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Original Paper

Enhancement of Chemotherapeutic Drug Toxicity to Human Tumour Cells *In Vitro* by a Subset of Non-steroidal Anti-inflammatory Drugs (NSAIDs)

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The effect on cytotoxicity of combining a range of clinically important non-steroidal anti-inflammatory drugs (NSAIDs) with a variety of chemotherapeutic drugs was examined in the human lung cancer cell lines DLKP, A549, COR L23P and COR L23R and in a human leukaemia line HL60/ADR. A specific group of NSAIDs (indomethacin, sulindac, tolmetin, acemetacin, zomepirac and mefenamic acid) all at non-toxic levels, significantly increased the cytotoxicity of the anthracyclines (doxorubicin, daunorubicin and epirubicin), as well as teniposide, VP-16 and vincristine, but not the other vinca alkaloids vinblastine and vinorelbine. A substantial number of other anticancer drugs, including methotrexate, 5-fluorouracil, cytarabine, hydroxyurea, chlorambucil, cyclophosphamide, cisplatin, carboplatin, mitoxantrone, actinomycin D, bleomycin, paclitaxel and camptothecin, were also tested, but displayed no synergy in combination with the NSAIDs. The synergistic effect was concentration dependent. The effect appears to be independent of the cyclo-oxygenase inhibitory ability of the NSAIDs, as (i) the synergistic combination could not be reversed by the addition of prostaglandins D₂ or E₂; (ii) sulindac sulphone, a metabolite of sulindac that does not inhibit the cyclooxygenase enzyme, was positive in the combination assay; and (iii) many NSAIDs known to be cyclo-oxygenase inhibitors, e.g. meclofenamic acid, diclofenac, naproxen, fenoprofen, phenylbutazone, flufenamic acid, flurbiprofen, ibuprofen and ketoprofen, were inactive in the combination assay. The enhancement of cytotoxicity was observed in a range of drug sensitive tumour cell lines, but did not occur in P-170-overexpressing multidrug resistant cell lines. However, in the HL60/ADR and COR L23R cell lines, in which multidrug resistance is due to overexpression of the multidrug resistance-associated protein MRP, a significant increase in cytotoxicity was observed in the presence of the active NSAIDs. Subsequent Western blot analysis of the drug sensitive parental cell lines, DLKP and A549, revealed that they also expressed MRP and reverse-transcription-polymerase chain reaction studies demonstrated that mRNA for MRP was present in both cell lines. It was found that the positive NSAIDs were among the more potent inhibitors of [³H]-LTC₄ transport into inside-out plasma membrane vesicles prepared from MRP-expressing cells, of doxorubicin efflux from preloaded cells and of glutathione-S-transferase activity. The NSAIDs did not enhance cellular sensitivity to radiation. The combination of specific NSAIDs with anticancer drugs reported here may have potential clinical applications, especially in the circumvention of MRP-mediated multidrug resistance. © 1998 Elsevier Science Ltd. All rights reserved.

Key words: non-steroidal anti-inflammatory drugs (NSAIDs), multidrug resistance-associated protein (MRP), circumvention, multidrug resistance (MDR), chemotherapy, cancer, treatment

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INTRODUCTION

INTRINSIC OR acquired resistance is an important limitation on the effectiveness of chemotherapy. Classical multidrug resistance (MDR) is characterised by cross-resistance to a range of chemically unrelated drugs. A number of mechanisms have been described to explain the development of MDR in a variety of cell types, including overexpression of transmembrane glycoprotein pumps, reduced expression of topoisomerase II and alterations in levels of metabolic enzymes, particularly of glutathione-S-transferases. The best characterised cause of MDR is overexpression of the *MDR1* gene which encodes the transmembrane protein P-glycoprotein (P-170) [1]. This protein acts as an ATP-dependent efflux pump for a range of cytotoxic agents, reducing their intracellular level. More recently, other novel proteins have been reported that are responsible for non-P-170-mediated MDR. The discovery of a MDR-associated protein (MRP) which confers MDR on a resistant variant of the H69 lung cell line was reported in 1992 [2]. MRP, which has a broad expression pattern in human tissues and solid tumour cell lines [3], has been characterised as a 190 kDa protein which from the deduced amino acid sequence was classified as a member of the superfamily of ABC transmembrane transporters [2]. P-170 is also a member of this superfamily, but the two proteins demonstrate only approximately 15–19% sequence homology. Both proteins share similar drug efflux profiles with significant differences, e.g. MRP does not confer a significant level of resistance to paclitaxel or vinblastine. Another distinction is that MRP can render cells resistant to some heavy metal anions, including arsenite, arsenate and trivalent and pentavalent antimonials, but not to other metal ions such as cadmium [4]. There are also differences in the verapamil sensitivity of MRP-overexpressing cell lines compared with cells whose resistance profile is due to P-170 [5]. Reduced drug accumulation and enhanced drug efflux is usually observed in drug-selected cells that overexpress MRP, although the mechanism by which MRP acts is not definitively understood [6]. MRP can act as an efflux pump for glutathione conjugates and it was originally thought that glutathione conjugation was an important step in the efflux of cytotoxic drugs from cells [7]. Recent reports have observed that unaltered cytotoxic drugs can be transported into inverted vesicles prepared from a MRP-overexpressing cell line, suggesting that glutathione conjugation may not be essential for efflux via MRP [8]. MDR due to P-170 expression can be circumvented by a variety of chemical agents, e.g. verapamil and cyclosporin, but clinical trials utilising these agents have proved to be disappointing, due in part to adverse side-effects caused by the combination [9]. To date, very little information has been reported on circumventing MRP-mediated MDR [10–13], especially with agents that may be useful clinically.

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently used medicinal drugs. They are utilised primarily as analgesics, anti-inflammatories and anti-platelets and their side-effects have been well studied. Their main known mode of action is through inhibition of the cyclo-oxygenase-mediated production of prostaglandins, but this is not thought to be sufficient to explain their wide variety of actions [14]. There is currently widespread interest in their potential roles as cancer chemopreventive agents [15] and NSAIDs have also been shown to potentiate tumour radio-response [16]. A number of studies have investigated the

possibility of NSAID-mediated enhancement of chemotherapeutic drug toxicity specifically combining flurbiprofen or indomethacin with methotrexate or melphalan [17–19]. Several other NSAIDs have been used in combination with a number of cytotoxic drugs, including an examination of the interaction of mefenamic acid, diflunisal, sulindac or indomethacin with cyclophosphamide, melphalan, or carmustine [20] and a reported enhancement of vincristine and doxorubicin cytotoxicity by indomethacin in human pulmonary adenocarcinoma cells [21]. A recent report indicates that indomethacin may reverse MRP-mediated efflux of doxorubicin and vincristine via inhibition of MRP pumping and inhibition of glutathione-S-transferase [22]. However, none of these studies has covered a comprehensive range of NSAIDs and anticancer agent classes.

This paper describes the first extensive screen of commercially available NSAIDs with anticancer drugs and discusses the potential clinical benefits of such combinations.

MATERIALS AND METHODS

Cell lines

All cells were grown at 37°C in an atmosphere of 5% CO₂ in antibiotic-free medium and were passaged two to three times per week. DLKP, a human lung squamous cell line was cultured in ATCC medium consisting of a 50:50 Hams F-12:DMEM solution supplemented with 5% fetal calf serum (FCS), and 2 mM glutamine [23]. A 300-fold resistant variant of DLKP, DLKP-A, which overexpresses P-170 [24], and a *MDR1* transfectant of DLKP, DLKP pHaMDR1/A #2, which is 15-fold resistant to doxorubicin (S. Coyle, Dublin City University, Ireland) were cultured in ATCC medium. A549, a human lung adenocarcinoma cell line, was cultured in DMEM supplemented as above. COR L23P and R (parental and MRP-overexpressing resistant variants, respectively), kindly donated by P. Twentyman, Cambridge, U.K., were cultured in RPMI 1640 medium supplemented with 10% FCS. These cell lines grew as monolayers.

Two human leukaemia cell lines were kindly donated by M. Center, University of Kansas, U.S.A.: HL60 which is sensitive to a range of anticancer drugs, and a variant thereof, HL60/ADR that has been selected for increased resistance to doxorubicin and which overexpresses MRP. These suspension cells were grown in RPMI 1640, supplemented with 10% FCS and 2 mM L-glutamine in a 5% CO₂ incubator at 37°C.

Chemicals

Doxorubicin and epirubicin (Farmitalia); daunorubicin (Rhone-Poulenc Rorer); carboplatin, cisplatin, and 5-fluorouracil (David Bull Labs); paclitaxel, etoposide (VP-16), and vincristine (Bristol-Myers Squibb Pharmaceuticals Ltd); mitoxantrone (Lederle); cytarabine (Upjohn) and bleomycin (Lundbeck) were all supplied as solutions and were diluted for use in culture medium. Actinomycin D, camptothecin, chlorambucil and methotrexate (Sigma) were dissolved in dimethyl sulphoxide (DMSO) before being diluted to their working concentrations in culture medium. A stable prodrug of cyclophosphamide, mafosfamide cyclohexylamine, was kindly provided by Asta Chemical Co. (Germany) and was dissolved in culture medium.

All the NSAIDs and all other chemicals (except sulindac sulphide and sulphone which were prepared as described in [25]) were purchased from Sigma (U.K.). Acetaminophen, acemetacin, fenoprofen, flufenamic acid, flurbiprofen,

indomethacin, ketoprofen, meclofenamic acid, mefenamic acid, phenylbutazone, piroxicam, sulindac and zomepirac were all dissolved at 10 mg/ml in DMSO before being diluted to their working concentrations with culture medium. Ibuprofen and diclofenac were dissolved in ethanol at 10 mg/ml before being diluted. Aspirin, aurothioglucose, chloroquine, levamisole, naproxen and tolmetin all dissolved directly in the culture medium.

In vitro toxicity testing

For the monolayer cell lines, miniaturised *in vitro* assays were used to determine drug toxicity. On day 1, cells were seeded at 1×10^3 cells/well in a 96-well plate and left to attach overnight. Drug was added on day 2, and the assay was terminated on day 7. Cell number was then evaluated by using one of two colorimetric endpoint assays: the acid phosphatase assay [26] or the crystal violet dye elution assay [27]. All assays were performed in triplicate.

For the suspension cells, the percentage of apoptotic cells within the cell population following drug exposure was used to quantify toxicity. Cells were plated in fresh medium at a concentration of 10^6 cells/ml in a 24-well plate (Greiner) and drugs were then added to the wells. A 200 μ l sample from each well was taken after 4 and 8 h. Samples were cytospun for 2 min at 500 rpm using a Shandon Cytofuge3. After air drying, the cytopspins were stained using Rappi-Diff II (Langanbach), dried and mounted in DPX (BDH). Slides were studied on an Optiphot microscope (Nikon) fitted with a $40\times$ objective, and visualised on a monitor via a Mitsubishi CCD-100E colour CCD-camera and a $0.6\times$ relay lens. Nuclear fragmentation was used as a morphological marker for apoptosis and a minimum of 200 cells were assessed in at least three different microscopic fields. Results were expressed as the percentage of apoptotic cells within the population. Each experiment was repeated in duplicate. In all cases where DMSO was used as the solvent, the final concentration in the well was $<0.1\%$ which was non-toxic in the assays used.

Miniaturised radiation assays

To determine the sensitivity of the cells to radiation, the cells were plated on to a 96-well plate, at a cell density of 10^3 cells/well/50 μ l medium. As the complete assay plate was exposed to a single dose of radiation, a separate plate was used for each radiation dose. The plates were incubated overnight at 37°C , in $5\% \text{ CO}_2$, to allow cell attachment. Following this, 50 μ l of the relevant NSAID was added. Each well was adjusted to 100 μ l final volume with medium. Within 2 h of the addition of the NSAID, the cells were irradiated as monolayers, at room temperature, with a single defined radiation dose from a linear accelerator, at a dose rate of 2.5–2.6 Gy/min. A 1 cm thick tissue-equivalent bolus was placed on top of the plate to ensure dose homogeneity. Following radiation exposure, the cells were maintained at 37°C in $5\% \text{ CO}_2$ for a further 7 days. Cell number was determined by acid phosphatase analysis [26].

Preparation of inside-out vesicles from plasma membrane of HL60/ADR cells

Inside-out plasma membrane vesicles were prepared essentially as previously described [28]. Cells ($1\text{--}2 \times 10^9$ cells) were harvested by centrifugation and washed once with ice-cold phosphate buffered saline (PBS). The cell pellet was

diluted 40-fold in hypotonic buffer (0.5 mM sodium phosphate, pH 7.0, 0.1 mM ethylene glycol-aminoethyl-tetraacetic acid (EGTA) and 0.1 Mm (phenyl methyl sulphonyl fluoride (PMSF) and stirred gently at 4°C for 1.5 h. The resulting cell lysate was centrifuged at 100 000 *g* for 30 min at 4°C . The subsequent pellet was resuspended in hypotonic buffer (10 ml) and homogenised with a Braun homogeniser. The homogenate was then diluted with 10 ml incubation buffer (250 mM sucrose/10 mM Tris-HCl, pH 7.4). The diluted homogenate was layered over 38% sucrose/10 mM Tris-HCl, pH 7.4 and centrifuged at 100 000 *g* for 30 min at 4°C . Following centrifugation, the interphase was collected, diluted with 20 ml incubation buffer and centrifuged at 100 000 *g* for 30 min at 4°C . The pellets were resuspended in 0.3 ml incubation buffer and vesicles were formed by passing the resuspended pellets through a 27G needle 20 times, using a 1 ml syringe. Aliquots (50 μ l) of the vesicle mixture (5 mg/ml) were stored at -80°C .

Vesicle transport assay using LTC₄

ATP-dependent transport of [^3H]LTC₄ into the membrane vesicles was measured by a rapid filtration method using a Millipore sampling manifold. The membrane vesicles were thawed at 37°C before use and kept on ice. The reaction components consisted of 0.25 mM sucrose/10 mM Tris-HCl pH 7.4/1 mM ATP/10 mM MgCl₂/10 mM creatine phosphate/100 μ g/ml creatine kinase and 10 nM [^3H]LTC₄ (NEN), with 50 μ g of the inside-out vesicle preparation, in a final volume of 110 μ l. This suspension was incubated at 37°C , with gentle mixing and aliquots (20 μ l) were taken up to 3 min. Those aliquots were diluted in 1 ml of ice-cold incubation buffer before being applied to 0.22 μ m Millipore GSWP nitrocellulose filters, presoaked in ice-cold incubation buffer, under vacuum. The filters were subsequently washed with 3 ml ice-cold incubation buffer and absorbed radioactivity was measured using a scintillation counter (Beckman). In control experiments, ATP was replaced with 5'-AMP to determine the ATP-dependent transport.

Assay for glutathione-S-transferase activity

Total cellular glutathione-S-transferase activity was assayed, using 1-chloro-2,4-dinitrobenzene as a substrate, according to the method of Habig and Jakoby [29]. Because of spectrophotometric interference at 340 nm, caused by the majority of the NSAIDs, the assays were all performed at 360 nm, without any apparent effect on the rate of the control reaction.

Western blot analysis

Western blotting for MRP detection was performed using whole cell extracts, except for DLKP cells for which a plasma membrane vesicle preparation (see above) was used. The protein concentration of each fraction was estimated by the Pierce bicinchoninic acid (BCA) protein assay [30]. The proteins were separated using 7.5% sodium dodecyl sulphate (SDS)-polyacrylamide gels in a discontinuous buffer system according to the method of Laemmli [31]. Western blotting was performed by the method of Towbin and colleagues [32] using PVDF Western blotting membranes (Boehringer Mannheim). Following protein transfer, the nitrocellulose sheets were placed in blocking buffer 5% non-fat dried milk in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) for 2 h prior to the addition of the primary antibody. The primary

antibody used was an antihuman MRP monoclonal, Clone MRP1 (TCS Biologicals). The secondary antibody was a horseradish peroxidase-linked antibody (Dako) which was detected by ECL (Amersham).

Reverse transcriptase–polymerase chain reaction (RT–PCR)

RT-PCR was performed as described previously [33].

Doxorubicin efflux studies

DLKP-7 cells were seeded into 75 cm² flasks at 0.5×10^6 cells/10 ml ATCC medium. The cells were incubated for 2 days, the ATCC medium was removed and fresh medium containing doxorubicin (0.5 µg/ml) was added. Following a 2 h incubation, this medium was removed, the flasks were washed twice with PBS, 10 ml ATCC medium or ATCC medium containing the test NSAID was added and the cells were incubated for a further 4 h. The cells were trypsinised and the pellet was frozen at –20°C.

Quantification of doxorubicin in cells

The amount of doxorubicin in the cells was quantified using liquid–liquid extraction and reverse-phase HPLC analysis. The frozen cell pellets were thawed and resuspended in 100 µl of water/100 µl of 33% silver nitrate and mixed for 5 min. Three hundred microlitres of methanol, containing 1.5 µg of daunorubicin as an internal standard (IS) and 1.3 ml of acetonitrile were then added and the tubes were mixed for a further 5 min. The mixture was transferred to glass tubes and kept at 4°C for 1 h. The tubes were then centrifuged at 4000 rpm for 15 min to pellet any solid material. A sample of 1.1 ml of the supernatant was removed to HPLC autosampler vials (Labquip, Ireland). The solvent was evaporated at room temperature under a stream of nitrogen gas.

The extract was resuspended in 50 µl of mobile phase 1 (MP1): 32 ml of 0.1 M phosphoric acid mixed with 244 ml of ultrapure water, pH 2.3 (adjusted with 1 N potassium hydroxide) to which 124 ml of acetonitrile was added and the final mixture was degassed in a sonicating bath for 5 min. The second mobile phase (MP2) consisted of acetonitrile. The

resuspended sample (20 µl) was automatically injected into the HPLC system (Beckman System Gold 507 autosampler, 125 pump and 166 detector). The mobile phase was pumped through a C18 reversed-phase Prodigy 5 µm particle size ODS-3 column (Phenomenex, U.K.) and absorbance was measured at 253 nm. For the first 14 min of the chromatographic run, MP1 was pumped at 0.5 ml/min. Between 14 and 20 min, the column was switched to MP2. At 20 min, the column was switched back to MP1 and the flow rate increased to 1 ml/min. At 30 min, the flow rate was brought back to 0.5 ml/min. The run continued for a further 5 min giving a total run time of 35 min. The peak area of doxorubicin was divided by the area of the IS peak and the ratio plotted against a concentration series for spiked doxorubicin samples, to quantify the amount of doxorubicin present.

Statistical analysis

Data were analysed using Calcsyn, a Windows software package for dose effect analysis from Biosoft (Cambridge, U.K.). The program provides combination index (CI) values which are a quantitative measure of drug interaction in terms of an additive (CI = 1), synergistic (CI < 1) or antagonistic (CI > 1) effect for a given endpoint of the assay used, adapted from Chou and Talalay [34].

RESULTS

NSAID-induced enhancement of chemotherapeutic drug toxicity in drug sensitive cells

The ability of a range of NSAIDs (Table 1), to enhance the cytotoxicity of a variety of chemotherapeutic drugs (Table 2) was determined using *in vitro* human tumour cells. It was found that only a limited number of NSAIDs, specifically indomethacin, sulindac, tolmetin, acemetacin, zomepirac and mefenamic acid, all at non-toxic concentrations, significantly enhanced the cytotoxicity of certain chemotherapeutic drugs, namely the anthracyclines (doxorubicin, daunorubicin and

Table 1. Summary of non-steroidal anti-inflammatory drugs (NSAIDs) screened with chemotherapeutic drugs

Acemetacin*	(40 µg/ml)
Aspirin	(150 µg/ml)
Diclofenac	(12 µg/ml)
Fenoprofen	(50 µg/ml)
Flufenamic acid	(6 µg/ml)
Flurbiprofen	(6 µg/ml)
Ibuprofen	(16 µg/ml)
Indomethacin*	(2.5 µg/ml)
Ketoprofen	(50 µg/ml)
Meclofenamic acid	(8 µg/ml)
Mefenamic acid*	(6 µg/ml)
Naproxen	(50 µg/ml)
Phenylbutazone	(12 µg/ml)
Piroxicam	(20 µg/ml)
Sulindac*	(6 µg/ml)
Tolmetin*	(25 µg/ml)
Zomepirac*	(40 µg/ml)

*NSAIDs positive for synergy when combined with chemotherapeutic drugs. The values in parentheses are the highest non-toxic concentration used for each of the NSAIDs on the A549 cell line.

Table 2. List of the chemotherapeutic drugs screened

Actinomycin D	(250 ng/ml)
Bleomycin	(1.0 µg/ml)
Camptothecin	(1.2 ng/ml)
Carboplatin	(1.75 µg/ml)
Chlorambucil	(0.5 µg/ml)
Cisplatin	(0.5 µg/ml)
Cyclophosphamide	(0.75 µg/ml)
Cytarabine	(10 ng/ml)
Daunorubicin*	(12.0 ng/ml)
Doxorubicin*	(12.0 ng/ml)
Epirubicin*	(12.0 ng/ml)
Etoposide (VP-16)*	(150 ng/ml)
5-Fluorouracil	(1.0 µg/ml)
Hydroxyurea	(10 ng/ml)
Methotrexate	(25 ng/ml)
Mitoxantrone	(0.75 ng/ml)
Paclitaxel	(1.0 ng/ml)
Teniposide (VM-26)*	(12.5 ng/ml)
Vinblastine	(750 pg/ml)
Vincristine*	(2.0 ng/ml)
Vinorelbine	(5.0 ng/ml)

*Chemotherapeutic drugs positive for synergy when combined with non-steroidal anti-inflammatory drugs (NSAIDs). The values in parentheses are the IC₅₀ values for each of the anticancer agents on the DLKP cell line.

epirubicin), vincristine, teniposide and VP-16. For the other chemotherapeutic drugs listed in Table 2, there was no toxicity enhancement, as was the case with the inactive NSAIDs listed in Table 1. The significance of the synergistic combination was analysed as described in Materials and Methods and representative data are shown in Table 3. The data demonstrated a significant synergistic effect of combining the positive NSAIDs and cytotoxic drugs. This effect of the NSAIDs was concentration dependent (Figure 1) and while the NSAIDs were always used at non-toxic levels the magnitude of the enhancement was greater with increasing NSAID concentration, as manifested in the CI values. The other NSAIDs found to be positive in our screening assays gave similar results: in the presence of doxorubicin (15 ng/ml) A549 cell survival was 65%, but when used in the combination assay with non-toxic concentrations of mefenamic acid (6 µg/ml), acemetacin (40 µg/ml) or zomepirac (40 µg/ml) the percentage cell survival decreased to 16, 19 and 25%, respectively.

While investigating the mechanism by which these specific NSAIDs were acting as chemosensitisers, we were able to rule out cyclo-oxygenase inhibition, a property common to most NSAIDs, for the following reasons. Sulindac, one of the positive NSAIDs, is metabolised *in vivo* to a sulphide which has cyclo-oxygenase inhibitory ability and to a sulphone which does not. Both these metabolites were positive in our tests and to similar degrees of significance as the parent molecule, i.e. doxorubicin (15 ng/ml) in combination with sulindac (6.0 µg/ml), sulindac sulphide (1.4 µg/ml) and sulindac sulphone (19.5 µg/ml) had CI values of 0.368, 0.328 and 0.176, respectively, in the A549 cell line. Additionally, we cultured the human lung carcinoma cell line DLKP with a drug/NSAID combination (doxorubicin (10 ng/ml)/indomethacin (2.5 µg/ml)) plus PgD₂ or PgE₂, at 0.2, 2, 20 and 200 ng/ml, and found no reversal of the synergistic effect. Addition of arachidonic acid (2.8 and 0.28 µg/ml) did not alter the enhancement of toxicity of doxorubicin (10 ng/ml) by indomethacin (2.5 µg/ml) in A549 cells. These results, combined with the fact that not all NSAIDs are positive in our system, strongly contra-indicate cyclo-oxygenase inhibition as the mechanism by which this effect occurs.

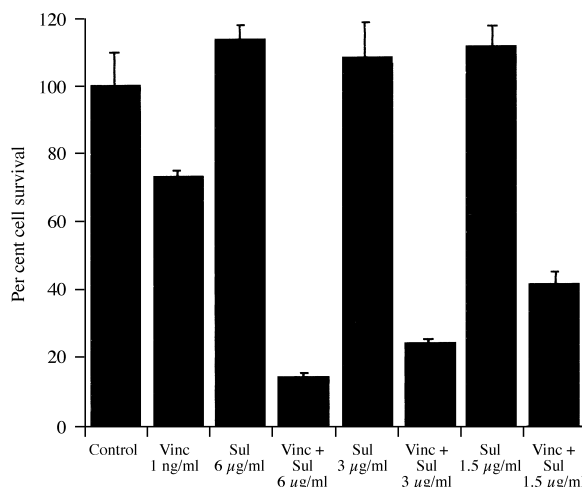


Figure 1. Effect of co-treating DLKP cells with vincristine (Vinc) and a range of non-toxic concentrations of sulindac (Sul). For Vinc + Sul 6 µg/ml, CI=0.42; Vinc + Sul 3 µg/ml, CI=0.49 and Vinc + Sul 1.5 µg/ml, CI=0.62. The assays were performed as described in Materials and Methods. The data shown are means \pm S.D. for a minimum of three experiments.

Effect of the combination of NSAIDs with drugs or radiation on MDR cell lines

We subsequently expanded the range of cell lines screened to include some MDR variants. No enhancement of chemotherapeutic drug cytotoxicity in the *MDR1* overexpressing cell line DLKP-A, nor in a *MDR1* transfectant of DLKP, was observed (data not shown). However, in two MRP-positive MDR cell lines, COR L23R and HL60/ADR, there was a significant increase in drug toxicity in the presence of the positive NSAIDs (Figure 2a,b). MRP confers resistance to a similar anticancer drug menu as P-170, with the notable exceptions of paclitaxel and vinblastine [4] which mirrored the results of our screening (Table 2).

At concentrations of the NSAIDs that caused negligible or low levels of DLKP or DLKP-A cell kill, but which caused enhanced cytotoxicity in DLKP cells in the *in vitro* toxicity tests described above, neither sulindac nor indomethacin

Table 3. Demonstration of a synergistic combination of selected non-steroidal anti-inflammatory drugs (NSAIDs) with certain chemotherapeutic drugs in A549 and DLKP cells

	A549 cells				DLKP cells			
	No NSAID	Indomethacin (2.5 µg/ml)	Sulindac (6.0 µg/ml)	Tolmetin (25 µg/ml)	No NSAID	Indomethacin (2.5 µg/ml)	Sulindac (6.0 µg/ml)	Tolmetin (25 µg/ml)
No anticancer agent	100 \pm 0.0	98.38 \pm 5.0	96.01 \pm 5.36	99.24 \pm 10.3	100 \pm 0.0	94.76 \pm 5.3	98.40 \pm 7.7	98.53 \pm 8.1
Doxorubicin*	60.60 \pm 7.9	21.00 \pm 3.6 (0.251)	35.27 \pm 6.4 (0.368)	31.82 \pm 2.7 (0.357)	81.52 \pm 4.5	N.D.	30.98 \pm 3.2 (0.449)	29.09 \pm 3.6 (0.467)
Vincristine*	67.33 \pm 6.0	16.78 \pm 2.2 (0.301)	28.47 \pm 3.5 (0.462)	N.D.	76.71 \pm 14.1	N.D.	13.68 \pm 0.8 (0.241)	30.75 \pm 3.3 (0.593)
VP-16*	72.20 \pm 6.3	53.45 \pm 7.9 (0.916)	46.59 \pm 4.6 (0.681)	N.D.	47.95 \pm 1.1	23.07 \pm 1.9 (0.534)	18.82 \pm 1.7 (0.463)	31.49 \pm 0.6 (0.745)
Paclitaxel*	69.84 \pm 8.1	76.83 \pm 7.0 (2.234)	70.37 \pm 8.1 (1.539)	65.96 \pm 4.9 (1.349)	79.63 \pm 2.7	68.78 \pm 7.2 (1.227)	70.29 \pm 7.9 (1.233)	N.D.

Data are expressed as percentage cell survival \pm standard deviation for a minimum of three determinations. Values in parentheses are the combination index (CI) values for the combination, calculated as described in Materials and Methods. *Doses: A549 cells: doxorubicin, 20 ng/ml; vincristine, 8 ng/ml; VP-16, 100 ng/ml; paclitaxel, 0.5 ng/ml. DLKP cells: doxorubicin, 10 ng/ml; vincristine 1 ng/ml; VP-16, 150 ng/ml, paclitaxel, 0.8 mg/ml. N.D., not determined.

increased cellular sensitivity to radiation-induced cell kill; this was noted in cases where the NSAID was present throughout the radiation exposure, and in cases where the NSAID was added immediately following radiation exposure.

Enhanced toxicity of heavy metal compounds in combination with NSAIDs

Overexpression of MRP can also confer resistance to heavy metals, particularly arsenate, arsenite and antimonial compounds, but not to cadmium [4]. The DLKP cells were chemosensitised to sodium arsenate and potassium antimonyl tartrate, but not to cadmium chloride (Table 4), which once again mirrors the reported profile for MRP-expressing cells [4].

Western blot and RT-PCR analysis

The results from the COR L23R and HL60/ADR cell lines suggested that MRP inhibition may be involved in the syner-

gistic combination of the drugs and the NSAIDs. As MRP overexpression in those MDR cell lines has been well characterised [35, 36], the expression of MRP in all the cell lines utilised in this report was compared. The expression of MRP was observed in cell lysates from A549, in agreement with a recent publication [37], but not in equivalent preparations from DLKP when examined by immunoblot (Figure 3). However, in plasma membrane vesicle preparations from DLKP, expression of MRP was observed (Figure 3). There have been no previous studies on MRP expression in DLKP. Associated RT-PCR studies indicated that mRNA for MRP was present in both the DLKP and A549 cell lines (Figure 4).

LTC₄ transport into inverted vesicles from HL60/ADR and DLKP

To determine if the NSAIDs were acting directly on the MRP pump, the uptake of [³H]-LTC₄, a glutathione-conjugate which is very efficiently transported by MRP, into inside-out plasma membrane vesicles prepared from the MRP-overexpressing HL60/ADR cell line or from the DLKP cell line, was examined using a rapid filtration procedure [28]. HL60, the parental line from which HL60/ADR was derived, has been demonstrated to possess relatively low rates of LTC₄ transporting ability [38]. The HL60/ADR vesicles provide a relatively pure preparation of functional MRP for performing direct inhibition studies. These data suggest that the positive NSAIDs, especially sulindac and indomethacin, have MRP pump inhibitory activity, whereas inactive NSAIDs, naproxen and piroxicam, do not (Table 5).

Glutathione-S-transferase assays

MRP can act as an efflux pump for glutathione conjugates and it has been suggested that glutathione metabolism is an important factor in drug efflux from MRP-overexpressing cell lines [39]. As indomethacin is known to be an inhibitor of glutathione-S-transferases [40, 41], we tested the other NSAIDs in a total glutathione-S-transferase assay [29], using a DLKP cell extract and the results are shown in Table 6. The most inhibitory NSAID in the assay was piroxicam, which was negative in the combination assays, followed by those NSAIDs that were positive for a synergistic effect. These data suggest that the NSAIDs do possess glutathione-

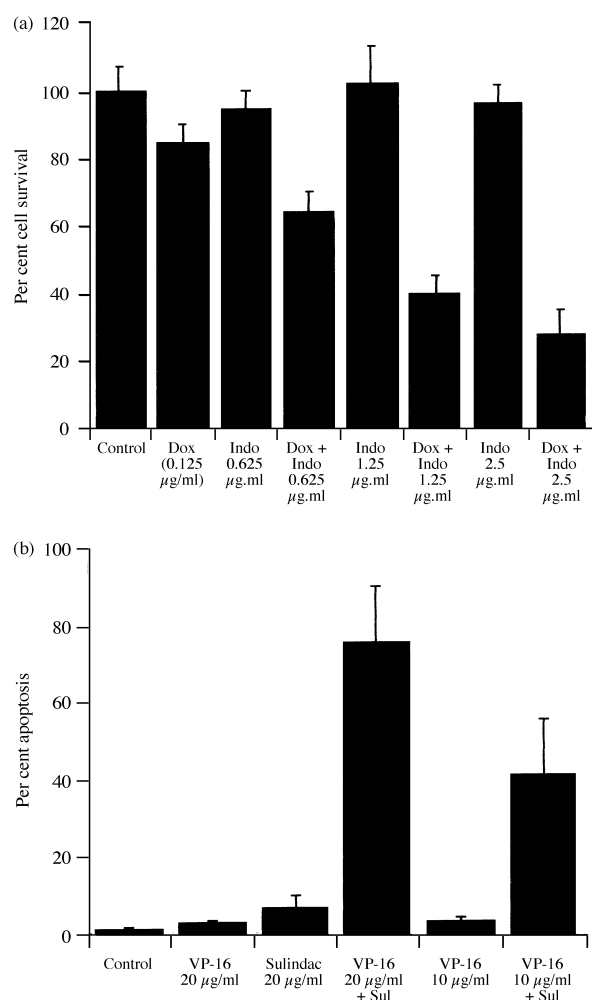


Figure 2. Demonstration of synergy in (a) a MRP positive human lung tumour cell line, and (b) a MRP positive human leukaemia cell line. COR L23R cells were co-treated with doxorubicin (Dox) at 0.125 µg/ml, and a range of non-toxic concentrations of indomethacin (Indo). For Dox+Indo 0.625 µg/ml, CI=0.57; Dox+Indo 1.25 µg/ml, CI=0.41 and Dox+Indo 2.5 µg/ml, CI=0.40. The assays are set-up as described in Materials and Methods. HL60/ADR cells were co-treated with VP-16: 20 µg/ml or 10 µg/ml and sulindac (Sul) at 20 µg/ml and % apoptosis was determined as described in Materials and Methods. The data shown are means ± S.D. for a minimum of three experiments.

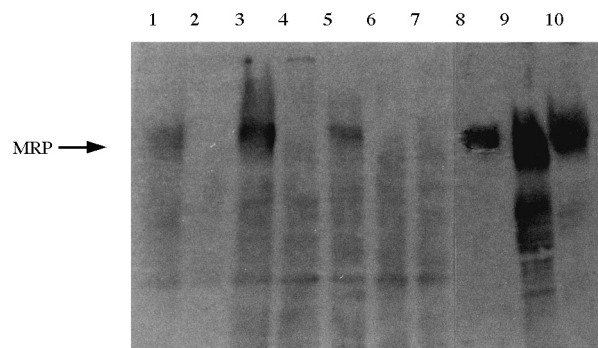


Figure 3. Crude cell lysates (15 µg protein) of HL60/ADR (lane 1), HL60 (lane 2), COR L23R (lanes 3 and 10), COR L23 P (lane 4), A549 (lane 5), DLKP (lane 6) and DLKP-A (lane 7), and plasma membrane vesicles (50 µg protein) prepared from DLKP (lane 8) and HL60/ADR (lane 9) were probed for MRP with antibody MRPr1 (1/40 dilution) and detected using horseradish peroxidase-linked rabbit and anti-rat secondary antibody and enhanced chemiluminescence.

Table 4. Effect of cotreating DLKP cells with heavy metal compounds and an active (sulindac) or an inactive (naproxen) non-steroidal anti-inflammatory drug (NSAID)

	No NSAID	Sulindac (6 µg/ml)	Naproxen (50 µg/ml)
No heavy metal	100.0 ± 0.0	98.40 ± 7.7	95.81 ± 0.7
Sodium arsenate (1.5 µg/ml)	45.94 ± 2.0	19.90 ± 1.2 (0.332)	44.02 ± 4.3 (1.164)
Potassium antimonyl tartrate (7.5 µg/ml)	39.98 ± 3.3	9.91 ± 0.9 (0.354)	41.74 ± 1.1 (1.059)
Cadmium chloride (150 ng/ml)	51.88 ± 3.3	50.06 ± 2.0 (1.218)	N.D.

Data given are percentage cell survival ± S.D. for a minimum of three experiments. Combination index (CI) values, as described in Materials and Methods, are given in parentheses. The NSAID concentrations were non-toxic (cell survival > 95%) in all cases. N.D., not determined.

S-transferase inhibitory ability, but at concentrations that are higher than listed serum concentrations for these drugs. The ability of the NSAIDs to accumulate within the cells is obviously a factor in analysing the significance of these data. Preliminary data (Table 7) indicate that piroxicam is poorly accumulated by HL60/ADR cells, and this may explain its lack of activity in the combination assay, despite a high level

of glutathione-S-transferase inhibition in cell lysates. Interestingly, these results also indicate a greater accumulation of both NSAIDs in the ADR variant of the HL60 cell line. The reason for this difference in accumulation is unclear, but may be due to changes in membrane permeability properties between the two cell lines, perhaps associated with the different levels of MRP in the membrane.

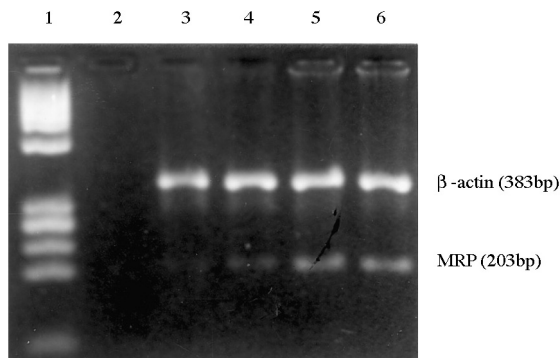


Figure 4. Agarose gel electrophoresis of RT-PCR product for MRP mRNA from DLKP (lanes 3 and 4) and A549 (lanes 5 and 6). Lanes 1 and 2 are marker and H₂O respectively.

Table 5. Effect of selected non-steroidal anti-inflammatory drugs (NSAIDs) on transport of [³H]-LTC₄ into inside-out vesicles from HL60/ADR and DLKP cells

NSAID	Concentration	HL60/ADR cells (% inhibition)	DLKP cells (% inhibition)
None	—	0	0
Indomethacin	98 µM	70	N.D.
	49 µM	20	N.D.
	100 µM	N.D.	62
	50 µM	N.D.	15
Sulindac	89 µM	69	N.D.
	45 µM	25	N.D.
Tolmetin	0.863 mM	50	N.D.
	0.432 mM	22	N.D.
Naproxen	2.2 mM	15.5	N.D.
	100 µM	N.D.	0
Piroxicam	0.302 mM	0	N.D.

Relative ATP-dependent transport rates shown are expressed as a percentage of untreated control, taken as 100%, by subtracting the rate in the presence of AMP, which was used as a blank. Similar results were obtained with different membrane preparations. N.D., not determined.

Table 6. Effect of non-steroidal anti-inflammatory drugs (NSAIDs) on glutathione-S-transferase activity in cell extracts prepared from DLKP cells. Glutathione-S-transferase activity was assessed using the assay of Habig and Jakoby [29]

NSAID	Concentration (mM)	% inhibition*
Piroxicam	0.125	51
Acemetacin	0.25	58
Zomepirac	0.25	56
Sulindac	0.25	42
Tolmetin	0.4	55
Indomethacin	0.4	54
Diclofenac	0.75	54
Flufenamic acid	0.75	41.2
Fenoprofen	1.0	22.5
Ibuprofen	1.0	18.5
Naproxen	1.0	17
Fenbufen	1.0	15.6
Ketoprofen	1.0	11.2
Levamisole	1.0	0

*Assays were performed at 360 nm rather than 340 nm because of spectrophotometric interference at that wavelength. Relative transport rates shown are expressed as a percentage of untreated control, taken as 100%. Similar results were obtained with different cell extracts repeated a minimum of three times.

Table 7. Piroxicam and indomethacin accumulation within the HL60 and HL60/ADR cell lines

Cell line	Piroxicam/10 ⁶ cells	Indomethacin/10 ⁶ cells
HL60	0.22 ± 0.07 ng	3.3 ± 0.2 ng
HL60/ADR	1.50 ± 0.32 ng	10.9 ± 0.9 ng

HL60 and HL60/ADR cells were cultured in 175 cm² flasks. The culture medium was removed and replaced with medium containing either piroxicam or indomethacin at 5 µg/ml for 2 h, the cells washed twice with phosphate buffered saline and then spun down. The pelleted cells were then analysed by separate HPLC methods for piroxicam or indomethacin ([42] and [43], respectively). Data are expressed as the non-steroidal anti-inflammatory drug (NSAID) content of 10⁶ cells ± S.D. for a minimum of three determinations.

Table 8. Inhibition of doxorubicin efflux from cells treated with indomethacin relative to untreated and piroxicam-treated cells

NSAID	% doxorubicin content at 0 h*	% of original doxorubicin content remaining at 4 h*
Control	100	64 ± 5
Piroxicam	100	67 ± 10
Indomethacin	100	109 ± 4

*DLKP cells were seeded into 75 cm² flasks at 0.5 × 10⁶ cells/10 ml ATCC medium. The cells were incubated for 2 days, the ATCC medium was then removed and fresh medium containing doxorubicin (0.5 µg/ml) was added. Following a 2 h incubation, this medium was removed (0 h), the flasks were washed twice with phosphate buffered saline, 10 ml ATCC medium or ATCC medium containing the test NSAID was added, and the cells were incubated for a further 4 h (4 h). The cells were trypsinised and the pellet was frozen at -20°C. The NSAIDs used were indomethacin (10 µg/ml) and piroxicam (20 µg/ml). Data are expressed as the % doxorubicin content ± S.D. of 10⁶ cells relative to the control at 0 h (which was 289 ± 35 ng doxorubicin/10⁶ cells) for a minimum of three determinations.

Doxorubicin efflux studies

Reduced drug accumulation and enhanced drug efflux is usually observed in drug-selected cells that overexpress MRP [6]. We examined this by preloading cells with doxorubicin and studying the direct effect of an active (indomethacin) and an inactive (piroxicam) NSAID on the efflux of doxorubicin from DLKP cells. The level of doxorubicin within the indomethacin-treated cells remained constant during the 4 h of the experiment, whereas in the control and piroxicam-treated cells, the level of doxorubicin in the cells gradually decreased (Table 8). The results demonstrate that export of doxorubicin from MRP-expressing cells can be inhibited by indomethacin.

DISCUSSION

The results presented here demonstrate that in a variety of human tumour cell lines, specific NSAIDs, i.e. indomethacin, sulindac, tolmetin, acemetacin, zomepirac and mefenamic acid, but not others, enhance the cytotoxicity of certain anticancer drugs. All the positive anticancer drugs, the anthracyclines (doxorubicin, daunorubicin and epirubicin), as well as vincristine, VP-16 and teniposide, are substrates for MRP. The synergistic combination was effective in cell lines in which MDR was due to MRP overexpression and in more drug sensitive cell lines which were subsequently shown to express MRP. The toxicity enhancement effect was also seen with the heavy metals, arsenate and antimony, but not with cadmium compounds, supporting an MRP-based mechanism. Furthermore, paclitaxel and vinblastine (unlike vincristine) are inactive in the combination assays, as would be expected from the reported MRP substrate profile [4]. In cell lines whose MDR was P-170 mediated, no synergy was observed.

The precise mechanism by which MRP renders cells resistant to cytotoxic insults has not been definitively established, but it is believed to act as an efflux pump for GSH conjugates of xenobiotics [28], although recent results support the possibility of unconjugated drug export [8]. Using inside-out vesicles prepared from the plasma membrane of HL60/ADR, which overexpress MRP, or DKLP cells, active NSAIDs (sulindac, indomethacin and tolmetin) have been shown to

inhibit the uptake of LTC₄, a MRP substrate. The concentration of tolmetin that caused 50% inhibition in the HL60/ADR cells is high relative to the other active NSAIDs, indicating that factors, such as solubility, membrane penetration and affinity for the pump, are important. Tolmetin is the only water-soluble NSAID that is positive and this factor may explain its lack of potency in the transport assay, where the lipid composition of the vesicles may inhibit the effectiveness of this agent.

In whole cells, there is believed to be an interaction between glutathione metabolism and the action of MRP. As MRP is an extremely effective transporter of glutathione conjugates [44], and drug transport in MRP- but not P-170-overexpressing MDR tumour cell lines can be regulated by intracellular GSH levels [39], we examined the effect of NSAIDs on glutathione-S-transferase activity and found that the active, but not the inactive, NSAIDs inhibited glutathione-S-transferase activity. One exception is piroxicam, which is an effective glutathione-S-transferase inhibitor, but is inactive in the combination assay. This fact may be explained by the observation that piroxicam was poorly accumulated within cells relative to indomethacin (Table 7).

The efflux of doxorubicin from cells treated with indomethacin was significantly retarded relative to untreated cells or cells treated with the inactive NSAID piroxicam. It would appear from this cumulative evidence that the positive NSAIDs have direct inhibitory potential against the activity of MRP. Therefore, our results suggest the possibility that the active NSAIDs inhibit MRP-mediated drug efflux by direct inhibition of the pump and/or by inhibiting metabolism of drugs prior to MRP-mediated efflux. Recent results in MRP-overexpressing cell lines for one of the active NSAIDs (indomethacin) are also in agreement with the data presented here [22].

The enhancement effect is not due to the cyclo-oxygenase inhibitory activity of the NSAIDs as: (i) many NSAIDs known to be cyclo-oxygenase inhibitors do not show synergistic activity; (ii) sulindac sulphone, a metabolite of sulindac, is active although it does not possess cyclo-oxygenase inhibitory activity and (iii) the addition of PgD₂ or PGE₂ does not reverse the effect. Previous reports in the literature showing a beneficial combination of cytotoxic drugs and NSAIDs may be unrelated to the work presented here, as synergy between NSAIDs, in many cases using flurbiprofen or indomethacin, with drugs such as methotrexate, which was inactive in our system, are involved [18]. The radiation study provided uniformly negative results indicating that DNA damage caused by the radiation and/or cellular repair mechanisms are not affected by the NSAIDs studied.

The fact that the combination was effective in MRP-expressing cell lines, at clinically relevant concentrations, was especially interesting, as very few studies have reported specific MRP-circumventing agents to date. Preliminary data on transplantable mouse tumours indicate that the effect may also operate *in vivo* (R. O'Connor, E. Moran and M. Clynes, Dublin City University). Additional studies with multicellular spheroids [45] may be useful as a further guide to the potential therapeutic value of these findings. These specific MRP inhibitors may avoid some of the adverse side-effects on, e.g. brain and kidney tissue, which are observed with modulators of P-170 [9] many of which inhibit both P-170 and MRP. The fact that MRP is expressed at such a low level in DLKP (i.e. it was only visible on Western blots when a concentrated

plasma membrane preparation was used), indicates that a small amount of functional MRP is sufficient to render cells less sensitive to chemotherapeutic drugs, but that this low-level resistance can be circumvented using these specific compounds. MRP expression has been reported in a wide variety of human tumours, at mRNA and protein level, by a number of groups [46–49] and will need further investigation, particularly in the light of the recent publication detailing the existence of MRP homologues [50]

A combination of anticancer agents with these NSAIDs (which are already used in cancer care as pain-control agents) might, based on the data presented here, be a useful advance in chemotherapy of MRP-positive tumours.

- Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 1993, **62**, 385–427.
- Cole SPC, Bhardwaj G, Gerlach JH, *et al.* Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992, **258**, 1650–1654.
- Kruh GD, Gaughan KT, Godwin A, Chan A. Expression pattern of MRP in human tissues and adult solid tumor cell lines. *J Natl Cancer Inst* 1995, **87**(16), 1256–1258.
- Cole SPC, Sparks KE, Fraser K, *et al.* Pharmacological characterization of multidrug-resistant MRP-transfected human tumor cells. *Cancer Res* 1994, **54**, 5902–5910.
- Breuninger LM, Paul S, Gaughan K, *et al.* Expression of multidrug resistance-associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution. *Cancer Res* 1995, **55**, 5342–5347.
- Loe DW, Deeley RG, Cole SPC. Biology of the multidrug resistance-associated protein, MRP. *Eur J Cancer* 1996, **32A**(6), 945–957.
- Muller M, Meijer C, Zaman GJR, *et al.* Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc Natl Acad Sci* 1994, **91**, 13033–13037.
- Paul S, Breuninger LM, Tew KD, Shen H, Kruh GD. ATP-dependent uptake of natural product cytotoxic drugs by membrane vesicles establishes MRP as a broad specificity transporter. *Proc Natl Acad Sci* 1996, **93**, 6929–6934.
- Fisher GA, Lum BL, Hausdorff J, Sikic BI. Pharmacological considerations in the modulation of multidrug resistance. *Eur J Cancer* 1996, **32A**(6), 1082–1088.
- Versantvoort CHM, Broxterman HJ, Lankelma J, Feller N, Pinedo HM. Competitive inhibition by genistein and ATP dependence of daunorubicin transport in intact MRP overexpressing human small cell lung cancer cells. *Biochem Pharmacol* 1994, **48**, 1129–1236.
- Gekeler V, Boer R, Ise W, Sanders KH, Schachtele C, Beck J. The specific bisindolylmaleimide PKC-inhibitor GF109203X efficiently modulates MRP-associated multiple drug resistance. *Biochem Biophys Res Commun* 1995, **206**, 119–126.
- Gekeler V, Ise W, Sanders KH, Ulrich W-R, Beck J. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun* 1995, **208**(1), 345–352.
- Gollapudi S, Thadepalli F, Kim CH. Difloxacin reverses multidrug resistance in HL60/AR cells that overexpress the multidrug resistance-related protein (MRP) gene. *Oncology Res* 1995, **7**(5), 213–225.
- Amin AR, Vyas P, Attur M, *et al.* The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. *Proc Natl Acad Sci* 1995, **92**, 7926–7930.
- Alberts DS, Hixson L, Ahnen D, *et al.* Do NSAIDs exert their colon cancer chemoprevention activities through the inhibition of mucosal prostaglandin synthetase? *J Cell Biochem* 1994, **S22**, 18–23.
- Furuta Y, Hunter N, Barkley Jr T, Hall E, Milas L. Increase in radioresponse of murine tumors by treatment with indomethacin. *Cancer Res* 1988, **48**, 3008–3013.
- Powles TJ, Alexander P, Millar JL. Enhancement of anti-cancer activity of cytotoxic chemotherapy with protection of normal tissues by inhibition of PG synthesis. *Biochem Pharmacol* 1978, **27**, 1389–1392.
- Bennett A, Gaffen JD, Melhuish PB, Stamford IF. Studies on the mechanism by which indomethacin increases the anticancer effect of methotrexate. *Br J Pharmacol* 1987, **91**, 229–235.
- Gaffen JD, Chambers EA, Bennett A. The effects of dipyridimadole and indomethacin on methotrexate cytotoxicity in LoVo colon cancer cells. *J Pharm Pharmacol* 1989, **41**, 350–352.
- Teicher BA, Korbut TT, Menon K, Holden SA, Ara G. Cyclooxygenase and lipoxygenase inhibitors as modulators of cancer therapies. *Cancer Chemother Pharmacol* 1994, **33**, 515–552.
- Kobayashi S, Okada S, Yoshida H, Fujimura S. Indomethacin enhances the cytotoxicity of VCR and ADR in human pulmonary adenocarcinoma cells. *Tohoku J Exp Med* 1997, **181**, 361–370.
- Draper MP, Martell RL, Levy SB. Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein. *Br J Cancer* 1997, **75**(6), 810–815.
- Law E, Gilvarry U, Lynch V, Gregory B, Grant G, Clynes M. Cytogenetic comparison of two poorly differentiated human lung squamous cell carcinoma lines. *Cancer Genet Cytogenet* 1992, **59**(2), 111–118.
- Clynes M, Redmond A, Moran E, Gilvarry U. Multiple drug-resistance in variant of a human non-small cell lung carcinoma cell line, DLKP-A. *Cytotechnology* 1992, **10**, 75–89.
- Hucker HB, Stauffer SC, White SD, *et al.* Physiologic disposition and metabolic fate of a new anti-inflammatory agent, cis-5-fluoro-2-methyl-1(p-(methylsulfinyl)-benzylidenyl)-indene-3-acetic acid in the rat, dog, rhesus monkey, and man. *Drug Metab Dispos* 1973, **1**(6), 721–736.
- Martin A, Clynes M. Acid phosphatase: endpoint for in vitro toxicity tests. *In vitro Cell Develop Biol* 1991, **27A**, 183–184.
- Martin A, Clynes M. Comparison of 5 microplate colorimetric assays for *in vitro* cytotoxicity testing and cell proliferation assays. *Cytotechnology* 1993, **11**, 49–58.
- Ishikawa T, Wright CD, Ishizuka H. GS-X pump is functionally overexpressed in cis-diamminedichloroplatinum(II)-resistant human leukemia HL-60 cells and down-regulated by cell differentiation. *J Biol Chem* 1994, **269**(46), 29085–29093.
- Habig WH, Jakoby WB. Glutathione S-transferases (rat and human). *Methods Enzymol* 1981, **77**, 218–231.
- Smith P, Krohn R, Hermanson G, *et al.* Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985, **150**, 76–85.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, **227**, 680–685.
- Towbin HI, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedures and some applications. *Proc Natl Acad Sci* 1979, **76**, 4350–4354.
- Moran E, Cleary I, Larkin AM, *et al.* Co-expression of MDR-associated markers, including P-170, MRP and LRP and cytoskeletal proteins, in three resistant variants of the human ovarian carcinoma cell line, OAW42. *Eur J Cancer* 1997, **33**(4), 652–660.
- Chou T-C, Talalay P. Analysis of combined drug effects: a new look at a very old problem. *Trends Pharmacol Sci* 1983, **4**, 450–454.
- Barrand MA, Heppel-Parton AC, Wright KA, Rabbitts PH. A 190 k protein overexpressed in non-P-glycoprotein containing MDR cells and its relation to the MRP gene. *J Natl Cancer Inst* 1994, **86**, 110–117.
- Zhu Q, Center MS. Cloning and sequence analysis of the promoter region of the MRP gene of HL60 cells isolated for resistance to adriamycin. *Cancer Res* 1994, **54**, 4488–4492.
- Berger W, Elbling L, Hauptmann E, Micksche M. Expression of the multidrug resistance-associated protein (MRP) and chemoresistance of human non-small cell lung cancer cells. *Int J Cancer* 1997, **73**, 84–93.
- Jedlitschky G, Leier I, Buchholz U, Center M, Keppler D. ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. *Cancer Res* 1994, **54**, 4833–4836.

39. Versantvoort CHM, Broxterman HJ, Bagrij T, Scheper RJ, Twentyman PR. Regulation by glutathione of drug transport in multidrug-resistant human lung tumour cell lines overexpressing multidrug resistance-associated protein. *Br J Cancer* 1995, **72**, 82–89.
40. Hall A, Robson CN, Hickson ID, Harris AL, Proctor SJ, Cattar AR. Possible role of inhibition of glutathione S-transferase in the partial reversal of chlorambucil resistance by indomethacin in a Chinese hamster ovary cell line. *Cancer Res* 1989, **49**, 6265–6268.
41. Mosialou E, Morgenstern R. Inhibition studies on rat liver microsomal glutathione transferase. *Chem-Biol Interactions* 1990, **74**, 275–280.
42. Dixon JS, Lowe JR, Galloway DB. Rapid method for the determination of either piroxicam or tenoxicam in plasma using high performance liquid chromatography. *J Chromatog* 1984, **310**, 455–459.
43. Johnson AG, Ray JE. Improved high-performance liquid chromatographic method for the determination of indomethacin in plasma. *Therapeutic Drug Monitoring* 1992, **14**, 61–65.
44. Zaman GJR, Lankelma J, Van Tellingen O, *et al.* Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. *Proc Natl Acad Sci* 1995, **92**, 7690–7694.
45. Kerbel RS, St. Croix B, Florenes VA, Rak J. Induction and reversal of cell adhesion-dependent multicellular drug resistance in solid breast tumors. *Hum Cell* 1996, **9**(4), 257–264.
46. Flens M, Izquierdo MA, Scheffer GL, *et al.* Immunochemical detection of the multidrug resistance-associated protein (MRP) in tumor cells by monoclonal antibodies. *Cancer Res* 1994, **54**, 4557–4563.
47. Flens M, Zaman GJR, Van der Valk P, *et al.* Tissue distribution of the multidrug resistance protein. *Am J Pathol* 1994, **148**, 1237–1247.
48. Zhan Z, Sandor VA, Gamelin E, *et al.* Expression of the multidrug resistance-associated protein gene in refractory lymphoma: quantitation by a validated polymerase chain reaction assay. *Blood* 1997, **89**(10), 3795–3800.
49. Kubo H, Sumizawa T, Koga K, *et al.* Expression of multidrug resistance-associated protein (MRP) gene in urothelial carcinomas. *Int J Cancer* 1996, **69**(6), 488–494.
50. Kool M, Haas M de, Scheffer GL, *et al.* Analysis of expression of cMOAT(MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* 1997, **57**(16), 3537–3547.