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Verapamil-stimulated glutathione transport by the multidrug resistance-associated protein (MRP1) in leukaemia cells

Karen V. Cullen[a], Ross A. Davey[b], Mary W. Davey[a,*]

[a]*Cell and Molecular Biology Department, University of Technology, Sydney, Gore Hill 2065, Australia*
[b]*Bill Walsh Cancer Research Laboratories, Royal North Shore Hospital, St Leonards, NSW 2065, Australia*

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Abstract

Multidrug resistance mediated by the multidrug resistance-associated protein MRP1 is associated with decreased drug accumulation, which is in turn dependent on cellular glutathione. We have reported that verapamil, an inhibitor of drug transport, caused a decrease in cellular glutathione in CCRF-CEM/E1000 MRP1-overexpressing leukaemia cells (Biochem Pharmacol 55;1283–9, 1998). We now demonstrate that other inhibitors of MRP1-mediated drug transport (e.g. MK571, indomethacin, genistein, and nifedipine) deplete cellular glutathione in these leukaemia cells (>30% decrease; $P < 0.01$) while having no effect on the parental CCRF-CEM cells. However, treatment with etoposide or vincristine (at similar molar concentrations) caused a 20% decrease in glutathione. Verapamil-stimulated glutathione transport correlated with MRP1 expression in a series of drug-resistant cells, and glutathione was quantitatively recovered in the extracellular media. Further, verapamil-stimulated glutathione transport was rapid (50% decrease in 10 min), dose-dependent, and inhibited by vanadate, an inhibitor of ATPase activity, but not by sulphobromophthalein (BSP) or methionine, inhibitors of hepatic glutathione transporters. Incubation of CCRF-CEM/E1000 cells in 25 mM glutathione not only showed that verapamil-mediated efflux occurred against the concentration gradient, but also demonstrated the MRP1-mediated uptake of glutathione ($P < 0.01$ compared to the parental CCRF-CEM cells), which was not inhibited by vanadate. These results demonstrate that while MRP1 transports glutathione in the presence of inhibitors of drug transport, there is no convincing evidence for co-transport of glutathione with drug. They further demonstrate that MRP1 mediates the facilitated transport of glutathione into the MRP1-overexpressing CEM/E1000 cells, suggesting that MRP1 may play a major role in cellular glutathione homeostasis.

Keywords: Multidrug resistance-associated protein; MRP1; Verapamil; Glutathione; CEM leukaemia cells

1. Introduction

The ATP-binding cassette (ABC) transport proteins are a rapidly expanding superfamily of membrane proteins that mediate the energy-dependent transport of many compounds both in and out of cells [1]. While most members transport one or a specific class of compound, the multidrug transporters, P-glycoprotein, and MRP1 (multidrug resistance-associated protein) transport a wide variety of structurally and functionally different compounds, including natural product drugs. By mediating enhanced drug efflux, these two transport proteins confer resistance on cancer cells, and expression of these proteins has been associated with the failure of chemotherapy in the treatment of cancer [2]. Since development of drug resistance remains a major obstacle in the successful treatment of cancer, understanding the mechanism of action of these drug transport proteins and the search for inhibitors of drug transport is of great clinical importance.

A diverse variety of compounds have been identified that specifically inhibit P-glycoprotein-mediated drug transport by binding to P-glycoprotein and preventing drug efflux [3]. While many of these compounds also inhibit MRP1-mediated drug transport, their effect is usually not as great as on P-glycoprotein-expressing cells [4]. Verapamil, one of the first identified inhibitors of P-glycoprotein-mediated drug efflux [5], also inhibits MRP1-mediated efflux [6], showing

* Corresponding author. Tel.: +61-2-9514-4065; fax: +61-2-9514-4026.

E-mail address: Mary.Davey@uts.edu.au (M. Davey).

Abbreviations: MRP1, multidrug resistance-associated protein; BSO, buthioninesulphoximine; MDR, multidrug resistance; MTT, (3,-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; BSP, sulphobromophthalein; GST, glutathione *S*-transferase; and CFTR, cystic fibrosis transmembrane conductance regulator.

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that although these proteins exhibit only about 15% homology, their transport properties may be similar. However, early studies on MRP1-overexpressing cells demonstrated that BSO, an inhibitor of glutathione synthesis, was able to prevent the efflux of drug from MRP1-expressing cells while having no effect on P-glycoprotein-expressing cells [7]. This implicated the tripeptide glutathione in MRP1-mediated drug resistance. Glutathione plays a central role in cellular defence against toxic agents by conjugation to xenobiotics and their subsequent transport out of the cell in an ATP-dependent process. While it is clear that MRP1 may transport glutathione conjugates, including leukotriene C_4, there is no evidence for drug conjugation in MRP1-expressing MDR cells. However, studies using membrane vesicles have demonstrated that drug transport by MRP1 is dependent on the presence of glutathione [8,9].

Although a role for glutathione in MRP1-mediated transport was supported by the findings that MRP1 knockout mice and cell lines both have increased cellular levels of glutathione [10], over-expression of MRP1 is not consistently associated with decreased cellular glutathione. While some multidrug-resistant cells show decreased glutathione, the multidrug-resistant CEM/E1000 human leukaemia cells show no change in cellular glutathione, although they are approximately 200-fold resistant to etoposide and have high expression of MRP1 [11]. We have previously reported that treatment of MRP1-overexpressing CEM/E1000 cells with verapamil was associated with the depletion of cellular glutathione, although verapamil had no effect on glutathione levels in the P-glycoprotein-expressing CEM/VLB_{100} cells [11]. This suggests that sensitisation by verapamil in MRP1-expressing cells may be due to changes in glutathione, rather than a direct inhibition of drug efflux. Subsequently, it was demonstrated that verapamil was able to inhibit MRP1-mediated LTC_4 transport in membrane vesicles, but only in the presence of glutathione [12]. While studies using membrane vesicles may be useful in the analysis of the kinetic properties of transport, they lack the cellular topology and glutathione metabolism associated with MRP1-mediated drug transport. We have therefore extended our studies on the CEM/E1000 MRP1-overexpressing cell line derived from the CCRF-CEM T-cell leukaemia cells to further examine the relationship between verapamil, glutathione, and MRP1-mediated drug transport.

2. Materials and methods

2.1. Drugs

Daunorubicin was purchased from Delta West, epirubicin from Pharmacia, and vincristine from David Bull Laboratories. Etoposide (powder) was from Sigma Chemical Co. Etoposide was prepared as a stock solution of 20 mg/mL in DMSO.

2.2. Cell lines

The CCRF-CEM (CEM) human leukaemia cell line [13] and the epirubicin-selected CEM/E50, CEM/E100, and CEM/E1000 [14] multidrug-resistant sublines were grown in RPMI-1640 medium (Trace Biosciences, Sydney), supplemented with 10% foetal calf serum (Trace), 20 mM HEPES (Trace), and 10 mM $NaHCO_3$ at 37° in a humidified atmosphere with 5% CO_2. The multidrug-resistant sublines were maintained in the absence of drug, and resistance was stabilised by drug treatment for 3 days every 6 weeks (CEM/E50 subline with 50 ng/mL of epirubicin, CEM/E100 subline with 100 ng/mL of epirubicin, and CEM/E1000 subline with 1000 ng/mL of epirubicin). Exponentially growing cells were used for all experiments. All cultures were mycoplasma-free.

2.3. Cytotoxicity assays

Sensitivity to drugs was determined in triplicate using the MTT assay as previously described [11]. Cells were seeded at a density of 5×10^4 cells/well and viability determined after 4 days using MTT (Sigma). The IC_{50} was determined as the drug concentration which resulted in a 50% reduction in cell viability. Relative resistance was calculated by dividing the IC_{50} obtained for the resistant subline by the IC_{50} obtained for the CEM parental cell line. Reversal of resistance was determined by incubating the cells in the absence and presence of 10 μM verapamil, 20 μM nifedipine, 70 μM diltiazem, 10 μM BSO, 50 μM indomethacin, 20 μM genistein (all from Sigma), or 50 μM MK571 (a gift from Merck Sharpe & Dohme), in a cytotoxicity assay. The IC_{50} was determined and fold reversal was calculated by dividing the IC_{50} for the cells incubated with drug alone by the IC_{50} for the cells incubated with the drug in the presence of the reversing agent.

For the 1-hr cytotoxicity assays, cells were exposed to drug for 1 hr, after which they were resuspended in fresh culture medium, plated in triplicate at a density of 5×10^4 cells/well, and incubated as described above.

2.4. Daunorubicin accumulation

Cell-associated daunorubicin was measured by flow cytometry in the absence (control) and presence of 10 μM verapamil, using 5×10^5 cells in duplicate as previously described [7].

2.5. Glutathione determination

Total intracellular glutathione was determined using a modification of the colorimetric method of Suzakake *et al.* [15] as previously described [11]. Briefly, exponentially growing cells (10^7) were harvested by centrifugation (800 *g* for 5 min) and resuspended in 20 mL of culture medium for treatment with all drugs and modulators, with the exception

of vincristine, for which cells were resuspended in 1 mL fresh culture medium. Cells were incubated in 50 mL round-bottom tubes in the absence (control) and presence of modulator or drug for 1 hr at 37° with 5% CO_2. For treatment with BSO (50 μM) and glutathione (25 mM), cells were incubated for 3 hr at 37° with 5% CO_2.

To examine the effect of potential inhibitors of glutathione export, cells were preincubated for 15 min with 200 μM BSP (Sigma), or for 1 hr with 5 mM methionine (Sigma) or 2 mM vanadate (Sigma), followed by a 1-hr incubation with or without 10 μM verapamil. The pH of the culture medium was adjusted to pH 7.3.

After the treatments, cells were washed twice with PBS, lysed in 600 μL water, and the protein precipitated by the addition of 50 μL of 30% sulphosalicyclic acid (SSA) to 450 μL of the lysate. After 15 min on ice, protein-free supernatants were collected. Glutathione was determined using a reaction mixture containing 20 μL lysate or standard (15 μg/mL in 3% SSA), 20 μL triethanolamine buffer, pH 8.0 (1 M), 120 μL NADPH (0.3 mM), and 20 μL 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, 6 mM). After 3 min at 30°, the reaction was started by the addition of 20 μL (2 units) glutathione reductase. Absorbency was monitored in a multiwell microplate reader (BioRad) at 405 nm for 6 min and glutathione levels calculated. All reagents were from Sigma. All glutathione concentrations are the means of at least triplicate determinations.

To determine extracellular glutathione, exponentially growing cells (10^7) were harvested by centrifugation (800 *g* for 5 min) and resuspended in 1 mL PBS. In some experiments, cells were preincubated for 15 min with acivicin (250 μM; Sigma), an inhibitor of the plasma membrane associated γ-glutamyltransferase, before the addition of verapamil (10 μM) for 1 hr. Following the incubation, supernatants were collected and glutathione levels were determined.

2.6. Statistical analysis

All experiments were repeated at least twice and differences determined using the Student's *t*-test. Significance was determined at $P < 0.05$.

3. Results

3.1. Depletion of cellular glutathione by verapamil

Reports that MRP1-mediated drug resistance was specifically modulated by treatment with BSO demonstrated the importance of cellular glutathione in multidrug resistance mediated by this transporter. We previously reported that treatment of the MRP1-overexpressing CEM/E1000 cells with verapamil resulted in depletion of cellular glutathione [11]. Fig. 1 shows that for the CEM/E1000 subline, this depletion by verapamil was dose-dependent, and that glutathione was significantly reduced after treatment for 1 hr with doses as low as 1 μM verapamil. This rapid decrease was confirmed in Fig. 2A, which shows that treatment of the CEM/E1000 cells with 10 μM verapamil, a non-toxic dose, caused a 50% decrease in cellular glutathione within 10 min, without any change in the parental CEM cells. After 2 hr, the CEM/E1000 were approximately 80% depleted of

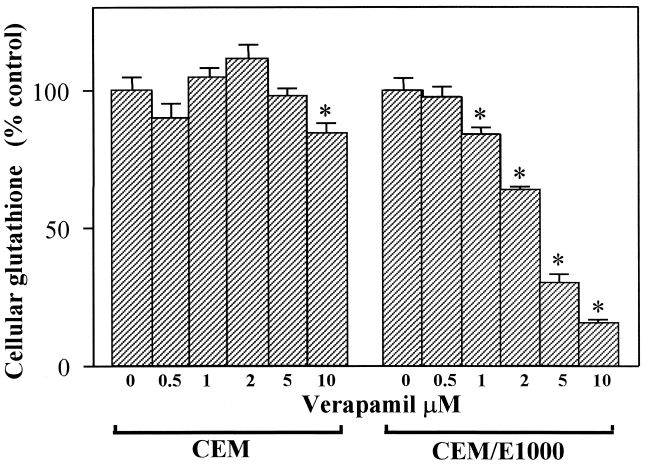

Fig. 1. Effect of verapamil concentration on cellular glutathione. CEM cells and MRP1-overexpressing CEM/E1000 cells were incubated without or with verapamil for 1 hr after which cellular glutathione was determined. Results are presented as % control untreated CEM cells; error bars are the standard deviation of duplicate samples; * indicates a significant difference ($P < 0.05$) from the untreated CEM cells using the Student's *t*-test.

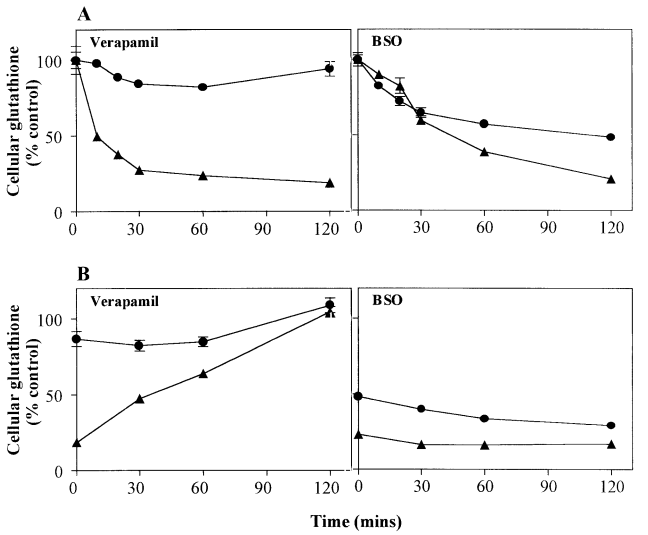

Fig. 2. (A) Time-course of glutathione depletion by verapamil and BSO. CEM cells (●) and MRP1-overexpressing CEM/E1000 cells (▲) were incubated with verapamil (10 μM) or BSO (50 μM), and cellular glutathione determined at the times indicated. (B) Time-course of glutathione recovery after treatment. CEM cells (●) and MRP1-overexpressing CEM/E1000 cells (▲) were incubated with verapamil (10 μM) or BSO (50 μM), after which the modulator was removed and the cells incubated in fresh culture media. Cellular glutathione was determined at the times indicated. Results are presented as % control untreated CEM cells; error bars are the standard deviation of duplicate samples.

glutathione, while the CEM cells showed only a 10% reduction. These levels were maintained in cells exposed to verapamil for 24 hr (not shown). This contrasts with the decrease in cellular glutathione after BSO treatment where glutathione depletion, due to lack of synthesis, was slow with no significant difference over the first 30 min between the cell lines. While there was a greater decrease in the CEM/E1000 cells after 2-hr treatment with BSO, cellular glutathione in both the CEM and CEM/E1000 was the same after 24-hr treatment and <10% of the level in untreated cells (not shown).

Removal of the verapamil from the cells showed that recovery of the cellular glutathione in the CEM/E1000 cells was also rapid, with the level increasing by 2-fold within 30 min and the glutathione was completely restored by 2 hr (Fig. 2B). This again contrasts with BSO treatment, which showed no increase in glutathione over this time following removal of BSO (Fig. 2B). Recovery of glutathione after removal of verapamil was prevented by addition of 50 μM BSO, indicating that the recovery was due to synthesis of glutathione and not transport from the culture medium (not shown).

To confirm that the verapamil-induced decrease in cellular glutathione was due to MRP1-mediated transport, we examined the effect of verapamil in a series of multidrug-resistant cells (CEM/E50, CEM/E100, CEM/E1000) with increasing multidrug resistance associated with increasing MRP1 expression [14]. These sublines show increasing resistance to epirubicin (Fig. 3A), but no change in the enzyme GST (not shown), or in total cellular glutathione (Fig. 3B). Depletion of glutathione by 10 μM verapamil correlated with increased expression of MRP1 in the CEM/E100 and CEM/E1000 sublines (Fig. 3C). Further, the depleted cellular glutathione was quantitatively recovered from the culture medium after treatment with verapamil (Fig. 3D). The recovery of glutathione in the culture media was not affected by the inclusion of acivicin (250 μM), an inhibitor of the plasma membrane-associated γ-glutamyltransferase, indicating there was little degradation and recycling of the glutathione by these cells (not shown).

GSH transporters are of major importance in maintaining glutathione homeostasis and several have been identified particularly in liver. BSP, an inhibitor of canalicular hepatic glutathione transport, and methionine, an inhibitor of sinusoidal glutathione transport [16,17], failed to inhibit the verapamil-induced depletion of cellular glutathione (Fig. 4). However, vanadate, an inhibitor of the ATPase activity of P-glycoprotein and MRP1, completely inhibited this cellular glutathione efflux (Fig. 4).

To confirm that the MRP1-dependent glutathione efflux in the presence of verapamil was due to active transport, efflux was determined in culture medium containing a pharmacological concentration of glutathione (25 mM). Fig. 5A shows that for the CEM/E1000 cells, the verapamil-dependent glutathione efflux still occurred against a glutathione concentration gradient, while this treatment caused little

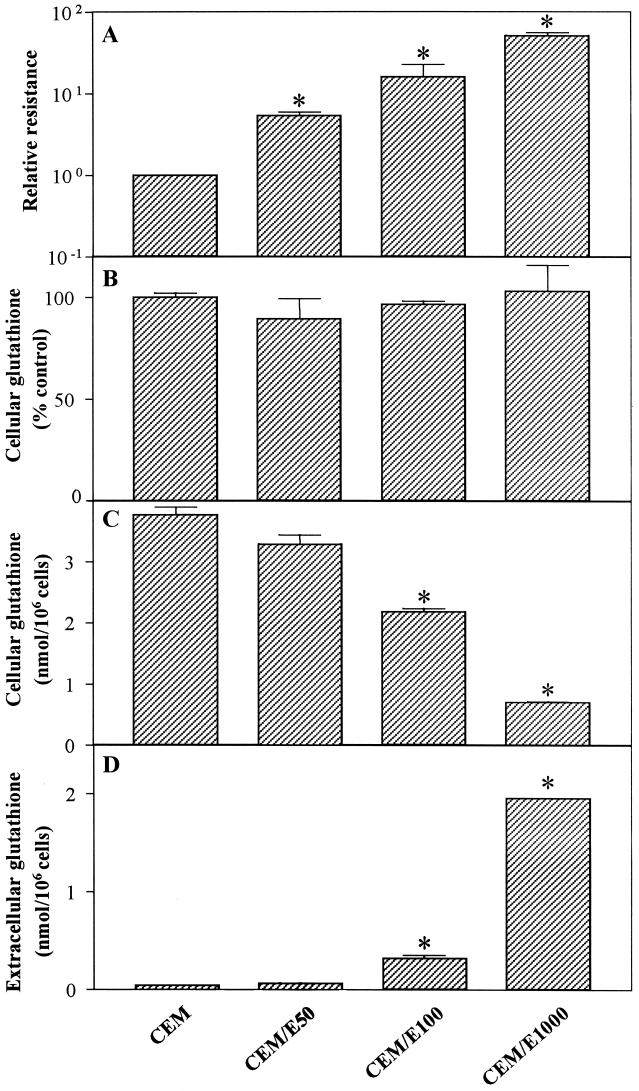

Fig. 3. Effect of verapamil on MRP1-overexpressing sublines. (A) The cytotoxicity of epirubicin in CEM cells and MDR sublines was determined and the relative resistance calculated. Results are presented compared to the parental CEM cells (relative resistance 1). (B) The parental CEM cells and the MDR sublines were harvested in exponential growth and the cellular glutathione determined as described in Methods. Results are presented as % control CEM cells. (C) CEM cells and the MRP1-overexpressing sublines were treated with verapamil (10 μM) for 1 hr after which cells were harvested and cellular glutathione determined. (D) CEM cells and the MRP1-overexpressing sublines were treated with verapamil (10 μM) for 1 hr after which the media were assayed for glutathione. All experiments were repeated at least twice. Error bars are the standard deviation of duplicate samples; * indicates a significant difference ($P < 0.05$) from the CEM cells using the Student's *t*-test.

change in the CEM cells. Incubation for 2 hr in medium containing 25 mM glutathione increased the intracellular glutathione level in the CEM/E1000 cells by approximately 1.5-fold ($P < 0.05$), with little effect on the parental CEM cells, demonstrating that MRP1 may also be involved in the uptake of glutathione. This MRP1-dependent increase in intracellular glutathione was not inhibited by vanadate, suggesting this uptake was not associated with MRP1-mediated

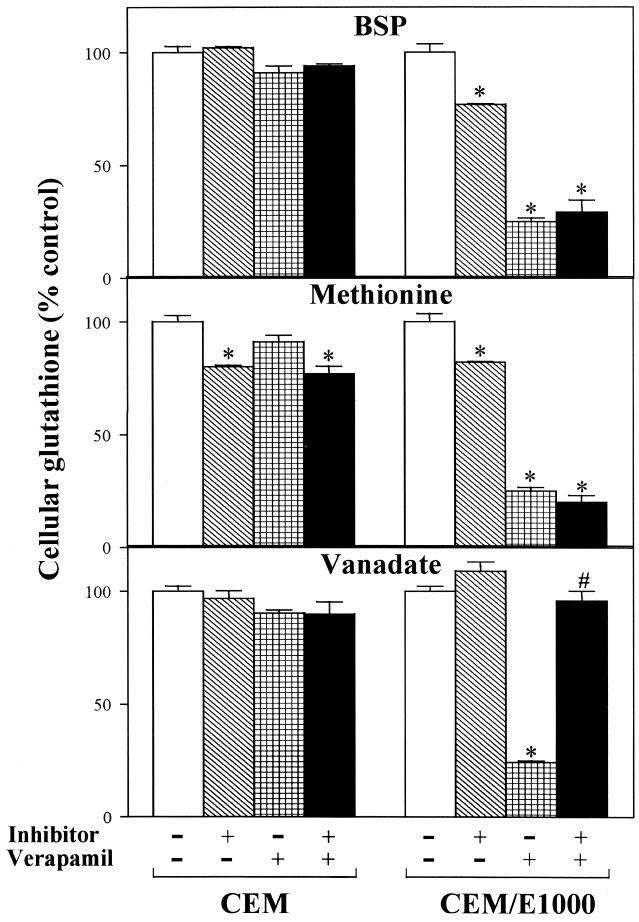

Fig. 4. Effect of inhibitors on glutathione export stimulated by verapamil. Cells were incubated in the absence or presence of 200 μM BSP (15 min), 5 mM methionine (1 hr), or 2 mM vanadate (1 hr) as indicated and the cellular glutathione determined after a 1-hr incubation with or without verapamil (10 μM). Results are presented as % control untreated CEM cells; error bars are the standard deviation of duplicate samples; * indicates a significant difference ($P < 0.05$) from the untreated CEM cells using the Student's *t*-test. # indicates a significant increase ($P < 0.05$) from the verapamil-treated cells using the Student's *t*-test.

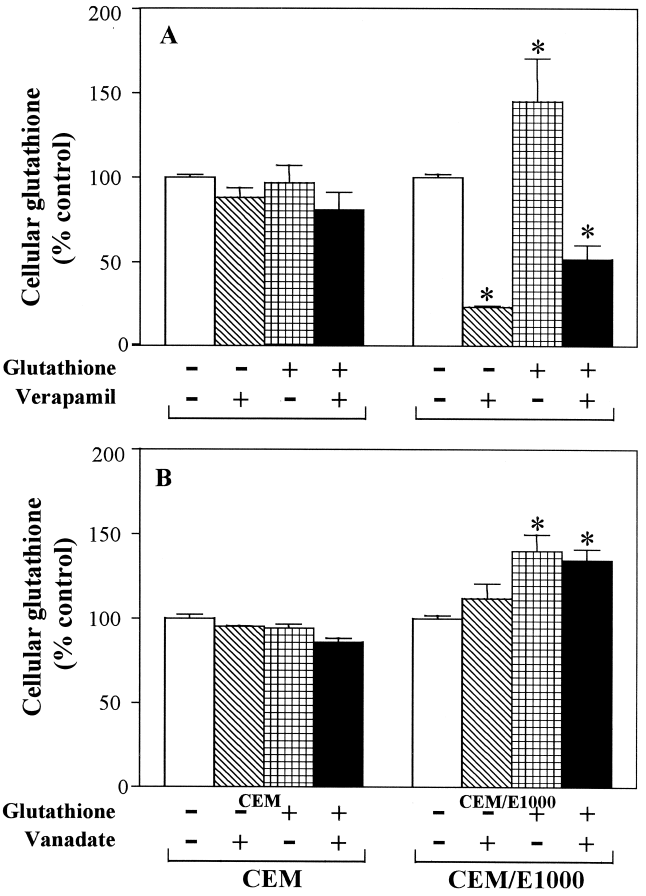

Fig. 5. Effect of extracellular glutathione on glutathione transport. (A) Cells were incubated in the absence or presence of 25 mM glutathione for 2 hr. Verapamil was added for 1 hr (as indicated) after which the cellular glutathione was determined. (B) Cells were incubated in the absence or presence of 25 mM glutathione with or without vanadate (as indicated) after which the cellular glutathione was determined. Results are presented as % control untreated CEM cells; error bars are the standard deviation of duplicate samples; * indicates a significant difference ($P < 0.05$) from the untreated CEM cells using the Student's *t*-test.

ATP hydrolysis, but MRP1 may be passively involved (Fig. 5B).

3.2. Relationship between glutathione depletion and MDR

To determine if the efflux of glutathione affects daunorubicin accumulation or cytotoxicity, daunorubicin treatment of cells in the presence of verapamil (10 μM) was compared with daunorubicin treatment of cells pretreated with verapamil for 1 hr, and the verapamil removed before the addition of daunorubicin. Fig. 6 shows that while daunorubicin accumulation was significantly increased in the presence of verapamil, there was little effect on drug accumulation in cells pretreated with verapamil. This was consistent with the rapid recovery of cellular glutathione on removal of verapamil (Fig. 1). This increase in intracellular drug resulted in increased cytotoxicity (Fig. 6B). Thus, the level of cellular glutathione is inversely related to the level of daunorubicin accumulation and proportional to daunorubicin cytotoxicity.

3.3. Effect of MRP1 inhibitors on cellular glutathione

Table 1 summarises the effect of inhibitors of MRP1 on cellular glutathione and daunorubicin resistance. Treatment for 1 hr with non-cytotoxic doses of the different inhibitors all produced a decrease in cellular glutathione, while having little effect on the glutathione levels in the parental CEM cells. Genistein reduced cellular glutathione to 24%, while MK571 and indomethacin reduced cellular glutathione to 69% and 51% respectively. All of the modulators had a similar effect on daunorubicin cytotoxicity, with the CEM/E1000, which were 25-fold resistant to daunorubicin, sensitised 2–4 fold, suggesting that sensitisation was due to changes in glutathione (Table 1).

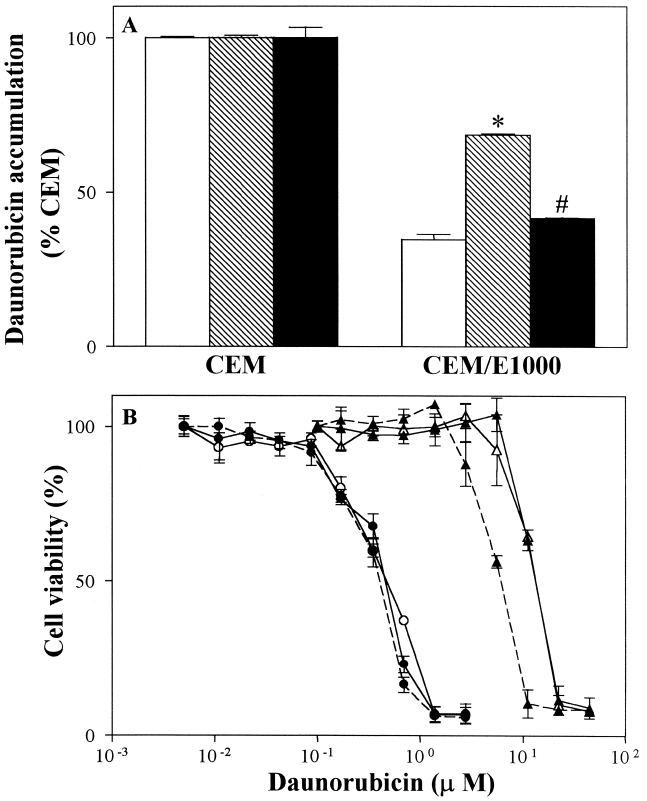

Fig. 6. Correlation of daunorubicin accumulation and cytotoxicity with cellular glutathione. (A) Daunorubicin accumulation. Cells were incubated for 1 hr with 1 μM daunorubicin in the absence of verapamil (open bar), the presence of verapamil (hatched bar), or cells pretreated for 1 hr with verapamil, after which cell-associated daunorubicin was determined by flow cytometry. Results are presented as % control untreated CEM cells; error bars are the standard deviation of duplicate samples; * indicates a significant increase ($P < 0.05$) in DNR accumulation compared to untreated CEM/E1000 cells; # indicates a significant decrease in daunorubicin accumulation in the CEM/E1000 cells pretreated with verapamil compared to cells incubated with daunorubicin in the presence of verapamil. (B) Daunorubicin cytotoxicity. CEM (●) and CEM/E1000 (▲; △) cells were incubated with daunorubicin in the absence (———) or presence (– – – –) of 10 μM verapamil for 1 hr, or cells were pretreated with 10 μM verapamil for 1 hr before incubation with DNR in the absence of verapamil (CEM, ❍; CEM/E1000, △). Following drug treatment, cells were resuspended in fresh culture medium and incubated for 4 days, after which cell viability was determined as described in Methods. Points are the means of triplicate wells; error bars are the standard deviation.

Table 2
Effect of drugs on cellular glutathione

Modulator	Concentration	Glutathione (% Control)	
		CEM	CEM/E1000
Daunorubicin	—	100 ± 9.7	100 ± 6.1
	1 μM	106.3 ± 13.3	104.2 ± 5.9
Vincristine	—	100 ± 6.1	100 ± 5.0
	50 μM	97.5 ± 1.0	80.2 ± 1.5*
	200 μM	104.2 ± 4.4	77.9 ± 0.6*
Etoposide	—	100 ± 11.3	100 ± 7.0
	50 μM	91.1 ± 2.1	91.1 ± 2.0
	200 μM	95.4 ± 2.3	77.1 ± 0.7*

Glutathione levels are calculated % control, and are the means of ≥two independent experiments.

* Indicates a significant decrease in cellular glutathione ($P < 0.05$) compared to untreated cells using the Student's *t*-test.

Compared to these MRP1 inhibitors, treatment of the CEM/E1000 cells with drugs was less effective at reducing glutathione (Table 2). Vincristine at the same molar concentration (50 μM) reduced glutathione to 80%, while etoposide had no effect on cellular glutathione at this concentration, although a higher concentration of etoposide (200 μM) caused a reduction to 80% in cellular glutathione. Daunorubicin (1 μM) had no effect on cellular glutathione, while higher doses (5 μM) were toxic to both cell lines.

4. Discussion

From this comparative study of the CEM and CEM/E1000 cells, we conclude that in intact cells, MRP1 actively transports glutathione in the presence of several reversing agents including verapamil, nifedipine, indomethacin, and genistein (Table 1). The evidence supporting this is that verapamil treatment caused a rapid and dose-dependent depletion of cellular glutathione (Figs. 1 and 2). This verapamil-dependent export occurred against a glutathione concentration gradient (Fig. 5) and was energy-dependent, as demonstrated by vanadate inhibition of the glutathione export (Fig. 4C).

Table 1
Effect of modulators of drug transport on cellular glutathione and drug resistance

Modulator	Glutathione (% Control)		IC_{50} (daunorubicin)	
	CEM	CEM/E1000	CEM	CEM/E1000
Control	100 ± 2.9	100 ± 2.9	0.08 ± 0.01	2.0 ± 0.7
Nifedipine 20 μM	96.4 ± 2.4	54.1 ± 1.2*	0.07 ± 0.01	0.7 ± 0.01** (3.0)[a]
Diltiazem 70 μM	89.0 ± 9.6	26.7 ± 4.5*	0.07 ± 0.01	0.4 ± 0.1** (4.6)
MK571 50 μM	97.4 ± 0.8	69.0 ± 9.1*	0.10 ± 0.03	0.9 ± 0.2** (2.4)
Indomethacin 50 μM	99.4 ± 4.4	51.4 ± 12*	0.05 ± 0.01	0.4 ± 0.07** (5.0)
Genistein 50 μM	93.4 ± 2.2*	23.7 ± 0.5*	0.07 ± 0.01	0.5 ± 0.1** (4.4)

Results are presented as the mean of two independent experiments.

[a] Fold reversal in daunorubicin resistance.

* Indicates a significant decrease in cellular glutathione ($P < 0.05$).

** Indicates a significant fold reversal of drug resistance ($P < 0.05$).

That this verapamil-dependent glutathione export is via MRP1 is supported by the following evidence. First, glutathione export correlated with MRP1 expression. Verapamil-dependent glutathione efflux was minimal in both the CEM cells and the CEM/E50 subline with basal MRP1 expression, and was progressively increased with increasing MRP1 expression in the CEM/E100 and CEM/E1000 sublines (Fig. 3). Second, glutathione export was not via other known glutathione transporters. *P*-glycoprotein expression was not detectable in the CEM, CEM/E100, or CEM/E1000 cells [14], and BSP and methionine, inhibitors of glutathione transport from the canalicular or sinusoidal surfaces of hepatocytes [16], did not inhibit verapamil-dependent glutathione export. These compounds also inhibit the glutathione transport from leukemia cells associated with induction of apoptosis by anti-fas [18] or drug [19], dissociating MRP1 from these cellular activities. While other studies have implicated MRP1 in glutathione transport, it is usually suggested that glutathione transport is associated with the transport of xenobiotics [20]. However, this does not appear to be the case in the CEM/E1000 subline, where verapamil stimulates glutathione export in the absence of verapamil transport since the CEM cells and the CEM/E1000 subline have a similar sensitivity to verapamil (unpublished results), suggesting that verapamil is not transported by MRP1. Loe and coworkers [12] have also reported that verapamil is not a substrate for MRP1 transport.

Increased MRP1 expression in the CEM/E1000 cells was not only associated with the verapamil-stimulated efflux of glutathione, but was also associated with an increase in the uptake of glutathione (Fig. 5). This uptake was not inhibited by vanadate, and uptake was only evident at high extracellular concentrations of glutathione (25 mM), suggesting that MRP1 is involved in the facilitated diffusion of glutathione or it may act as a pore for glutathione. It is conceivable that such a pore could also facilitate the passive movement of glutathione out of the cell, as reported for the closely related CFTR [21]. The functional similarity between these two proteins is confirmed by the demonstration that high-affinity substrates of MRP1 also block chloride transport by CFTR [22].

It therefore appears that there are two mechanisms of transport of glutathione by MRP1: the verapamil-dependent active transport of glutathione and the passive permeability of glutathione. This is consistent with reported changes in cellular glutathione with MRP1 expression. The decreased glutathione export in MRP1 knockout cells relative to the wild-type cells [20] would represent loss of the passive, concentration-dependent transport of glutathione, whereas the variable glutathione decreases reported in the presence of drugs or modulators of drug transport [23] would reflect verapamil-stimulated active transport. Both active and passive transport of glutathione by MRP1 is also consistent with studies using inside-out membrane vesicles. Drug uptake into MRP1-containing membrane vesicles was rapid and saturable and dependent on the presence of glutathione, but glutathione accumulation was slow and independent of drug [12], suggesting two different functions of glutathione in MRP1-mediated transport. Some of the confusing transport data from isolated membrane vesicles may also be explained by the dual transport role of MRP1, as this would complicate the interpretation of any stoichiometric relationship between glutathione and drug transport. The energy-independent transport of glutathione may explain this lack of correlation between drug and glutathione transport. This is further supported by the energy-independent transport of glutathione by the closely related protein MRP2 [24,25].

The finding that many inhibitors of MRP1-mediated drug transport are able to deplete cellular glutathione (Table 1) suggests that sensitisation to drug is due to decreased glutathione, with the level of cellular glutathione regulating daunorubicin accumulation and cytotoxicity (Fig. 6). These results are consistent with the proposal that MRP1 has at least two binding sites, one with affinity for drugs and the second with affinity for glutathione [26], and glutathione is required for co-transport with drug. However, the fact that high drug concentrations did not cause large changes in glutathione while low doses of modulators caused large changes in cellular glutathione (Tables 1 and 2) argues against the co-transport of drug and glutathione. It may equally be argued that increased daunorubicin accumulation in the presence of verapamil is a result of verapamil blocking drug efflux, and glutathione causes conformational changes required for drug efflux rather than for co-transport. As the nucleotide binding domains of MRP1, unlike those of P-glycoprotein, are functionally unequal [27], each domain may potentially be involved in the transport of different compounds, circumventing the requirement for co-transport of drug and glutathione.

While the transport of glutathione needs to be confirmed in other MRP1-overexpressing cells, the high, stable expression of MRP1 in the CEM/E1000 cells in the absence of drug allows for clear determination of the cellular glutathione efflux associated with verapamil treatment. The finding that many inhibitors of MRP1-mediated drug transport are able to deplete cellular glutathione (Table 1) and MRP1 mediates both active and passive transport of glutathione suggests that MRP1 may be important in determining the cellular response to xenobiotics through its influence on cellular glutathione. We propose that the role of MRP1 is to regulate intracellular glutathione by its ability to transport glutathione.

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