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# Evidence That the Multidrug Resistance Protein (MRP) Functions as a Co-Transporter of Glutathione and Natural Product Toxins[1]

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## Abstract

**The *MRP* (multidrug resistance protein) gene, a member of the ubiquitous superfamily of ATP-binding cassette transporters, is associated with the multidrug resistance of mammalian cells to natural product anticancer agents. We have previously shown that abrogation of MRP expression by gene targeting leads to hypersensitivity to several drugs. In two independently produced MRP double knockout clones, the baseline export of glutathione (GSH) was one-half that of wild-type embryonic stem (ES) cells. The export of GSH from wild-type ES cells, but not from the MRP double knockout clones, increased in the presence of etoposide (VP-16) and sodium arsenite, accompanied by equivalent decreases in intracellular levels of GSH. In the two MRP double knockout clones, the intracellular steady-state concentration of etoposide was twofold greater than that in wild-type cells. Depletion of intracellular GSH by D,L-buthionine sulfoximine increased the intracellular accumulation of radiolabeled etoposide in parental ES cells up to the level present in the two MRP knockout clones but did not change etoposide levels in the MRP knockout clones. These observations provide evidence that: (*a*) MRP exports GSH physiologically, presumably in association with an endogenous compound(s); (*b*) baseline MRP expression protects cells from the toxic effects of xenobiotics by effluxing the xenobiotics and GSH from the intracellular compartment into the extracellular medium by a co-transport mechanism; and (*c*) disruption of the gene encoding MRP abrogates the co-transport of xenobiotics and GSH.**

## Introduction

Resistance of tumor cells to antineoplastic agents is a frequent cause of failure in the chemotherapeutic treatment of disseminated cancers. Resistance to natural product drugs, such as etoposide and doxorubicin, often presents as MDR.[3] To date, two proteins have been shown to be capable of conferring MDR in humans, the 170-kDa P-glycoprotein, encoded by the *MDR1* gene (standard gene symbol, *PGY1*), and the 190-kDa MRP, encoded by the *MRP* gene (1, 2). Both MDR1 and MRP belong to the ATP-binding cassette transporter superfamily (2) and act as energy-dependent efflux pumps, decreasing the intracellular concentration of drugs. An 88% amino acid identity has been shown for human and murine MRPs, and both proteins can induce MDR when introduced into drug-sensitive cells by transfection (3, 4). The *MRP* gene is ubiquitously expressed, with high levels present in a variety of human and murine tissues (2, 3).

MRP has been suggested to be the GS-X pump, an ATP-dependent export pump for multivalent organic anions such as cysteinyl leukotrienes, GSH disulfide, and various other GSH *S*-conjugates (5). This suggestion was based on the finding that in membrane vesicles isolated from MRP-transfected or -overexpressing cell lines, MRP exhibited high-affinity transport of cysteinyl leukotrienes as well as other GSH and glucuronide conjugates but not of GSH alone in its reduced form (6, 7).

To gain an understanding of the physiological mechanism of action of MRP, we have generated sublines of ES cells in which both copies of the *MRP* gene have been disrupted, resulting in total abrogation of MRP expression (8). The abrogation of MRP expression resulted in hypersensitivity to many natural toxins, including the epipodophyllotoxin derivatives VP-16 and VM-26, sodium arsenite, vincristine, and the anthracyclines doxorubicin and daunorubicin, with single MRP knockout cells displaying an intermediate level of sensitivity (8). This pattern of hypersensitivity resembled the pattern of resistance expressed by MRP-transfected cell lines (3, 4). In this paper, we report that MRP co-transports GSH and toxins by relocating them from the intracellular compartment to the extracellular medium.

## Materials and Methods

**Cell Growth Characteristics.** Undifferentiated W9.5 ES cells and MRP knockout clones were maintained in gelatinized tissue culture dishes in DMEM supplemented with 15% heat-inactivated fetal bovine serum, 2 mM glutamine, 0.1 mM β-mercaptoethanol, and 1000 units/ml recombinant leukemia-inhibitory factor (Life Technologies, Inc., Grand Island, NY) at 37°C in an atmosphere of 10% $CO_2$ in air.

**Electroporation and Selection of ES MRP−/−2 Knockout Clone.** The preparation of the gene-targeting construct, electroporation into W9.5 ES cells, and selection and screening of the clones, first by PCR analysis and then by DNA blot analysis, were performed as described previously (8). The second MRP double knockout clone, ES MRP−/−2, was obtained by exposing a single knockout clone different from that used to generate ES MRP−/−1 to high concentrations of G418 for 2 weeks.

**Analysis of Drug Sensitivities.** The hypersensitivity patterns of the knockout cell lines were determined using a tetrazolium salt-based microtiter plate assay as described previously (8). The 96-well plates were inoculated with 3000 cells/well in a volume of 100 μl in complete medium. Twenty-four h later, drugs were added at various concentrations. Control wells were included for each drug that consisted of the respective solvents. Seventy-two h later, 333 μg/ml 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and 25 μM phenazine methosulfate were added, and incubations were continued for 2 h at 37°C. In the presence of the electron-coupling reagent phenazine methosulfate, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium is reduced by dehydrogenase enzymes found in metabolically active cells into a formazan product that is readily soluble in tissue culture medium. The formazan product was quantified by measurement of the absorbance at 490 nm. At least five drug concentrations were used to determine $IC_{50}$ values, and each drug concentration was replicated in six wells for each individual experiment. Hypersensitivity factors were calculated as the ratio of the $IC_{50}$ of wild-type ES cells:$IC_{50}$ of MRP double knockout ES clones.

**Accumulation of [$^3$H]Etoposide.** The intracellular accumulation of radiolabeled etoposide was measured by methodology described earlier (9). The viability of cells after exposure to etoposide was assessed by the exclusion of trypan blue. At the indicated times after the onset of drug treatment, 100 μl of

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[3] The abbreviations used are: MDR, multidrug resistance; MRP, multidrug resistance protein; ES, embryonic stem cells; GST, glutathione *S*-transferase; BSO, D,L-buthionine sulfoximine; GSH, glutathione; GSSG, oxidized GSH.

cell suspension were added to a tube containing 10 ml of ice-cold PBS, and cells were collected by centrifugation. An aliquot of the supernatant was saved for measurement of extracellular radioactivity, and the pellet was resuspended in 60 μl of ice-cold PBS and placed in an oil stop tube consisting of a 400-μl Beckman microfuge tube containing 125 μl of oil (16% Fisher 0121 light paraffin oil and 84% Dow Corning 550 silicon fluid, with a final specific gravity of 1.04 g/ml) layered over 30 μl of 15% trichloroacetic acid and immediately centrifuged for 30 s at 10,000 × *g* using a Beckman model B microfuge. Microfuge tubes were cut through the oil layer, and the lower parts were placed in glass miniscintillation vials. After the addition of 500 μl of distilled water, vials were vortexed vigorously, 5 ml of scintillation mixture were added, and radioactivity therein was measured by scintillation spectrometry. Zero-time binding, measured by adding radiolabeled etoposide to cells prechilled on ice, was always less than 5%. The intracellular volume was calculated for untreated cells and for cells exposed to different concentrations of etoposide using $^3H_2O$ to determine the total water space, and [$^{14}$C]inulin was used to calculate the extracellular space.

**Flow Cytometric Analysis of GSH-bimane Conjugate.** Monochlorobimane (Molecular Probes, Eugene, OR) was dissolved in ethanol. The cellular fluorescence was analyzed using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). Cells were excited at 356 ± 6 nm, and emission was collected through a 460 ± 35 nm bandpass filter. To standardize the assay, the mean fluorescence of Hoechst UV calibration beads (Flow Cytometry Standards Corp., Research Triangle Park, NC) was recorded, and the mean cellular fluorescence was compared to that of the beads. A minimum of 20,000 cells was analyzed for each sample.

**Measurement of GSH Levels.** To measure the export of GSH, 2–3 × $10^6$ cells growing in 6-well plates were washed with HBSS and incubated in 0.5 ml of HBSS at 37°C. At different time intervals, the solution was aspirated, centrifuged at 12,500 × *g* for 30 s, and assayed for GSH according to the recycling method of Tietze (10). To measure GSSG, GSH was derivatized with 2-vinylpyridine before the sample was applied to the recycling reaction. To measure intracellular GSH, cells were detached by incubation with 0.05% trypsin for 2 min at 37°C, centrifuged, and resuspended in 3% (w/v) 5-sulfosalicylic acid in water. After centrifugation, 100-μl aliquots of the supernatant were incubated at 20°C with or without 2 μl of 2-vinylpyridine for 1 h; 18 μl of triethanolamine (1:3, v/v, in $H_2O$) were then added to bring the pH to 7.0–7.5, and GSH was assayed as described above.

## Results and Discussion

**Hypersensitivity Pattern of Two MRP Double Knockout Cell Lines.** We have previously reported the development and characterization of a MRP double knockout clone, designated here as ES MRP−/−1, from MRP single knockout clone 24 (8). To exclude the possibility that random genotypic changes rather than the complete abrogation of MRP expression were responsible for the MRP knockout phenotype, we have developed a second MRP double knockout clone from an independently isolated MRP single knockout clone. The methodology used for the targeted disruption of the MRP locus has been described previously (8). MRP single knockout clone 4 was expanded *in vitro* and exposed to 3 mg/ml G418 for 2 weeks to select for MRP double knockout clones. Six clones selected in high G418 were subjected to DNA blot analyses as described previously (8); five of the clones were found by DNA blot analysis to have undergone correct homologous recombination in both alleles, and one of them, designated clone ES MRP−/−2, was analyzed by Western blotting and reverse transcription-PCR as described previously (8) and was found to not express MRP (data not shown), in a manner analogous to clone ES MRP−/−1. ES MRP−/−2 cells displayed a level of hypersensitivity to the podophyllotoxin derivatives etoposide and teniposide, to the vinca alkaloid vincristine, to the anthracyclines doxorubicin and daunorubicin, and to sodium arsenite, similar to that previously reported for ES MRP−/−1 cells (8), whereas sensitivity to the cytotoxic action of colchicine and 1-β-D-arabinofuranosylcytosine was not altered (Table 1). The fact that two independently isolated MRP double knockout clones display the same pattern of hypersensitivity relative to that of the wild-type cell line demonstrates that the abrogation of MRP expression is uniquely responsible for the observed phenotype. Hypersensitivity of both ES MRP−/−1 and ES MRP−/−2 clones to the cytotoxicity of the heavy metals sodium arsenate and antimony potassium tartrate but not cadmium chloride was also observed. Although MRP has a high degree of homology with the yeast *YCF-1* gene, which confers resistance to cadmium, the observed pattern of hypersensitivity to heavy metals is not surprising, given the fact that MRP-transfected cells acquire resistance to arsenicals and antimonials but not to cadmium (11).

**Sensitivity to Alkylating Agents and Transport of GSH *S*-Conjugates of Monochlorobimane.** Drug transport studies in membrane vesicles from MRP-transfected cells have shown that leukotriene C4, an endogenous GSH *S*-conjugate, and monochloromonoglutathionyl melphalan are potential substrates for MRP (6, 7). GSH *S*-conjugation of alkylating agents is a well-known detoxification pathway. Increases in expression of GST after transfection of various GST cDNAs impart a 2- to 3-fold level of resistance to many

Table 1 *Sensitivity of wild-type ES and of the MRP double knockout clones ES MRP−/−1 and ES MRP−/−2 to various cytotoxic agents*

| Agent | $IC_{50}$[a] | | | Hypersensitivity factor[b] |
|---|---|---|---|---|
| | ES | ES MRP−/−1 | ES MRP−/−2 | |
| Etoposide | 145 ± 12 | 36 ± 6[c] | 36 ± 4[c] | 4.0; 4.1 |
| Teniposide | 34 ± 8 | 8 ± 1.5[c] | 9 ± 1[c] | 4.2; 3.8 |
| Vincristine | 4 ± 0.6 | 1.9 ± 0.07[c] | 1.9 ± 0.2[c] | 2.1; 2.1 |
| Doxorubicin | 30 ± 2 | 17 ± 1.5[c] | 16 ± 2[c] | 1.8; 1.9 |
| Daunorubicin | 32 ± 4 | 20 ± 4[c] | 18 ± 2[c] | 1.6; 1.8 |
| Colchicine | 55 ± 7 | 41 ± 6 | 45 ± 7.5 | 1.3; 1.2 |
| Cytosine arabinoside | 410 ± 35 | 410 ± 45 | 430 ± 40 | 1.0; 1.0 |
| Sodium arsenite | 7,100 ± 700 | 2,400 ± 250[c] | 2,300 ± 180[c] | 3.0; 3.1 |
| Sodium arsenate | 48,400 ± 5,000 | 21,500 ± 2,000[c] | 26,900 ± 3,500[c] | 2.2; 1.8 |
| Antimony potass. tartrate | 7,700 ± 600 | 1,540 ± 170[c] | 1,230 ± 140[c] | 5.0; 6.2 |
| Cadmium chloride | 13,000 ± 1,500 | 14,000 ± 1,000 | 12,000 ± 1,200 | 0.9; 1.1 |
| Chlorambucil | 3,300 ± 300 | 3,000 ± 250 | 2,800 ± 200 | 1.1; 1.2 |
| Melphalan | 1,300 ± 150 | 1,475 ± 100 | 1,570 ± 120 | 0.9; 0.8 |
| Cisplatin | 800 ± 100 | 900 ± 50 | 850 ± 100 | 0.9; 0.9 |
| Dichloroethane (mM) | 8 ± 0.6 | 7.5 ± 0.5 | 8.3 ± 0.8 | 1.1; 1.0 |
| Chloroiodoethane (mM) | 5 ± 0.5 | 4.5 ± 0.5 | 5.5 ± 0.7 | 1.1; 0.9 |

[a] The $IC_{50}$ value for each agent was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay and is expressed as the nM concentration, unless otherwise indicated. The values shown are means ± SE of 4–11 independent experiments. Statistical significance was determined using an unpaired Student's *t* test.

[b] Hypersensitivity factors were calculated as the ratio of the $IC_{50}$ of wild-type ES cells:$IC_{50}$ of MRP double-knockout ES cells. The initial number represents the value for ES MRP−/−1, and the second number represents the value for ES MRP−/−2.

[c] Values obtained with the MRP double knockout ES cell lines are significantly different from those obtained with the wild-type ES cell line ($P < 0.05$).

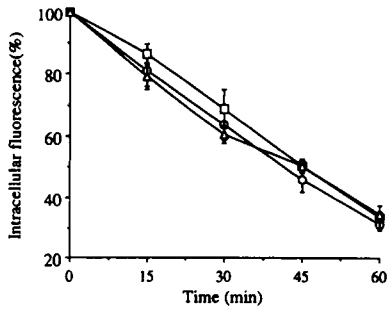


Fig. 1. Efflux of GSH-bimane conjugate from wild-type and MRP double knockout ES clones. Cells were incubated with 40 μM monochlorobimane for 10 min at 37°C and washed, and the efflux of the GSH-bimane conjugate was monitored by flow cytometry as a decrease in intracellular fluorescence over a 60-min time course at 37°C. Data are expressed as the percentage of intracellular fluorescence at efflux time 0. *Points*, the means of four independent experiments; *bars*, SE. ○, wild-type ES cells; □, ES MRP−/−1; △, ES MRP−/−2.

alkylating agents, including chlorambucil, cisplatin, and melphalan (12). The export of the conjugated drugs by the GS-X pump is presumed to be a crucial step in this detoxification pathway (5). Therefore, we have determined whether baseline MRP expression has the capacity to exert a protective role against the toxicity of multiple alkylating agents. As shown in Table 1, no difference in sensitivity to chlorambucil, melphalan, and cisplatin existed between wild-type ES cells and the two MRP double knockout cell lines. It is possible, however, that MRP exports GSH *S*-conjugates of alkylating agents out of the cells, but because the conjugates have already been detoxified, their export does not result in additional protection from cytotoxicity. The physiological importance of the export of GSH *S*-conjugates may be emphasized in cases in which the GSH conjugation reaction turns to bioactivation as opposed to detoxification (5). This is the case for the haloethanes 1,2-dichloroethane and 1-chloro-2-iodoethane. These compounds are activated to DNA-reactive electrophiles by GSH conjugation via GST. In the case of 1-chloro-2-iodoethane, the DNA-reactive electrophile may also be formed by a direct nonenzymatic reaction, due to the fact that iodide is a much better leaving group than chloride. As shown in Table 1, no difference in sensitivity existed between wild-type ES cells and the two MRP double knockout cell lines for these compounds.

To analyze more directly whether MRP transports GSH *S*-conjugates of alkylating agents, we compared the rate of efflux of monochlorobimane by wild-type and the two MRP knockout cell lines. In murine cells, monochlorobimane binds to GSH via a GST-mediated reaction, producing a fluorescent conjugate (13). At 37°C, a plateau of intracellular fluorescence was reached for monochlorobimane concentrations ranging from 10–80 μM between 5 and 10 min in both wild-type and knockout cell lines (data not shown). Cells were incubated with 40 μM monochlorobimane for 10 min at 37°C and washed, and efflux of the GSH-bimane conjugate was monitored by flow cytometry as a decrease in intracellular fluorescence over a 60-min time course at 37°C. As shown in Fig. 1, no differences in the rate of efflux of the GSH-bimane conjugate were found between wild-type ES cells and the two MRP double knockout cell lines, with about 50% of the drug being effluxed by 40 min. These findings collectively demonstrate that baseline MRP expression does not exert a protective role against the toxicity of alkylating agents. Because it is possible that multiple GS-X pumps (14) with different substrate preferences are present in ES cells, our findings do not exclude the possibility that MRP transports certain GSH *S*-conjugates in the parental cell line.

**BSO-induced GSH Depletion Increases Etoposide Accumulation in Wild-Type but not in MRP Double Knockout Cells.** Etoposide constitutes a major obstacle to the hypothesis that MRP is only a transporter of GSH *S*-conjugates, because GSH conjugates of etoposide are not known to exist. Furthermore, although MRP-mediated transport has been observed for glucuronosyl-etoposide in membrane vesicles from MRP-transfected cells (7), glucuronide conjugation of etoposide seems to take place mainly in the liver. We have previously reported that in the MRP−/−1 double knockout clone, the steady-state levels of [$^3$H]etoposide were about two times higher than those in parental ES cells (8). A similar increase in the steady-state concentration of etoposide was also found in ES MRP−/−2 cells (Fig. 2). We have also previously shown that the intracellular accumulation of etoposide is linearly concentration dependent in the range of 2–10 μM. When cells were exposed to 4 μM [$^3$H]etoposide, the radiolabeled drug began to accumulate very early in the MRP knockout clones, occurring to a greater extent in the knockout clones than in wild-type ES cells, with a difference observable after 60 s, and reaching a plateau in all of the cell lines at about 10 min (Fig. 2). The effects of BSO-induced GSH depletion on the intracellular accumulation of radiolabeled etoposide were evaluated in wild-type ES and in ES MRP−/−1 and ES MRP−/−2 cell clones. BSO is an irreversible inhibitor of γ-glutamylcysteine synthetase, the first enzyme in the biosynthetic pathway of GSH. Exposure to 50 or 80 μM BSO for 24 h reduced the GSH levels in wild-type ES cells from 1.47 ± 0.27 to 0.26 ± 0.03 and 0.10 ± 0.03 mM, respectively; in ES MRP−/−1 cells from 2.05 ± 0.26 to 0.37 ± 0.04 and 0.14 ± 0.02 mM, respectively; and in ES MRP−/−2 cells from 2.01 ± 0.16 to 0.35 ± 0.05 and 0.13 ± 0.02 mM, respectively. Under these conditions, cell survival was unaffected by BSO, as demonstrated by the finding that 95–100% of the cells excluded trypan blue at the end of the treatment, and after wash-out of external BSO, cell numbers, measured by a tetrazolium salt-based microtiter plate assay after growth in drug-free medium for

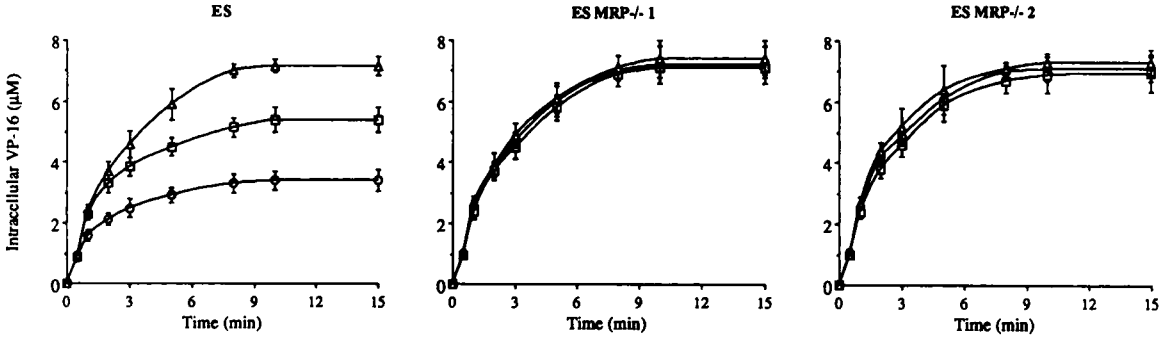


Fig. 2. Effects of BSO-induced GSH depletion on the accumulation of [$^3$H]etoposide in wild-type and MRP double knockout ES clones. Untreated control cells and cells treated with BSO for 24 h were incubated with 4 μM [$^3$H]etoposide, and intracellular radioactivity was measured at different times thereafter as described in "Materials and Methods." Exposure to 50 or 80 μM BSO for 24 h reduced the GSH levels by 80–85% and 90–95%, respectively, in both wild-type ES cells and in ES MRP−/−1 and ES MRP−/−2 cell clones. *Points*, the means of three to five independent experiments; *bars*, SE. ○, no BSO; □, 50 μM BSO; △, 80 μM BSO.

Table 2 *Exported and intracellular levels of GSH in wild-type ES and MRP double knockout clones*

| Cell lines | Exported GSH[a] (pmol/$10^6$ cells/h) | Exported GSSG (pmol/$10^6$ cells/h) | Intracellular GSH[a] (mM) | Intracellular GSSG (mM) |
|---|---|---|---|---|
| ES | 77.0 ± 2.7 | <1 | 1.47 ± 0.27 | <0.05 |
| ES MRP−/−1 | 37.3 ± 1.6 | <1 | 2.05 ± 0.26 | <0.05 |
| ES MRP−/−2 | 40.6 ± 1.6 | <1 | 2.01 ± 0.16 | <0.05 |

[a] Data represent the mean of four to six experiments run in triplicate ± SE.

2 additional days, were 90–100% of control untreated cells. The effects of BSO treatment on VP-16 accumulation were determined by exposing cells to BSO for 24 h, followed by removal of extracellular BSO and incubation of cells in medium containing 4 μM [$^3$H]etoposide for different periods of time. As shown in Fig. 2, pretreatment of wild-type ES cells with 50 or 80 μM BSO increased the steady-state concentration of etoposide by 60 and 110%, respectively, whereas it did not affect the steady-state concentration of etoposide in the MRP knockout cell lines. In wild-type cells, 80 μM BSO increased the accumulation of etoposide to the levels present in the knockout cell lines. Consistent with these findings, BSO treatment has been reported to result in a complete reversal of the MDR phenotype in lung carcinoma cells transfected with a MRP cDNA expression vector (15) and in a partial reversal in MRP-overexpressing drug-selected MCF7/VP cells (16). BSO also enhanced the therapeutic efficacy of doxorubicin *in vivo* against MRP-overexpressing tumors (17). Furthermore, a direct interaction between GSH and MRP was recently demonstrated by the observation that etoposide, vincristine, and GSH stimulated the vanadate-induced trapping of 8-azidoATP by MRP (18).

**MRP-mediated Export of GSH.** A possible interpretation of our results and of those of others (6, 7, 15–18) is that MRP, besides transporting certain GSH *S*-conjugates, also co-transports GSH and drug(s). To gain evidence in support of this possibility, we compared the rate of export of GSH into the extracellular medium by wild-type ES cells and by ES MRP−/−1 and ES MRP−/−2 cells and have found that, in the absence of drugs, wild-type ES cells exported GSH at approximately double the rate occurring with ES MRP−/−1 and ES MRP−/−2 cells (Table 2). Thus, the expression of the MRP gene seems to account for more than one-half of the baseline GSH export. GSH export may be underestimated if these cells have substantial amounts of γ-glutamyltranspeptidase (19), which rapidly degrades GSH at the level of the exoplasmic portion of the plasma membrane. No significant export of GSSG was detected in any of the cell lines.

The finding of a MRP-mediated export of GSH is in agreement with the observation that MRP-transfected cells export two times more GSH into the extracellular medium than do parental cells (15). Export of GSH from eukaryotic cells in culture has been extensively documented in studies carried out in the presence of BSO (19, 20), but the protein(s) responsible for this export has not yet been identified, and the physiological significance of this phenomenon is presently unknown. Our data provide evidence that MRP is partially responsible for the export.

The multiple essential functions of intracellular GSH make it extremely important for every cell in the body to maintain high intracellular levels (0.1–10 mM). In addition, because a considerable

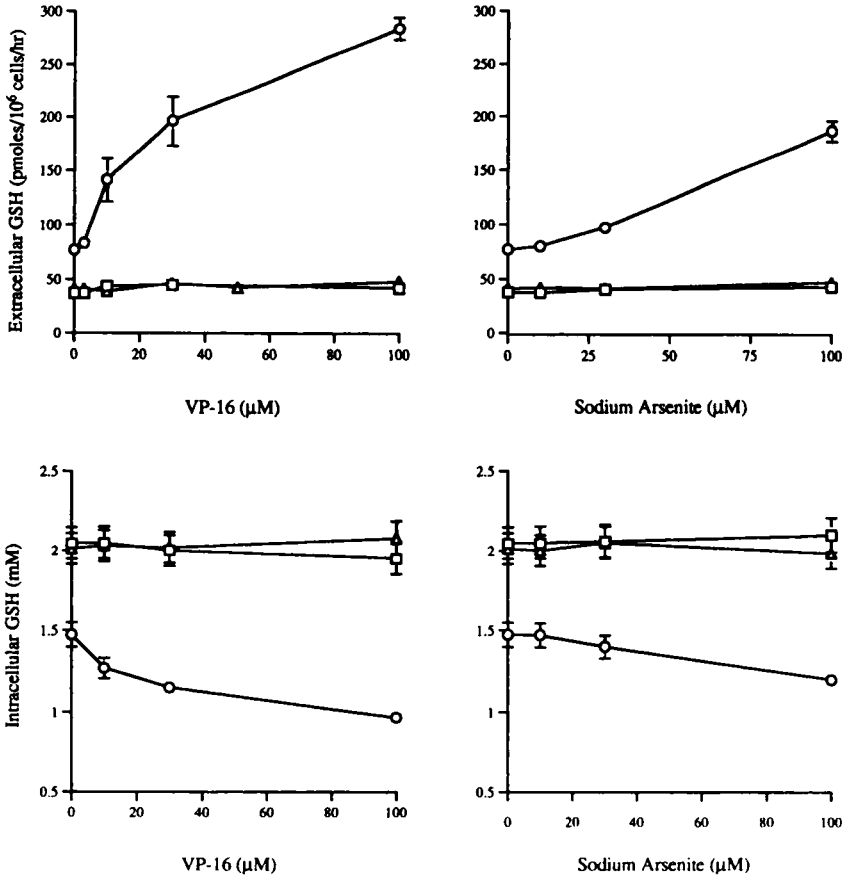


Fig. 3. Effects of sodium arsenite and etoposide on exported and intracellular concentrations of GSH from wild-type and MRP double knockout ES clones. Cells were incubated with different concentrations of etoposide or sodium arsenite in 0.5 ml of HBSS, and intracellular GSH was analyzed after 1 h as described in "Materials and Methods." The rate of export of GSH was calculated by measuring the concentration of GSH in the extracellular solution every 30 min for 3 h. *Points*, the means of three to five independent experiments; *bars*, SE (shown only when they are larger than the *points*). ○, wild-type ES cells; □, ES MRP−/−1; △, ES MRP−/−2.

amount of energy is required to keep most of the cellular GSH in the reduced state, the release of GSH from cells must involve an important function. MRP-mediated GSH export may contribute to fueling the production of cysteine, an amino acid that is exceedingly toxic when present in cells at high concentrations, and may also be of general importance in protecting the surface of cells from oxidants (20).

Studies in membrane vesicles from MRP-overexpressing cells have shown that reduced GSH is not a MRP substrate, nor does it inhibit the transport of other compounds (6). Thus, it is probable that the MRP-mediated export of GSH occurs only in association with an endogenous metabolite present in the intracellular compartment. The nature of this endogenous metabolite is presently unknown. The fact that, in the absence of the MRP protein, the two knockout clones still exhibit a basal release of GSH suggests that there is at least one other MRP-independent system responsible for GSH release into the extracellular milieu. This conclusion is in agreement with the observation that HeLa cells, which do not express MRP, transport GSH (20, 21).

GSH release increased markedly after exposure to natural exogenous compounds such as sodium arsenite and etoposide. Thus, exposure of wild-type cells to 10–100 $\mu$M etoposide or 30–100 $\mu$M sodium arsenite for 1 h resulted in a great increase in GSH export and a simultaneous decrease in intracellular GSH; whereas, in contrast, no change in GSH concentration was observed in the two MRP double knockout cell lines (Fig. 3). The possibility that, instead of a specific GSH exporter, nonspecific leakage of GSH occurred due to cell lysis produced by drug toxicity is unlikely, because after exposure to these agents for 1 h, more than 99% of the cells were viable. Furthermore, the hypersensitivity of the knockout clones to the toxicities of etoposide and sodium arsenite suggests that if GSH export was due to a toxic event, it should be more evident in the knockout clones than in the parental ES clone. No GSSG release from either wild-type or MRP double knockout ES cell lines was detected after treatment with sodium arsenite or etoposide (data not shown). In addition, no release of either GSH or GSSG was observed (besides the baseline level of export) in any of the three cell lines after exposure for 2 h to 10, 30, or 100 $\mu$M monochlorobimane or 100 $\mu$M cisplatin (data not shown), implying that co-transport of these compounds and GSH does not occur.

It has been proposed that arsenite and GSH form a transient $As(SG)_3$ complex (22); if this were the form of arsenite transported by MRP, GSSG should have been detected after dissociation of the complex in the extracellular medium rather than GSH. Consistent with our findings, Zaman *et al.* (15) reported that exposure to 100 $\mu$M sodium arsenite increased the rate of export of GSH 10-fold from MRP-transfected lung cancer cells and only 5-fold from the parental counterpart. In wild-type ES cells, the decrease in intracellular GSH after treatment with etoposide or sodium arsenite was equivalent to the amount of GSH exported in the extracellular medium (Fig. 3). For example, after 1 h of exposure of $10^6$ ES cells to 100 $\mu$M etoposide, 275 pmol of GSH were exported, and the intracellular amount of GSH decreased by 270 pmol. These observations suggest that under these experimental conditions, over a short period of time, there are no homeostatic mechanisms able to compensate for the loss of GSH. We have found a slightly higher intracellular concentration of GSH in untreated knockout clones than in untreated wild-type ES cells (Table 2). Accordingly, in many tissues from mice with a homozygous disruption of the MRP gene, the GSH content is significantly increased compared to those of wild-type animals (23).

Although MRP can transport some GSH *S*-conjugates, such as leukotriene C4, our findings and those of others (15, 24) demonstrate that MRP also catalyzes the co-transport of GSH and drug. The evidence in support of this conclusion includes: (*a*) a MRP-mediated export of GSH is present in untreated wild-type ES cells; (*b*) the concentration-dependent etoposide and arsenite-induced efflux of GSH from ES cells is totally abrogated in MRP knockout cells; (*c*) the amount of GSH exported into the extracellular medium is equivalent to the loss of intracellular GSH after exposure to etoposide or sodium arsenite for 1 h; (*d*) the depletion of intracellular GSH by treatment with BSO increases the intracellular accumulation of etoposide in wild-type ES cells but not in MRP double knockout cells, and, moreover, BSO has been shown by others to reverse the MRP-mediated MDR phenotype both *in vitro* and *in vivo* (15, 17); and (*e*) whereas P-glycoprotein-enriched membrane vesicles have been shown to directly transport several unconjugated chemotherapeutic drugs, reports on the binding and transport of unmodified forms of the drugs by MRP have been contradictory (24, 25). Interestingly, vincristine transport by MRP-enriched membrane vesicles was demonstrable only in the presence of reduced GSH (24). Also, the ability of vincristine and vinblastine to inhibit leukotriene C4 transport also was enhanced by GSH (24). Therefore, the co-transport of drug(s) and GSH provides a plausible explanation for the observation that overexpression of MRP confers resistance to drugs such as etoposide, doxorubicin, and vincristine, which are not known to undergo major modification in cells. It is not clear at this time whether a molecular interaction between the drug and GSH is required, but if this is the case, the complex must be easily dissociable, because the exported GSH is still present in the extracellular medium as free GSH.

The data presented in this report demonstrate the existence of a link between two of the most studied mechanisms of resistance to anticancer agents, the ATP-dependent membrane export of drugs and the GSH detoxification pathway. A systematic assessment of the co-transport properties of ATP-binding cassette transporters may open a new chapter in the search for the physiological functions of the GSH system.

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