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Retroviral transfer of MRP1 and γ-glutamyl cysteine synthetase modulates cell sensitivity to L-buthionine-*S*,*R*-sulphoximine (BSO): new rationale for the use of BSO in cancer therapy

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Abstract

MRP1 (multidrug resistance protein 1) co-exports glutathione (GSH) and drug(s) and exports GSH, glucuronide, and sulphate-conjugated drugs. Human Fly-eco fibrosarcoma cells producing the MRP1-expressing retrovirus SF91MRP (Fly-eco MRP1), as well as 3T3 cells transduced with SF91MRP (3T3/MRP1), presented a decrease in intracellular GSH levels, as measured by two different methods. The enhanced export of GSH caused by the overexpression of MRP1 was partially counterbalanced by an increased rate of GSH synthesis. Fly-eco MRP1 and 3T3/MRP1 were hypersensitive to the GSH-depleting and cytotoxic activities of L-buthionine-*S*,*R*-sulphoximine (BSO), compared with their parental counterparts. In addition, the potentiation by BSO of the cytotoxic activity of chlorambucil and doxorubicin in Fly-eco MRP1 cells was greater than in parental Fly-eco cells. Although the turnover time of GSH, i.e. the theoretical time in which the entire GSH pool is resynthesised, was approximately 50% faster in Fly-eco MRP1 cells than in parental cells, this was not sufficient to fully restore the intracellular GSH level. In addition, *mrp1* (−/−) mice were resistant to the GSH-depleting activity of intraperitoneally (i.p.) injected BSO, compared with *mrp1* (+/+) mice. Co-transfer of the cDNAs for MRP1 and the heavy subunit of γ-glutamyl cysteine synthetase (GCS) resulted in increased intracellular GSH levels and in high-level resistance to the GSH-depleting and cytotoxic activities of BSO. These data, and in particular the elevated single-agent cytotoxicity of BSO, provide a new rationale for the use of BSO in the treatment of MRP1-overexpressing tumours.

Keywords: Multidrug resistance protein; Glutathione; Retrovirus; BSO; γ-Glutamyl cysteine synthetase

1. Introduction

The *MRP1* gene, discovered in 1992 and associated with the multidrug resistance of mammalian cells to natural product anticancer agents [1], is the first identified member of the MRP family, that consists of at least six genes [2]. MRP1 exports the endogenous GSH-S-conjugate leukotriene C4 and several glutathione (GSH), glucuronide and sulphate conjugates. In 1995, Zaman and colleagues reported that MRP1-transfected cells exported 2 times more GSH into the extracellular medium than parental cells and exposure to sodium arsenite further increased the rate of export of GSH [3]. We subsequently found that the baseline expression of MRP1 protects embryonic stem cells from the toxic effects of xenobiotics by co-transport of GSH and the xenobiotic from the intracellular compartment into the extracellular medium [4,5]. Furthermore, when the gene encoding MRP1 was disrupted in two sublines by homologous recombination, the co-transport mechanism was abrogated [5]. The abrogation of MRP1 expression in mice resulted in increased levels of GSH in organs like lung, kidney and colon, that normally express relatively high levels of MRP1, but not in organs that express very low levels of MRP1, e.g. the liver [6].

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We have recently assembled two retroviral vectors, expressing MRP1 alone (SF91MRP) or MRP1 and the heavy subunit of γ-glutamyl cysteine synthetase (γ-GCS), the rate-limiting enzyme of GSH biosynthesis (SF91GCS-MRP) [7]. Producer cells and 3T3 fibroblasts transduced with either SF91MRP or SF91GCS-MRP were resistant to antimony potassium tartrate, sodium arsenite, doxorubicin and VP-16, all MRP1 substrates. However, only bicistronic producers and 3T3 fibroblasts transduced with SF91GCS-MRP were also resistant to the alkylating agents, melphalan, chlorambucil and cis-platin [7]. In this paper, we have investigated the effects of retroviral vector-mediated overexpression of MRP1 and γ-GCS on intracellular GSH metabolism and on cellular sensitivity to the γ-GCS inhibitor, BSO.

2. Materials and methods

2.1. Materials

[3-^{13}C]-L-Cysteine was purchased from Cambridge Isotopes, Inc. (Woburn, MA, USA). [3,3′-$^{13}C_2$]-Cystine was prepared by stirring a slightly basic solution of [3-^{13}C]-L-cysteine in an open container for 72 h. Cystine and methionine-free Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma Chemical Company (St. Louis, MO, USA). This medium was supplemented with 200 μM methionine and 200 μM [3,3′-$^{13}C_2$]-L-cystine. Solid-phase extraction cartridges (C18) were from Burdick & Jackson, Inc. (Muskegon, MI, USA). Monobromobimane was purchased from Calbiochem (La Jolla, CA, USA).

2.2. Cell growth characteristics

3T3 mouse fibroblasts and Fly-eco packaging cells, derived from the HT-1080 human fibrosarcoma cell line [8] were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine at 37 °C in an atmosphere of 10% CO_2 in air.

2.3. Viral transduction of 3T3 fibroblasts

Retroviral supernatant (1.5 mL) harvested from the producer Fly-eco clones was applied to 10^6 3T3 fibroblasts in the log phase of growth in the presence of 22 μg/ml polybrene in complete medium. After 24 h, cells were washed with phosphate-buffered saline (PBS), and incubated in virus-free medium with 3 μM antimony potassium tartrate, a lethal dose for parental 3T3 fibroblasts. The choice of antimony as a selecting agent is partially due to the fact that, not being mutagenic, it cannot induce random mutations in the viral genome, thus reducing the risk of generating replication-competent virus or of unwanted contributions to the drug-resistant phenotype. After selection, the cells were grown in the absence of antimony, and the resistant phenotype was found to be stable for over 10 passages in culture.

2.4. Analysis of drug sensitivity

The sensitivity to BSO of the producer clones and of the virus-transduced 3T3 cell lines was determined using a tetrazolium salt-based microtitre plate assay [9]. The 96-well plates were inoculated with 3000 Fly-eco cells/well or 2000 3T3 cells/well in a volume of 100 μl in complete medium. BSO was added 24 h after plating of the cells. To analyse the effects of the combination of BSO with chlorambucil or doxorubicin, cells were exposed to BSO 24 h after plating. 24 h later, chlorambucil or doxorubicin were added for two additional days. Control wells were included for each drug which consisted of the respective solvents. 72 h after the addition of BSO, the drug-containing medium was removed, and 100 μl of fresh medium containing 333 μg/ml of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and 25 μM phenazine methosulphate (PMS) were added and the incubations continued for 2 h at 37 °C. In the presence of the electron coupling reagent, PMS, MTS is reduced by dehydrogenase enzymes found in metabolically active cells into a formazan product that is readily soluble in tissue culture medium [9]. The formazan product was quantified by measurement of the absorbance at 490 nm.

2.5. Measurement of intracellular GSH by an enzymatic assay

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-sulphosalicylic acid in water. After centrifugation, 18 μl of triethanolamine (1:3, v/v, in H_2O) were added to 100 μl aliquots of the supernatant to bring the pH to 7.0–7.5 and GSH was assayed according to the recycling method [10]. Briefly, the rate of 2-nitro-5-thiobenzoic acid formation was monitored at 412 nm, and was found to be linear for all of the samples analysed. The glutathione concentration was evaluated by comparison with the rate of a standard curve generated with known amounts of GSH.

2.6. Measurement of fluorescence of GSH–bimane conjugates

Cells were detached by incubation with 0.05% trypsin for 2 min at 37 °C, centrifuged and resuspended in PBS. Monochlorobimane (Molecular Probes, Eugene, OR, USA) was prepared as 4 mM solution in 100% ethanol, and added at a final concentration of 40 μM. In murine cells, monochlorobimane binds to glutathione via a

glutathione-*S*-transferase-mediated reaction, producing a fluorescent conjugate [11]. At 40 μM of monochlorobimane, a plateau of intracellular fluorescence is reached in most murine cells within 15 min, the level of fluorescence being proportional to the intracellular level of glutathione [11]. In fact, at 22 °C, a plateau of intracellular fluorescence was reached between 5 and 11 min after exposure of the cells to 40 μM monochlorobimane. After incubation for 15 min at 22 °C, the fluorescence of the GSH-bimane conjugate was measured using a FACStar Flow cytometer (Becton-Dickinson, San Jose, CA, USA). Cells were excited at 356±6 nm and emission was collected through a 460±35 nm bandpass filter. In order to standardise the assay, the mean fluorescence of Hoechst ultraviolet (UV) calibration beads (Flow Cytometry Standards Corp., Research Triangle Park, NC, USA) was recorded and the mean cellular fluorescence compared with that of the beads. A minimum of 20 000 cells were analysed for each sample.

2.7. Measurement of GSH synthesis

GSH synthesis in cells growing in monolayer was determined by a slight modification of the methods detailed earlier in Ref. [12]. At 24 and 4 h prior to feeding the cells with isotope labelled medium, the cells were fed with fresh, unlabelled medium. This ensures the cells are well supplied with all nutrients when fresh, isotope labelled medium is introduced. In all experiments, cells were in the log-phase of growth. Immediately before the addition of labelled medium, cells were washed with Hank's Balanced Salt Solution to remove traces of the unlabelled medium. DMEM/10% FBS containing [3,3′-^{13}C]-L-cystine was added and the cells were incubated for periods of between 1 and 24 h. Cells were harvested by trypsinisation, suspended in PBS containing 20 μM acivicin, treated with monobromobimane and extracted with perchloric acid. The extract was adjusted to pH 3 with KOH and the supernatant applied to a solid-phase extraction cartridge. The cartridge was washed with 0.2% acetic acid to remove unwanted metabolites and the GSH-bimane adduct was eluted from the cartridge using 30% methanol in water. Before nuclear magnetic resonance (NMR) spectroscopy, methanol was removed under a stream of nitrogen and the samples lyophilised.

2.8. NMR spectroscopy

NMR spectroscopy was performed at 11.75 T on a Varian Unity 500 NMR spectrometer at the Duke University Medical Center. The conditions used were outlined previously in Ref. [12]. Lyophilised cell extracts for NMR spectroscopy were taken up in 0.65 ml of D_2O for analysis.

2.9. Isotope incorporation rate

The fractional enrichment of GSH was determined from NMR data. The content of reduced GSH was determined by the high performance liquid chromatography (HPLC) assay of the monobromobimane derivative [13]. All HPLC assays were performed using a Zorbax RX C18 column (Hewlett-Packard) on a Waters (Milford, MA, USA) Millennium system equipped with a Model 474 fluorescence detector. The incorporation rate was determined based on the fractional enrichment and the content of reduced GSH [12]. For each cell line, replicate measurements of the percent label incorporation were performed for periods ranging from 1 to 8 h. Since linear increases in label incorporation were observed for at least 2 h for each cell line, the isotopic enrichments determined in extracts after a 2-h exposure to labelled medium were used to calculate the rate of synthesis as outlined in the Materials and methods section.

2.10. BSO Treatment of mrp1 (+/+) and mrp1 (−/−) mice

The targeted disruption of the *mrp1* locus and generation of *mrp1* (−/−) mice were previously reported Ref. [6]. Heterozygous mice (129×c57/BL6 genotype) were interbred to generate homozygous mice. Sex- and age-matched 8–10-week-old mice of the F5 generation were employed in the present study. Mice were maintained in an environmentally controlled room at 22 °C with 50% relative humidity and a 12-h-light, 12-h-dark cycle and received acidified water and food *ad libitum*. No expression of the MRP1 protein was observed in all examined tissues and organs of the *mrp1* (−/−) mice, particularly in the liver and lung [6]. BSO (three doses of 800 mg/kg every 4 h) was dissolved in saline and administered by intraperitoneal (i.p.) injection (150 μl). GSH was measured in washed blood erythrocytes by the enzymatic recycling method 30 min after the last dose of BSO.

3. Results

We have previously reported the cloning and production of SF91MRP and SF91GCS-MRP retroviral vectors from human Fly-eco fibrosarcoma cells [7]. Supernatants from Fly-eco MRP producer clones were used to transduce 3T3 cells. The producer clones, Fly-eco MRP1 and Fly-eco MRP1c (previously named Fly-eco MRP4), contained only one copy of the provirus per cell, detected by Southern blotting after digestion with *NdeI*, which cuts once in the vector, while Fly-eco MRP1a (previously Fly-eco MRP2) and Fly-eco MRP1b (previously Fly-eco MRP3) contained two copies of the provirus per cell. On average, 3T3 MRP1

and 3T3 MRP1a (previously 3T3 MRP2) contained 2.4 and 3 copies of MRP1 provirus per cell, respectively ([7] and data not shown). We have previously reported an increase in expression of the MRP1 protein, measured by flow cytometry, using the MRPr1 monoclonal antibody [7].

3.1. GSH levels

In the four independently-derived MRP1 producer clones, Fly-eco MRP1-MRP1c, intracellular GSH levels, measured during the logarithmic phase of growth by a modification of the recycling method of Tietze [10], were lower than in parental Fly-eco cells, with a decrease ranging from 36 to 50% (Fig. 1). To exclude that the lower GSH levels of the producer clones could be at least partially due to the production of the viral vector, independently from the expression of the *MRP1* gene, we measured the GSH level of a Fly-eco clone (Fly-eco MRP0) that produced a MRP1 viral vector unable to express the MRP1 protein because of an aberrant ATG 33 nucleotide upstream of the putative MRP1 initiation codon [7]. We found that its GSH level was unchanged compared with parental Fly-eco cells (Fig. 1). Analogously, in two independently derived MRP1-transduced 3T3 cell lines, intracellular GSH levels during the logarithmic phase of growth were lower than in the parental 3T3 cells, with a decrease of 24–38% (Fig. 1). The lower GSH concentration could not be explained by differences in cell kinetics, because the doubling time and the fraction of S-phase cells in the MRP1-overexpressing sublines were not different from those of the parental cell lines (data not shown). Due to the similarity in the levels of MRP1 expression among the four Fly-eco GCS-MRP1 clones ([7] and data not shown), no correlation between copies of provirus per cell, MRP1 expression and intracellular levels of GSH was possible. As further evidence of an MRP1-induced decrease in intracellular GSH, we exposed 3T3 cells and their respective MRP1-transduced counterparts to monochlorobimane. In murine cells, monochlorobimane binds to GSH via a GSH-*S*-transferase-mediated reaction, producing a fluorescent conjugate [11]. Cells were incubated with 40 μM monochlorobimane for 15 min, and the fluorescence of GSH–bimane conjugates was measured. As shown in Fig. 2, the intracellular fluorescence of 3T3 MRP1 and 3T3 MRP1a was lower than the fluorescence of 3T3 cells. The ratio of the mean fluorescence intensity between 3T3 MRP1 and 3T3 was 0.64, and between 3T3 MRP1a and 3T3 was 0.68. In 3T3 cells transduced with SF91GCS-MRP, the fluorescence of GSH-bimane conjugates was higher than that of 3T3 cells with a ratio between the mean channel of fluorescence of 3T3 GCS-MRP and 3T3 cells of 3.2. This is in agreement with our previous finding by an enzymatic assay of increased GSH levels in both GCS-MRP producers and in 3T3 cells transduced with SF91GCS-MRP, compared with their respective parental cells [7]. Therefore, the increased GSH production sustained by the overexpression of γ-GCS was more than adequate to compensate for the GSH loss due to the MRP-mediated export of cellular GSH into the extracellular medium.

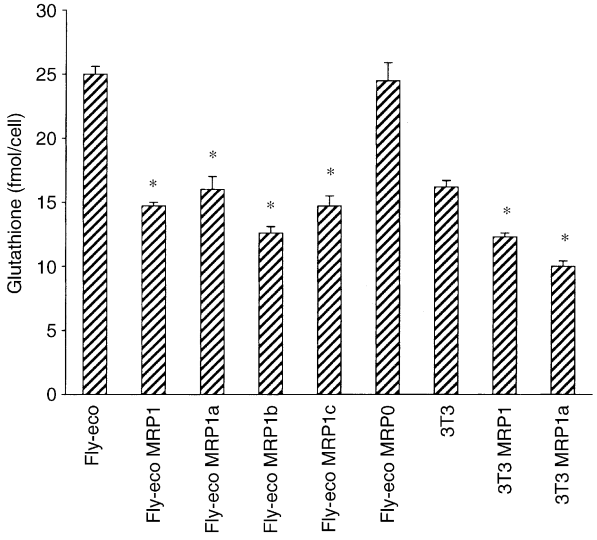

Fig. 1. Levels of intracellular glutathione (GSH) in Fly-eco and 3T3 cells. Cells were grown to the same density and intracellular GSH was extracted and measured by the enzymatic recycling method as detailed in Materials and methods. Fly-eco multidrug resistance protein 0 (MRP0) is a Fly-eco clone that produces a MRP1 viral vector unable to express the MRP1 protein. Data are the means of 4 to 6 separate experiments. Bars, standard deviation (S.D.) *, significantly different from parental cells ($P < 0.05$, unpaired Student's *t*-test).

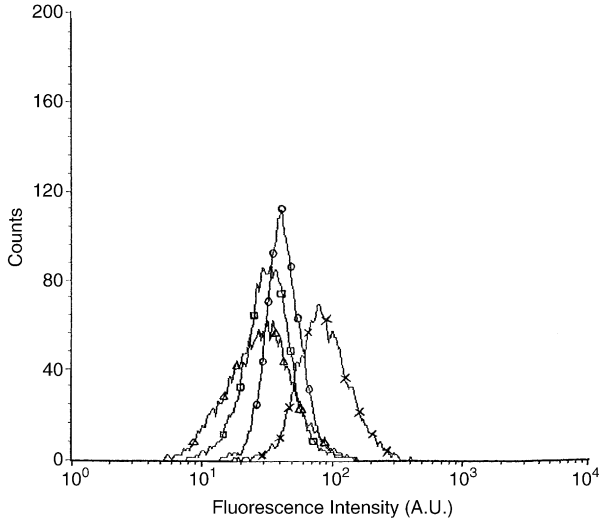

Fig. 2. Mean fluorescence intensity of parental 3T3 cells and of 3T3 cells transduced with SF91MRP or SF91GCS-MRP retroviral vectors after staining with 40 μM monochlorobimane for 15 min at room temperature. 3T3 (○); 3T3 MRP1 cells (□); 3T3 MRP1a cells (△); 3T3 GCS-MRP1 cells (x). A.U., arbitrary units.

3.2. GSH synthesis

To measure the actual rates of glutathione synthesis, Fly-eco, Fly-eco MRP1 and Fly-eco GCS-MRP1 growing in monolayer were treated with medium containing [3,3′-^{13}C]-cystine. Cells were extracted at various time points and the ^{13}C-enrichments were determined by ^{1}H NMR spectroscopy. The rates of GSH synthesis, calculated as detailed in Materials and methods, are given in Table 1. Although steady-state levels of GSH were lower, the rate of GSH synthesis were increased in Fly-eco MRP1 compared with Fly-eco cells. These data indicate that, due to the combination of lower intracellular GSH concentrations and rapid synthesis from cystine, Fly-eco MRP1 have a higher rate of turnover than parental Fly-eco cells. The turnover time provides a theoretical time in which the entire GSH pool can be resynthesised in a particular cell, and is calculated by dividing the total GSH content per cell, by the rate of GSH synthesis. As shown in the right column of Table 1, the turnover time was greatly reduced in Fly-eco MRP1 compared with Fly-eco cells. Fly-eco GCS-MRP1 cells, overexpressing both MRP1 and γ-GCS, presented a higher steady-state GSH level, an increased synthetic rate and an equivalent turnover time compared with parental Fly-eco cells (Table 1). These data indicate that the overexpression of γ-GCS is sufficient to cope with the increased MRP1-mediated export of GSH from Fly-eco cells.

3.3. BSO treatment of cells

Exposure of cells to BSO for 18–48 h caused a more profound depletion of GSH in Fly-eco MRP1 cells than in parental Fly-eco cells (Fig. 3). Thus, after exposure of Fly-eco MRP1 to 10 μM BSO for 48 h, the level of intracellular GSH dropped to levels similar to those of Fly-eco cells exposed to a 10-fold higher concentration of BSO for the same time. However, it should be considered that the basal levels of GSH are higher in Fly-eco parental cells than in Fly-eco MRP1. A more profound BSO-induced loss of GSH also occurred in 3T3 MRP1 cells, compared with parental 3T3 cells (Fig. 4). Thus, when untransduced 3T3 cells were exposed to 10 μM BSO for 18 h, intracellular GSH decreased by 78.5%, while exposure of 3T3 MRP1 to 10 μM BSO for 18 h resulted in a 97.6% decrease in intracellular GSH. That inhibition of GSH synthesis by BSO resulted in a more rapid and pronounced GSH depletion in Fly-eco MRP1 and 3T3 MRP1 cells, compared with the respective wild-type cells, further demonstrates that enhanced GSH utilisation and efflux in the MRP1-overexpressing cells account for the lowered steady-state GSH concentrations. We then investigated whether the profound depletion of GSH

Table 1
Rates of GSH synthesis in Fly-eco, Fly-eco MRP1 and Fly-eco GCS-MRP1 cells

Cell line	Rate (fmol/cell/h)	Turnover time[a] (h)
Fly-eco	1.72±0.08	26.7±3.7
Fly-eco MRP1	2.12±0.06*	12.6±0.6*
Fly-eco GCS-MRP1	2.27±0.19**	25.9±1.6***

[a] Turnover time = GSH (fmol/cell)/rate (fmol/cell/h).
* Significantly different from parental Fly-eco ($P<0.005$).
** Significantly different from parental Fly-eco ($P<0.05$).
*** Significantly different from Fly-eco MRP1($P<0.005$), but not from parental Fly-eco cells.

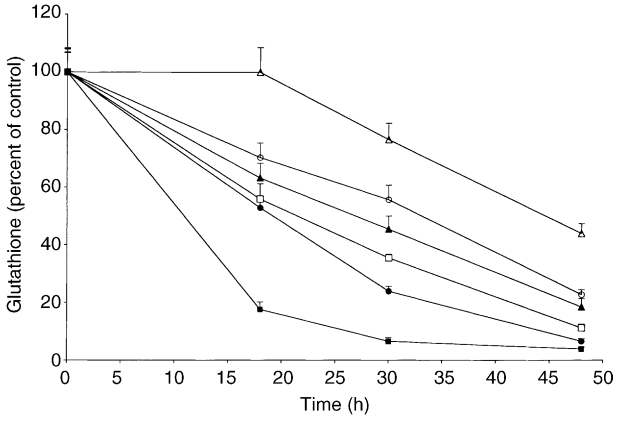

Fig. 3. The effect of 10 μM (empty symbols) and 100 μM L-buthionine-*S*,*R*-sulphoximine (BSO) (solid symbols) on the intracellular levels of glutathione (GSH) in parental Fly-eco (○), Fly-eco MRP1 (□), and Fly-eco GCS-MRP1 cells (△). Cells were exposed to BSO for 0, 18, 30 and 48 h and intracellular GSH was extracted and measured as detailed in Materials and methods. Data are the means of 3 to 5 separate experiments. Bars, S.D.

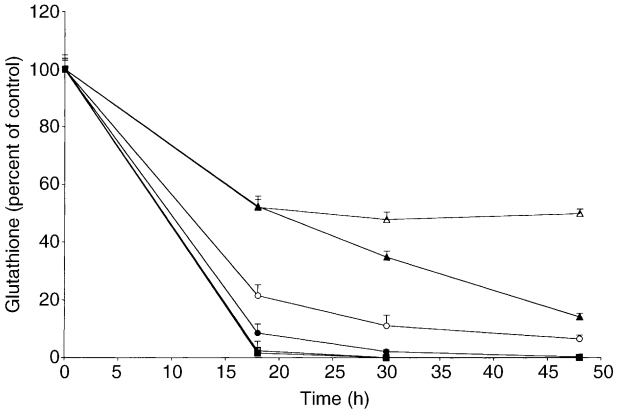

Fig. 4. The effect of 10 μM (empty symbols) and 100 μM L-buthionine-*S*,*R*-sulphoximine (BSO) (solid symbols) on the intracellular levels of GSH in parental 3T3 (○), 3T3 MRP1 (□) and in 3T3 GCS-MRP1 cells (△). Cells were exposed to BSO for 0, 18, 30 and 48 h and intracellular GSH was extracted and measured as detailed in Materials and methods. Data are the means of 3–4 separate experiments. Bars, standard deviation (S.D.).

induced by BSO in Fly-eco MRP1 and 3T3/MRP1 cells translated into hypersensitivity to the cytotoxic activity of BSO. Fig. 5 shows that continuous exposure to concentrations of BSO below equivalent steady-state plasma levels reported in adult human trials [14] resulted in decreased survival of Fly-eco MRP1, 3T3 MRP1 and 3T3 MRP1a, compared with wild-type cells. The similar pattern observed in Fly-eco MRP1 producer cells and in 3T3 cells transduced with the MRP1-expressing retroviral vector suggested that overexpression of MRP1, but not production of the viral vector, was responsible for the phenotypic changes observed. To exclude the possibility that the BSO hypersensitivity of the producer clone could be at least partially due to production of the viral vector, independently from the expression of the *MRP1* gene, we analysed the sensitivity to BSO of Fly-eco MRP0. We found that its sensitivity to BSO was unchanged compared with parental Fly-eco cells (Fig. 5). We then investigated whether pre-treatment with BSO potentiated the cytotoxic activity of the alkylating agent, chlorambucil and of the MRP1 substrate, doxorubicin. Fig. 6 shows that pretreatment with BSO potentiated the cytotoxic activity of chlorambucil and doxorubicin in both Fly-eco and Fly-eco MRP1. However, the potentiating effect of BSO was much greater in Fly-eco MRP1 cells than in the parental cells.

Exposure of Fly-eco GCS-MRP1 (Fig. 3) and 3T3/GCS-MRP1 (Fig. 4) to 10 μM BSO for 18–48 h, caused a modest decrease in intracellular GSH, such that at 48 h the intracellular GSH levels were similar to the level of their respective untreated parental cells. Exposure of Fly-eco GCS-MRP1 (Fig. 3) and 3T3/GCS-MRP1 (Fig. 4) to 100 μM BSO resulted in a time-dependent decrease in intracellular GSH. However, the GSH level at 48 h was still higher than in their respective parental counterparts exposed for the same period of time to a 10-fold lower BSO concentration. The level of resistance to the GSH-depleting activity of BSO correlated with resistance of Fly-eco GCS-MRP1 and 3T3/GCS-MRP1 to the cytotoxic activity of BSO (Fig. 5). When Fly-eco and 3T3 cells were continuously exposed for 72 h to BSO, the IC50s were 7 and 2.7 μM, respectively. For Fly-eco GCS-MRP1 and 3T3 GCS-MRP1 cells, the IC50s were 90 and 26 μM, respectively, yielding a 13- and a 10-fold level of resistance compared with their parental cell lines.

3.4. *BSO treatment of* mrp1*(−/−) mice*

To further investigate the relationship between MRP1 and sensitivity to BSO, we have compared the effect of BSO on the GSH level of blood erythrocytes from *mrp1* (+/+) and *mrp1* (−/−) mice. We have previously reported that tissue levels of GSH are elevated in *mrp1* (−/−) mice, especially in those tissues that are known to physiologically express high levels of *MRP1*. In particular, blood erythrocytes of *mrp1* (−/−) have 50–60% higher levels of intracellular GSH than erythrocytes of *mrp1* (+/+) mice ([6] and Table 2). I.p. treatment of *mrp1* (+/+) mice with BSO for 8 h resulted in a decrease in the intracellular GSH content of blood erythrocytes ($P<0.05$, unpaired Student's *t*-test), while it did not change the GSH content in *mrp1* (−/−) mice (Table 2), strongly suggesting that decreased GSH efflux

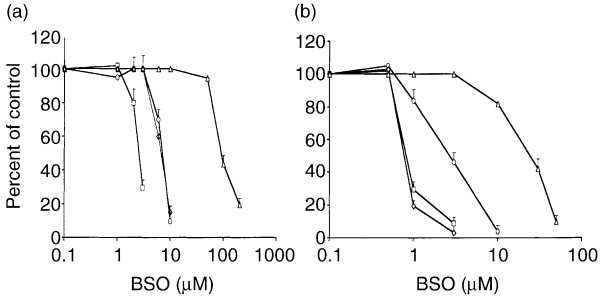

Fig. 5. The effect of various concentrations of L-buthionine-*S*,*R*-sulphoximine (BSO) on the growth of a. Fly-eco (○), Fly-eco MRP1 (□), Fly-eco GCS-MRP1 (△) and Fly-eco MRP0 (◇); b. 3T3 (○), 3T3 MRP1 (□), 3T3 MRP1a (◇) and 3T3 GCS-MRP1 (△) cells. After 3 days of continuous exposure, the sensitivity of cells to the various drugs was measured by the MTS/PMS assay and expressed as a percent of the absorbance value of cells treated with the solvent alone (ranging from 1.0 to 1.5 $\triangle A_{490nm}$). Data are the means of four separate experiments. Bars standard deviation (S.D.) (show only when they are larger than the points).

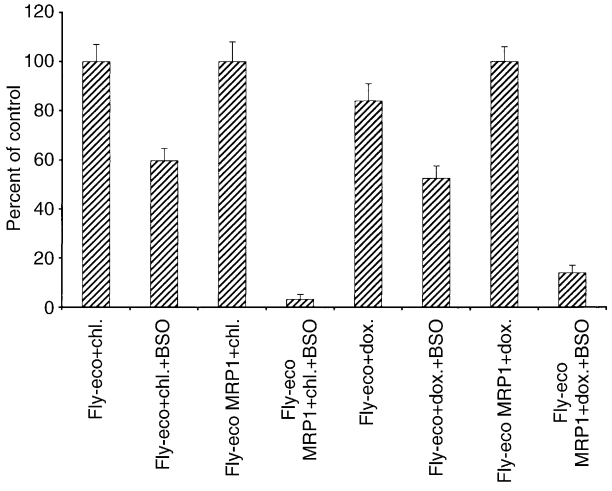

Fig. 6. The effect of pre-treatment with 3 μM BSO on the cytotoxic activity of 50 μM chlorambucil (chl) and 200 nM doxorubicin (dox) in Fly-eco and Fly-eco MRP1 cells. Chlorambucil or doxorubicin were added 24 h after exposure to BSO, and the incubation with the drugs were continued for 2 additional days. The sensitivity of cells to the various drugs was measured by the MTS/PMS assay and expressed as a percent of the absorbance value of cells treated with the solvent alone (ranging from 1.0 to 1.5 ΔA_{490nm}). Data are the means of 3–4 separate experiments. Bars, standard deviation (S.D.) (shown only when they are larger than the points).

Table 2
Effect of BSO on glutathione level of red blood cells of *mrp1* (+/+) and *mrp1* (−/−) mice

	Glutathione (nmol/mg)
mrp1 (+/+)	1.1±0.12
mrp1 (+/+)+BSO[a]	0.75±0.14*
mrp1 (−/−)	1.65±0.49
mrp1 (−/−)+BSO	1.57±0.21

[a] Mice (4/group, matched for sex and age) were treated intraperitoneally (i.p.) with three doses of 800 mg/kg BSO every 4 h, and the glutathione content of red blood cells was analysed 30 min after the last dose of BSO.

* Significantly different from *mrp1* (+/+) ($P<0.05$).

in *mrp1* (−/−) mice accounts for the higher basal intracellular GSH levels.

4. Discussion

The present study demonstrates that the overexpression of MRP1 results in decreased intracellular GSH levels. Our observation is in agreement with previous reports from Schneider and colleagues [15] and Gamcsik and colleagues [16] that MRP1-overexpressing MCF7/VP cells present a decreased intracellular GSH level compared with parental MCF7 cells, and with the findings by O'Brien and colleagues [17] that MRP1-transfected cells have lower intracellular GSH concentrations. Our findings are consistent with the notion that MRP1 is a co-transporter of GSH and drug(s) [5] and with the observation that *mrp1* (−/−) mice have increased levels of GSH in several organs [6]. Due to the multiple essential functions of GSH, the maintenance of a certain intracellular level of GSH, above a minimal hypothetical threshold, may be critical for cell homeostasis and, under certain stress conditions, ultimately result in altered differentiation or cell death. The increased GSH synthetic rate observed in Fly-eco MRP1 cells may represent an adaptation response to cope with the increased export of GSH. Presumably, the increase in MRP1 expression in the absence of an increased GSH synthetic rate would result in cell death. As suggested by Borst [2], this may explain why several research groups, including ours, have found it difficult to establish *in vitro* drug-resistant cell lines that express high levels of MRP1. The MRP1-induced decrease in intracellular GSH may be dangerous in gene therapy approaches, where haematopoietic stem cells are transduced with MRP1-expressing retroviral vectors to make the bone marrow resistant to a wide spectrum of anticancer agents [7,18]. Other members of the MRP family, i.e. MRP2 and MRP5, have recently been shown to secrete GSH into the extracellular medium [2,19]. Thus, their overexpression might result in decreased intracellular GSH levels as well. However, the observation that GSH levels were above normal levels in cells producing the bicistronic γ-GCS-MRP1 retroviral vector and in cells transduced with γ-GCS-MRP1 [7], suggests that the strategy of co-expressing γ-GCS and MRP1 may be a valid solution to this problem. Whether the increased GSH production triggered by the overexpression of γ-GCS would be critical to sustain the enhanced MRP1 efflux activity in early haematopoietic cells, although conceivable, needs to be verified in appropriate experimental models.

The co-expression of MRP1 and of γ-GCS resulted in an increased GSH synthetic rate, increased steady-state GSH intracellular levels and normalisation of the turnover time of GSH. Our findings are in agreement with those of O'Brien and colleagues [17]. They have previously reported that increased expression through single cDNA transfection of γ-GCS (regulatory plus catalytic subunits), and MRP1 increased the intracellular steady-state levels of GSH. In certain types of cancer, the coordinated overexpression of MRP1 and γ-GCS has been reported [20–22]. Again, this may reflect the fact that some tumour cells overexpressing MRP1 can only survive when also upregulating γ-GCS. It is conceivable that the coordinated expression of MRP1 and γ-GCS occurs only under certain stress conditions, presumably as part of a gradual adaptive stress response, as suggested by Tew and colleagues [23].

In the present paper, we have also shown for the first time that MRP1-overexpressing cells were hypersensitive to the γ-GCS inhibitor, BSO. The hypersensitivity was due to the increased rate of export of intracellular GSH, because: (i) BSO induced a more profound loss of GSH in MRP1 producers and in MRP1-transduced cells than in their parental counterparts; (ii) cells transduced with the γ-GCS-MRP1 bicistronic retroviral vector were not hypersensitive to BSO, but rather resistant; (iii) *in vivo* BSO treatment resulted in a significant decrease in the intracellular GSH content of blood erythrocytes of *mrp1* (+/+) mice, but not of *mrp1* (−/−) mice.

The lower steady-state level of GSH in Fly-eco MRP1 and 3T3/MRP1 cells did not result in hypersensitivity to alkylating agents [7]. However, pretreatment with BSO resulted in a greater potentiation of chlorambucil in Fly-eco MRP1 cells than in parental Fly-eco cells. In addition, pretreatment with BSO resulted in a complete reversal of doxorubicin resistance in Fly-eco MRP1 cells. *In vitro* reversal by BSO of the multidrug resistance phenotype in cells overexpressing MRP1 and *in vivo* potentiation of the therapeutic efficacy of doxorubicin by BSO against MRP1-overexpressing experimental tumours were previously reported [24,25]. Furthermore, depletion of intracellular GSH by BSO significantly enhanced the cytotoxic activity of alkylating agents, platinating compounds, and irradiation [26]. Phase I clinical trials of BSO and melphalan have documented the possibility of achieving a consistent

GSH depletion in tumours with little toxicity [26]. Since the concentrations of BSO employed in the present study are well below equivalent steady-state plasma levels of BSO reported in adult human trials [14], the preferential cytotoxic effect of BSO towards MRP1-overexpressing cells could be exploited in cancer therapy.

Co-expression of MRP1 and of the catalytic subunit of γ-GCS resulted in resistance rather than hypersensitivity to the GSH-depleting and cytotoxic activity of BSO. However, we have preliminary evidence that the overexpression of MRP1 and of both catalytic and regulatory subunits of γ-GCS does not result in high-level resistance to BSO (G. Rappa and A. Lorico, unpublished data). Thus, it is conceivable that the overexpression of the catalytic subunit of γ-GCS created an imbalance between the two subunits of γ-GCS, making an excess of catalytic subunit available for sequestration of BSO, without severe consequences for the cell homeostasis.

In many types of human cancer, MRP1 is expressed at relatively high levels [27], while it is expressed at relatively low levels in human bone marrow cells [28]. Before passing to clinical testing of BSO in MRP1-overexpressing tumours, our finding of BSO hypersensitivity of retrovirally transduced MRP1-overexpressing cells needs to be confirmed in appropriate MRP1-overexpressing tumour models. However, it is intriguing that a very high sensitivity to the direct cytotoxic activity of BSO was observed in a panel of neuroblastoma cell lines [29], particularly in those with *N-myc* amplification, which are known to have very high levels of MRP1 gene expression [30]. In addition, *in vivo* direct antitumour activity of BSO alone on neuroblastoma xenografts was previously reported by Busse and colleagues [31].

Our data suggest that in certain types of MRP1-overexpressing tumours, where compensatory homeostatic mechanisms of the GSH pathway are not so efficient to raise GSH steady-state levels above normal, BSO may exert a direct anticancer effect and at the same time modulate the anticancer activity of MRP1 substrates and alkylating agents. The possibility to further increase the level of differential toxicity between tumour and host bone marrow by transducing haematopoietic precursors with a bicistronic γ-GCS-MRP1 retroviral vector is currently being investigated in our laboratory.

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References

1. Cole SP, Bhardwaj G, Gerlach JH, *et al*. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line [see comments]. *Science* 1992, **258**, 1650–1654.
2. Borst P, Evers R, Kool M, Wijnholds J. The multidrug resistance protein family. *Biochim Biophys Acta* 1999, **1461**, 347–357.
3. Zaman GJ, Lankelma J, van Tellingen O, *et al*. Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. *Proc Natl Acad Sci USA* 1995, **92**, 7690–7694.
4. Lorico A, Rappa G, Flavell RA, Sartorelli AC. Double knockout of the MRP gene leads to increased drug sensitivity in vitro. *Cancer Res* 1996, **56**, 5351–5355.
5. Rappa G, Lorico A, Flavell RA, Sartorelli AC. Evidence that the multidrug resistance protein (MRP) functions as a co-transporter of glutathione and natural product toxins. *Cancer Res* 1997, **57**, 5232–5237.
6. Lorico A, Rappa G, Finch RA, Yang D, Flavell RA, Sartorelli AC. Disruption of the murine MRP (multidrug resistance protein) gene leads to increased sensitivity to etoposide (VP-16) and increased levels of glutathione. *Cancer Res* 1997, **57**, 5238–5242.
7. Rappa G, Lorico A, Hildinger M, Fodstad O, Baum C. Novel bicistronic retroviral vector expressing gamma-glutamylcysteine synthetase and the multidrug resistance protein 1 (MRP1) protects cells from MRP1-effluxed drugs and alkylating agents. *Hum Gene Ther* 2001, **12**, 1785–1796.
8. Cosset FL, Takeuchi Y, Battini JL, Weiss RA, Collins MK. High-titer packaging cells producing recombinant retroviruses resistant to human serum. *J Virol* 1995, **69**, 7430–7436.
9. Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* 1991, **3**, 207–212.
10. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969, **27**, 502–522.
11. Hedley DW, Chow S. Evaluation of methods for measuring cellular glutathione content using flow cytometry. *Cytometry* 1994, **15**, 349–358.
12. Gamcsik MP. 13C-Isotopic enrichment of glutathione in cell extracts determined by nuclear magnetic resonance spectroscopy. *Anal Biochem* 1999, **266**, 58–65.
13. Millis KK, Lesko SA, Gamcsik MP. Formation, intracellular distribution and efflux of glutathione-bimane conjugates in drug-sensitive and-resistant MCF-7 cells. *Cancer Chemother Pharmacol* 1997, **40**, 101–111.
14. O'Dwyer PJ, Hamilton TC, LaCreta FP, *et al*. Phase I trial of buthionine sulfoximine in combination with melphalan in patients with cancer. *J Clin Oncol* 1996, **14**, 249–256.
15. Schneider E, Yamazaki H, Sinha BK, Cowan KH. Buthionine sulphoximine-mediated sensitisation of etoposide-resistant human breast cancer MCF7 cells overexpressing the multidrug resistance-associated protein involves increased drug accumulation. *Br J Cancer* 1995, **71**, 738–743.
16. Gamcsik MP, Dubay GR, Cox BR. Increased rate of glutathione synthesis from cystine in drug-resistant MCF-7 cells. *Biochem Pharmacol* 2002, **63**, 843–851.
17. O'Brien M, Kruh GD, Tew KD. The influence of coordinate overexpression of glutathione phase II detoxification gene products on drug resistance. *J Pharmacol Exp Ther* 2000, **294**, 480–487.
18. Machiels JP, Govaerts AS, Guillaume T, *et al*. Retrovirus-mediated gene transfer of the human multidrug resistance- associated protein into hematopoietic cells protects mice from chemotherapy-induced leukopenia. *Hum Gene Ther* 1999, **10**, 801–811.

19. Evers R, de Haas M, Sparidans R, *et al*. Vinblastine and sulfinpyrazone export by the multidrug resistance protein MRP2 is associated with glutathione export. *Br J Cancer* 2000, **83**, 375–383.
20. Ishikawa T, Bao JJ, Yamane Y, *et al*. Coordinated induction of MRP/GS-X pump and gamma-glutamylcysteine synthetase by heavy metals in human leukemia cells. *J Biol Chem* 1996, **271**, 14981–14988.
21. Kuo MT, Bao J, Furuichi M, *et al*. Frequent coexpression of MRP/GS-X pump and gamma-glutamylcysteine synthetase mRNA in drug-resistant cells, untreated tumor cells, and normal mouse tissues. *Biochem Pharmacol* 1998, **55**, 605–615.
22. Kuo MT, Bao JJ, Curley SA, Ikeguchi M, Johnston DA, Ishikawa T. Frequent coordinated overexpression of the MRP/GS-X pump and gamma-glutamylcysteine synthetase genes in human colorectal cancers. *Cancer Res* 1996, **56**, 3642–3644.
23. Tew KD, O'Brien M, Laing NM, Shen H. Coordinate changes in expression of protective genes in drug-resistant cells. *Chem Biol Interact* 1998, **111–112**, 199–211.
24. Vanhoefer U, Cao S, Minderman H, *et al*. d,l-Buthionine-(S,R)-sulfoximine potentiates in vivo the therapeutic efficacy of doxorubicin against multidrug resistance protein-expressing tumors. *Clin Cancer Res* 1996, **2**, 1961–1968.
25. Versantvoort CH, Withoff S, Broxterman HJ, *et al*. Resistance-associated factors in human small-cell lung-carcinoma GLC4 sub-lines with increasing adriamycin resistance. *Int J Cancer* 1995, **61**, 375–380.
26. Bailey HH. L-S,R-Buthionine sulfoximine: historical development and clinical issues. *Chem Biol Interact* 1998, **111–112**, 239–254.
27. Nooter K, Westerman AM, Flens MJ, *et al*. Expression of the multidrug resistance-associated protein (MRP) gene in human cancers. *Clin Cancer Res* 1995, **1**, 1301–1310.
28. Legrand O, Perrot JY, Tang R, *et al*. Expression of the multidrug resistance-associated protein (MRP) mRNA and protein in normal peripheral blood and bone marrow haemopoietic cells. *Br J Haematol* 1996, **94**, 23–33.
29. Anderson CP, Tsai JM, Meek WE, *et al*. Depletion of glutathione by buthionine sulfoxine is cytotoxic for human neuroblastoma cell lines via apoptosis. *Exp Cell Res* 1999, **246**, 183–192.
30. Norris MD, Bordow SB, Marshall GM, Haber PS, Cohn SL, Haber M. Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma. *N Engl J Med* 1996, **334**, 231–238.
31. Busse E, Bartsch O, Kornhuber B. Non-myelotoxic antitumour effects of L-dopa, buthionine sulphoximine and tamoxifen on neuroblastoma cells in vitro and in vivo. *J Cancer Res Clin Oncol* 1991, **117**, 449–453.