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Structure–activity relationship of indomethacin analogues for MRP-1, COX-1 and COX-2 inhibition: identification of novel chemotherapeutic drug resistance modulators

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Abstract

We report the screening of analogues of indomethacin to investigate the structure–activity relationship (SAR) of indomethacin-mediated multidrug resistance associated protein-1 (MRP-1) inhibition. By examining the activities of compounds with minor variations of the parent structure, we were able to separate MRP-1, glutathione-S-transferase (GST), cyclooxygenase (COX)-1 and COX-2 inhibitory activities. Combination cytotoxicity assays were utilised to identify agents which possess synergistic potential in MRP-1-expressing cell lines. MRP-1 Inside Out Vesicles (IOVs) were utilised to demonstrate the ability of the indomethacin analogues to inhibit the pump directly. Most of the indomethacin analogues active as MRP-1 inhibitors were poor GST inhibitors when compared with the GST-inhibitory activity of indomethacin. Two of the MRP-1 inhibitory analogues were found to have no COX-1 inhibitory activity and low COX-2 inhibitory activity, suggesting potentially reduced clinical toxicity. One MRP-1 inhibitory indomethacin analogue was also found to have low COX-1 inhibitory activity, but significant COX-2 inhibitory activity, making this analogue again interesting in terms of low potential toxicity, but with the possibility of direct inhibitory effects on tumour growth.

Keywords: Cancer; MRP; NSAID; Resistance circumvention; Indomethacin; GST; Cyclooxygenase; Structure–activity relationship; COX-1; COX-2

1. Introduction

The development of drug resistance is one of the most significant obstacles to effective treatment of cancer. Elucidation of the mechanisms determining inherent or chemotherapy-induced resistance in human tumours is therefore an important challenge [1].

Multidrug resistance in model systems has been shown to be conferred by different integral membrane proteins including the 170 kDa P-glycoprotein (Pgp) [2] and the 190 kDa multidrug resistance associated protein (MRP) [3]. These proteins belong to the adenosine triphosphate (ATP) binding cassette (ABC) family of transport proteins. Since the discovery of MRP (now termed MRP-1), an additional six homologues of MRP (MRP2–MRP7) have been described [4,5].

MRP-1 can confer cellular resistance to natural product drugs, including anthracyclines, some vinca alkaloids, and epipodophyllotoxins [6,7]. This 190 kDa multidrug resistance-associated protein mediates the ATP driven unidirectional transport of a broad range of neutral, as well as anionic, compounds across cellular membranes [8]. MRP-1 can transport drugs conjugated to glutathione (GSH) out of the cell [9] and can actively co-transport GSH and unmodified vincristine, possibly via an interaction with leukotriene C_4 (LTC_4) binding site(s) [4,8].

The anti-inflammatory action of non-steriodal anti-inflammatory drugs (NSAIDs) is due to reduced synthesis of prostaglandins through inhibition of the enzyme prostaglandin endoperoxide synthase or cyclooxygenase (COX) which exists in two isoforms and which transforms arachidonic acid, liberated by phospholipase A_2 (PLA_2) to prostaglandins [10]. COX-1 is constitutively produced and is involved in regulating

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Table 1
List of the analogues of indomethacin investigated in this study. Both analogue number and chemical nomenclature are given for each compound

Compound	Mol. Wt.	Chemical description
Indo.	358	1-(4-Chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid
4	344	1-(4-Chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid
5	391	1-(4-Methylthiobenzyl)-5-methoxy-2-methylindole-3-acetic acid
7	388	1-(4-Bromobenzyl)-5-methoxy-2-methylindole-3-acetic acid
8	261	1-Benzylindole-3-acetic acid
9	296	1-(4-Chlorobenzyl)indole-3-acetic acid
10	341	1-(4-Bromobenzyl)indole-3-acetic acid
11	292	1-(4-Methoxybenzyl)indole-3-acetic acid
13	308	1-Benzyl-5-methoxy-2-methylindole-3-acetic acid
14	327	1-(4-Fluorobenzyl)-5-methoxy-2-methylindole-3-acetic acid
16	344	1-(3-Chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid
17	344	1-(2-Chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid
18	327	1-(3-Fluorobenzyl)-5-methoxy-2-methylindole-3-acetic acid
19	329	1-(4-Chlorobenzyl)-5-methoxyindole-3-acetic acid
20	314	1-(4-Chlorobenzyl)-2-methylindole-3-acetic acid
21	322	1-Benzoyl-5-methoxy-2-methylindole-3-acetic acid
22	402	1-(4-Bromobenzoyl)-5-methoxy-2-methylindole-3-acetic acid
23	372	Methyl 1-(4-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetate
24	447	*N*-Tolyl 1-(4-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetamide
25	328	1-(4-Chlorobenzoyl)-2-methylindole-3-acetic acid
26	370	4-[3(Amidomethyl)-2-methyl-1-(phenylmethyl)- indol-5-yl]oxy]butanoic acid
27	384	4-[3(Amidomethyl)-2-ethyl-1-(phenylmethyl)- indol-5-yl]oxy]butanoic acid
28	406	[3-[3(Amidomethyl)-2-methyl-1-(phenylmethyl)-indol-5 yl]oxy]propyl]phosphonic acid
29	420	[3-[3(Amidomethyl)-2-ethyl-1-(phenylmethyl)-indol-5-yl]oxy]propyl]phosphonic acid

Mol. Wt., molecular weight.

normal cellular processes, such as gastrointestinal (GI) cytoprotection, vascular homeostasis and renal function [11]. In contrast, COX-2, identified as an inducible synthase [12], is generally undetectable in most normal tissues. However, its expression can be increased dramatically after exposure of fibroblasts, vascular smooth muscle or endothelial cells to growth factors, hypoxia, phorbol esters, cytokines and by lipopolysaccharides (LPS) in monocytes/macrophages [13]. Evidence suggests that the gastrointestinal toxicity associated with NSAID use is primarily the result of inhibition of COX-1 and anti-inflammatory effects are largely due to the inhibition of COX-2 [14]. The expression of COX-2 in a number of cancer types, including Familial Adenomatous Polyposis (FAP) has been associated with increased tumorigenesis [15]. COX-2 is also associated with angiogenesis and may therefore represent a specific target for anti-tumour therapy [16].

A number of NSAIDs (indomethacin, sulindac, tolmetin, acemetacin, zomepirac and mefenamic acid) at non-toxic levels, have been demonstrated by our group [17] to have the ability to significantly enhance the cytotoxicity of a number of anti-cancer drugs (including doxorubicin, daunorubicin, epirubicin, teniposide, VP-16 and vincristine) *in vitro* when co-administered in cell lines which express MRP-1. These results are consistent with other published data [18,19]. In addition, two sulindac metabolites, sulindac sulphide and sulindac sulphone, were found to be active in the combination toxicity assay. As previously described, sulindac sulphone is, by definition, not a NSAID due to the fact that it is not a COX inhibitor [20].

Similarities in the chemical structures of the NSAIDs which act as chemosensitising agents suggested that there might be specific structural requirements for the synergistic combination effect. Hence, a number of compounds, structurally related to indomethacin, were synthesised and screened to further investigate the structure–activity relationship (SAR) of indomethacin-mediated MRP inhibition (Table 1) [21].

2. Materials and methods

2.1. Cell lines

DLKP (a human lung squamous carcinoma), A549 (a human lung adenocarcinoma), COR L23P (a human large cell lung cancer), COR L23R (an anthracycline-resistant variant of COR L23), HL60 (a human promyelocytic leukaemia) and HL60/ADR (an anthracycline-resistant variant of HL60) cells were cultured as previously described in Ref. [17].

2.2. Chemicals

Doxorubicin (Pharmacia, UK), cisplatin, and 5-fluorouracil (David Bull Labs, UK), etoposide (VP-16), and

vincristine (Bristol-Myers Squibb Pharmaceuticals Ltd., UK) were all supplied as solutions and were diluted for use in culture medium. Indomethacin was purchased from Sigma (UK) and was dissolved at 5 mg/ml in dimethyl sulphoxide (DMSO) before being diluted to its working concentration with culture medium. The indomethacin analogues were synthesised in our laboratories [21] and were dissolved in the same manner as indomethacin. All compounds synthesised were fully characterised spectroscopically and analytically.

2.3. In vitro *toxicity testing*

Cytotoxicity testing of drugs and drug combinations was measured by colormetric assays as previously described [17,22,23]. Briefly, on day 1, cells were seeded at 1×10^3 cells/well in a 96-well plate and left to attach overnight in a 5% CO_2 incubator at 37 °C. The appropriate concentrations of drug and/or compound were added to the plate on day 2, and the assay was terminated on day 7. All assays were performed in triplicate.

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

Inside out plasma membrane vesicles were prepared from HL60/ADR cells as previously described in Refs. [17,24]. Cells ($1–2\times10^9$ 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-tetra-acetic 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. 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 interface 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 27 G needle 20 times, using a 1-ml syringe. Aliquots (50 μl) of the vesicle mixture (5 mg/ml) were stored at −80 °C.

2.5. *Vesicle transport assay using LTC_4*

ATP-dependent transport of [^{3}H] LTC_4 into the membrane vesicles was measured by a rapid filtration method, using a Millipore sampling manifold as previously described in Ref. [17]. 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 $MgCl_2$/10 mM creatine phosphate/100 mg/ml creatine kinase and 10 nM [^{3}H]LTC_4 (Dupont-NEN, USA), with 50 mg of the inside-out vesicle preparation, in a final volume of 110 μl. This suspension was incubated at 37 °C, with gentle mixing and aliquots were taken up to 3 min. Those aliquots were diluted in ice-cold incubation buffer before being applied to 0.22 mm Millipore GSWP nitrocellulose filters, presoaked in ice-cold incubation buffer, under vacuum. The filters were subsequently washed with ice-cold incubation buffer and absorbed radioactivity was measured using a scintillation counter (Beckman). In control experiments, ATP was replaced with adenosine monophosphate (AMP) to determine the ATP-dependent transport.

2.6. *Assay for glutathione-S-transferase activity*

Total cellular glutathione-S-transferase (GST) activity was assayed, using 1-chloro-2,4-dinitrobenzene as a substrate, according to the method of Habig and Jakoby [25]. Because of spectrophotometric interference at 340 nm, caused by indomethacin and analogues, the assays were performed at 360 nm, without any apparent effect on the measurement of the rate of the reaction.

2.7. *Doxorubicin/indomethacin/indomethacin analogue efflux studies*

DLKP cells were seeded into 75-cm^2 flasks at 0.5×10^6 cells/10 ml American Type Culture Collection (ATCC) medium. The cells were incubated for 2 days, the ATCC medium was removed and fresh medium containing doxorubicin (10 μM), indomethacin/indomethacin analogue (28 μM) or a combination of both compound and drug were 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 5 h. The cells were trypsinised, counted and the pellet was then frozen at −20 °C.

2.8. *Quantification of doxorubicin in DLKP cells*

The level of doxorubicin in DLKP cells was quantified using liquid–liquid extraction and reverse-phase high performance liquid chromatography (HPLC) analysis as previously described in Ref. [17].

2.9. *Quantification of indomethacin in DLKP cells*

The frozen pellets were thawed and resuspended in glass tubes in 1000 μl ultra high purity (UHP)/100 μl mefenamic acid (10 μg/ml)/500 μl 1 M citrate buffer (pH 3.0)/7 ml dichloromethane. The tubes were mixed for 10

min and were then centrifuged at 4000 rotations per minute (r.p.m.) for 15 min. 1.1 ml was removed from the dichloromethane phase (bottom phase) and added to the HPLC autosampler vials. The vial contents were evaporated under nitrogen and resuspended in 50 μl of HPLC mobile phase. Measurement of the test samples by HPLC was carried out as previously described in Refs. [17,26].

2.10. Measurement of inhibition of COX-1 by indomethacin and analogues

The spectrophotometric assay, for measuring COX-1 activity, has been previously described in Refs. [27,28]. In brief, COX-1 (Cayman Chemical, Ann Arbor, MI, USA) was incubated with 100 μM arachidonic acid (Cayman Chemical, Ann Arbor, MI, USA) and co-factors (0.5 mM glutathione, 0.5 mM hydroquinone, 0.625 μM haemoglobin and 1.25 mM $CaCl_2$ in 100 mM Tris–HCl, pH 7.4) at 37 °C for 20 min in the presence of various NSAIDs or their solvent (1% DMSO final concentration). 12 μg/ml of indomethacin and analogues were added to the reaction mixture to assess the ability of these compounds to inhibit COX-1 enzyme activity (preliminary experiments indicated that 12 μg/ml inhibited approximately 50% COX-1 activity under the conditions used in this assay). The reaction was terminated by the addition of trichloroacetic acid. Enzyme activity was measured by the thiobarbituric colour reaction of malonaldehyde formed in the reaction and determined by a spectrophotometer at 530 nm.

2.11. Measurement of inhibition of COX-2 by indomethacin and analogues

A prostaglandin E_2 (PGE_2) specific enzyme-linked immunosorbent assay (ELISA) (Cayman Chemicals, Ann Arbor, MI, USA) was utilised to determine the COX-2 inhibitory activity of indomethacin and analogues. Cells were seeded at high density (2.5×10^5 cells per well) in 6 well plates. (The cell line, A549, a human lung adenocarcinoma, was chosen for the experiment as it was demonstrated by Asano and colleagues [29], that COX-2 is the constitutive and dominant isoform in unstimulated and stimulated cultured human lung epithelial cells. A549 cells express *COX-2* mRNA and/or protein when they are stimulated with epidermal growth factor or pro-inflammatory cytokines such as interleukin-1β (IL-1β). The plates were incubated overnight in serum-containing media. The media was removed after this incubation and washed twice with fresh media. The compounds of interest (such as NSAID (10 nM) and IL-1β (Sigma, UK) (10 ng/ml)) were then added to the cells. Control wells were treated with media only. After 24 h, the media was removed from the wells, placed into appropriately labelled eppendorfs and stored at −80 °C. Samples were then analysed using the PGE_2 enzyme immunoassay kit. Concentrations of PGE_2 present in the samples were determined from a standard curve of absorbance at 405 nm versus PGE_2 concentration.

2.12. Statistical analysis of combination toxicity assays

Results obtained from the analysis of data using the fractional method were confirmed using a computer package for multiple drug effect analysis, 'Dose–Effect Analysis with Microcomputers' (Biosoft, UK) [30]. 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 [31]. CI values presented in this paper were calculated using a wide range of concentrations and related effects for both anticancer drug and indomethacin/indomethacin analogues.

3. Results

3.1. Combination toxicity assays

The combination toxicity assay was the preliminary biological assay carried out on the compounds to assess if the analogues of indomethacin (at non-toxic concentrations) were capable of potentiating the toxicity of doxorubicin in MRP-expressing cell lines. DLKP cells were used in the combination toxicity assay as this cell line had previously been shown to express MRP-1 [17]. Of the 23 indomethacin analogues analysed, 9 had doxorubicin toxicity-enhancing ability in DLKP cells (Table 2).

The first of the compounds to be produced, **8**, **9**, **10**, **11**, were very dissimilar to indomethacin and were inactive in the screening assay. A number of *N*-benzyl analogues of indomethacin were then produced. In the combination toxicity assays, the potentiation activity was maintained after removing the carbonyl group alone from the indomethacin structure (**4**). However, removal of the methyl (**19**) and/or methoxy (**20**) and/or halogen groups (**13**) from the structure rendered the benzyl compounds inactive. When chlorine was replaced with bromine (**7**) or fluorine (**14**), the analogues were still active in the combination toxicity assay. But when this chlorine was replaced with a methylthio substituent (**5**) the compound was rendered inactive. Moving chlorine from the *para*- to the *ortho*- (**17**) or *meta*- (**16**) position in the benzyl series resulted in inactive analogues. Analogue **18** proved to be the only exception to these findings in that when fluorine was changed from the *para*- to the *meta*- position the compound was still active in the combination toxicity assay.

Table 2
CI values for indomethacin and indomethacin analogues with doxorubicin in DLKP cells[a]

Compound (μg/ml)	NSAID + doxorubicin CI[b] value
Indo (2.5)	0.557
4 (5)	0.483
5 (10)	1.000
7 (10)	0.437
13 (10)	1.020
14 (10)	0.563
16 (10)	1.035
17 (5)	1.000
18 (15)	0.340
19 (5)	1.074
20 (5)	1.044
21 (5)	0.609
22 (5)	0.566
23 (5)	1.024
24 (2.5)	1.286
25 (5)	0.550
26 (20)	0.551
27 (50)	0.397
28 (25)	1.385
29 (50)	1.050

NSAID, non-steroidal anti-inflammatory drug.

[a] The highest non-toxic concentrations of indomethacin and analogues are shown in brackets. Data are expressed as combination index (CI) values for the combination as described in the Materials and Methods. All combination data were obtained from triplicate determinations.

[b] CI, Combination Index: (CI = 1) additive, (CI < 1) synergistic or (CI > 1) antagonistic.

In the *N*-benzoyl series, two compounds were developed in which the acetic acid side chains were manipulated to form indomethacin methyl ester (**23**) and indomethacin tolyl amide (**24**). Both these compounds were more difficult to dissolve in DMSO than indomethacin due to removal of the carboxylic acid, and were inactive in the combination toxicity assay. Analogues **21** and **22**, in which the chlorine was removed completely (**21**) or was replaced with bromine (**22**) were found to be active in the combination toxicity assay. Analogue **25** in which the methoxy substituent was removed was also found to be active.

Only six of the positive indomethacin analogues could be used in the combination toxicity assay at concentrations comparable to indomethacin and the results of these combinations are summarised in Table 3.

As indomethacin is a known phospholipase A_2 (PLA_2) inhibitor [32], an indomethacin analogue (**27**) was generated, the structure of which was similar to indomethacin and based on the structures of known PLA_2 inhibitors [21]. This compound was positive in the combination toxicity assay, but at concentrations approximately 10 times higher than those required for indomethacin to enhance toxicity. Replacing the ethyl substituent in **27** with a methyl group (**26**) resulted in a compound that was also positive in the combination toxicity assay, but only at 8 times the concentration used for indomethacin. A phosphonic acid analogue of **26**, (**28**), and an ethyl derivative of **28**, (**29**), were inactive in this assay.

Table 3
Combination of indomethacin or indomethacin analogues at 2.5 μg/ml (concentrations are also given in mM for direct molar comparison), with doxorubicin in DLKP cells[a]

	Molar concentration in assay (mM)	NSAID + doxorubicin CI[b] value
Indomethacin	0.0070	0.557
4	0.0075	0.619
7	0.0064	0.571
14	0.0076	0.754
21	0.0080	0.619
22	0.0062	0.566
25	0.0075	0.544

NSAID, non-steroidal anti-inflammatory drug.

[a] Data are expressed as combination index (CI) values for the combination as described in the Materials and Methods. All combination data were obtained from triplicate determinations.

[b] CI = Combination Index: (CI = 1) additive, (CI < 1) synergistic or (CI > 1) antagonistic.

A number of the analogues, active in the combination toxicity assay in DLKP cells, were analysed in combination toxicity assays in the COR L23 parental (low, but detectable MRP-1 levels) and resistant cell lines (high MRP-1 levels) (Table 4). Indomethacin, **27**, **21** and **22**, in combination with doxorubicin, vincristine or VP-16 (where determined) resulted in an increase in the toxicity of these chemotherapeutic drugs in the COR L23R cells. There was also evidence of some increase in the toxicity of the same drugs in the COR L23 parental cell line.

3.1.1. GST assays

Only three of the indomethacin analogues, **21**, **22** and **25**, which were active in the combination toxicity assay, were also positive in the GST assay (Table 5). Interestingly, these analogues are all benzoyl derivatives of indomethacin. The results indicate that **22** was a stronger inhibitor of GST than indomethacin. The remainder of the indomethacin analogues that were positive in the combination toxicity assay were not as good GST inhibitors as indomethacin.

3.1.2. Inside out vesicle assays (IOVs)

The influence of indomethacin and indomethacin analogues on multidrug resistance associated protein-1 (MRP-1) activity in HL60/ADR IOVs was investigated by measuring the ability of the compounds to inhibit the transport of [^{3}H]-LTC_4.

The results of the IOV assay indicate that all of the compounds positive in the combination toxicity assay

Table 4
Synergistic combination of indomethacin or indomethacin analogues, at their highest non-toxic concentrations (bracketed values), with doxorubicin, vincristine, VP-16 and 5-FU in COR L23R cells (a) and COR L23S parental cells (b)[a]

COR L23R	No anticancer agent	Doxorubicin (250 ng/ml)	Vincristine (12 ng/ml)	VP-16 (2.5 μg/ml)	5-FU (2.0 μg/ml)
No NSAID	100±0.0	46.7±3.1	57.5±15.1	34.5±5.3	15. 5±1.2
Indo (2.5 μg/ml)	101.0±4.3	16.1±5.7	16.9±3.0	9.9±3.4	14.0±5.0
21 (5μg/ml)	103.0±0.9	5.8±1.0	15.2±1.5	9.8±1.1	14.9±1.4
22 (5 μg/ml)	102.0±2.0	9.7±0.2	11.5±3.7	10.1±5.1	15.7±3.5
27 (50 μg/ml)	97.5±5.8	13.0±1.4	ND	ND	ND
28 (2.5 μg/ml)	103.2±6.4	47.0±1.4	ND	ND	ND
COR L23S	No anticancer agent	Doxorubicin (20 ng/ml)	Vincristine (1ng/ml)	VP-16 (0.1μg/ml)	5-FU (0.4μg/ml)
No NSAID	100±0.0	45.8±10.1	57.5±15.1	23.4±2.8	29.0±1.1
Indo (2.5 μg/ml)	101.6±5.6	31.9±6.4	11.3±3.2	15.5±6.7	27.7±7.0
21 (5 μg/ml)	103.0±5.6	17.2±1.3	12.9±1.2	16.2±1.3	28.4±3.1
22 (5 μg/ml)	102.0±11.7	24.6±8.6	19.0±2.36	16.8±4.4	28.2±9.3
27 (50 μg/ml)	97.5±5.9	34.8±5.0	ND	ND	ND
28 (2.5 μg/ml)	98.1±6.0	36.3±4.2	ND	ND	ND

Indo, indomethacin; 5-FU, 5-fluorouracil; NSAID, non-steroidal anti-inflammatory drug; ND, not determined.
[a] Data are expressed as % cell survival ± standard deviation for a minimum of three determinations.

were inhibitors of MRP (Table 6). The most active MRP inhibitors were indomethacin analogues **22**, **14**, **7** and **27**, followed closely by **21**. One of the analogues, **18**, which was very active in the combination toxicity assay was not a very strong inhibitor of [^{3}H]-LTC_4 transport in the IOV assay, although it was still a more potent inhibitor than analogue **28**, which was used as the negative agent in this assay, as it was inactive in the combination toxicity assay.

3.1.3. Inhibition of COX-1 and COX-2 by indomethacin and indomethacin analogues

Indomethacin is considered to be a non-selective inhibitor of COX-1 and COX-2 [33]. The indomethacin analogues were analysed to assess if they displayed any COX selectivity *in vitro*. The results of the COX-1 assay (Table 7) indicate that, of the compounds positive in the combination toxicity assay, **21** and **25** had similar COX-1 inhibitory ability to indomethacin. Analogue **22** proved to be a better COX-1 inhibitor than indomethacin, while analogues **4**, **7**, **14** and **26** were weak inhibitors of COX-1. However, two of the indomethacin analogues **18** and **27** had almost no COX-1 inhibitory activity. The results for **18** and the *para*-form of this compound, **14**, suggests that moving the halogen from the *para*- to the *meta*-position resulted in a loss of COX-1 inhibitory activity.

The results obtained from the COX-2 assays indicated that indomethacin and analogues **4, 21, 22** and **25** were the most capable of inhibiting COX-2-mediated PGE_2 production, with analogue **22** being the most potent inhibitor (Table 8). Analogues **7** and **14** were weaker inhibitors of PGE_2 production and analogues **27**, **18** and **26** were the least active inhibitors of COX-2.

Table 5
Glutathione-S-transferase (GST) assay results showing the % inhibition of production of glutathione conjugates by indomethacin and indomethacin analogues (±standard deviation (S.D.))[a]

Compound	Molarity of compound in test solution (mM)	Average% inhibition
Indomethacin	0.9	93.1±3.5
4	1.0	7.2±1.0
5	0.9	0.0±0.0
7	1.0	15.1±2.7
13	1.1	17.6±2.3
14	1.0	21.2±2.6
16	1.0	2.0±0.2
17	1.0	46.7±0.7
18	1.0	8.2±0.8
19	1.0	0.1±0.1
20	1.1	4.0±0.3
21	1.1	70.9±4.7
22	0.9	94.3±2.6
23	0.9	0.1±0.1
24	0.8	0.1±0.1
25	1.0	74.0±3.8
26	0.8	12.1±0.9
27	0.8	13.4±1.2
28	0.8	9.7±1.4
29	0.8	11.7±2.1

[a] All results are the average of a minimum of two readings for each compound from a minimum of three assay repeats. Data is expressed as % inhibition relative to an untreated control (negative control). % Inhibition values are expressed as a percentage of untreated control, taken as 100%.

3.1.4. Doxorubicin efflux studies using HPLC analysis

Reduced drug accumulation and enhanced drug efflux is usually observed in drug-selected cells that over-express MRP-1 [34]. To investigate the possibility that indomethacin and the active analogues may also be

substrates for MRP-1 drug efflux, analysis was carried out on DLKP cells treated with a combination of doxorubicin and indomethacin or indomethacin analogues, using quantification by HPLC. An analogue of indomethacin, which was positive in the combination toxicity assay (**18**) and a negative analogue (**28**) were used in this assay.

Table 6
Effect of indomethacin and selected analogues on the transport of [^{3}H]-LTC_4 into inside-out vesicles from HL60/ADR cells (±standard deviation (S.D.))[a]

Compound	Molarity of compound in test solution (mM)	Average% Inhibition
Indomethacin	46.0	83.1±10.4
4	47.6	57.7±4.7
7	47.2	66.9±7.7
14	50.1	67.1±13.5
18	50.1	26.8±5.2
21	51.0	64.4±7.8
22	41.0	85.0±2.8
25	50.2	60.4±8.0
26[b]	13.3	65.1±19.5
27	41.8	85.0±9.0
28	39.8	0.0±0.8
29	38.2	22.5±5.6

[a] The relative adenosine triphosphate (ATP)-dependent rates are expressed as a percentage of untreated control, taken as 100%, by subtracting the rate in the presence of adenosine monophosphate (AMP), which was used as the blank.% inhibition was calculated using the following formula: (LTC_4 uptake@T180 s $\frac{(\text{untreated}) - \text{Level of } LTC_4 \text{ @ T180 s (+compound)}}{LTC_4 \text{ uptake @ T180 s (untreated)}} \times 100$. Data given are from a minimum of three assay repeats.

[b] Indomethacin analogue **26** proved very difficult to dissolve in the reaction mixture.

Table 7
Cyclooxygenase-1 (COX-1) assay results showing the % inhibition of COX-1 activity (±standard deviation (S.D.)) by indomethacin and indomethacin analogues[a]

Compound (12 μg/ml)	% Inhibition of COX-1
Indomethacin	60.9±12.2
4	16.3±12.4
7	24.6±5.8
14	26.6±9.9
18	0.9±3.6
21	59.9±13.2
22	79.0±11.3
25	57.3±12.9
26	22.1±8.2
27	−0.1±7.4
28	−7.5±0.3
29	1.7±8.1
No compound (control)	0.0
With DMSO (no compound)	−1.3±9.0

DMSO, dimethyl sulphoxide.

[a] All results are the average of a minimum of three assay repeats. Data is expressed as% inhibition relative to an untreated control.

After the initial 2-h loading period, there was a greater accumulation of doxorubicin in DLKP cells treated with doxorubicin + indomethacin or doxorubicin + analogue **18** (Table 9). When the levels of indomethacin and analogue **18** were maintained for a further 5 h only a slight decrease in the levels of doxorubicin was observed.

Table 8
Effect of indomethacin and analogues on production of prostaglandin E_2 (PGE_2) by cyclooxygenase-2 (COX-2)[a]

Treatment	% Inhibition of production of PGE_2 by COX-2
Cell control	0.0±0.0
IL-1β (10 ng/ml)	0.0±0.0
Indomethacin (10 nM) + IL-1β	86.1±5.9
4 (10 nM) + IL-1β	78.1±11.1
7 (10 nM) + IL-1β	44.1±12.2
14 (10 nM) + IL-1β	40.1±18.5
18 (10 nM) + IL-1β	13.6±10.6
21 (10 nM) + IL-1β	80.5±12.7
22 (10 nM) + IL-1β	88.1±4.4
25 (10 nM) + IL-1β	79.6±12.2
26 (10 nM) + IL-1β	24.3±8.1
27 (10 nM) + IL-1β	14.7±14.9
28 (10 nM) + IL-1β	48.0±0.8
29 (10 nM) + IL-1β	0.2±0.3

[a] Results are represented as means ±standard deviations (S.D.) for duplicate determinations carried out on three separate occasions. Inhibition is expressed as a percentage of untreated control (Interleukin-1β (IL-1β) (10 ng/ml)), taken as 100%

Table 9
Doxorubicin levels (±standard deviation (S.D.)) in DLKP cells treated with doxorubicin alone versus DLKP cells treated with doxorubicin and indomethacin/indomethacin analogues combined[a]

Time	Treatment	Average Dox. content (ng/million cells)
T0	Doxorubicin alone	125.0±13.3
T5	Doxorubicin alone	105.9±7.6
T0	Doxorubicin + indomethacin	144.6±7.1
T5	(+) Indomethacin	131.3±7.6
T5	(−) Indomethacin	99.3±9.8
T0	Doxorubicin + **18**	137.1±13.2
T5	(+) **18**	129.0±13.1
T5	(−) **18**	104.4±4.3
T0	Doxorubicin + **28**	124.4±15.8
T5	(+) **28**	102.7±6.8
T5	(−) **28**	93.2±0.9

T0, time point immediately after the initial 2-h loading period; T5, time point 5 h after the initial 2-h loading period; (+) indomethacin/analogue, flasks re-fed with medium containing indomethacin or analogue after the initial 2-h loading period; (−) indomethacin/analogue, flasks re-fed with medium only after the initial 2-h loading period.

[a] After the initial loading period, samples were taken at T0 and the remaining flasks were re-fed with either fresh media, indomethacin or the relevant indomethacin analogue to assess if maintaining the level of the compound in the cells would have an effect on the accumulation of doxorubicin in the cells. The cells were incubated for a further 5 h. Data shown are the average of three separate determinations.

However, there was a decreased amount of doxorubicin after 5 h in those cells treated with doxorubicin alone or with the analogue **28**. The results demonstrate that the export of doxorubicin from MRP-1-expressing cells can be inhibited by indomethacin and its active analogues.

3.1.5. Indomethacin efflux studies

Indomethacin efflux was also analysed, using HPLC quantification, in cells treated with indomethacin alone or doxorubicin and indomethacin combined. After the initial 2-h loading period, there was a greater accumulation of indomethacin in DLKP cells treated with indomethacin plus doxorubicin. When the levels of doxorubicin were maintained for a further 90 min only a small decrease in the levels of indomethacin was observed. We had previously determined that, in the absence of any inhibitor, levels of indomethacin fell below levels that could be measured 120 min after cessation of a 2-h incubation (data not shown). Therefore, the level of indomethacin in the cells was assessed after 90 min incubation (Table 10). The results indicate that the level of indomethacin in the cells treated with a combination of indomethacin and doxorubicin is higher than the level of indomethacin in those cells treated with indomethacin alone. Indomethacin levels were even lower in cells that had been exposed to doxorubicin plus indomethacin and had both agents removed for the 90-min incubation. This suggests that in the presence of doxorubicin the efflux of indomethacin from the cell is greatly reduced.

A qualitative summary of all of the results is expressed in Table 11.

Table 10
Indomethacin levels (±standard deviation (S.D.)) in DLKP cells treated with indomethacin alone versus DLKP cells treated with doxorubicin and indomethacin combined[a]

Time	Treatment	Average Indo content (ng/million cells)
T0	Indomethacin alone	10.1±0.7
T90 min	Indomethacin alone	3.0±0.8
T0	Indomethacin + doxorubicin	14.8±2.8
T90 min	(+) Doxorubicin	9.9±2.5
T90 min	(−) Doxorubicin	1.9±0.1

T0, time point immediately after the initial 2-h loading period; T90 min, time point 90 min after the initial 2-h loading period; (+) doxorubicin, flasks re-fed with medium containing doxorubicin after the initial 2-h loading period; Indo, indomethacin; (−) doxorubicin, flasks re-fed with medium only after initial 2-h loading period.

[a] Data shown are the average of three separate determinations.

Table 11
Summary table of all results from investigations of the structure–activity relationship (SAR) of indomethacin

	Combination toxicity assay	GST	IOV	COX-1	COX-2
Indomethacin	+++	+++	+++	++	+++
4	+++	−	++	+	+++
5	−	−			
7	+++	−	++(+)	+	++
13	−	±			
14	+++	±	+++	+	++
16	−	−			
17	−	±			
18	+++	−	+	−	±
19	−	−	−		
20	−	−	−		
21	+++	++	++(+)	++	+++
22	+++	+++	+++	+++	+++
23	−	−	−		
24	−	−	−		
25	+++	++	++	++	+++
26	+++	−	++	+	+
27	+++	−	+++	−	±
28	−	−	−	−	++
29	−	−	+/−	±	−

GST, glutathione-S-transferase; IOV, inside out vesicle assays; COX, cyclooxygenase. +++ Very strong positive; ++ strong positive; + positive; ±weakly positive; −, negative.

4. Discussion

The results obtained from the combination toxicity assay using an MRP-1 expressing cell line, DLKP, indicated that the doxorubicin resistance circumvention activity of indomethacin and analogues was concentration-dependent and that a number of substituents on the indomethacin structure were critical for the potentiating ability of the compounds. Changing the structure from an *N*-benzoyl to an *N*-benzyl compound (which allows freer rotation around the bonds joining the two ring systems) did not affect inhibitory activity. In the benzyl and the benzoyl series of indomethacin analogues, the structural requirements differ. Alterations to the benzyl structure indicated that the presence of the halogen was necessary for the potentiating activity of *N*-benzyl-indomethacin derivatives and could not be replaced with a non-halogen substituent. The activity of benzyl indomethacin was retained when the chlorine was replaced with bromine, but only if the halogen was in the *para*-position; however, the fluoro-derivative was active whether fluorine was in the *para*- or the *meta*-positions. Perhaps this is due to the smaller size of the fluorine substituent, which may have less impact on interaction with the active site of MRP-1 than the larger chlorine or bromine substituents. Further investigations of *N*-benzyl-indomethacin derivatives determined that both the methoxy and methyl groups were necessary for the activity of this compound. Alterations to the halogen in benzoyl-indomethacin indicated that this substituent was not required for the potentiating activity of the compound. Furthermore, removal of the methoxy

substituent from the benzoyl indomethacin structure did not render the compound inactive.

The active analogues also showed synergy in other cell lines possessing MRP-1 indicating that the effect is not cell line-specific.

Compounds known to be PLA_2 inhibitors were tested, but only two of these were capable of potentiating the toxicity of doxorubicin. These were less toxic to the cells, but also had to be used at higher concentrations to produce the same potentiation effect. These results indicated that the potentiating ability of the NSAIDs was unlikely to involve PLA_2 inhibition.

Indomethacin or indomethacin analogues might exert their toxicity-enhancing effect on the chemotherapeutic drug through inhibition of the activity of GST. Glutathione conjugates are transported very effectively by MRP-1 and these glutathione conjugates are formed by the GST enzyme. Indomethacin is also a classic inhibitor of GST. Results demonstrated varying ability among the indomethacin analogues to inhibit GST. Of the indomethacin analogues shown to have the ability to potentiate the toxicity of doxorubicin, only the benzoyl analogues were good GST inhibitors. The remaining indomethacin analogues demonstrated insignificant or no GST inhibitory ability which indicates that the potentiating activity of the analogues on the chemotherapeutic drugs is not through inhibition of GST.

Using inside-out vesicles prepared from the plasma membrane of HL60/ADR cells, which overexpress MRP-1, we demonstrated that the active indomethacin analogues had the ability to inhibit the uptake of the MRP-1 substrate [^{3}H] LTC_4 into the vesicles indicating a direct interaction between the analogues and MRP-1.

The efflux of doxorubicin from DLKP cells treated with indomethacin or active indomethacin analogues was significantly retarded relative to untreated cells or cells treated with an inactive indomethacin analogue. Conversely, doxorubicin also inhibited the efflux of indomethacin in these cells suggesting that this effect is due to direct competitive inhibition of the export pump.

A number of the active indomethacin analogues were found to potentiate the toxicity of MRP-1 substrates, doxorubicin, VP-16 and vincristine in an MRP-1-overexpressing cell line, COR L23R. There was a lower potentiation of the same drugs in the parental COR L23 cell line when combined with the active analogues. MRP-1 is expressed in both the COR L23 variants [5], although at relatively low levels in the COR L23 parental cells. MRP-1 is also expressed at a low level in DLKP cells (i.e. it was only visible on Western blots when a concentrated plasma membrane preparation was used [17]), but it is clearly functional as indicated by our data. We also demonstrated that indomethacin and the active analogues were unable to potentiate the toxicity of 5-FU in the COR L23 parental and resistant cells. 5-FU is not a substrate for MRP-1, and this result further supports the idea that the action of the active analogues is through an interaction with MRP-1.

The variation between the magnitude of toxicity synergism seen between the different inhibitors and different chemotherapy drugs (and in different cell lines) suggests that there may be quantitative differences in the activity of inhibitors with different MRP substrates. For example, there is little difference between the synergistic activity of any of the agents combined with etoposide, whereas **21** may be a better potentiator of doxorubicin activity in COR L23R and COR L23S cells, while **25** is more active in DLKP cells. This may reflect the interactions of the different physiochemical environments in each cell causing minor modifications in substrate and inhibitor specificity, and/or inhibition of other efflux pumps, the expression of which varies from one cell line to another.

We had previously reported that the enhancement effect is not due to the COX inhibitory activity of NSAIDs [17]. However, we analysed the indomethacin analogues for COX-1 and COX-2 inhibitory activity to assess if any of the active analogues were potentially capable of enhancing chemotherapeutic drug toxicity without the potential for damage that is associated with NSAIDs, due to COX-1 inhibition. The *N*-benzoyl-indomethacin analogues were effective at inhibiting COX-1 and COX-2, while the *N*-benzyl indomethacin analogues were weak COX-1 inhibitors. This suggests that the carbonyl group in indomethacin may be important for COX-1 inhibition. Of these benzyl analogues, only **4** was equi-potent to the benzoyl analogues at inhibiting COX-2. It seems that removing the chlorine from the benzene ring, changing its position from *para*-, or replacing it with another halogen, affected the ability of the compound to inhibit COX-2.

Of particular interest are those active indomethacin analogues with reduced COX-1 inhibitory ability, as they might provide an approach by which the toxicity of the anticancer drugs could be enhanced without the gastrointestinal toxic side-effects associated with indomethacin. In particular, analogues **18** and **27**, *N*-benzyl indomethacin analogues, active in the combination toxicity assay, were less toxic than indomethacin and demonstrated no COX-1 and low COX-2 inhibitory activity. Indomethacin analogue **4** also demonstrated very low COX-1 inhibitory ability but, in contrast to **18** and **27**, was also a very potent COX-2 inhibitor. This suggests that **4** is worthy of clinical evaluation due to strong MRP-1 inhibitory activity combined with possible lower toxic side-effects than indomethacin with additional ability to suppress tumour growth due to inhibition of COX-2 [35].

Taken together, the results also show that it is possible to distinguish molecular structures which give better MRP inhibition or better COX inhibition and although some of the structural determinants are similar, sufficient differences exist to permit the design of specific inhibitors of individual proteins.

Overall, these results have demonstrated that indomethacin and a number of indomethacin analogues have the ability to potentiate (via MRP-1 inhibition) the toxicity of a number of clinically important chemotherapeutic drugs at non-toxic concentrations. For certain cancers, where drug resistance is a result of MRP-1-overexpression, these active analogues are promising as potentiators of the toxicity of chemotherapeutic drugs potentially enhancing existing treatments for cancer patients.

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