac 1 control	100.06	9.81
Adriamycin 1 + Etod	73.78	6.48
Adriamycin 2 control	97.16	6.85
Adriamycin 2 + Etod	91.97	8.64
Adriamycin 3 control	102.96	12.16
Adriamycin 3 + Etod	98.21	10.35
Adriamycin 1 control	76.82	4.18
Etodolac 2 control	99.26	5.05
Adriamycin 1 + Etod	74.69	5.00
Adriamycin 2 control	92.83	5.12
Adriamycin 2 + Etod	93.73	5.29
Adriamycin 3 control	97.17	6.57
Adriamycin 3 + Etod	94.46	5.54
Adrianycin 1 control	66.27	5.49
Etodolac 3 control	94.60	5.86
Adriamycin 1 + Etod	66.91	3.19
Adriamycin 2 control	85.42	6.36
Adrianycin 2 + Etod	89.53	6.18
Adriamycin 3 control	91.12	4.93
Adriamycin 3 + Etod	90.87	5.02

Table 3.5.9: Survival of DLKP cells in the presence of various concentrations of adriamycin and etodolac (Etod). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P16), Vincristine and Acemetacin

Vincristine conc. 1 :- 0.002 μg/mL
Vincristine conc. 2 :- 0.001 μg/mL
Vincristine conc. 3 :- 0.003 μg/mL

Acemetacin conc. 1 :- 37.50 μg/mL
Acemetacin conc. 2 :- 18.30 μg/mL
Acemetacin conc. 3 :- 7.50 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	10.08	1.63
Acemetacin 1 control	71.83	5.24
Vincristine 1 + Ace	5.31	0.33
Vincristine 2 control	77.40	5.05
Vincristine 2 + Ace	12.33*	2.50
Vincristine 3 control	87.22	5.16
Vincristine 3 + Ace	20.39*	6.57
Vincristine 1 control	11.58	2.72
Acemetacin 2 control	93.39	9.08
Vincristine 1 + Ace	0.72	0.48
Vincristine 2 control	87.54	7.87
Vincristine 2 + Ace	3.83*	0.76
Vincristine 3 control	95.19	10.36
Vincristine 3 + Ace	17.90*	3.52
Vincristine 1 control	17.57	3.88
Acemetacin 3 control	98.02	5.94
Vincristine 1 + Ace	0.71	0.33
Vincristine 2 control	91.09	6.08
Vincristine 2 + Ace	12.09*	1.61
Vincristine 3 control	101.06	17.61
Vincristine 3 + Ace	51.13*	6.13

Table 3.5.10: Survival of DLKP cells in the presence of various concentrations of vincristine and acemetacin (Ace). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 17), Vincristine and Indomethacin

Vincristine conc. 1 :- 0.0014 µg/mL
Vincristine conc. 2 :- 0.0010 µg/mL
Vincristine conc. 3 :- 0.0007 µg/mL

Indomethacin conc. 1 :- 2.50 µg/mL
Indomethacin conc. 2 :- 1.40 µg/mL
Indomethacin conc. 3 :- 0.70 µg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	31.10	3.25
Indomethacin 1 control	95.07	5.06
Vincristine 1 + Indo	1.60*	0.49
Vincristine 2 control	66.26	5.51
Vincristine 2 + Indo	3.02*	0.66
Vincristine 3 control	87.39	6.64
Vincristine 3 + Indo	10.52*	1.05
Vincristine 1 control	34.71	5.90
Indomethacin 2 control	98.84	5.97
Vincristine 1 + Indo	1.87*	0.39
Vincristine 2 control	64.28	4.17
Vincristine 2 + Indo	4.34*	0.90
Vincristine 3 control	86.50	8.15
Vincristine 3 + Indo	17.48*	2.99
Vincristine 1 control	37.27	6.72
Indomethacin 3 control	95.81	9.08
Vincristine 1 + Indo	2.21*	0.44
Vincristine 2 control	68.00	8.13
Vincristine 2 + Indo	7.75*	1.66
Vincristine 3 control	89.35	12.88
Vincristine 3 + Indo	36.24*	7.61

Table 3.5.11: Survival of DLKP cells in the presence of various concentrations of vincristine and indomethacin (Indo). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 21), Vincristine and Mefenamic acid

Vincristine conc. 1 :- 0.0015 µg/mL
Vincristine conc. 2 :- 0.0012 µg/mL
Vincristine conc. 3 :- 0.0070 µg/mL

Mefemamic acid conc. 1 :- 7.60 µg/mL
Mefemamic acid conc. 2 :- 4.00 µg/mL
Mefemamic acid conc. 3 :- 2.00 µg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	70.51	5.23
Mefenamic acid 1 control	96.46	16.65
Vincristine 1 + Mef	2.33*	0.42
Vincristine 2 control	88.77	14.47
Vincristine 2 + Mef	6.36*	0.90
Vincristine 3 control	98.26	11.96
Vincristine 3 + Mef	56.45*	10.26
Vincristine 1 control	76.27	7.28
Mefenamic acid 2 control	96.21	10.27
Vincristine 1 + Mef	10.28*	2.12
Vincristine 2 control	85.95	9.71
Vincristine 2 + Mef	34.88*	4.99
Vincristine 3 control	95.21	11.36
Vincristine 3 + Mef	86.93	7.39
Vincristine 3 control	82.43	5.63
Mefenamic acid 3 control	100.93	10.49
Vincristine 1 + Mef	41.44*	6.11
Vincristine 2 control	94.02	7.81
Vincristine 2 + Mef	80.57	7.76
Vincristine 3 control	99.03	8.42
Vincristine 3 + Mef	95.84	7.04

Table 3.5.12: Survival of DLKP cells in the presence of various concentrations of vincristine and mefenamic acid (Mef). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 18), Vincristine and Sulindac

Vincristine conc. 1 :- 0.002 µg/mL
Vincristine conc. 2 :- 0.001 µg/mL
Vincristine conc. 3 :- 0.005 µg/mL

Sulindac conc. 1 :- 7.60 µg/mL
Sulindac conc. 2 :- 3.90 µg/mL
Sulindac conc. 3 :- 2.00 µg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	74.08	7.23
Sulindac 1 control	94.26	9.30
Vincristine 1 + Sul	6.23*	1.02
Vincristine 2 control	92.62	6.89
Vincristine 2 + Sul	26.67*	3.09
Vincristine 3 control	99.87	10.28
Vincristine 3 + Sul	87.24	7.32
Vincristine 1 control	74.37	6.20
Sulindac 2 control	100.05	13.96
Vincristine 1 + Sul	9.43*	1.30
Vincristine 2 control	97.97	8.13
Vincristine 2 + Sul	51.75*	3.61
Vincristine 3 control	100.70	9.73
Vincristine 3 + Sul	97.98	7.72
Vincristine 1 control	75.37	5.35
Sulindac 3 control	103.56	11.67
Vincristine 1 + Sul	25.86*	3.71
Vincristine 2 control	97.13	5.95
Vincristine 2 + Sul	86.06	8.10
Vincristine 3 control	103.56	8.10
Vincristine 3 + Sul	102.79	8.85

Table 3.5.13: Survival of DLKP cells in the presence of various concentrations of vincristine and sulindac (Sul). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 19), Vincristine and Tolmetin

Vincristine conc. 1 :- 0.0015 μg/mL
Vincristine conc. 2 :- 0.0012 μg/mL
Vincristine conc. 3 :- 0.0007 μg/mL

Tolmetin conc. 1 :- 26.80 μg/mL
Tolmetin conc. 2 :- 13.40 μg/mL
Tolmetin conc. 3 :- 6.50 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	54.86	4.56
Tolmetin 1 control	93.11	6.16
Vincristine 1 + Tol	5.93*	1.08
Vincristine 2 control	68.57	6.31
Vincristine 2 + Tol	16.00*	2.09
Vincristine 3 control	90.16	5.59
Vincristine 3 + Tol	65.45*	4.75
Vincristine 1 control	52.08	4.23
Tolmetin 2 control	97.40	5.60
Vincristine 1 + Tol	12.70*	2.14
Vincristine 2 control	66.33	4.34
Vincristine 2 + Tol	26.76*	2.24
Vincristine 3 control	90.35	6.41
Vincristine 3 + Tol	79.28	6.08
Vincristine 1 control	55.21	5.50
Tolmetin 3 control	98.93	7.29
Vincristine 1 + Tol	25.66*	3.88
Vincristine 2 control	67.27	5.34
Vincristine 2 + Tol	44.73	4.93
Vincristine 3 control	90.90	7.01
Vincristine 3 + Tol	87.43	9.70

Table 3.5.14: Survival of DLKP cells in the presence of various concentrations of vincristine and tolmetin (Tol). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 19), Vincristine and Zomepirac

Vincristine conc. 1 :- 0.0015 μg/mL
Vincristine conc. 2 :- 0.0012 μg/mL
Vincristine conc. 3 :- 0.0006 μg/mL

Zomepirac conc. 1 :- 37.20 μg/mL
Zomepirac conc. 2 :- 17.20 μg/mL
Zomepirac conc. 3 :- 8.40 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Vincristine 1 control	61.37	18.65
Zomepirac 1 control	78.95	5.67
Vincristine 1 + Zom	1.73*	0.46
Vincristine 2 control	82.09	4.70
Vincristine 2 + Zom	2.90*	0.67
Vincristine 3 control	96.31	6.32
Vincristine 3 + Zom	22.28*	4.80
Vincristine 1 control	66.19	5.86
Zomepirac 2 control	91.60	4.53
Vincristine 1 + Zom	2.03*	0.28
Vincristine 2 control	86.79	6.11
Vincristine 2 + Zom	4.46*	0.48
Vincristine 3 control	95.30	7.73
Vincristine 3 + Zom	33.89*	3.96
Vincristine 1 control	62.79	5.89
Zomepirac 3 control	93.54	6.39
Vincristine 1 + Zom	1.99*	0.39
Vincristine 2 control	81.20	4.47
Vincristine 2 + Zom	4.67*	0.80
Vincristine 3 control	95.75	5.51
Vincristine 3 + Zom	43.63	5.93

Table 3.5.15: Survival of DLKP cells in the presence of various concentrations of vincristine and zomepirac (Zom). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 21), Vincristine and Etodolac

Vincristine conc. 1 :- 0.0015 µg/mL
Vincristine conc. 2 :- 0.0012 µg/mL
Vincristine conc. 3 :- 0.0007 µg/mL

Etodolac conc. 1 :- 15.20 µg/mL
Etodolac conc. 2 :- 7.60 µg/mL
Etodolac conc. 3 :- 3.70 µg/mL

Treatment	**% Cell Survival**	**S.D.**
Cell control	100	
Vincristine 1 control	64.35	13.21
Etodolac 1 control	83.94	5.48
Vincristine 1 + Etod	72.87	11.71
Vincristine 2 control	72.38	11.37
Vincristine 2 + Etod	80.86	9.41
Vincristine 3 control	87.30	8.76
Vincristine 3 + Etod	84.36	8.51
Vincristine 1 control	69.82	7.15
Etodolac 2 control	98.67	8.15
Vincristine 1 + Etod	82.34	8.25
Vincristine 2 control	79.34	6.68
Vincristine 2 + Etod	91.48	10.35
Vincristine 3 control	94.30	8.83
Vincristine 3 + Etod	96.18	7.28
Vincristine 1 control	66.03	5.10
Etodolac 3 control	99.79	6.69
Vincristine 1 + Etod	71.52	4.27
Vincristine 2 control	77.77	7.32
Vincristine 2 + Etod	82.26	6.10
Vincristine 3 control	94.12	8.09
Vincristine 3 + Etod	97.44	4.79

Table 3.5.16: Survival of DLKP cells in the presence of various concentrations of vincristine and etodolac (Etod). Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

3.6 Effect of exogenous prostaglandin addition on NSAID mediated chemotherapeutic drug toxicity enhancement.

The A549 cell line produces a variety of eicosanoids such as PgE_2, $PgF_{2\alpha}$, TxB_2 and 15-HETE (Asano *et al.*, 1996; Claria *et al.*, 1996). It is estimated that PgE_2 and $PgF_{2\alpha}$ can account for approximately 80% of total eicosanoid production by this cell line (Hubbard *et al.*, 1988). NSAIDs are believed to exert their anti-inflammatory, antipyretic and analgesic properties as a result of their inhibition of the prostaglandin H synthase (PGHS) enzyme. This inhibition ultimately results in a reduction in prostanoid synthesis. For this reason an investigation was conducted in order to see if NSAID-mediated inhibition of prostanoid synthesis was involved in the enhancement of drug toxicity effect. This investigation involved co-treatment of cells with cytotoxic drug, NSAID and PgE_2 or PgD_2 using the protocol described in Section 2.7.3. PgD_2 is not known to be produced by A549 under stimulated or unstimulated conditions and so for this reason served as a negative control for these experiments. The results are summarised in Table 3.6.1 with sample data provided in Table 3.6.2 for adriamycin and indomethacin in combination with PgE_2, and Table 3.6.3 for adriamycin and indomethacin in combination with PgD_2. From Table 3.6.2 it can be seen that indomethacin at a non-toxic level enhanced the toxicity of adriamycin (10 ng/mL) from approximately 10% to 66% but simultaneous exogenous addition of 0.60 μg/mL PgE_2 had no significant effects on cell survival. This concentration of PgE_2 is non-toxic and is significantly higher than the levels of PgE_2 produced by A549 even under stimulated conditions. Lowering the concentration of exogenously added PgE_2 in these experiments to more physiologically relevant levels (A549 can produce 150 pg PgE_2/mL under IL-1β stimulated conditions; Croxtall and Flower, 1994) was found to have no effect on the combined toxicity of adriamycin and indomethacin. The fact that the exogenous addition of the eicosanoid (PgE_2) which is predominantly formed by the A549 cell line had no effect on NSAID mediated drug toxicity enhancement, strongly suggested that NSAIDs possessed drug toxicity enhancing abilities independently of any inhibitory effects on the synthesis of these eicosanoids. This was confirmed directly by further studies

detailed in Section 3.7 and 3.8. Related experiments using other NSAIDs or cytotoxic drugs generated similar data (Table 3.6.1).

Drug	NSAID	Eicosanoid	Reversal of toxicity enhancement*
Adriamycin	Indomethacin	PgE_2	(-)
Adriamycin	Sulindac	PgE_2	(-)
Vincristine	Indomethacin	PgE_2	(-)
Vincristine	Sulindac	PgE_2	(-)
Adriamycin	Indomethacin	PgD_2	(-)
Adriamycin	Sulindac	PgD_2	(-)
Vincristine	Indomethacin	PgD_2	(-)
Vincristine	Sulindac	PgD_2	(-)

* Toxicity enhancement is defined as a level of toxicity achieved with the drug plus NSAID combination, which is significantly greater than the level of toxicity predicted by the fractional product method. A (-) in Table 3.6.1 indicates that this toxicity level was unchanged by Pg treatment.

Table 3.6.1: Investigation into the potential for toxicity enhancement effect reversal with eicosanoid (PgE_2 or PgD_2) treatment of A549.

A549 (P 104) : Adriamycin and Indomethacin and PgE_2

Adriamycin conc. :- 0.01 μg/mL

Indomethacin conc. :- 2.37 μg/mL

Prostaglandin E_2 conc. 1 :- 0.60 μg/mL
Prostaglandin E_2 conc. 2 :- 0.06 μg/mL

Treatment	% Cell Survival	S.D.
Cell Control	100	
Adriamycin control	90.41	5.39
Indomethacin control	95.75	9.55
PgE_2 1 control	97.78	7.45
Adriamycin + Indo	33.66	2.74
Adriamycin + PgE_2 1	89.48	7.32
Indo + PgE_2 1	99.89	11.26
Adriamycin + Indo + PgE_2 1	35.67	3.30
Adriamycin control	89.35	10.17
Indomethacin control	101.44	11.54
PgE_2 2 control	103.37	13.93
Adriamycin + Indo	36.34	4.68
Adriamycin + PgE_2 2	89.28	7.83
Indo + PgE_2 2	101.15	11.98
Adriamycin + Indo + PgE_2 2	29.64	4.74

Table 3.6.2: Survival of A549 cells in the presence of adriamycin, indomethacin (Indo) or PgE_2. Survival is represented as a % of the control untreated cells ± S.D. Results are the average of triplicate determinations using the experimental protocol detailed in Section 2.7.3.

A549 (P 100) : Adriamycin and Indomethacin and PgD_2

Adriamycin conc. :- 0.01 μg/mL

Indomethacin conc. :- 2.40 μg/mL

Prostaglandin D_2 conc. 1 :- 0.60 μg/mL

Prostaglandin D_2 conc. 2 :- 0.06 μg/mL

Treatment	**% Cell Survival**	**S.D.**
Cell control	100	
Adriamycin control	70.11	6.16
Indomethacin control	74.76	5.02
PgD_2 conc. 1 control	97.81	3.48
Adriamycin + Indo	27.89	1.89
Adriamycin + PgD_2 1	75.06	4.49
Indo + PgD_2 1	72.15	2.08
Adr + Indo + PgD_2 1	25.39	1.47
Cell control	100	
Adriamycin control	67.83	7.73
Indomethacin control	98.15	6.31
PgD_2 conc. 2 control	100.94	6.56
Adriamycin + Indo	17.79	2.84
Adriamycin + PgD_2 2	70.03	8.57
Indo + PgD_2 2	101.94	5.12
Adr + Indo + PgD_2 2	19.01	2.1

Table 3.6.3: Survival of A549 cells in the presence of adriamycin, indomethacin (Indo) or PgD_2. Survival is represented as a % of the control untreated cells ± S.D. Results are the average of triplicate determinations using the experimental protocol detailed in Section 2.7.3

3.7 *In vitro* toxicity assay screening of non-NSAID inhibitors of the arachidonic acid cascade.

NSAIDs are known inhibitors of prostanoid biosynthesis due to their inhibitory effects on the PGHS enzyme. Investigations were conducted to elucidate if non-NSAID-mediated inhibition of prostanoid synthesis could contribute to the toxicity enhancement effect. This investigation involved using inhibitors of the critical steps of the arachidonic acid cascade. As described in Section 1.3, the most critical step in eicosanoid biosynthesis involves the liberation of arachidonic acid from cell membranes as a result of phospholipase (PL) activity. In the A549 cell line, cytosolic PLA_2 is the most critical PL involved in this process (Croxtall *et al.*, 1996). Inhibition of PLA_2 activity causes inhibition of the synthesis of prostanoids and HETEs and this inhibition can be caused by agents such as 4-bromophenacyl bromide, manoalide and quinacrine. If inhibition of prostanoid synthesis was critical for achievement of chemotherapeutic drug toxicity enhancement, then one would expect that total inhibition of eicosanoid synthesis due to the presence of a PL inhibitor would be sufficient to achieve the toxicity enhancement effect. As can be seen from Table 3.7.1 and Tables 3.7.2, 3.7.5 and 3.7.6, none of the PLA_2 inhibitors studied (4-bromophenacyl bromide, manoalide and quinacrine) had any effects on the toxicity of adriamycin. These compounds were added at non-toxic concentrations rather than at concentrations known to be effective for total PLA_2 inhibition. The specific effects of these PLA_2 inhibitors on PLA_2 activity at the concentrations used in these experiments was not investigated. This was not performed as it was required to add these agents at relatively non-toxic concentrations, which potentially affected cell growth in an equivalent fashion to that achieved in the presence of an NSAID. As expected the PLA_2 inhibitors were highly toxic (quinacrine IC_{50} of 4.0 μg/mL). The concentrations reported in the literature as being effective for total PLA_2 inhibition (quinacrine inhibits macrophage membrane-associated PLA_2 activity by 20% at 400 μg/mL; Lister *et al.*, 1989) were highly toxic for A549 cells . As it was unclear as to the extent of prostanoid synthesis inhibition required for the toxicity enhancement effect to occur, it was decided to use the PLA_2 inhibitors at non-toxic doses. The use of 4-bromophenacyl bromide, manoalide and quinacrine

at these concentrations may be questionable for the purposes of this experiment as the exact extent of PL inhibition was unknown.

NSAIDs are not believed to exert any direct inhibitory effects on the lipoxygenase (LOX) catalysed branch of the AA cascade as described in Section 1.4. Screening was carried out using a 5- and 12-LOX inhibitor, esculetin, in order to see if inhibition of the LOX catalysed branch was sufficient to enhance chemotherapeutic drug toxicity. As can be seen from Table 3.7.4, inhibition of 5-LOX and 12-LOX due to the presence of non-toxic levels of esculetin had no effect on the toxicity of adriamycin in the A549 cell line. This result illustrates that as expected the LOX catalysed branch of the AA cascade was not involved in the NSAID-mediated drug toxicity enhancement effect. As described in Section 1.3.1, PGHS is known to exist in constitutive and inducible forms. NSAIDs vary in their ability to preferentially inhibit either or both of the PGHS-1 and PGHS-2 isoforms. The NSAIDs which had been identified from *in vitro* toxicity screening as possessing the ability to enhance the toxicity of a suitable chemotherapeutic drug in the A549 and DLKP cell lines are known to inhibit both PGHS-1 and PGHS-2 (Frolich, 1997). It was also known that aspirin, an NSAID with negligible effects on PGHS-2, was unable to enhance the toxicity of adriamycin or vincristine. This suggested that preferential inhibition of the PGHS-2 isozyme may have been important in achievement of the drug toxicity enhancement effect by a given NSAID. Dexamethasone has a large variety of cellular effects including inhibition of induction of PGHS-2 mRNA synthesis (Croxtall and Flower, 1994). From Table 3.7.3 it can be seen that this compound had no effects on adriamycin toxicity. This indicates that PGHS isozyme preference of an NSAID may not be critical for the ability of that NSAID to enhance drug toxicity. This result is in no way definitive as dexamethasone has a wide variety of effects on cellular metabolism.

Drug	Inhibitor	Inhibitor description	Toxicity enhancement*
Adriamycin	4-Bromophenacyl bromide	Phospholipase A_2 inhibitor	(-)
Adriamycin	Dexamethasone	PGHS-2 synthesis inhibitor	(-)
Adriamycin	Esculetin	5- and 12-lipoxygenase inhibitor	(-)
Adriamycin	Manoalide	Phospholipase A_2 inhibitor	(-)
Adriamycin	Quinacrine	Phospholipase A_2 inhibitor	(-)

* Toxicity enhancement is scored with a (+) if a level of toxicity is achieved with a drug plus inhibitor combination, which is significantly greater than the level of toxicity predicted by the fractional product method. A (-) indicates no significant toxicity enhancement effects.

Table 3.7.1: Investigation into potential ability of inhibitors of critical steps of the arachidonic acid cascade to enhance the toxicity of adriamycin.

A549 (P102), Adriamycin and 4-Bromophenacyl bromide

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.008 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

4-Bromophenacyl bromide conc. 1 :- 1.25 μg/mL
4-Bromophenacyl bromide conc. 2 :- 0.90 μg/mL
4-Bromophenacyl bromide conc. 3 :- 0.60 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	69.71	5.32
4BrPhAcBr 1 control	83.16	12.79
Adriamycin 1 + 4-BrPhAcBr	53.58	5.26
Adriamycin 2 control	89.79	11.83
Adriamycin 2 + 4-BrPhAcBr	67.39	6.12
Adriamycin 3 control	97.35	12.06
Adriamycin 3 + 4-BrPhAcBr	70.45	9.17
Adriamycin 1 control	59.52	8.11
4BrPhAcBr 2 control	78.88	13.62
Adriamycin 1 + 4-BrPhAcBr	58.90	10.20
Adriamycin 2 control	86.95	12.12
Adriamycin 2 + 4-BrPhAcBr	61.33	12.59
Adriamycin 3 control	95.21	13.24
Adriamycin 3 + 4-BrPhAcBr	67.13	14.05
Adriamycin 1 control	66.21	4.23
4BrPhAcBr 3 control	90.33	7.80
Adriamycin 1 + 4-BrPhAcBr	55.00	6.54
Adriamycin 2 control	90.19	6.13
Adriamycin 2 + 4-BrPhAcBr	86.74	8.04
Adriamycin 3 control	96.00	6.59
Adriamycin 3 + 4-BrPhAcBr	85.27	8.52

Table 3.7.2: Survival of A549 cells in the presence of adriamycin and 4-bromophenacyl bromide (4-BrPhAcBr). Survival is represented as a % of the control untreated cells ± S.D. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P104), Adriamycin and Dexamethasone

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.007 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

Dexamethasone conc. 1 :- 0.020 μg/mL
Dexamethasone conc. 2 :- 0.010 μg/mL
Dexamethasone conc. 3 :- 0.005 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	68.39	5.09
Dexamethasone 1 control	49.58	3.14
Adriamycin 1 + Dex	37.47	6.44
Adriamycin 2 control	86.64	8.57
Adriamycin 2 + Dex	38.69	1.83
Adriamycin 3 control	93.77	11.18
Adriamycin 3 + Dex	44.44	3.09
Adriamycin 1 control	62.90	8.86
Dexamethasone 2 control	48.65	5.26
Adriamycin 1 + Dex	46.39	9.98
Adriamycin 2 control	82.07	12.56
Adriamycin 2 + Dex	36.95	10.23
Adriamycin 3 control	71.06	9.74
Adriamycin 3 + Dex	43.08	2.92
Adriamycin 1 control	67.36	5.29
Dexamethasone 3 control	64.89	4.11
Adriamycin 1 + Dex	36.07	2.37
Adriamycin 2 control	85.91	8.81
Adriamycin 2 + Dex	50.36	2.58
Adriamycin 3 control	95.21	13.78
Adriamycin 3 + Dex	59.86	4.23

Table 3.7.3: Survival of A549 cells in the presence of adriamycin and dexamethasone (Dex). Survival is represented as a % of the control untreated cells ± S.D. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P103), Adriamycin and Esculetin

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.007 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

Esculetin conc. 1 :- 1.90 μg/mL
Esculetin conc. 2 :- 0.95 μg/mL
Esculetin conc. 3 :- 0.50 μg/mL

Treatment	**% Cell Survival**	**S.D.**
Cell control	100	
Adriamycin 1 control	84.78	3.99
Esculetin 1 control	89.01	4.65
Adriamycin 1 + Esc	68.03	3.90
Adriamycin 2 control	93.16	5.58
Adriamycin 2 + Esc	81.97	4.46
Adriamycin 3 control	97.09	5.36
Adriamycin 3 + Esc	89.89	5.91
Adriamycin 1 control	66.11	4.85
Esculetin 2 control	100.90	8.07
Adriamycin 1 + Esc	71.62	3.78
Adriamycin 2 control	88.75	6.33
Adriamycin 2 + Esc	88.77	8.54
Adriamycin 3 control	92.85	4.99
Adriamycin 3 + Esc	92.63	3.86
Adriamycin 1 control	79.47	5.32
Esculetin 3 control	100.05	7.99
Adriamycin 1 + Esc	78.99	3.25
Adriamycin 2 control	90.71	5.84
Adriamycin 2 + Esc	93.61	4.60
Adriamycin 3 control	98.51	5.87
Adriamycin 3 + Esc	97.90	8.22

Table 3.7.4: Survival of A549 cells in the presence of adriamycin and esculetin (Esc). Survival is represented as a % of the control untreated cells ± S.D. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 97) : Adriamycin and Manoalide

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.0075 μg/mL
Adriamycin conc. 3 :- 0.0040 μg/mL

Manoalide conc. 1 :- 0.10 μg/mL
Manoalide conc. 2 :- 0.035 μg/mL
Manoalide conc. 3 :- 0.015 μg/mL

Treatment	**% Cell Survival**	**S.D.**
Cell control	100	
Adriamycin 1 control	71.06	4.82
Mano 1 control	100.79	8.11
Adr 1 + Mano	84.67	5.79
Adr 2 control	83.47	11.85
Adr 2 + Mano	101.93	11.54
Adr 3 control	101.81	10.92
Adr 3 + Mano	100.19	8.23
Adriamycin 1 control	77.33	9.41
Mano 2 control	103.69	9.20
Adr 1 + Mano	76.32	6.65
Adr 2 control	83.36	8.17
Adr 2 + Mano	102.24	10.44
Adr 3 control	104.51	12.35
Adr 3 + Mano	104.00	12.11
Adriamycin 1 control	75.67	10.40
Mano 3 control	97.41	11.69
Adr 1 + Mano	81.23	11.29
Adr 2 control	84.53	8.40
Adr 2 + Mano	96.29	13.05
Adr 3 control	95.64	10.00
Adr 3 + Mano	98.09	10.55

Table 3.7.5: Survival of A549 cells in the presence of adriamycin and manoalide (Mano). Survival is represented as a % of the control untreated cells ± S.D. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P110), Adriamycin and Quinacrine

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.007 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

Quinacrine conc. 1 :- 1.00 μg/mL
Quinacrine conc. 2 :- 0.50 μg/mL
Quinacrine conc. 3 :- 0.30 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	91.06	21.55
Quinacrine 1 control	96.46	20.08
Adriamycin 1 + Quin	82.37	23.38
Adriamycin 2 control	99.23	21.12
Adriamycin 2 + Quin	87.60	23.13
Adriamycin 3 control	100.24	17.09
Adriamycin 3 + Quin	89.74	21.78
Adriamycin 1 control	88.57	9.40
Quinacrine 2 control	106.56	10.20
Adriamycin 1 + Quin	90.25	11.82
Adriamycin 2 control	101.56	14.38
Adriamycin 2 + Quin	97.23	28.08
Adriamycin 3 control	97.41	10.31
Adriamycin 3 + Quin	93.03	8.77
Adriamycin 1 control	85.29	22.37
Quinacrine 3 control	102.30	12.37
Adriamycin 1 + Quin	102.22	19.30
Adriamycin 2 control	106.45	12.41
Adriamycin 2 + Quin	111.55	16.39
Adriamycin 3 control	111.20	15.10
Adriamycin 3 + Quin	123.53	11.48

Table 3.7.6: Survival of A549 cells in the presence of adriamycin and quinacrine (Quin). Survival is represented as a % of the control untreated cells ± S.D. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

3.8 PgE_2 synthesis by A549 cells exposed to chemotherapeutic drugs and NSAIDs

As described in Section 1.4, NSAIDs are believed to exert their therapeutic effects by means of their inhibition of the PGHS enzymes. A study was carried out to investigate if co-treatment of the A549 cell line with a cytotoxic drug and NSAID, such as adriamycin and indomethacin caused any significant alterations in prostanoid production which could contribute to enhancement of drug toxicity. A PgE_2 ELISA assay was used to quantify the PgE_2 production by A549 cells during treatment with cytotoxic drugs and NSAIDs. The experimental protocol used in these experiments is detailed in Section 2.8. In summary, cells were seeded at a density of 2.5 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. Aliquots of media for PgE_2 analysis were removed after 24 hours incubation in the agents of interest. The results of this study are summarised in Table 3.8.1. In the unstimulated state A549 cells produced only approximately 5 pg PgE_2 per 10^5 cells whereas IL-1β stimulated A549 cells produced approximately 304 pg PgE_2 per 10^5 cells. From Table 3.8.1 it can be seen that the unstimulated PgE_2 production by A549 was practically negligible when one corrects for errors and the media blank (media which had not been exposed to cells). The level of PgE_2 synthesis by A549 cells was unchanged from unstimulated PgE_2 levels following treatment with adriamycin (0.5 μg/mL) or Taxol (35.9 ng/mL). These concentrations of drug were optimised for use with the cell densities used in these experiments. At these concentrations, the drugs caused equivalent levels of cell kill to that achieved with the concentrations of drug used in the *in vitro* toxicity assay screening experiments (approximately 30% toxicity after 7 days). The results suggests that, under these conditions, prostanoid production was unaffected by chemotherapeutic drug treatment. In the A549 cell line, adriamycin toxicity was enhanced in the presence of an NSAID such as indomethacin. The toxicity of Taxol was known to be unaffected by NSAID treatment (Section 3.2). No significant difference in PgE_2 synthesis was found between adriamycin treated, Taxol treated or untreated cells, all of which produced negligible levels of PgE_2 (Table 3.8.1). For this reason it was not surprising to find that indomethacin or

flurbiprofen treatment (39.50 μg/mL and 39.90 μg/mL respectively) had no effect on adriamycin treated, Taxol treated or untreated cells.

Treatment	Prostaglandin E_2 production, ($pg/10^5$ cells)
Media blank	3.90 ± 2.00
Media treated cells	5.10± 2.50
Adriamycin (0.46 μg/mL)	5.00 ± 3.30
Indomethacin (39.50 μg/mL)	4.00 ± 4.10
Adriamycin + Indomethacin	3.50 ± 3.80
Flurbiprofen (39.90 μg/mL)	4.30 ± 2.90
Adriamycin + Flurbiprofen	6.50 ± 4.90
Taxol (35.90 ng/mL)	7.20 ± 3.70
Indomethacin (39.50 μg/mL)	3.50 ± 2.80
Taxol + Indomethacin	5.20 ± 4.20
IL-1β (10 ng/mL)	304.00 ± 65.60

Table 3.8.1: PgE_2 production by A549 cells in the presence of cytotoxic drugs and NSAIDs. Cells were seeded at 2.5 x 10^5 cells in 6-well plates and incubated for 24 hours in the compounds of interest. Aliquots of media were then removed and analysed for PgE_2 content as described in Section 2.8. Results are represented as means ± S.D for duplicate determinations carried out on two separate occasions.

3.9 Ability of sulindac sulfide and sulfone to enhance adriamycin and vincristine toxicity in the A549 cell line.

Sulindac sulfide is the active metabolite of sulindac *in vivo* and is a potent inhibitor of PGHS. Sulindac sulfone is an oxidation product of sulindac sulfoxide and is known to be at least 5000-fold less potent than the sulfide for inhibiting cyclooxygenase (Piazza *et al.*, 1997a). These compounds were analysed in *in vitro* toxicity screening assays in combination with adriamycin and vincristine. It was found that these sulindac analogues enhanced the toxicity of adriamycin and vincristine. The results from these experiments have already been provided in Tables 3.3.4 and 3.3.10 for sulindac sulfide and Tables 3.3.5 and 3.3.11 in the case of sulindac sulfone. This immediately suggests that potential ability for inhibition of PGHS activity is not necessary for an NSAID to enhance the toxicity of a suitable chemotherapeutic drug. This is due to the fact that sulindac sulfone, which is devoid of PGHS inhibitory activity, enhances the toxicity of adriamycin and vincristine to the same extent found in the case of the potent PGHS inhibitor sulindac sulfide. From the data it can be seen that sulindac sulfide enhances adriamycin and vincristine toxicity at concentrations equivalent to those used in the case of sulindac (approximately 7 to 1 μg/mL). Sulindac sulfone was found to be effective when used at relatively high concentrations (from 20 to 5 μg/mL). The IC_{50} values for sulindac sulfide and sulfone in A549 are 12 ± 1 and 40 ± 5 μg/mL respectively.

3.10 Effect of drug treatment on PGHS-2 isozyme expression and PgE_2 production in the A549 cell line.

There is evidence to suggest that drug treatment can have an influence on the arachidonic acid cascade (discussed in Section 4.6). Many tumor types produce eicosanoids in elevated levels relative to related normal tissue. These eicosanoids can have a variety of roles in promoting tumor cell survival (Section 1.3.2), but the potential involvement of eicosanoids in promoting cell survival in the presence of chemotherapeutic drugs has not been extensively investigated. It was known from investigations into the variations in PgE_2 levels during NSAID mediated chemotherapeutic drug toxicity enhancement (Section 3.8), that under the experimental conditions examined, cytotoxic drug treatment had no effects on the PgE_2 synthesis by A549. The study detailed in Section 3.8 was relatively limited as it only investigated drug concentrations relevant for the NSAID-mediated drug toxicity enhancement effect. In addition it also only investigated PgE_2 production after a 24 hour period. For this reason a series of experiments were conducted to investigate the effects on the arachidonic acid cascade of treating A549 cells with various concentrations of chemotherapeutic drugs for varying lengths of time. A549 can produce PgE_2 in relatively high levels under suitable conditions. Elevations in PgE_2 synthesis in A549 are attributable to induction of the PGHS-2 isozyme. A549 has not been shown to express significant levels of PGHS-1 mRNA or protein under stimulated (phorbol ester stimulation) or unstimulated conditions, (Asano *et al.*, 1996). One ideally would aim to analyse all eicosanoids produced by A549 when attempting to investigate the effect of chemotherapeutic drug treatment on the arachidonic acid cascade. Due to the obvious analytical difficulties in attempting to simultaneously quantify PgE_2, $PgF_{2\alpha}$, TxB_2 and 15-HETE in cell culture media, studies focused upon PgE_2 production by A549. The protein levels of the PGHS isozyme responsible for the synthesis of PgE_2, namely PGHS-2 were also examined.

Three related experiments were conducted into the effect of drug treatment on PgE_2 synthesis and PGHS-2 protein levels. The first experiment investigated variations in the levels of PGHS-2 protein and PgE_2 production during treatment with 0.35 μg/mL adriamycin for various periods of time. An adriamycin concentration of 0.35 μg/mL adriamycin was shown to be non-toxic over a 24 hour period at the cell density used in these experiments (Section 2.9). If cells were permitted to grow for a period of 7 days in this concentration of drug, it was found that approximately 70% of A549 cells were killed relative to a control non-drug treated cells. The results of these studies are summarised in Table 3.10.1 and Figure 3.10.1. As can be seen from the data, adriamycin treatment (0.35 μg/mL) for 2, 4 or 24 hours caused no significant increases in PgE_2 production relative to control (media treated) cells. No PGHS-2 protein was found in unstimulated media treated cells or in drug treated cells (Figure 3.10.1). A549 cells treated with 3.0 ng/mL IL-1β for 24 hours produced high levels of PgE_2 (approximately 490 $pg/10^6$ cells) and PGHS-2 protein as expected.

Sample Number	Treatment	PgE_2, (pg / 10^6 cells)	PGHS-2 protein levels*
1	Adriamycin for 2 hours	1.20 ± 2.10	(-)
2	Adriamycin for 4 hours	2.30 ± 1.50	(-)
3	Adriamycin for 8 hours	1.20 ± 2.30	(-)
4	Adriamycin for 24 hours	0.20 ± 2.30	(-)
5	3.0 ng/mL IL-1β for 24 hours	490 ± 56.80	(++)
6	Media treated cells	2.20 ± 1.80	(-)

* High level PGHS-2 expression is signified by a (++) and moderate PGHS-2 expression is signified by a (+). No PGHS-2 expression if signified by a (-). For the PgE_2 ELISA it was found that media which had not been exposed to cells contained 6.00 ± 1.20 pg PgE_2 /20 mL media. All PgE_2 data in the above table has been corrected for this amount of PgE_2.

Table 3.10.1: Effect of adriamycin treatment (0.35 μg/mL) on PgE_2 and PGHS-2 protein levels produced by A549. PgE_2 concentrations are represented as the mean ± S.D. for duplicate determinations carried out on two separate occasions.

Figure 3.10.1: PGHS-2 expression in IL-1β control (lane 5), adriamycin (lanes 1-4) and untreated A549 cells (lane 6). Corresponding PgE_2 data for each sample is shown in Table 3.10.1. The details of the incubation times and concentrations of the various agents are provided in Table 3.10.1.

From the results shown with IL-1β in Table 3.10.1 and Figure 3.10.1 it was obvious that PgE_2 synthesis and PGHS-2 protein synthesis induction was readily detectable after a period of 24 hours incubation with a suitable agent. For this reason all subsequent experiments involved analysis after a 24 hour incubation period with the compound of interest. The experiment shown in Table 3.10.1 and Figure 3.10.1 investigated the effect of only one adriamycin concentration on PgE_2 and PGHS-2 levels. For this reason a subsequent experiment was conducted to elucidate if there was any potential concentration-dependent relationship between drug treatment and eicosanoid production by A549. Cells were treated with a variety of adriamycin concentrations for a period of 24 hours. Adriamycin concentrations of 0.25, 0.35 and 0.45 μg/mL were investigated. These drug concentrations would cause 60, 75 and 85% toxicity respectively after a 7 day incubation period in drug. As can be seen from Table 3.10.2 adriamycin treatment at these concentrations had negligible effects on PgE_2 synthesis or PGHS-2 protein levels (Figure 3.10.2). Cells treated with 3 ng/mL IL-1β produced high levels of PgE_2 (approximately 620 pg/10^6 cells) and PGHS-2. Co-treatment of cells with adriamycin and IL-1β was found to have variable effects on the levels of PgE_2 synthesis and PGHS-2 protein synthesis. No relationship was found to exist between drug concentration and ability to vary IL-1β-induced PgE_2 synthesis. The variation in PgE_2 levels found with varying concentrations of adriamycin, in the adriamycin and IL-1β co-treatment regime was found to be more a result of

variation in the PgE_2 synthetic capabilities of A549 cells rather than a result of a significant influence of adriamycin on IL-1β induced PgE_2 synthesis. From Figure 3.10.2 it can be seen that this was the case, as co-treatment of cells with adriamycin and IL-1β had no significant effects on PGHS-2 synthesis relative to IL-1β treated cells.

Sample Number	Treatment	PgE_2, (pg/10^6 cells)	PGHS-2 protein levels*
1	Media treated cells	14.80 ± 10.60	(-)
2	0.25 μg/mL adriamycin	10.00 ± 10.90	(-)
3	0.35 μg/mL adriamycin	9.01 ± 5.30	(-)
4	3 ng/mL IL-1β	620.00 ± 46.90	(++)
5	0.25 μg/mL adriamycin + 3 ng/mL IL-1β	770.00 ± 80.14	(++)
6	0.35 μg/mL adriamycin + 3 ng/mL IL-1β	790.20 ± 99.70	(++)
7	0.45 μg/mL adriamycin + 3 ng/mL IL-1β	527.10 ± 100.78	(++)

* High level PGHS-2 expression is signified by a (++) and moderate PGHS-2 expression is signified by a (+). No PGHS-2 expression if signified by a (-). For the PgE_2 ELISA it was found that media which had not been exposed to cells contained 6.00 ± 1.20 pg PgE_2 /20 mL media. All PgE_2 data in the above table has been corrected for this level of PgE_2.

Table 3.10.2: Effect of adriamycin treatment for 24 hours on PgE_2 and PGHS-2 protein levels produced by A549. PgE_2 concentrations are represented as the mean ± S.D. for duplicate determinations carried out on two separate occasions.

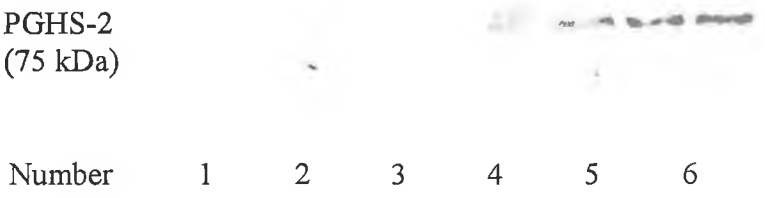

Figure 3.10.2: PGHS-2 expression in A549 cells treated for 24 hours with 3 ng/mL IL-1β (lane 4), media (lane 1), 0.25 and 0.35 μg/mL adriamycin (lanes 2 and 3) or IL-1β and adriamycin (lanes 5-7). Corresponding PgE_2 data for each sample is shown in Table 3.10.2.

A drug from a totally separate mechanistic category of chemotherapeutic drugs, VP-16 (an epipodophyllotoxin) has been shown to be activated to a toxic metabolite as a result of PGHS activity (discussed in Section 4.6). In order to investigate if PGHS activity in A549 was altered in the presence of this cytotoxic drug, the experiment documented in Table 3.10.2 and Figure 3.10.2 was performed using VP-16 in place of adriamycin. Cells were treated with 2.5, 0.5 and 0.1 μg/mL VP-16 which are non-toxic over 24 hours but which would cause 70, 40 and 20% toxicity respectively if growth was permitted to continue for 7 days. The results are represented in Table 3.10.3 and Figure 3.10.3. As can be seen, VP-16 at the three concentrations analysed had no effects on PgE_2 synthesis or PGHS-2 levels. IL-1β treated cells produced approximately 510 pg PgE_2 per 10^6 cells and high levels of PGHS-2. VP-16 had no significant effects on IL-1β induced PgE_2 or PGHS-2 levels. Variations in PgE_2 levels formed during co-incubation with VP-16 and IL-1β can be attributed to non-specific variability in PgE_2 synthesising abilities of A549 cells rather than a specific effect on PgE_2 synthesis.

Sample Number	Treatment	PgE_2, ($pg/10^6$ cells)	PGHS-2 protein levels*
1	2.50 μg/mL VP-16	14.00 ± 10.00	(-)
2	0.50 μg/mL VP-16	12.10 ± 12.30	(-)
3	0.10 μg/mL VP-16	6.70 ± 9.30	(-)
4	3 ng/mL IL-1β	510.30 ± 98.10	(++)
5	2.50 μg/mL VP-16 + 3 ng/mL IL-1β	667.80 ± 85.30	(++)
6	0.50 μg/mL VP-16 + 3 ng/mL IL-1β	603.20 ± 109.80	(++)
7	Media treated cells	11.80 ± 10.70	(-)

* High level PGHS-2 expression is signified by a (++) and moderate PGHS-2 expression is signified by a (+). No PGHS-2 expression if signified by a (-). For the PgE_2 ELISA it was found that media which had not been exposed to cells contained 6.00 ± 1.20 pg PgE_2 /20 mL media. All PgE_2 data in the above table has been corrected for this amount of PgE_2.

Table 3.10.3: Effect of VP-16 treatment for 24 hours on PgE_2 and PGHS-2 protein levels produced by the A549 cell line. PgE_2 concentrations are represented as the mean ± S.D. for duplicate determinations carried out on two separate occasions.

Figure 3.10.3: PGHS-2 expression in A549 cells treated for 24 hours with 3 ng/mL IL-1β (lane 4), 2.50, 0.50 or 0.10 μg/mL VP-16 (lanes 1, 2 and 3), media (lane 7) or VP-16 and IL-1β (lanes 5 and 6). Corresponding PgE_2 data for each sample is shown in Table 3.10.3.

3.11 PGHS-2 isozyme expression and PgE_2 production in DLKP and DLKP derived resistant variants.

The investigations detailed in Section 3.10 focused upon the effect of chemotherapeutic drug treatment on PgE_2 production and PGHS-2 expression in the A549 drug sensitive cell line. There have been isolated reports of drug resistant tumors with altered eicosanoid synthesising abilities (Section 4.7). For this reason, PgE_2 and PGHS-2 were analysed in the case of two drug-selected cell lines DLKP-VP3 and DLKP-VP8. These cell lines are derived from the drug sensitive DLKP cell line, (Heenan, 1994). The VP-16 selected VP3 and VP8 cell lines were chosen for analysis as there is evidence to suggest that PGHS can be involved in the activation of VP-16 to a metabolically active form, (Section 4.7). If this was the case and if a cell developed VP-16 resistance, one would predict that alterations in PGHS levels in resistant cell line relative to the parental sensitive cells could develop as a possible mechanism of desensitising the cell to drug. As in Section 3.10 only PGHS-2 and not PGHS-1 was investigated. Asano *et al.*, (1996) reported that PGHS-2 is the dominant PGHS isoform in unstimulated and stimulated lung tumor cell lines *in vitro*. The levels of PgE_2 synthesis by the DLKP, DLKP-VP3 and DLKP-VP8 cell lines under a variety of conditions was also investigated. This particular eicosanoid was chosen for analysis as it is the most commonly formed eicosanoid in lung cancer cell lines (Hubbard *et al.*, 1988). Figure 3.11.1 shows that neither DLKP, VP-3 or VP-8 express PGHS-2 protein when stimulated with 3 ng/mL IL-1β whereas A549 cells demonstrated high level expression of this protein. DLKP, VP-3 and VP-8 produce negligible levels of PgE_2 under these conditions whereas A549 cells produce approximately 200 $pg/10^6$ cells.

Figure 3.11.1: PGHS -2 expression in IL-1β (3 ng/mL) treated DLKP (lane 1), DLKP-VP3 (lane 2), DLKP-VP8 (lane 3) and A549 (lane 4) cells. Corresponding PgE_2 data for each sample is shown in Table 3.11.1.

Sample Number	Treatment	PgE_2, (pg/10^6 cells)	PGHS-2 protein levels*
1	DLKP (3 ng/mL IL-1β)	4.00 ± 11.00	(-)
2	DLKP-VP3 (3 ng/mL IL-1β)	8.16 ± 4.20	(-)
3	DLKP-VP8 (3 ng/mL IL-1β)	9.80 ± 2.40	(-)
4	A549 (3 ng/mL IL-1β)	205.30 ± 68.10	(++)

* High level COX-2 expression is signified by a (++) and moderate PGHS-2 expression is signified by a (+). No PGHS-2 expression if signified by a (-). For the PgE_2 ELISA it was found that media which had not been exposed to cells contained 6.00 ± 1.20 pg PgE_2 /20 mL media. All PgE_2 data in the above table has been corrected for this amount of PgE_2.

Table 3.11.1: Effect of IL-1β treatment for 24 hours on PgE_2 and PGHS-2 levels in DLKP, DLKP-VP3, DLKP-VP8 and A549. PgE_2 concentrations are represented as the mean ± S.D. for duplicate determinations carried out on two separate occasions.

As no significant levels of PGHS-2 could be detected in DLKP, DLKP-VP3 or DLKP-VP8 after IL-1β treatment it was decided that in subsequent experiments, PgE_2 levels would be analysed before analysing PGHS-2 levels. This was due to the fact that increases in the levels of PgE_2 should correlate with increases in PGHS-2 protein synthesis (as found in Section 3.10). These experiments are detailed in Tables 3.11.2 to 3.11.4. In the first of these experiments DLKP cells were treated with adriamycin, IL-1β or a combination of these agents. It was

found that treatment of DLKP cells with 0.15 or 0.25 μg/mL adriamycin (non-toxic over 24 hours but causing 40% and 60% toxicity respectively if growth was allowed to continue for 7 days) had no effect on PgE_2 levels. Treatment with 3 ng/mL and 5 ng/mL IL-1β also had no effect on PgE_2 levels. Co-treatment with cytotoxic drug and IL-1β had no effect on PgE_2 levels (which were practically negligible). IL-1β treated A549 cells produced approximately 360 pg PgE_2 per 10^6 cells over the same period. This experiment served as a reference for equivalent experiments in the VP-3 and VP-8 drug selected cell lines. The results of these experiments are summarised in Tables 3.11.3 and 3.11.4 for DLKP-VP3 and VP-8 respectively. DLKP-VP3 cells produced negligible levels of PgE_2 in the unstimulated state. Treatment of these cells with adriamycin (14 and 22 μg/mL which cause 40 and 70% toxicity over a 7 day period in this cell line) had no effects on PgE_2 synthesis. In addition no significant levels of PgE_2 were formed after IL-1β treatment or after co-treatment with adriamycin and IL-1β. Similar results were found in the case of the DLKP-VP8 cell line (Table 3.11.4). These results illustrated that DLKP, VP-3 and VP-8 cells do not produce PgE_2 or PGHS-2 protein in the unstimulated, IL-1β stimulated or drug treated state.

Treatment for 24 hours	**PgE_2, ($pg/10^6$ cells)***
Media treated cells	11.60 ± 9.70
0.15 μg/mL adriamycin	11.50 ± 11.70
0.25 μg/mL adriamycin	8.40 ± 11.50
3 ng/mL IL-1β	13.50 ± 12.80
5 ng/mL IL-1β	17.70 ± 13.70
0.25 μg/mL adriamycin + 3 ng/mL IL-1β	17.70 ± 15.00
A549 control (3 ng/mL IL-1β)	362.10 ± 25.10

* For the PgE_2 ELISA it was found that media which had not been exposed to cells contained 6.00 ± 1.20 pg PgE_2 /20 mL media. All PgE_2 data in the above table has been corrected for this amount of PgE_2.

Table 3.11.2: Characterisation of eicosanoid synthesising ability of DLKP in the presence of various concentrations of adriamycin or IL-1β for 24 hours. PgE_2 concentrations are represented as the mean ± S.D. for duplicate determinations carried out on two separate occasions.

Treatment for 24 hours	PgE_2, (pg/10^6 cells)*
Media treated cells	10.20 ± 19.40
14 μg/mL adriamycin	8.50 ± 18.70
22 μg/mL adriamycin	9.20 ± 21.50
3 ng/mL IL-1β	10.20 ± 12.60
5 ng/mL IL-1β	13.70 ± 15.70
14 μg/mL adriamycin + 3 ng/mL IL-1β	12.20 ± 25.00
A549 control (3 ng/mL IL-1β)	430.20 ± 45.90

* For the PgE_2 ELISA it was found that media which had not been exposed to cells contained 6.00 ± 1.20 pg PgE_2 /20 mL media. All PgE_2 data in the above table has been corrected for this amount of PgE_2.

Table 3.11.3: Characterisation of eicosanoid synthesising ability of DLKP-VP3 in the presence of various concentrations of adriamycin or IL-1β for 24 hours. PgE_2 concentrations are represented as the mean ± S.D. for duplicate determinations carried out on two separate occasions.

Treatment for 24 hours	PgE_2, (pg/10^6 cells)*
Media treated cells	9.60 ± 10.10
14 μg/mL adriamycin	4.60 ± 16.30
28 μg/mL adriamycin	8.60 ± 20.40
3 ng/mL IL-1β	11.20 ± 13.20
5 ng/mL IL-1β	8.70 ± 12.30
14 μg/mL adriamycin + 3 ng/mL IL-1β	11.20 ± 15.10
A549 control (3 ng/mL IL-1β)	850.60 ± 82.30

* For the PgE_2 ELISA it was found that media which had not been exposed to cells contained 6.00 ± 1.20 pg PgE_2 /20 mL media. All PgE_2 data in the above table has been corrected for this amount of PgE_2.

Table 3.11.4: Characterisation of eicosanoid synthesising ability of DLKP-VP8 in the presence of various concentrations of adriamycin or IL-1β for 24 hours. PgE_2 concentrations are represented as the mean ± S.D. for duplicate determinations carried out on two separate occasions.

3.12 Indomethacin accumulation in DLKP and DLKP-A.

The NSAID-mediated enhancement of chemotherapeutic drug toxicity had only been shown to occur in the DLKP and not in the Pgp-overexpressing cell line, DLKP-A, (Duffy *et al.*, 1997). An experiment was carried out to investigate the possibility that one of the reasons for the toxicity enhancement effect not occurring in resistant cells was that the NSAID was not entering or being retained within resistant cells such as DLKP-A. The details of the design of this experiment are provided in the Section 2.12, but the experiment essentially consisted of treating cells with [^{14}C]-labelled indomethacin for varying lengths of time and then monitoring the levels of labelled indomethacin retained by the cells. It was found experimentally that there was rapid accumulation of labelled indomethacin by the cells (reaching maximum accumulation by approximately 5 minutes), and that there was no significant difference between the level of labelled indomethacin accumulated by DLKP or DLKP-A.

Incubation time, (min)	DLKP Indomethacin accumulation, (ng/10^6 cells)	DLKP-A Indomethacin accumulation, (ng/10^6 cells)
0	0.0 ± 10.0	0.0 ± 10.0
4	200.0 ± 20.0	210.0 ± 25.0
60	225.0 ± 15.0	220.0 ± 30.0
120	240.0 ± 10.0	240.0 ± 25.0
240	245.0 ± 25.0	240.0 ± 30.0

Table 3.12.1: Time course of [^{14}C]-Indomethacin accumulation in DLKP and DLKP-A cells treated as described in Section 2.12. Data is represented as the mean (± S.D.) for triplicate samples carried out on two separate occasions.

3.13 Investigation into MRP-mediated transport of LtC_4 into IOVs isolated from DLKP in the presence or absence of NSAIDs.

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.*, 1997). 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, (Section 1.5.2.3). This suggested that co-treatment of MRP-expressing cells with an MRP-substrate drug and an NSAID with the ability to interfere with MRP's drug pumping ability may have resulted in increased retention of drug within the cell. This ultimately would then cause an enhancement of cytotoxic drug induced cell kill. One significant problem with this theory was the fact that the toxicity enhancement effect was found to occur in DLKP, but MRP protein 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. Isolation of inside-out vesicles (IOVs) from DLKP was performed in order to isolate the plasma membranes from these cells and specifically target Western blotting analysis to the area in which the MRP molecule may have been localised. In addition, isolation of IOVs allowed investigation of potential glutathione-conjugate transport activity in DLKP via the glutathione conjugate pump, MRP.

The experimental protocol used for isolation of the IOVs from DLKP is detailed in Section 2.10. It was found that MRP was detectable by Western blotting, in IOVs isolated from DLKP (Section 3.17.4). The level of MRP protein in DLKP was significantly lower than the levels found in IOVs isolated from HL60-Adr. This result indicated that DLKP possessed low levels of MRP protein and that this level may have been functionally effective for drug transport out of the cell. Inhibition of the pumping activity due to treatment with a suitable NSAID ultimately resulted in increased drug accumulation levels and an enhancement in chemotherapeutic drug toxicity. Transport assays were performed with [^{3}H]-LtC_4 and the IOVs isolated from DLKP. The glutathione conjugate, LtC_4 was found to

be transported into the vesicles in an ATP dependent manner. This indicated that a glutathione-conjugate pump was active in DLKP. The initial rate of transport of [^{3}H]-LtC_4 into DLKP derived IOVs was 0.8 ± 0.2 pmol/mg protein/minute and in HL60-ADR IOVs was 4.0 ± 1.0 pmol/mg/minute (with maximum LtC_4 accumulation of 2.0 ± 0.3 and 7.0 ± 1.0 pmol/mg protein respectively). All of the above [^{3}H]-LtC_4 transport data expressed above is the mean ± S.D. for duplicate determinations carried out on two separate occasions using different vesicle preparations from a cell line on each occasion. The initial rate of [^{3}H]-LtC_4 transport and maximum accumulation of [^{3}H]-LtC_4 are believed to be dependent on the amount of the relevant transporter in the preparation. From Western blotting results it was demonstrated that MRP protein levels in HL60-ADR IOVs were significantly higher than in DLKP derived IOVs (Section 3.17.4). This strongly suggested that the [^{3}H]-LtC_4 transporter in DLKP was MRP.

The influence of various NSAIDs on MRP activity in HL60-ADR IOVs has been described by Duffy *et al.*, (1997). No data had been previously available for IOVs isolated from the DLKP cell line. From Figure 3.13.3 it can be seen that concentrations of 100 and 50 μM indomethacin inhibit [^{3}H]-LtC_4 transport into HL60-ADR IOVs by 80 and 50% respectively. Naproxen (Figure 3.13.4) did not exert any significant inhibitory effects on MRP activity at 100, 50 or 10 μM concentrations. Similar results were found in the case of DLKP-derived IOVs. Concentrations of 100 and 50 μM indomethacin (Figure 3.13.5) inhibited [^{3}H]-LtC_4 transport into DLKP-derived IOVs by approximately 60 and 35% respectively. Naproxen (Figure 3.13.6) did not exert significant effects on [^{3}H]-LtC_4 transport into DLKP-derived IOVs at 100, 50 or 10 μM. These results illustrated that an NSAID such as indomethacin may act as a potent inhibitor of MRP-mediated drug efflux activity in DLKP and so ultimately cause an enhancement in chemotherapeutic drug induced cell kill. NSAIDs such as naproxen, do not exert any significant effects on MRP transport activity and so do not influence chemotherapeutic drug toxicity.

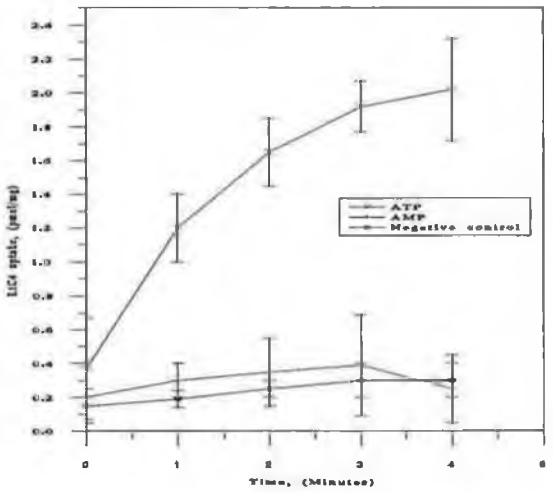

Figure 3.13.1: Time course of LtC_4 transport into IOVs isolated from the DLKP cell line over time in the presence of ATP, AMP or in the absence of ATP and AMP (negative control). Data points represent the means (± S.D.) of duplicate determinations.

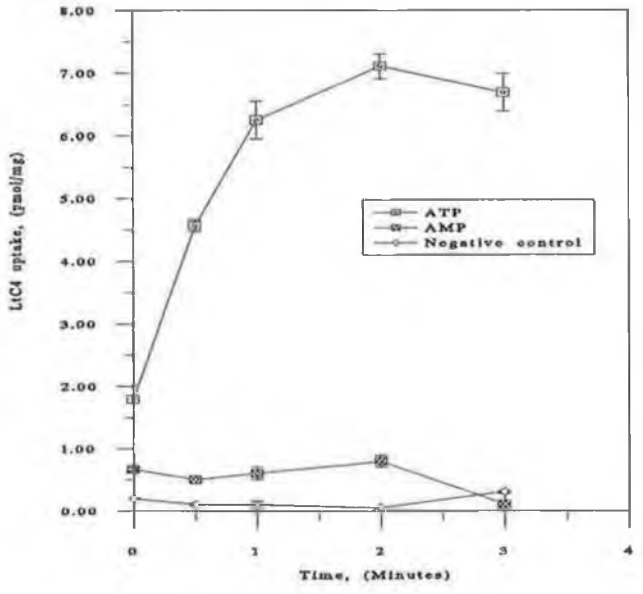

Figure 3.13.2: Time course of transport of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or in the absence of ATP and AMP (negative control). Data points represent the means (± S.D.) of duplicate determinations.

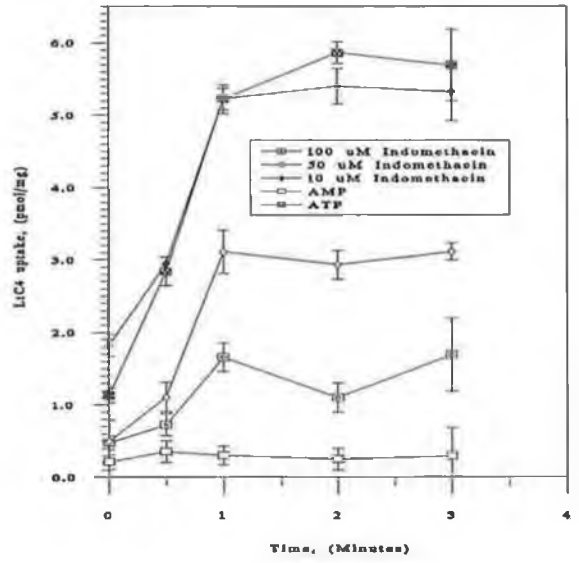

Figure 3.13.3: Inhibition of ATP dependent transport of LtC_4 into IOVs isolated from the HL60-ADR cell line caused due to the presence of ATP and 100, 50 or 10 μM indomethacin. Data points represent the means (± S.D.) of duplicate determinations.

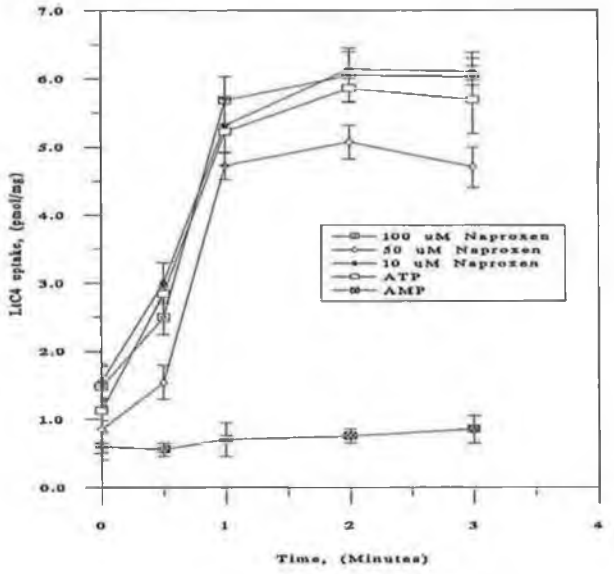

Figure 3.13.4: Inhibition of ATP dependent transport of LtC_4 into IOVs isolated from the HL60-ADR cell line caused due to the presence of ATP and 100, 50 and 10 μM naproxen. Data points represent the means (± S.D.) of duplicate determinations.

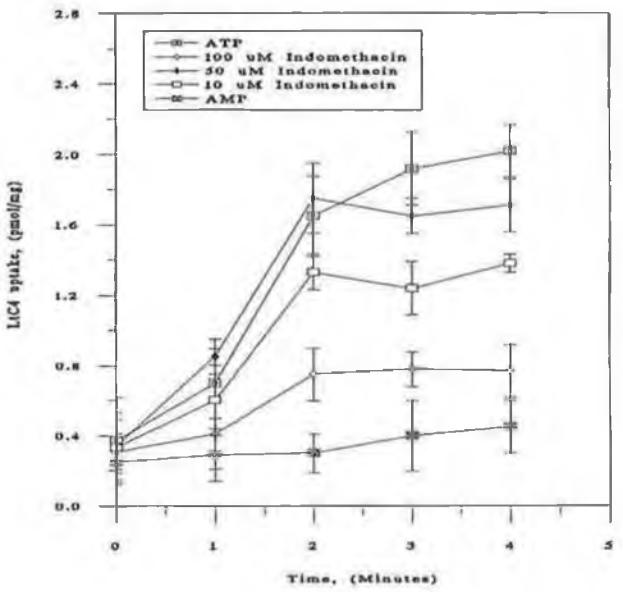

Figure 3.13.5: Inhibition of ATP dependent transport of LtC_4 into IOVs isolated from the DLKP cell line caused due to the presence of ATP and 100, 50 or 10 μM indomethacin. Data points represent the means (± S.D.) of duplicate determinations.

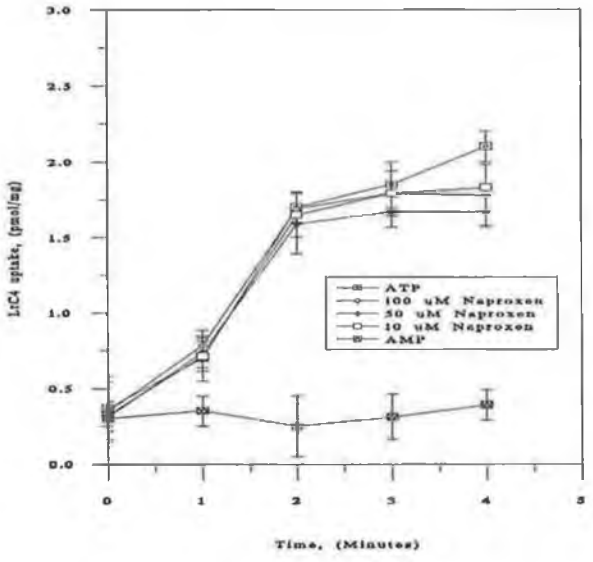

Figure 3.13.6: Inhibition of ATP dependent transport of LtC_4 into IOVs isolated from the DLKP cell line caused due to the presence of ATP and 100, 50 or 10 μM naproxen. Data points represent the means (± S.D.) of duplicate determinations.

3.14 Verification of the identity of the LtC_4 transporter in IOVs isolated from the DLKP cell line.

Western blot analysis of the proteins present in IOVs isolated from DLKP, demonstrated that MRP protein was present in the DLKP cell line (Section 3.17.4). From the data presented in Section 3.13, it can be seen that DLKP derived IOVs demonstrate ATP-dependent transport of the glutathione-conjugate, [^{3}H]-LtC_4. This characteristic is typical of MRP-expressing cell lines such as HL60-ADR. These results suggested that DLKP contains low levels of MRP in the plasma membrane which is isolated following purification of plasma membrane derived-IOVs. In addition, the results indicated that this glutathione conjugate pump transported [^{3}H]-LtC_4 in an ATP dependent fashion. The presence of MRP in DLKP derived IOVs was obvious from Western blotting results, but the specific identity of the [^{3}H]-LtC_4 transporter in DLKP IOVs required further investigation. This transporter could potentially be any of a variety of MRP isoforms or alternatively an unrelated LtC_4 transporter. For this reason a number of experiments were conducted to verify the identity of the LtC_4 transporter in DLKP derived IOVs.

3.14.1 Enhancement of drug toxicity in the presence of inhibitors of MRP activity in the DLKP cell line.

All cell lines with which NSAID mediated chemotherapeutic drug toxicity enhancement have been demonstrated have been shown to express MRP protein (Duffy *et al.*, 1997). The DLKP cell line possesses significant levels of MRP protein in the plasma membrane. Evidence suggests that the NSAID mediated toxicity enhancement effect is achieved by NSAIDs interfering with MRP-catalysed transport of cytotoxic drugs (Figures 3.13.5 and 3.13.6). These cytotoxic drugs, under normal conditions may serve as substrates for transport by MRP out of the cell. If the [^{3}H]-LtC_4 transporter in DLKP IOVs was MRP then one would predict that an agent which strongly interferes with [^{3}H]-LtC_4 transport activity would also interfere with MRP activity. Interference with MRP

activity should result in the enhancement of the toxicity of a suitable drug in the DLKP cell line. The leukotriene receptor antagonist, MK571 is a potent inhibitor of [^{3}H]-LtC_4 transport by MRP (Section 3.16) to the interior of IOVs. If the [^{3}H]-LtC_4 transporter in DLKP IOVs was MRP, one would predict that MK571 should interfere with MRP activity to an extent comparable to that found in the case of a suitable NSAID. This should ultimately result in an enhancement of toxicity of a suitable cytotoxic drug.

From the data presented in Tables 3.14.1.1 and 3.14.1.2 it can be seen that MK571 enhances the toxicity of adriamycin and VP-16 in the DLKP cell line. This effect was found to occur over a range of MK571 concentrations. The extent of adriamycin and VP-16 toxicity enhancement is comparable to that achieved with NSAIDs such as indomethacin (Tables 3.2.2 and 3.2.10). The concentration of MK571 required for achievement of the toxicity enhancement effect is comparable to that required for NSAIDs. For example, indomethacin is a potent enhancer of adriamycin toxicity when used at 2.5 μg/mL or 7 μM (Table 3.5.4) and MK571 potently enhances adriamycin toxicity even at 5 μg/mL or 9 μM. MK571 did not enhance the toxicity of Taxol (Table 3.14.1.3). Taxol is not believed to be readily transported by MRP and so inhibition of MRP by MK571 should have little effect on Taxol efflux from cells. MK571 also had comparable effects on adriamycin and VP-16 toxicity in a second MRP expressing cell line, A549 (Tables 3.14.1.4 and 3.14.1.5). No MRP negative cell line was analysed in order to see if the effects of MK571 on drug toxicity were isolated to MRP expressing cell lines.

The bisindolylmaleimide, GF109203X is believed to be a selective inhibitor of protein kinase C. This compound has been shown to interfere with MRP activity (Gekeler *et al.*, 1995b). This compound is relatively non-specific as regards interference with drug efflux activity as it has been shown to have effects on Pgp as well as MRP activity, (Gekeler *et al.*, 1995b). GF109203X was shown to enhance the toxicity of adriamycin and VP-16 (Tables 3.14.1.6 and 3.14.1.7). This compound also enhanced the toxicity of the non-MRP substrate drug Taxol (Table 3.14.1.8). This indicates that as expected this compound was not selective for MRP activity but due to interference in protein kinase C activity, had a range

of cellular effects which resulted in enhancement of the toxicity of a variety of MRP and Pgp substrate drugs. The results obtained using MK571 strongly indicated that the [^{3}H]-LtC_4 transporter in DLKP was MRP. In order to obtain more direct evidence of the identity of [^{3}H]-LtC_4 transporter in DLKP, this transport was characterised directly in order to identify MRP like characteristics. The results from these investigations are provided in Section 3.14.2.

DLKP (P 27), Adriamycin and MK571

Adriamycin conc. 1 :- 0.010 μg/mL
Adriamycin conc. 2 :- 0.005 μg/mL
Adriamycin conc. 3 :- 0.003 μg/mL

MK751 conc. 1 :- 15.10 μg/mL
MK751 conc. 2 :- 8.40 μg/mL
MK751 conc. 3 :- 5.00 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	67.17	5.81
MK571 conc. 1 control	38.89	3.01
Adriamycin 1 + MK571	15.78*	1.72
Adriamycin 2 control	102.70	10.97
Adriamycin 2 + MK571	37.37	3.95
Adriamycin 3 control	105.32	9.80
Adriamycin 3 + MK571	45.98	5.95
Adriamycin 1 control	82.35	7.86
MK571 conc. 2 control	100.62	14.84
Adriamycin 1 + MK571	26.84*	4.67
Adriamycin 2 control	99.27	9.06
Adriamycin 2 + MK571	88.90	13.24
Adriamycin 3 control	104.60	12.12
Adriamycin 3 + MK571	101.47	9.01
Adriamycin 1 control	80.21	8.23
MK571 conc. 3 control	99.24	12.18
Adriamycin 1 + MK571	32.98*	5.08
Adriamycin 2 control	95.42	8.84
Adriamycin 2 + MK571	96.36	11.36
Adriamycin 3 control	104.16	14.71
Adriamycin 3 + MK571	100.39	12.64

Table 3.14.1.1: Survival of the DLKP cell line in the presence of various concentrations of adriamycin and MK571. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 27), VP-16 and MK571

VP-16 conc. 1 :- 0.13 μg/mL
VP-16 conc. 2 :- 0.07 μg/mL
VP-16 conc. 3 :- 0.03 μg/mL

MK751 conc. 1 :- 15.10 μg/mL
MK751 conc. 2 :- 8.40 μg/mL
MK751 conc. 3 :- 5.00 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
VP-16 1 control	45.81	3.70
MK571 conc. 1 control	36.67	3.23
VP-16 1 + MK571	8.16*	0.85
VP-16 2 control	89.76	8.93
VP16 2 + MK571	21.65*	1.83
VP-16 3 control	99.76	14.26
VP-16 3 + MK571	33.25	2.90
VP-16 1 control	49.97	4.32
MK571 conc. 2 control	103.75	10.43
VP-16 1 + MK571	7.08*	2.23
VP-16 2 control	98.76	18.71
VP16 2 + MK571	45.08*	4.23
VP-16 3 control	102.21	15.48
VP-16 3 + MK571	85.53	19.35
VP-16 1 control	44.82	4.88
MK571 conc. 3 control	95.71	20.71
VP-16 1 + MK571	6.62*	2.15
VP-16 2 control	93.47	20.48
VP16 2 + MK571	51.04*	8.87
VP-16 3 control	101.90	17.60
VP-16 3 + MK571	85.72	22.39

Table 3.14.1.2: Survival of the DLKP cell line in the presence of various concentrations of VP-16 and MK571. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 30), Taxol and MK571

Taxol conc. 1 :- 1.20 ng/mL
Taxol conc. 2 :- 0.60 ng/mL
Taxol conc. 3 :- 0.30 ng/mL

MK751 conc. 1 :- 15.10 µg/mL
MK751 conc. 2 :- 8.00 µg/mL
MK751 conc. 3 :- 5.00 µg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Taxol 1 control	54.70	5.00
MK571 conc. 1 control	37.64	4.11
Taxol 1 + MK571	18.74	1.99
Taxol 2 control	44.93	3.32
Taxol 2 + MK571	18.70	2.63
Taxol 3 control	95.60	16.43
Taxol 3 + MK571	35.66	8.05
Taxol 1 control	54.81	6.15
MK571 conc. 2 control	89.51	12.55
Taxol 1 + MK571	40.26	8.50
Taxol 2 control	44.22	2.91
Taxol 2 + MK571	40.81	3.72
Taxol 3 control	95.71	16.01
Taxol 3 + MK571	85.52	15.73
Taxol 1 control	56.50	5.07
MK571 conc. 3 control	98.27	12.78
Taxol 1 + MK571	56.92	6.15
Taxol 2 control	43.60	3.85
Taxol 2 + MK571	47.45	4.36
Taxol 3 control	96.36	12.54
Taxol 3 + MK571	91.37	6.58

Table 3.14.1.3: Survival of the DLKP cell line in the presence of various concentrations of Taxol and MK571. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 98), Adriamycin and MK571

Adriamycin conc. 1 :- 0.010 µg/mL
Adriamycin conc. 2 :- 0.005 µg/mL
Adriamycin conc. 3 :- 0.003 µg/mL

MK751 conc. 1 :- 15.10 µg/mL
MK751 conc. 2 :- 8.40 µg/mL
MK751 conc. 3 :- 5.00 µg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	62.83	8.85
MK571 conc. 1 control	16.63	1.57
Adriamycin 1 + MK571	12.35	2.60
Adriamycin 2 control	86.11	7.04
Adriamycin 2 + MK571	14.73	2.76
Adriamycin 3 control	95.95	5.70
Adriamycin 3 + MK571	15.16	3.08
Adriamycin 1 control	69.98	8.65
MK571 conc. 2 control	79.75	4.68
Adriamycin 1 + MK571	22.18*	6.80
Adriamycin 2 control	87.17	10.06
Adriamycin 2 + MK571	50.22	16.80
Adriamycin 3 control	94.94	8.42
Adriamycin 3 + MK571	64.72	24.99
Adriamycin 1 control	57.95	14.11
MK571 conc. 3 control	93.36	9.79
Adriamycin 1 + MK571	25.86*	3.41
Adriamycin 2 control	81.80	7.71
Adriamycin 2 + MK571	61.73	14.86
Adriamycin 3 control	85.92	8.71
Adriamycin 3 + MK571	77.48	20.87

Table 3.14.1.4: Survival of the A549 cell line in the presence of various concentrations of adriamycin and MK571. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

A549 (P 98), VP-16 and MK571

VP-16 conc. 1 :- 0.13 μg/mL
VP-16 conc. 2 :- 0.07 μg/mL
VP-16 conc. 3 :- 0.03 μg/mL

MK751 conc. 1 :- 15.10 μg/mL
MK751 conc. 2 :- 8.40 μg/mL
MK751 conc. 3 :- 5.00 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
VP-16 1 control	54.55	6.81
MK571 conc. 1 control	12.42	2.03
VP-16 1 + MK571	11.59	1.67
VP-16 2 control	66.99	10.46
VP16 2 + MK571	12.58	2.56
VP-16 3 control	87.08	10.88
VP-16 3 + MK571	13.15	1.74
VP-16 1 control	51.07	7.56
MK571 conc. 2 control	78.35	9.56
VP-16 1 + MK571	22.72*	2.31
VP-16 2 control	81.27	8.91
VP16 2 + MK571	32.93*	3.92
VP-16 3 control	92.47	8.91
VP-16 3 + MK571	57.98	7.84
VP-16 1 control	66.87	4.81
MK571 conc. 3 control	93.59	7.88
VP-16 1 + MK571	29.67*	5.03
VP-16 2 control	54.05	4.51
VP16 2 + MK571	52.74	5.09
VP-16 3 control	68.15	6.12
VP-16 3 + MK571	70.88	6.21

Table 3.14.1.5: Survival of the A549 cell line in the presence of various concentrations of VP-16 and MK571. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 32), Adriamycin and GF109203X

Adriamycin conc. 1 :- 0.015 μg/mL
Adriamycin conc. 2 :- 0.008 μg/mL
Adriamycin conc. 3 :- 0.004 μg/mL

GF109203X conc. 1 :- 4.00 μg/mL
GF109203X conc. 2 :- 2.20 μg/mL
GF109203X conc. 3 :- 0.90 μg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Adriamycin 1 control	48.66	5.75
GF109203X conc. 1 control	101.86	15.27
Adriamycin 1 + GF109203X	8.07*	0.99
Adriamycin 2 control	98.01	15.67
Adriamycin 2 + GF109203X	69.51*	7.04
Adriamycin 3 control	101.32	12.46
Adriamycin 3 + GF109203X	99.51	15.95
Adriamycin 1 control	57.14	7.03
GF109203X conc. 2 control	101.07	9.59
Adriamycin 1 + GF109203X	10.70*	1.52
Adriamycin 2 control	101.60	12.91
Adriamycin 2 + GF109203X	96.00	12.53
Adriamycin 3 control	108.86	15.16
Adriamycin 3 + GF109203X	96.61	12.81
Adriamycin 1 control	70.08	7.97
GF109203X conc. 3 control	99.68	14.12
Adriamycin 1 + GF109203X	22.12*	2.56
Adriamycin 2 control	97.43	16.00
Adriamycin 2 + GF109203X	99.16	16.14
Adriamycin 3 control	103.84	16.38
Adriamycin 3 + GF109203X	101.33	16.28

Table 3.14.1.6: Survival of the DLKP cell line in the presence of various concentrations of adriamycin and GF109203X. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 27), VP-16 and GF109203X

VP-16 conc. 1 :- 0.13 mg/mL
VP-16 conc. 2 :- 0.07 mg/mL
VP-16 conc. 3 :- 0.03 mg/mL

GF109203X conc. 1 :- 4.00 mg/mL
GF109203X conc. 2 :- 2.20 mg/mL
GF109203X conc. 3 :- 0.90 mg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
VP-16 1 control	84.00	12.62
GF109203X conc. 1 control	91.88	8.44
VP-16 1 + GF109203X	30.28*	3.26
VP-16 2 control	94.44	9.05
VP-16 2 + GF109203X	63.96*	6.37
VP-16 3 control	98.93	9.41
VP-16 3 + GF109203X	80.85	11.86
VP-16 1 control	87.24	7.63
GF109203X conc. 2 control	108.42	10.34
VP-16 1 + GF109203X	39.96*	3.36
VP-16 2 control	99.41	10.74
VP-16 2 + GF109203X	78.40	7.69
VP-16 3 control	105.48	12.33
VP-16 3 + GF109203X	103.34	12.95
VP-16 1 control	79.33	10.04
GF109203X conc. 3 control	109.71	10.76
VP-16 1 + GF109203X	59.73*	5.54
VP-16 2 control	94.84	9.13
VP-16 2 + GF109203X	94.54	9.10
VP-16 3 control	102.92	12.50
VP-16 3 + GF109203X	103.59	10.11

Table 3.14.1.7: Survival of the DLKP cell line in the presence of various concentrations of VP-16 and GF109203X. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

DLKP (P 32), Taxol and GF109203X

Taxol conc. 1 :- 1.20 ng/mL
Taxol conc. 2 :- 0.60 ng/mL
Taxol conc. 3 :- 0.30 ng/mL

GF109203X conc. 1 :- 4.00 mg/mL
GF109203X conc. 2 :- 2.20 mg/mL
GF109203X conc. 3 :- 0.90 mg/mL

Treatment	% Cell Survival	S.D.
Cell control	100	
Taxol 1 control	90.97	10.74
GF109203X conc. 1 control	97.19	11.69
Taxol 1 + GF109203X	48.33*	5.34
Taxol 2 control	99.98	12.05
Taxol 2 + GF109203X	93.96	7.56
Taxol 3 control	102.36	13.34
Taxol 3 + GF109203X	94.00	8.26
Taxol 1 control	90.47	10.16
GF109203X conc. 2 control	95.35	8.63
Taxol 1 + GF109203X	85.00	12.16
Taxol 2 control	93.81	9.70
Taxol 2 + GF109203X	98.01	10.88
Taxol 3 control	99.14	10.94
Taxol 3 + GF109203X	97.18	15.06
Taxol 1 control	100.08	17.94
GF109203X conc. 3 control	100.32	18.55
Taxol 1 + GF109203X	79.72	11.16
Taxol 2 control	96.54	15.16
Taxol 2 + GF109203X	102.23	14.48
Taxol 3 control	105.17	19.36
Taxol 3 + GF109203X	102.20	17.87

Table 3.14.1.8: Survival of the DLKP cell line in the presence of various concentrations of Taxol and GF109203X. Survival is represented as a % of control untreated cells ± S.D. Statistically significant results are indicated with an asterisk. Results are the average of triplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

3.14.2 Quantification of V_{max} and K_m parameters for LtC_4 transport in the DLKP and HL60-ADR cell lines.

The characteristic V_{max} and K_m values for [^{3}H]-LtC_4 transport have been reported for a variety of MRP-expressing cell lines. In MRP-transfected HeLa cells, K_m and V_{max} for [^{3}H]-LtC_4 transport have been shown to be 105 nM and 530 pmol/mg/min respectively, (Loe *et al.*, 1996b). Experiments were conducted in order to use Lineweaver and Burk analysis to quantify these [^{3}H]-LtC_4 transport parameters in the DLKP and HL60-ADR cell lines. The protocol for these experiments and procedure used for calculation of kinetic parameters are detailed in Section 2.11.1 . As can be seen from Figure 3.14.2.1, transport of LtC_4 was concentration dependent and saturatable at high concentrations. Regression analysis performed for double reciprocal plots of $1/V_{initial}$ ($V_{initial}$ being initial LtC_4 transport rate in pmol/mg/min) versus 1/S (S being substrate concentration) allowed more accurate determination of the V_{max} and K_m values (Y axis intercept of $1/V_{max}$ and X axis intercept of $-1/K_m$). From Figure 3.14.2.2, the regression equation for LtC_4 transport by DLKP was found to be $y = 4013.83x + 3.14x10^{10}$ with a regression coefficient of 0.99. This is reflected in a V_{max} value of 32 pmol/mg/min and K_m of 127 nM for LtC_4 transport in the DLKP cell line. When values were averaged for two independent experiments with a given IOV preparation, V_{max} and K_m values of 30 ± 2 pmol/mg/min and 114 ± 13 nM were obtained. Similar analysis was performed with HL60-ADR derived IOVs (Figures 3.14.2.3 and 3.14.2.4). The regression analysis of double reciprocal plots for this cell line yielded an equation of $y = 472.16x + 8.08x10^9$ with a regression coefficient of 0.99. This predicts V_{max} and K_m values for HL60-ADR of 123 pmol/mg/min and 58 nM respectively. When values were averaged for two independent experiments with a given IOV preparation, V_{max} and K_m values of 211 ± 89 pmol/mg/min and 84 ± 26 nM were obtained. The V_{max} value is particularly dependent on the quantity of MRP in the IOV preparation. DLKP and HL60-ADR transport LtC_4 with a V_{max} of 30 ± 2 and 211 ± 89 pmol/mg/min respectively. From Western blotting experiments conducted with samples of IOVs from these cell lines (Figure 3.17.4.1), it was obvious that HL60-ADR derived

IOVs contained very high levels of MRP relative to DLKP derived IOVs. The V_{max} value was shown to be approximately 7 fold higher in HL60-ADR than in DLKP. This indicated that the LtC_4 transporter in DLKP may be MRP. The K_m values for DLKP and HL60-ADR are 114 ± 13 nM and 84 ± 26 nM respectively. As described above, there is significant variability in the K_m values quoted in the literature for MRP mediated LtC_4 transport. These values can vary from 35 nM (Paul *et al.*, 1996c) to 120 nM (Pulaski *et al.*, 1996). The K_m values found for LtC_4 transport in DLKP and HL60-ADR derived IOVs show significant agreement with these values. This data is presented as the mean ± S.D. of duplicate determinations carried out with a given vesicle preparation on two separate occasions. It was found that it was not possible to obtain consistently repeatable results for K_m and V_{max} using different vesicle preparations from a given cell line. In these experiments it was found that a 3-fold variation in the values of K_m and V_{max} could be found experimentally depending on the specific preparation of IOVs from a given cell line used. This was primarily a result of the intrinsic experimental difficulties experienced in determining accurate transport parameters while working with relatively concentrated lipid solutions. In addition, the variation in the purity of separate IOV preparations also significantly influenced the accurate determination of kinetic parameters. For this reason, the values for K_m and V_{max} described above for DLKP and HL60-ADR IOVs may only be regarded as being specific only for the actual IOV preparations analysed. The implications of these results are discussed in Section 4.9.2.

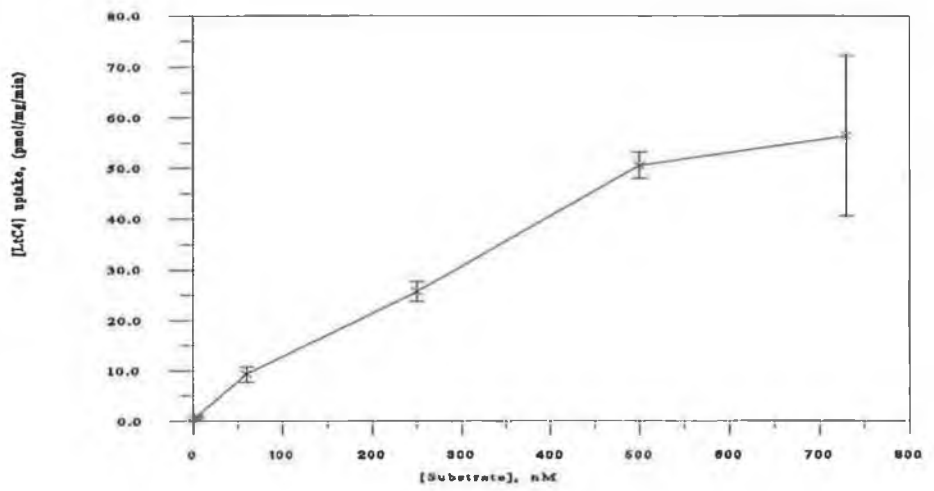

Figure 3.14.2.1: Effect of LtC_4 concentration on initial rate of uptake of [^{3}H]-LtC_4 into DLKP IOVs. Initial rate of uptake was determined after an incubation time of 1 minute at 37°C over the range of substrate (LtC_4) concentrations illustrated above. Data points represent the means (± S.D) of duplicate determinations in a single experiment.

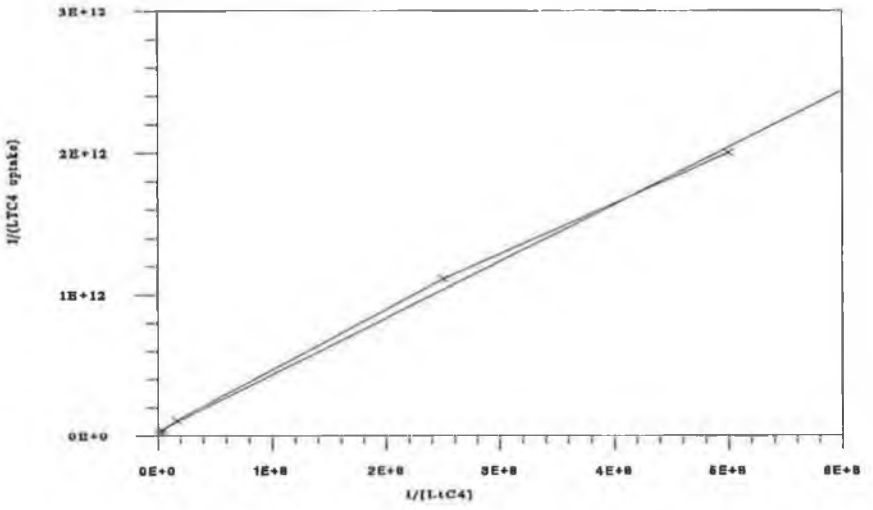

Figure 3.14.2.2: Double reciprocal plot used for Lineweaver-Burk analysis of [^{3}H]-LtC_4 uptake in DLKP IOVs and determination of K_m and V_{max} transport parameters. Data points represent the means of duplicate determinations in a single experiment only.

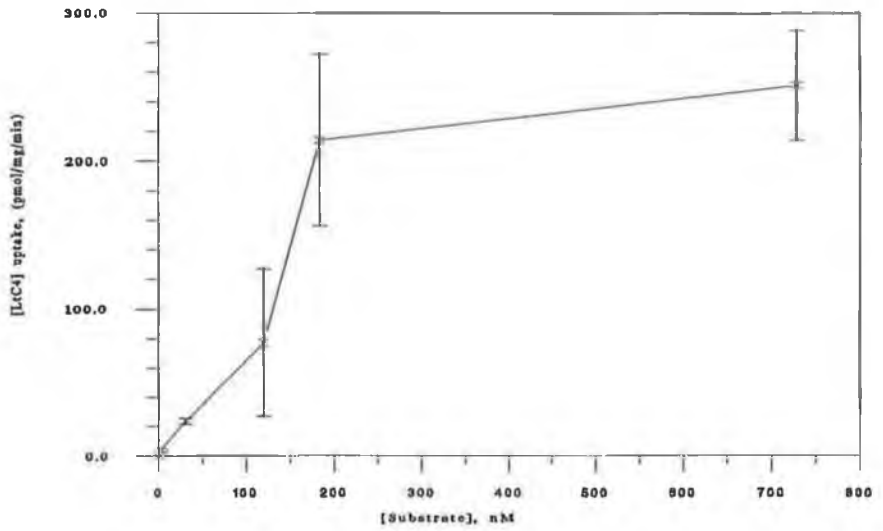

Figure 3.14.2.3: Effect of LtC_4 concentration on initial rate of uptake of [^{3}H]-LtC_4 into HL60-ADR IOVs. Initial rate of uptake was determined after an incubation time of 1 minute at 37°C over the range of substrate (LtC_4) concentrations illustrated above. Data points represent the means (± S.D) of duplicate determinations in a single experiment.

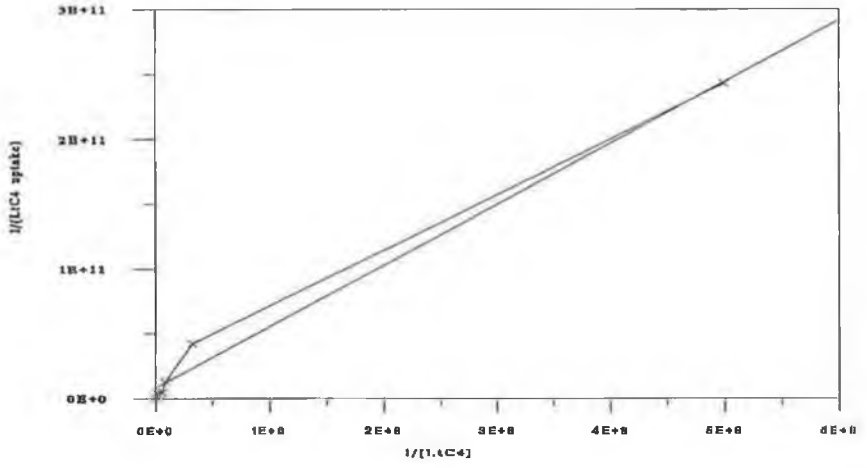

Figure 3.14.2.4: Double reciprocal plot used for Lineweaver-Burk analysis of [^{3}H]-LtC_4 uptake in HL60-ADR IOVs and determination of K_m and V_{max} transport parameters. Data points represent the means of duplicate determinations in a single experiment only.

3.15: Transport of cytotoxic drugs by MRP in HL60-ADR IOVs

The glutathione conjugate pump, MRP is believed to play a role in drug transport but there has been little evidence of direct transport of the drugs to which this transporter is believed to confer resistance. Paul *et al.*, (1996c) reported the MRP-mediated transport of [^{3}H]-labelled, unconjugated, natural product drugs such as VP-16, vincristine and daunorubicin but not of Taxol into IOVs isolated from MRP-transfected 3T3 fibroblasts. Stride *et al.*, (1997) demonstrated the transport into vesicles derived from MRP-transfected cells of [^{3}H]-vincristine in the presence of glutathione. Loe *et al.*, (1996b) also reported the transport of [^{3}H]-labelled vincristine into IOVs from MRP-transfected cells in the presence of glutathione. This group also reported that colchicine, adriamycin, daunorubicin, Taxol, vincristine, vinblastine and VP-16 were poor inhibitors of [^{3}H]-LtC_4 transport in MRP-containing IOVs. The inhibition of [^{3}H]-LtC_4 transport by vincristine, vinblastine and Taxol was enhanced, particularly in the case of vincristine and vinblastine, in the presence of reduced glutathione (GSH). This was suggested to be a result co-transport of GSH and a suitable MRP substrate drug. The majority of studies in the area of drug transport by MRP are carried out using MRP-transfected cell lines rather than drug selected MRP-overexpressing cell lines. For this reason, a number of related studies were conducted using the MRP-overexpressing drug selected HL60-ADR cell line. The initial study investigated if cytotoxic drugs which have been proposed as being substrates for MRP, could be transported by MRP in HL60-ADR IOVs. The effect of GSH on this transport was then examined.

3.15.1 Cytotoxic drug mediated inhibition of MRP activity.

It is practically impossible to conclusively define the cytotoxic drug substrate menu for MRP transport (Section 1.5.2.3). It is generally believed that MRP has the ability to transport drugs including adriamycin, vincristine, VP-16, daunorubicin and epirubicin. MRP-transfected or drug selected MRP-overexpressing cell lines often show low level resistance to Taxol (under 2-fold relative to the parental cells) but are generally sensitive to cisplatin. This suggests that MRP does not have the ability to transport cisplatin but may have low levels of Taxol transport ability. If a cytotoxic drug could be transported by MRP, one would predict that when added at the appropriate concentration, these compounds would compete for MRP-mediated transport to the interior of IOVs. The ability of vincristine, adriamycin, Taxol and cisplatin to inhibit transport of [^{3}H]-LtC_4 by MRP was investigated for this reason. These experiments involved co-incubating IOVs with [^{3}H]-LtC_4 and a suitable concentration of cytotoxic drug. In all cases, efforts were made to maintain the final concentration of solvent containing the drug under 0.5%, a concentration above which interference in [^{3}H]-LtC_4 transport due to presence of solvent could occur. From Figure 3.15.1.1 it can be seen that 106 μM and 211 μM vincristine inhibited the steady state, ATP dependent [^{3}H]-LtC_4 transport to the interior of HL60-ADR IOVs by approximately 50% relative to control IOVs incubated in ATP only. A concentration of 27 μM vincristine inhibited this transport by under 15%. This was as expected as one would assume that an MRP substrate drug should interfere with MRP activity when added at an appropriate cellular concentration. Figure 3.15.1.2 illustrates that surprisingly only at 250 μM adriamycin was significant inhibition of [^{3}H]-LtC_4 transport achieved (approximately 20% of control). Cisplatin was completely ineffective at inhibiting [^{3}H]-LtC_4 transport into IOVs (Figure 3.15.1.4). As expected, Taxol (Figure 3.15.1.3) was not found to inhibit [^{3}H]-LtC_4 transport at 100, 50 or 1 μM.

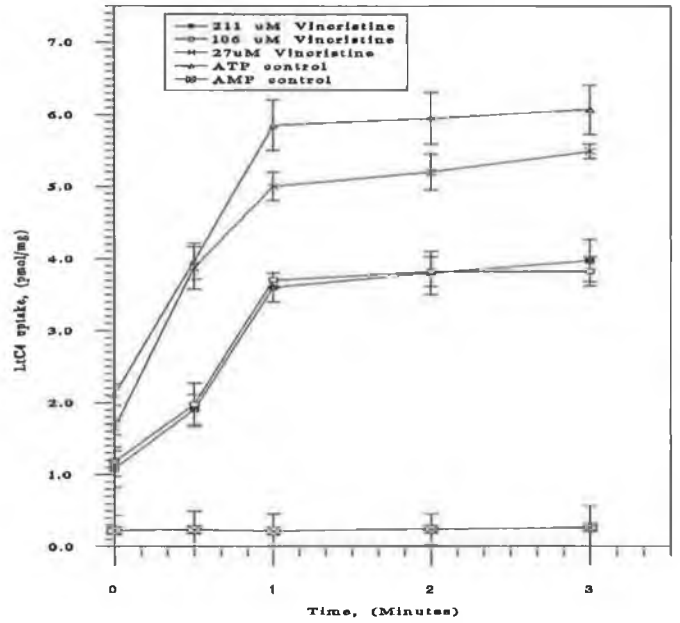

Figure 3.15.1.1: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and the individual concentrations of vincristine indicated above. Data points represent the means (± S.D.) of duplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

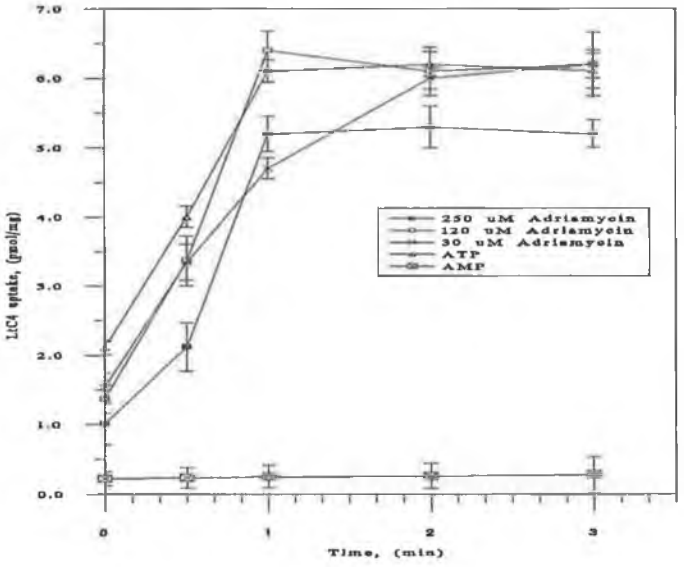

Figure 3.15.1.2: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and the individual concentrations of adriamycin indicated above. Data points represent the means (± S.D.) of duplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

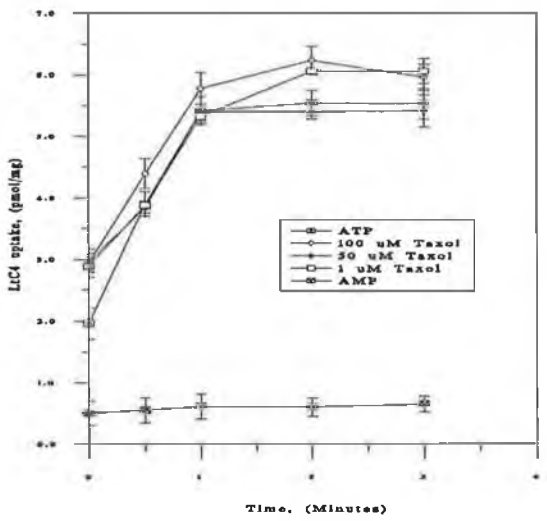

Figure 3.15.1.3: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and the individual concentrations of Taxol indicated above. Data points represent the means (± S.D.) of duplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

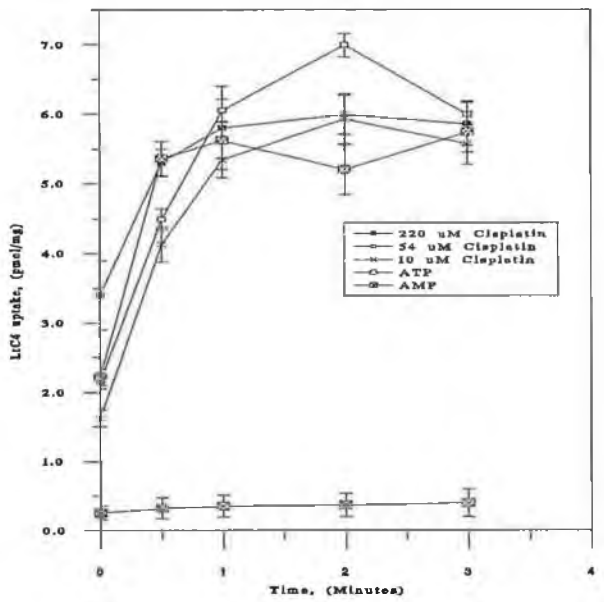

Figure 3.15.1.4: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and the individual concentrations of cisplatin indicated above. Data points represent the means (± S.D.) of duplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

3.15.2 Inhibition of MRP mediated transport of LtC_4 in the presence of cytotoxic drugs and glutathione in HL60-ADR IOVs.

The transport of [^{3}H]-LtC_4 into HL60-ADR IOVs in the presence of various concentrations of glutathione was investigated in order to see if this tripeptide interfered with the activity of the glutathione conjugate pump, MRP. From Figure 3.15.2.1 it can be seen that ATP-dependent MRP-mediated transport of LtC_4 was practically completely inhibited in the presence of 5.6 mM reduced glutathione (GSH). The level of inhibition was 70% of the level of transport achieved for the ATP treated control in the presence of 3.7 mM GSH. Concentrations of GSH below 1.9 mM had no inhibitory effects on [^{3}H]-LtC_4 transport. Previous evidence suggested that GSH in an unconjugated state is not a substrate for MRP transport. Leier *et al.* (1996) failed to show any direct transport of [^{3}H]-labelled GSH into MRP containing IOVs, when added at a concentration of 100 μM. Muller *et al.*, (1994) demonstrated that 5 mM GSH did not affect [^{3}H]-LtC_4 transport into IOVs isolated from MRP-transfected non-small cell lung carcinoma cell line SW1573/S1. Loe *et al.*, (1996b) stated that GSH in their experimental models did not inhibit LtC_4 transport even at 5 mM, a concentration stated as being the highest intracellular concentration achievable *in vivo*. The results shown in Figure 3.15.2.1 obviously differ from the statements made by Loe *et al.*, (1996b) and Muller *et al.*, (1994).

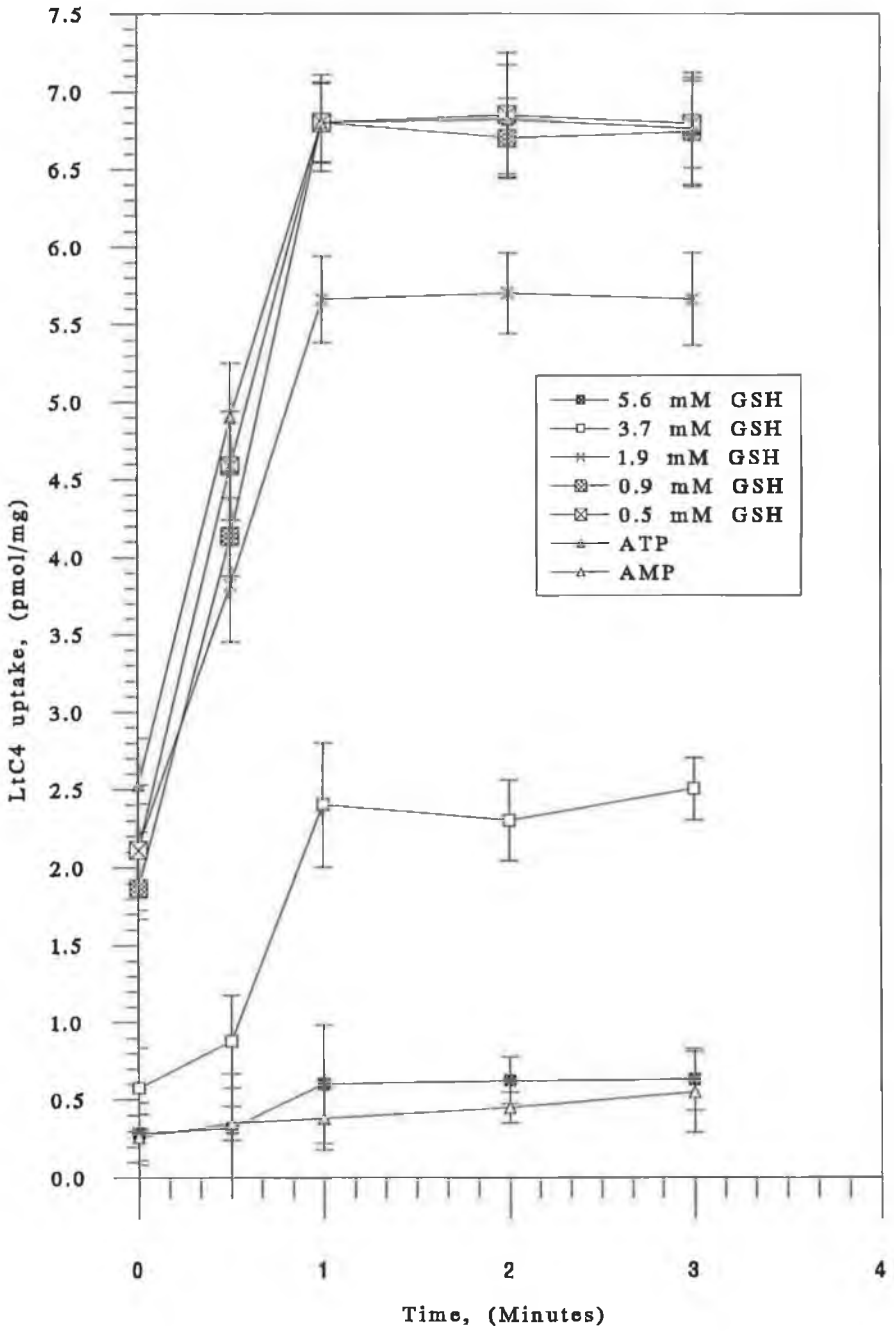

Figure 3.15.2.1: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and the individual concentrations of glutathione (GSH) indicated above. Data points represent the means (± S.D.) of duplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

From Figure 3.15.2.1 it can be seen that a concentration of 5 mM GSH totally inhibited LtC_4 transport. If one co-incubated HL60-ADR IOVs with vincristine or cisplatin, one would predict that irrespective of whatever drug was present, [^{3}H]-LtC_4 transport would be totally inhibited. From Figure 3.15.2.2 it can be seen that this was the case found experimentally. Cisplatin at 109 μM had no significant effects on [^{3}H]-LtC_4 transport but addition of 5 mM GSH completely inhibited LtC_4 transport into HL60-ADR IOVs. The influence of 1 mM GSH, which has no significant effects on [^{3}H]-LtC_4 transport in IOVs, was then investigated in the case of vincristine, adriamycin, cisplatin and Taxol. In Figure 3.15.2.3 it can be seen that in the presence of 1 mM GSH, [^{3}H]-LtC_4 uptake in the presence of 110 μM vincristine was significantly reduced from a steady state accumulation level of 4.5 pmol/mg protein to below 2.0 pmol/mg protein. From Figure 3.15.2.4, it can be seen that the level of [^{3}H]-LtC_4 accumulation in the presence of 250 μM adriamycin (a concentration of drug which reduces LtC_4 accumulation by approximately 15% relative to control IOVs) was only slightly affected by the addition of 1 mM GSH. This was also found to be the case for 100 μM cisplatin (Figure 3.15.2.5). [^{3}H]-LtC_4 accumulation in the presence of 50 μM Taxol was unaffected by the presence of 1 mM GSH (Figure 3.15.2.6). These results agree with a number of the findings of Stride *et al.*, (1997) and Loe *et al.*, (1996b) for MRP-transfected cells. The importance of the illustration of inhibition of LtC_4 transport by vincristine but not adriamycin, cisplatin or Taxol is discussed in Section 4.10.1.

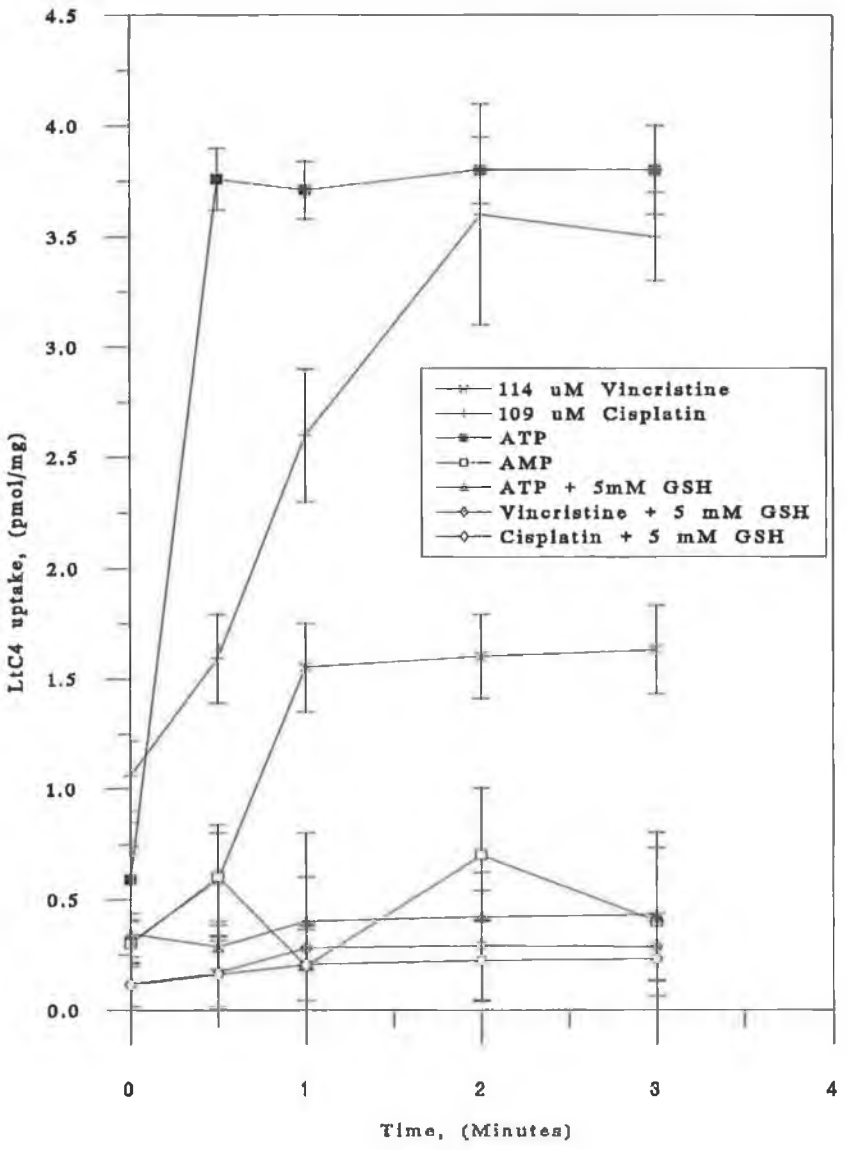

Figure 3.15.2.2: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and the concentrations the of drugs indicated above. IOVs were also incubated in the presence of 5 mM glutathione (GSH) and either ATP, ATP and vincristine, ATP and cisplatin or ATP and Taxol as indicated. Data points represent the means (± S.D.) of duplicate determinations.

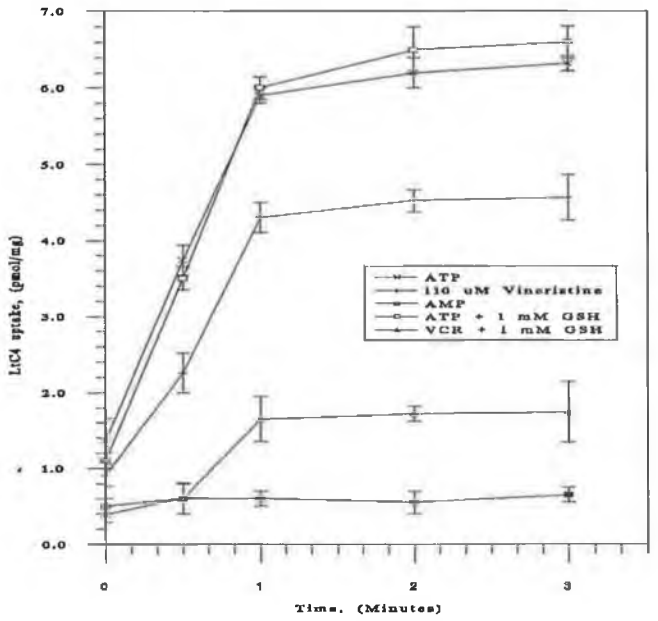

Figure 3.15.2.3: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and vincristine. IOVs were also incubated in the presence of 1 mM glutathione (GSH) and ATP, or 1 mM GSH and ATP and vincristine as indicated. Data points represent the means (± S.D.) of duplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

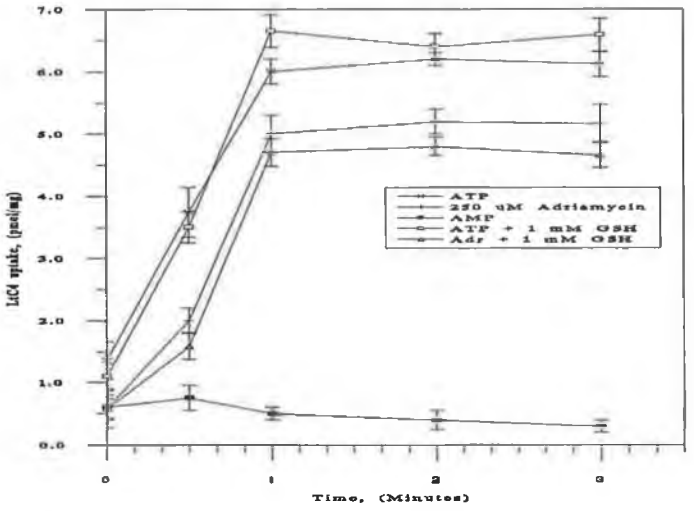

Figure 3.15.2.4: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and adriamycin. IOVs were also incubated in the presence of 1 mM glutathione (GSH) and ATP, or 1 mM GSH and ATP and adriamcyin as indicated. Data points represent the means (± S.D.) of duplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

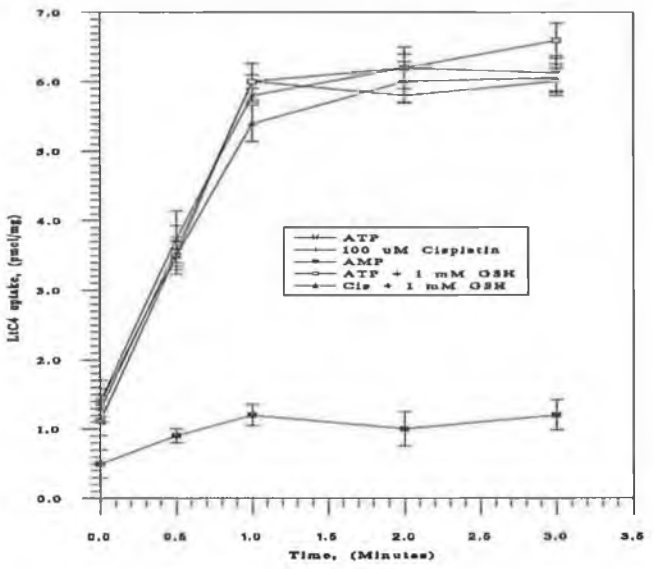

Figure 3.15.2.5: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and cisplatin. IOVs were also incubated in the presence of 1 mM glutathione (GSH) and ATP, or 1 mM GSH and ATP and cisplatin as indicated. Data points represent the means (± S.D.) of duplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

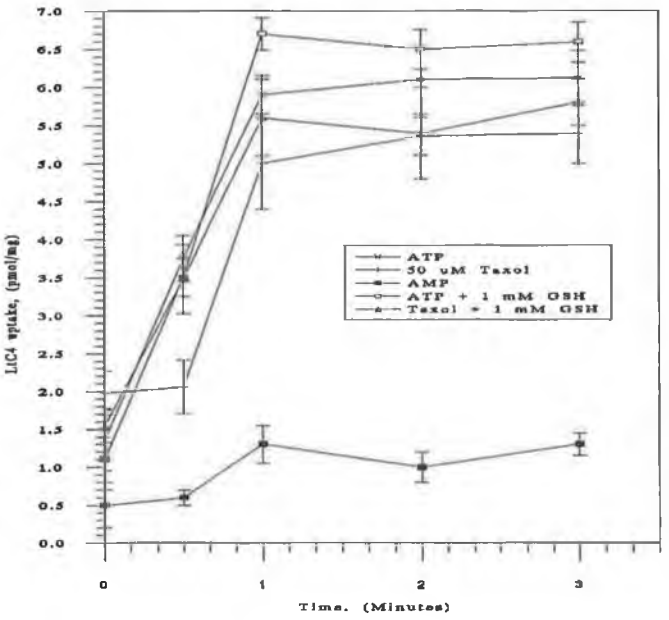

Figure 3.15.2.6: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and Taxol. IOVs were also incubated in the presence of 1 mM glutathione (GSH) and ATP, or 1 mM GSH and ATP and Taxol as indicated. Data points represent the means (± S.D.) of duplicate determinations in a single experiment. Similar results were obtained in at least one additional experiment.

3.15.3 Transport of natural product unconjugated cytotoxic drugs by MRP in HL60-ADR IOVs.

As described earlier, MRP-overexpressing cell lines have been shown to be resistant to a range of natural product cytotoxic drugs such as vincristine, adriamycin and VP-16. The mechanism by which MRP transports these drugs is unclear. MRP is a transporter of glutathione conjugates but natural product chemotherapeutic drugs are not believed to undergo glutathione conjugation. Stride *et al.*, (1997) and Loe *et al.*, (1996b) have shown that [^{3}H]-labelled vincristine was actively transported in IOVs derived from MRP-transfected cells only in the presence of 1, 3 or 5 mM glutathione. These groups suggested that some form of co-transport of vincristine and GSH into IOVs may have been responsible for the active transport of drug under suitable conditions. In order to investigate these findings in a drug selected MRP-overexpressing cell line, similar experiments were carried out using HL60-ADR IOVs.

The use of [^{3}H]-vincristine in the IOV transport assay system required modification of the protocol used for [^{3}H]-LtC_4 transport analysis (Section 2.11.2) in order to minimise non-specific binding of vincristine to the filters used to retain the IOVs. As can be seen from Figure 3.15.3.1, these modifications of the procedure did not significantly interfere with the detection of ATP dependent transport of [^{3}H]-LtC_4 to the interior of IOVs. The levels of LtC_4 transport found using the modified protocol were lower than obtained using the standard [^{3}H]-LtC_4 assay protocol. The reduction in detectable [^{3}H]-LtC_4 active transport (approximately 4 fold) caused by modification of the protocol to minimise non-specific drug binding, was significantly less significant than the reduction in non-specific binding of [^{3}H]-vincristine (reduced from 50% non-specific binding under standard assay conditions to under 2% using the modified protocol) to the filters. The reduction in detectable [^{3}H]-LtC_4 active transport was primarily attributable to the filters used, which were of 1 μm nominal pore size. The average size of IOVs is generally regarded as varying from 1.5 to 0.5 μm and so one could assume significant losses of IOVs using these filters. As can be seen from Figure 3.15.3.1, 10 mM GSH totally inhibited [^{3}H]-LtC_4 uptake into HL60-ADR IOVs.

A concentration of 0.5 mM GSH had no significant effects on LtC_4 transport in the presence of ATP, but 2.7 mM inhibited transport by approximately 20%.

From Figure 3.15.3.2, it can be seen that HL60-ADR IOVs were not shown to transport [^{3}H]-vincristine in a time dependent manner either in the presence of ATP or AMP. The addition of GSH at a concentration of 2.7 mM had no effect on [^{3}H]-vincristine transport in the presence of ATP. All results have been corrected for non-specific binding of [^{3}H]-vincristine to the filters. The conditions used in the experiment represented in Figure 3.15.3.2 were modified in a further experiment where [^{3}H]-vincristine transport was studied in the presence of a low and high concentration of glutathione (0.5 and 10 mM). In addition, the quantity of IOV protein was increased (from 50 to 100 μg) in order to see if this increased [^{3}H]-vincristine transport to a detectable level . The results of this experiment are summarised in Figure 3.15.3.3. As can be seen there were no significant differences in [^{3}H]-vincristine transport between ATP and AMP treated HL60-ADR IOVs. The addition of 0.5 mM GSH which has no effect on MRP activity or 10 mM GSH which totally inhibits MRP activity had no significant effects on the transport of [^{3}H]-vincristine in the presence of ATP. The minor increases in vincristine accumulation shown in Figures 3.15.3.2 and 3.15.3.3 during the various treatments are not a result of any specific transport of [^{3}H]-vincristine.

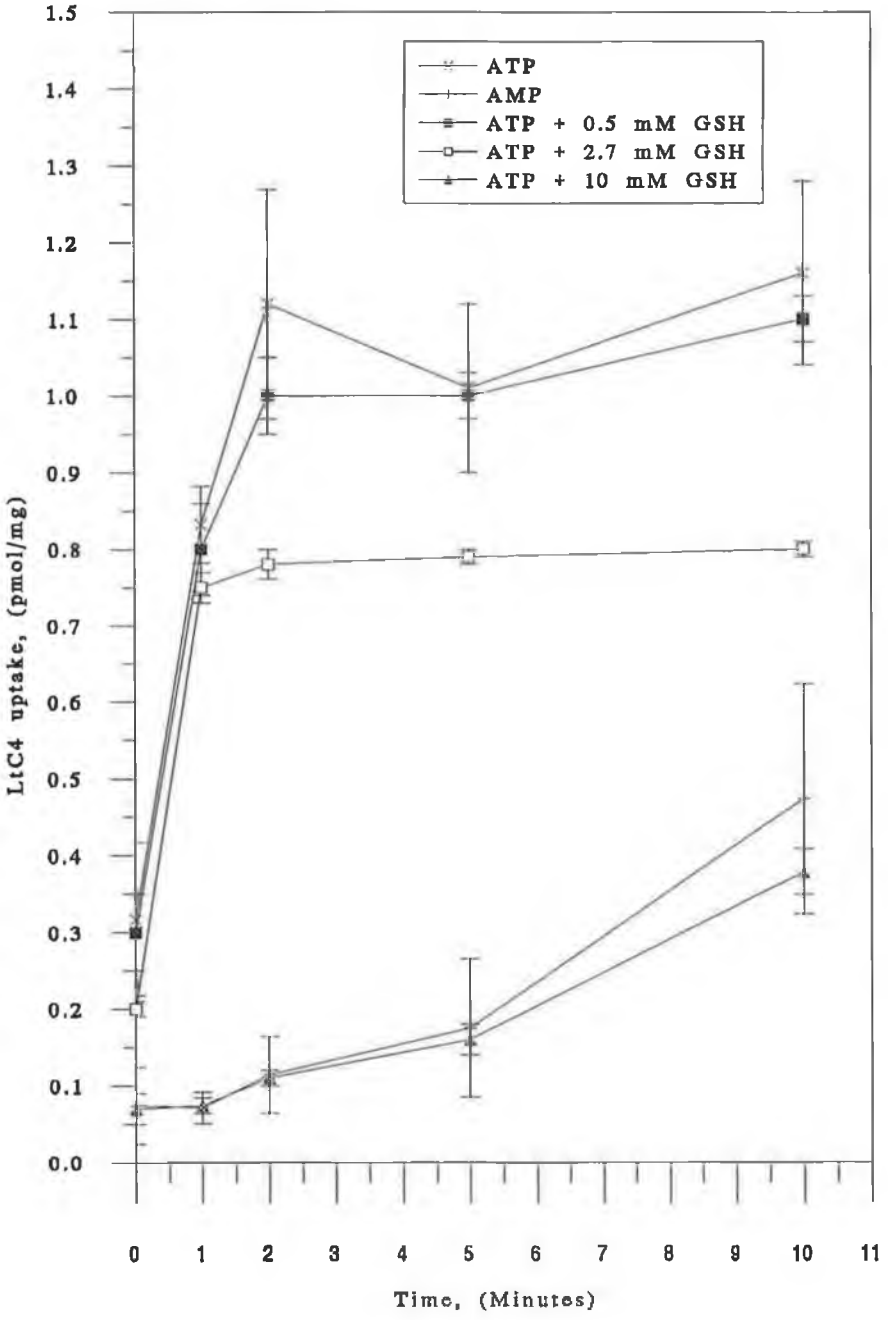

Figure 3.15.3.1: Time course of LtC_4 transport into IOVs isolated from the HL60-ADR cell line in the presence of ATP, AMP or a combination of ATP and glutathione (GSH). Data points represent the means (± S.D.) of duplicate determinations. Quantification of uptake of LtC_4 was performed using glass fibre filters of a 1 μm nominal diameter.

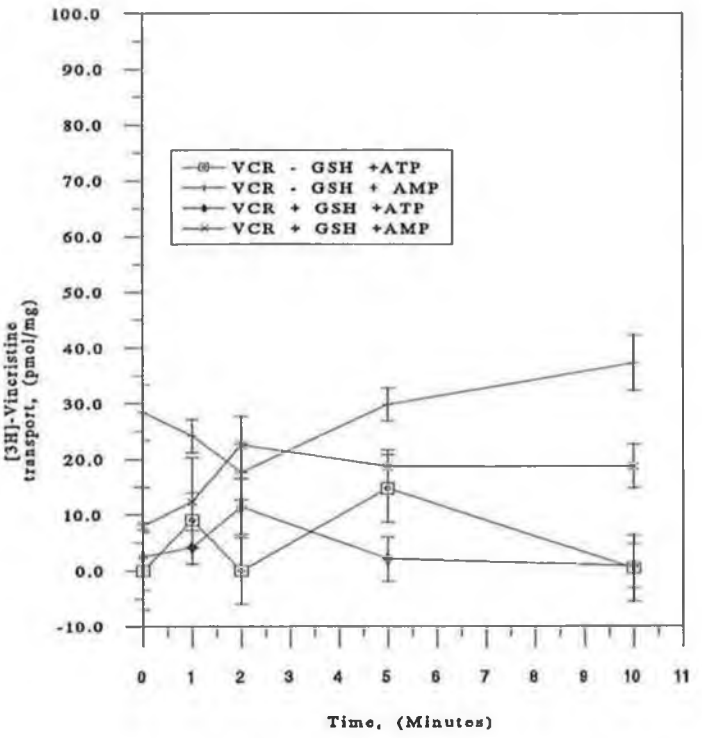

Figure 3.15.3.2: Investigation into [^{3}H]-vincristine transport into HL60-ADR IOVs in the presence of ATP, AMP, 2.7 mM glutathione (GSH) or ATP and AMP in combination with 2.7 mM GSH. Quantification of uptake of [^{3}H]-vincristine was performed using glass fibre filters of a 1 μm nominal diameter. Data points represent the means (± S.D.) of duplicate determinations carried out on two separate occasions.

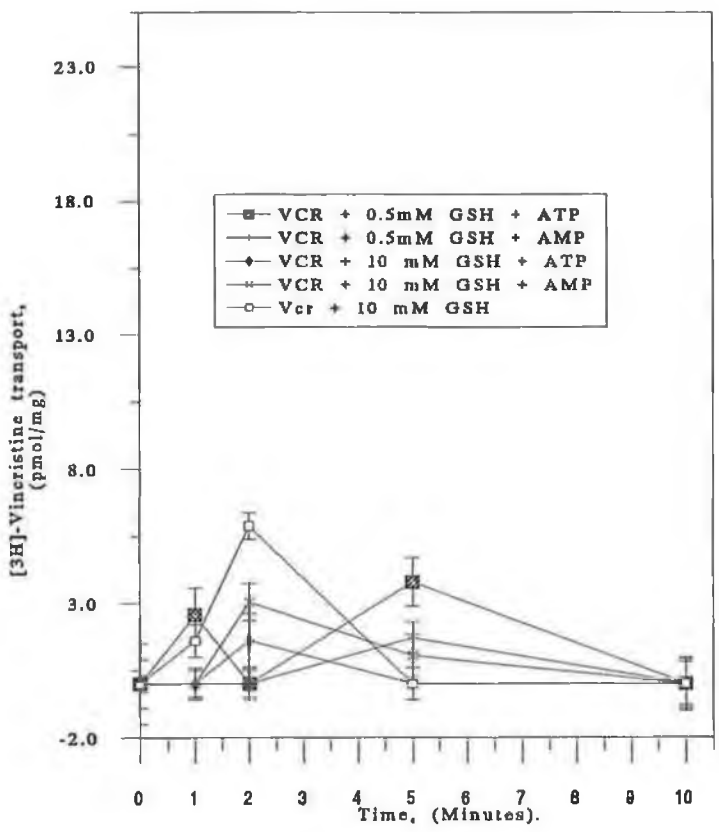

Figure 3.15.3.3: Investigation into [^{3}H]-vincristine transport into HL60-ADR IOVs (100 μg IOV protein) in the presence of ATP or AMP in combination with 0.5 or 10 mM glutathione (GSH). Quantification of uptake of [^{3}H]-vincristine was performed using glass fibre filters of a 1 μm nominal diameter. Data points represent the means (± S.D.) of duplicate determinations carried out on two separate occasions.

3.16 Inhibition of LtC_4 transport by MRP into HL60-ADR IOVs due to the presence of cyclosporin A, verapamil or MK571.

In Section 3.15.1 and 3.15.2 it was shown that cytotoxic agents such as vincristine can inhibit [^{3}H]-LtC_4 transport when added at high concentrations. These concentrations are very high and may appear to be unlikely to reflect the cellular concentrations achieved with these agents. The circumventing agents cyclosporin A and verapamil are known to exert inhibitory activities on MRP activity. MK571 is believed to be the most potent inhibitor of MRP available (Section 1.5.2.4). From studies in the DLKP cell line, (Section 3.14.1) it was known that MK571 enhanced the toxicity of the MRP substrate drugs adriamycin and VP-16. The toxicity of the non-MRP substrate drug, Taxol was unaffected by the presence of MK571. In order to quantify the potency of MK571 as an inhibitor of MRP, the inhibition characteristics of this compound in the HL60-ADR IOV model for MRP activity was examined. From Figure 3.16.1 it can be seen that 50, 10 and 1 μM concentrations of cyclosporin A inhibited [^{3}H]-LtC_4 uptake into HL60-ADR IOVs by MRP by approximately 60, 50 and 40% respectively at steady state. Figure 3.16.2 illustrates that verapamil is relatively ineffective as an inhibitor of [^{3}H]-LtC_4 transport. Concentrations of 50, 10 and 1 μM verapamil only achieve in the range of under 20% inhibition of [^{3}H]-LtC_4 transport relative to control ATP treated IOVs. The levels of inhibition of [^{3}H]-LtC_4 uptake into HL60-ADR IOVs achieved by cyclosporin and verapamil are significantly lower than achieved in the presence of the leukotriene receptor antagonist MK571. From Figure 3.16.3 it can be seen that 50 μM MK571 totally eliminates ATP dependent [^{3}H]-LtC_4 transport into HL60-ADR IOVs. Concentrations of 50 μM MK571 reduced ATP-dependent transport by approximately 90-95% and 1 μM caused a reduction in [^{3}H]-LtC_4 uptake by 20-30%. These results show that cyclosporin A is a more potent inhibitor of MRP than verapamil. MK571 is significantly more potent an inhibitor of MRP activity than either of these circumventing agents.

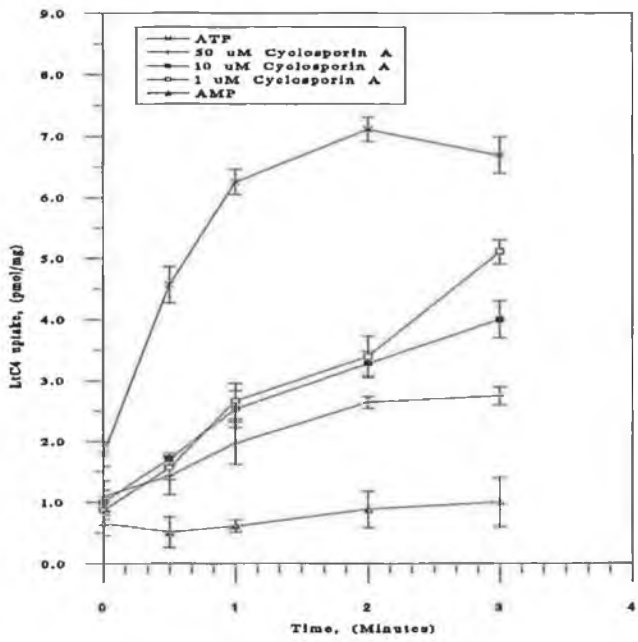

Figure 3.16.1: Time course of LtC_4 accumulation in HL60-ADR IOVs over time in the presence of ATP, AMP or ATP and the concentrations of cyclosporin A indicated above. Data points represent the means (± S.D.) of duplicate determinations. Similar results were obtained in at least one additional experiment.

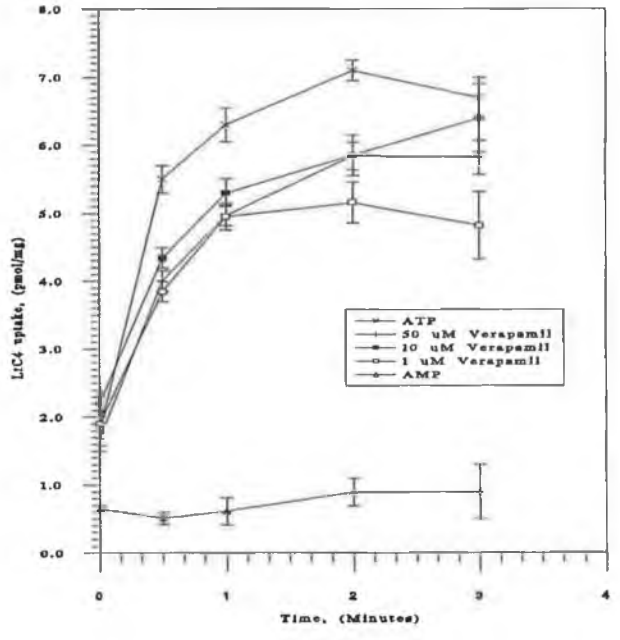

Figure 3.16.2: Time course of LtC_4 accumulation in HL60-ADR IOVs over time in the presence of ATP, AMP or ATP and the concentrations of verapamil indicated above. Data points represent the means (± S.D.) of duplicate determinations. Similar results were obtained in at least one additional experiment.

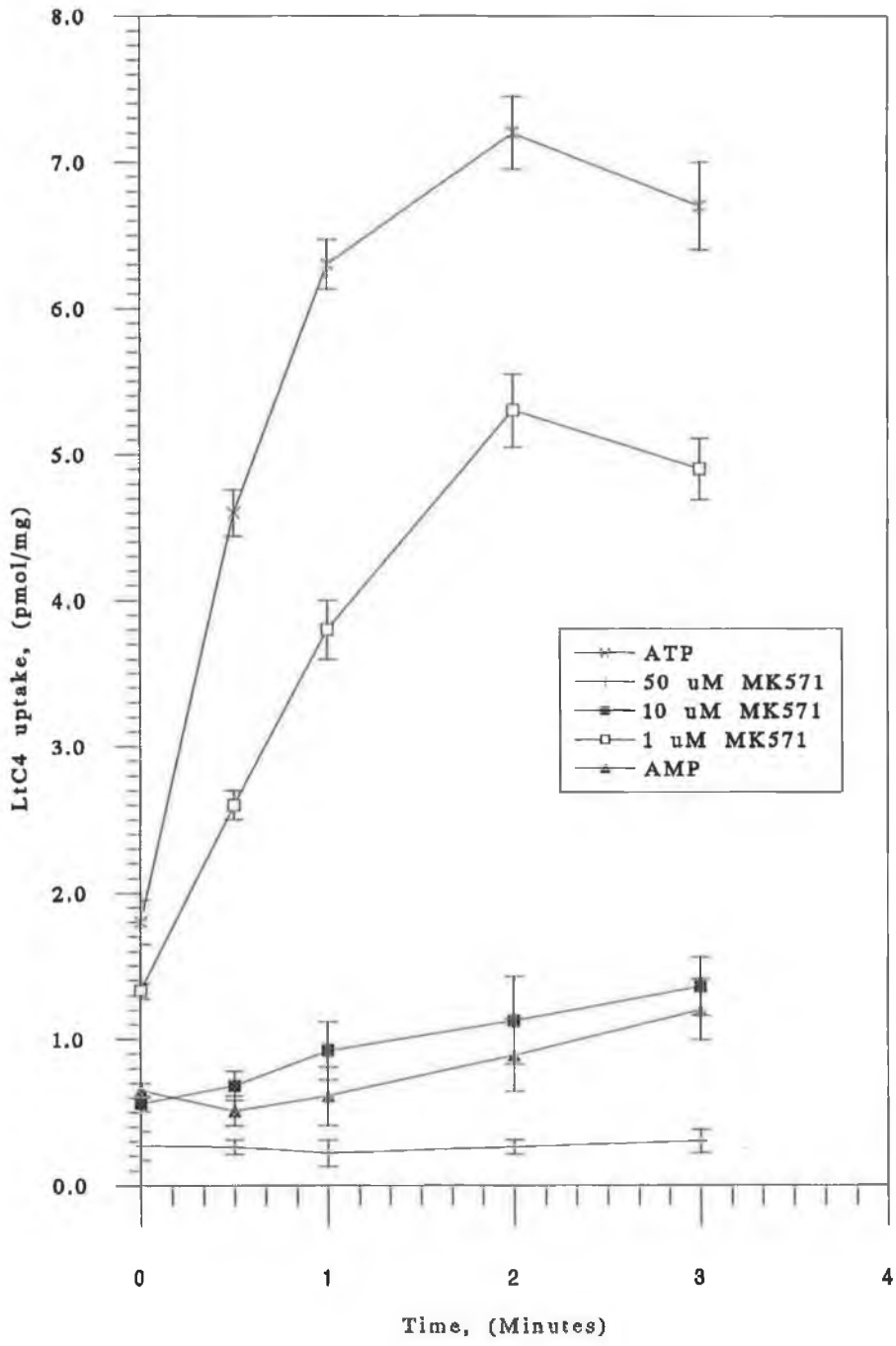

Figure 3.16.3: Time course of LtC_4 accumulation in HL60-ADR IOVs over time in the presence of ATP, AMP or ATP and the concentrations of MK571 indicated above. Data points represent the means (± S.D.) of duplicate determinations. Similar results were obtained in at least one additional experiment.

3.17 Investigation into the cytotoxic drug accumulation characteristics of the DLKP-A10 multidrug-resistant human lung tumor cell line.

The DLKP-A10 cell line is a drug resistant variant of DLKP obtained by continuous adriamycin exposure of the multidrug resistant cell line, DLKP-A. DLKP-A10 cells can survive in 17 μM (10 μg/mL) adriamycin, at cell densities appropriate for drug selection in 75 cm^2 flasks. The IC_{50} of this cell line in a standard toxicity assay is approximately 6.5 μM, a factor of over 800-fold more resistant than the DLKP parental cells. The MDR circumventing agents verapamil and cyclosporin A have been shown to significantly increase the level of accumulation of adriamycin and vincristine in the DLKP-A10 cell line (Cleary *et al.*, 1997). This level of accumulation is significantly higher than the levels present in DLKP-A10 in the absence of circumventing agent, but intracellular drug levels are still lower than the levels accumulated in the DLKP cell line. In the human lung squamous carcinoma cell line, SKMES-1/ADR it has been shown that both verapamil and cyclosporin A completely restored the accumulation deficit found in the resistant cells, to the levels of the drug sensitive parental cells, SKMES-1. The DLKP-A10 cell line overexpresses the P-glycoprotein (Pgp) drug transporter, but if resistance was totally due to this resistance mechanism, one would predict complete reversal of the accumulation deficit upon addition of a suitable concentration of verapamil or cyclosporin A (as found for the SKMES-1/ADR cell line). No significant increases in MRP protein were found in whole cell extracts of the DLKP-A10 cell line relative to the parental DLKP cells. This resistant cell line was noted to possess unusually high levels of vesicle formation within the cytosol (Cleary *et al.*, 1997). It was found that drug became localised in these vesicles, which was believed to contribute to a reduction in nuclear accumulation of drug. The results of the studies conducted by Cleary *et al.*, (1997) suggested that the DLKP-A10 cell line possessed an ATP-dependent vesicular sequesterisation system with contributed to the inability of verapamil and cyclosporin to reduce the accumulation deficit in this cell line. A number of MRP-expressing drug resistant cell lines have been shown to possess high levels of intracellular vesicles, (Marquardt and Center, 1992). For this reason, IOVs were

isolated from the DLKP-A10 cell line. Isolation of plasma membrane derived IOVs would not necessarily mean isolation of the proteins present in the intracellular vesicles present in DLKP-A10, but because of membrane trafficking to the plasma membrane, one could assume that a significant fraction of these vesicles would ultimately become retained in the plasma membrane. Analysis of the MRP activity in the DLKP-A10 cell line could provide some indication as to whether or not MRP contributed to the drug accumulation deficit found in this cell line in the presence of verapamil or cyclosporin A. In advance of isolation of IOVs from DLKP-A10, drug accumulation studies were carried out in order to examine the specific details of the accumulation deficit in this cell line.

3.17.1 Adriamycin accumulation characteristics in the DLKP, DLKP-A10, SKMES-1 and SKMES-1/ADR cell lines.

Figure 3.17.1.1 illustrates that in the presence of 10 μM adriamycin, DLKP cells accumulated high levels of adriamycin (approximately 4 nmoles adriamycin per 10^6 cells after 4 hours incubation in drug). No saturation in accumulation in this drug sensitive cell line was found even after 4 hours incubation in drug. The DLKP-A10 cell line accumulated very low levels of adriamycin relative to the parental DLKP cells. This resistant cell line only accumulated approximately 200 pmoles adriamycin per 10^6 cells after 4 hours incubation in drug. Steady state accumulation was achieved after 1-2 hours incubation in drug. Addition of cyclosporin A (8 μM) or verapamil (60 μM) only partially reversed the accumulation deficit. Both of these MDR modulators increased cellular adriamycin accumulation levels in DLKP-A10 to approximately 1400 pmol per 10^6 cells (35% of drug accumulation levels in the DLKP cell line). Steady state accumulation levels of adriamycin in the presence of these MDR modulators was achieved by 3 hours incubation in drug. These results reflected the findings of Cleary, (1995). Cleary, (1995) also demonstrated that the accumulation deficit in DLKP-A10 was not affected by increasing the concentration of verapamil or cyclosporin. In Figure 3.17.1.2 it can be seen that in the Pgp-overexpressing cell line SKMES-1/ADR, cyclosporin A (8 μM) and verapamil (60 μM) completely reversed the accumulation deficit to the levels achieved in the parental SKMES-1 cell line. The SKMES-1/ADR cell line accumulated only approximately 300 pmol adriamycin per 10^6 cells after 4 hours incubation in drug. The parental SKMES-1 cells accumulated approximately 1700 pmol per 10^6 cells after 4 hours incubation in 10 μM adriamycin. The drug resistance of SKMES-1/ADR is believed to be predominantly Pgp-mediated, (Cleary, 1995). As a result, drug accumulation levels in the presence of the potent Pgp modulators, verapamil or cyclosporin A were increased to the levels achieved in the drug sensitive SKMES-1 cells.

Antimycin A is a metabolic inhibitor which inhibits ATP production by interfering with electron transport from ubiquinone to cytochrome C in the electron transport chain. Sodium azide is also an inhibitor of the electron transport chain (inhibits reduction of oxygen by cytochrome oxidase). Cleary *et al.*, (1997) showed that under specific experimental conditions, antimycin A but not sodium azide reversed the accumulation deficit in the DLKP-A10 cell line. The explanation given by Cleary *et al.*, (1997) for the ability of the metabolic inhibitor antimycin A but not sodium azide to reverse the drug accumulation deficit in DLKP-A10, involved the fact that antimycin A inhibited the electron transport chain at an earlier stage than sodium azide. For this reason, this was believed to result in elimination of the adriamycin accumulation deficit in the DLKP-A10 cell line over the 3 hour time period studied. In order to investigate these findings, similar studies were conducted, the results of which are presented in Figure 3.17.1.3. The details of the protocol used in this experiment are provided in Section 2.15. This experimental protocol involved seeding cells at a density of 0.5 x 10^6 cells per 75 cm^2 flask. Following an incubation period of 48 hours, cells were washed with PBS and incubated in glucose free media for two hours. After this time cells were treated with the relevant concentrations of adriamycin (10 μM), antimycin A (20 μM) or sodium azide (10 mM) in glucose free medium. Cellular accumulation of adriamycin was then determined by HPLC as described in Section 2.15. From Figure 3.17.1.3 it can be seen that even in glucose free media, DLKP-A10 only accumulated approximately 500 pmol adriamycin per 10^6 cells after 3 hours incubation in drug. Steady state accumulation was achieved after 1 hour. The DLKP cell line accumulated approximately 1600 pmol adriamycin per 10^6 cells after 3 hours incubation. From Figure 3.17.1.3 it can be seen that 20 μM antimycin A caused a level of adriamycin accumulation in DLKP-A10 comparable to that achieved in the DLKP cell line. Sodium azide (10 mM) appeared to only partially reverse the accumulation deficit in DLKP-A10 (Figure 3.17.1.3). The primary reason for significant errors associated with the data is the toxicity of these metabolic inhibitors in glucose free media. Obviously interference with critical steps in the electron transport chain due to the presence of a metabolic inhibitor such as antimycin A has extremely detrimental effects on cellular

metabolism. Even after 30 minutes incubation in antimycin A or sodium azide, cells appear shocked and after a period of 4 hours, toxic effects begin to occur and cells undergo cell death. The differences between DLKP and DLKP-A10 adriamycin accumulation are relatively small (approximately 3 fold difference after 3 hours in glucose free media versus 20 fold in glucose containing media) and this makes the variation associated with the data quite significant. Due to the levels of variation in analysis of drug accumulation levels by HPLC, errors of ± 500 pmol adriamycin per 10^6 cells are possible at high drug concentration (such as in Figure 3.17.1.1 for DLKP at all timepoints). Even considering the levels of errors associated with the data it is clear that antimycin A reversed the accumulation deficit in DLKP-A10 and that sodium azide was only partially effective for this purpose. The potential ability of 2 μM antimycin A to reverse the adriamycin accumulation deficit in the DLKP-A10 cell line in glucose containing media was also investigated. From Figure 3.17.1.4 it can be seen that under these conditions, antimycin A was totally ineffective for reversal of the drug accumulation deficit in the DLKP-A10 cell line. This suggests that the abilities of antimycin A to increase adriamycin accumulation in the DLKP-A10 cell line were partially a result of the effects of this metabolic inhibitor on ATP production.

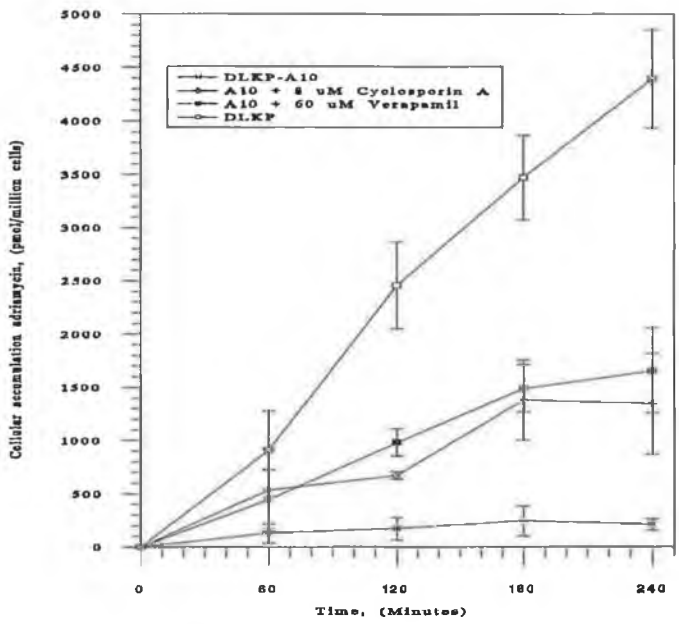

Figure 3.17.1.1: The effect of cyclosporin A (8 μM) and verapamil (60 μM) on adriamycin accumulation in the DLKP-A10 and DLKP cell lines over time. Data points represent the means (± S.D.) of duplicate determinations.

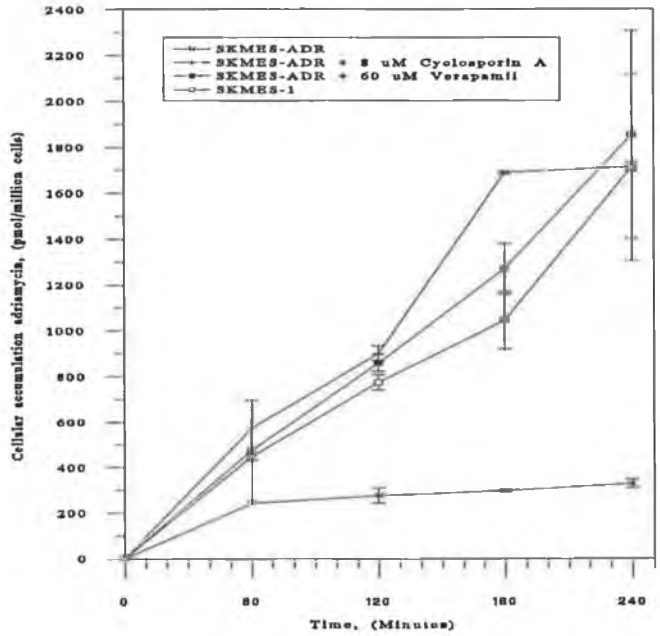

Figure 3.17.1.2: The effect of cyclosporin A (8 μM) and verapamil (60 μM) on adriamycin accumulation in the SKMES-1/ADR and SKMES-1 cell lines over time. Data points represent the means (± S.D.) of duplicate determinations.

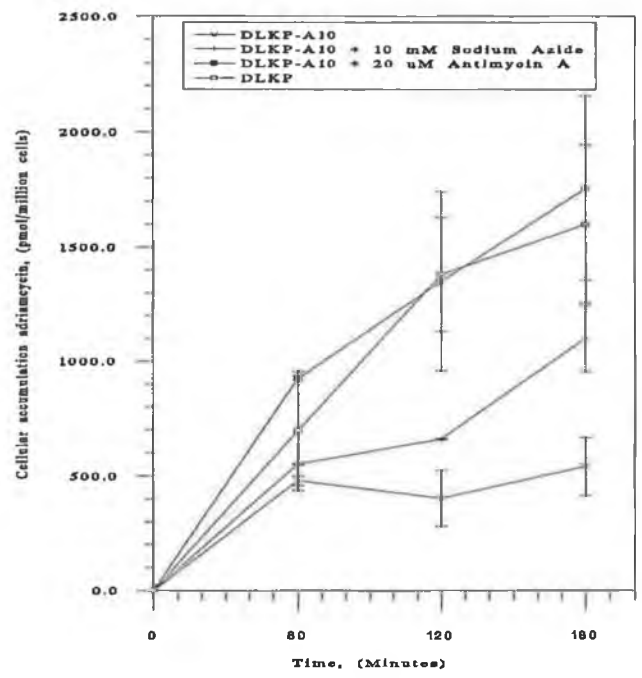

Figure 3.17.1.3: The effect of antimycin A (20 μM) and sodium azide (10 mM) on adriamycin accumulation in the DLKP-A10 and DLKP cell lines under glucose free conditions. Data points represent the means (± S.D.) of duplicate determinations.

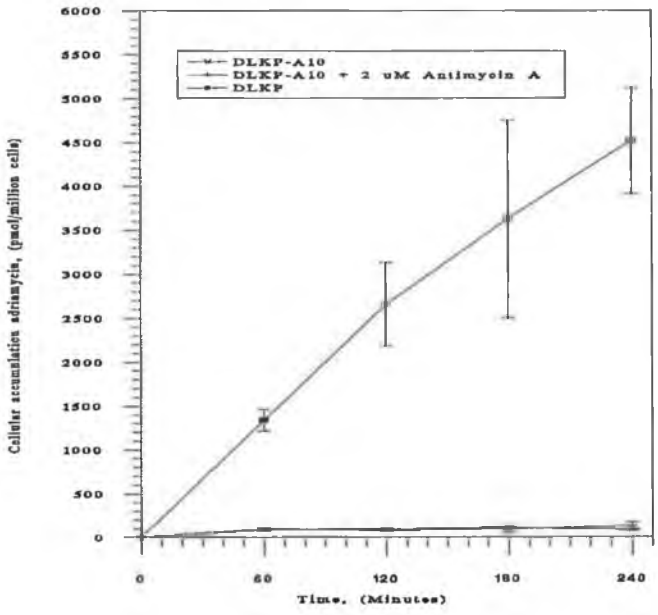

Figure 3.17.1.4: The effect of antimycin A (2 μM) on adriamycin accumulation in the DLKP-A10 and DLKP cell lines under glucose containing conditions. Data points represent the means (± S.D.) of duplicate determinations.

3.17.2 Transport of LtC_4 into DLKP-A10 derived IOVs and inhibition of this transport by antimycin A, sodium azide, cyclosporin A and verapamil.

DLKP-A10 IOVs were isolated using the protocol detailed in Section 2.10. The [^{3}H]-LtC_4 transporting abilities of this pump were then analysed and the results are summarised in Figure 3.17.2.1. As can be seen DLKP-A10 derived IOVs transported [^{3}H]-LtC_4 in an ATP dependent manner with an initial rate of 0.6 ± 0.2 pmol per mg IOV protein per minute and achieved a [^{3}H]-LtC_4 accumulation level of 1.8 ± 0.3 pmol/mg protein by 4.0 minutes. These values are the means ± S.D. of duplicate determinations carried out using two individually prepared vesicle preparations. The DLKP cell line transports [^{3}H]-LtC_4 with an initial rate of 0.8 ± 0.2 pmol per mg IOV protein per minute (maximum accumulation of 2.0 ± 0.2 pmol/mg) and the MRP overexpressing cell line HL60-ADR transports [^{3}H]-LtC_4 with an initial rate of 4.0 ± 1.0 pmol per mg IOV protein per minute (maximum accumulation of 7.0 ± 1.0 pmol/mg). Western blotting results demonstrated lower levels of MRP in DLKP-A10 derived IOVs compared to levels found in DLKP IOVs (Section 3.17.4).

As described in Section 3.17.1, the metabolic inhibitor antimycin A but not sodium azide was shown by Cleary *et al.*, (1997) to eliminate the accumulation deficit in the DLKP-A10 cell line. If the effects of antimycin A on ATP production were solely responsible for reversal of the accumulation deficit in DLKP-A10, one would expect sodium azide to have comparable effects. As this was not the case, an investigation was carried out to test if antimycin A had significantly different effects on [^{3}H]-LtC_4 transport and so MRP activity in DLKP-A10 IOVs. The effects of these agents on this activity was compared to the MDR modulators cyclosporin A and verapamil, which as shown in Section 3.17.1 were only partially effective as agents for reduction of the drug accumulation deficit in DLKP-A10. These circumventing agents exert inhibitory effects on Pgp and MRP, but are poor inhibitors of MRP relative to agents such as MK571 (Section 3.16). From Figure 3.17.2.2 it can be seen that 50 and 10 μM

cyclosporin A cause inhibition of ATP-dependent LtC_4 uptake into DLKP-A10 IOVs by approximately 80 and 60% respectively. A concentration of 1 μM cyclosporin A was without significant effects on [^{3}H]-LtC_4 uptake into these IOVs. Verapamil (Figure 3.17.2.3) was less potent an inhibitor of [^{3}H]-LtC_4 transport into DLKP-A10 derived IOVs as 50 and 10 μM reduced the levels of uptake by less than 20%. A concentration of 1 μM verapamil had no effects on [^{3}H]-LtC_4 uptake into DLKP-A10 IOVs. Antimycin A (as shown in Figure 3.17.2.4) was a potent inhibitor of [^{3}H]-LtC_4 transport activity at 50 μM (approximately 60% inhibition of ATP dependent [^{3}H]-LtC_4 transport) and 10 μM (over 30% inhibition of [^{3}H]-LtC_4 transport). This is relatively surprising as this compound was not previously believed to exert any direct inhibitory effects on MRP or other related transporter molecules. The fact that sodium azide (Figure 3.17.2.5) exerted minor inhibitory effects on [^{3}H]-LtC_4 transport into DLKP-A10 IOVs (but only at a concentration of 50 μM) indicated that these metabolic inhibitors could either interfere with the relevant [^{3}H]-LtC_4 transporter directly or simply interfere with the experimental model used (such as the ATP regenerating system involving creatine phosphate and creatine kinase). The investigations detailed in Section 3.17.3 demonstrated that antimycin A did not affect the ATP regenerating system in IOV transport assays. From Figure 3.17.2.6 it can be seen that the ability of antimycin A to inhibit [^{3}H]-LtC_4 transport is not restricted to the DLKP-A10 cell line. A concentration of 50 μM antimycin A was found to inhibit [^{3}H]-LtC_4 transport into HL60-ADR IOVs by 60-70%. This strongly suggested that this metabolic inhibitor exerted inhibitory effects on MRP-mediated transport of [^{3}H]-LtC_4 into DLKP-A10 and HL60-ADR IOVs.

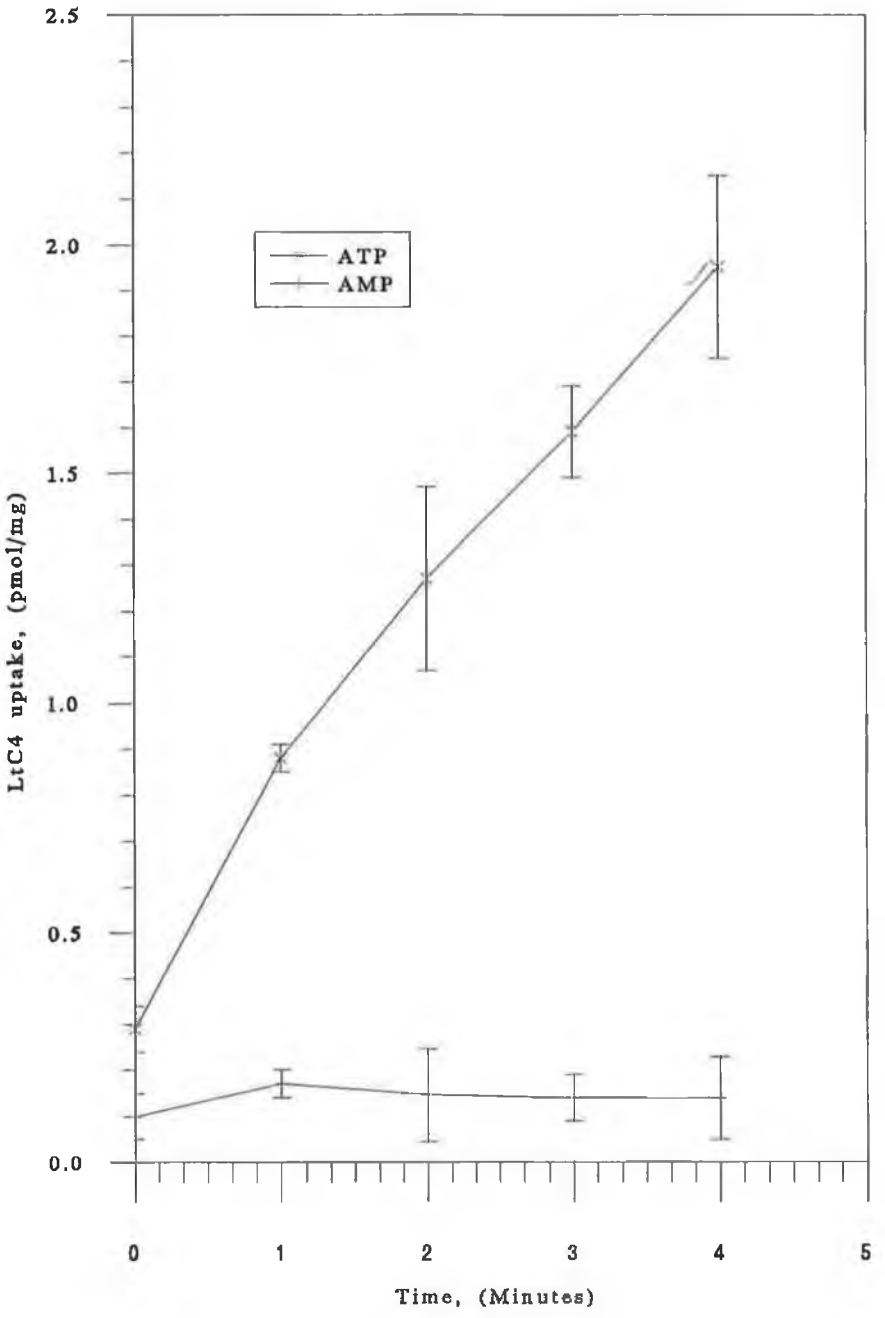

Figure 3.17.2.1: Time course of LtC_4 transport into IOVs from the DLKP-A10 cell line in the presence of ATP or AMP. Data points represent the means (± S.D.) of duplicate determinations.

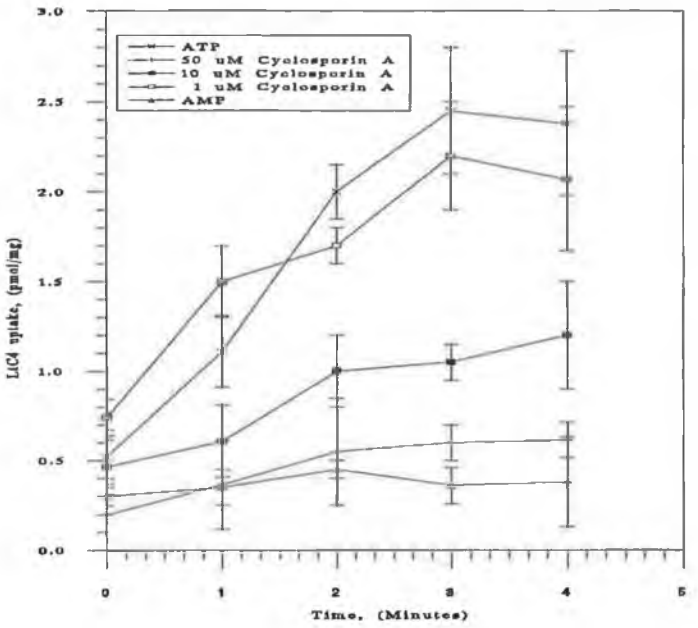

Figure 3.17.2.2: Time course of LtC_4 transport into IOVs from the DLKP-A10 cell line in the presence of ATP or AMP or ATP and 50, 10 and 1 μM cyclosporin A. Data points represent the means (± S.D.) of duplicate determinations. Similar results were obtained in at least one additional experiment.

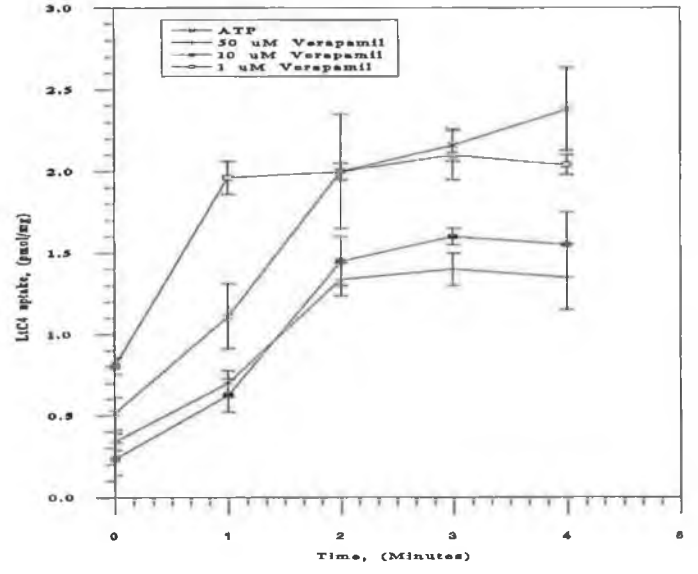

Figure 3.17.2.3: Time course of LtC_4 transport into IOVs from the DLKP-A10 cell line in the presence of ATP or AMP or ATP and 50, 10 and 1 μM verapamil. Data points represent the means (± S.D.) of duplicate determinations. Similar results were obtained in at least one additional experiment.

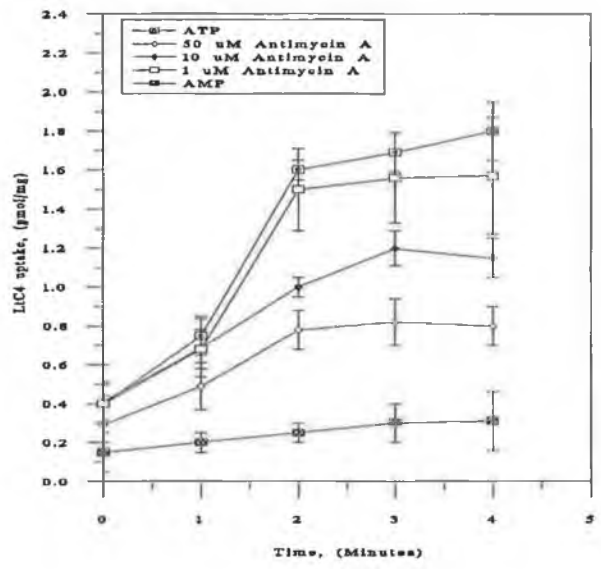

Figure 3.17.2.4: Time course of LtC_4 transport into IOVs from the DLKP-A10 cell line in the presence of ATP or AMP or ATP and 50, 10 and 1 μM antimycin A. Data points represent the means (± S.D.) of duplicate determinations. Similar results were obtained in at least one additional experiment.

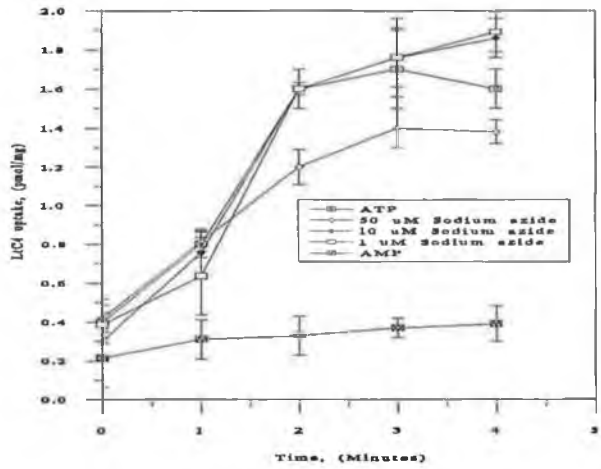

Figure 3.17.2.5: Time course of LtC_4 transport into IOVs from the DLKP-A10 cell line in the presence of ATP or AMP or ATP and 50, 10 and 1 μM sodium azide. Data points represent the means (± S.D.) of duplicate determinations. Similar results were obtained in at least one additional experiment.

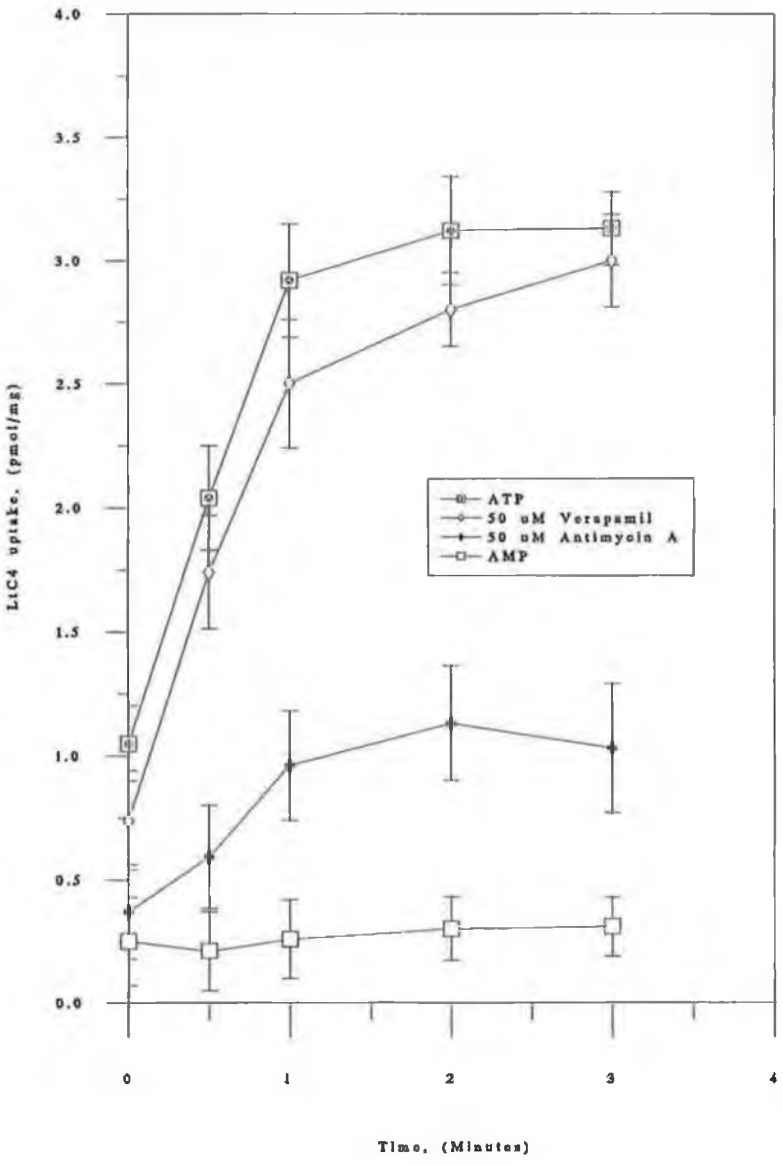

Figure 3.17.2.6: Time course of LtC_4 transport into IOVs from the HL60-ADR cell line in the presence of ATP or AMP or ATP and 50 μM verapamil or antimycin A. Data points represent the means (± S.D.) of duplicate determinations. Similar results were obtained in at least one additional experiment.

3.17.3 Influence of verapamil and antimycin A on ATP levels during LtC_4 transport into HL60-ADR IOVs.

In order to investigate if the inhibition of [^{3}H]-LtC_4 transport caused by the presence of antimycin A was a result of interference with the ATP-regenerating system in the [^{3}H]-LtC_4 transport model, ATP levels in the system were monitored under a variety of conditions. A bioluminescent ATP assay was used to quantify the levels of ATP present at various timepoints (Section 2.14). The ATP regenerating system in IOV transport assays involves the regeneration of ATP using creatine phosphate, creatine kinase and ATP. From Figure 3.17.3.1 it can be seen that in the absence of creatine kinase, ATP levels in the reaction mixture were significantly reduced by 30 minutes, with levels reduced practically to zero by 3-5 hours. This experiment was only performed with HL60-ADR IOVs as it was known that antimycin A exerted comparable inhibitory effects on [^{3}H]-LtC_4 transport into IOVs from both the DLKP-A10 and HL60-ADR cell lines. The HL60-ADR derived IOVs exhibited significantly higher capacities for [^{3}H]-LtC_4 transport than found in DLKP-A10 derived IOVs (Section 3.17.2). For this reason, the rate of ATP utilisation would be accordingly higher in HL60-ADR derived IOVs. This facilitated the rapid detection of variations in ATP levels in the reaction mixture under various conditions. In the presence of creatine kinase (Figure 3.17.3.1), ATP levels remained relatively constant in the reaction mixture. In the presence of 50 μM verapamil or 50 μM antimycin A, ATP levels appeared to remain relatively constant up to a period of 3 hours. After a period of 5 hours, ATP levels were slightly higher in reaction mixtures containing verapamil or antimycin A treated IOVs, relative to a reaction mixture containing untreated IOVs. These results illustrated that antimycin A did not exert any specific effects on the ATP-regenerating system in the IOV transport model and so the influence of this metabolic inhibitor on [^{3}H]-LtC_4 transport into DLKP-A10 and HL60-ADR derived IOVs was a result of direct inhibitory effects on the [^{3}H]-LtC_4 transport exhibited by IOVs from these cell lines.

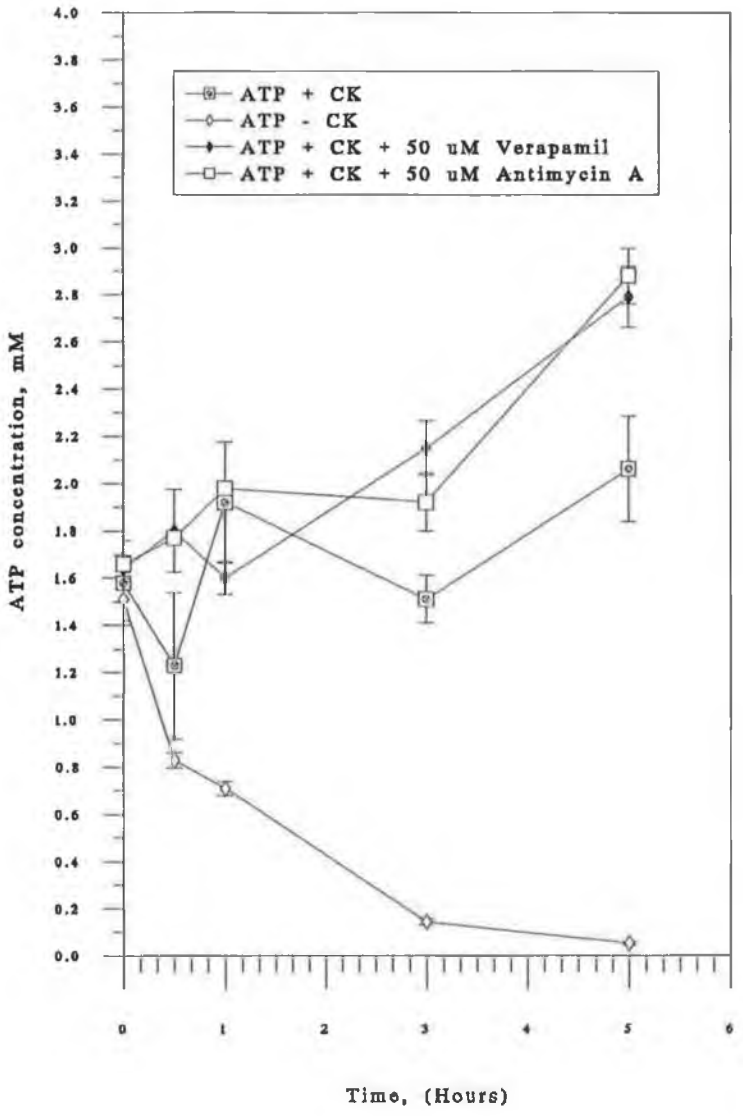

Figure 3.17.3.1: Time course of the effect of 50 μM verapamil and 50 μM antimycin A on ATP levels during LtC_4 transport into HL60-ADR IOVs. IOVs were incubated in the presence of ATP and creatine kinase (CK), ATP and CK and verapamil, ATP and CK and antimycin A, or ATP without CK. Data points represent the means (± S.D.) of duplicate determinations.

3.17.4 Western blot analysis of MRP levels in DLKP-A10 derived inside-out vesicles.

The protein levels of MRP in DLKP-A10 IOVs were analysed by Western blotting. As can be seen from Figure 3.17.4.1, DLKP, DLKP-A10 and HL60-ADR IOVs contained MRP protein. The HL60-ADR IOVs possessed extremely high levels of the 190 kDa MRP protein. As described in Section 2.9.6, the extremely sensitive Pierce SuperSignal Chemiluminescent substrate method was used for analysing MRP expression in DLKP and resistant variants of DLKP. The HL60-ADR IOVs contained such high levels of MRP that the Pierce SuperSignal Chemiluminescent substrate method was over sensitive as a strong signal was evident at all positions along the HL60-ADR IOV lane on the gel (Figure 3.17.4.1). Reducing protein loading of HL60-ADR IOVs from 20 to 2 μg showed that the chemiluminescent signal was predominantly due to the 190 kDa protein, MRP. From Figure 3.17.4.1 it can also be seen that DLKP IOVs express significant levels of MRP. As predicted from the levels of [^{3}H]-LtC_4 transport activity exhibited by DLKP relative to HL60-ADR derived IOVs (Section 3.13), DLKP IOVs contain significantly lower levels of MRP protein than found in HL60-ADR IOVs. DLKP-A10 derived IOVs also contain MRP but the levels present are lower than found in IOVs from DLKP. DLKP-A10 IOVs exhibit lower [^{3}H]-LtC_4 transporting abilities (initial rate of 0.6 ± 0.2 pmol per mg IOV protein per minute and maximum accumulation of 1.8 ± 0.3 pmol/mg) than found in the case of DLKP IOVs (initial rate of 0.8 ± 0.2 pmol per mg IOV protein per minute and maximum accumulation of 2.0 ± 0.3 pmol/mg). For this reason, one would have predicted from the [^{3}H]-LtC_4 transport characteristics of IOVs from the DLKP and DLKP-A10 cell lines, that DLKP-A10 IOVs possessed only slightly lower levels of MRP protein than DLKP derived IOVs. From Figure 3.17.4.1 it can be seen that the difference in MRP protein levels between DLKP and DLKP-A10 is larger than one would have assumed from the [^{3}H]-LtC_4 transport characteristics of these cell lines. The presence of the 116 kDa band in the DLKP, DLKP-A10 and HL60-ADR samples was not found to be present in a negative control blot performed using HL60-ADR IOVs.

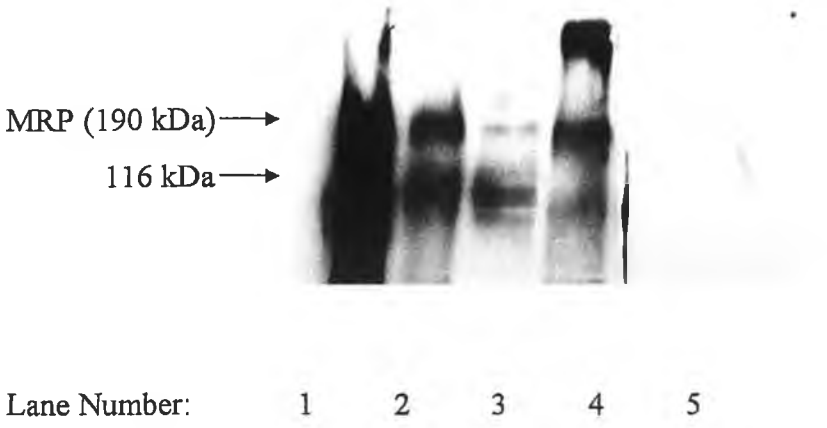

Figure 3.17.4.1: Western blot detection of MRP protein in IOVs isolated from the HL60-ADR (lanes 1 and 4), DLKP (lane 2) and DLKP-A10 (lane 3) cell lines. A quantity of 20 μg protein was loaded in each case with the exception of lane 4, where 2 μg HL60-ADR IOV protein was loaded. A negative control is shown in lane 5 where the nitrocellulose containing 20 μg HL60-ADR IOV protein was exposed to TBS instead of primary antibody.

3.18 MRP expression and LtC_4 transport characteristics in clonal populations isolated from the MDR cell line DLKP-A.

Heenan *et al.*, (1997) isolated 9 clonal subpopulations from the Pgp overexpressing cell line DLKP-A. The drug resistant DLKP-A cell line (approximately 250-fold resistant to adriamycin relative to the parental DLKP cells) overexpresses Pgp and possesses reduced levels of topoisomerase II (Topo II) relative to levels found in DLKP. The clonal subpopulations isolated from the DLKP-A cell line exhibit various levels of drug resistance. The drug resistance mechanisms responsible for this drug resistance have been thoroughly studied by Heenan *et al.*, (1997) and have been shown to primarily involve Pgp and Topo II. Until recently it was believed that DLKP did not express MRP protein, and this was also believed to be the case for DLKP-A and DLKP-A derived resistant clones. It is now known that DLKP expresses MRP and that this transporter is responsible for [^{3}H]-LtC_4 transport into IOVs from this cell line. The possible presence of MRP protein in DLKP-A derived clones was investigated by means of isolation of IOVs from the DLKPA2B, 11B, 6B and 5F clones of DLKP-A. These studies were carried out with two main objectives. Firstly these studies served as in investigation into MRP levels and activities in drug resistant variants of DLKP. Resistant variants of the DLKP cell line preferentially overexpress Pgp protein rather than MRP protein. This study therefore served as an investigation into the possibility that MRP protein expression would become down-regulated as resistance and Pgp expression developed. The second main objective of this study was to attempt to explain if MRP contributed to the findings of Heenan *et al.*, (1997) which showed that two clones (DLKPA6B and 11B) which had practically identical drug resistance profiles, possessed significant differences in drug resistance mechanisms.

As can be seen from Figure 3.18.1, IOVs isolated from DLKPA2B transported [^{3}H]-LtC_4 in an ATP dependent fashion with an initial rate of 0.5 ± 0.2 pmol/mg protein per minute with [^{3}H]-LtC_4 uptake saturated by 4 minutes at 1.5 ± 0.2 pmol/mg protein. DLKPA11B IOVs also transported [^{3}H]-LtC_4 in an ATP

dependent fashion (Figure 3.18.2) with an initial rate of 0.6 ± 0.3 pmol/mg protein/minute with [^{3}H]-LtC_4 uptake saturated by 3 minutes at 1.1 ± 0.2 pmol/mg protein. DLKPA5F derived IOVs exhibited relatively high levels of LtC_4 transport. [^{3}H]-LtC_4 transport occurred at an initial rate of 1.3 ± 0.2 pmol/mg protein/minute with saturation at 2.3 ± 0.2 pmol [^{3}H]-LtC_4 per mg protein, occurring after 2 minutes (Figure 3.18.3). DLKPA6B exhibited comparable levels of [^{3}H]-LtC_4 transporting abilities as was found in the case of DLKPA2B and 11B. The DLKPA6B derived IOVs transported [^{3}H]-LtC_4 with an initial rate of 0.7 ± 0.3 pmol LtC_4 per mg IOV protein per minute (Figure 3.18.4). Saturation in LtC_4 uptake by DLKPA6B IOVs occurred at a level of 1.0 ± 0.2 pmol/mg protein. The rate and LtC_4 accumulation data described above is the mean ± S.D. for duplicate determinations carried out using two separate IOV preparations from a given cell line.

As can be seen in Figure 3.18.5, IOVs from the DLKPA5F, 6B, 2B and 11B cell lines express MRP protein. The levels of MRP protein present in DLKP derived IOVs are comparable to the levels found in DLKPA5F IOVs. Substantial levels of MRP were also found to be present in DLKPA6B derived IOVs. In the case of IOVs from the DLKPA11B cell line, significant MRP protein expression was also found to be present but DLKPA2B-derived IOVs only expressed very low levels of this protein. The presence of the 116 kDa band in the case of all samples was not found to be present in a negative control blot performed using HL60-ADR, IOVs where the nitrocellulose was exposed to TBS instead of rat anti-MRP monoclonal antibody. A negative control with rat serum was not performed. No evidence was obtained to suggest that the 116 kDa was in any way specific for MRP or some degraded form of MRP and so was attributed to non-specific binding of the antibody to protein in the IOV preparations.

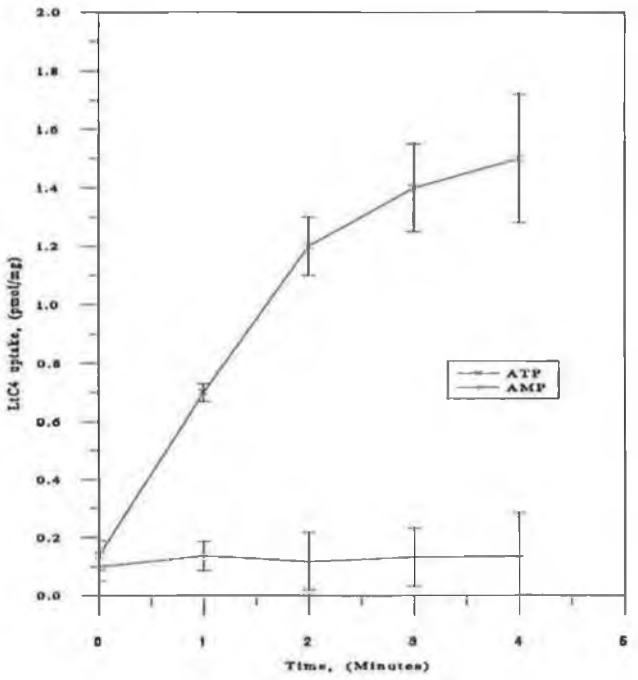

Figure 3.18.1: Time course of LtC_4 transport into IOVs isolated from the DLKPA2B cell line in the presence of ATP or AMP. Data has been corrected for non-specific binding of LtC_4 which occurred in the absence of ATP and AMP. Data points represent the means (± S.D.) of duplicate determinations.

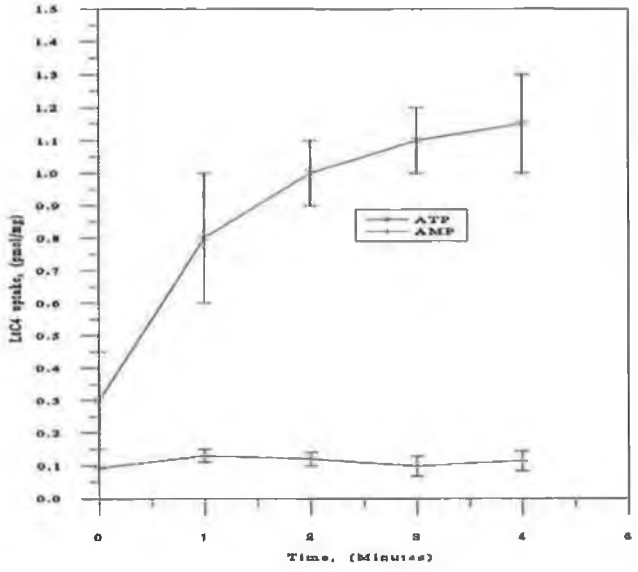

Figure 3.18.2: Time course of LtC_4 transport into IOVs isolated from the DLKPA11B cell line in the presence of ATP or AMP. Data has been corrected for non-specific binding of LtC_4 which occurred in the absence of ATP and AMP. Data points represent the means (± S.D.) of duplicate determinations

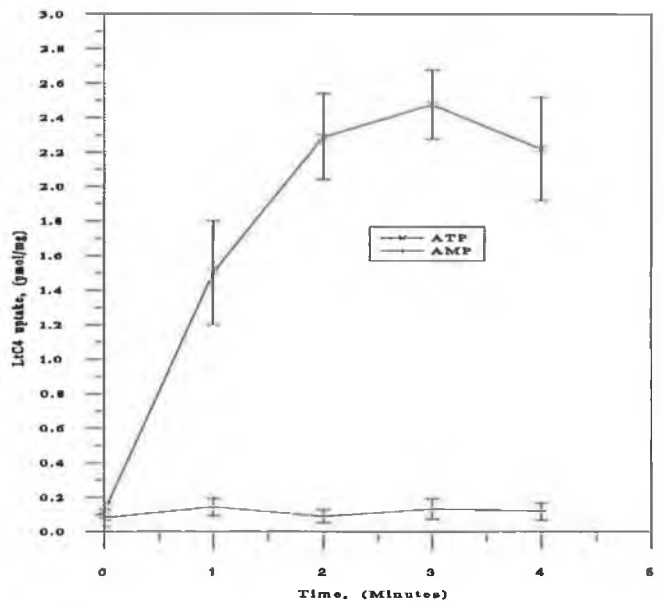

Figure 3.18.3: Time course of LtC_4 transport into IOVs isolated from the DLKPA5F cell line in the presence of ATP or AMP. Data has been corrected for non-specific binding of LtC_4 which occurred in the absence of ATP and AMP. Data points represent the means (± S.D.) of duplicate determinations.

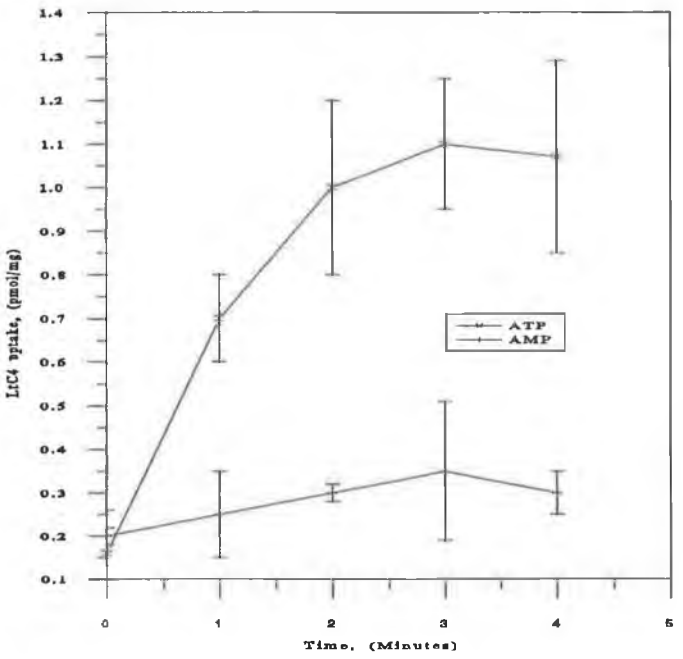

Figure 3.18.4: Time course of LtC_4 transport into IOVs isolated from the DLKPA6B cell line in the presence of ATP or AMP. Data has been corrected for non-specific binding of LtC_4 which occurred in the absence of ATP and AMP. Data points represent the means (± S.D.) of duplicate determinations.

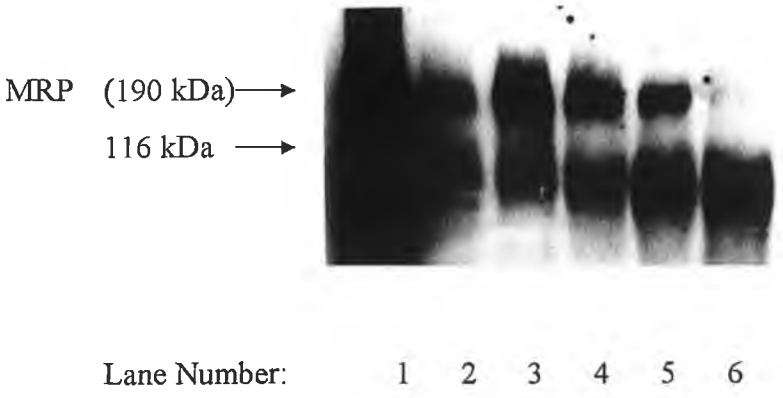

Figure 3.18.5: Western blot detection of MRP protein in IOVs isolated from the HL60-ADR (lane 1), DLKPA11B (lane 2), DLKP (lane 3), DLKPA5F (lane 4), DLKPA6B (lane 5) and DLKPA2B (lane 6) cell lines.

4.0 Discussion

4.1 General discussion

In cancer chemotherapy, tumors often develop resistance to the chemotherapeutic drugs with which they are being treated. Combination chemotherapy was initially introduced in an attempt to overcome this situation but it was found that in certain cases, tumors could develop which were cross-resistant to a range of structurally and functionally unrelated cytotoxic drugs. Multidrug resistance (MDR) is the term given to the phenomenon in which cancer cells become resistant to a range of chemotherapeutic agents with dissimilar structures and mechanisms of action. Tumor cells can be selected *in vitro* for resistance to a particular cytotoxic drug by continual or pulsed exposure to the drug. In many cases these cells develop the multidrug resistance phenotype described above. MDR can be mediated by overexpression of drug transporter molecules such as P-glycoprotein (Pgp). The Pgp molecule is a 170 kDa plasma membrane protein with twelve transmembrane domains and two intracellular ATPase active sites (Section 1.5). It is an energy dependent broad specificity pump with the ability to transport drugs such as the vinca alkaloids (including vinblastine and vincristine), epipodophyllotoxins (such as VP-16), anthracyclines (including doxorubicin and daunorubicin), taxanes (such as Taxol) and miscellaneous drugs such as mitoxantrone and actinomycin D, (Fisher *et al.*, 1996).

The calcium channel blocker verapamil was the first agent shown to be an effective inhibitor of Pgp-mediated drug efflux (Section 1.5.1.4). A number of modulators of Pgp activity have subsequently been developed and a selection of these modulators have been studied in clinical trials, (Fisher and Sikic, 1995). The results of these trials have been relatively disappointing as it has been found that the currently available Pgp modulators lack the potency to completely reverse the MDR phenotype at clinically achievable concentrations. One of the major factors in the failure of Pgp modulators as potential drug resistance circumventing agents is the fact that these compounds can cause extreme toxic side effects at the high doses needed for inhibition of the drug efflux activity of Pgp.

Overexpression of Pgp by tumor cells is by no means the only mechanism by which tumor cells can develop multidrug resistance. Cells that overexpress Pgp may simultaneously possess other resistance mechanisms including alterations in the nuclear enzyme topoisomerase II (Topo II) or expression of additional drug efflux pumps such as the multidrug resistance-associated protein, (MRP). Overexpression of MRP is sufficient to confer multidrug resistance on cells *in vitro,* (Cole *et al.*, 1992). MRP is a 1531 amino acid, 190 kDa glycoprotein that is overexpressed in a number of non-Pgp expressing cell lines (Section 1.5.2). It has also been detected at the protein level in various normal human tissues such as the lung, bladder, stomach, colon, adrenal gland, ovary, pancreas, heart, placenta, kidney, spleen and liver, (Cole *et al.*, 1992; Zaman *et al.*, 1993). Only a limited number of efficient modulators of MRP activity are currently available (Section 1.5.2.4). The tyrosine kinase inhibitor genistein has been demonstrated to modulate daunorubicin accumulation in human small cell lung cancer cells, (Versantvoort *et al.*, 1994). The bisindoylmaleimide protein kinase C inhibitor GF109203X and the leukotriene D_4 receptor antagonist MK571 have also been shown to modulate MRP activity, (Gekeler *et al.* 1995a, b). DL-buthionine-sulphoximine, (BSO) has also been shown to modulate MRP-mediated multidrug resistance. The γ-glutamyl-cysteine synthase enzyme is strongly inhibited by BSO, and inhibition of this enzyme can cause a depletion of cellular glutathione levels. Other compounds such as the ATPase inhibitor sodium orthovanadate and the organic anion transport inhibitor, probenecid, have also been shown to have MRP modulating activity, (Twentyman and Versantvoort, 1996).

The clinical relevance of the MRP molecule in multidrug resistance in cancer has not been fully elucidated, but it has been shown that MRP can be present in abnormally elevated levels in various forms of chemotherapy resistant leukaemias (Zhou *et al.*, 1995). In addition, even if MRP is clinically important, it is not known if the MRP-modulating agents listed briefly above could be used to modulate MRP-mediated resistance *in vivo*. It is possible that these compounds could cause serious side effects particularly when added at the high concentrations required for modulation of MRP activity (Section 1.5.2.4).

This thesis describes the use of NSAIDs as modulators of the MRP drug efflux pump in drug sensitive MRP-expressing cell lines. NSAIDs are among the most commonly prescribed medicines and have been comprehensively characterised *in vivo*. For this reason these compounds are ideal candidates for use in practically any chemotherapeutic regime. The NSAIDs identified in *in vitro* toxicity screening assays as MRP-modulating agents were used at clinically relevant concentrations. This should be reflected in the concentrations of NSAID used for modulation of MRP *in vivo* being relatively non-toxic for normal cells. Modulation of MRP-mediated efflux of drug *in vivo* may cause similar side effects to those seen in trials involving modulation of Pgp mediated drug efflux due to the fact that MRP, like Pgp, is expressed in normal tissues. In addition, the therapeutic advantage of modulating MRP activity in tumors *in vivo* is not fully clear (Twentyman and Versantvoort, 1996). The use of non-toxic doses of well characterised agents such as NSAIDs, would be an effective tool with which to discover if modulating MRP activity could be significantly advantageous in cancer chemotherapy.

The illustration of NSAID-mediated chemotherapeutic drug toxicity enhancement in drug sensitive cell lines such as DLKP, confirms the importance of the activity of drug efflux pumps in these cell lines. The identity of these efflux pumps and their relevance in drug resistance in DLKP was investigated in detail in this thesis. The isolation of plasma membrane-derived inside out vesicles (IOVs) provided evidence for the first time that the DLKP cell line expresses significant levels of MRP. The existence of MRP in drug resistant variants of the DLKP cell line could potentially explain the fact that the drug accumulation and resistance characteristics of many of these cell lines cannot be completely explained solely by the levels of P-glycoprotein and topoisomerase II present. The DLKP cell line preferentially develops Pgp-mediated drug resistance during drug selection. The variation in MRP levels and activities during the development of Pgp mediated drug resistance in drug resistant variants of DLKP was investigated in order to see if the expression and activity of MRP became down-regulated as Pgp expression and drug resistance developed. The IOV assay system was also used to investigate the role of MRP in the unusual drug accumulation characteristics found in a drug resistant variant of DLKP, DLKP-A10.

4.2 *In vitro* toxicity assay screening with indomethacin and sulindac in combination with various chemotherapeutic drugs in the A549 cell line.

Initial screening experiments in the A549 cell line demonstrated that the NSAIDs, indomethacin and sulindac, had the ability to enhance the toxicity of adriamycin (Tables 3.2.2 and 3.2.3). In an attempt to characterise if this effect was limited only to adriamycin, *in vitro* toxicity assay screening was expanded to include a variety of chemotherapeutic drugs from the various therapeutic categories (Table 3.2.1). It was found that indomethacin and sulindac enhanced the toxicity of adriamycin, daunorubicin, epirubicin, vincristine and VP-16. This indicated that there were specific structural or mechanistic characteristics required for a drug to have toxicity enhanced in the presence of indomethacin or sulindac. All of the drugs listed above, which were found to have toxicity enhanced in the presence of indomethacin or sulindac, are believed to be substrates for Pgp and MRP, (Cole *et al.*, 1994). Taxol is also a substrate of Pgp, but is not believed to be a high affinity substrate of MRP. This indicated that only MRP had a role in the toxicity enhancement effect, as only the toxicity of MRP substrate drugs was enhanced in the presence of indomethacin or sulindac.

In order to partially validate this theory, the heavy metals sodium arsenate and potassium antimony tartrate were tested in combination with indomethacin and tolmetin in the A549 cell line (see Section 3.4 for details). These heavy metals are normally toxic to cells but they can also serve as substrates for MRP-mediated transport, (Cole *et al.*, 1994). It was found experimentally that sodium arsenate toxicity was enhanced in the presence of indomethacin and tolmetin (an NSAID with the ability to enhance the toxicity of a suitable cytotoxic drug as described in Section 3.3). This suggested that these NSAIDs inhibited the transport of the heavy metal out of the cell and so enhanced the level of cell kill achieved by a given concentration of sodium arsenate. This result was complicated by the fact that the toxicity of another heavy metal, potassium antimony tartrate, was unaltered by either indomethacin or tolmetin treatment. From Tables 3.4.3 and 3.4.4 it can be seen that there was an

inconsistency in the level of cell kill achieved between Tables 3.4.3 and 3.4.4 in the presence of all control concentrations of potassium antimony tartrate used. This suggests that further investigations with potassium antimony tartrate would be required in order to conclusively answer if the toxicity of this compound was affected by the presence of indomethacin or tolmetin. The fact remains that sodium arsenate toxicity was enhanced in the presence of indomethacin or tolmetin. This suggests that the toxicity of an MRP substrate (not necessarily a chemotherapeutic drug) can be enhanced in the presence of a suitable NSAID.

4.3 *In vitro* toxicity assay screening with NSAIDs in combination with adriamycin and vincristine in the A549 cell line.

The data from the *in vitro* toxicity assay drug screening experiments (Section 3.2), indicated that MRP-mediated transport was a critical factor in the NSAID-mediated chemotherapeutic drug toxicity enhancement effect. The precise nature of the relationship between MRP-mediated drug transport and NSAID treatment was not clear from the *in vitro* toxicity assay screening data. It also remained to be discovered if all NSAIDs had this toxicity enhancing ability or if there were specific structural or mechanistic characteristics required for an NSAID to have the toxicity enhancing abilities possessed by indomethacin and sulindac. For this reason an elaborate screen of a wide range of NSAIDs was performed (Section 3.3).

It was found that acemetacin, indomethacin, mefenamic acid, sulindac, sulindac sulfide, sulindac sulfone, tolmetin and zomepirac enhanced the toxicity of adriamycin and vincristine when present at an appropriate relatively non-toxic concentration. All but one of the NSAIDs found to have drug toxicity enhancing abilities, have common structural characteristics and were classified as heteroarylacetic acids by Korolkovas, (1988). The remaining NSAID with toxicity enhancing abilities, mefenamic acid is a fenamate NSAID. Not all heteroarylacetic acid NSAIDs possess toxicity enhancing abilities. For example, the heteroarylacetic acid, etodolac, did not enhance the toxicity of adriamycin or vincristine (Table 3.3.1).

The potential for development of additional toxicity enhancing NSAID analogues is quite encouraging. From the above list of toxicity enhancing NSAIDs, only indomethacin, mefenamic acid, sulindac and tolmetin were initially found to possess this ability. Acemetacin is a pro-drug of indomethacin, sulindac sulfide and sulfone are analogues of sulindac and zomepirac is a structural analogue of tolmetin. Screening of these analogues demonstrated that these compounds also possessed chemotherapeutic drug toxicity enhancing abilities. This indicates that it may be possible to develop NSAID analogues from a suitable NSAID which may not necessarily possess potent anti-inflammatory or analgesic properties, but which may actually possess chemotherapeutic drug toxicity enhancing abilities.

There did not initially appear to be any mechanistic similarities between the activities of these specific toxicity-enhancing NSAIDs. All possessed varied abilities to inhibit prostaglandin H synthase (cyclooxygenase) activity and so prostaglandin synthesis. The level of this ability varied from high levels of inhibitory activity, (indomethacin) to no inhibitory activity, (sulindac sulfone). In addition, NSAIDs with potent inhibitory activities on the cyclooxygenase enzyme such as flurbiprofen did not possess the ability to enhance adriamycin or vincristine toxicity (Table 3.3.1). There did not appear to be any relationship between the ability of an NSAID to enhance toxicity and the ability of that NSAID to preferentially inhibit either the PGHS-1 or PGHS-2 isozymes. For example indomethacin preferentially inhibits PGHS-1 (IC_{50} ratio of inhibition of PGHS-2 to PGHS-1 of 9:1). Some NSAIDs which do not possess drug toxicity enhancing abilities (such as ibuprofen) have a similar preference for PGHS-1 (IC_{50} ratio of inhibition of PGHS-2 to PGHS-1 of 11:1). These ratios were developed by Gierse *et al.*, (1995) in an *in vitro* cell-free enzyme system. The PGHS-1/PGHS-2 ratios found for intact cells (Vane and Botting, 1996) show similarities to ratios developed using the cell free enzyme systems of Gierse *et al.*, (1995). The other toxicity enhancing NSAIDs show varying levels of preferential inhibition of PGHS isozymes but no correlation was found between preferential inhibition of PGHS-1 or PGHS-2 and ability of a given NSAID to enhance drug toxicity. Dexamethasone has a variety of effects in cells amongst which is the ability to inhibit PGHS-2 protein expression with little effect on PGHS-1. It was found experimentally that

dexamethasone did not possess drug toxicity enhancing abilities (Table 3.7.1). This further indicated that the elimination of PGHS activity was not sufficient to achieve the toxicity enhancement effect

4.4 *In vitro* toxicity assay screening with various NSAIDs for identification of NSAIDs with an ability to enhance the toxicity of adriamycin and vincristine in the DLKP cell line.

The *in vitro* toxicity assay screening results obtained using A549 were compared to results obtained in a second drug sensitive cell line, DLKP. It was found that as was the case in the A549 cell line, only acemetacin, indomethacin, mefenamic acid, sulindac, tolmetin and zomepirac enhanced the toxicity of adriamycin or vincristine in DLKP cells (Section 3.5). The ability of sulindac sulfide and sulfone to enhance adriamycin and vincristine toxicity was not investigated in the case of the DLKP cell line. The results indicated that the toxicity enhancement effect was not isolated to the A549 cell line. The NSAID-mediated chemotherapeutic drug toxicity enhancement effect must for this reason have involved a fundamental drug resistance mechanism found in drug sensitive cell lines. DLKP can be cultured in the presence of serum or alternatively in a fully defined serum free medium formulation, (Section 2.5). Screening was carried out under serum free conditions in order to investigate the possibility that the toxicity enhancement effect was serum or growth factor dependent. It was found that even under serum free conditions, adriamycin toxicity was enhanced in the presence of indomethacin, sulindac and tolmetin. Adriamycin toxicity was unaffected by naproxen treatment under serum free conditions, (Table 3.5.2).

4.5 Investigation into the role of the arachidonic acid cascade in NSAID-mediated chemotherapeutic drug toxicity enhancement.

The NSAID-mediated toxicity enhancement effect was believed to be dependent on some form of inhibitory activity being exerted on the drug efflux activity of the MRP pump. The manner in which NSAIDs influenced MRP-mediated drug transport was not obvious from the *in vitro* toxicity assay screening results. From initial screening studies it was obvious that the effect was not totally dependent on PGHS inhibition as relatively potent PGHS inhibitors such as flurbiprofen did not enhance drug toxicity. As discussed in Section 4.3, there was also no relationship between the extent of preferential inhibition of PGHS isozymes and the ability of an NSAID to enhance drug toxicity.

A number of reports have described an influence of cytotoxic drugs on the arachidonic acid cascade and this topic will be dealt with in more detail in Section 4.6. It is known that MRP is a transporter of certain eicosanoids such as LtC_4 and prostaglandin conjugates, (Jedlitschky *et al.*, 1996; Ishikawa, 1992), and that eicosanoids such as the cytotoxic prostaglandin PgA_1 can interfere with MRP activity, (Hollo *et al.*, 1996). Treatment of cells with a suitable NSAID could possibly enhance the production of an eicosanoid which may act as a substrate for MRP. Inhibition of PGHS could cause a decrease in prostaglandin production and a resultant increase in the synthesis of other eicosanoids due to increased arachidonic acid availability in the absence of PGHS activity. If these eicosanoids were substrates for MRP, then NSAID treatment would result in competition for MRP-mediated transport between the cytotoxic drug and the eicosanoid. This would ultimately result in an increase in cellular drug accumulation and an enhancement of chemotherapeutic drug toxicity. This theory was weakened by the fact that the potent PGHS inhibitors such as flurbiprofen did not enhance the toxicity of the MRP substrate drugs in A549 (Table 3.3.1). A number of related studies were carried out in an attempt to conclusively discount all possibilities of even a minor involvement of eicosanoids and PGHS in the toxicity enhancement effect. The first of these studies (results provided in Section 3.6) involved an attempt at reversing the

toxicity enhancement effect by exogenous addition of prostaglandins. A549 predominately produces PgE_2 and so if NSAIDs had any significant effects on eicosanoid production in this cell line, it should reflect partially in an alteration in PgE_2 production. If the toxicity enhancement effect was not reversed by PgE_2 addition, it indicated simply that this particular eicosanoid was not critical for the effect to occur. A related investigation in this area involved the use of non-NSAID inhibitors of the arachidonic acid cascade. The major drawback of the study described above involved the fact that it examined only one particular eicosanoid namely PgE_2. Inhibition of PGHS by non-NSAID agents could cause alterations in the eicosanoid profile formed by a cell, in a manner comparable to that achieved by NSAIDs. In this way it would be possible to investigate if alterations in the eicosanoid profile formed by a cell as a result of PGHS inhibition was involved in NSAID-mediated toxicity enhancement. This study involved using inhibitors of critical steps of the arachidonic acid cascade such as the liberation of arachidonic acid by phospholipase A_2. If PGHS inhibition and so eicosanoid synthesis inhibition was important for drug toxicity enhancement, then it would be probable that total elimination of eicosanoid synthesis by phospholipase A_2 inhibition could also have an influence on drug toxicity. The final study in this area involved the use of sulindac sulfide (a potent PGHS inhibitor) and sulindac sulfone (devoid of significant PGHS inhibitory activity) in *in vitro* toxicity assay screening experiments (Section 3.9). If an NSAID analogue without PGHS inhibitory activity could enhance drug toxicity, this would provide conclusive proof that the toxicity enhancement effect was totally independent of NSAID-mediated inhibition of PGHS.

4.5.1 Investigation into the potential reversal of NSAID-mediated drug toxicity enhancement as a result of exogenous PgE_2 or PgD_2 addition.

The results from these experiments are provided in Section 3.6. As can be seen from Table 3.6.1, PgE_2 or PgD_2 were unable to reverse the NSAID-mediated toxicity enhancement effect irrespective of whether adriamycin or vincristine were used in combination with indomethacin or sulindac. All experiments were repeated using a range of prostaglandin concentrations, which ranged from relatively low (60 ng/mL) to high PgE_2 concentrations (up to 0.60 μg/mL). A549 cells in the stimulated state can produce 150 pg PgE_2 per 10^5 cells per mL, (Croxtall and Flower, 1994). Obviously the concentrations of PgE_2 added exogenously in these experiments are significantly higher than the concentrations to which A549 cells are exposed even after stimulation with an agent such as IL-1β. Lowering the concentrations of exogenously added PgE_2 or PgD_2 to a concentration of 150 pg/mL was also found to be without effect on NSAID mediated toxicity enhancement (data not shown). PgD_2 was included as a negative control for these experiments as it is not normally produced by A549 cells under stimulated or unstimulated conditions (Hubbard *et al.*, 1988). These results indicate that changes in extracellular levels of PgE_2 have little if any influence on NSAID-mediated toxicity enhancement. These results were later confirmed by the results found by PgE_2 ELISA, (Section 3.8).

4.5.2 *In vitro* toxicity assay screening with non-NSAID inhibitors of the arachidonic cascade

These experiments served to investigate if non-NSAID inhibitors of PGHS could also cause an enhancement of drug toxicity. Inhibitors of critical steps in the arachidonic cascade were combined with cytotoxic drugs in an attempt to see if altering the eicosanoid profile produced by the cells could also potentially contribute to enhancing drug toxicity. From Table 3.7.1 it can be seen that dexamethasone was screened in combination with adriamycin and vincristine. This glucocorticoid has a wide variety of effects in cells including inhibition of PGHS-2 protein synthesis (Masferrer *et al.*, 1994). It was found that dexamethasone did not affect the toxicity of adriamycin in toxicity assay screening experiments. This indicated that elimination of PGHS-2 activity was not sufficient for achievement of enhancement of adriamycin toxicity. The limiting step in the arachidonic acid cascade involves the liberation of arachidonic acid from the cell membrane as a result of cytosolic phospholipase A_2, (PLA_2) activity. Interference with the activity of this enzyme can cause a total block in eicosanoid synthesis by the cells. A variety of PLA_2 inhibitors were used in screening experiments, namely 4-bromophenacyl bromide, manoalide and quinacrine, and none of these inhibitors had any effects on adriamycin toxicity (Table 3.7.1). A dual 5- and 12-lipoxygenase inhibitor, esculetin, was also screened in combination with adriamycin and vincristine. The 5-lipoxygenase enzyme is the critical enzyme in the pathway responsible for leukotriene synthesis (Section 1.3). Leukotrienes such as LtC_4 can be transported out of cells by molecules such as MRP but inhibition of this enzyme had no effect on adriamycin toxicity in the A549 cell line. This cell line is not believed to express significant levels of 5-, 12- or 15-LOX, (Claria *et al.*, 1996) and so these findings were as expected.

4.5.3 Variations in PgE_2 levels produced by A549 cells during the NSAID mediated toxicity enhancement effect.

Quantification of the levels of PgE_2 produced by A549 in the presence of an NSAID and cytotoxic drug was performed using a PgE_2 ELISA assay. From Table 3.8.1 it can be seen that there were no significant alterations in PgE_2 levels produced by A549 cells in the presence of adriamycin or Taxol alone or in the presence of these drugs in combination with indomethacin or flurbiprofen. All compounds were added at a concentration equivalent to the concentration used for screening in the *in vitro* toxicity assays. Adriamycin toxicity in A549 cells is known to be enhanced in the presence of indomethacin but not flurbiprofen (Table 3.3.1). Taxol toxicity is known to be unchanged by indomethacin treatment (Table 3.2.1). There was no alteration in the levels of PgE_2 formed by A549 cells treated with any of the agents listed above. The PGHS-2 stimulating compound IL-1β, served as a positive control for these experiments and caused significant stimulation of PgE_2 synthesis, (Table 3.8.1). These results demonstrated that under the conditions examined, there were no unexpected alterations in the levels of PgE_2 produced by A549 cells as a result of NSAID and drug treatment. These results agree with the results obtained from the prostaglandin reversal experiments, (Section 4.5.1).

4.5.4 *In vitro* toxicity assay screening with sulindac sulfide and sulfone in combination with adriamycin and vincristine in the A549 cell line.

As described earlier, sulindac sulfide is a potent PGHS inhibitor but sulindac sulfone is devoid of PGHS inhibitory activity. Section 3.9 provides the details of the screening experiments with these compounds. It was found that these sulindac analogues enhanced the toxicity of both adriamycin and vincristine to an extent comparable to that found in the case of sulindac (Tables 3.2.3 and 3.2.9). This result with sulindac sulfone is highly significant as it illustrates that a compound which is practically devoid of PGHS inhibitory activity can enhance the toxicity of adriamycin and vincristine. This obviously implies that the potential ability of NSAIDs to inhibit PGHS is totally

unrelated to their ability to enhance drug toxicity. This result, when combined with the results from the studies detailed in Sections 4.5.1 to 4.5.3 conclusively demonstrated that the arachidonic acid cascade had no involvement in the NSAID-mediated toxicity enhancement effect.

4.6 Effect of chemotherapeutic drug treatment on PGHS isozyme expression and PgE_2 production in the A549 cell line.

The results from the experiments discussed in Section 4.5 demonstrated that drug treatment had no effect on PgE_2 production by A549 cells under the specific conditions examined. An investigation was subsequently conducted into potential relationships between the arachidonic acid cascade and chemotherapeutic drug treatment under a variety of conditions of tumor cell growth. It is widely known that tumor cells *in vivo* and *in vitro* can produce abnormally high levels of eicosanoids (Section 1.3.1). In many cases, tumors *in vivo* are treated with cytotoxic drugs but surprisingly few studies have examined the relationship between chemotherapeutic drug treatment and eicosanoid production. Tisdale, (1977) described the effects of a number of anti-tumor agents on the activity of PGHS from sheep seminal vesicles. It was shown that chlorambucil inhibited PgE_2 and $PgF_{2\alpha}$ synthesis by PGHS in a cell free system. A number of reports have indirectly carried out studies into the area of eicosanoids in cancer chemotherapy. One such study showed that an advanced human ovarian cancer which was unresponsive to chemotherapy, produced more eicosanoids than tumors responding to chemotherapy, (Bauknecht *et al.*, 1985). The involvement of PGHS in the bioactivation of various xenobiotics is discussed in Section 1.3.1 but there is some evidence to suggest that PGHS activity can be involved in bioactivation of a select number of chemotherapeutic drugs. Studies by Smith and Kehrer, (1991) suggested that PGHS activity could be involved in the bioactivation of cyclophosphamide. Lau *et al.*, (1987) investigated arachidonic acid metabolism in a variety of human lung tumor cell lines. This group found that two well differentiated non-small cell lung adenocarcinoma cell lines (NCI-H322 and NCI-H358) were shown to be relatively sensitive to the cytotoxic effects of mitoxantrone. These cell lines were shown to

possess PGHS activity which resulted in PgE_2 formation. Two small cell lung carcinoma cell lines (NCI-H69 and NCI-H128) were also analysed and shown to be devoid of PGHS activity and PgE_2 synthetic ability. These cell lines which lacked detectable PGHS activity were shown to be unaffected by mitoxantrone treatment. This group also reported that PGHS present in ram seminal vesicular microsome preparations activated mitoxantrone to a highly reactive metabolite which had a strong affinity for binding to cellular macromolecules. Hamuro *et al.*, (1996) reported on PgE_2 production by a number of sublines derived from a cyclophosphamide and lentinan (an anti-tumor polysaccharide used in immunotherapy) resistant murine fibrosarcoma. These sublines exhibited cyclophosphamide and lentinan resistance relative to the parental cells used to establish the original tumor in B10.D2 mice. In addition, these sublines were resistant to 5-fluorouracil. All chemo-immunotherapy resistant cell lines showed enhanced PgE_2 synthesis relative to the parental cells. Observations of this nature have not been limited to chemotherapy or chemo-immunotherapy. Sochanowicz and Szumiel, (1996) reported that increased PgE_2 synthesis occurred in radiation resistant murine leukaemic lymphoblasts following radiation and ionophore stimulation. This PgE_2 synthesis was not found to occur in the case of related radiation sensitive murine leukaemic lymphoblasts. This suggested that PgE_2 was involved in cellular radiosensitivity modification which caused resistant cells to be protected against radiation damage by PgE_2.

In order to investigate if chemotherapeutic drug treatment had any influence on the arachidonic acid cascade in human lung cell lines, a number of experiments were carried out using the A549 cell line. This cell line expresses negligible levels of PGHS-1 and is easily induced to express PGHS-2 and produce PgE_2 (Asano *et al.*, 1996). If there was an alteration in the level of eicosanoid production as a result of drug treatment, one would assume that in A549, the levels of PgE_2 and PGHS-2 would vary accordingly. Three related experiments were carried out using the A549 cell line. The first experiment investigated if there were any differences in PGHS-2 expression or PgE_2 production over a 24 hour time period as a result of treatment of cells with a relatively high dose of adriamycin (0.35 μg/mL). The second experiment involved treating cells with a variety of adriamycin concentrations for a period of 24 hours. The

final experiment served as an investigation into the variations in the levels of PGHS-2 and PgE_2 during treatment with a range of VP-16 concentrations.

The results and experimental details of the first experiment are provided in Table 3.10.1. A high concentration of adriamycin (0.35 μg/mL) was used which was non-toxic over 24 hours but which caused approximately 85% toxicity over a period of 7 days. It was found that there were no significant alterations in PGHS-2 protein expression or PgE_2 production during adriamycin treatment even after a period of 24 hours (Table 3.10.1 and Figure 3.10.1). The time period of 24 hours was found to be sufficient for PGHS-2 and PgE_2 (approximately 490 pg PgE_2 per 10^6 cells) synthesis induction, as demonstrated by cells treated with IL-1β (Figure 3.10.1). As this time period was sufficient for PGHS-2 and PgE_2 synthesis induction, a study was carried out to investigate if varying the concentration of adriamycin had any effect on PGHS-2 protein or PgE_2 levels. From Table 3.10.2, it can be seen that concentrations of adriamycin which caused 60-85% cell kill (0.25 and 0.35 μg/mL adriamycin respectively) over 7 days but which were non-toxic over 24 hours, had no significant effects on PGHS-2 protein synthesis induction or PgE_2 synthesis. Combining adriamycin treatment with IL-1β treatment resulted in variable non-significant effects on PgE_2 synthesis by A549 cells. The variation in PgE_2 levels formed by A549 in the presence of adriamycin and IL-1β was a result of variations in PgE_2 synthesising abilities of sub-populations of A549 cells. It was not a result of adriamycin influencing PGHS-2 activity in any way. From Figure 3.10.2 it was obvious that PGHS-2 levels were unchanged in the presence of IL-1β and adriamycin (at all concentrations analysed) from the levels present in IL-1β treated A549 cells.

The precise mechanism by which the epipodophyllotoxin, VP-16 exerts cytotoxic effects is unclear (Section 1.2.3). Loike and Horwitz, (1976) reported that the presence of a free 4'-OH group in the phenolic ring of VP-16 is essential for VP-16 induced DNA damage. It is believed that the metabolic activation of VP-16 occurs with the formation of a phenoxyl radical and ultimate conversion to a VP-16 quinone derivative, (Yalowich *et al.*, 1996). This bioactivation of VP-16 is critical for VP-16

and topoisomerase II induced DNA damage, and can be catalysed in part by the peroxidase activity of PGHS, (Haim *et al.*, 1987). For this reason, an investigation was conducted into the influence of VP-16 treatment on PGHS-2 expression and PgE_2 formation in A549 cells (Table 3.10.3 and Figure 3.10.3). From this data it can be seen that treatment of A549 cells with concentrations of VP-16 which can cause 70, 40 and 20% toxicity over a period of 7 days (2.5, 0.5 and 0.1 μg/mL VP-16 respectively) had no significant effects on PgE_2 synthesis or PGHS-2 expression after 24 hours. IL-1β treated A549 cells produced high levels of PGHS-2 and PgE_2. Co-treatment of cells with VP-16 (2.5 or 0.5 μg/mL) and IL-1β was found to have variable effects on PgE_2 synthesis. As was the case for adriamycin (Table 3.10.2), these effect were not VP-16 specific but were simply a result of variations in PgE_2 synthesising abilities of A549 cells. In summary, these experiments demonstrated that cytotoxic drug treatment (either with adriamycin or VP-16) had no influence on the levels of PgE_2 or PGHS-2 protein synthesis by the human lung adenocarcinoma cell line, A549.

4.7 Investigation into PGHS isozyme expression and PgE_2 production in DLKP and VP-16 selected drug resistant variants

Although the arachidonic acid cascade is reasonably well characterised for A549, this is not the case for the human squamous cell carcinoma cell line, DLKP. Drug selected multidrug resistant variants have been selected from DLKP, (Clynes *et al.*, 1992). Two such variants, DLKP-VP3 and DLKP-VP8 were selected by culturing the drug sensitive cell line, DLKP in gradually increasing concentrations of VP-16, (Heenan, 1994). These drug resistant variants are sufficiently resistant to grow in concentrations of 3 and 8 μg/mL VP-16 respectively. The IC_{50} values for adriamycin in the DLKP, DLKP-VP3 and DLKP-VP8 cell lines are 0.02, 1.30 and 3.90 μg/mL respectively. The DLKP-VP3 and DLKP-VP8 cell lines were used in a series of experiments to investigate if drug selected resistant cells showed alterations in their eicosanoid synthesising abilities relative to the parental drug sensitive DLKP cells (Section 3.11). The VP-16 selected resistant variants were chosen for analysis in preference to resistant variants selected with a different drug because of the potential role of PGHS in VP-16 activation (Section 4.6). If cells were selected for VP-16 resistance and if PGHS was involved in the activation of VP-16 to the form of the drug responsible for DNA damage, then cells which were resistant to VP-16 could potentially possess low levels of PGHS protein. Alternatively PGHS activity could be in some way lower in VP-16 resistant cells than found in the parental drug sensitive cells. Either of these possibilities could result in a reduction in PGHS catalysed VP-16 activation in resistant cells and so permit survival of these cells in relatively high concentrations of VP-16. This effect could only be a relatively minor contributor to the drug resistance levels of these cell lines. Heenan, (1994) demonstrated that DLKP-VP3 and DLKP-VP8 possess elevated levels of Pgp and reduced levels of Topo II protein relative to DLKP cells. Due to overexpression of the drug efflux pump Pgp, these resistant cells would accumulate low levels of drug relative to their sensitive counterparts. Reduced levels of Topo II would result in a reduction in efficacy of VP-16 induced DNA damage and cell kill. For this reason, if VP-16 resistant cells overexpressed Pgp and possessed reduced levels of Topo II, the contribution of reduced levels of PGHS or low activity PGHS, to VP-16 resistance may be relatively small. Drug resistance is often dependent

upon contributions from various drug resistance mechanisms. Even though a given cell may overexpress Pgp and possess reduced levels of Topo II, alterations in PGHS levels or activities could potentially be critical factors contributing in some way to this drug resistance.

As the eicosanoid synthesising capacity of DLKP had not been previously characterised, it was decided that as for A549, the levels of PGHS-2 protein expression and PgE_2 production would be studied. The PGHS-2 isozyme and PgE_2 are expressed in a large percentage of human lung cell lines (Asano *et al.*, 1996; Hubbard *et al.*, 1988) and so the levels of PGHS-2 and PgE_2 produced by DLKP and VP-16 drug resistant variants were examined. No other aspects of the arachidonic acid cascade were examined. As can be seen from Table 3.11.1 and Figure 3.11.1, A549 cells treated with IL-1β produced high levels of PgE_2 (approximately 205 pg/10^6 cells) and PGHS-2 protein. DLKP did not produce significant levels of PgE_2 or express PGHS-2 protein during IL-1β treatment. This result is quite significant with relevance to the NSAID mediated toxicity enhancement effect. If DLKP does not express PGHS-2, this indicates that NSAIDs enhance drug toxicity in DLKP independently of any effects on this PGHS isozyme. From Figure 3.11.1 and Table 3.11.1 it can be seen that the DLKP-VP3 and DLKP-VP8 cell lines did not differ from DLKP in their PGHS-2 expressing or PgE_2 synthesising abilities in the presence of IL-1β. This illustrated that VP-16 selected cells do not express PGHS-2 as a result of IL-1β treatment.

The DLKP-VP3 and DLKP-VP8 cell lines have been shown by Heenan, (1994) to be resistant to VP-16 (58 and 100-fold more resistant than DLKP) and adriamycin (90 and 270-fold more resistant than DLKP). Interestingly these VP-16 selected cells developed a higher level of resistance to adriamycin than to VP-16. Both DLKP-VP3 and DLKP-VP8 cell lines expressed higher protein levels of Pgp than the parental DLKP cell line. In addition, these resistant cells expressed less Topo II than DLKP. Relatively surprisingly, DLKP-VP3 cells expressed higher levels of Pgp and accumulated less adriamycin in drug accumulation experiments than the DLKP-VP8 cell line (Heenan, 1994). The higher level of adriamycin resistance in DLKP-VP8

(approximately 3 fold higher than found for DLKP-VP3) was explained by Heenan, (1994) as being partially a result of lower levels of Topo II in DLKP-VP8 relative to DLKP-VP3. For this reason, in the presence of the Topo II poison adriamycin, DLKP-VP8 cells could survive high intracellular adriamycin concentrations because of the low levels of Topo II possessed by this cell line. DLKP-VP3 cells accumulate lower intracellular levels of adriamycin than DLKP-VP8 because of high levels of Pgp expression. These DLKP-VP3 cells are more sensitive to this relatively low intracellular adriamycin concentration than DLKP-VP8 cells are to a relatively high adriamycin concentration because of higher levels of the Topo II enzyme in the DLKP-VP3 cell line. The DLKP-VP3 and DLKP-VP8 cell lines are also resistant to vincristine (1090 and 1740 fold more resistant than DLKP). This vinca alkaloid is not associated with Topo II mediated toxicity and is believed to act as a substrate for the Pgp drug efflux pump. If Pgp and Topo II were the only explanations for the levels of drug resistance in DLKP-VP3 and DLKP-VP8 one would predict that DLKP-VP3 would have lower levels of adriamycin and higher levels of vincristine resistance than found in DLKP-VP8. As this was not the case, it was possible that other mechanisms were involved in drug resistance in these cell lines. For this reason, an investigation was conducted into the potential involvement of the arachidonic acid cascade in this drug resistance. PGHS-2 protein synthesis is inducible by a wide variety of intracellular and extracellular stimuli including IL-1β, lipopolysaccharide and retinoic acid (Williams and DuBois, 1996). The PGHS-2 promoter possesses a xenobiotic response element, but the compounds involved in influencing transcription by means of this site have not yet been fully characterised (Williams and DuBois, 1996). It was known from the experiment detailed in Table 3.11.1 that PGHS-2 did not appear to be expressed in DLKP or VP-16 selected variants. An investigation was then carried out to determine if cytotoxic drugs such as adriamycin rather than IL-1β, influenced PGHS-2 transcription by influencing binding to the xenobiotic response element of the PGHS-2 promoter. This had not been shown not to be the case in A549 (Section 3.10) but it was not known if this was the case in resistant cell lines such as DLKP-VP3 and DLKP-VP8 which were insensitive to IL-1β stimulation of PGHS-2 synthesis (Figure 3.11.1). As PGHS-2 protein synthesis induction was found to be reflected in PgE_2 synthesis (Section 3.10) it was decided to initially analyse PgE_2 levels under a variety

of treatment conditions. This would provide a general indication of any PGHS-2 synthesis or activity and so if results were encouraging from initial experiments, then PGHS-2 protein levels could then be analysed.

From the resistance profiles of the DLKP-VP3 and DLKP-VP8 cell lines it was clear that even though these cells had been selected for VP-16 resistance, they exhibited a higher fold resistance to adriamycin. For these reasons, the investigation into the involvement of the arachidonic acid cascade in the unusual drug resistance characteristics of these cell lines was carried out using adriamycin rather than VP-16. The experiments with adriamycin involved treating DLKP, DLKP-VP3 and DLKP-VP8 cells with various concentrations of drug and IL-1β and then monitoring the resultant levels of PgE_2 synthesis. From Table 3.11.2 it can be seen that treating DLKP with 0.15 or 0.25 μg/mL adriamycin had no effects of PgE_2 production by this cell line. Treatment of this cell line with various IL-1β concentrations also had no effects on PgE_2 production but treatment of A549 with 3 ng/mL IL-1β stimulated significant PgE_2 production by this cell line. Co-treatment of DLKP cells with adriamycin and IL-1β had no unexpected stimulatory effects on PgE_2 synthesis. Similar results were found with DLKP-VP3 and DLKP-VP8 cells treated with adriamycin, IL-1β or co-treated with adriamycin and IL-1β (Tables 3.11.3 and 3.11.4). The drug concentrations used in these experiments for DLKP, DLKP-VP3 and DLKP-VP8 were non-toxic over the period of analysis (24 hours) but caused 40 to 70% cell kill over 7 days. These results suggested that the aspects of the arachidonic acid cascade examined in these studies were not involved in the unusual drug resistance profiles of the DLKP-VP3 and DLKP-VP8 cell lines. As described in Section 1.5 the drug resistance profile of a drug selected cell line can be dependent upon the simultaneous influence of a wide variety of resistance mechanisms including Pgp, MRP, LRP, TAP, Topo II and GST. The unusual drug resistance profiles of the DLKP-VP3 and DLKP-VP8 cell lines should be explainable by further investigation into the influence of these mechanisms on the individual drug resistance levels of these cell lines.

4.8 NSAID mediated drug toxicity enhancement studies in DLKP

As described earlier, it was known from the *in vitro* toxicity assay screening experiments that MRP-mediated transport of cytotoxic drugs was potentially a critical element of the toxicity enhancement effect. A number of difficulties existed with this hypothesis, primarily that MRP protein expression could not be detected in whole cell extracts of the DLKP cell line but yet the NSAID mediated drug toxicity effect could be demonstrated in this cell line. If MRP was the critical factor in achieving the NSAID mediated drug toxicity enhancement effect in a given cell line, then this drug transporter should have been expressed in DLKP. The investigations into this area are described in Section 4.8.2. It was known that in non-MRP expressing drug resistant cell lines such as the Pgp overexpressing cell line DLKP-A, the toxicity enhancement effect did not occur (Duffy *et al.*, 1997). It was not known if this was totally due to Pgp pumping the cytotoxic drug out of the cell or if it was also a result of the NSAID not entering or alternatively being transported out of these resistant cells. A series of experiments were performed to investigate the possibility that differences in NSAID transporting abilities between the drug sensitive cell line, DLKP and the drug resistant cell line. DLKP-A may have been involved in the inability of NSAIDs such as indomethacin to enhance drug toxicity in DLKP-A.

4.8.1 Assessment of extent of NSAID accumulation in the DLKP and DLKP-A cell lines.

From Section 3.12 it was found that both DLKP and DLKP-A cells accumulated [^{14}C]-indomethacin very rapidly and the level of this accumulation was saturated by 4 minutes and unchanged from this level after a period of 4 hours. These results agree significantly with the results of Beaven and Bayer, (1980) who showed that rat hepatoma cells accumulated indomethacin very rapidly with a steady state accumulation level being reached by 3 to 10 minutes. The NSAID transport experiments detailed in Section 3.12 demonstrated that there were no significant

differences in [^{14}C]-indomethacin accumulation between DLKP and DLKP-A. For this reason it was suggested that the toxicity enhancement effect could not be demonstrated in Pgp-overexpressing cells such as DLKP-A because NSAIDs such as indomethacin were without significant effects on Pgp activity. Pgp overexpressing drug resistant cells which were co-treated with a drug and NSAID mixture such as adriamycin and indomethacin would, if this was the case, continue to efflux drug to an extent comparable to that found in the absence of the NSAID.

4.8.2 Investigation into MRP expression and activity in DLKP.

As described in Section 4.4, Western blotting analysis failed to demonstrate expression of MRP protein in whole cell extracts of DLKP. This obviously contradicted the suggestion that NSAIDs interfered with MRP-mediated drug efflux and in this way facilitated chemotherapeutic drug toxicity enhancement. In an attempt to explain the apparent absence of MRP from the DLKP cell line, inside-out vesicles (IOVs) were isolated from this cell line. This was carried out firstly to see if targeting the analysis to a point of localisation of MRP (in the plasma membrane) would permit detection of MRP protein by Western blotting. In addition, the isolation of IOVs from DLKP would facilitate assessment of MRP-like activity in DLKP IOVs. It was found experimentally (Section 3.17.4) that MRP was detectable by Western blotting in 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. This suggests that DLKP possesses low levels of MRP and that this level may be functionally effective for drug transport out of the cell. Inhibition of the drug efflux activity of MRP due to treatment with a suitable NSAID ultimately resulted in the chemotherapeutic drug toxicity enhancement effect. The results illustrated in Figures 3.13.3 and 3.13.4 demonstrate that chemotherapeutic drug toxicity enhancing NSAIDs such as indomethacin significantly inhibit MRP-mediated transport of [^{3}H]-LtC_4 into HL60-ADR IOVs. NSAIDs which do not possess drug toxicity enhancing abilities such as naproxen, do not significantly affect MRP activity. This was also found in the case of DLKP derived IOVs (Figures 3.13.5 and 3.13.6). This strongly suggests that

the ability of NSAIDs such as indomethacin to enhance the toxicity of potential MRP substrate chemotherapeutic drugs, is a result of these NSAIDs inhibiting the drug efflux activity of MRP. From Section 3.13 it can also be seen that DLKP IOVs actively transport [^{3}H]-LtC_4 to their interior at a lower initial rate and lower maximum accumulation than found in the case of HL60-ADR IOVs (initial rates of 0.8 ± 0.2 and 4.0 ± 1.0 pmol LtC_4 per mg protein per minute respectively and maximum accumulations of 2.0 ± 0.3 and 7.0 ± 1.0 respectively). In order to verify that the [^{3}H]-LtC_4 transporter present in DLKP-derived IOVs was MRP, a number of further investigations were carried out as detailed in Section 3.14.

4.9 Verification of the identity of the LtC_4 transporter in IOVs from the DLKP cell line.

As described in Section 4.8.2, DLKP exhibited MRP-like activity where [^{3}H]-LtC_4 was actively transported into IOVs isolated from this cell line. Western blotting analysis (Section 3.17.4), of the proteins present in these IOVs demonstrated that MRP was present at low levels relative to IOVs from the MRP overexpressing cell line, HL60-ADR. The transporter responsible for [^{3}H]-LtC_4 transport into DLKP derived IOVs could potentially be any of the isoforms of MRP described in Section 1.5.2.5 or alternatively an unrelated leukotriene transporter. In order to identify the LtC_4 transporter in DLKP IOVs, a number of related investigations were conducted.

4.9.1 Enhancement of drug toxicity in the presence of inhibitors of MRP activity in the DLKP cell line.

As described in Section 3.14.1, experimental evidence suggests that the NSAID-mediated toxicity enhancement effect is achieved by means of NSAIDs interfering with transport of cytotoxic drugs by MRP. The drugs in question may, under normal conditions act as substrates for MRP-catalysed efflux out of the cell. The toxicity enhancement effect was known to occur in the DLKP cell line in the presence of a suitable cytotoxic drug and NSAID combination. If the transporter of [^{3}H]-LtC_4 into DLKP derived IOVs was MRP, then one may make the assumption that any agent which interfered with [^{3}H]-LtC_4 transport and so MRP transport activity, should also enhance the toxicity of a suitable cytotoxic drug in this cell line. This would be a result of the agent in question interfering with MRP-mediated drug efflux out of the cell. This would cause an increase in intracellular drug concentrations which ultimately would result in an enhanced cell kill for a given dose of cytotoxic drug. The leukotriene receptor antagonist MK571 is a potent inhibitor of [^{3}H]-LtC_4 transport by MRP (Section 3.16), as shown in HL60-ADR IOVs. If the [^{3}H]-LtC_4 transporter in DLKP IOVs was MRP then one would predict that MK571, which is believed to possess only leukotriene

receptor antagonising activities, should interfere with MRP activity and so give rise to an enhancement in the cell kill achieved by a suitable cytotoxic drug. From Section 3.14.1 it can be seen that MK571 enhances the toxicity of adriamycin and VP-16 in the DLKP cell line. These cytotoxic agents are believed to be substrates for MRP and so MK571 mediated interference in their active efflux from the cell by MRP would potentially result in enhanced cell kill for a given dose of drug. Taxol is not believed to be a high affinity substrate for MRP, and as shown in Table 3.14.1.3 the toxicity of this compound was unaffected by co-treatment with MK571. These results suggest that interference with MRP activity in DLKP resulted in an enhancement of cell kill to an extent comparable to that found with NSAIDs such as indomethacin. In addition it was found that MK571 was found to be relatively toxic at the higher doses analysed (approximately 15 μg/mL), as shown in Section 3.14.1. This may suggest that this leukotriene receptor antagonist interferes with a critical aspect of cellular metabolism in DLKP. This was also found in the case of the A549 cell line, where 5 μg/mL MK571 (a non-toxic dose) enhanced the toxicity of 10 ng/mL adriamycin and 7 ng/mL VP-16 (Tables 3.14.1.4 and 3.14.1.5). Increasing the concentration of MK571 to 15 μg/mL caused approximately 70-80% cell kill in the absence of cytotoxic drug. In summary, the *in vitro* toxicity assay results with MK571 provided evidence that the [^{3}H]-LtC_4 transporter in DLKP was MRP.

The bisindolylmaleimide GF109203X is also believed to exert inhibitory effects on MRP activity due to effects on protein kinase C. It was found (Tables 3.14.1.6, 3.14.1.7 and 3.14.1.8) that this compound enhanced the toxicity of the MRP substrates adriamycin and VP-16 but also of the non-MRP substrate Taxol, in the DLKP cell line. Due to the effects of this compound on protein kinase C, one would predict a range of effects on cellular metabolism (including Pgp inhibition) in DLKP. This obviously is reflected in enhancement of the toxicity of drugs of various specificity's for the various drug transport mechanisms possessed by DLKP.

4.9.2 Comparison of V_{max} and K_m parameters for LtC_4 transport in the DLKP and HL60-ADR cell lines.

In Section 3.14.2 it was shown that HL60-ADR IOVs transported LtC_4 with a V_{max} value of 211 ± 89 pmol/mg/min and K_m of 84 ± 26 nM. The LtC_4 transporter in DLKP derived IOVs actively transported LtC_4 with a V_{max} value of 30 ± 2 pmol/mg/min and K_m of 114 ± 13 nM. The characteristic V_{max} and K_m values for LtC_4 transport in MRP expressing IOVs have been quoted in the literature for a variety of cell lines. Loe *et al.*, (1996b) demonstrated that MRP transfected HeLa cells transport LtC_4 with V_{max} and K_m values of 529 pmol/mg/min and 105 nM respectively. Pulaski *et al.*, (1996) found a K_m value of 120 nM for LtC_4 transport by MRP in human erythroleukemia cell derived IOVs. Paul *et al.*, (1996c) found that MRP transfected NIH 3T3 cells transported LtC_4 with a K_m of 35 nM and a V_{max} of 59 pmol/mg/min. The V_{max} value is believed to be a reflection of the quantity of MRP present in the IOV preparation. In the case of DLKP and HL60-ADR, one would predict that if MRP was responsible for LtC_4 transport in DLKP derived IOVs one would predict significantly higher protein levels of MRP in HL60-ADR IOVs relative to DLKP derived IOVs. Western blotting protein analysis of IOVs from these two cell lines is described in Section 3.17.4. The fact that HL60-ADR IOVs accumulated LtC_4 with a relatively low V_{max} value compared to values quoted in the literature for MRP transfected cell lines may simply result from differences in the protocol used for isolation of the IOVs. Only one individual IOV isolation procedure was used for the isolation of DLKP and HL60-ADR IOVs. For this reason, comparison of V_{max} values for LtC_4 transport into IOVs from these cell lines is justifiable for the purposes of attempting to approximately correlate MRP protein levels in the IOVs and the V_{max} values of these IOVs. From the combination of the Western blotting data which showed significantly higher levels of MRP in HL60-ADR IOVs relative to DLKP IOVs, and the V_{max} data which is a factor of 7 times higher in HL60-ADR, the results provide some indication that MRP is responsible for LtC_4 transport in DLKP.

A wide range of values have been quoted in the literature for K_m values in MRP mediated transport of LtC_4. These can range from 35 nM (Paul *et al.*, 1996c) to over 118 nM (Pulaski *et al.*, 1996) with significant errors (± 30 nM) generally associated with the data. The K_m values for DLKP and HL60-ADR are 114 ± 13 nM and 84 ± 26 nM respectively. The K_m value obtained for these cell lines are within the range of values quoted for MRP mediated transport of LtC_4. The experiments performed for determination of K_m and V_{max} were performed in duplicate with a given vesicle preparation. Extremely high quality data is necessary in order to obtain accurate values for Lineweaver and Burk type analyses. Minor variability in data between replicates resulted in major shifts in the intercepts of the regression line with the X- and Y-axes. This ultimately resulted in significant variation in the K_m and V_{max} values found by regression analysis. There are considerable difficulties in obtaining accurate kinetic parameter data when using IOVs, due to the basic experimental difficulties encountered in working with concentrated lipid solutions. As described in Section 3.14.2, the data is represented as the mean ± S.D. for duplicate determinations in two separate experiments using one vesicle preparation from the relevant cell line. It was not found to be possible to obtain consistently repeatable results in subsequent experiments using separately prepared IOV preparations, as the values of K_m and V_{max} could in some cases vary by a factor of 3-fold between these IOV preparations from a given cell line. This may have been a reflection of the purity of the IOV preparations used in these experiments. Irrespective of the explanation, the lack of consistent repeatability in these experiments indicates that the accuracy of the K_m and V_{max} values described above may be questioned. Even considering this fact, this does not mean that the results shown above are not an accurate reflection of the kinetic parameters for a given high purity preparation of IOVs from a cell line such as analysed in the experiments described above. For the IOV preparations analysed in these experiments it may be reasonably justifiable to state from the kinetic parameter data determined experimentally that MRP was responsible for [^{3}H]-LtC_4 transport in the preparation of HL60-ADR IOVs analysed. From the data, it could also be proposed that the [^{3}H]-LtC_4 transporter in the DLKP-derived IOV preparation analysed in this experiment was MRP.

The design of the experiments described in Section 3.14.2 was relatively poor for the accurate determination of kinetic parameters. The Lineweaver-Burk plots in Section 3.14.2 have the disadvantage of compressing the data points at high substrate concentrations into a small region and emphasising the points at lower substrate concentrations. This may have contributed to significant variation in kinetic parameters between different IOV preparations from a given cell line as the majority of data points were determined using intermediate to high substrate concentrations. The experiment was designed so that saturation in LtC_4 accumulation at high substrate concentrations could be demonstrated (Figure 3.14.2.1 and 3.14.2.3). The direct use of this data which involved only a small number of lower substrate concentrations, for double-reciprocal plots resulted in the majority of the data being compressed into a small region (Figures 3.14.2.2 and 3.14.2.4). For this reason, if further investigations were to be performed in this area, it would be necessary to design the experiment so that a sufficient number of low substrate concentrations were analysed for accurate Lineweaver-Burk analysis. An alternative would be to use an Eadie-Hofstee plot for analysis of the data (where initial rate of uptake is plotted against the rate divided by substrate concentration). This has not been performed for the data in Section 3.14.2, but it has the advantage of not compressing data obtained using higher substrate concentrations into a small region. The only conclusive way to identify the transporter responsible for [^{3}H]-LtC_4 transport in DLKP IOVs would be to carry out photoaffinity labelling experiments. These experiments would involve incubating [^{3}H]-LtC_4 with IOVs and irradiating the mixture at 312 nm. Following a rapid freezing step, [^{3}H]-LtC_4 would become irreversibly bound to the relevant transporter molecule. The molecular weight of the [^{3}H]-LtC_4 bound protein in the IOVs could then be easily determined following gel electrophoresis of the proteins and autoradiography. If MRP was responsible for [^{3}H]-LtC_4 binding, one would predict that all autoradiographic signal resulting from the presence of [^{3}H] would be present at a position corresponding to a 190 kDa protein.

4.10 Transport of cytotoxic drugs by MRP into HL60-ADR IOVs

The glutathione conjugate transporter MRP is believed to be involved in the transport of cytotoxic drugs. There has been little evidence of transport of unconjugated cytotoxic drugs by MRP with the exceptions of groups such as Stride *et al.*, (1997), Paul *et al.*, (1996c) and Loe *et al.*, (1996b). In addition, the MRP pump is known to transport glutathione conjugates but drugs which are believed to be transported by MRP are not known to readily undergo glutathione conjugation within cells. MRP-overexpressing cells are often sensitive to cisplatin, a drug for which glutathione conjugation is known to occur. In order to investigate the possible transport of cytotoxic drugs by MRP in a drug selected rather than transfected cell line, two main approaches were taken. The first approach involved indirectly examining drug transport by MRP by studying the inhibition of MRP transport in HL60-ADR IOVs which occurred in the simultaneous presence of the high affinity MRP substrate, [^{3}H]-LtC_4 and various cytotoxic drugs. The second and more direct approach involved monitoring the transport of [^{3}H]-labelled vincristine into HL60-ADR IOVs by MRP.

4.10.1 Influence of glutathione and cytotoxic drugs on LtC_4 transport into HL60-ADR IOVs by MRP.

From Section 3.15.1 it can be seen that vincristine, adriamycin, cisplatin and Taxol were examined for inhibitory effects on [^{3}H]-LtC_4 transport into HL60-ADR IOVs. As described above, if a given drug was transported by MRP, one would predict that co-incubation of IOVs in [^{3}H]-LtC_4 and cytotoxic drug would result in competition for transport by MRP. Increasing the concentration of drug should cause a reduction in the accumulation of [^{3}H]-LtC_4 within the IOVs, due to increased competition for transport by MRP. The ability of a given drug to inhibit [^{3}H]-LtC_4 transport into IOVs should not be regarded as being solely a result of competition for transport. Addition of an agent to the IOV preparation could potentially influence any of the critical aspects of system necessary for MRP

transport of [^{3}H]-LtC_4. For this reason if an exogenously added agent interfered with the ATP regenerating system or osmotic pressure in the assay system, [^{3}H]-LtC_4 accumulation in IOVs would be significantly reduced. In the case of this series of experiments and in all other experiments examining [^{3}H]-LtC_4 transport in the presence of inhibitory agents, the concentration of solvent used for dissolution of the compound of interest was maintained below 0.5% whenever possible. At this level, [^{3}H]-LtC_4 transport inhibition was affected only by the agent in question. It should be noted that comparison of inhibition of [^{3}H]-LtC_4 uptake into IOVs by various agents is quoted in all subsequent discussions using steady state accumulation levels rather than initial rates although both initial and steady state accumulation levels were monitored. In order to fully characterise the inhibition of [^{3}H]-LtC_4 transport into IOVs by a particular agent it would be necessary to determine the initial rate of [^{3}H]-LtC_4 transport at a variety of [^{3}H]-LtC_4 and inhibitor concentrations so that Lineweaver-Burk plots could be developed for various concentrations of inhibitor. This would then allow elucidation of the K_i value (concentration of inhibitor which reduced maximum LtC_4 accumulation rate by 50%) as well as the specific nature of the inhibition of LtC_4 transport caused by the compound in question. In preference to carrying out an elaborate study of this nature, it was decided to express the inhibition of [^{3}H]-LtC_4 transport caused due to the presence of a compound as a percentage of steady state accumulation of [^{3}H]-LtC_4 in the absence of this inhibitor. This did not facilitate the determination of a K_i value or the elucidation of the nature of inhibition caused by a particular compound but this method did permit the rapid approximation of the inhibitory effects of these compounds on [^{3}H]-LtC_4 transport to the interior of IOVs.

Section 3.15.1 demonstrated that vincristine was found to be a relatively potent inhibitor of [^{3}H]-LtC_4 transport into HL60-ADR IOVs. A concentration of approximately 100 μM vincristine reduced ATP-dependent [^{3}H]-LtC_4 transport by approximately 50% relative to an ATP treated control. Adriamycin was a relatively poor inhibitor of [^{3}H]-LtC_4 transport, with 250 μM adriamycin achieving only approximately 20% inhibition relative to ATP treated control

IOVs. Cisplatin was totally ineffective even at 220 μM as an inhibitor of [^{3}H]-LtC_4 transport into IOVs. Taxol was ineffective as an inhibitor of [^{3}H]-LtC_4 transport into HL60-ADR IOVs even at the high concentrations of 100, 50 and 1 μM. The results obtained experimentally with the HL60-ADR drug selected MRP overexpressing cell line, agree significantly with the results reported by Loe *et al.*, (1996b) for an MRP-transfected cell line. This group demonstrated that at a concentration of 100 μM vincristine, MRP activity was inhibited by 50% and that cisplatin was without effect on [^{3}H]-LtC_4 transport. Vincristine is believed to be a substrate for MRP-mediated efflux from cells and so the results found experimentally would suggest that this drug competed with [^{3}H]-LtC_4 for binding to the substrate binding site of MRP. This ultimately caused a reduction in detectable accumulated levels of [^{3}H]-LtC_4 in HL60-ADR IOVs. Cisplatin is not believed to act as a substrate for MRP and for this reason had no effects on [^{3}H]-LtC_4 transport into IOVs. The relatively poor ability of adriamycin (believed to be an MRP substrate) to inhibit [^{3}H]-LtC_4 transport is relatively surprising and differs from the results of Loe *et al.*, (1996b) where it was demonstrated that adriamycin at 100 μM inhibited LtC_4 transport by 60-70%. There is no evidence to suggest that MRP has higher affinity for vincristine rather than adriamycin. These drugs are of comparable potency as anti-tumor agents. MRP transfected cell lines often show comparable levels of resistance to adriamycin and vincristine (Section 1.5.2.3). The explanation for the inability of adriamycin to inhibit [^{3}H]-LtC_4 transport to the same extent achieved by vincristine requires further investigation. Taxol was found to be an ineffective inhibitor of [^{3}H]-LtC_4 transport into HL60-ADR IOVs. This also conflicts with the results found by Loe *et al.*, (1996b) in their MRP-transfected cell line model. This may be a result of differences between IOVs from MRP-transfected and drug selected MRP-overexpressing cells. In the experiment illustrated in Figure 3.15.2.6, Taxol was dissolved in ethanol, and final ethanol concentration was maintained below 0.5%, a concentration which was without any influence on [^{3}H]-LtC_4 transport. Taxol is administered *in vivo* in a concentrate form containing dehydrated alcohol and polyethoxylated castor oil. It was found experimentally with HL60-ADR IOVs that Taxol in this form totally inhibited [^{3}H]-LtC_4 transport at 100, 50 and 1 μM (data not shown). Although the

final concentration in the incubation mixture of ethanol and castor oil was below 0.5%, it was obvious that at the concentration added, these compounds caused significant alterations in incubation mixture viscosity and possibly osmotic pressure. An appropriate drug solvent control was not available in order to investigate the effects of the combination of ethanol and castor oil on [^{3}H]-LtC_4 transport. For this reason, it was decided to use a Taxol stock which was dissolved in ethanol only in all further experiments. The details of the Taxol formulation used by Loe *et al.*, (1996b) are not provided but it is possible that the Taxol used by this group in these experiments was the form which is provided dissolved in ethanol and castor oil. If this was the case, it could explain the high levels of MRP transport inhibition achieved in the presence of low concentrations of Taxol. In the absence of data for a vehicle control it is not possible to state if this Taxol mediated inhibition was a result of Taxol acting as a substrate for MRP or simply a result of the Taxol formulation used.

Obviously the concentrations of cytotoxic drugs used in these studies are extremely high relative to the concentrations of these drugs used to treat cells *in vitro*. These concentrations may be relevant in terms of intracellular drug concentrations where drug becomes concentrated within the cell in intracellular compartments. Taxol is not regarded as a high affinity substrate for MRP but is an extremely potent cytotoxic agent. For this reason, cells are normally treated with lower concentrations of Taxol to achieve a given level of cell kill, than would be added in the case of a less potent cytotoxic agent such as vincristine. For example, in the A549 cell line, 15 nM vincristine and 40 nM adriamycin achieve approximately 40% cell kill. A dose of 0.5 nM of Taxol (30 fold lower than vincristine) achieves a similar level of cell kill in this cell line. As a result of this fact, it would be more relevant to compare levels of inhibition of [^{3}H]-LtC_4 transport due to the presence of a given cytotoxic drug at the relative intracellular concentrations to which cells are exposed. If vincristine causes a particular level of [^{3}H]-LtC_4 transport inhibition at a given concentration, then comparison of this concentration with a 30-fold lower concentration of Taxol may be more relevant for attempting to correlate with relevant intracellular concentrations of drug. The cytotoxic drugs examined in these studies were low potency inhibitors of [^{3}H]-

LtC_4 when examined in comparison to cyclosporin A or MK571. As described in Section 3.16, cyclosporin A is a relatively potent inhibitor (up to 60% of steady state accumulation levels) of MRP mediated [^{3}H]-LtC_4 transport at 50, 10 and 1 μM. Verapamil was found to inhibit MRP activity in HL60-ADR IOVs at very low levels at steady state even at a concentration of 50 μM (Figure 3.16.2). There have been conflicting reports in the literature as regards the potency of verapamil and cyclosporin A as inhibitors of MRP activity as discussed in Section 1.5.2.4. Cole *et al.*, (1994) reported that cyclosporin A enhanced adriamycin toxicity to a more minor extent in MRP-transfected HeLa cells than was found in the case of verapamil. Hollo *et al.*, (1996) reported that cyclosporin A was two fold more potent than verapamil as an inhibitor of MRP mediated transport of calcein into HL60-ADR cells. The results from Section 3.16 provide evidence that cyclosporin A is more potent an inhibitor of MRP activity. The potency of these non-selective MDR modulating agents is extremely low when compared to the leukotriene receptor antagonist MK571. This compound practically completely inhibits [^{3}H]-LtC_4 transport into HL60-ADR IOVs at concentrations of 10 and 50 μM. At a concentration of 1 μM MK571, [^{3}H]-LtC_4 uptake into HL60-ADR IOVs was reduced by 20-30 %.

Relatively surprising results emerged from investigations into the effects of reduced glutathione (GSH) on [^{3}H]-LtC_4 uptake into HL60-ADR IOVs. As shown in Figure 3.15.2.1 a concentration of approximately 6 mM GSH was found to totally inhibit ATP dependent [^{3}H]-LtC_4 uptake. Only concentrations of GSH below 2 mM were without inhibitory effects on the ATP dependent uptake of [^{3}H]-LTC_4 into HL60-ADR IOVs. It is believed that GSH is not a substrate for MRP transport (Leier *et al.*, 1996) which is relatively surprising considering the high affinity of GS-conjugates for transport by MRP. In addition GSH was stated by Loe *et al.*, (1996b) and Muller *et al.*, (1994) to be without effects on MRP at 5 mM. The inhibitory effects of GSH on [^{3}H]-LtC_4 accumulation could possibly be a result of active transport of this tripeptide by MRP although there has been no previous evidence of transport of unconjugated GSH by MRP. Alternatively, GSH when added in high concentrations could simply bind to or block the

substrate binding site of MRP without necessarily being transported by the MRP molecule. This would then result in a reduction in accessibility for [^{3}H]-LtC_4 to the MRP binding site, causing a reduction in [^{3}H]-LtC_4 accumulation. These possibilities require further investigation.

An investigation into the inhibition of [^{3}H]-LtC_4 transport into HL60-ADR IOVs in the presence of GSH and various cytotoxic drugs was then conducted. As described earlier cytotoxic drugs in their unconjugated state have not been widely shown to be transported by the glutathione conjugate transporter MRP. In addition cytotoxic drugs which are believed to act as substrates for MRP have not been shown to undergo glutathione conjugation in cells. It has been suggested that cellular GSH metabolism is a critical element of MRP activity in MRP expressing cells (Section 1.5.5.2) and for this reason, the inhibition characteristics of various concentrations of cytotoxic drugs on [^{3}H]-LtC_4 transport into HL60-ADR IOVs was investigated. From Figure 3.15.2.2 it can be seen that in the presence of 5 mM GSH, ATP dependent transport of [^{3}H]-LtC_4 was totally eliminated. In the presence of this concentration of GSH, vincristine and cisplatin treated IOVs also have all ATP dependent LtC_4 transport eliminated as expected. In the presence of 1 mM GSH, ATP dependent [^{3}H]-LtC_4 transport was unchanged from the levels achieved in the absence of 1 mM GSH. The presence of 1 mM GSH had no effects on the uptake of [^{3}H]-LtC_4 into HL60-ADR IOVs treated with 100 μM cisplatin. A concentration of 100 μM cisplatin in the absence of GSH also had no effects on ATP dependent [^{3}H]-LtC_4 transport (Figure 3.15.2.5). The addition of 1 mM GSH had no effects on the level of inhibition of [^{3}H]-LtC_4 uptake achieved by 50 μM Taxol (Figure 3.15.2.6). From Figure 3.15.2.4 it can be seen that a concentration of 250 μM adriamycin inhibited [^{3}H]-LtC_4 uptake into HL60-ADR IOVs by 15-20%. A very small increase in inhibition of this transport was achieved in the presence of 1 mM GSH (Figure 3.15.2.4). The increase in inhibition of ATP dependent [^{3}H]-LtC_4 transport into IOVs was found to occur to a highly significant extent in the case of 110 μM vincristine (Figure 3.15.2.3). In the presence of 110 μM vincristine [^{3}H]-LtC_4 uptake into HL60-ADR IOVs was approximately 4 pmol per mg IOV protein at steady state.

In the presence of 1 mM GSH and 110 μM vincristine, this steady state ATP dependent accumulation was reduced by over 50% to under 2 pmol per mg IOV protein. As 1 mM GSH was without effects on ATP-dependent MRP activity, one may assume (although there could be various possible explanations) that the synergistic inhibition of [^{3}H]-LtC_4 uptake in the presence of vincristine and 1 mM GSH was a result of increased transport of vincristine, GSH or some form of conjugate of both of these agents. The fact that 1 mM GSH was without effects on the levels of inhibition of [^{3}H]-LtC_4 uptake into IOVs achieved by 100 μM cisplatin and 50 μM Taxol suggests that transport of vincristine by MRP may explain the results of Figure 3.15.2.3. In the absence of the cellular enzymes required for glutathione conjugation, the IOV transport results suggest that glutathione conjugation is not strictly necessary to achieve transport of vincristine. It is possible that some form of co-transport of vincristine and GSH occurs. Active transport of GSH into IOVs is suggested from the data of Figure 3.15.2.1. Alternatively MRP could possess a number of substrate binding sites, one of which recognises and binds to GSH. [^{3}H]-LtC_4 could be transported by MRP because of the high affinity of this glutathione conjugate for a specific substrate binding site on MRP. The GSH binding site does not need to be occupied for transport of [^{3}H]-LtC_4 by MRP to occur. When GSH and an agent that could bind to one of the additional substrate binding sites on MRP (such as vincristine but not Taxol) were present, this agent could be transported by MRP because of the fact that both the GSH and the substrate binding sites would be simultaneously occupied. This would then result in the overall level of [^{3}H]-LtC_4 transport into the IOVs to be reduced because of the increased competition between vincristine and [^{3}H]-LtC_4 for active transport by MRP. This synergistic inhibition of [^{3}H]-LtC_4 uptake by an MRP-substrate drug and GSH may be specific for only a select number of drugs. Loe *et al.*, (1996b) showed that in MRP-transfected cells, 1 mM GSH caused an increase in the level of inhibition of LtC_4 uptake caused by 100 μM vincristine and vinblastine but not adriamycin.

4.10.2 Influence of glutathione on cytotoxic drug transport into HL60-ADR IOVs by MRP.

A limited number of reports have shown the direct transport of unconjugated drugs by MRP in MRP-transfected cells. In order to investigate this phenomenon in MRP-overexpressing drug selected cells, the transport of [^{3}H]-vincristine into HL60-ADR IOVs was examined. As described in Section 3.15.3, the protocol used for these experiments was developed so as to minimise non-specific binding of [^{3}H]-vincristine to the filters used to retain the IOVs. This modified protocol was still sufficient for detection of ATP dependent transport by MRP of a suitable substrate (such as LtC_4) into HL60-ADR IOVs (Figure 3.15.3.1). From Figure 3.15.3.2 it can be seen that no specific ATP-dependent active transport of [^{3}H]-vincristine was found either in the presence or absence of 2.7 mM GSH. This concentration of GSH inhibited ATP-dependent MRP activity by over 40% (Figure 3.15.3.1). The levels of transport of [^{3}H]-vincristine were unchanged in the presence of this concentration of GSH. This strongly suggests that this drug was not actively transported by MRP. This absence of active transport of [^{3}H]-vincristine by HL60-ADR IOVs was found to occur even when the levels of IOV were doubled from the levels normally used in the IOV assay system, to 100 μg vesicle protein (Figure 3.15.3.3). Under these conditions, significant [^{3}H]-vincristine uptake into HL60-ADR IOVs was negligible in the presence of ATP or AMP. The addition of a concentration of GSH which totally inhibited MRP activity (10 mM) had no effects on [^{3}H]-vincristine accumulation within HL60-ADR IOVs. This was also found to be the case with 0.5 mM GSH, a concentration of GSH which is known to be without significant effects on MRP activity. The illustration of vincristine transport by the indirect study discussed in Section 4.10.1 in the presence of 1 mM GSH, and the inability to detect this transport by the direct [^{3}H]-vincristine method may have a relatively simple explanation. In the indirect studies, vincristine was added at a final concentration of 100 μM. In the direct studies of Section 3.15.3, [^{3}H]-vincristine was added at 200 nM. Concentrations of vincristine over 200 nM may be necessary to fulfil whatever criteria are required for active transport of drug in the presence or

absence of GSH. The use of a concentration of [^{3}H]-vincristine of 100 μM is not possible in practical terms as it would require very high levels of [^{3}H] but as already described this concentration of drug may be relevant as an intracellular concentration of drug if drug became localised or compartmentalised in specific regions of the cell. This possibility could be relatively easily investigated using the ATP assay described in Section 2.14. As was demonstrated in Section 3.17.3, in the presence of a suitable substrate and in the absence of creatine kinase, ATP levels decrease because of active transport of the substrate by MRP to the interior of the IOVs. In the absence of a suitable substrate, active transport would not occur and so ATP levels would remain constant. For this reason, it would be possible to monitor the active transport by MRP of agents such as vincristine. For these experiments it would be necessary to include appropriate controls to ensure that ATP hydrolysis occurred as a result of active transport rather than as a result of non-specific degradation. The main advantage of using this system would be that it would eliminate the technical difficulties experienced in attempting to minimise non-specific binding of high concentrations of [^{3}H]-vincristine to filters.

4.11.1 Investigation into the cytotoxic drug accumulation characteristics of the DLKP-A10 multidrug-resistant human lung tumor cell line.

The DLKP-A10 cell line is a drug resistant variant of DLKP and is derived from the DLKP-A cell line. This highly resistant cell line (over 800 fold more resistant to adriamycin than DLKP) was selected in 17 μM adriamycin. The DLKP cell line accumulated over 4000 pmol adriamycin per million cells after 4 hours incubation (Figure 3.17.1.1). The resistant DLKP-A10 cells accumulated under 300 pmol adriamycin per million cells after 4 hours incubation in drug-containing media. Treatment of DLKP-A10 cells with 8 μM cyclosporin A or 60 μM verapamil was only partially effective at reversing the drug accumulation defect in DLKP-A10. These compounds caused DLKP-A10 cells to accumulate over 1300 pmol adriamycin per million cells, with a steady state in drug accumulation levels reached after 3 hours incubation in drug. If the only mechanism of resistance responsible for resistance and so reduced drug accumulation levels in DLKP-A10 involved Pgp, one would predict that addition of these circumventing agents would cause complete reversal of the drug accumulation deficit. This is illustrated in the case of the SKMES-1 and SKMES-1/ADR cell lines (Figure 3.17.1.2). The low levels of adriamycin accumulation in the SKMES-1/ADR cell line (250 pmol adriamycin per 10^6 cells) appeared to be solely a result of the Pgp-mediated drug efflux capabilities of this cell line. Inhibition of Pgp activity due to treatment of SKMES-1/ADR with 8 μM cyclosporin A or 60 μM verapamil completely restored adriamycin accumulation levels to the levels achieved in the case of the SKMES-1 parental drug sensitive cell line. The SKMES-1 cell line is more resistant to adriamycin (IC_{50} of 25 nM) than the DLKP cell line (IC_{50} of 9 nM) and so this may explain the low levels of adriamycin accumulation found in the case of SKMES-1 when compared to DLKP (2 nmol adriamycin per 10^6 SKMES-1 cells after 4 hours as compared to 4 nmol adriamycin per 10^6 DLKP cells after 4 hours incubation in drug containing media). In summary, cyclosporin A and verapamil were found to possess the ability to reverse the accumulation deficit in resistant cells where Pgp is believed to be solely responsible for drug efflux, such as in SKMES-1/ADR. The inability of these agents to reverse the accumulation

defect in DLKP-A10 suggested that a non-Pgp-related mechanism of drug transport is active in this cell line. These results agreed with findings of Cleary *et al.*, (1997).

In order to investigate the ATP dependence of this drug transport mechanism in the DLKP-A10 cell line, transport was investigated in the presence of 10 mM sodium azide and 20 μM antimycin A. It was found that these metabolic inhibitors were highly toxic at these and at even lower concentrations to DLKP-A10 and DLKP after a period of 4 hours. Exposure of the cells to these compounds for periods of under 3 hours did not cause any obvious toxic effects, but cells could be seen to be in a shocked state by 3-4 hours. The experiments with these metabolic inhibitors were conducted under glucose free conditions as described in Section 2.15. As can be seen in Figure 3.17.1.3, DLKP accumulated significantly more adriamycin after 4 hours incubation in 10 μM adriamycin than the resistant DLKP-A10 cell line (1500 and 500 pmol adriamycin per 10^6 cells respectively). This illustrates that even within the glucose free conditions used in this experiment, the drug transport mechanisms responsible for the low levels of drug accumulation in the DLKP-A10 cell line continued to perform with comparable efficiency to that achieved under glucose containing conditions. From Figure 3.17.1.3 it can be seen that treatment of the DLKP-A10 cell line with sodium azide or antimycin A under glucose deficient conditions, caused a significant increase in the accumulation of adriamycin in this resistant cell line. The metabolic inhibitor antimycin A completely reversed the accumulation defect in the DLKP-A10 cell line by causing the cells to accumulate levels of adriamycin comparable to those found in DLKP in glucose free conditions. Sodium azide also caused a reasonably high level of adriamycin accumulation in the DLKP-A10 cell line under these conditions. These findings agreed with results of similar experiments performed by Cleary *et al.*, (1997). The results described above demonstrated that the drug transport mechanism responsible for the low levels of adriamycin accumulation in the DLKP-A10 cell line was energy dependent and could be reversed completely in the presence of antimycin A. The effects of antimycin A were dependent on the ability of this compound to interfere with ATP production in DLKP-A10. Treatment of DLKP-A10 cells with this compound under glucose

containing conditions had no effects on the accumulation of adriamycin as shown in Figure 3.17.1.4. As antimycin A was believed to be more effective than sodium azide in achieving reversal of the accumulation deficit in DLKP-A10 and as both of these compounds should have comparable effects on ATP production, further experiments were conducted in order to see if antimycin A and not sodium azide had some direct influences on the drug transport mechanisms in DLKP-A10.

4.11.2 Transport of LtC_4 into DLKP-A10 derived IOVs and inhibition of this transport by antimycin A, sodium azide, cyclosporin A and verapamil.

A number of drug resistant MRP-expressing cell lines have been shown to exhibit high levels of intracellular vesicle formation (Marquardt and Center, 1992). It has been suggested that MRP can be involved in transport of drug into these cytoplasmic vesicles in order to facilitate eventual efflux of drug from the cell. The DLKP-A10 cell line was shown by Cleary *et al.*, (1997) to exhibit unusually high levels of vesicle formation within the cytosol. It was also found that drug became localised within these vesicles and that these vesicles were involved in sequestering drug away from the nucleus and potentially out of the cell (Cleary *et al.*, 1997) The formation of intracellular vesicles by DLKP-A10 suggested that MRP was present in this cell line. Cleary *et al.*, (1997) demonstrated that MRP protein was not detectable in whole cell extracts of the DLKP-A10 cell line. As has already been described, MRP protein had not been detected in the DLKP cell line until active MRP protein was shown to be present in IOVs isolated from this cell line. This indicated that the isolation of IOVs from the DLKP-A10 cell line would illustrate if even very low levels of active MRP protein were present in these cells.

IOVs were isolated from DLKP-A10 in order to investigate if MRP activity contributed to the unusual drug accumulation characteristics of this cell line. The results presented in Section 3.17.2 demonstrate that IOVs from the DLKP-A10 cell line exhibit significant levels of [^{3}H]-LtC_4 transport and so MRP-like activity. From Section 3.17.2 it was demonstrated that IOVs from this cell line transport [^{3}H]-LtC_4 in an ATP dependent fashion with an initial rate of 0.6 ± 0.2 pmol per mg per minute. This transport becomes saturated by 2.5 minutes at a level of 1.8 ± 0.3 pmol/mg protein. From Section 3.13 it was demonstrated that DLKP derived IOVs transport [^{3}H]-LtC_4 with an initial rate of approximately 0.8 ± 0.2 pmol per mg per minute (maximum accumulation of 2.0 ± 0.3 pmol/mg). From Section 3.17.4 it was shown that DLKP IOVs contained higher levels of the 190 kDa protein MRP, than had been found in DLKP-A10 IOVs. The presence of a 116 kDa band in all Western blots performed using the rat MRPr1 monoclonal antibody (TCS Biologicals, ZUMC-201) had not been found previously with other batches of this antibody. A negative control blot (with HL60-ADR IOVs) using PBS in place of primary antibody did not exhibit the 116 kDa or any other banding. A negative control using rat serum was not performed. The low molecular weight of this protein indicates that the 116 kDa banding is unlikely to be attributable to the presence of MRP. For this reason the 116 kDa banding must be attributed to non-specific binding of the antibody.

Demonstration of the presence of active MRP protein in the DLKP-A10 cell line suggested that this drug resistance mechanism may have exerted an influence on the unusual adriamycin accumulation characteristics of this cell line. As described in Section 4.11.1, these unusual adriamycin accumulation characteristics firstly involved the inability of cyclosporin A and verapamil to completely reverse the drug accumulation deficit in DLKP-A10. In addition, the ability of antimycin A and the inability of sodium azide under glucose free conditions to reverse the accumulation deficit in DLKP-A10 could not be explained on the basis of the levels of Pgp possessed by this cell line. If MRP activity in DLKP-A10 cells contributed to these adriamycin accumulation characteristics one would predict that antimycin A was a more potent modulator of MRP activity than sodium

azide, cyclosporin A or verapamil. This prediction was complicated by the fact that the ability of antimycin A to reduce the accumulation deficit in DLKP-A10 was partially dependent on the ability of this compound to inhibit ATP production. MRP activity was analysed in DLKP-A10 derived IOVs in the presence of antimycin A, sodium azide, verapamil and cyclosporin A. From Figure 3.17.2.2 it can be seen that 50 and 10 μM cyclosporin A caused inhibition of [^{3}H]-LtC_4 uptake into DLKP-A10 IOVs by 80 and 60% respectively. Verapamil exhibited low levels of inhibitory effects on [^{3}H]-LtC_4 transport by DLKP-A10 IOVs (Figure 3.17.2.3). A concentration of 50 μM antimycin A inhibited LtC_4 accumulation in DLKP-A10 IOVs by approximately 50-60% and a 10 μM concentration of this compound achieved 30-40% inhibition. From Figure 3.17.2.5 it can be seen that sodium azide also inhibited [^{3}H]-LtC_4 transport when present at a 50 μM concentration (approximately 20% inhibition of transport). The inhibitory effects of antimycin A on [^{3}H]-LtC_4 transport were not a result of interference in the ATP regenerating system (Figure 3.17.3.1) and so were a direct result of inhibitory effects on the MRP transporter molecule.

The results of the studies on the effects of antimycin A on [^{3}H]-LtC_4 transport into DLKP-A10 IOVs demonstrated that this metabolic inhibitor also exerted significant inhibitory effects on MRP activity. The other metabolic inhibitor examined, sodium azide was significantly less potent an inhibitor of the activity of MRP in IOVs from this cell line. The ability of antimycin A to reduce the drug accumulation deficit in DLKP-A10 was partially dependent on the ability of this compound to inhibit ATP production. Under glucose containing conditions, antimycin A had no effect on adriamycin accumulation in DLKP-A10 cells (Figure 3.17.1.4). The results suggested that the abilities of antimycin A to simultaneously inhibit ATP production and MRP activity resulted in this compound reducing the drug accumulation deficit in DLKP-A10. Sodium azide only partially reversed this deficit because of the fact that although it inhibited ATP synthesis, this metabolic inhibitor exerted relatively insignificant effects on MRP activity in DLKP-A10. The combination of the Western blotting results of Cleary *et al.*, (1997), which showed very high levels of Pgp protein in DLKP-A10, and the Western blotting

results of Figure 3.17.4.1 which showed relatively low levels of MRP in DLKP-A10, suggest that Pgp overexpression is the dominant drug resistance mechanism present. The expression of low levels of MRP may become influential on the drug accumulation characteristics of DLKP-A10 only when ATP levels in cells become depleted during growth under glucose free conditions in the presence of antimycin A or sodium azide. The dual ability of antimycin A to inhibit ATP production and to inhibit MRP activity could potentially explain the ability of this compound to eliminate the drug accumulation deficit in DLKP-A10.

In order provide further evidence for this possibility, it would be necessary to perform three related experiments. In the first of these experiments, it would be necessary to demonstrate that IOVs from the SKMES-1/ADR cell line did not possess MRP activity. In the absence of MRP, the potent Pgp modulators, verapamil and cyclosporin A would be predicted to eliminate the accumulation deficit in this cell line, as had been found experimentally (Figure 3.17.1.2). In the second experiment, it would be necessary to demonstrate that the levels of adriamycin accumulated in DLKP cells were significantly increased in the presence of antimycin A under glucose free conditions. The DLKP cell line is known to express active MRP protein (Figure 3.17.4.1) and so significant interference in the activity of MRP should result in an increase in drug accumulation in this cell line. Cleary, (1995) demonstrated that this is in fact the case. DLKP cells were found to accumulate approximately 444 pmol adriamycin per 10^6 cells after 3 hours incubation in 10 μM adriamycin under glucose free conditions. Under these conditions and in the additional presence of 10 μM antimycin A, DLKP cells accumulated approximately 980 pmol adriamycin per 10^6 cells. In the third and final experiment, an established inhibitor of MRP such as MK571 (Section 3.16) would be used under conditions where DLKP-A10 cells were deprived of ATP. If DLKP-A10 was deprived of ATP and if MRP activity was simultaneously inhibited due to the presence of MK571, then one would predict that the drug accumulation deficit in DLKP-A10 would be eliminated. Under conditions where DLKP-A10 was not deprived of ATP, MK571 should have no effect on the levels of drug accumulated by DLKP-A10. This would be a

result of the fact that Pgp activity would be unaffected by the treatment with MK571 and so would actively efflux drug from the cell.

The dual ability of antimycin A to inhibit ATP production and to inhibit MRP activity may be proven to explain the ability of this compound to eliminate the drug accumulation deficit in DLKP-A10. This compound may have potential for use as an MRP inhibitor as was demonstrated with HL60-ADR IOVs in Figure 3.17.2.6. This compound would be predicted to enhance the toxicity of drugs such as adriamycin in MRP expressing cell lines such as DLKP. It would not significantly influence drug accumulation characteristics and drug toxicity in Pgp overexpressing cell lines under standard culture conditions as there is no evidence to suggest that this compound has any inhibitory effects on Pgp activity.

4.12 MRP expression and LtC_4 transport characteristics in clonal populations isolated from the MDR cell line DLKP-A.

As described in Section 3.18, Heenan *et al.*, (1997) isolated 9 clonal subpopulations from the Pgp-overexpressing cell line DLKP-A. This cell line is an adriamycin-selected variant of DLKP. The DLKP-A cell line (250-fold resistant to adriamycin relative to DLKP cells) overexpresses Pgp protein and possesses reduced levels of topoisomerase II (Topo II) protein. The drug resistance mechanisms responsible for the various levels of drug resistance in the DLKP-A clones had previously been believed to only involve Pgp and Topo II. It is now known that DLKP expresses MRP in the plasma membrane (Section 3.17.4). The possible existence of MRP in DLKP-A derived clones was investigated by means of isolation of IOVs from the DLKPA2B, 11B, 6B and 5F cell lines. These studies were carried out with two main objectives. The first objective involved an investigation into MRP protein levels and activities in these drug resistant variants of DLKP. The DLKP cell line preferentially develops Pgp-mediated resistance rather than MRP-mediated resistance. This study therefore examined the possibility that MRP expression would become down regulated if Pgp expression became up-regulated in a given clone. The second main objective of this study was to attempt to explain the findings of Heenan *et al.*, (1997) which demonstrated that the adriamycin accumulation characteristics of two clones two clones (DLKPA6B and 11B) did not correlate with the levels of Pgp protein possessed by these clones. The DLKPA6B cell line expressed extremely low levels of Pgp (lower than the levels present in DLKP) relative to DLKPA11B but yet after a period of 4 hours, DLKPA6B and DLKPA11B accumulated comparable levels of adriamycin (approximately 200 pmol/10^6 cells). The levels of adriamycin accumulation in these clones were significantly less than the levels accumulated by DLKP over the same period (approximately 600 pmol/10^6 cells) which indicated the expression of an active drug efflux mechanism in these drug resistant clones.

From the results presented in Section 3.18 it was demonstrated that IOVs from all clones examined exhibited ATP-dependent transport of [^{3}H]-LtC_4 to their interior. IOVs isolated from DLKPA2B transported [^{3}H]-LtC_4 in an ATP dependent fashion with an initial rate of 0.5 ± 0.2 pmol/mg protein/minute with [^{3}H]-LtC_4 uptake saturated by 4 minutes at 1.5 ± 0.2 pmol/mg protein. DLKPA11B IOVs also transported [^{3}H]-LtC_4 in an ATP dependent fashion with an initial rate of 0.6 ± 0.3 pmol/mg protein/minute with [^{3}H]-LtC_4 uptake saturated by 3 minutes at 1.1 ± 0.2 pmol/mg protein. DLKPA5F derived IOVs exhibited relatively high levels of [^{3}H]-LtC_4 transport. [^{3}H]-LtC_4 transport occurred at an initial rate of 1.3 ± 0.2 pmol/mg protein/minute with a maximal accumulation of 2.3 ± 0.2 pmol LtC_4 per mg protein, occurring after 2 minutes. The DLKPA6B cell line was found to possess comparable levels of [^{3}H]-LtC_4 transporting abilities to those found in DLKPA11B. The DLKPA6B derived IOVs transported [^{3}H]-LtC_4 with an initial rate of approximately 0.7 ± 0.3 pmol LtC_4 per mg IOV protein per minute. Maximal accumulation (1.0 ± 0.2 pmol LtC_4 per mg IOV protein) of [^{3}H]-LtC_4 by DLKPA6B IOVs occurred after an incubation period of 2 minutes. Western blotting protein analysis demonstrated that DLKP and DLKPA5F IOVs contained reasonably comparable levels of MRP. DLKPA6B IOVs contained marginally higher protein levels of MRP than found in DLKPA11B IOVs. The protein levels of MRP in DLKPA2B IOVs were found to be extremely low. The results of [^{3}H]-LtC_4 transport and Western blotting experiments are shown in summary in Table 4.12.1. All [^{3}H]-LtC_4 transport data is expressed as the mean ± S.D. of duplicate determinations carried out on two separate occasions, using different IOV preparations from the relevant cell line on each occasion.

	DLKP	DLKP-A2B	DLKP-A11B	DLKP-A5F	DLKP-A6B
MRP protein levels in IOVs	High	Very low	Intermediate	High	Intermediate
Pgp protein levels in cells*	Low	Intermediate	High	Very high	Very low
Initial LtC_4 transport rate (pmol/mg/min)	0.8 ± 0.2	0.5 ± 0.2	0.6 ± 0.3	1.3 ± 0.2	0.7 ± 0.3
LtC_4 accumulation after 4 minutes (pmol/mg)	2.0 ± 0.3	1.5 ± 0.2	1.1 ± 0.2	2.3 ± 0.2	1.0 ± 0.2
Adriamycin fold resistance *	1.0 ± 0.1	37.0 ± 3.7	84.0 ± 8.4	331 ± 33.1	95.0 ± 9.5
Adriamycin accumulation after 4 hours (pmol/10^6 cells)*	618 ± 39	424 ± 31	204 ± 12	160 ± 12	232 ± 12
Cisplatin fold resistance*	1.0 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	1.4 ± 0.1	0.7 ± 0.1

* Data obtained from Heenan *et al.*, (1997)

Table 4.12.1: Summary of MRP protein levels and LtC_4 transport characteristics of IOVs isolated from DLKP and clonal subpopulations of the drug resistant DLKP variant DLKP-A. The levels of MRP protein in IOVs from the various cell lines are illustrated in Figure 3.18.5. Densitometry has not been used to quantify the protein levels of MRP or Pgp present but instead the level protein expression as determined by Western blotting is illustrated in the above table according to the band intensity at the position corresponding to the molecular weight of MRP (190 kDa) or Pgp (170 kDa).

The primary objective of this study was to examine if MRP protein levels were related to the protein levels of Pgp in a clone with a known level of adriamycin resistance. From Table 4.12.1 it can be seen that DLKPA2B cells express higher levels of Pgp protein and lower levels of MRP protein than is found to be the case for DLKP. DLKPA11B cells express higher levels of Pgp and MRP protein than DLKPA2B. DLKPA5F cells express higher levels of Pgp and MRP than is found to be the case for DLKPA11B and DLKPA2B. The levels of MRP protein in DLKPA5F are higher than present in any of the other clones and these levels are comparable to the levels of MRP present in DLKP. These results may be partially explained by the levels of adriamycin resistance of these clones (Table 4.12.1).

The following discussion ignores the potential influences on drug resistance of the levels of Topo II protein possessed by the various clones. The primary aim of this study was to determine if MRP protein expression was influenced in some way by the levels of Pgp protein expression in the various clones. It was not designed to determine the precise contribution of either of these transporters to the overall level of drug resistance of the various clones. The DLKPA2B clone was the least resistant of the clones (approximately 37 fold more resistant to adriamycin than DLKP). In this clone, Pgp overexpression may be sufficient to permit survival of DLKPA2B in relatively low adriamycin concentrations. For this reason, MRP protein synthesis could be down-regulated from the levels present in DLKP, as the levels of Pgp present would be more than sufficient for efflux of the levels of drug to which this cell line would be exposed. The DLKPA11B cell line was significantly (84-fold) more resistant to adriamycin than DLKP. In this clone, the levels of Pgp and MRP protein were higher than was found in the case of DLKPA2B. Although increased levels of Pgp would facilitate a higher level of resistance in DLKPA11B compared to DLKPA2B, an increase in the level of MRP protein from the levels in DLKPA2B could also simultaneously contribute to an increase in the level of resistance to adriamycin. In the absence of significant MRP protein expression by a clone with a level of adriamycin resistance equivalent to DLKPA11B, one would predict that the levels of Pgp in this clone would have to be higher than found in DLKPA11B. The DLKPA5F clone was highly resistant to adriamycin (330 fold more resistant than DLKP). This clone expressed higher levels of MRP and Pgp protein than DLKPA11B. The higher levels of Pgp protein in DLKPA5F compared to DLKPA11B, contributed to DLKPA5F possessing a higher level of adriamycin resistance than DLKPA11B. The higher levels of MRP protein in DLKPA5F could also simultaneously contribute to the relatively high level of adriamycin resistance of this cell line. In summary, the results suggest that in the low level adriamycin resistant clone DLKPA2B, increased levels of Pgp expression were sufficient to permit survival of these cells in low adriamycin concentrations. For this reason MRP protein expression could be effectively down regulated without any detrimental effects on the ability of this clone to survive in the presence of low adriamycin concentrations. In clones with higher levels of adriamycin resistance such as

DLKPA11B and DLKPA5F, high levels of Pgp protein expression contributed to the abilities of these clones to survive in high adriamycin concentrations. In addition, the simultaneous expression of increased levels of MRP also contributed to this resistance. The significance as regards drug resistance, of small variations in MRP protein levels between the various clones may be questionable because of the fact that only very low levels of the protein are present in any of the clones. It should be remembered that NSAID-mediated interference in the activity of the low levels of MRP present in DLKP was sufficient to increase drug-induced cell kill. For this reason, even minor alterations in the levels of MRP protein, when combined with alterations in the levels of other drug resistance mechanisms such as Pgp, could have significant influences on the level of resistance of a clone to a given drug.

The second main objective of these studies was to attempt to explain the adriamycin accumulation characteristics of the DLKPA6B and DLKPA11B cell lines. The DLKPA6B cell line was omitted from the previous discussion as the levels of adriamycin resistance of this clone were particularly unusual considering the levels of Pgp and Topo II possessed by this cell line. This cell line was shown by Heenan *et al.*, (1997) to express lower levels of Pgp protein than was found in the case of DLKP. In addition this cell line expressed similar levels of Topo II protein compared to the levels found in DLKP. It was surprising for these reasons to find that DLKPA6B exhibited comparable levels of adriamycin resistance to those found in the case of DLKPA11B (Table 4.12.1) as DLKPA11B was known to express more Pgp and less Topo II protein than DLKP. This suggested that a non-Pgp and Topo II related mechanism was involved in the levels of drug resistance of DLKPA6B. The fact that DLKPA6B and DLKPA11B accumulated comparable levels of adriamycin after a period of 4 hours (Table 4.12.1) suggested that in the absence of significant levels of Pgp in DLKPA6B, a drug efflux pump may have been present in DLKPA6B which compensated for low levels of Pgp in this cell line. From Table 4.12.1 and Figure 3.18.5 it can be seen that DLKPA6B and DLKPA11B contain comparable levels of MRP protein in their plasma membrane. For this reason it can be stated that MRP is not responsible for the unusual drug accumulation characteristics of DLKPA6B. The

identity of the drug transporter molecule involved in the active efflux of drug in DLKPA6B requires further investigation.

From Table 4.12.1 it can be seen that IOVs from a number of the clones exhibit significantly different levels of [^{3}H]-LtC_4 transport than was found in DLKP. If MRP was totally responsible for [^{3}H]-LtC_4 transport in IOVs from these clones, one would assume from the [^{3}H]-LtC_4 transport data that MRP protein levels in 2B, 11B and 6B derived IOVs would be slightly lower than in DLKP IOVs which in turn would be lower than the levels found in DLKPA5F IOVs. From Figure 3.18.5 it was shown that this was not the case. These results could be used to suggest that a non-MRP1 gene product LtC_4 transporter was responsible for the unusual [^{3}H]-LtC_4 transporting abilities of IOVs derived from the various clones. From Figure 3.18.5 it can be seen that HL60-ADR IOVs possess extremely high levels of MRP protein. A study performed by Kool *et al.*, (1997) demonstrated that HL60-ADR cells contained very high levels of MRP-1 mRNA and practically negligible levels of the other MRP analogues analysed. This suggested that the monoclonal antibody used for detection of MRP (Figure 3.18.5) showed high affinity for the MRP-1 gene product (specificity for MRP related isoforms not known). The presence of high levels of MRP-1 protein in HL60-ADR is reflected in an initial rate of [^{3}H]-LtC_4 transport of 4.0 ± 1.0 pmol/mg protein/minute with a saturation value of 7.0 ± 1.0 pmol LtC_4/mg protein reached after 1-2 minutes. The only clone with a relatively high LtC_4 transport ability was found to be DLKPA5F (Table 4.12.1). From Figure 3.18.5 it can be seen that IOVs from DLKPA5F contained slightly lower levels of MRP-1 protein than found in DLKP derived IOVs. Table 4.12.1 illustrates that DLKPA5F derived IOVs possess higher capabilities for [^{3}H]-LtC_4 transport than is found in the case of DLKP. The DLKPA6B cell line was found to express marginally lower protein levels of MRP-1 than found in DLKP or DLKPA5F. This was reflected in relatively low [^{3}H]-LtC_4 transport capabilities. IOVs isolated from the DLKPA2B cell line were found to possess very low levels of MRP (Figure 3.18.5). Surprisingly these IOVs exhibited an initial rate of [^{3}H]-LtC_4 transport of 0.5 ± 0.2 pmol/mg/min, a rate comparable to that achieved in the case of DLKPA6B and DLKPA11B.

The possible presence of a non-MRP1 gene product [^{3}H]-LtC_4 transporter is strongly suggested by the [^{3}H]-LtC_4 transport and Western blotting results obtained in the case DLKPA5F and DLKPA2B. The various clones of DLKP-A are known from Western blotting studies to express the MRP-1 gene product, and so even if an additional [^{3}H]-LtC_4 transporter was present in any of these clones, the contribution of any individual transporter to overall [^{3}H]-LtC_4 transport and ultimately to drug resistance, becomes extremely difficult to conclusively determine. If a non-MRP1 gene product, glutathione conjugate pump was involved in [^{3}H]-LtC_4 transport in the various clones, the specific identity of this transporter is not fully clear from the experimental data. The DLKPA2B, 11B, 6B and 5F clones are 0.6, 0.7, 0.6 and 1.39 fold resistant to cisplatin relative to the parental DLKP cell line (which is designated a fold resistance value of 1). The cMOAT protein is a product of the MRP-2 gene and has been shown by Kool *et al.*, (1997) to be elevated in several cell lines selected for cisplatin resistance and in a select number of adriamycin selected non-small cell lung cancer cell lines. This group provided some evidence that expression levels of this transporter correlated with the cisplatin but not adriamycin resistance levels of these cell lines. If the cMOAT protein contributed to [^{3}H]-LtC_4 transport simultaneously with MRP-1 mediated [^{3}H]-LtC_4 transport in the clones one would predict that there was little cMOAT in the DLKPA6B and DLKP11B clones. This is due to the fact that the levels of [^{3}H]-LtC_4 transport in these clones were even lower than the levels predicted from the levels of MRP-1 possessed by these clones relative to DLKP. The DLKPA5F clone possessed comparable levels of MRP-1 protein to those found in DLKP, but if cMOAT was present, simultaneous MRP-1 and cMOAT activity could have contributed to the higher levels of [^{3}H]-LtC_4 transport in DLKPA5F than was found in the case of DLKP derived IOVs. The DLKPA2B cell line expressed very little MRP-1 in the plasma membrane and so if simultaneous cMOAT and MRP-1 expression was found, then DLKPA2B IOVs must be assumed to contain reasonably high levels of cMOAT which resulted in a relatively high levels of [^{3}H]-LtC_4 transport into the IOVs. These assumptions would predict that DLKPA6B and 11B would possess a comparable level of cisplatin resistance to that found in the case of DLKP. In addition this would also

predict that DLKPA2B and DLKPA5F would be more resistant to cisplatin than was found in the case of DLKP. From Table 4.12.1 it can be seen that this is not found to be the case. Attempting to correlate levels of drug resistance with a specific drug resistance mechanism is questionable because of the many complex interacting systems involved in resistance to a given drug. It is particularly questionable in this particular case of cisplatin resistance of the DLKP-A clones because of the very small differences in fold resistance of these clones relative to DLKP (fold resistance ranging only from 0.6 to 1.4). For this reason there is no evidence currently available to suggest that cMOAT in any way contributes to [^{3}H]-LtC_4 transport in the various clones. The potential contribution of MRP analogues to [^{3}H]-LtC_4 transport and to various aspects of drug resistance of the DLKP-A clones requires further investigation.

5.0 Conclusions

The *in vitro* toxicity assay screening performed with the A549 cell line demonstrated that the toxicity of a specific group of chemotherapeutic drugs was enhanced in the presence of a suitable NSAID. A number of key results were crucial in the elucidation of the mechanism involved in this effect. These results are summarised in the following points:

1. The cytotoxicity of adriamycin, vincristine, daunorubicin, epirubicin and VP-16 in A549 was enhanced in the presence of non-toxic levels of a suitable NSAID. The toxicity's of carboplatin, Taxol and 5-fluorouracil were unaffected by the presence of these NSAIDs.
2. Only a limited number of NSAIDs were found to enhance the toxicity of this select profile of chemotherapeutic drugs namely indomethacin, sulindac, tolmetin, acemetacin, sulindac sulfide, sulindac sulfone, zomepirac and mefenamic acid.
3. This toxicity enhancement effect was found to occur to a comparable extent in the human lung squamous carcinoma cell line, DLKP. All NSAIDs found to enhance drug toxicity in A549 were also found to enhance drug toxicity in DLKP.
4. The chemotherapeutic drugs described above are believed to act as substrates for the multidrug resistance-associated protein (MRP). Heavy metals such as sodium arsenate are known to be transported by MRP. The toxicity of this compound was found to be enhanced in the presence of an NSAID such as indomethacin.
5. The ability of the NSAIDs listed above to enhance chemotherapeutic drug toxicity was demonstrated to be independent of any effects on prostaglandin H_2 synthase (PGHS). This was conclusively demonstrated by the ability of sulindac sulfone, a metabolite of sulindac which is devoid of inhibitory effects on PGHS to enhance the toxicity of a suitable cytotoxic drug. The extent of chemotherapeutic drug toxicity enhancement was comparable to that achieved by the potent PGHS inhibitor, sulindac sulfide.

6. Interference in MRP-mediated drug efflux by a given NSAID appeared to explain the NSAID-mediated enhancement of chemotherapeutic drug toxicity. The major weakness in this theory was the fact that MRP protein had not been detected by Western blot analysis of whole cell extracts of the DLKP cell line. As the toxicity enhancement effect was known to occur in this cell line, one would have predicted significant levels of MRP protein to be present in this cell line. Isolation of plasma membrane derived inside-out vesicles (IOVs) from DLKP demonstrated the presence of active MRP protein in this cell line. It was found that NSAIDs such as indomethacin, which can potently enhance the toxicity of MRP substrate drugs, have the ability to interfere with MRP activity (as assessed by LtC_4 transport capabilities) in DLKP IOVs. NSAIDs without chemotherapeutic drug toxicity enhancing abilities such as naproxen, were demonstrated to be without effect on LtC_4 transport in DLKP IOVs. These results suggested that MRP was present in the plasma membrane of DLKP cells and that this drug transporter was effective in aiding survival of these cells against the cytotoxic effects of chemotherapeutic drugs. Treatment of DLKP with NSAIDs such as indomethacin interfered with this drug resistance mechanism and ultimately caused an enhancement in chemotherapeutic drug toxicity.

   The presence of an active drug resistance mechanism in DLKP may appear relatively surprising as this cell line is regarded as being drug sensitive. The existence of low levels of MRP in DLKP is not surprising when one considers that this cell line is also known to express Pgp protein at low levels (Heenan *et al.*, 1997).

The use of NSAIDs such as indomethacin as modulators of MRP-related drug resistance *in vivo*, has the potential to be more successful for the development of clinically successful chemotherapeutic regimes than has been achieved with existing modulators of multidrug resistance such as cyclosporin A or verapamil. MDR modulators such as verapamil have not proven to be effective modulators of

MDR drug resistance mechanisms because these compounds are often required to be present at high concentrations to achieve their MDR modulating effects. It has not been generally possible to achieve these high modulator concentrations in patient's serum without unacceptable side effects. NSAIDs such as indomethacin have been shown *in vitro* to enhance chemotherapeutic drug toxicity at concentrations of even below 1 μg/mL (3 μM). Indomethacin is rapidly absorbed from the gastrointestinal tract following ingestion. Once absorbed it is almost entirely bound to plasma proteins and can reach a peak plasma concentration of 0.5-1 μg/mL, 2 hours after administration of a 25 mg dose, with a half life of 2-3 hours, (Flower *et al.*, 1980). Indomethacin has been chosen as an example, but from the *in vitro* data it may be suggested that it may be possible to achieve the NSAID concentrations *in vivo* that have been demonstrated to be sufficient for MRP-modulatory effects *in vitro*.

Although the explanation for the NSAID-mediated drug toxicity enhancement effect is based upon interference in drug efflux by MRP, the mechanism by which chemotherapeutic drugs are transported by MRP is currently unclear. No evidence of direct transport of [^{3}H]-vincristine by MRP into HL60-ADR derived IOVs was found experimentally. It was demonstrated that vincristine significantly inhibited [^{3}H]-LtC_4 transport by MRP into HL60-ADR IOVs, suggesting the occurrence of competition for transport by MRP or competition for binding to the substrate binding site of MRP. The inability to demonstrate direct [^{3}H]-vincristine transport in HL60-ADR IOVs was believed to be a result of the possibility that very high vincristine concentrations were required for MRP to exhibit significant transport of this drug. Adriamycin was found to inhibit [^{3}H]-LtC_4 transport at high drug concentrations only, (200 μM). Cisplatin and Taxol were not found to significantly inhibit [^{3}H]-LtC_4 transport by MRP even when present at high concentrations. Taxol and cisplatin are not believed to be high affinity substrates for MRP, whereas vincristine and adriamycin are believed to be substrates for this glutathione conjugate pump. These results suggested that MRP did possess some abilities to bind to unconjugated natural product drugs such as vincristine when present at very high concentrations. The HL60-ADR cell line was selected for

resistance in 186 nM adriamycin but only concentrations of over 200 μM adriamycin were found to inhibit [^{3}H]-LtC_4 transport by MRP. Intracellular concentrations of 200 μM adriamycin may appear to be extremely high for consideration as being an achievable intracellular concentration but these concentrations may be relevant if drug somehow became localised in a subcellular compartment or organelle. If MRP was localised in a membrane adjacent to this region, it would effectively be exposed to this extremely high concentration of drug. The leukotriene D_4 receptor antagonist, MK571 was found to potently inhibit [^{3}H]-LtC_4 transport by MRP at concentrations as low as 10 μM. This demonstrated that cytotoxic drugs such as vincristine are relatively poor inhibitors of [^{3}H]-LtC_4 transport by MRP.

It was previously believed that reduced glutathione (GSH) was not a substrate for transport by the glutathione conjugate pump MRP, (Leier *et al.*, 1996). Loe *et al.*, (1996b) stated that a concentration of 5 mM GSH was without effects on the [^{3}H]-LtC_4 transport activity of MRP in IOVs isolated from MRP-transfected cells. It was found experimentally with HL60-ADR IOVs that MRP activity was potently inhibited even in the presence of 4 mM GSH. Concentrations of GSH below 1.9 mM GSH were found to be without significant effects on the [^{3}H]-LtC_4 transport activity of MRP. These inhibitory effects of GSH on [^{3}H]-LtC_4 accumulation in HL60-ADR IOVs may have been a result of active transport of this tripeptide by MRP although there has been no previous experimental evidence to suggest that this occurs. An equally possible alternative was that GSH competitively interfered with the binding of [^{3}H]-LtC_4 to the substrate binding site of MRP. The chemotherapeutic drug which was found to be most inhibitory for [^{3}H]-LtC_4 transport by MRP was found to be vincristine. The inhibitory effects of vincristine on [^{3}H]-LtC_4 transport were found to be enhanced in the presence of 1 mM GSH. The inhibitory effects of the non-MRP substrate drugs Taxol and cisplatin were unaffected by the presence of 1 mM GSH. This may suggest that co-transport of vincristine and GSH may have occurred. An alternative explanation involves the possibility that MRP possesses a number of substrate binding sites, one of which recognises GSH. Even in the absence of GSH, a

glutathione conjugate such as [^{3}H]-LtC_4 could be transported by MRP once bound to the relevant specific substrate binding site. When GSH and an agent that could bind to one of the additional substrate binding sites on MRP (such as vincristine but not Taxol) were present, this agent could be transported by MRP because of the fact that both the GSH and the substrate binding sites would be simultaneously occupied. This ultimately would result in the overall level of [^{3}H]-LtC_4 transport into the IOVs to be reduced because of the increased competition between vincristine and [^{3}H]-LtC_4 for active transport by MRP. The specific mechanism responsible for the ability of GSH to enhance the ability of vincristine to inhibit [^{3}H]-LtC_4 transport requires further investigation. The results do suggest that the presence of GSH may cause active transport of drugs such as vincristine by MRP. The inhibitory effects of very high concentrations of vincristine and adriamycin may have been a result of these drugs simply binding MRP and so blocking access for [^{3}H]-LtC_4. The non-MRP substrate drugs, Taxol and cisplatin did not possess the ability to bind to MRP and so did not exert any effects on the level of [^{3}H]-LtC_4 transport There was no direct evidence to suggest that drugs such as vincristine were actually transported by MRP under conditions where GSH was absent. The increase in the ability of vincristine (due the presence of GSH), to exhibit relatively potent inhibitory effects on [^{3}H]-LtC_4 transport by MRP suggested that GSH is required for transport of vincristine by MRP. These results may indicate that in the case of MRP-expressing cells, that glutathione metabolism may play a key role in the efficacy of MRP as a drug transporter. 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 synthesis caused an inhibition in the drug efflux activity of MRP in MRP-overexpressing cell lines. The results of the studies by Versantvoort *et al.*, (1995) and the results of the studies described above suggest that one should consider GSH metabolism when investigating the influence of MRP on drug resistance of a given cell line. Examination of MRP protein expression and activity in a given cell line may give some indication of the influence of this glutathione conjugate pump in the drug resistance of a cell line. If a cell line was in any way deficient in GSH metabolism, then the ability of MRP to transport drugs such as vincristine could be

substantially reduced. For this reason, both MRP protein expression and GSH metabolism should be examined in any experiments attempting to develop a conclusive explanation for the involvement of MRP in the drug resistance of any given cell line.

The detection of active MRP protein in DLKP cells suggested that some of the drug resistance characteristics of DLKP related drug selected cell lines may have been explainable by the presence of MRP in these drug resistant cell lines. The DLKP-A10 cell line was established by exposure of DLKP-A cells to gradually increasing concentrations of adriamycin. This cell line was known to overexpress P-glycoprotein (Pgp) and to accumulate less adriamycin over time than DLKP cells. This was also known to occur in the case of the SKMES-1/ADR (drug resistant) cell line, which was derived from the drug sensitive, SKMES-1 cell line. The accumulation deficit was found to be reversed in SKMES-1/ADR cells by treatment with the modulators of P-glycoprotein (Pgp), verapamil and cyclosporin A. The accumulation deficit was found to be only partially reversed with these compounds in the case of DLKP-A10 cells. This indicated that non-Pgp-related drug efflux mechanisms were active in DLKP-A10 cells. The significant levels of intracellular vesicle formation by this cell line suggested that this highly resistant cell line may have possessed significant levels of MRP as intracellular vesicle formation has been documented as being a feature of a number of MRP-expressing cell lines. The only compound identified as having the ability to completely reverse the accumulation deficit in DLKP-A10 was found to be the metabolic inhibitor, antimycin A. This effect could not be achieved using the metabolic inhibitor sodium azide and so the effects of antimycin A on DLKP-A10 cells were not believed to be solely dependent on inhibition of ATP production. The ability of antimycin A to inhibit ATP production was necessary for achievement of reversal of the accumulation deficit in DLKP-A10 as antimycin A had no effects on drug accumulation under conditions where cells were not deprived of ATP. Isolation of plasma membrane derived IOVs from DLKP-A10 demonstrated that this cell line expressed MRP protein and that the levels of MRP were lower than those present in DLKP. IOVs from the DLKP-A10 cell line exhibited slightly lower levels of [^{3}H]-LtC_4 transport than found in the case of

IOVs from the DLKP cell line. It was found that antimycin A was a surprisingly potent inhibitor of MRP activity in DLKP-A10 IOVs but was no more inhibitory of [^{3}H]-LtC_4 transport activity than cyclosporin A. Sodium azide was found to only slightly inhibit [^{3}H]-LtC_4 transport activities of DLKP-A10 IOVs at relatively high concentrations. The effects of the metabolic inhibitor antimycin A were found to be independent of any inhibitory effects on the ATP regenerating system in the IOV transport assay model. The results suggested that the abilities of antimycin A to simultaneously inhibit ATP production and MRP activity resulted in this compound reducing the drug accumulation deficit in DLKP-A10. Sodium azide only partially reversed this deficit because of the fact that although it inhibited ATP synthesis, this metabolic inhibitor exerted relatively insignificant effects on MRP activity in DLKP-A10. The expression of low levels of MRP protein in DLKP-A10 may be influential on the drug accumulation characteristics of DLKP-A10 only when ATP levels in these cells became depleted. The ability of antimycin A to inhibit ATP production and MRP activity reduced the influence of MRP on the drug accumulation characteristics of DLKP-A10 and so under glucose free conditions, caused elimination of the drug accumulation deficit in this cell line. Antimycin A was found to possess relatively potent inhibitory effects on MRP and so may have potential for use as a modulator of MRP activity in non-Pgp overexpressing cell lines. The possibility of using antimycin A as a modulator of MRP-mediated drug resistance *in vitro* requires further investigation, but the extreme toxicity caused by even low concentrations of antimycin A suggests that this compound may not be entirely suitable for this application.

The identification of active MRP protein in DLKP suggested that MRP may have an involvement in the drug accumulation and resistance characteristics of clonal subpopulations isolated from the DLKP-A cell line. It was found that all of the clones analysed contained active MRP protein. In a clone with a low level of adriamycin resistance,(DLKPA2B) it was found that Pgp expression increased and MRP expression decreased from the levels present in DLKP. It was suggested that this was a result of high levels of Pgp protein expression being sufficient to facilitate survival of DLKPA2B in low adriamycin concentrations. For this reason MRP protein expression could be down-regulated without any detrimental effects

on the ability of this clone to survive in low adriamycin concentrations. In clones of higher adriamycin resistance such as DLKPA11B and DLKPA5F it was found that Pgp and MRP protein expression was upregulated from the levels present in DLKPA2B. The simultaneous upregulation of MRP protein synthesis in these clones may have contributed to the level of adriamycin resistance of these clones. The differences in MRP protein levels between the various clones were relatively small. None of the clones expressed more MRP protein than DLKP. The fact that small levels of MRP protein were found to exist in the various clones does not necessarily mean that MRP did not influence the drug accumulation and drug resistance characteristics of these clones. Even though these clones generally contain Pgp protein, MRP may simultaneously exert some influences on the ability of drugs to be retained within the various clones. The adriamycin accumulation characteristics of the DLKPA6B and DLKPA11B cell lines did not correlate with the levels of Pgp protein possessed by these clones, (Heenan *et al.*, 1997). The DLKPA6B cell line expressed extremely low levels of Pgp relative to DLKPA11B but after 4 hours these clones accumulated comparable levels of adriamycin. These results suggested that a non-Pgp drug efflux pump may have been involved in the ability of DLKPA6B and DLKPA11B to accumulate comparable levels of adriamycin. It was found that these clones contained comparable levels of MRP protein. These levels were found to be lower than the protein levels present in DLKP. For these reasons, it was demonstrated that MRP was not the reason for the ability of DLKPA6B and DLKPA11B to accumulate comparable levels of adriamycin despite large differences in Pgp protein expression. The presence of non-MRP1 gene product LtC_4 transporters was suggested from the levels of LtC_4 transport achieved by the DLKPA5F and DLKPA2B clones. The protein levels of MRP in DLKPA2B were found to be extremely low but yet IOVs from this clone exhibited significant [^{3}H]-LtC_4 transport activity. The [^{3}H]-LtC_4 transporting properties of DLKPA5F were higher than would be predicted from the protein levels of MRP-1 in the IOVs from this clone. The specific identity of the putative non-MRP1 gene product [^{3}H]-LtC_4 transporters in the various clones was not obvious from the experimental data obtained to date. Further investigation in this area will be required in order to elucidate if non-MRP1 transporters are present in the DLKP-A related clones.

The demonstration of NSAID mediated enhancement of chemotherapeutic drug toxicity in MRP-expressing cell lines may indicate that NSAIDs could potentially be used in the clinical treatment of certain cancers in order to selectively enhance the cytotoxicity of chemotherapy. The possibility of including NSAIDs in chemotherapeutic regimes is currently being intensively investigated by researchers at the NCTCC. The investigations into the role of MRP in the NSAID-mediated enhancement of chemotherapeutic drug toxicity effect uncovered a number of potentially important features of the roles of drug efflux pumps in human cancer cell lines. These findings have already been discussed but some of the future work emanating from these findings are discussed briefly in the following points.

1. The mechanism of drug transport by MRP remains to be fully elucidated. No direct evidence for active transport of [^{3}H]-vincristine was found either in the presence or absence of GSH but this may have simply been a result of the concentrations of [^{3}H]-vincristine used. These were the maximum concentrations of drug that could be used experimentally because at higher concentrations, non-specific binding of [^{3}H]-vincristine to the filters became problematic. In preference to examining the transport of [^{3}H]-vincristine, it could be possible to monitor transport of unlabelled vincristine into IOVs using the ATP assay described in Section 2.14. In the presence of active transport of a suitable substrate and in the absence of creatine kinase, ATP levels significantly decrease (Figure 3.17.3.1). For this reason, it would be possible to indirectly monitor the active transport of any possible substrate of MRP such as vincristine. It would obviously be necessary to include appropriate controls for these experiments to ensure that ATP hydrolysis was specifically occurring as a result of transport of a substrate by MRP. This system would facilitate the rapid identification of compounds with an ability to be actively transported by MRP.

2. The explanation for the apparent increases in vincristine transport in the presence of GSH requires further investigation. As already described this could be a result of some form of co-transport of GSH and vincristine or alternatively a result of multiple substrate binding sites on MRP. If the apparent increases in vincristine transport in the presence of GSH were a reflection of the situation found for drug efflux in cells, then this effect should not be limited to vincristine and should be shared by all potential MRP substrate drugs. This suggestion needs to be examined in more detail.

3. The studies carried out into the involvement of MRP in the drug accumulation and drug resistance characteristics of MRP in DLKP-related drug resistant variants concentrated totally on protein levels and [^{3}H]-LtC_4 transport abilities of this transporter. As already described, GSH metabolism appears to be a critical factor in the involvement of MRP in drug resistance. For this reason it would be necessary to analyse the characteristics of GSH metabolism in these drug resistant variants of DLKP if one was attempting to conclusively explain the involvement of MRP in the drug resistance of one of these variants. The analyses carried out using the drug resistant DLKP-A10 cell line demonstrated that the metabolic inhibitor antimycin A may have effects on MRP activity. Further investigations will be necessary in order to identify if this compound could have effects on MRP drug efflux activity in non-Pgp overexpressing cell lines.

6.0 References

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7.0 Appendices

Appendix 7.1 Abbreviations

AA	Arachidonic acid
ABC	ATP binding cassette
Ace	Acemetacin
ADR	Adriamycin
AMP	Adenosine 5'-monophosphate
ATCC	American Tissue Culture Collection
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSO	DL-Buthionine-[S,R]-sulfoximine
CDK	Cyclin dependent kinase
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
Cis	Cisplatin
CK	Creatine kinase
CML	Chronic myelocytic leukaemia
COX	Cyclooxygenase or Prostaglandin H synthase
CsA	Cyclosporin A
Da	Dalton
Dauno	Daunorubicin
DMEM	Dulbeccos modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamininetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
Epi	Epirubicin
FCS	Foetal calf serum
GSH	Reduced glutathione
GST	Glutathione-S-transferase
HCl	Hydrochloric acid
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
HETE	Hydroxyperoxy fatty acid
HPLC	High pressure liquid chromatography
IC_{50}	Drug concentration sufficient for achievement of 50% cell kill
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 resistance-related protein
Lt	Leukotriene
MDR	Multidrug resistance
Mef	Mefenamic acid
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex

mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance associated protein
MT	Metallothionein
NaCl	Sodium chloride
NADPH	Nicotine adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NEAA	Non-essential amino acids
NSAID	Nonsteroidal antiinflammatory drugs
NSCLC	Non-small cell lung carcinoma
OD	Optical Density
P	Passage
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
Pg	Prostaglandin
PGHS	Cyclooxygenase or Prostaglandin H synthase
PgI_2	Prostacyclin
Pgp	P-glycoprotein
PMSF	Phenylmethylsulphonyl fluoride
psi	Pounds per square inch
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SCLC	Small cell lung carcinoma
SD	Standard deviation
SDS	Sodium dodedecyl sulphate
Sul	Sulindac
TAP	Transporter associated with antigen presentation
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
TGF-ß	Transforming growth factor ß
Tol	Tolmetin
Topo II	DNA topoisomerase II
Tris	Tris(hydroxymethyl)aminomethane
Tx	Thromboxane
UV	Ultraviolet
Vcr	Vincristine
Ver	Verapamil
VM-26	Tenopside
V_{max}	Maximum rate of reaction when substrate present at saturation levels
VP-16	Etoposide
Zom	Zomepirac

Appendix 7.2 Molecular weights

Compound	*Molecular Weight*
Acemetacin	414
Adriamycin	580
Antimycin A	549
Aspirin	180
ATP	509
Cisplatin	300
Cyclosporin A	1203
Daunorubicin	564
Epirubicin	572
Fenoprofen	523
5-Fluoruracil	130
Glutathione (GSH)	307
Indomethacin	358
Ketoprofen	254
Leukotriene C_4	626
Mefenamic acid	241
Mitoxantrone	517
MK571	537
Naproxen	230
Prostaglandin D_2	353
Prostaglandin E_2	353
Sodium azide	65
Sucrose	342
Sulindac	356
Taxol	854
Tolmetin	315
Verapamil	455
Vincristine	825
VP-16	589
VP-16	589
Zomepirac	314

Appendix 7.3 IC_{50} values for DLKP and DLKP-A10

Cell line	Cisplatin IC_{50}, (μM)*	Fold resistance
DLKP	1.125 ± 0.15	1
DLKP-A10	0.90 ± 0.12	0.8

*Values are the average of triplicate *in vitro* toxicity assays performed on two separate occasions.