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The Relationship Between Modulation of MDR and Glutathione in MRP-Overexpressing Human Leukemia Cells

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ABSTRACT. Multidrug resistance-associated protein (MRP) causes multidrug resistance (MDR) involving the anthracyclines and epipodophyllotoxins. Many studies show modulation of anthracycline levels and cytotoxicity in MRP-overexpressing cells, but there is limited data on the modulation of etoposide levels and cytotoxicity in MRP-overexpressing or in P-glycoprotein-expressing cells. Etoposide accumulation was 50% reduced in both the CEM/E1000 MRP-overexpressing subline and the CEM/VLB_{100} P-glycoprotein-expressing subline compared to the parental CEM cells, correlating with similar resistance to etoposide (200-fold) of the two sublines. For the CEM/VLB_{100} subline, the P-glycoprotein inhibitor SDZ PSC 833, but not verapamil, was able to increase etoposide accumulation and cytotoxicity. For the CEM/E1000 subline, neither SDZ PSC 833 nor verapamil had any effect on etoposide accumulation. However, verapamil caused a 4-fold sensitization to etoposide in this subline, along with an 80% decrease in cellular glutathione ($P < 0.05$). Buthionine sulfoximine (BSO), which depletes glutathione, also caused a 2.5-fold sensitization to etoposide with no effect on accumulation in the CEM/E1000 subline. In contrast, SDZ PSC 833 was able to increase daunorubicin accumulation in the CEM/E1000 subline ($P < 0.05$), but had no effect on daunorubicin cytotoxicity, or cellular glutathione. These results show that modulation of etoposide cytotoxicity in MRP-overexpressing cells may be through changes in glutathione metabolism rather than changes in accumulation and confirm that changes in drug accumulation are not related to drug resistance in MRP-overexpressing cells. BIOCHEM PHARMACOL **55**;8:1283–1289, 1998.

KEY WORDS. multidrug resistance; multidrug resistance-associated protein; P-glycoprotein; glutathione; verapamil; SDZ PSC 833

Despite improvement in chemotherapy strategies, the development of MDR§ still remains the major problem in the use of chemotherapy to cure cancer. MDR is characterized by cross-resistance to a wide variety of functionally distinct, lipophilic natural product drugs, and is associated with decreased cellular drug accumulation, and with the expression of a 170-kDa plasma membrane glycoprotein, P-glycoprotein [1]. P-glycoprotein is a member of the ATP-binding cassette superfamily of proteins and is proposed to confer MDR by acting as a drug efflux pump.

More recently expression of another member of the ATP-binding cassette superfamily, the MRP, has also been shown to confer MDR to natural product drugs with the notable exception of paclitaxel [2]. It is thought that MRP may mediate multidrug resistance by effluxing cytotoxic drugs in a similar manner to P-glycoprotein. However, characterization of the mechanism of action of MRP has remained elusive as variability in drug accumulation, in drug transport and in intracellular drug distribution have been reported in different cell lines and in MRP-transfected cells. Furthermore, while numerous "reversing agents" or "chemosensitizers," such as verapamil and SDZ PSC 833, are capable of inhibiting the efflux activity of P-glycoprotein and reversing cellular resistance to cytotoxic agents, their effect on MRP is variable and at best only modest [3].

Studies in MRP-overexpressing cells indicate that glutathione metabolism is involved in MRP-mediated MDR [4], and it has even been suggested that MRP is the glutathione S-conjugate (GS-X) pump [5]. Although membrane vesicles from cells overexpressing MRP have shown that MRP may function in the ATP-dependent transport of many glutathione conjugates including leukotriene C_4 [6] and also in the transport of the anticancer drug conjugate glucuronosyl-etoposide, there is no evidence for the transport of unmodified cytotoxic drugs by MRP [7]. However Loe and coworkers [8] have reported stimulation of vinc-

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§ *Abbreviations:* MDR, multidrug resistance; MRP, multidrug resistance-associated protein; BSO, buthionine sulfoximine; MTT, 3,-[4,5-dimethylthiazo-2-yl]-2,5-diphenyltetrazolium bromide.

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ristine transport in vesicles from MRP-transfected HeLa cells in the presence of glutathione.

The role of glutathione metabolism in MRP-mediated drug transport is, therefore, not clear. While membrane vesicle transport studies have identified potential substrates for MRP-mediated transport, they lack the cellular topology and associated glutathione metabolism of cells which are undoubtedly important factors for drug transport. The CEM/E1000 MRP-overexpressing subline of the CCRF-CEM cells [4], which has similar resistance to the anthracycline daunorubicin as the well characterized P-glycoprotein-expressing CCRF-CEM/VLB_{100} subline [9] is, therefore, an ideal cellular model in which to study the relationship between MRP, glutathione, and drug resistance compared with that associated with P-glycoprotein expression. Since MRP-overexpressing cells are highly resistant to etoposide as well as to the anthracyclines, the effects of the well characterized modulators of P-glycoprotein on verapamil and SDZ PSC 833 etoposide resistance and cellular glutathione were investigated along with the effects of BSO, an inhibitor of glutathione synthesis. These results were compared with the effects of these modulators on daunorubicin accumulation and cytotoxicity in the MDR sublines.

MATERIALS AND METHODS

Drugs

Daunorubicin and etoposide were from David Bull Laboratories. Etoposide (powder) was obtained from Sigma Chemical Co. and was prepared as a stock solution of 20 mg/mL in DMSO.

Cell Lines

The CCRF-CEM (CEM) human leukemia cell line [10] and its epirubicin selected CEM/E1000 [4] and vinblastine selected CEM/VLB_{100} [9] multidrug-resistant sublines were grown in RPMI 1640 medium (Trace) supplemented with 10% fetal calf serum (Trace), 20 mM of HEPES (Trace), and 10 mM of $NaHCO_3$ at 37° in a humidified atmosphere with 5% CO_2. The sublines were maintained in the absence of drug, and resistance was stabilized by drug treatment for 3 days every 6 weeks (CEM/E1000 subline with 1000 ng/mL of epirubicin; CEM/VLB_{100} with 100 ng/mL of vinblastine). Exponentially growing cells were used for all experiments. All cultures were free of mycoplasma.

Cytotoxicity Assays

Sensitivity to drugs was determined in triplicate using the MTT assay as previously described [11]. Cells were seeded at a density of 4×10^4 cells per well, and were incubated at 37° in a humidified atmosphere with 5% CO_2 in the presence of drug for 4 days prior to the addition of 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 cells in the absence and presence of 10 μM verapamil (Sigma), 1 μM of SDZ PSC 833 (a gift from Sandoz, Sydney, Australia) or 10 μM of BSO (Sigma) in a cytotoxicity assay. The IC_{50} was determined and fold reversal was calculated by dividing the IC_{50} for cells incubated with drug alone by the IC_{50} for cells incubated with the drug in the presence of reversing agent.

Etoposide Accumulation

Cells were harvested by centrifugation (800 *g* for 5 min) and resuspended in fresh culture medium at a final concentration of 2.0×10^6 cells/mL. For treatment with verapamil (10 μM or 50 μM) or SDZ PSC 833 (2 μM or 10 μM) cells were incubated for 5 min at 37° before the addition of 1 μM or 20 μM of [^{3}H]etoposide (Moraveck Biochemicals) for 60 min. For BSO treatment, cells were incubated for 1 hr with 10 μM or 50 μM of BSO before addition of etoposide for 1 hr. The incubation was terminated by the addition of 1-mL cells to 3 mL of ice-cold phosphate buffered saline, pH 7.2 (PBS). Samples were centrifuged at 4°, washed once with ice-cold PBS, lysed in 100 μL of 1% SDS, and radioactivity was determined.

Daunorubicin Accumulation

Cell-associated daunorubicin was measured by flow cytometry using 5×10^5 cells in duplicate as previously described [4].

Glutathione Determination

Total intracellular glutathione was determined using a modification of the colorimetric method of Suzakake *et al.* [12]. Exponentially growing cells (10^7) were harvested by centrifugation (800 *g* for 5 min) and resuspended in 20 mL of medium. Cells were incubated in the absence (control) and presence of verapamil (10 μM or 50 μM) or SDZ PSC 833 (2 μM or 10 μM) for 60 min at 37° with 5% CO_2. For treatment with BSO, cells were incubated for 2 hr with 10 μM or 50 μM of BSO. Cells were washed twice with PBS, lysed in 600 μL of water and vortexed. Fifty microliters of 30% sulfosalicyclic acid was added to 450 μL of the lysate and samples vortexed. After 15 min on ice, protein-free supernatants were collected by centrifugation (12000 *g* for 2 min). Glutathione was determined using a reaction mixture containing 20 μL of lysate or standard (15 μg/mL in 3% sulfosalicyclic acid), 20 μL of triethanolamine buffer, pH 8.0 (1 M), 120 μl of NADPH (0.3 mM) and 20 μl of 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) of glutathione reductase. Absorbency was monitored in a multiwell microplate reader (BioRad, Sydney, Australia) at 405 nm for 6 min and glutathione levels

calculated. All reagents were from Sigma. All glutathione concentrations were the mean of at least triplicate determinations and all experiments were repeated at least twice.

Western Blot Analysis of Topoisomerase II

Cell lysates were sheered using a 23-gauge needle and subjected to electrophoresis on a Tris-glycine 4–15% gradient gel (BioRad). Blots were developed using a polyclonal antibody (clone MAC 5.3) which recognizes both topoisomerase II α and β, kindly provided by Dr. W. Beck, University of Illinois. Membranes were re-developed using an anti-β-actin antibody (Sigma) and topoisomerase II quantitated using the Microtek ScanMaker III and the Molecular Analyst program (BioRad).

Statistical Analysis

All experiments were performed at least twice and significance was determined using Student's *t*–test.

RESULTS

Characterization of Etoposide Resistance

Etoposide is a lipophilic drug, the pharmacological preparation being administered in a solvent containing polyethylene glycol 300 and polysorbate 80. Because similar solvents have been reported to sensitize P-glycoprotein-expressing cells to the cytotoxic effects of drugs [13], the cytotoxicity of this preparation was compared to that of etoposide dissolved in DMSO in both the MRP-overexpressing and the P-glycoprotein-expressing MDR sublines. Figure 1 shows that the solvent had no effect on etoposide cytotoxicity in the parental CEM cells. However, the IC_{50} of the pharmacological etoposide in the P-glycoprotein-expressing CEM/VLB_{100} cells was 11.9 ± 2.5 μM ($N = 7$), 8-fold more sensitive than etoposide in DMSO (IC_{50} of etoposide in DMSO 96.1 ± 17.2 μM, $N = 7$). Further, the MRP-overexpressing CEM/E1000 cells were 3.5-fold sensitized by the pharmacological preparation (IC_{50} 37.9 ± 6.5 μM, $N = 8$; IC_{50} of etoposide in DMSO 131.8 ± 35.0 μM, $N = 8$). This shows that both sublines were sensitized to etoposide by the pharmacological preparation and both sublines exhibit approximately 200-fold resistance to etoposide in DMSO. Etoposide in DMSO was used in all experiments to eliminate any solvent effect, at concentrations of DMSO which were not toxic to the cells.

Western blot analysis showed that resistance to etoposide in the CEM/E1000 and CEM/VLB_{100} sublines was not due to changes in topoisomerase II (data not shown). Additionally there was no significant difference in the levels of the topoisomerase II α and β isoforms of the drug resistant sublines compared to those of the parental CEM cells.

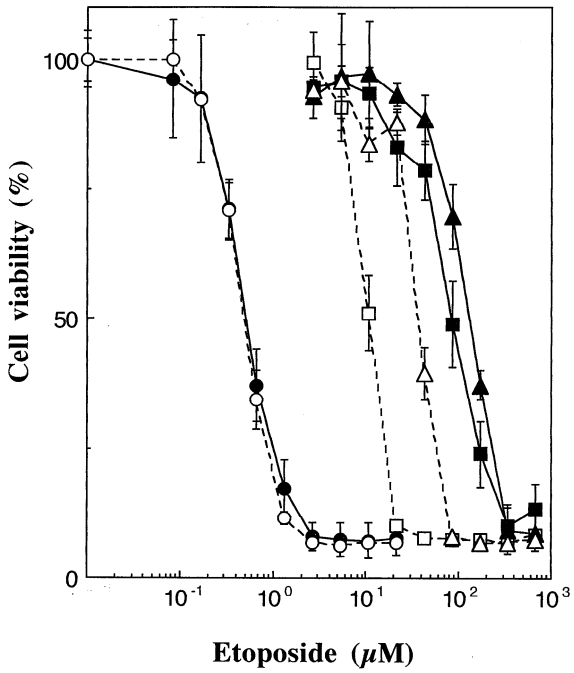

FIG. 1. Effect of solvent on etoposide cytotoxicity. The cytotoxicity of etoposide was determined in the CEM cells (○, ●), the CEM/E1000 subline (△, ▲) and the CEM/VLB_{100} subline (□, ■) with a pharmacological preparation of etoposide (open symbols and broken lines) or DMSO (solid symbols and lines). The assay presented is representative of >8 experiments. Points are means of triplicate determinations and the error bars represent the standard deviation.

Modulation of Etoposide Accumulation and Resistance

Accumulation of etoposide was decreased to the same level in both the MRP-overexpressing and the P-glycoprotein-expressing MDR sublines compared to the accumulation in the parental CEM cells (approximately 45% of the CEM accumulation; Fig. 2B). This accumulation did not change over 4 hr incubation (not shown). There was no effect of verapamil on etoposide accumulation (Fig. 2B). However, Fig. 2A shows that verapamil sensitized the CEM/E1000 subline 2.5-fold ($P < 0.05$; $N = 3$) to etoposide but had no effect on etoposide cytotoxicity in the CEM/VLB_{100} subline. Treatment with verapamil (10 μM, Fig. 2C) was also associated with an 80% decrease in cellular glutathione in the CEM/E1000 cells ($P < 0.01$, $N = 2$), but had no effect on the other cell lines. Similar results were obtained with 50 μM verapamil ($P < 0.01$, $N = 2$; not shown).

In contrast to verapamil, SDZ PSC 833 was able to significantly increase etoposide accumulation ($P < 0.05$, $N = 3$) in the P-glycoprotein-expressing CEM/VLB_{100} subline (Fig. 3B) that correlated with decreased resistance to etoposide in the presence of SDZ PSC 833 (40-fold sensitization, $P < 0.01$, $N = 2$; Fig. 3A). SDZ PSC 833 had no effect on etoposide resistance or accumulation in the CEM/E1000 MRP-overexpressing subline (Fig. 3A, B) or cellular glutathione (Fig. 3C).

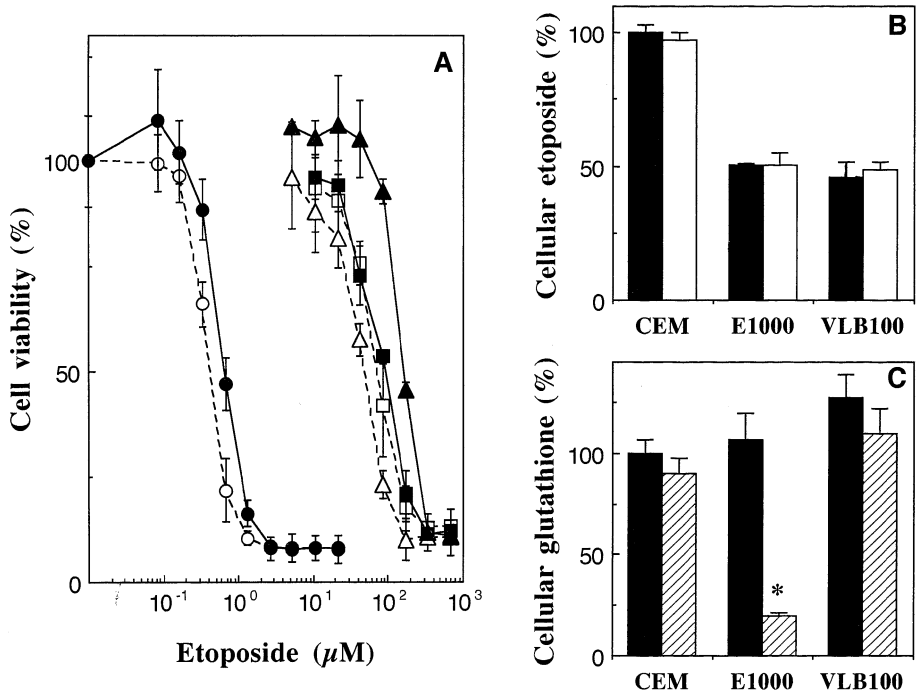

FIG. 2. Effect of verapamil treatment on etoposide cytotoxicity and accumulation, and on cellular glutathione. A) The cytotoxicity of etoposide was determined in the absence (solid lines) or presence (broken lines) of 10 μM of verapamil in the parental CEM cells (○, ●) and the MDR sublines CEM/E1000 (△, ▲) and CEM/VLB_{100} (□, ■). The experiment was repeated at least three times and a representative result is shown. Points are the mean of triplicate determinations and the error bars represent the standard deviation. B) Cells were incubated in duplicate without (solid bars) or with 10 μM of verapamil (open bars) for 60 min with 1 μM of [^{3}H]etoposide, and the accumulation of etoposide determined relative to the untreated CEM cells (100%). Error bars represent the standard deviation ($N = 3$). C) Cells were incubated in duplicate without (solid bars) or with 10 μM of verapamil (hatched bars) for 60 min and the glutathione levels determined relative to the untreated CEM cells (100%). Error bars represent the standard deviation of at least triplicate glutathione determinations. *$P < 0.01$ compared with untreated cells ($N = 2$).

Effect of BSO on Etoposide Accumulation and Cytotoxicity

Figure 4C shows that while treatment with BSO significantly depletes glutathione in the parental cells and the drug resistant sublines ($P < 0.05$, $N = 3$ for all cells), this was not associated with any changes in etoposide accumulation (Fig. 4B). Treatment with BSO (Fig. 4A) sensitized the CEM/E1000 subline to etoposide (3.1-fold; $P < 0.02$, $N = 3$) but had no significant effect on the parental CEM cells (1.9-fold; $N = 3$) and the CEM/VLB_{100} subline (1.7-fold; $N = 3$).

Modulation of Daunorubicin Accumulation and Resistance

Figure 5 shows that daunorubicin cytotoxicity was similar in both the MRP-overexpressing CEM/E1000 and the P-glycoprotein-expressing CEM/VLB_{100} sublines (IC_{50} 1.6 ± 0.5 μM and 1.9 ± 0.5 μM respectively; $N = 11$), approximately 50-fold resistant relative to the CEM cells (IC_{50} 0.03 ± 0.01 μM; $N = 21$). This resistance was associated with decreased daunorubicin accumulation in both MDR sublines, which was restored in the presence of SDZ PSC 833 (Fig. 5B; $P < 0.05$, $N = 3$). However, SDZ PSC 833 had no effect on daunorubicin cytotoxicity in the MRP-overexpressing CEM/E1000 subline, in contrast to its effect on daunorubicin cytotoxicity in the CEM/VLB_{100} cells which showed a 21-fold sensitization (Fig. 5A; $P < 0.05$, $N = 3$).

DISCUSSION

Both the MRP-overexpressing CEM/E1000 and the P-glycoprotein-expressing CEM/VLB_{100} were similarly resistant to etoposide (Fig. 1). The effect of the solvent for the preparation of etoposide on cytotoxicity suggests that the variability reported on etoposide resistance in MDR cells may be related to the solvent used in the preparation of the drug. The lack of effect of the solvent on the parental CEM cells indicates this effect was specific for both MRP-overexpressing and P-glycoprotein-expressing cells. A similar effect has been reported for cremophore EL on P-glycoprotein-expressing cells [13].

Etoposide accumulation was not modulated by the classical P-glycoprotein modulator verapamil (Fig. 2B) or by SDZ PSC 833 (Fig. 3B) in the MRP-overexpressing CEM/E1000 cells, suggesting that if decreased etoposide accumulation, due to drug efflux, is mediating MDR in these cells, then etoposide has a higher affinity for MRP than verapamil or SDZ PSC 833. Consequently, the modulation of

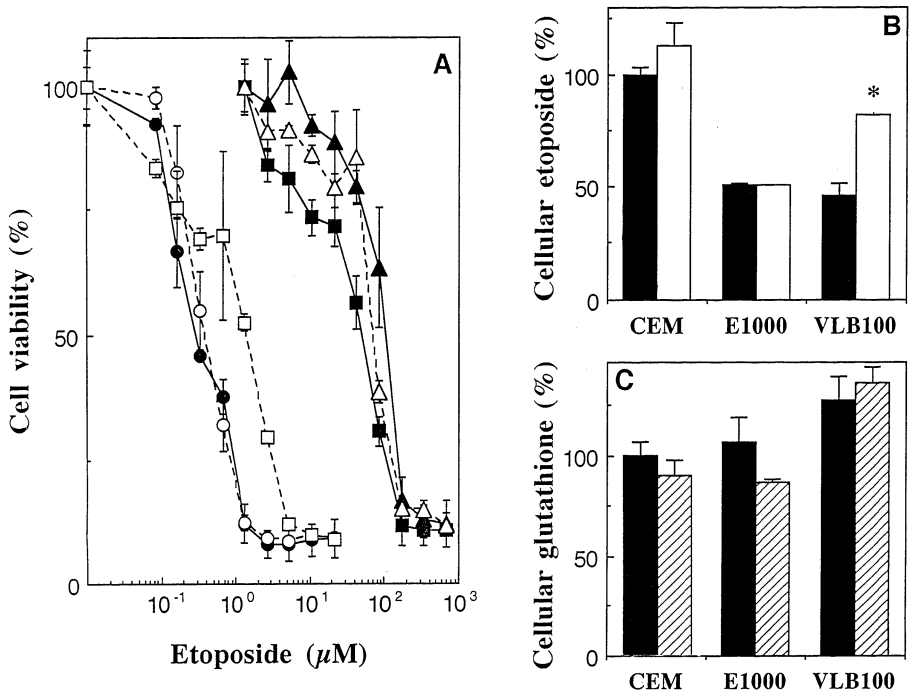

FIG. 3. Effect of SDZ PSC 833 treatment on etoposide cytotoxicity and accumulation, and on cellular glutathione. A) The cytotoxicity of etoposide was determined in the absence (solid lines) or presence (broken lines) of 1 μM of SDZ PSC 833 in the parental CEM cells (○, ●), and the MDR sublines CEM/E1000 (△, ▲) and CEM/VLB$_{100}$ (□, ■). The experiment was repeated twice and a representative result is shown. Points are the mean of triplicate determinations and the error bars represent the standard deviation. B) Cells were incubated in duplicate without (solid bars) or with 2 μM of SDZ PSC 833 (open bars) for 60 min with 1 μM of [^{3}H]etoposide, and the accumulation of etoposide determined relative to the untreated CEM cells (100%). Error bars represent the standard deviation. *$P < 0.05$ compared with untreated cells ($N = 3$). C) Cells were incubated in duplicate without (solid bars) or with 2 μM of SDZ PSC 833 (hatched bars) for 60 min and the glutathione levels determined relative to the untreated CEM cells (100%). Error bars represent the standard deviation of at least triplicate glutathione determinations ($N = 2$).

resistance to etoposide by verapamil in the CEM/E1000 cells (Fig. 2A) is not attributable to the competitive inhibition of MRP by verapamil. Rather, this reversal is more likely associated with the depletion of glutathione by verapamil (Fig. 2C).

It has been proposed that glutathione may be required for the maintenance of MRP activity from experiments showing transport of vincristine into MRP-containing membrane vesicles only in the presence of glutathione [8]. This is consistent with the demonstration that glutathione depletion by treatment with BSO was associated with reversal of daunorubicin resistance in MRP-overexpressing cells [4]. BSO treatment was also able to increase the daunorubicin accumulation in the MRP-overexpressing cells. For etoposide, however, sensitization by treatment with BSO was only modest (Fig. 4A) and was not associated with any changes in etoposide accumulation (Fig. 4B), although glutathione levels were decreased over 80% in the CEM/E1000 cells by this treatment (Fig. 4C). It is possible that in the case of etoposide, glutathione itself is not associated with MRP-mediated drug transport. Resistance to etoposide in MRP-overexpressing cells may be predominantly due to conjugation to glucuronide, consistent with reports that glucuronosyl-etoposide is transported into MRP-containing vesicles [7].

How verapamil depletes glutathione specifically in the MRP-overexpressing resistant subline is unknown, but as verapamil is a calcium channel antagonist, it may deplete cellular glutathione indirectly by altering other cellular processes required for glutathione synthesis. It would be interesting to determine whether verapamil has a similar effect on other MRP-overexpressing MDR cells, since depletion of glutathione by verapamil may account for the variation of modulation by verapamil of MRP-mediated multidrug resistance. A recent report of decreased cellular glutathione by the nucleoside transport inhibitor dipyridamole in MRP-overexpressing lung cancer cells suggests that such a response may be common for modulators of MRP-mediated MDR [14].

It is interesting to note that verapamil had no effect on etoposide accumulation (Fig. 2B) or cytotoxicity (Fig. 2A) in the CEM/VLB$_{100}$ subline, although SDZ PSC 833 was able to increase etoposide accumulation (Fig. 3B) and restore etoposide sensitivity in the P-glycoprotein-expressing cells (Fig. 3A). This makes etoposide unique in the group of P-glycoprotein-effluxed MDR drugs, since other drugs (*Vinca* alkaloids, anthracyclines) show sensitivity to both verapamil and SDZ PSC 833. This result may account for proposals that etoposide is not a classic MDR drug, and may not be effluxed by P-glycoprotein [15, 16].

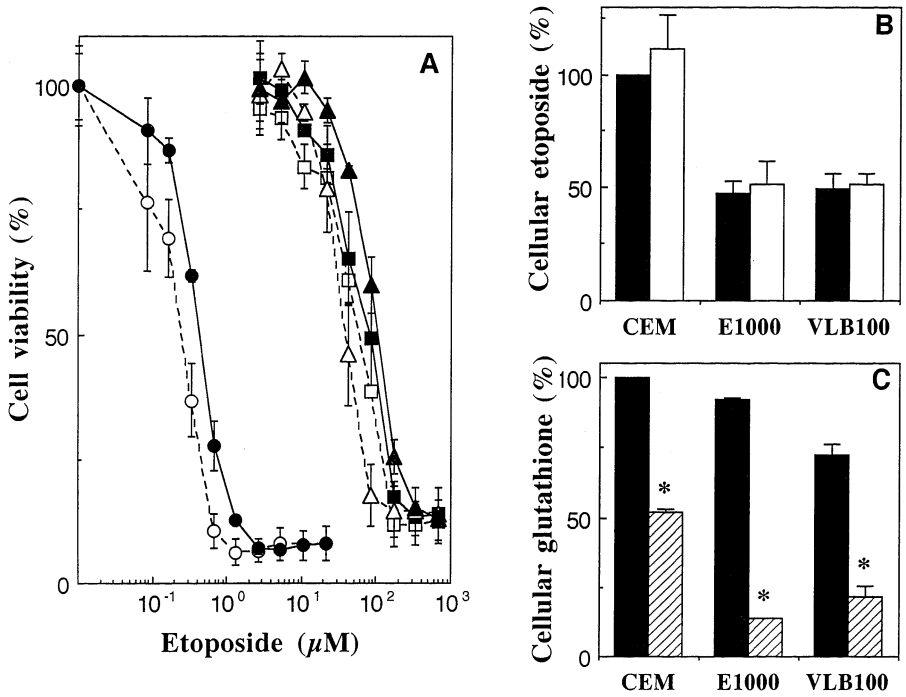

FIG. 4. Effect of BSO treatment on etoposide cytotoxicity and accumulation, and on cellular glutathione. A) The cytotoxicity of etoposide was determined in the absence (solid lines) or presence (broken lines) of 10 μM of BSO in the parental CEM cells (○, ●), and the MDR sublines CEM/E1000 (△, ▲) and CEM/VLB_{100} (□, ■). The experiment was repeated at least three times and a representative result is shown. Points are the mean of triplicate determinations and the error bars represent the standard deviation. B) Cells were incubated in duplicate without (solid bars) or with 10 μM of BSO (open bars) for 2 hr before addition of 20 μM of [^{3}H]etoposide for 1 hr, and the accumulation of etoposide determined relative to the untreated CEM cells (100%). Error bars represent the standard deviation ($N = 4$). C) Cells were incubated in duplicate without (solid bars) or with 10 μM of BSO (hatched bars) for 2 hr and the glutathione levels determined relative to the untreated CEM cells (100%). Error bars represent the standard deviation of at least triplicate glutathione determinations. *$P < 0.05$ compared with untreated cells ($N = 3$).

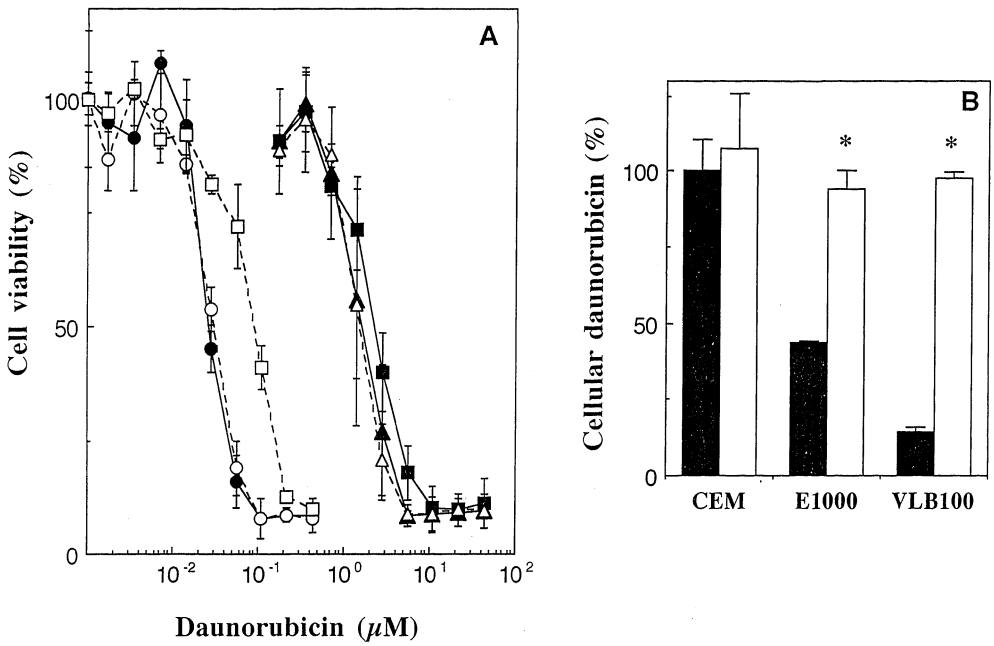

FIG. 5. Effect of SDZ PSC 833 on daunorubicin cytotoxicity and accumulation. A) The cytotoxicity of daunorubicin was determined in the absence (solid lines) or presence (broken lines) of 1 μM of SDZ PSC 833 in the parental CEM cells (○, ●), and the MDR sublines CEM/E1000 (△, ▲) and CEM/VLB_{100} (□, ■). The experiment was repeated at least three times and a representative result is shown. Points are the mean of triplicate determinations and the error bars represent the standard deviation. B) Cells were incubated in duplicate without (solid bars) or with 2 μM of SDZ PSC 833 (open bars) for 60 min with 1 μM of daunorubicin, and the accumulation of daunorubicin determined relative to the untreated CEM cells (100%). *$P < 0.05$ compared with untreated cells ($N = 3$).

The lack of correlation between drug accumulation and cytotoxicity in MRP-overexpressing cells, as found with etoposide, is also evident for daunorubicin modulation by SDZ PSC 833 (Fig. 5). While SDZ PSC 833 was able to restore daunorubicin accumulation in the CEM/E1000 subline (Fig. 5B), there was no effect on cytotoxicity (Fig. 5A), suggesting that although SDZ PSC 833 was able to inhibit drug efflux, the daunorubicin was still not able to reach its target to cause cytotoxicity, and may be sequestered into intracellular vesicles as has been reported for MRP-overexpressing cells [17]. This would, however, require different forms and/or activities of MRP in the plasma membrane compared to the MRP in intracellular vesicles since the former was inhibited by SDZ PSC 833 while the intracellular MRP was not inhibited. Alternatively, this competitor may not have access to the intracellular MRP.

This lack of relationship between drug accumulation and the effect of modulators was also reported for another anthracycline, idarubicin [18]. The CEM/E1000 cells were 30-fold resistant to idarubicin, but there was no change in drug accumulation compared with the parental CEM cells, suggesting that resistance to idarubicin was not associated with decreased drug accumulation. However, verapamil, SDZ PSC 833 and BSO were all able to increase idarubicin accumulation. This leads to the conclusion that modulation of drug accumulation in MRP-overexpressing cells is not due to competitive inhibition of MRP-mediated drug efflux.

While the function of MRP in mediating MDR is still unclear, it is apparent that in MRP-overexpressing cells, changes in drug accumulation do not necessarily result in increased drug cytotoxicity, suggesting that MRP is not a drug efflux pump like P-glycoprotein. The ability of verapamil to selectively deplete glutathione in the MRP-overexpressing CEM/E1000 subline further implicates glutathione in the modulation of MRP-mediated MDR through a mechanism not simply related to glutathione concentration. Understanding this interaction between glutathione and MRP-mediated drug resistance is of great importance because the lack of correlation between drug accumulation and cytotoxicity will provide many problems for both the clinical trials with modulators/chemosensitizers to reverse multidrug resistance and for implementation of functional assays to detect multidrug resistance in tumor cells.

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