D,L-Buthionine-(S,R)-sulfoximine Potentiates in Vivo the Therapeutic Efficacy of Doxorubicin against Multidrug Resistance Protein-expressing Tumors

Udo Vanhoefer, Shousong Cao, Hans Minderman, Karoly Toth, Basil S. Skenderis II, Marilyn L. Slovak, and Youcef M. Rustum

Department of Experimental Therapeutics, Grace Cancer Drug Center [U. V., S. C., H. M., K. T., Y. M. R.] and Department of Surgical Oncology [B. S. S.], Roswell Park Cancer Institute, Buffalo, New York 14263, and Department of Cytogenetics, City of Hope National Medical Center, Duarte, California 91010 [M. L. S.]

ABSTRACT

Intracellular glutathione (GSH) has been implicated as a regulatory determinant of multidrug resistance protein (MRP) function. The objective of the present study was to evaluate in vivo the ability of D,L-buthionine-(S,R)-sulfoximine (D,L-BSO), a potent inhibitor of GSH biosynthesis, to reverse MRP-mediated drug resistance to doxorubicin. Athymic nude mice (nu/nu) bearing advanced parental human fibrosarcoma HT1080 and MRP-expressing HT1080/DR4 tumors were treated with the maximum tolerated dose of doxorubicin (10 mg/kg, i.v. push). This therapy produced an overall response rate of 50% (20% complete response and 30% partial response) in mice bearing parental HT1080 xenografts, whereas no significant antitumor activity against HT1080/DR4 tumors was observed. Treatment of mice bearing HT1080 and HT1080/DR4 xenografts with a continuous i.v. infusion of non toxic doses of D,L-BSO (300 and 600 mg/kg/day) produced a 60% reduction of GSH plasma levels and greater than 95% reduction in GSH tumor levels in both parental and multidrug-resistant tumors; however, this treatment possessed no in vivo antitumor activity by itself. Under these treatment conditions, a combination of D,L-BSO with the maximum tolerated dose of doxorubicin administered at 24 h during a 48-h i.v. infusion of D,L-BSO completely restored the response of MRP-expressing HT1080/DR4 tumors to doxorubicin (overall response rate, 63%; complete response rate, 38%) with no potentiation of host toxicity. The D,L-BSO-induced in vivo reversal of MRP-mediated drug resistance correlated in vitro with the restoration of intracellular doxorubicin retention in cultured HT1080/DR4 cells. Depletion of GSH by D,L-BSO in drug-sensitive HT1080 tumors that do not express MRP did not alter the in vivo response to doxorubicin. Using the same treatment schedule, dose, and administration of doxorubicin with and without D,L-BSO in nude mice bearing P-170 glycoprotein-expressing A2780/Dx5 tumors, no potentiation of the therapeutic index of doxorubicin was found, demonstrating the in vivo selectivity of D,L-BSO-induced GSH depletion on MRP function. The data reported herein indicate that in vivo function of MRP as a mediator of doxorubicin resistance requires the presence of sufficient GSH pools. D,L-BSO may provide an example of an effective in vivo modulator of MRP-mediated drug resistance.

INTRODUCTION

MDR has been associated with overexpression of a 190-kDa efflux pump of the ABC superfamily of transporter proteins (1–5) termed MRP (1). Transfection of a MRP cDNA expression vector into HeLa, SW-1573, and NIH/3T3 cells conferred a MDR phenotype (6–8) associated with alterations in drug accumulation and retention (7–9).

Several agents capable of reversing Pgp-associated MDR, such as verapamil and cyclosporine analogues, have entered clinical trials; however, drug resistance-modifying agents effective in reversal of MRP-mediated MDR are limited (10–14).

Recent studies indicate that MRP is not only an export pump for GSH conjugates (15, 16) but also for glucuronidated xenobiotics (i.e., glucuronosyl-etoposide, Ref. 17). In addition, MRP has been related to the multispecific organic anion transporter (16), thus, conjugation or complex formation of drugs with GSH or negative-charged compounds has been proposed as well. However, the requirement of drug conjugation or complex formation for MRP-mediated drug transport remains controversial (18–20).

In vitro, inhibition of intracellular GSH synthesis by D,L-BSO resulted in increased drug accumulation and retention of anthracyclines (18–20), vincristine (21, 22), etoposide (21), and rhodamine (18) in MRP- but not Pgp-mediated MDR (18–20, 23). Although the mechanisms of involvement of the GSH pathway in MRP-mediated MDR are still poorly understood, these in vitro studies suggest that sufficient intracellular GSH levels are required for MRP-mediated drug transport of at least several xenobiotics.

We recently established MRP-overexpressing xenografts of the multidrug-resistant human fibrosarcoma cell line...
HT1080/DR4 (3, 24) in athymic nude mice (nu/nu) highly resistant to doxorubicin and partially cross-resistant to paclitaxel treatment (14). Pyridine analogue PAK-104P (25), a potent modulator of MRP- and Pgp-mediated MDR function (26, 27), restored in vivo drug sensitivity to doxorubicin (partially) and to paclitaxel (completely, Ref. 14).

The present study investigated the in vivo selectivity of D,L-BSO-induced GSH depletion by comparing its effect on the therapeutic efficacy of doxorubicin against parental HT1080 and multidrug-resistant MRP-expressing HT1080/DR4 xenografts and Pgp- but not MRP-expressing A2780/Dx5 xenografts.

MATERIALS AND METHODS

Mice. Eight to 12-week-old female athymic nude mice (nu/nu; body weight, 20–25 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were kept (five mice/cage) under specific pathogen-free conditions with water and food ad libitum.

Tumor. The human fibrosarcoma cell line HT1080 (parental) and the MDR subline HT1080/DR4 (MRP- and LRP-positive, Pgp-negative) have been characterized earlier (3, 24, 28). Monolayer cultures of HT1080 and HT1080/DR4 cell lines were maintained in Eagle’s MEM supplemented with Earle’s balanced salt solution, 10% heat-inactivated FCS, nonessential amino acids, and L-glutamine. HT1080 and HT1080/DR4 xenografts were established as described previously (14). Nonnecrotic tumor tissue of approximately 50 mg was transplanted s.c. (via trocar), and drug treatments were initiated when tumor sizes reached 150–200 mg.

The human ovarian cancer cell line A2780/wt (parental) and the MDR subline A2780/Dx5 [Pgp-positive (29), MRP-negative data not shown] have been characterized previously. A2780 and A2780/Dx5 xenografts were established in athymic nude mice by injection of 10⁶ cells s.c. Drug treatments were initiated when tumor sizes reached 150–200 mg.

Immunohistochemical Characterization of MRP Expression. Tumor tissue of HT1080 and HT1080/DR4 tumors was processed for paraffin sections (5 μm) after 10% neutral buffered formalin fixation. Microwave antigen retrieval was applied in 6 M urea, and nonspecific staining was blocked with 0.03% casein in PBS as described previously (30). MRP-specific binding of mouse mAb MRPM6 (1.0 μg/ml, Ref. 31; a gift from Prof. R. J. Scheper, Free University Hospital, Amsterdam, the Netherlands) was visualized using a polyclonal biotinylated secondary antibody and streptavidin-peroxidase reagent (Shandon-Lipshaw, Pittsburgh, PA) using 3,3′-diaminobenzidine (Sigma, St. Louis, MO) as chromogen. Isotype-matched mouse IgG1 (Sigma) served as a negative control.

Drug Treatment. Doxorubicin (Farmitalia Carla Erba S.P.A., Milan, Italy) was dissolved in sterile 0.9% saline and injected i.v. at the MTD of 10 mg/kg, D,L-BSO (Sigma Co.) was dissolved in sterile 0.9% saline, filtered through a 0.2-μm polysulfone membrane filter (Whatman, Maidstone, United Kingdom), and administered by 48-h continuous i.v. infusion at a dose of 300 mg/kg/day and 600 mg/kg/day starting at 24 h before doxorubicin administration. All solutions were prepared fresh and used immediately. MTD was defined as the maximum dose that could be administered to tumor-bearing mice without causing drug-related lethality and body-weight loss more than 20%. All studies were performed in accordance with Institutional Animal Care and Use Committee guidelines.

Tumor Measurements and Body Weight. Tumor weight (milligrams) was determined by measurements of the longest axis (L) and short axis (W) of each tumor with a vernier caliper and calculated assuming unit density by the following formula: \( \frac{1}{2} (L \times W^2) \). RTV was calculated using the formula \( \text{RTV} (\%) = \frac{V_f}{V_i} \times V_i \times 100 \), where \( V_f \) represents the tumor volume on day x and \( V_i \) represents the initial tumor volume. As a general policy, mice were sacrificed when the tumor sizes exceeded 2.0 g. To evaluate drug toxicity, body weights of mice were recorded at the time of tumor measurements.

Antitumor Activity. Antitumor activity was assessed by maximum inhibitory rate of the RTV of the treated over the untreated controls. Tumor doubling time was defined as the mean time for the tumor to reach twice the initial size. PR was defined as a reduction in tumor size greater than 50%, and CR was defined as the inability to detect a tumor by palpation at the initial site of the tumor appearance for more than 60 days posttreatment (cures).

GSH Determination. GSH levels in cultured cells, murine plasma, and xenografts were measured according to the GSH reductase recycling method of Griffith (32). To determine GSH content in tumor tissue, samples were washed immediately in ice-cold PBS after tumor excision, then homogenized in 10 volumes of ice-cold 125 mm phosphate buffer (pH 7.4) using 5 strokes of a polytron (Kinematica Inc., Luzern, Switzerland). Samples were centrifuged at 120,000 × g for 20 min at 2°C, and the supernatant was deproteinized with a final concentration of 2% (w/v) sulfosalicylic acid (Sigma). Absorbance was measured at 412 nm for 5 min at 30°C using a computer-connected spectrophotometer (BIO-TEK Instruments, Inc., Winooski, VT). GSH concentrations were calculated by reference to a standard curve that was run with each batch of samples. Results are expressed as nanomoles/10⁶ cells, micromoles/fiber plasma, and nanomoles/gram tumor weight.

Quantitative Flow Cytometric Determination. Drug efflux studies in cultured HT1080 and HT1080/DR4 cells were performed as described previously (14, 27). In brief, cells in exponential growth were pretreated for 24 h with 1–100 μM D,L-BSO or drug-free medium, then exposed for 2 h at 37°C to equicytotoxic concentrations of doxorubicin using the IC₅₀ of HT1080 cells (0.2 μM doxorubicin) and HT1080/DR4 cells (55.0 μM doxorubicin), respectively, or for 20 min to a noncytotoxic equimolar concentration of Rh-123 (13.0 μM; Molecular Probes, Eugene, OR). Samples were then immediately after drug exposure (zero time, drug accumulation) and after 4 h in drug-free medium (drug retention) in the presence or absence of D,L-BSO. Previous studies from our laboratory have shown that the efflux of doxorubicin and Rh-123 in HT1080/DR4 cells is time-dependent (27). Cellular drug concentrations were determined by analyzing cells with a FACSscan (Becton Dickinson, San Jose, CA). Excitation wavelength was 488 nm, and doxorubicin emission was captured using a 650 long-pass filter, whereas Rh-123 emission was captured using a 530/30 nm band-pass filter. Results were calculated and analyzed with Win List software (Verity Software House, Topsham, ME). Cellular
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Fig. 1  Immunohistochemical analysis for MRP expression (mAb MRPm6) on paraffin sections (×600). Nude mice bearing HT1080 (A) and HT1080/DR4 xenografts (B), respectively. C, isotype control (IgG1) for MRPm6.

drug concentrations were calculated as mean Arbitrary Fluorescence Units (A.F.U.).

**Statistical Analysis.** The differences between the mean values were analyzed for significance using the unpaired two-tailed Student’s t test for independent samples; P < 0.05 was considered to be statistically significant.

**RESULTS**

**Immunohistochemical Analysis of MRP Expression.** Expression of MRP in HT1080/DR4 xenografts during repeated tumor passages was confirmed by immunohistochemical analysis on paraffin sections. Applying the MRP-specific mAb MRPm6, a strong membrane-specific immunostaining was found in approximately 70% of the tumor cells in HT1080/DR4 xenografts, whereas no staining was observed in the parental HT1080 xenografts (Fig. 1). The MRP expression is consistent with a previously reported Western blot analysis of these xenografts using a different MRP-specific mAb (MRPr1, Ref. 14). The heterogeneity and intensity of MRP expression in HT1080/DR4 xenografts grown in nude mice and in tumor cells grown as monolayer culture were similar.

**GSH Concentrations in Plasma and Tumor Tissue.** The plasma and tumor tissue levels of GSH in athymic nude mice bearing HT1080 and HT1080/DR4 xenografts were quantitated at 24 h during a continuous i.v. infusion of nontoxic doses of d,l-BSO (300 and 600 mg/kg/day, respectively; Fig. 2).

The plasma level of GSH in control animals was 12.1 ± 1.9 μM. Reduction of plasma GSH by d,l-BSO was dose-dependent and significant at 300 mg/kg/day (P < 0.05) and 600 mg/kg/day (P < 0.01; Fig. 2A). The data indicated that with 600 mg/kg d,l-BSO, the plasma concentration of GSH was reduced by 60%.

The GSH levels in tumor tissue of HT1080 and HT1080/DR4 xenografts of untreated mice were significantly different (P < 0.001) with concentrations of 1.0 and 1.7 μM/g tumor tissue, respectively. Using d,l-BSO at a dose of 600 mg/kg/day the tumor GSH pools were reduced by 95% in HT1080 and 98% in HT1080/DR4 tumors, respectively (Fig. 2B). The data in Fig. 2 demonstrate that d,l-BSO at a dose of 600 mg/kg/day achieved a greater reduction of GSH levels in tumor tissue than in plasma.

**Antitumor Activity.** We have demonstrated previously that treatment of nude mice with the MTD of doxorubicin (10 mg/kg, i.v. push) had no antitumor activity against HT1080/DR4 tumor (14).

The data in Fig. 2 indicate that the reduction of GSH plasma and tumor levels was a function of the d,l-BSO dose. The significant higher GSH concentrations in resistant HT1080/DR4 tumors (1.7 versus 1.0 μM/g in parental HT1080 tumors) were reduced by >98% (Fig. 2B). The therapeutic efficacy of doxorubicin (MTD) in HT1080 and HT1080/DR4 xenografts was evaluated after depletion of cellular GSH by d,l-BSO, and the results are summarized in Table 1 and Fig. 3. The data show that although d,l-BSO dramatically reduced GSH concentrations in both tumors, modulation of the antitumor activity of doxorubicin by d,l-BSO was only achieved in MRP-expressing HT1080/DR4

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Fig. 2. **In vivo** depletion of GSH levels after treatment with d,l-BSO at a dose of 300 mg/kg and 600 mg/kg for 24 h as an i.v. continuous infusion in murine plasma (A) and in tumor tissue of HT1080 and HT1080/DR4 xenografts (B). The results are presented as the mean ± SD of measurements in duplicate of four xenografts/group.

**Table 1** **In vivo** effect of d,l-BSO on the therapeutic efficacy of doxorubicin in athymic nude mice bearing HT1080 and HT1080/DR4 xenografts

Drug treatments were conducted as follows: doxorubicin, 10 mg/kg, i.v. push (MTD); d,l-BSO, 300 mg/kg/day and 600 mg/kg/day continuous i.v. infusion for 48 h starting 24 h before doxorubicin treatment. Eight to 10 mice/group were treated. Experiments were performed in duplicate; no treatment-related death occurred. MIR, maximum inhibitory rate; TD, tumor doubling time in days; MWL, maximum weight loss (% of initial body weight).

<table>
<thead>
<tr>
<th>Drug treatments</th>
<th>HT1080 MIR (%)</th>
<th>TD (days)</th>
<th>CR (%)</th>
<th>PR (%)</th>
<th>MWL (%)</th>
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<td>2.6 ± 1.0</td>
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<td>-</td>
<td>1.2 ± 1.0</td>
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<tr>
<td>d,l-BSO (600 mg)</td>
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<tr>
<td>d,l-BSO (600 mg)</td>
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<td>20</td>
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<td>12.1 ± 3.4</td>
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<table>
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<tr>
<th>Drug treatments</th>
<th>HT1080/DR4 MIR (%)</th>
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<th>CR (%)</th>
<th>PR (%)</th>
<th>MWL (%)</th>
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<tr>
<td>d,l-BSO (600 mg)</td>
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<tr>
<td>d,l-BSO (300 mg)</td>
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<tr>
<td>d,l-BSO (600 mg)</td>
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<td>38</td>
<td>25</td>
<td>25</td>
<td>15.4 ± 2.0</td>
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* P < 0.01 doxorubicin versus doxorubicin + d,l-BSO.
Fig. 3. Induction and time kinetic of tumor response (CR and PR rate) in athymic nude mice (nu/nu) bearing parental HT1080 (A and B) and multidrug-resistant MRP-expressing HT1080/DR4 xenografts (C and D). PR: CR. ■ D.L-BSO at a dose of 600 mg/kg/day was administered by iv. continuous infusion for 48 h. Doxorubicin was given at the MTD (10 mg/kg) iv. push at 24 h. Eight to 10 mice were treated/group. Experiments were performed in duplicate: no treatment-related death occurred.

Fig. 4. Antitumor activity of doxorubicin alone and in combination with D.L-BSO against parental A2780 (A) and multidrug-resistant Pgp-expressing A2780/Dx5 (B) xenografts in athymic nude mice (nu/nu). Treatments were conducted as follows: no drug treatment. ○: doxorubicin 10 mg/kg iv. push (MTD). ●: combination of D.L-BSO (600 mg/kg/day for 48 h iv. infusion) and doxorubicin (10 mg/kg iv. push at 24 h). □.

Data confirm the in vivo selectivity of D.L-BSO-induced GSH depletion on MRP-function.

MRP-mediated in Vitro Doxorubicin Resistance and Drug Retention. The in vitro effect of D.L-BSO-induced GSH depletion on MRP-mediated doxorubicin efflux was
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Fig. 5. Relationship between GSH pools (○), doxorubicin retention (●), and Rh-123 (■) in parental HT1080 (A) and resistant HT1080/DR4 (B) cells. Cells were preincubated for 24 h with 1–100 μM D,L-BSO or drug-free medium. Drug retention of doxorubicin was evaluated using equicytotoxic concentrations [IC50 HT1080, 0.2 μM; IC50 HT1080/DR4, 55.0 μM; Ref. 14], whereas drug retention of Rh-123 was studied using a noncytotoxic equimolar concentration (13.0 μM). Drug retention is expressed as percentage of mean Arbitrary Fluorescence Units (A.F.U.); intracellular GSH content is expressed as percentage of untreated controls. The results are presented as the mean ± SD of three independent experiments.

DISCUSSION

Recent in vitro studies demonstrated that inhibition of intracellular GSH synthesis by D,L-BSO in MRP-expressing tumor cells resulted in restoration of drug retention and reversal of in vitro drug resistance (13, 18–21, 33).

We have recently established the MRP-expressing human fibrosarcoma xenograft HT1080/DR4 and documented expression of MRP and in vivo resistance to doxorubicin (14). The present study demonstrated that: (a) with the highest nontoxic dose of D,L-BSO (600 mg/kg), the plasma and tumor tissue concentrations of GSH were reduced by 60 and 98%, respectively (Fig. 2); data consistent with inhibition of γ-glutamylcysteine synthetase activity by D,L-BSO (34); (b) in the absence of MRP expression (HT1080 xenograft), dramatic in vivo decrease in GSH content of both plasma and human tissues by D,L-BSO was not sufficient to modulate the antitumor activity of doxorubicin. The total response rates to doxorubicin ± D,L-BSO of mice bearing HT1080 tumors were similar (Table 1; Fig. 3, A and B); (c) significant decrease >98% of tumor tissue GSH levels by D,L-BSO in MRP-expressing HT1080/DR4 tumors (Fig. 2B) resulted in complete in vivo reversal of resistance to doxorubicin (Fig. 3, C and D), associated with restoration of intracellular drug retention (Fig. 5). The dose of 600 mg/kg D,L-BSO in combination with the MTD dose of doxorubicin seems to be maximal because the total body-weight loss was less than 15% (Table 1), the limit set for reversible toxicity; and (d) D,L-BSO had no affect on the therapeutic efficacy of doxorubicin in nude mice bearing Pgp-expressing A2780/Dx5 xenografts (Fig. 4B). Preliminary in vitro experiments performed in our laboratory showed that D,L-BSO did not modulate the cytotoxicity of doxorubicin in MDR1 cDNA-transfected cells (data not shown), confirming previous reports that demonstrated the lack of modulatory activity of D,L-BSO on Pgp-mediated MDR (18–20, 23).

Although the importance of MRP in clinical drug resistance has not been fully established, overexpression of MRP mRNA and MRP protein was recently reported in clinical tumor samples (35–37). Phase I clinical trials demonstrated that D,L-BSO can safely be given to patients at doses ranging from 1.5–17.0 g/m² i.v. (38, 39). The doses of D,L-BSO administered to mice in the present study (300 mg/kg and 600 mg/kg) capable of complete in vivo reversal of MRP-mediated resistance to doxorubicin are equivalent to 900 and 1800 mg/m² in man, doses that can be clinically administered with acceptable toxicity.
The role of the GSH pathway in MRP-mediated drug efflux has not been completely elucidated. It has been shown that reduction of intracellular GSH by d,L-BSO had no effect on MRP gene and protein expression (18) and did not alter ATP-dependent efflux of calcein by MRP (19), results that are not consistent with the possibility that consumption of ATP or alterations of the plasma membrane by oxidative stress may be associated with inhibition of MRP function. Furthermore, exposure to GSH ethyl ester restored MRP function after d,L-BSO treatment in vitro (18), demonstrating that the modulation of MRP function by d,L-BSO is directly related to intracellular GSH concentrations. Using inside-out-oriented membrane vesicles of MRP-transfected HeLa/T5 cells, unmodified doxorubicin and daunorubicin seemed not to be substrates for MRP-mediated drug transport (17), contrasting with findings of Paul et al. (40). However, thus far, stable conjugates of anthracyclines with GSH have not been detected in substantial amounts in MDR cells (41).

Recently, glutathionyl-melphalan has been identified as substrate for MRP-mediated drug transport (17). Gluthathione S-transferase activity is increased 1.6-fold in resistant HT1080/DR4 cells (42), and melphalan may serve as substrate for gluthathione S-transferase-mediated GSH conjugation (41, 43). However, we found no cross-resistance to melphalan in HT1080/DR4 cells (resistance factor, 0.9; data not shown) nor to cisplatin, results that are consistent with the lack of significant cross-resistance to alkylating agents in drug-selected MRP-expressing cells (5, 33, 44). Additional in vitro evidence has been generated demonstrating the role of GSH in the maintenance of MRP function, mediating the transport of several xenobiotics (20–22).

In conclusion, d,L-BSO provides an example of an effective and selective in vivo modulator of MRP-mediated MDR. A significant reduction of tumor tissue GSH concentrations by d,L-BSO was necessary to induce complete reversal of resistance to doxorubicin in nude mice bearing MRP-expression tumors. Additional preclinical studies on MRP-transfected cells are needed to understand the mechanism of GSH involvement in MRP-mediated MDR. Because d,L-BSO may potentiate side effects of anthracyclines (i.e., heart toxicity), additional preclinical toxicity studies are warranted. To evaluate the clinical utility of d,L-BSO as a modulator of doxorubicin resistance, clinical trials should be directed to patients with tumors that significantly express MRP (i.e., chronic lymphocytic leukemia, relapsed acute leukemia, non-small cell lung cancer, and esophageal squamous cell carcinoma, Refs. 35–37).

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