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Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein

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Summary Decreased accumulation of the fluorescent dye BCECF [2′, 7′-bis-(2-carboxyethyl)-5-(6)- carboxyfluorescein] characterized murine and human multidrug-resistant cell lines overexpressing the multidrug resistance protein (MRP). Indomethacin (10 μM), a known cyclo-oxygenase and glutathione-S-transferase inhibitor as well as a modulator of anion transport, increased accumulation and blocked efflux of BCECF in MRP-expressing murine and human cells. The drug did not affect P-glycoprotein (P-gp)-mediated export of rhodamine 123. The indomethacin effect on BCECF efflux was not reversed by the addition of exogenous prostaglandins, suggesting that the drug acts by a mechanism other than decreasing prostaglandin synthesis. Indomethacin also increased multidrug susceptibility of both murine and human cell lines overexpressing MRP, but not those displaying P-gp-associated resistance. In addition, indomethacin modulated the decreased vincristine accumulation in cells expressing MRP, but not in those expressing P-gp. These data suggest that indomethacin is a specific inhibitor of MRP, possibly functioning by inhibition of glutathione-S-transferase or, alternatively, by direct competition with the drug at the transport site.

Keywords: BCECF; chemosensitizer; modulator; transport inhibitors

Multidrug resistance in mammalian cells has been associated with altered drug transport. Active efflux or altered intracellular sequestration has been attributed to membrane-associated P-glycoprotein (P-gp) or the multidrug resistance protein (MRP) (Inaba et al, 1979; Zamon et al, 1994). We have previously demonstrated that murine and human cell lines that overexpressed MRP also showed altered accumulation and increased efflux of the free acid form of the fluorescent dye BCECF (Draper et al, 1996). BCECF is loaded into the cells as the membrane-permeable acetoxymethyl (AM) ester, BCECF-AM. This lipophilic ester derivative can cross cell membranes, entering the cytoplasm and various organelles, where the AM ester is hydrolysed by intracellular esterases to release BCECF free acid, which is normally retained by the cell. We used the altered BCECF accumulation phenotype of cell lines overexpressing MRP to look for drugs that could modulate MRP function. One of the drugs examined first was indomethacin as it has previously been reported to modulate efflux of BCECF from cultured epithelial cell monolayers, although the mechanism is unknown (Collington et al., 1992). We report here on the ability of indomethacin, a member of the non-steroidal family of anti-inflammatory drugs (NSAID), to specifically modulate MRP-mediated resistance.

MATERIALS AND METHODS

Cell culture

The murine erythroleukaemia (MEL) cell line, PC4, and its drug-resistant derivatives were cultured in basal medium Eagle (BME) containing 10% fetal bovine serum as previously described (Slapak et al, 1994). Human HL60 and HL60 derivative cell lines (kindly provided by M. Center, Kansas State University) were grown in RPMI media containing 10% fetal bovine serum, 5 mM glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. All cultures were incubated in a Revco Ultima incubator set at 5% carbon dioxide.

Fluorescence measurements

Cells were washed once in phosphate-buffered saline (PBS) containing 1 mg ml^{-1} glucose (PBS–glucose) and resuspended in either minimal essential medium (MEM), equilibrated in a 5% carbon dioxide atmosphere lacking phenol red or PBS–glucose, as indicated, at a concentration of 2.5×10^6 cells ml^{-1}. The dye 2′, 7′-bis-(2-carboxyethyl) -5-(6)-carboxyfluorescein acetoxymethylester (BCECF-AM) (Molecular Probes, Eugene, OR, USA) was added at a concentration of 2 μM, and the cells were incubated at 37°C for 15 min. Cells were then pelleted by centrifugation, the supernatant fluid removed and the cell pellet placed on ice. Cells, resuspended in fresh MEM or PBS–glucose at a concentration of 2.5×10^6 cells ml^{-1}, were analysed on a Hitachi F-2000 Fluorescence spectrophotometer (Tokyo, Japan) at excitation wavelengths 439 nm and 505 nm and the emission measurements at 535 nm. Experiments designed to examine the accumulation of 2 μM rhodamine were conducted in a similar manner except that an excitation wavelength of 500 nm and an emission wavelength of 534 nm were used.

Analysis of drug efflux

Equal numbers of cells were incubated with BCECF-AM as described above except that 10 μM indomethacin was added to normalize the dye loading among the cell lines. Cells were then

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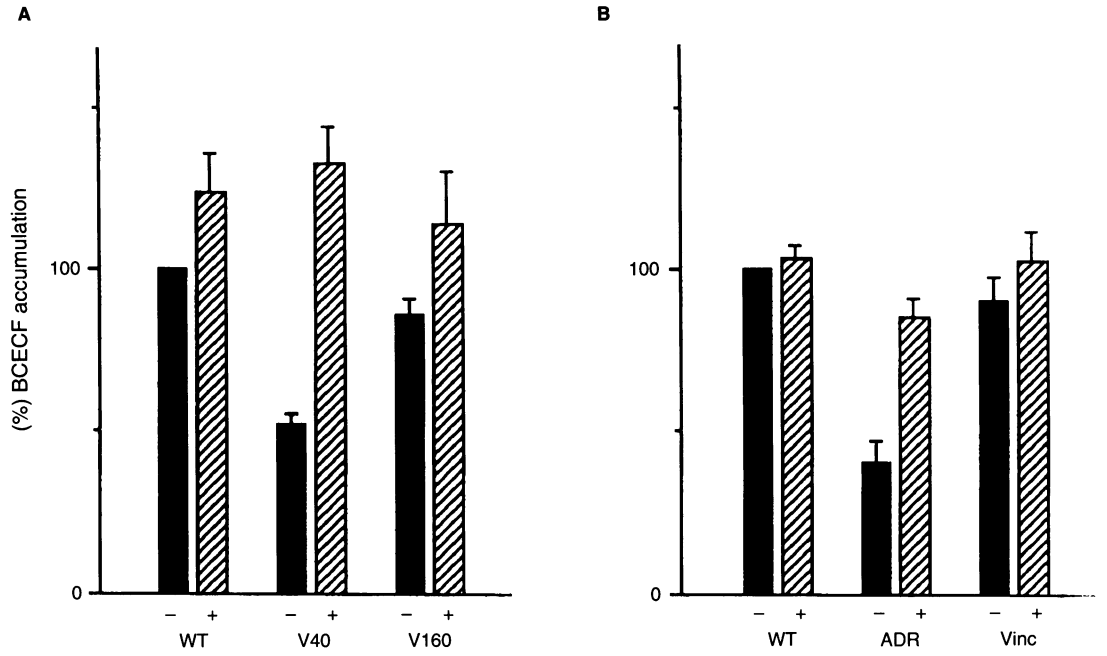

Figure 1 Effect of indomethacin on BCECF accumulation in human and murine leukaemia cell lines overexpressing MRP and/or P-gp. Data are the mean ± s.d. of at least three experiments reported as percentage accumulation of BCECF compared with the appropriate wild type (WT) control (without indomethacin). Accumulation of BCECF was assayed by fluorescence measurements, performed as described in Materials and methods. + and – symbols indicate the presence or absence of indomethacin (10 μM). (**A**) Murine cell lines: PC4-WT (WT), PC-V40 (V40) and PC-V160 (V160). (**B**) Human cell lines: HL60-WT (WT), HL60/ADR (ADR) and HL60/Vinc (Vinc)

washed in PBS, collected by centrifugation and resuspended in PBS–glucose with or without 10 μM indomethacin and placed in a shaking water bath at 37°C. Samples, taken at the indicated time points, were centrifuged to collect the cells and kept on ice before assaying by fluorescence spectroscopy for the amount of dye retained, as described above.

Drug susceptibility studies

Sensitivity of each cell line to the chemotherapeutic agents, vincristine, doxorubicin or etoposide, was determined using the 48-h MTT colorimetric assay (Mosmann, 1983; Slapak et al, 1990). Briefly, 1 ml of 2×10^4 cells, with or without indomethacin (10 μM), were seeded into a 24-well plate containing increasing concentrations of the chemotherapeutic agent. After a 48-h incubation, cells were assayed for viability by the MTT assay. The IC_{50} (dose of drug which reduced the final absorbance to 50% of control) was read from the dose–response curves.

Vincristine steady-state accumulation studies

Vincristine (VCR) accumulation was assayed in 10^6 cells ml^{-1} after a 60 min incubation at 37°C in PBS–glucose (1 mg ml^{-1}) using 25 nM [G-^{3}H]vincristine sulphate (6.6–8.6 Ci $mmol^{-1}$, Amersham, Arlington Heights, IL, USA) in the absence or presence of indomethacin (10 μM). Cell-associated radioactivity was determined after centrifugation of samples (2×10^5 cells) through silicone oil in microfuges tubes previously prepared with 20 *u*l of formic acid overlaid with 200 μl of silicone oil (density = 1.035–1.046, Nye Lubricants, New Bedford, MA USA). After freezing the tubes, the tips were severed (containing the frozen formic acid/cell pellet) and the cell-associated radioactivity was determined by scintillation counting.

RESULTS

Effect of indomethacin on BCECF-AM accumulation in murine or human cells expressing MRP and/or P-gp

We have previously shown that multidrug-resistant human and murine cell lines expressing the MRP have a decreased accumulation of the fluorescent dye BCECF, reflecting increased dye efflux (Draper et al, 1996). We examined the effect of various compounds on BCECF accumulation in MRP-overexpressing murine (PC4) (Slapak et al, 1994) and human (HL60) (Krishnamachary et al, 1994) leukaemia cell lines. One such compound, indomethacin (10 μM), caused a dramatic increase in the accumulation of BCECF in both the murine (PC-V40) and the human (HL60/ADR) MRP-expressing cell lines, normalizing drug accumulation to wild type levels (Figure 1). The murine PC4-WT and PC-V160 (overexpressing both P-gp and MRP) (Slapak et al, 1994) also showed an increase in BCECF accumulation, however, neither HL60WT nor HL60/Vinc (a P-gp-overexpressing cell line) (McGrath et al, 1989) showed an increase in BCECF accumulation. This pattern suggested activity against cells expressing MRP. The indomethacin-mediated increase in BCECF accumulation in PC4-WT could be the result of the low levels of MRP expressed in these cells (Slapak et al, 1994). Indomethacin appeared to have a greater effect on the MRP-overexpressing murine cells than on the human cell lines. Use of indomethacin at concentrations less than 1 μM caused little change in BCECF accumulation (data not shown).

A 1-h incubation of cells (PC4-WT, PC4-V40 and PC4-V160) with 10 μM indomethacin did not detectably alter the intracellular glutathione (GSH) content (e.g. PC4-V40; control = 2.5 ± 0.15 nmol 10^{-6} cells, indomethacin = 2.4 ± 0.25 nmol 10^{-6} cells). Indomethacin is a potent inhibitor of cyclo-oxygenase activity.

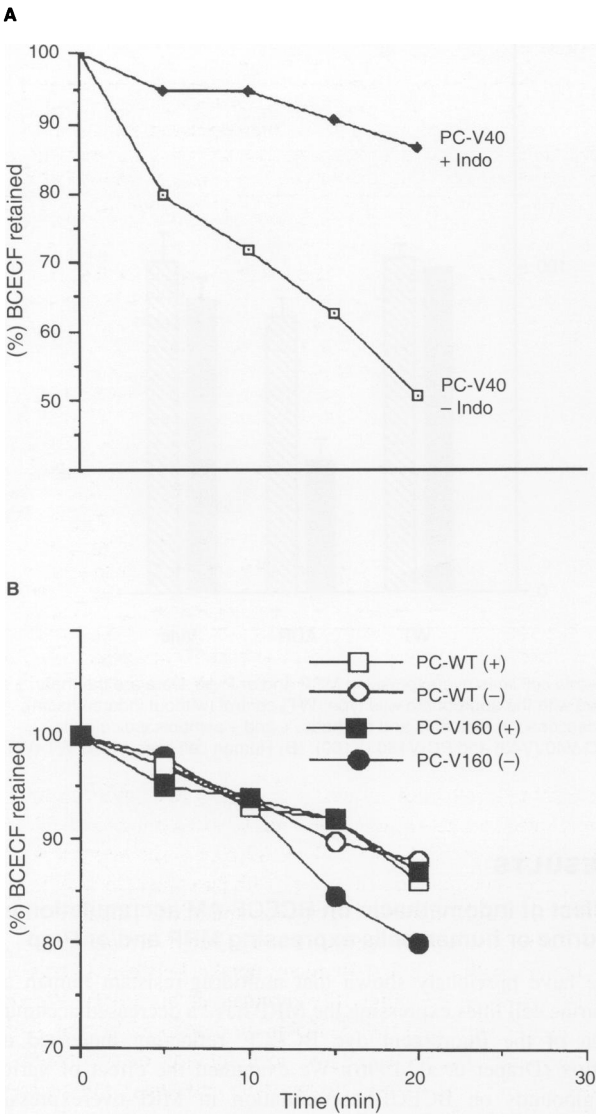

Figure 2 Effect of indomethacin on the efflux of BCECF. Efflux experiments were performed as described in Materials and methods. Data are the mean of three experiments and are expressed as a percentage of BCECF present at time zero. By paired *t*-tests, the values for PC-V40 (+) indomethacin versus (–) indomethacin at the 20-min time point were significant at $P \leq 0.05$. + and – indicate the presence or absence of indomethacin (10 μM)

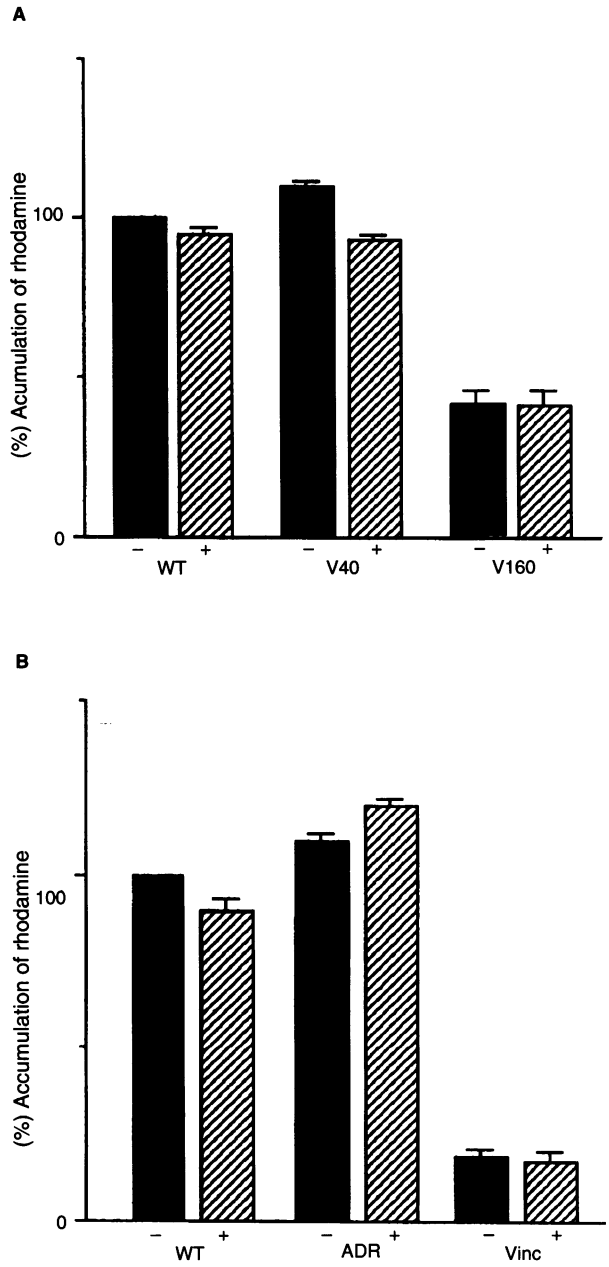

Figure 3 Rhodamine accumulation in murine (**A**) and human (**B**) cell lines expressing MRP and/or P-gp. Accumulation of rhodamine was performed as described in Materials and methods. Data are the mean ± s.d. of at least three experiments. Cells were incubated without (–) or with (+) 10 μM indomethacin. Results are presented as percentage accumulation compared with the appropriate wild type control in the absence of indomethacin. (**A**) PC-WT (WT), PC-V40 (V40) and PC-V160 (V160); (**B**) HL60-WT (WT), HL60/ADR (ADR) and HL60/Vinc (Vinc)

However, inhibition of cyclo-oxygenase did not appear to be the mechanism behind the modulation of BCECF accumulation as the addition of exogenous prostaglandins (0.1 μM PGE1 or PGE2) did not detectably modify the altered BCECF accumulation in the MRP-overexpressing cell lines, nor did they ameliorate the effect of 10 μM indomethacin on BCECF accumulation in these cell lines (data not shown).

Effect of indomethacin on efflux of BCECF by multidrug-resistant murine cell lines

We examined whether the indomethacin-mediated increased accumulation of BCECF was due to inhibition of dye efflux. Murine cell lines were loaded with BCECF in the presence of 10 μM indomethacin which normalized the dye loading among the cell lines. Cells were then washed in PBS–glucose to remove any extracellular BCECF-AM derivative or indomethacin and allowed to incubate in BCECF-AM-free PBS–glucose at 37°C in the presence or absence of indomethacin (10 μM). At various times, samples were taken to determine the dye levels in the cells. The presence of indomethacin inhibited the efflux of dye from the PC-V40 cell line (Figure 2A). A small effect was noted in PC-V160 which overexpresses MRP as well as P-gp (Slapak et al, 1994). These findings indicate that the increased accumulation of BCECF in cells incubated with indomethacin likely occurred via inhibition of efflux of the free-acid form of BCECF.

Table 1 Effect of indomethacin on drug susceptibility in murine and human cell lines

Cell line (ABC transporter)	IC_{50}[a] Dox[b] (fold resistance)[c]		VCR[b] (fold resistance)[c]	
	Control	+Indomethacin	Control	+Indomethacin
Murine				
PC4-WT	ND	ND	2.5 ± 0.26 (1)	1.6 ± 0.35 (0.64)
PC-V40 (MRP)	ND	ND	167 ± 17 (67)	31 ± 9.5 (12)
PC-V160 (MRP and P-gp)	ND	ND	330 ± 60 (132)	350 ± 87 (140)
PC4-80 (P-gp)[d]	ND	ND	78 ± 7 (31)	102 ± 39 (41)
Human				
HL60WT	43 ± 5.7 (1)	36 ± 5.7 (.93)	1.03 ± 0.03 (1)	0.63 ± 0.08 (.61)
HL60/ADR (MRP)	4333 ± 570 (100)	1000 ± 115 (25)	22 ± 2 (21)	2.8 ± 0.7 (2.7)
HL60/Vinc (P-gp)	ND	ND	900 ± 60 (873)	950 ± 70 (922)

[a]IC_{50} (ng ml^{-1}) as determined by 48-h MTT assay. Data are the mean ± s.d. of three experiments. [b]DOX, doxorubicin; VCR, vincristine. [c]Fold resistance compared with wild type cells assayed without indomethacin. Fold resistance was calculated by dividing the mean IC_{50} value by that for the parental cells (PC4-WT or HL60-WT). [d]Doxrubicin-selected P-gp-expressing cell line (Slapak et al, 1994). ND, not determined.

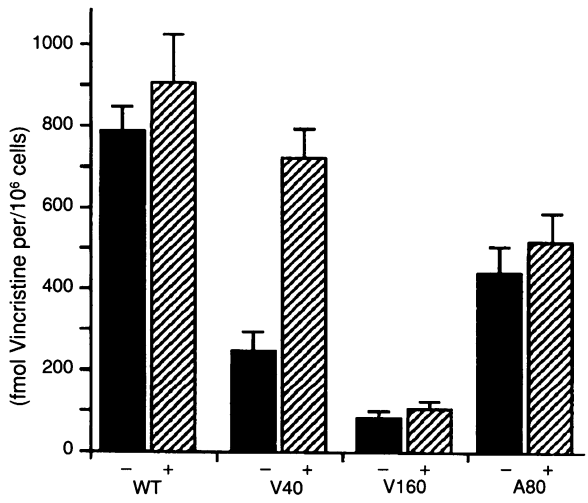

Figure 4 Effect of indomethacin on vincristine accumulation in murine erythroleukaemia cell lines overexpressing the MRP and/or P-gp. Data are the mean ± s.d. of at least three experiments reported as fmol of drug 10^{-6} cells. Accumulation of vincristine was assayed as described in Materials and methods. + and – symbols indicate the presence or absence of indomethacin (10 μM). Murine cell lines: PC4-WT (WT), PC-V40 (MRP overexpressing) (V40), PC-V160 (MRP and P-gp overexpressing) (V160) and PC4-80 (P-gp overexpressing) (A80)

Effect of indomethacin on rhodamine transport

Rhodamine 123 has previously been shown to be a substrate for transport by P-gp (Tapiero et al, 1984). We examined the ability of cells expressing MRP or P-gp to accumulate rhodamine. As expected, the P-gp-expressing murine (PC-V160) and human (HL60/Vinc) cell lines showed a markedly decreased accumulation of rhodamine (Figure 3A and B). In contrast, the MRP-expressing lines (PC-V40 and HL60/ADR) showed no altered rhodamine accumulation compared with parental cells and, in fact, exhibited a slight increase in drug accumulation. This is in accord with previous results showing no altered accumulation of rhodamine, within the first 30 min of uptake, between parental and MRP-expressing cell lines (Twentyman et al, 1994). We then examined the effect of indomethacin on rhodamine accumulation. Indomethacin did not affect the accumulation of rhodamine by either MRP- or the P-gp-overexpressing cell lines compared with controls (Figure 3).

Effect of indomethacin on drug susceptibility of murine and human cell lines

We examined the ability of indomethacin to function as a chemosensitizing agent. As determined by the MTT assay, the IC_{10} for indomethacin was 50 μM for the wild-type and resistant murine cell lines and 20 μM for all the human cell lines. At a concentration of 10 μM, no toxicity was noted. In a 48-h MTT cell viability assay, 10 μM indomethacin reversed MRP-mediated vincristine resistance in both the murine and human cell lines (Table 1). As little doxorubicin resistance is expressed by the murine PC-V40, we examined the effect of indomethacin on doxorubicin resistance in the HL60/ADR cell line which overexpresses MRP and was originally established in doxorubicin. Against HL60/ADR indomethacin produced a dramatic chemosensitization to doxorubicin (Table 1). Indomethacin had no effect on P-gp-mediated vincristine resistance, further suggesting its specificity as a MRP modulator (Table 1). Of note, indomethacin had no effect on PC-V160 (expressing both MRP and P-gp) which is in accord with its previously noted P-gp-dominant phenotype (Figure 3) (Draper et al, 1996).

Effect of indomethacin on vincristine accumulation

We evaluated the effect of indomethacin on a 60-min vincristine accumulation in the murine PC4-WT, PC-V40 (MRP expressing), PC-V160 (MRP and P-gp expressing) and the doxorubicin-selected P-gp-expressing PC4-80 cell lines. Indomethacin modulated the altered vincristine accumulation only in the PC-V40 (MRP expressing) cell line (Figure 4). Slight increases in vincristine accumulation were noted in the other cell lines, consistent with the BCECF accumulation data (Figure 1). This slight increase could be due to the effect of indomethacin on the low basal level of the MRP found in these cells.

DISCUSSION

These studies show a modulation of drug transport in human and murine cells expressing MRP by indomethacin, a non-steroidal anti-inflammatory drug. Few reports describe compounds that specifically influence MRP-mediated multidrug resistance. One study, using the tyrosine kinase inhibitor genistein, demonstrated the modulation of daunorubicin accumulation in human small-cell lung cancer cells. The high concentration of genistein (200 μM) prohibited the use of this compound in a cell-proliferation assay (Versantvoort et al, 1994). Recently, Gekeler et al, have shown that MRP-associated resistance is efficiently modulated by the bisindolylmaleimide PKC inhibitor GF 109203X and the LTD_4-receptor antagonist MK571 (Gekeler et al, 1995*a*,*b*). In both cases, modulation of MRP-mediated resistance was shown using the MTT assay.

Depletion of cellular glutathione (GSH) levels with DL-buthionine (S,R)-sulphoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase, has been shown to modulate MRP-mediated resistance (Versantvoort et al, 1995; Zaman et al, 1995). The chemosensitizing effects of BSO are presumed to be due to the cellular depletion of GSH. In the vincristine-selected cell lines, we have demonstrated a reversal of vincristine and etoposide resistance by 25 μM BSO (Draper et al, 1996). However, the transport of some molecules, such as BCECF and calcein, is not affected by GSH depletion (Draper et al, 1996; Feller et al, 1995). The reason for the chemosensitization in response to depletion of intracellular GSH is unknown. It seems unlikely that the drugs are being transported as GSH-modified adducts, as no modified drugs can be detected (Zaman et al, 1995). This leaves open the possibility that drug transport is directly affected by GSH or that the transport of GSH or other GSH-modified compounds acts in some manner to facilitate transport of the chemotherapeutic agents.

In this present study, we have used the altered accumulation of the fluorescent dye BCECF to identify a modulator of multidrug resistance in MRP-overexpressing cell lines. Indomethacin both increased the accumulation of BCECF (Figure 1) and vincristine (Figure 4) and sensitized MRP-overexpressing cells to vincristine and doxorubicin (Table 1). It appears to have little effect on the function of P-gp as it failed to modulate P-gp-mediated altered accumulation of rhodamine (Figure 3) and vincristine (Figure 4) and failed to alter the drug resistance of both human and murine P-gp-overexpressing cell lines (Table 1). These data suggest that indomethacin is a specific modulator of MRP.

Indomethacin is a well-known inhibitor of prostaglandin synthesis and has also been shown to be a potent inhibitor of glutathione-S-transferase (Primiano and Novak, 1993). It has previously been used to enhance the anti-cancer activity of chlorambucil (Hall et al, 1989; Yang et al, 1992) and has also been shown to give partial reversal of methotrexate and cholate efflux in L1210 cells (Henderson et al, 1994).

The mechanism behind indomethacin's ability to chemosensitize MRP-overexpressing cells remains unknown at this time. As the addition of exogenous prostaglandins did not restore MRP function, it seems unlikely that indomethacin is functioning by inhibiting prostaglandin synthesis. This is in agreement with previous studies which looked at the effect of indomethacin on the efflux of BCECF in non-drug-resistant human and canine cell lines (Collington et al, 1991, 1992). Indomethacin also does not appear to be functioning by altering cellular GSH content as we could find no ability of the drug to alter GSH levels. There are two likely explanations for indomethacin function. First indomethacin could be interacting directly with MRP, functioning as a competitive inhibitor. Indomethacin has a pKa of 4.1, so that at physiological pH the drug would be in an anionic form (Tonnessen et al, 1989). MRP is known to display preference for more hydrophilic compounds and has been shown to transport amphiphilic organic anions (Cole et al, 1994; Leier et al, 1994). Its ability to block BCECF efflux suggests such an activity. However, as the MRP- and P-gp-expressing cell lines displayed no difference in the toxicity level of indomethacin, it does not seem likely that the drug is being effluxed; however, indomethacin may be binding to MRP and inhibiting efflux in another manner. Alternatively indomethacin may be functioning by inhibiting the function of GST, which may, like GSH, be necessary for proper drug efflux. Indomethacin has been shown to function as an inhibitor of class μ glutathione-S-transferases at a concentration of 1 μM (Primiano and Novak, 1993). Further, in a chlorambucil-resistant cell line demonstrating a 40-fold increase in an alpha class GST, indomethacin functioned as a chemosensitizing agent (Hall et al, 1989).

The clinical relevance of MRP is presently unknown. However, increases in P-gp and MRP have been reported in different types of leukaemias refractory to chemotherapy (Goasguen et al, 1993; Zhou et al, 1995). The concentration of indomethacin needed to increase drug susceptibility is clinically relevant (10 μM) and indomethacin is known to be well tolerated (Tonnessen et al, 1989; Statkevich et al, 1993). This suggests that indomethacin may be a valuable adjunct to chemotherapy to block MRP-mediated resistance as well as, in conjunction with a P-gp modulator, to deal with cells expressing both efflux proteins.

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ABBREVIATIONS

MRP, multidrug resistance protein; P-gp, P-glycoprotein; BCECF-AM, the fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethylester; NSAID, non-steroidal anti-inflammatory drug.

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