ABSTRACT

Resistance of tumor cells to chemotherapeutic drugs may be due to several mechanisms within a single cell line. Resistance to doxorubicin in the human multidrug resistant breast cancer cell line, MCF-7 Adr$, has been attributed to increased glutathione (GSH) S-transferase and GSH peroxidase activity, as well as to increased expression of the mdr1 gene product, P-glycoprotein. We studied the potentiation of doxorubicin activity in these cells by buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, and by verapamil and trans-flupenthixol, agents which interact with P-glycoprotein. Treatment with BSO enhanced the effect of doxorubicin by 1.5-fold, while verapamil or trans-flupenthixol caused a greater reversal of drug resistance. The combination of BSO with trans-flupenthixol produced no further potentiation of doxorubicin activity. However, the combination of BSO with verapamil and doxorubicin caused up to a 10-fold increment in antiproliferative effect. To explore the mechanism by which BSO interacted with this drug combination, we determined whether or not BSO might potentiate the effects of verapamil. These studies demonstrated that the effects of BSO were predominantly due to an increase in verapamil toxicity rather than to doxorubicin toxicity. In addition, when mice received concentrations of BSO in their drinking water sufficient to deplete GSH and were treated with verapamil, the calcium channel blocker was lethal to 9 of 12 mice receiving BSO compared to 1 of 10 control animals receiving verapamil alone. These studies demonstrate that BSO does not markedly increase the pharmacological effect of doxorubicin against MCF-7 Adr$ cells and suggest that alterations in GSH and related enzymes are not a major factor in drug resistance in this cell line. Furthermore, BSO can increase the toxicity of verapamil, a finding which may have important implications for clinical trials.

INTRODUCTION

Doxorubicin is a chemotherapeutic agent used for the treatment of many solid and hematologic malignancies (1). Clinical resistance is often acquired to DOX,4 as well as to many other anticancer drugs, resulting in their loss of therapeutic efficacy. Therefore, a major goal of cancer pharmacology is to elucidate the cellular and molecular basis of drug resistance, and to discover ways to reverse or circumvent it.

Multidrug resistance is one form of drug resistance which has been well characterized in vitro (2, 3). Cell lines selected for the MDR phenotype are resistant to many structurally and functionally dissimilar cytotoxic agents, including DOX and other anthracyclines, the Vinca alkaloids, the epipodophyllotoxins, and colchicine (4, 5). The basis for MDR is believed to be the overexpression of a M, 170,000 membrane glycoprotein (P-glycoprotein) which functions as a putative multidrug transporter, resulting in decreased cellular accumulation of cytotoxic drugs (4, 5). Transfection and expression of the P-glycoprotein gene, mdr1, in drug-sensitive cells reproduces MDR (6, 7). Elevated levels of mdr1 mRNA and P-gp have been found in human tumor samples, suggesting that MDR may also contribute to clinical drug resistance (5, 8, 9).

However, alternative mechanisms for cellular resistance to DOX have been described, and it is likely that clinical DOX resistance is a multifactorial process. For example, the DOX-selected MDR human breast cancer cell line, MCF-7 Adr$, has been shown to possess an amplified and overexpressed mdr1 gene (10), as well as a 7- to 12-fold increase in the activity of selenium-dependent GSH peroxidase and a 45-fold increase in the activity of GSH-S-transferase, the latter resulting from the increased expression of a single anionic (α) GST isozyme (11, 12). Alterations in the activity of GST and other GSH-related enzymes have been identified in several cell lines resistant to alkylating agents (13–15). Among its enzymatic activities, GST conjugates electrophilic xenobiotics to GSH, producing mercapturic acid metabolites that can be excreted in the urine (16). Also, GSTα possesses significant organic (non-selenium-dependent) peroxidase activity (11). Thus, GST may directly conjugate and inactivate DOX, and GSTα and GSH peroxidase may scavenge oxygen free radicals produced secondary to the reduction of DOX to its semiquinone by the cytochrome P-450 system (17). In fact, MCF-7 Adr$ cells demonstrate a reduced formation of hydroxyl radicals in the presence of DOX and an increased tolerance to superoxide compared to MCF-7 sensitive cells (12, 18). These findings suggest that alterations in GSTα or GSH peroxidase enzymes may contribute to DOX resistance, and may constitute one part of a multifactorial basis for DOX resistance in MCF-7 Adr$ cells.

Therefore, it was important to determine whether the combined pharmacological reversal of both GSH and P-gp-mediated resistance to DOX potentiated the cytotoxicity of DOX against MCF-7 Adr$ cells. Recently, several chemosensitizing agents have been identified which modulate GSH- and P-gp-associated drug resistance. The diphenylalkylamine, verapamil, and the thioxanthene, trans-flupenthixol, have been shown to antagonize P-gp-associated MDR by binding to P-gp and increasing drug accumulation (19–22). Buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase, causes a marked decrease in cellular GSH, thus decreasing the activity of GSH-dependent detoxifying enzymes (23). BSO has been shown to sensitize several tumor cell lines in vitro and in vivo to mephalan (24–27) and to cisplatin (27, 28) and DOX (27, 29) in vitro. In particular, it has been reported that BSO increases the activity of DOX in MCF-7 Adr$ cells in vitro (30, 31).

The combined use of BSO, a drug that depletes GSH, with verapamil, a drug that antagonizes P-gp, has been reported to...

Received 6/29/90; accepted 10/2/90.

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This work was supported by National Cancer Institute Grant CA43888 and CA08341 and by Grant CH-49507 from the American Cancer Society.

1 To whom requests for reprints should be addressed, at Section of Medical Oncology, P.O. Box 3333, Stanford University Medical Center, Stanford, CA 94305.

2 Present address, Department of Medicine, S-102C, Stanford University School of Medicine, Stanford, CA 94305.

3 Burroughs-Wellcome Scholar in Clinical Pharmacology.

4 The abbreviations used are: DOX, doxorubicin; BSO, buthionine sulfoximine; GSH, glutathione; GST, glutathione-S-transferase; MDR, multidrug resistance; P-gp, P-glycoprotein; OTZ, 2-oxothiazolidine-4-carboxylate; MCF-7 Adr$, multidrug-resistant subclone of MCF-7 cells.
completely restore sensitivity of MCF-7 AdrR cells to DOX, while each modulator alone was only partially effective (31). This suggested that MDR in this cell line is multifactorial, and can be fully reversed by a combination of appropriately targeted chemosensitizers. However, an important alternative was that BSO enhanced the intrinsic cytotoxicity of verapamil rather than DOX by decreasing the concentration of GSH.

Since the thiols constitute a major cellular mechanism for detoxification of drugs, modulation by BSO may significantly alter the clinical toxicity of many drugs to normal tissues, as well as to tumor cells, thus posing a potential problem for the use of BSO in combination with other chemotherapeutic or chemosensitizing drugs. Therefore, we have studied the effect of BSO on the sensitivity of MDR cells to both verapamil and DOX, have further analyzed the combined effects of BSO with verapamil and trans-flupenthixol on DOX resistance in MCF-7 AdrR cells, and have studied the effect of BSO on verapamil toxicity in mice.

MATERIALS AND METHODS

Cell Culture. MCF-7 human breast cancer cells, and the multidrug-resistant subclone MCF-7 AdrR were kindly supplied by Dr. Kenneth Cowan, National Cancer Institute, Bethesda, MD. They were maintained in exponential growth in Corning 75-cm² tissue culture flasks in RPMI 1640 medium supplemented with 5% fetal bovine serum, in a humidified atmosphere of 5% CO₂ and 95% air. MCF-7 AdrR cells were approximately 200-fold more resistant to doxorubicin than the parental cell line and maintained a stable MDR phenotype while grown in drug-free medium for a period of at least 3 months, after which time they were discarded. Sensitive and resistant cells had a doubling time of 24 h. Cell lines were routinely tested and found to be free of contamination by Mycoplasma or fungi.

In Vitro Drug Sensitivity. To determine the antiproliferative effect of various combinations of drugs we used a modified spectrophotometric assay as previously described (21). Briefly, 1 x 10⁴ cells were plated in each well of 96-well microtiter plates in 100 μl of medium. Cells were allowed to attach and grow for 24 h, at which time 50 μl BSO (Sigma), final concentration 200 μM, or medium alone was added. After an additional 24-h incubation, DOX (Sigma) in the absence or presence of various concentrations of verapamil (Sigma) or trans-flupenthixol (provided by Dr. J. Hytetl, Lundbeck) was added in 50 μl of medium, and cells were incubated for 48 h. For certain experiments, cells were exposed to 10 mM 1,2-oxithiazolidine-4-carboxylate for 2 h prior to and throughout the 48-h drug exposure, as previously described (29). These concentrations of BSO and OTZ alone had no effect on cell growth.

Drugs were dissolved in small amounts of sterile water or ethanol (final culture concentration, <0.1% ethanol) before dilution with medium. Controls were exposed to vehicle-containing medium. Medium was then decanted and cells were stained with 0.5% méthylène blue (Sigma) in 50% ethanol (w/v) and solubilized with sodium /V-lauroyl was then decanted and cells were stained with 0.5% méthylène blue was then decanted and cells were stained with 0.5% méthylène blue for 200 μM BSO for 24 h. However, under these conditions, verapamil alone was more toxic, producing up to 60% inhibition of cell growth in the absence of DOX. Treatment of MCF-7 AdrR cells with BSO alone caused a 1.5-fold increase in the antiproliferative effect of DOX.

To further study the effect of BSO on verapamil toxicity, the effect of various concentrations of verapamil alone on cellular growth was determined in BSO-treated and untreated cells. Pretreatment of MCF-7 AdrR cells with BSO caused a 33-fold increase in the antiproliferative effect of verapamil (Fig. 2A). Conversely, pretreatment of MCF-7 AdrR cells with OTZ, a drug that increases the synthesis of GSH (33), caused a 1.5-fold decrease in the sensitivity of MCF-7 AdrR cells to verapamil (Fig. 2A). The effect of verapamil with BSO on sensitive MCF-7 cells was also studied (Table 1). These cells were intrinsically more sensitive to verapamil than the resistant clone (50%
INCREASED TOXICITY OF VERAPAMIL BY BSO IN MULTIDRUG RESISTANCE

Fig. 1. Inhibition of the growth of MCF-7 AdrR cells by DOX with or without BSO in the absence and presence of verapamil or trans-flupenthixol (FPT); 10,000 cells were plated in each of 96-well microtiter plates in the presence or absence of 200 μM BSO. After a 24-h incubation, DOX in the absence or presence of various concentrations of verapamil or trans-flupenthixol was added, and cells were incubated for an additional 48 h. Cell growth was assessed by a spectrophotometric method as described in "Materials and Methods." Inhibition of cell growth was expressed as a percentage of vehicle-treated controls. Points, mean of quadruplicate determinations; SE < 5%.

Fig. 2. Effect of BSO and OTZ on the anti-proliferative effect of verapamil and trans-flupenthixol (FPT) on MCF-7 AdrR cells. Cell growth was determined as described in Fig. 1. Cells were exposed to 200 μM BSO for 24 h, or 10 mM OTZ for 2 h, prior to and throughout a 48-h exposure to verapamil or trans-flupenthixol. Inhibition of cell growth was expressed as a percentage of vehicle-treated control. Points, mean of quadruplicate determinations from a representative experiment; SE < 5%.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7/S</td>
<td>Verapamil</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Verapamil + BSO</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>trans-Flupenthixol</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>trans-Flupenthixol + BSO</td>
<td>9</td>
</tr>
<tr>
<td>MCF-7 AdrR</td>
<td>Verapamil</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Verapamil + BSO</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>trans-Flupenthixol</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>trans-Flupenthixol + BSO</td>
<td>14</td>
</tr>
</tbody>
</table>

* IC50, concentration of drug that reduced staining to 50% of control. Each value represents the mean of quadruplicate determinations having less than 10% variation between them.

inhibitory concentration, 80 μM versus 200 μM) and were sensitized by BSO by only 13%.

Fig. 1C demonstrates that 5 μM trans-flupenthixol produced a 20-fold potentiation of DOX activity in MCF-7 AdrR cells. At this concentration, trans-flupenthixol alone caused ≤10% inhibition of cell growth. BSO did not augment the antiproliferative effect of DOX in combination with trans-flupenthixol against MCF-7 AdrR (Fig. 1D). Pretreatment with BSO did not alter the toxicity of trans-flupenthixol alone in MCF-7 AdrR cells (Fig. 2B).

The effect of BSO, verapamil, and trans-flupenthixol on cellular accumulation of DOX is shown in Fig. 3. Sensitive MCF-7 cells accumulated 10-fold more DOX than resistant cells. The addition of trans-flupenthixol or verapamil increased accumulation of DOX in the MDR cells in a dose-dependent manner. Pretreatment of cells with BSO did not alter the
INCREASED TOXICITY OF VERAPAMIL BY BSO IN MULTIDRUG RESISTANCE

Fig. 1. Plot showing the effect of verapamil on cell proliferation. Each point represents the mean ± SD of 3-4 determinations from 2 separate experiments.

Fig. 2. Effect of BSO on total glutathione concentration in cell lines and tissues.

Table 2. Effect of BSO on total glutathione concentration in cell lines and tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>+ BSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>8.9 ± 1.8</td>
<td>5.6 ± 1.5</td>
</tr>
<tr>
<td>Heart</td>
<td>1.3 ± 0.3</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>Lung</td>
<td>1.9 ± 0.4</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.2 ± 0.2</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of 6 determinations from 2 separate experiments.

DISCUSSION

These studies demonstrate that the combination of a chemosensitizer with P-gp (verapamil or trans-flupenthixol) with a drug that depletes GSH (BSO) does not increase the sensitivity of MDR cells to DOX beyond that of the chemosensitizer alone (Fig. 1). Rather, the additional effect of BSO in this setting was explained by an increased toxicity of verapamil rather than DOX under conditions causing GSH depletion (Fig. 2). BSO had no additional effect on P-gp-mediated pathways, as no additional DOX accumulation was seen in BSO-treated cells (Fig. 3).

Our results also suggest that neither GSH peroxidase nor GST contribute in a major way to DOX resistance in MCF-7 Adr R cells, since BSO had little effect on DOX sensitivity (Fig. 1). In support of this conclusion are recent experiments in which the transfection of GST R into sensitive or MDR MCF-7 cells produced no further DOX resistance (34). However, our studies are in contrast to others in several respects. For example, both Kramer et al. (31) and Dusre et al. (30) found a greater sensitizing effect of BSO to MCF-7 Adr R cells than the present studies. These differences can be partially explained by differences in experimental conditions and in the assays used. For example, Kramer found a 4-fold potentiation of the effect of DOX by BSO by using measurements of DNA synthesis, and Dusre's group found 4- to 7-fold potentiation by measuring cytotoxicity. We found only a 1.5- to 2.0-fold enhancement by BSO in this setting was explained by an increased toxicity of verapamil rather than DOX.

The apparent increase in DOX toxicity caused by BSO in the presence of verapamil was actually due to an increased sensitivity to verapamil produced by GSH depletion. For example, BSO markedly increased the toxicity of verapamil to MCF-7 Adr R cells in the absence of DOX (Fig. 2A) but had no effect on the toxicity of verapamil in the presence of verapamil (Fig. 2B). BSO had no additional effect on P-gp-mediated pathways, as no additional DOX accumulation was seen in BSO-treated cells (Fig. 3).

Histological examination of the organs from animals treated with BSO and verapamil failed to reveal the site of acute toxicity produced by this combination.
mor cells (30). Yet, Fairchild et al. did not find a change in DOX sensitivity in MCF-7 cells overexpressing a transfected GST\textsuperscript{T} gene (34). It is likely that several components of drug detoxification systems are expressed in cell lines such as MCF-7 Adr\textsuperscript{R} (35), which are highly resistant to chemotherapeutic agents.

Overexpression of both P-gp and GST\textsuperscript{T} have been found in tissues from a variety of human tumors (36), giving impetus for the clinical use of chemosensitizers in combination to modulate multifactorial drug resistance. However, our findings suggested that these combinations might enhance the toxicity of drugs to the host as well. To test this possibility, we studied the effect of BSO on verapamil toxicity in mice and found that GSH depletion by BSO proved lethal to animals receiving this combination (Fig. 4). These results point out the potential dangers inherent in the pharmacological alteration of biochemical systems essential to normal tissues as well as to tumor cells.

The mechanism by which BSO enhanced verapamil toxicity is unproven but is likely to be explained by the metabolism of the calcium channel blocker. Verapamil undergoes extensive hepatic metabolism (80%) by the P-450 enzyme system. For example, the primary metabolism of verapamil is generally attributed to \textit{N}-dealkylation and \textit{O}-demethylation by Phase I cytochrome P-450 enzymes (37–39). The metabolic products of these oxidative reactions produce electrophilic, reactive compounds which may be detoxified by enzymatic conjugation to GSH or reduced by selenium or non-selenium-dependent GSH peroxidases (40, 41). Given the depletion of hepatic GSH (Table 2), it is likely that animals receiving BSO died from the effects of increased systemic concentrations of verapamil or from a toxic metabolite. The marked decrease in cardiac GSH might also diminish the ability of that organ to protect against these compounds.

The mechanism by which GSH depletion sensitizes cancer cells to verapamil is less clear, but is likely to involve decreased cellular inactivation of verapamil by GSH-dependent mechanisms. In support of this conclusion, MCF-7/S cells had lower GSH content than MCF-7 Adr\textsuperscript{R} cells (Table 2) and were more sensitive to the toxic effects of verapamil (Table 1).

Whereas the metabolism of verapamil is mediated by a GSH system, the metabolism of \textit{trans}-flupenthixol is not (42). Accordingly, cellular depletion of GSH by BSO did not affect the intrinsic toxicity of \textit{trans}-flupenthixol, nor alter its chemosensitizing properties in combination with DOX (Figs. 1 and 2; Table 1).

We conclude that the primary effect of BSO on MCF-7 Adr\textsuperscript{R} cells treated with DOX and verapamil is to increase the toxicity of verapamil but not DOX. Therefore, the clinical use of multiple biochemical modulators must be carefully screened in preclinical models to avoid unsuspected toxicities. If BSO is to be given in combination with drugs which modulate P-gp-associated MDR, it will be important to choose a chemosensitizer which is not metabolized by the GSH system.

ACKNOWLEDGMENTS

We thank K. H. Cowan for providing the MCF-7 Adr\textsuperscript{R} cell line and Mae Day for typing the manuscript.

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Effect of Buthionine Sulfoximine on Toxicity of Verapamil and Doxorubicin to Multidrug Resistant Cells and to Mice

James M. Ford, Jin-ming Yang and William N. Hait


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