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Influences of glutathione on anionic substrate efflux in tumour cells expressing the multidrug resistance-associated protein, MRP1

Tanya Bagrij, Antonios Klokouzas, Stephen B. Hladky*, Margery A. Barrand

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ, UK

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Abstract

The ATP-dependent transport of natural product drugs, e.g. vincristine, by multidrug resistance-associated protein (MRP1) requires reduced glutathione (GSH), whilst that of anionic substrates does not. The present results suggest, however, that GSH can modulate transport of anionic species. Efflux of fluorescent anionic substrates was measured from adherent MRP1-expressing human multidrug-resistant lung tumour cells, COR-L23/R, and drug-sensitive parental cells. As expected, much greater efflux of calcein, methylfluorescein-glutathione (GS-MF), and 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) was observed from the resistant cells. Unexpectedly, lowering GSH levels in COR-L23/R cells by inhibiting GSH synthesis with buthionine sulfoximine decreased efflux of calcein and of GS-MF (3-fold and 1.6-fold) but not efflux of BCECF. Transport of the anionic conjugate dinitrophenyl-glutathione ([^{3}H]DNP-SG) was investigated by following its uptake into inside-out plasma membrane vesicles prepared from the MRP1-expressing cells. At least 90% of the ATP-dependent uptake was blockable by the anti-MRP1 antibody QCRL-3 and 100 μM vincristine inhibited uptake but only in the presence of 1–3 mM GSH, suggesting MRP1 to be the protein primarily responsible for this transport. Agents shown to reduce efflux of calcein from resistant cells, i.e. indomethacin, MK-571, and probenecid, also inhibited [^{3}H]DNP-SG uptakes, consistent with MRP1 being responsible for export of calcein. At concentrations achievable within cells, GSSG (70 μM) inhibited uptake whereas GSH (1 and 3 mM) enhanced uptake. We suggest that variations in both GSH and GSSG levels within cells may affect MRP1-mediated anion transport.

Keywords: Glutathione; Multidrug resistance-associated protein; Calcein; Buthionine sulfoximine; Methylfluorescein-glutathione; 2′,7′-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BCECF

1. Introduction

The multidrug resistance-associated protein (MRP1), like the structurally related P-glycoprotein, is an ATP-dependent multidrug transporter that can efflux a number of anticancer drugs from tumour cells, thereby rendering them resistant to cytotoxic attack [1]. MRP1 has been implicated in certain types of clinical drug resistance and thus may be a suitable target for inhibition to improve the effectiveness of various chemotherapeutic strategies. The function of MRP1 in normal cells may be more closely related to its ability to transport anionic species, including heavy metal anions [2,3], glutathione, glucuronide, and sulphate conjugates [4,5], and the fluorescent indicators BCECF [6,7], GS-MF [8–10], and calcein. Calcein, together with its membrane-permeant non-fluorescent ester precursor, calcein-AM, has been used extensively as a means of detecting and monitoring MRP and P-glycoprotein functional activity in tumour cells [11–13].

Efflux of hydrophobic natural product anticancer drugs agents such as daunorubicin and vincristine from cells expressing MRP1 is thought to require GSH [14,15]. The nature of the involvement of GSH is not fully clarified, though co-transport of GSH is now believed to take place [16–18]. By contrast, uptake of either of the anions, DNP-SG or LTC4, into inside-out, MRP1-containing, plasma membrane vesicles has been studied extensively in the absence of GSH [4,19]. It has also been reported that

* Corresponding author. Tel.: +44-1223-334.019; fax: +44-1223-334.040.

E-mail address: sbhl@cam.ac.uk (S.B. Hladky).

Abbreviations: BCECF, 2′,7′-bis-(2-carboxyethel)-5-(and-6)-carboxyfluorescein; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; BSO, buthionine sulfoximine; calcein-AM, calcein acetoxymethyl ester; CMFDA, chloromethylfluorescein diacetate; DNP-SG, dinitrophenyl-*S*-glutathione; GS-MF, methylfluorescein-glutathione; MRP1, multidrug resistance-associated protein; and LTC4, leukotriene C_4.

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depletion of GSH has no effect on calcein accumulation in cells [11,12,14] or on the amount retained after an efflux period of 60 min [11].

We have looked at the time-course of the fluorescence changes found with cells preloaded with anionic species in small numbers of adherent cells [20]. Unexpectedly, reduction of GSH reduced the rate of efflux of two separate anionic species, calcein and GS-MF, but not a third, BCECF. We investigated this further using inside-out vesicles and found that GSH could increase the rate of DNP-SG transport. Our results suggest that, while GSH is not required, both GSH and its oxidised form GSSG can modify the transport of anionic substrates. Thus, changes to GSH status inside cells may influence their MRP1-mediated transport.

Preliminary reports of some of this work have been given previously [21–23].

2. Materials and methods

2.1. Chemicals and drugs

Calcein-AM, CMFDA, and BCECF-AM were obtained from Molecular Probes. BSO, 4′,6-diamidino-2-phenylindole (DAPI), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), GSH, Na_2ATP, TRIS (1 M, pH 7.4), and indomethacin were purchased from Sigma. MK-571 was a gift of Dr. Ford-Hutchinson (Merck Frosst Canada, Inc.) [24]. Solvents used were the following: anhydrous DMSO (41648, Fluka) containing 200 mg mL^{-1} pluronic F-127 (P-2443, Sigma) (for calcein-AM, CMFDA, and BCECF-AM), PBS (for BSO), water (for MK-571 and vincristine), ethanol (for indomethacin), methanol (for DTNB), and 0.2 M EDTA (for GSH). GSH solutions were freshly prepared immediately prior to experiments. Solutions were titrated to pH 7.4 using sodium hydroxide. All reagents were either tissue culture, analytical, or molecular biology grade as appropriate.

2.2. Tumour cell lines and culture conditions

Cells used were from the human large-cell lung carcinoma cell line COR-L23/P, which expresses only low levels of MRP1, and from its multidrug-resistant variant COR-L23/R, which is known to overexpress MRP1 but not P-glycoprotein [25]. The cells grow as attached monolayers. They were cultured in RPMI-1640 medium (Sigma) supplemented with 10% foetal calf serum, 2 mM glutamine, 100 IU mL^{-1} of penicillin and 100 μg mL^{-1} of streptomycin at 37° in 5% CO_2/95% air. The COR-L23/R cell line was maintained in the presence of doxorubicin (0.2 μg mL^{-1}), but cells were kept in drug-free medium for at least 48 hr before use in experiments.

2.3. Determination of efflux of calcein, GS-MF, and BCECF by fluorimetry

Cells were plated out on glass coverslips as described [20] at a density of 2.5×10^4 cells (COR-L23/P and COR-L23/R) and left 48 hr before experiments. To follow efflux, dye loading was first undertaken at 15° for 10 min (15 min in some series) in a buffer of the following composition: 135 mM NaCl, 4 mM KCl, 0.3 mM $CaCl_2$, 0.6 mM $MgSO_4$, 10 mM HEPES, 1.1 mM Na_2HPO_4, 0.6 mM KH_2PO_4, and 6 mM glucose, adjusted to pH 7.4 containing the membrane-permeant, non-fluorescent precursor of the dye, either 0.5 μM calcein-AM, 2.5 μM CMFDA, or 0.5 μM BCECF-AM. The cells were then placed into a cuvette containing dye- and precursor-free buffer. Changes in intracellular fluorescence were constantly monitored at 37° for 15–20 min at excitation wavelengths of 485 nm (for calcein), 490 nm (for GS-MF), and 440 nm (for BCECF) and with emission measurements at 530 nm. The measured fluorescences were corrected by subtraction of the fluorescence observed with the cells before loading and with the efflux medium without cells. The rate constant for efflux was estimated by linear unweighted least-squares fitting to the steepest portion of a plot of ln [fluorescence(t)/fluorescence(t_0)] versus t, where t is the time and t_0 is an arbitrary, convenient reference time (see e.g. Fig. 1).

2.4. GSH assay

GSH levels were measured by a spectrophotometric method modified from that of Sedlak and Lindsay [26]. Following typsinisation, 2–5 $\times$ 10^5 cells/sample were washed in PBS, pelleted, and then lysed in 100 μL EDTA (0.2 M). Protein was precipitated by addition of 20 μL of ice-cold 20% (v/w) trichloroacetic acid. The volume of this mixture was then made up to 200 μL with distilled water and the supernatant following centrifugation (Sanyo, MSE Micro Centaur) assayed for GSH content. Assays were carried out in 96-well plates, the incubation mixture in each well consisting of 100 μL of protein-free supernatant, 160 μL of Tris buffer (0.4 M, pH 8.9), and 4 μL of 5,5-dithiobis(2-nitrobenzoic acid), Ellman's reagent dissolved in methanol at a concentration of 3.4 mg mL^{-1}. The formation of 2-nitro-5-thiobenzoic acid (yellow product) was measured at 414 nm (Titertek Multiskan® MCC/340). Calibration curves of 0.5–3.35 nmol GSH, dissolved in 0.2 M EDTA were used as standards.

2.5. Estimation of intracellular esterase activity

Cell lysates were prepared from trypsinised COR-L23 cells lysed in 10 mM TRIS solution (at pH 7.4) containing protease inhibitors: 2 μg mL^{-1} of aprotinin, 5 μg mL^{-1} of leupeptin, and 80 ng mL^{-1} of pepstatin. Calcein-AM (0.5 μM) was then added to the lysates and cleavage at 37° monitored by measuring the increase of fluorescence over

time. The same cell number (1×10^6 cells/sample) was used with both types of cell lysate in order to allow comparison.

2.6. Measurement of intracellular ATP concentration

After trypsinisation to remove the cells from the culture flasks, the cells were washed and pelleted and intracellular ATP levels determined in trichloroacetic acid (10% v/v) extracts by spectrophotometric analysis using assay kit (Sigma, procedure no. 366-UV). This relies on the conversion of NADH to NAD in a two-step enzymatic process in which the amount of ATP limits the extent of the reaction. The amount of ATP originally present can be obtained from the decrease in absorbance at 340 nm. Calibration curves of 0–60 nmol of ATP were used as standards. As a positive control for decreased levels, cellular ATP was depleted by preincubation of the cells in HEPES medium without glucose and in the presence of 10 mM sodium azide and 6 mM 2-deoxy-glucose for 30 min.

2.7. Isolation of membrane vesicles and vesicular uptake of [^{3}H]DNP-SG

Vesicles were prepared and drug uptake assessed using the method of Ishikawa as adapted by Neo. Briefly COR-L23/R cells were lysed in ice-cold hypotonic buffer (1 mM Tris–HCl, pH 7.4, 2 μg mL^{-1} of aprotinin, 5 μg mL^{-1} of leupeptin, 80 ng mL^{-1} of pepstatin A) and then pelleted. Following homogenisation, the plasma membrane layer was obtained by separation on a sucrose gradient and mixed thoroughly with sucrose/Tris buffer (250 mM sucrose/10 mM Tris–HCl/protease inhibitors) to promote vesicle formation.

Vesicles (25 μg protein per reaction tube) were added to reaction tubes at 37° (prepared in triplicate) containing 250 mM sucrose, 10 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 100 μg mL^{-1} of creatine kinase, 3 μM [^{3}H]DNP-SG, and any drug being tested, to give a total volume of 110 μL. Samples for counting were taken at intervals up to 10 min, diluted in ice-cold stop solution (1 mL 250 mM sucrose/10 mM Tris–HCl, pH 7.4), filtered immediately through nitrocellulose filters (Whatman 0.2-μm pore size presoaked overnight in 3% w/v BSA) under light suction and washed three times with 1 mL ice-cold stop solution. Tracer retained on the filter was determined by liquid scintillation counting. Accumulation was also measured with ATP and the regenerating system (phosphocreatine and phosphocreatine kinase) replaced by equi-osmolar NaCl (16 mM). ATP-sensitive uptake was calculated as the difference. Results were normalised for protein content determined using a BCA Protein Assay kit (Pierce). [^{3}H]DNP-SG (specific activity around 0.4 Ci mmol^{-1}) was synthesised from [^{3}H]GSH (Dupont NEN, specific activity around 40 Ci mmol^{-1}), unlabelled GSH (Sigma), and 1-chloro-2,4-dinitrobenzene (Sigma) using glutathione *S*-transferase from equine liver (Sigma) as described by Ishikawa [27].

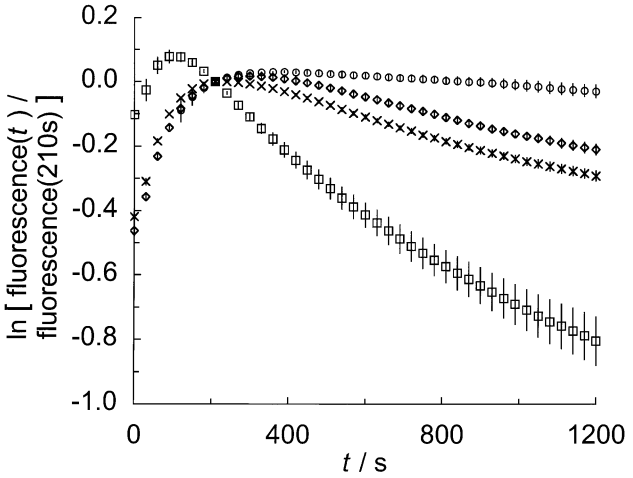

Fig. 1. Efflux of calcein from COR-L23/P and COR-L23/R cells and the effect of indomethacin. Efflux is much more rapid from resistant than from parent cells, and the efflux from resistant cells can be inhibited by indomethacin. The curves are normalised relative to a time point, 210 sec, after which conversion of precursors to fluorescent anion is substantially complete. The early increase in fluorescence presumably represents conversion of precursors already within the cells at the time of the temperature change and transfer of the cells to precursor-free medium. The rate constants for efflux calculated for each individual set of data are summarised in Table 1. Parent COR-L23/P (○) or resistant COR-L23/R (□, ×, ❋) cells were exposed to 0.5 μM calcein-AM for 15 min at 15° and were then placed in dye-free buffer at 37° ($t = 0$) and changes in fluorescence monitored over time. Treated cells were exposed to 10 μM (×) or 50 μM (❋) indomethacin for 30 min prior to loading and also during the loading and efflux periods. Values are shown as means ± SEM, N = 5.

3. Results

Significantly greater fractional loss of calcein, GS-MF, and BCECF was observed from the MRP1-overexpressing COR-L23/R cells than from the parental COR-L23/P cells (values for calcein shown in Fig. 1). This efflux from resistant cells was significantly reduced in the presence of 10 μM indomethacin (Fig. 1). Another MRP1 blocker, MK-571 [28], produced an essentially complete block of efflux (see Table 1). Probenecid (5 mM) has been shown previously to produce a similarly complete block of calcein efflux in these cells [14]. As expected from previous results, depletion of ATP had no effect on the accumulation of fluorescence of the parent cells during loading at 37°, but increased that of the resistant cells to values similar to those of the parent cells (data not shown).

To assess the effects of glutathione depletion, efflux was monitored using cells that had been pretreated for 24 hr with 25 μM BSO. Though similar levels of loading at 15° were achieved in treated and untreated cells, there was significant reduction in the rate constants for efflux of calcein (from 1.1×10^{-3} sec^{-1} to 0.35×10^{-3} sec^{-1}) and of GS-MF

Table 1
Rate constants for efflux

	Rate constant/sec^{-1}	SEM	N	*P*
Calcein				
Parent cells control	0.000059	0.000039	7	0.000041
Resistant cells control	0.0011	0.00012	21	
10 μM indomethacin	0.00034	0.000015	4	0.012
50 μM indomethacin	0.00028	0.000022	4	0.0075
BSO-treated	0.00035	0.000093	4	0.014
25 μM MK-571	0.00012	0.000028	3	0.0063
BCECF, resistant cells				
Control	0.0011	0.00016	5	
BSO-treated	0.0015	0.000044	9	0.01042
GSMF, resistant cells				
Control	0.00087	0.00010	6	
BSO-treated	0.00054	0.000060	5	0.026

P values were calculated relative to the resistant cell control for the corresponding anion using an unpaired equal variance *t*-test.

(from 8.7×10^{-4} to 5.4×10^{-4} sec^{-1}) but a small increase in the rate constant for BCECF (from 1.1×10^{-3} sec^{-1} to 1.5×10^{-3} sec^{-1}) following pretreatment with BSO (see Table 1 and Fig. 2).

Measurement of intracellular GSH content confirmed that blockade of GSH synthesis by BSO had produced large decreases in GSH, i.e. to about 25% of untreated controls, in both parental (untreated, N = 6: 1.30 ± 0.04 versus treated, N = 6: 0.35 ± 0.05 nmol per 10^5 cells) and resistant cells (untreated, N = 6: 0.51 ± 0.02 versus treated, N = 6: 0.11 ± 0.01 nmol per 10^5 cells). It was also noted that intracellular GSH levels were 2–3× lower in untreated resistant than in untreated sensitive cells (parental, N = 6: 1.30 ± 0.04 versus resistant, N = 6: 0.51 ± 0.02 nmol per 10^5 cells).

To determine if the observed decreases in efflux from COR-L23/R cells following BSO treatment might be due to changes in ATP, ATP levels were measured. No significant decreases were found in the samples prepared from cells pretreated for 24 hr with BSO, though lower ATP levels were detectable in cells exposed for 30 min to glucose-free buffer containing sodium azide and deoxyglucose (values in nmol/10^5 cells in parental cells of 3.32 ± 0.17 untreated versus 3.31 ± 0.16, BSO-treated N = 6 and 1.55 ± 0.23, ATP-depleted, N = 5; and in resistant cells of 2.65 ± 0.20 untreated versus 3.06 ± 0.36 BSO-treated, N = 6 and 1.37 ± 0.05, ATP-depleted, N = 6).

To investigate further the relation between anion transport by MRP1, GSH, and GSSG in COR-L23/R cells, transport studies with DNP-SG were conducted on inside-out plasma membrane vesicles prepared from these cells. The anti-MRP1 specific antibody, QCRL-3, which recognises a conformation-dependent epitope exposed on the cytoplasmic side of MRP1 [29], was able to inhibit more than 90% of the ATP-dependent uptake of the DNP-SG (Fig. 3). No inhibition was apparent with equivalent amounts of the isotype control immunoglobulin (mouse IgG). Vincristine

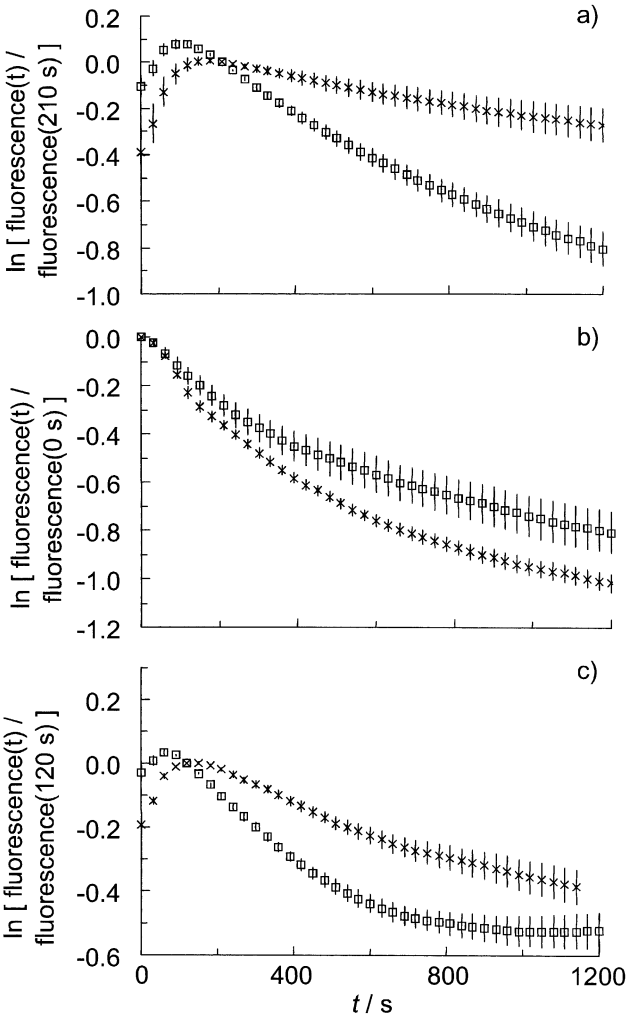

Fig. 2. Effects of BSO pretreatment on efflux of (a) calcein, (b) BCECF, and (c) GS-MF from COR-L23/R cells. For calcein and GS-MF, the logarithm of the fluorescence falls less rapidly with time after BSO treatment, while for BCECF the decrease becomes more rapid. The curves for each dye were normalised relative to a time after which conversion of precursors to fluorescent anion was substantially complete. BCECF conversion was apparently complete within the time taken to transfer the cuvette into the fluorimeter. Cells untreated (□) or treated (×) for 24 hr with 25 μM BSO were loaded by exposure to 0.5 μM calcein-AM, BCECF-AM, or CMFDA for 10 min at 15° and then transferred into a cuvette containing medium at 37° and the fluorescence monitored over time. Values (means ± SEM, N values in Table 1). The rate constants for efflux calculated for each individual set of data are summarised in Table 1.

alone (100 μM) did not block uptake [30], but 100 μM vincristine together with 1 or 3 mM GSH produced a marked inhibition (Fig. 4), similar to that reported previously for MRP1-mediated transport of LTC4 [17]. Inhibition by indomethacin and probenecid is shown by the data in Table 2.

The uptake of [^{3}H]DNP-SG into inside-out plasma membrane vesicles prepared from COR-L23/R cells was increased by 1 and 3 mM GSH but there was little change at 5 or 10 mM GSH (Fig. 5). The decrease previously reported at 5 mM [30] was caused by low pH of the GSH solution,

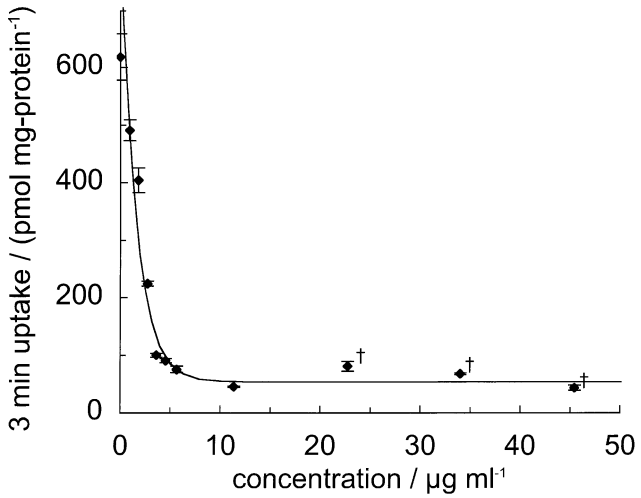

Fig. 3. Effects of anti-MRP1 antibody, QCRL-3, on [^{3}H]DNP-SG uptake into vesicles prepared from COR-L23/R cells. Preincubation with QCRL-3 inhibits more than 90% of the [^{3}H]DNP-SG uptake. Vesicles were preincubated with QCRL-3 for 15 min at room temperature. The temperature was then increased to 37° and [^{3}H]DNP-SG added (mean ± SEM, N = 3 to 6 except for points marked † for which N = 2 and the error bars indicate the range). The theoretical curve, a non-linear, weighted least-squares fit, assumes that the rate of loss of inhibitable activity is proportional to the concentration of the antibody and the amount of inhibitable activity remaining, i.e. that the inhibitable activity remaining during the uptake measurement decreases exponentially with the concentration of the antibody. The plateau of the curve corresponds to 7% of the activity not inhibitable by QCRL-3.

not by the GSH. In contrast to the reduced form of glutathione, the oxidised form, GSSG, showed a concentration-dependent inhibition of anionic conjugate uptake (Fig. 5) with a K_i of 75 ± 12 μM, N = 6, a value which is in line with those previously reported [17,31].

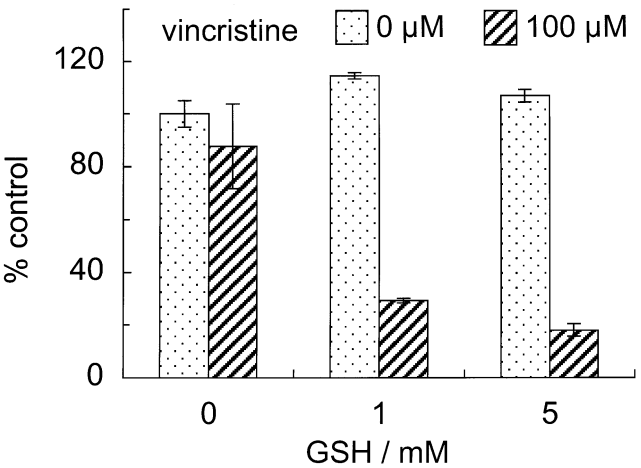

Fig. 4. Effects of vincristine with and without added GSH on [^{3}H]DNP-SG uptake into COR-L23/R-derived vesicles. Vincristine (100 μM) alone does not block uptake, but there is marked inhibition when 1 or 5 mM GSH is also present. GSH at 1 mM alone stimulates uptake of [^{3}H]DNP-SG (see Fig. 5). Uptake was measured over 5 min (mean ± SEM, N = 3). *P* values for the effect of vincristine were 0.42 (not significant) without GSH and <0.001 for 1 and 5 mM GSH (unpaired *t*-test).

Table 2
Inhibition of DNP-SG uptake into vesicles by probenecid and indomethacin

Concentration of inhibitor/μM	Uptake of [^{3}H]DNP-SG as % of control mean ± SEM (N)	
	Probenecid[a]	Indomethacin[b]
10	110 ± 7 (4)	82 ± 9 (4)
25		67 ± 23 (4)
50		30 ± 27 (4)
100	87 ± 5 (4)	11 ± 7 (4)
1000	36 ± 8 (6)	

[a] Data of S.K. Neo.
[b] Unpublished data of the late J. Chew.

4. Discussion

As expected, accumulation of fluorescence of calcein or BCECF was much higher in the parental COR-L23/P cells than in the resistant COR-L23/R cells when loaded at 37°. Although esterase activity detected in the parental cells was approximately twofold higher than in the resistant cells (data not shown), which could contribute towards the higher accumulation, the major factor accounting for the much larger difference in the fluorescences must be the simultaneous efflux of fluorescent dye or non-fluorescent ester during loading of resistant cells, as noted in many previous studies [11,12,14,32]. Therefore, to allow loading of both parent and resistant cells, the cells were maintained at 15° during exposure to the precursors of the fluorescent dyes,

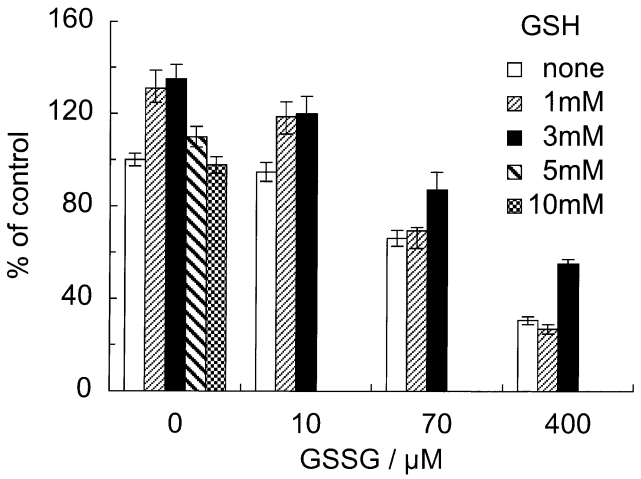

Fig. 5. Effects of GSH and GSSG on [^{3}H]DNP-SG uptake into vesicles prepared from COR-L23/R cells. GSH at 1 and 3 mM but not at 5 or 10 mM stimulates uptake of [^{3}H]DNP-SG. GSSG inhibits uptake of [^{3}H]DNP-SG. Values are shown as means ± SEM with N values of 10, 9, 6, 6, and 7 for no GSSG and 3 for all values with GSSG. The data for no GSSG include those shown in Fig. 4. *$P < 0.05$ and **$P < 0.01$ relative to the same concentration of GSSG without GSH (unpaired *t*-test). In 9 out of 9 experiments without GSSG, the values for 1 and 3 mM GSH each exceeded the value of the paired control without GSH. The *P* values for 70 and 400 μM GSSG relative to the same concentration of GSH without GSSG were <0.01, 0.024, <0.01, and <0.01 (unpaired *t*-test).

i.e. calcein-AM, CMFDA, and BCECF-AM. Following this loading protocol, a significantly greater fractional loss of all three fluorescent species was observed from the MRP1-overexpressing COR-L23/R cells than from the parental COR-L23/P cells, the efflux from resistant cells being significantly reduced in the presence of indomethacin, an agent known to block MRP1 activity [7,12,33,34] or in some circumstances to stimulate it [33]. MK-571, which blocks transport by MRP1 [28], produced an essentially complete block of efflux. Vincristine at 100 μM has been observed to inhibit efflux from SW-1573 cells transfected with MRP1 [11]. However, it was not possible in the present studies to test the effects of vincristine on calcein efflux in COR-L23/R cells, since at 100 μM concentration vincristine affected the cells in some way that greatly suppressed calcein accumulation. As expected, ATP depletion increased accumulation of fluorescence in resistant but not in parent cells during loading at 37°.

In contrast to expectations, glutathione depletion brought about by pretreatment for 24 hr with 25 μM BSO showed significant effects on efflux of the anionic species. Despite similar levels of loading at 15° in treated and untreated cells, there was significant reduction in the rate constants for efflux of calcein and of GS-MF, but not of BCECF, following pretreatment with BSO. This was not due to changes in ATP levels, since no significant decreases in ATP levels could be detected in the cells pretreated for 24 hr with BSO. Thus, efflux of calcein and of GS-MF but not BCECF appears to be inhibited by reduction in intracellular GSH levels.

An effect of GSH levels on the transport of calcein and GS-MF was unexpected, since results in previous studies have suggested that the accumulation of calcein is not dependent on the levels of GSH [11,12,14] and that the fraction of the preloaded calcein remaining in MRP-expressing cells 60 min after loading was the same in treated and untreated cells [11]. However, on closer inspection it is not clear that the methods employed in these previous studies would have detected the effects reported here. It has already been demonstrated that MRP1 transports the ester precursor, calcein-AM, with much higher affinity than it transports the anion [32], and that changes in export of the ester and hence in the rate of production of calcein within the cell rather than changes in the export of the anion determine the initial rate of increase in fluorescence [11,12,32]. Thus, it is not simple to make inferences about the efflux of anion from measurements of accumulation, and any inferences that are made describe efflux in the presence of competition from the precursor. In the only previous report on efflux of calcein from preloaded, BSO-treated cells [11], the efflux from the control untreated cells was largely complete in less than 30 min. Thus, while the substantial inhibition of transport observed for vincristine and probenecid was seen easily, inhibition by a relatively small factor may have prolonged the time required for loss of most of the calcein without markedly increasing the amount remaining after 60 min. In one of the three cell lines reported, HL60/ADR, 25% greater retention was observed, which might correspond to inhibition of efflux similar to that reported here. In the present experiments, we have measured the rate constant of loss for the fluorescent anion approximately 5 min after the end of loading. This rate constant was decreased by the BSO treatment. The reduced efflux of calcein after BSO treatment cannot be explained by loss of ATP since the ATP levels were unchanged. Nor was it likely to be due to a direct action of BSO on MRP1 activity, since previous studies using isolated inside-out membrane vesicles prepared from COR-L23/R cells have shown that 25 μM BSO has no direct inhibitory effect on uptake of [^{3}H]DNP-SG [30].

There are other explanations to account for the above results on GSH depletion in these cells. GSH, though not essential for anion transport, may nevertheless provide a modulatory effect on MRP1 and the extent of this inhibition may differ in different cell types. Alternatively, though MRP1 is clearly overexpressed in COR-L23/R cells, the possibility exists that another transporter present in the COR-L23/R but not COR-L23/P cells may efflux the fluorescent anions from the resistant cells at a much higher rate than COR-L23/R and it is this other transporter that is sensitive to GSH. Calcein is well retained in most cells (and its retention is often used as a test of cell viability), but whenever tested in MRP1-overexpressing cells marked efflux has always been found. Thus, either the calcein transport in these cells is a function of MRP1 or the calcein transporter is always coexpressed with MRP1. Strong evidence that MRP1 itself can transport calcein is provided by the observation of transport in SW-1573 MRP1-transfected cells, but not in the parent SW-1573 cell line or in SW-1573 cells transfected with P-glycoprotein [11,12].

To investigate further the relation between anion transport by MRP1, GSH, and GSSG in COR-L23/R cells, a number of transport studies with DNP-SG were conducted on inside-out plasma membrane vesicles prepared from these cells. Uptake of DNP-SG by these vesicles is ATP-dependent, with the rate observed in the absence of ATP less than 1% of the total [30]. To establish whether the uptake observed into the vesicles could be accounted for primarily by MRP1, the monoclonal antibody QCRL-3 was added to the uptake mixture. QCRL-3, which recognises a conformation-dependent epitope exposed on the cytoplasmic side of MRP1 [29], was able to inhibit more than 90% of the ATP-dependent uptake of the DNP-SG, suggesting strongly that MRP1, the isoform overexpressed in these cells, accounts for the conjugate transport. Consistent with this identification, 100 μM vincristine alone did not block [30], but 100 μM vincristine together with 1 or 3 mM GSH produced a marked inhibition similar to that reported previously for MRP1-mediated transport of LTC4 [17].

To date, all agents that have been found to reduce efflux of calcein have, if tested, also inhibited DNP-SG uptake into the inside-out plasma membrane vesicles consistent with the

MRP1 being responsible for the transport of calcein. We have shown here and previously that indomethacin, probenecid, and MK-571 all inhibit uptake of DNP-SG into vesicles prepared from COR-L23/R cells [30]. This is as expected from previous studies [4,19]. Indomethacin and MK-571 have also been found to inhibit transport of BCECF [7,35] and MK-571 the transport of GS-MF [10] in MRP1-expressing cells. The observations that indomethacin can enhance and at higher concentrations inhibit uptake of NEM-SG (*N*-ethylmaleimide glutathione) into vesicles made from MRP1-infected Sf9 cells [33] and that it inhibits GSH efflux from MRP1-transfected MDCKII cells [34] also confirm that indomethacin does affect MRP1 function.

The surprising finding was enhancement of [^{3}H]DNP-SG uptake into inside-out plasma membrane vesicles prepared from COR-L23/R cells in the presence of 1 and 3 mM GSH but concentration-dependent inhibition in the presence of the oxidised form, GSSG. Thus, because they are in the opposite direction, the effects of GSH on [^{3}H]DNP-SG uptake cannot be ascribed to effects of a minor contamination by GSSG. Similarly, the observed inhibition of efflux from BSO-treated, intact cells of calcein and GS-MF is in the opposite direction to that expected if the important effect of glutathione depletion were a decrease in GSSG.

Interaction of GSH with MRP1 in a way that could stimulate [^{3}H]DNP-SG uptake into vesicles and calcein efflux from COR-L23/R cells is consistent with the now extensive evidence that: GSH modulates both other transport functions and the ATPase activity of MRP1 [14,15,16–18,33,34,36–38]; GSH efflux is enhanced from MRP1-expressing cells [37,39] and this enhanced efflux can be inhibited by indomethacin and probenecid [34]; and that GSH in the presence of NaCl can stimulate uptake of LTC4 into vesicles prepared from MRP-expressing HL60/ADR cells [38]. However, stimulation by GSH of NEM-SG uptake into vesicles from Sf9 cells infected with MRP1 was sought but not observed [33]. Stimulation of MRP1-mediated transport of anions by GSH requires kinetic models for MRP1 transport with at least two binding sites. Evers *et al*. [34] have proposed a model in which one site (G-site) has a relatively high affinity for GSH and low affinity for the transported substrate, and the other (D-site) has a high affinity for substrate and a low affinity for GSH. They further propose that at low substrate concentrations, GSH occupies both sites leading to a slow rate of transport of GSH. In terms of this model, the present data could be explained if GSH competes with DNP-SG or calcein for the D-site while stimulating transport via the G-site.

The present results suggest that changes in the concentrations of GSH and its oxidised form GSSG inside cells may each influence MRP1-mediated anion transport. Furthermore, because depletion of total glutathione from cells may reduce both GSH and GSSG levels, the effects observed may depend on their relative levels in different cell types. Similarly, changes in both GSH and GSSG may be of some physiological consequence during or following hypoxia or oxidative stress, where increased inhibition of the transport of other anions by GSSG may play a significant part. Such a hypothesis certainly warrants further more extensive investigation.

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