Biochemical Pharmacology, Vol. 55, pp. 349–359, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00480-2

Potentiation of Treosulfan Toxicity by the Glutathione-Depleting Agent Buthionine Sulfoximine in Human Malignant Glioma Cells

The Role of Bcl-2

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ABSTRACT. Median survival of human malignant glioma patients is less than one year even with cytoreductive surgery and postoperative radiotherapy. Adjuvant chemotherapy has been rather ineffective. Here, we studied the potentiation by L-buthionine-[S,R]-sulfoximine (BSO), a glutathione-depleting agent, of anticancer drug actions on two human malignant glioma cell lines, LN-229 and T98G. LN-229 has wild-type p53 status, T98G is mutant for p53. Glutathione levels were depleted by BSO with similar kinetics in both cell lines. Only LN-229 cells were growth-inhibited by BSO. BSO had minor effects on the toxicity of doxorubicin, ACNU (1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chloroethyl)-3-nitrosourea, nimustine) and vincristine. BSO failed to alter teniposide or cytarabine toxicity. BSO induced prominent sensitization to the alkylating agent, treosulfan, in both cell lines, as assessed by viability assays, *in situ* DNA end labeling and quantitative DNA fragmentation. Treosulfan is thought to mediate toxicity via formation of reactive epoxides. In the absence of BSO, treosulfan had little acute cytotoxic and moderate antiproliferative effects. Synergistic glioma cell cytotoxicity induced by treosulfan and BSO was not associated with reactive oxygen species formation. Ectopic expression of bcl-2 did not alter basal glutathione levels but attenuated glutathione depletion induced by BSO. Bcl-2 provided only moderate protection from synergistic induction of glioma cell death by treosulfan and BSO. Glutathione depletion may play a role in BSO-mediated chemosensitization, but other mechanisms are probably involved as well. BSO may be a useful agent for glioma cell sensitization to specific chemotherapeutic drugs such as treosulfan. BIOCHEM PHARMACOL **55**;3:349–359, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. treosulfan; apoptosis; chemotherapy; malignant glioma; glutathione; BSO

Malignant gliomas are the most common intrinsic human brain tumors. Established therapeutic approaches include cytoreductive surgery and postoperative radiotherapy. This therapy results in median survival of less than one year. A minority of glioma patients benefit from adjuvant chemotherapy. The major drugs used for the adjuvant chemotherapy of malignant gliomas are the nitrosoureas, BCNU¶ (1,3-bis(2-chloroethyl)-1-nitrosourea, carmustine), ACNU (1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chloroethyl)-3-nitrosourea, nimustine), and, as part of the PCV (procarbacine, CCNU, vincristine) protocol, CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, lomustine). Part of the failure of chemotherapy may be caused by the blood-brain barrier which shields at least the tumor margins from cancer chemotherapy and by the common practice of concurrent steroid treatment which stabilizes the blood-brain barrier and provides protection from chemotherapy at the subcellular level [1].

The molecular mechanisms underlying intrinsic drug resistance of human malignant glioma cells may involve loss of wild-type p53 activity and enhanced expression of antiapoptotic proteins of the bcl-2 family [2–4] and of cytoprotective, drug-detoxifying enzymes including the glutathione system [5–7]. Thus, elevated glutathione levels or enhanced expression of glutathione transferase (RX: glutathione R-transferase; E.C. 2.5.1.18) have been attributed a role in glioma cell resistance to BCNU [5, 8] and the topoisomerase I inhibitor, camptothecin-11 [9]. Sensitization to BCNU has been observed in some glioma cells after preexposure to the glutathione-depleting agent BSO [8, 10], an inhibitor of glutathione synthase (γ-L-glutamyl-L-cysteine:glycine ligase, E.C. 6.3.2.3), the rate-limiting enzyme of glutathione synthesis. A similar BSO-based approach has been used to sensitize human glioma xenografts

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¶*Abbreviations:* ACNU, 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chloroethyl)-3-nitrosourea); BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BSO, L-buthionine-[S,R]-sulfoximine; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; DCF-Hr_2, 2′,7′-dichlorodihydrofluorescein diacetate.

Received 16 April 1997; accepted 5 August 1997.

to radiotherapy [11]. In contrast, BSO failed to induce relevant sensitization of rat glioma cells to ACNU, vinblastine, doxorubicin, or irradiation [12]. The purpose of the present study was to analyze the effects of BSO on glioma cell responses to cancer chemotherapy drugs with different modes of action *in vitro*.

MATERIALS AND METHODS

Materials

Treosulfan (L-threitol 1,4-bismethane sulfonate, dihydrobusulfan, OVASTAT®) (MW 278.3) was kindly provided by Medac. The drug was dissolved in H_2O and stored in aliquots at −20°. L-buthionine-[S,R]-sulfoximine (BSO) and all other reagents, unless indicated otherwise, were obtained from Sigma. 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-Hr_2) was from Molecular Probes.

Cell Culture

T98G human malignant glioma cells were obtained from the American Type Culture Collection (ATCC). LN-229 human malignant glioma cells were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland). LN-229 is wild-type for p53, T98G is mutant for p53 [13, 14]. The glioma cell lines were maintained in Dulbecco's minimal essential medium containing 10% foetal calf serum, 2 mM glutamine and 1% penicillin/streptomycine as previously described [15, 16]. Pooled murine bcl-2-transfected LN-229 and T98G cells were obtained by electroporation with the BMG mbcl-2 plasmid [2]. Repeated immunoblot analyses confirmed that ectopic bcl-2 expression remained stable during the course of the experiments. Mock-transfected cells carry the plasmid with the neomycin resistance gene but no cDNA insert and are herein referred to as neo control cells. The cells were regularly checked for mycoplasmic contamination.

Viability Assays

The cytotoxicity and growth inhibition experiments were performed in 96-well plates. In most experiments, the cells were pretreated with BSO for 15 hr and subsequently cotreated with BSO and cytotoxic drugs for 24 hr. Viability was assessed either at the end of drug exposure or, alternatively, the drugs were removed and the cells cultured in drug-free complete medium for 2 and 7 days. Cell numbers per well were 12×10^3 for experiments without recovery, 7.5×10^3 for LN-229 and 5×10^3 for T98G in experiments with recovery for 2 days, and 1.5×10^3 for both cell lines in experiments with recovery for 7 days. Cell survival and growth were determined by crystal violet assay. The cell culture medium was removed and surviving cells stained with 0.5% crystal violet in 20% methanol for 20 min at room temperature. The plates were washed extensively under running tap water, air-dried and optical density values read in an ELISA reader at 550 nm wave length. For trypan blue dye exclusion, detached and adherent cells were harvested by centrifugation and trypsinization, pooled, centrifuged, mixed with 0.1% trypan blue solution in Hank's balanced salt solution (V/V) for 5 min, and assayed for dye exclusion. DNA fragmentation was quantified by fluorometry as described [15]. Single and double strand DNA breaks at the single cell level were detected by *in situ* DNA end labeling [15, 17].

Determination of Glutathione Levels

Total glutathione levels (GSH and GSSG) were determined by an enzymatic microtiter assay as described [18]. Differential assessment of cytosolic and mitochondrial glutathione was achieved by adapting the digitonin disruption method described by Meredith and Reed [19] for the glioma cell lines. The purity of the cytosolic and mitochondrial fractions was ascertained using lactate dehydrogenase activity as a cytosolic marker and citrate synthase as a mitochondrial marker. Cross contamination according to these enzyme assays was below 4%.

Determination of Reactive Oxygen Species Formation

Reactive oxygen species were detected using DCF-Hr_2 fluorescence as previously described [20]. At different time points after drug treatment, the cells were incubated with DCF-Hr_2 (1 μg/mL) for 30 min, washed with PBS, and fluorescence read at 485 nm excitation and 530 nm emission in a CytoFluor 2350 plate reader (Millipore).

Immunoblot Analysis

Immunoblot analysis of bcl-2-transfected glioma cells was performed as described [2].

Statistical Analysis

EC_{50} values were obtained by linear regression analysis. Statistical comparisons were performed by *t*-test at $P = 0.05$, 0.01 and 0.001.

RESULTS

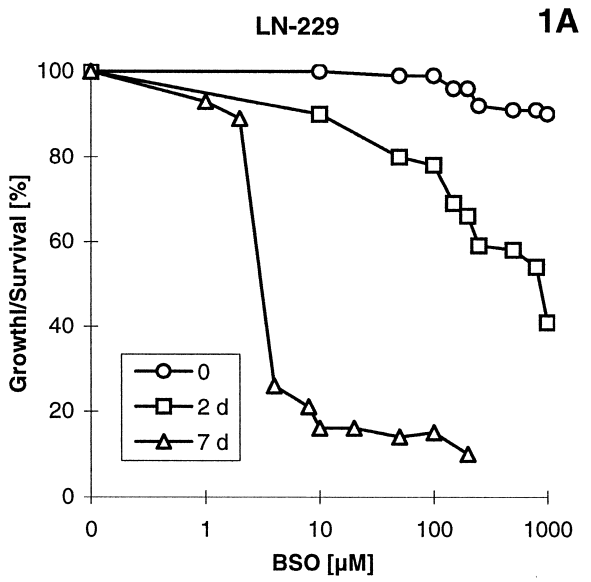
Effects of BSO on Viability and Glutathione Levels in LN-229 and T98G Human Malignant Glioma Cells

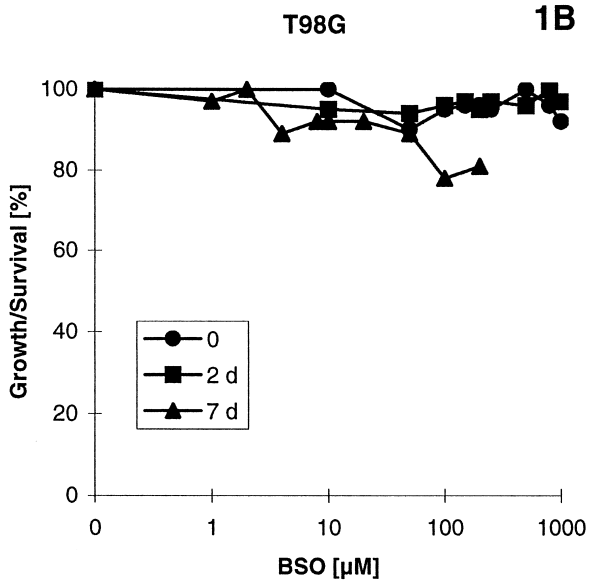
The purpose of this study was to evaluate whether the cytotoxic and antiproliferative actions of chemotherapeutic drugs on human malignant glioma cells can be augmented by a glutathione-depleting agent, BSO. The first set of experiments was designed to assess the effects of BSO on two well-characterized human glioma cell lines, LN-229 and T98G, when administered alone. We intended to cotreat with BSO and cytotoxic drugs for 24 hr and to pretreat with BSO sufficiently long to assure that glutathione levels were depleted at the beginning of cytotoxic drug exposure. Preliminary experiments resulted in the paradigm

eventually chosen, that is, 15 hr preexposure to BSO and 24 hr coexposure to BSO and cytotoxic drugs (see below). Concentrations of BSO up to 1 mM were not acutely cytotoxic to the glioma cells (Fig. 1A,B, circles). However, when BSO was removed after exposure for 39 hr and the cell cultures monitored for several days, prominent inhibitory effects of BSO became apparent in LN-229 cells (Fig. 1A). The loss of optical density readings in LN-229 cells treated with BSO for 39 hr and monitored for 2–7 days thereafter was mainly due to inhibition of proliferation and not to BSO-induced cytotoxicity as assessed by light microscopy and trypan blue dye exclusion (data not shown). In contrast, T98G were refractory to cytotoxic or antiproliferative effects of BSO even in long-term assays (Fig. 1B). Exposure to BSO for 15 hr resulted in a loss of glutathione exceeding 80%, with EC_{50} values of 1.5 ± 0.2 μM for LN-229 and 3.2 ± 0.4 μM for T98G cells. This difference was significant ($P < 0.05$, *t*-test) (Fig. 1C). Even excessive concentrations of BSO did not fully deplete intracellular glutathione stores. Differential assessment of cytosolic and mitochondrial glutathione showed that BSO preferentially depleted cytosolic glutathione, which accounted for more than 90% of total glutathione in both cell lines, whereas the mitochondrial glutathione pool was rather refractory to BSO-mediated glutathione depletion. However, after 15 hr exposure to BSO, the time frame shown in Fig. 1C, there was also a 50% loss of mitochondrial glutathione, suggesting that there was some transfer of glutathione from mitochondria to cytosol under conditions of severe cytosolic glutathione depletion. This contrasted with the effects of ethacrynic acid (100 μM), which rapidly depleted both cytosolic and mitochondrial glutathione within 2 hr (data not shown).

Selective Potentiation of Treosulfan Toxicity by BSO

LN-229 or T98G cells were either not pretreated or pretreated with BSO and then exposed for 24 hr to increasing concentrations of doxorubicin, ACNU, teniposide, cytarabine, vincristine, or treosulfan, in the absence or presence of BSO, using the three time schedules shown in Fig. 1A,B. The concentrations of BSO were chosen based on the data shown in Fig. 1A,B and were intended not to





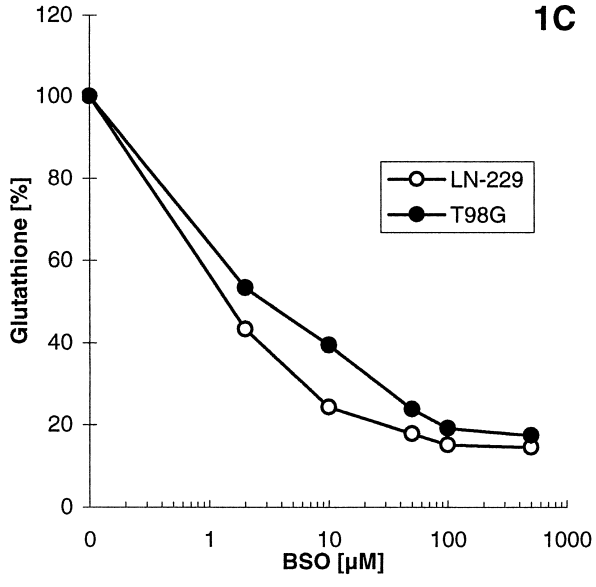


FIG. 1. Effects of BSO on viability and glutathione levels in LN-229 and T98G human malignant glioma cells. LN-229 (open symbols, A) or T98G (filled symbols, B) cells were treated with increasing concentrations of BSO for 39 hr. Cell survival and growth were assessed by crystal violet staining either immediately thereafter (circles) or after a recovery of 2 (squares) or 7 days (triangles). In Fig. 1C, glutathione levels were measured enzymatically (for details, see "Materials and Methods") in LN-229 (open circles) and T98G (filled circles) treated with increasing concentrations of BSO for 15 hr. The basal levels of glutathione were 4.6 ± 0.6 nmol/mg protein for LN-229 and 5.6 ± 1.5 nmol/mg protein for T98G cells. Data are expressed as mean percentages relative to untreated controls ($n = 5$, 1A,B; $n = 3$, 1C). SEM were below 5%.

TABLE 1. BSO-mediated sensitization to drug cytotoxicity and growth inhibition is drug-specific[a]

	LN-229 EC_{50} [μM]	LN-229 plus BSO EC_{50} [μM]	T98G EC_{50} [μM]	T98G plus BSO EC_{50} [μM]
Doxorubicin				
A	2 ± 0.1	1.3 ± 0.1*	4.6 ± 0.3	2.5 ± 0.2*
B	0.13 ± 0.04	0.07 ± 0.02	0.06 ± 0.01	0.06 ± 0.02
C	0.03 ± 0.004	0.013 ± 0.003	0.04 ± 0.007	0.03 ± 0.005
ACNU				
A	>900	690 ± 24^{+}	>900	>900
B	>240	>240	>240	>240
C	24 ± 3	17 ± 3	>80	75 ± 5^{+}
Teniposide				
A	>160	>160	>160	>160
B	0.9 ± 0.1	0.6 ± 0.1	1.6 ± 0.2	1.8 ± 0.2
C	0.8 ± 0.1	0.8 ± 0.1	0.1 ± 0.02	0.1 ± 0.01
Cytarabine				
A	>100	>100	>100	>100
B	21 ± 2	19 ± 3	37 ± 3	35 ± 1
C	12 ± 1	10 ± 1	9 ± 1	8 ± 1
Vincristine				
A	>50	>50	26 ± 2	2.5 ± 0.2**
B	0.013 ± 0.002	0.012 ± 0.002	0.012 ± 0.001	0.003 ± 0.002*
C	0.012 ± 0.002	0.01 ± 0.003	0.004 ± 0.0004	0.001 ± 0.0002*
Treosulfan				
A	>80	7.2 ± 0.3^{+}	>160	45 ± 5^{+}
B	21 ± 2	4 ± 0.5**	31 ± 3	18 ± 2*
C	5.7 ± 0.4	4.4 ± 0.2*	8.1 ± 0.4	3.1 ± 0.2**

[a] LN-229 or T98G human malignant glioma cells were pretreated with BSO for 15 hr and subsequently cotreated for 24 hr with BSO and increasing concentrations of either treosulfan, doxorubicin, ACNU, teniposide, cytarabine or vincristine. Survival and growth were determined either immediately after exposure (A) or after 2-day recovery (B) or after 7-day recovery (C). The BSO concentrations for LN-229 and T98G cells, respectively, were 1 mM for A, 10 μM (LN-229) and 1 mM (T98G) for B, and 2 μM (LN-229) and 50 μM (T98G) for C. These BSO concentrations had no effect when administered alone (see Fig. 1A,B). Data are expressed as means and SEM of EC_{50} values [μM] except for experiments where an EC_{50} was not achieved at meaningful concentrations of cytotoxic drugs ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, t-test, BSO-cotreated cells compared with controls exposed to cytotoxic drug only; $^{+}$indicates relevant difference that cannot be subjected to statistical analysis because EC_{50} values were not achieved).

have toxic effects of their own but to be sufficiently high to allow synergy to be detected. Table 1 indicates that there was moderate augmentation of doxorubicin actions in all three assays and of acute ACNU toxicity in LN-229 cells. Vincristine effects were significantly enhanced in T98G cells in all three paradigms but never in LN-229 cells. BSO had no effects on glioma cell responses to teniposide or cytarabine. Note that very high concentrations of BSO were required for the moderate augmentation of ACNU toxicity in LN-229 cells and doxorubicin toxicity in both cell lines, exceeding 100-fold the EC_{50} value for BSO-mediated glutathione depletion, raising the possibility that other mechanisms are involved in this BSO effect. BSO did not enhance the cytotoxicity or growth inhibition induced by any of these drugs to the same degree as that of treosulfan (Table 1). The actions of treosulfan on glioma cells in the absence of BSO are summarized in more detail in Fig. 2. Here, LN-229 or T98G cells were exposed to treosulfan for 2, 8 or 24 hr and viability assessed immediately thereafter or after 2 or 7 days. There was no acute toxicity even with prolonged exposure for 24 hr at 100 μM (Fig. 2A,B). However, toxic and antiproliferative effects of treosulfan became apparent with increasing time of observation (2–7 days) after treatment. These effects became more prominent with increasing times of exposure (2–24 hr) (Fig. 2C–F). Fig. 3 extends the data on treosulfan toxicity in the presence of BSO shown in Table 1 and serves to illustrate that there was potentiation of treosulfan toxicity by BSO over a broad range of concentrations of BSO (Fig. 3A–F) and treosulfan (Fig. 3G–L) in both cell lines. Next, we sought to determine the mode of cell death induced by coexposure to treosulfan and BSO. Enhanced toxicity induced by coexposure to BSO and treosulfan compared with exposure to either agent alone also became apparent at the level of DNA lesions detected by *in situ* DNA end labeling (Fig. 4A) and quantitative DNA fluorometry (Fig. 4B), consistent with apoptotic cell death. To further study the mechanisms underlying synergistic glioma cell killing induced by treosulfan and BSO, we measured the formation of reactive oxygen species in LN-229 or T98G cells exposed to BSO, treosulfan or both for 0–24 hr, as assessed by DCF-Hr_2 fluorescence [20]. In these experiments, free radical formation was measured at 2.5, 11 and 24 hr after exposure to concentrations of BSO and treosulfan that were strongly cytotoxic when coadministered. No increase in reactive oxygen species was detected. As a positive control, ethacrynic acid (10–100 μM), an agent that depletes cytoplasmic and mitochondrial glutathione levels, induced free radical formation, peaking within 30 and 180 min of exposure to levels exceeding the baseline by 800–900% (Table 2).

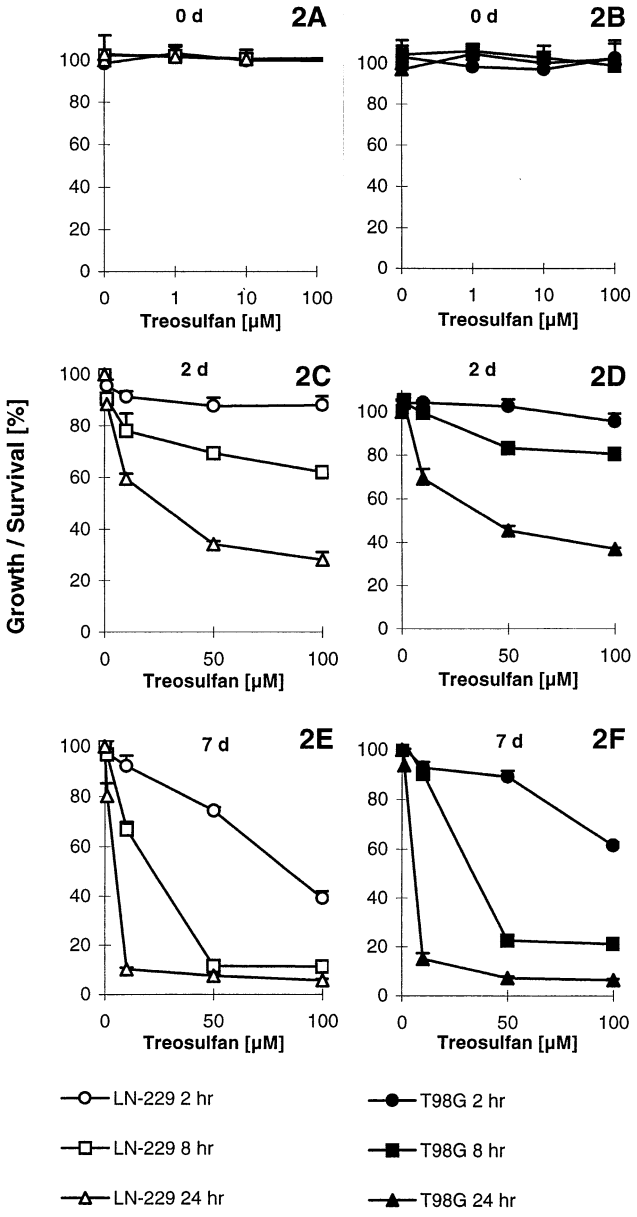


FIG. 2. Treosulfan inhibits the growth of human malignant glioma cells in a concentration- and time-dependent fashion. LN-229 (left panel, open symbols) or T98G (right panel, closed symbols) cells were exposed to increasing concentrations of treosulfan for 2 (circles), 8 (squares) or 24 hr (triangles). Glioma cell survival or growth was assessed either immediately after treosulfan exposure (upper panels) or after 2 (middle panels) or 7 days (bottom panels). Data are representative of triplicate experiments repeated three times with similar results and are expressed as mean percentages and SEM.

Effects of a Murine Bcl-2 Gene Transfer on Endogenous Glutathione Levels and BSO-Mediated Sensitization to Treosulfan

The bcl-2 protein plays a key role in tumor cell resistance to chemotherapy and has been suggested to act in an antioxidant pathway. Therefore, we investigated the role of bcl-2 in the sensitivity to treosulfan/BSO cotreatment. Both LN-229 and T98G cells express high levels of endogenous bcl-2 protein [15]. The generation of LN-229 and T98G cells engineered to express high levels of murine bcl-2 protein has been described [2] (Fig. 5A). Pooled bcl-2 transfectants have been shown to acquire partial resistance to CD95 antibodies, CD95 ligand and various cytotoxic drugs [2, 3]. They are also more resistant to the broad spectrum kinase inhibitor, staurosporine, a positive control for bcl-2-mediated protection from apoptosis [14] (Fig. 5B). In contrast to neural cell lines forced to express bcl-2 [21, 22], pooled bcl-2-transfected LN-229 and T98G cells did not have significantly higher intracellular glutathione levels than neo control cells (Fig. 5C). The mean glutathione values were 4.6 ± 0.6 and 5 ± 0.7 nmol/mg protein for neo and bcl-2 LN-229 cells ($n = 6$, $P = 0.27$, t-test) and 5.6 ± 1.5 and 5.7 ± 0.8 nmol/mg protein for neo and bcl-2 T98G cells ($n = 6$, $P = 0.76$, t-test). We then determined whether ectopic expression of bcl-2 would alter the kinetics of glutathione depletion after BSO challenge (Fig. 5D,E). There was no significant difference in the maximal degree of glutathione depletion achieved. However, there was a significant protective effect of bcl-2 at lower concentrations of BSO as shown in Fig. 5F. At 2.5 μM BSO, glutathione levels fell to 55 ± 5% in bcl-2 cells compared with 36 ± 1% in neo cells of the LN-229 cell line and to 91 ± 1% in bcl-2 cells instead of 53 ± 3% in neo cells of the T98G cell line ($P < 0.05$, t-test). The EC_{50} values for BSO-mediated glutathione depletion were significantly higher in bcl-2 cells than in neo control cells (LN-229 neo: 1.5 ± 0.1, LN-229 bcl-2: 3.4 ± 0.3, T98G neo: 3.1 ± 0.3, T98G bcl-2: 7.2 ± 0.4, $P < 0.05$, t-test). The antiproliferative effects of BSO on LN-229 cells (Fig. 1A) were unaffected by ectopic expression of bcl-2 (data not shown).

Next, we examined whether ectopic expression of bcl-2 modulated the cytotoxic and antiproliferative effects of treosulfan in the absence or presence of BSO. In the absence of BSO, bcl-2-transfected cells were as resistant to acute cytotoxic effects of treosulfan as neo control cells (Fig. 6A,B). With increasing length of recovery from treosulfan exposure, moderate cytoprotective effects of bcl-2 became apparent (Fig. 6C–F). Ectopic expression of bcl-2 did not prevent the dramatic BSO-mediated sensitization to acute treosulfan toxicity in either cell line (Fig. 6A,B). However, at low concentrations of treosulfan, the potentiating effect of BSO on drug toxicity was consistently less prominent in bcl-2-transfected than in neo control cells. This moderate bcl-2 effect was observed in all three paradigms of treosulfan actions, that is, assessment of drug effects immediately after exposure, after 2-day recovery or after 7-day recovery, and was overcome by increasing the concentrations of treosulfan.

DISCUSSION

Treosulfan is a bifunctional alkylating prodrug agent that is active against ovarian cancer in human patients and against human breast and small and non-small cell lung cancer xenografts in nude mice [23–25]. Treosulfan is structurally related to busulfan but differs in the mechanism of alkyla-

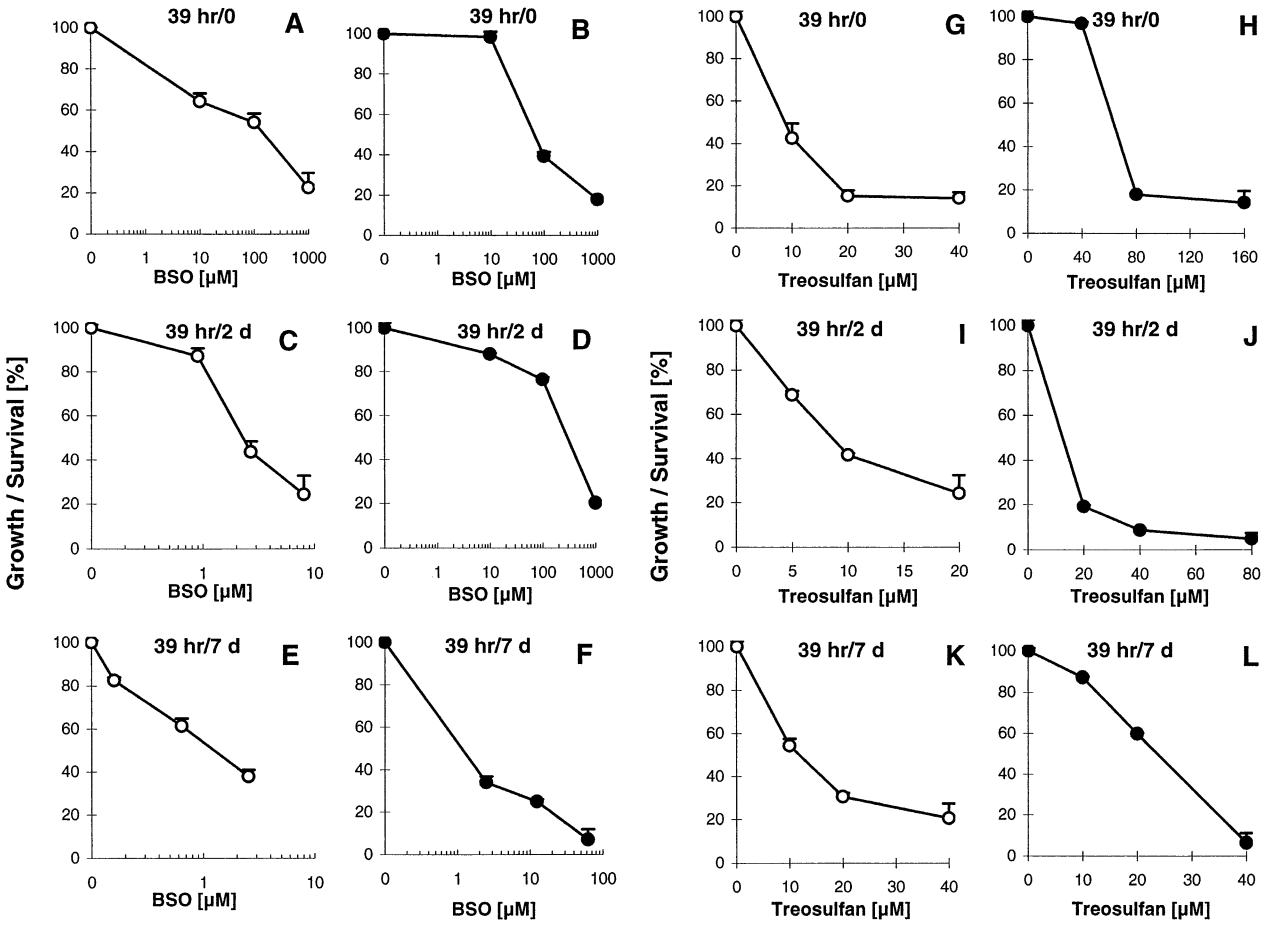


FIG. 3. BSO sensitizes human malignant glioma cells to treosulfan-induced cytotoxicity and growth inhibition. Left panels: LN-229 (A,C,E) or T98G (B,D,F) cells were pretreated with increasing concentrations of BSO for 15 hr and cotreated for 24 hr with increasing concentrations of BSO in the presence of a fixed concentration of treosulfan. The concentrations of treosulfan were 10 μM (A), 80 μM (B), 20 μM (C,D), 10 μM (E) and 40 μM (F). Survival was assessed immediately after treatment (A,B) or after recovery for 2 (C,D) or 7 (E,F) days. Right panels: LN-229 (G,I,K) or T98G (H,J,L) cells were pretreated with fixed concentrations of BSO for 15 hr and cotreated for 24 hr with fixed concentrations of BSO and increasing concentrations of treosulfan. The concentrations of BSO were 1 mM (G,H,J), 10 μM (I), 2 μM (K) or 50 μM (L). Survival was assessed immediately after treatment (G,H) or after recovery for 2 (I,J) or 7 days (K,L). Data are representative of triplicate experiments repeated three times with similar results and are expressed as mean percentages and SEM.

tion, in that reactive nucleophilic monoepoxides and diepoxides are thought to mediate treosulfan toxicity whereas methansulfonyloxy groups mediate busulfan toxicity. The present study shows that exposure to BSO depletes glutathione levels in human glioma cells to less than 20%. The residual glutathione detected after 15 hr exposure to BSO can be attributed to the mitochondrial glutathione pool that is rather refractory to BSO [8]. Coexposure of human malignant glioma cells to treosulfan and BSO induces strong synergistic antiglioma activity in acute cytotoxicity and growth inhibition assays (Fig. 3). The acute toxicity of treosulfan evolving in the presence of BSO fulfills criteria of apoptotic cell death (Fig. 4). The toxicity is not mediated by reactive oxygen species. Synergistic killing was achieved in a glioma cell line with wild-type p53 status, LN-229, and in a cell line with mutant p53, T98G [13, 14]. Ectopic expression of bcl-2 provides only modest protection from cotreatment with treosulfan and BSO (Figs. 5,6).

This is the first study to characterize treosulfan as a potentially effective antiglioma agent and the potentiation of treosulfan toxicity by BSO. We find that the potentiating effect of BSO is rather selective for treosulfan, since the toxicity of other drugs including ACNU and doxorubicin is either unaffected or only moderately enhanced by BSO (Table 1). A review of the literature shows that the consequences of BSO exposure and glutathione depletion for susceptibility to drug toxicity are both cell type- and drug-specific. Thus, expression of antisense cDNA to glutathione S-transferase in a human colon cancer cell line induced sensitization to adriamycin, cisplatin, melphalan and etoposide but not to taxol, vincristine, 5-fluorouracil or mitomycin C [26].

How BSO selectively induces glioma cell vulnerability to treosulfan toxicity is at present unclear. There may be more than one mechanism. Although BSO depleted glutathione levels at the concentrations required for potentiation of

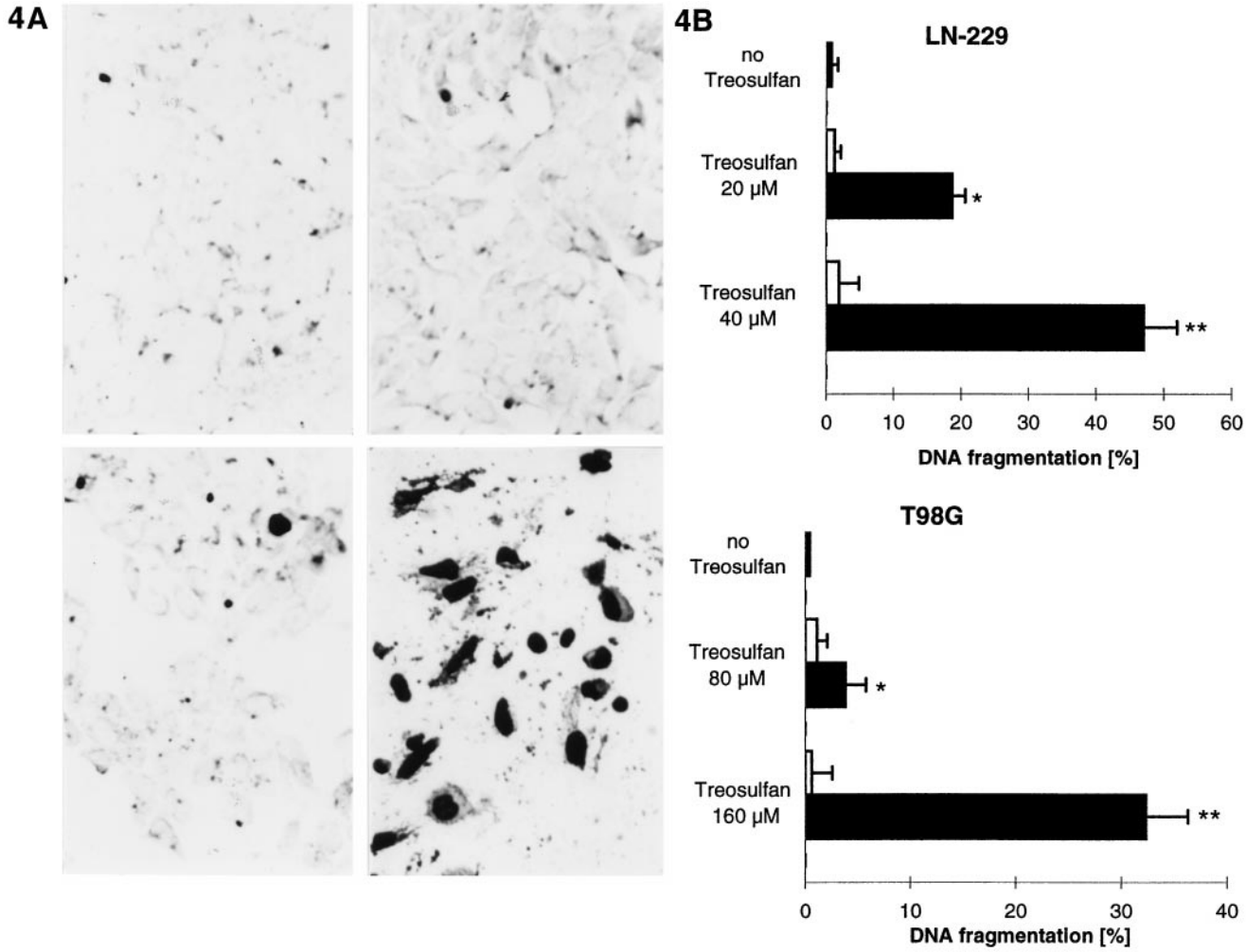


FIG. 4. Induction of DNA fragmentation in human malignant glioma cells by coexposure to treosulfan and BSO. A) LN-229 cells were either untreated (upper left) or exposed to 1 mM BSO (upper right) or 40 µM treosulfan (lower left) or both (lower right) for 24 hr. DNA breaks were detected by *in situ* DNA end labeling [17]. B) LN-229 (upper panel) or T98G (lower panel) cells were either untreated or exposed to treosulfan in the absence (open bars) or presence of 1 mM BSO (filled bars). DNA fragmentation was quantified fluorometrically as previously described [15]. Data are expressed as means and SEM of percent DNA fragmentation (n = 3, *$P < 0.05$, **$P < 0.01$, ANOVA; treosulfan plus BSO compared with either agent alone or untreated cells).

TABLE 2. Coexposure of glioma cells to treosulfan and BSO fails to induce the formation of reactive oxygen species

	Reactive Oxygen Species (%, arbitrary units)	
	2.5 hr	**11 hr**
Untreated	103 ± 3	106 ± 4
Treosulfan	99 ± 6	104 ± 2
BSO	104 ± 2	99 ± 5
Treosulfan plus BSO	101 ± 8	105 ± 3
	30 min	120 min
Ethacrynic acid	487 ± 18	874 ± 46

LN-229 cells were untreated or exposed to treosulfan (40 µM) or BSO (1 mM) or both for 2.5 or 11 hr or exposed to ethacrynic acid (10 µM) for 30 or 120 min. Data are expressed as mean percentages and SEM (n = 3) of increase in arbitrary fluorescence units over untreated controls that were normalized to 100 at the respective time points.

treosulfan toxicity in the glioma cells (Figs. 1C and 3), much higher concentrations of BSO were required to potentiate acute treosulfan toxicity than to deplete glutathione in T98G cells. However, these differences between the cell lines are no longer prominent when monitoring for long-term effects of treosulfan/BSO coexposure (Fig. 3E and F), clinically presumably the most relevant paradigm. Here, BSO potentiates the treosulfan effects in a concentration-dependent manner in a range of concentrations in both cell lines similar to those required for initial glutathione depletion (Fig. 1C). Thus, long-term effects of BSO on treosulfan toxicity can be linked to initial glutathione depletion, but potentiation of acute treosulfan toxicity is likely to involve other mechanisms.

We excluded enhanced formation of reactive oxygen species as the mediator of synergistic glioma cell killing by treosulfan and BSO. The pathway underlying synergy of treosulfan and BSO was also rather refractory to cytopro-

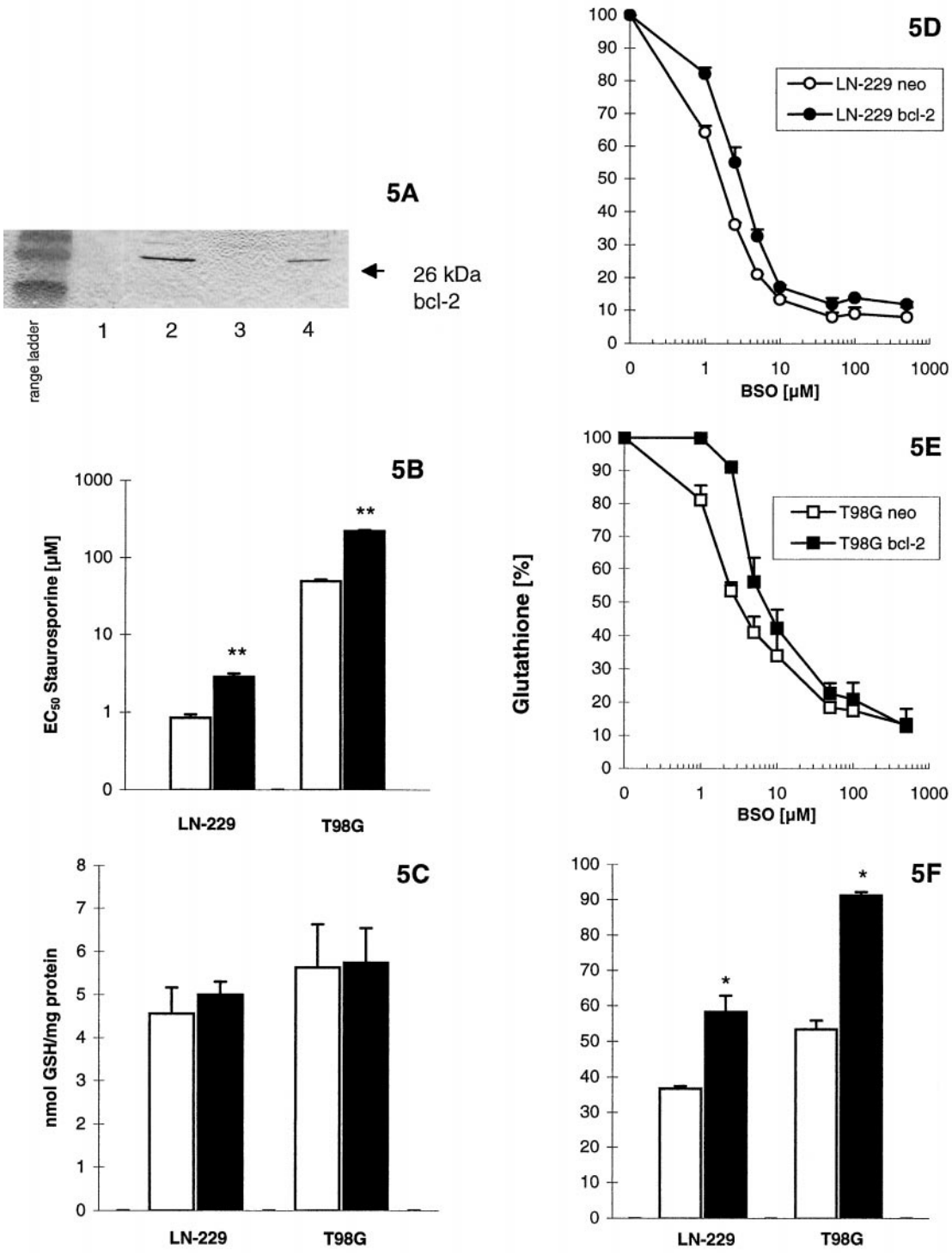


FIG. 5. Ectopic expression of a murine bcl-2 transgene does not affect basal glutathione levels but inhibits BSO-mediated glutathione depletion. A) Immunoblot analysis of murine bcl-2 protein expression in pooled neo- and bcl-2-transfected LN-229 and T98G cells was performed as described in "Materials and Methods" (lane 1, LN-229 neo; lane 2, LN-229, bcl-2; lane 3, T98G, neo; lane 4, T98G, bcl-2). Size markers of 16, 24 and 33 kDa are included on the left. A 26 kD protein is detected in bcl-2-transfected cells but not in neo cells [2]. B) Pooled neo- (open bars) and bcl-2-transfected (filled bars) LN-229 or T98G cells were exposed to increasing concentrations of staurosporine for 72 hr. Survival was assessed by crystal violet staining. EC_{50} values were determined by linear regression analysis ($n = 3$, $^{**}P < 0.01$, *t*-test). C) Basal glutathione levels were measured in pooled neo (open bars) or bcl-2 (filled bars)-transfected LN-229 or T98G glioma cells as described in "Materials and Methods." The differences were not significant ($n = 3$, $P > 0.05$, *t*-test), D, E, F) Pooled neo- (open symbols) or bcl-2-transfected (filled symbols) LN-229 (D) or T98G (E) cells were exposed to increasing concentrations of BSO for 15 hr. Glutathione levels were measured as described in "Materials and Methods." Data are expressed as mean percentages and SEM ($n = 3$). Fig. 5F shows the partial prevention of glutathione depletion in bcl-2-transfected glioma cells (filled bars) compared with neo cells (open bars) at a specific concentration of BSO (2.5 µM) ($^{*}P < 0.05$, *t*-test).

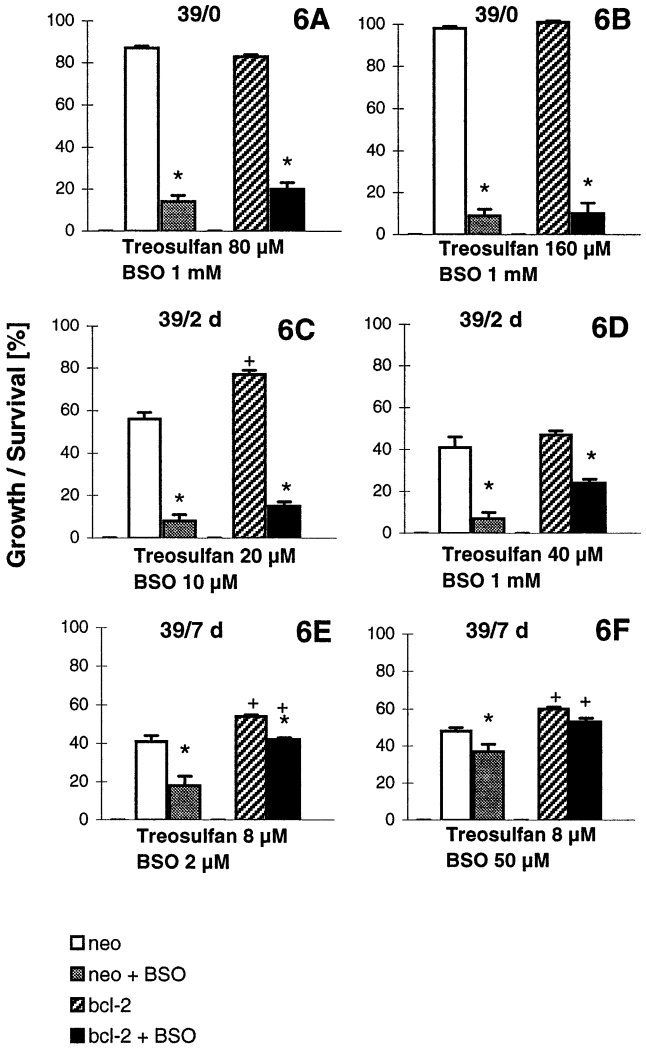


FIG. 6. Effects of murine bcl-2 gene transfer on BSO-augmented treosulfan toxicity. Neo- or bcl-2-transfected LN-229 (A, C, E) or T98G (B, D, F) cells were treated with treosulfan in the absence or presence of BSO, as indicated in the bar legend. BSO was administered as pretreatment for 15 hr and cotreatment with treosulfan for another 24 hr. Viability and growth were assessed by crystal violet staining either immediately after exposure (A, B) or after 2 (C, D) or 7 days (E, F). The concentrations of BSO were 1 mM (A, B, D), 10 µM (C), 2 µM (E) and 50 µM (F). The concentrations of treosulfan were 80 µM (A), 160 µM (B), 20 µM (C), 40 µM (D), and 8 µM (F). Data are expressed as mean percentages and SEM of survival or growth relative to untreated or BSO-treated controls ($P < 0.05$, *t*-test, *potentiation of drug effect by BSO, $^{+}$protective effect of bcl-2). In these experiments, the concentrations of BSO were devoid of toxic effects when applied alone (see Fig. 1).

tection afforded by bcl-2 (Fig. 6). The mechanisms by which bcl-2 blocks apoptosis and the role of reactive oxygen species in apoptosis with specific reference to glutathione are controversial [27–29]. Recent studies link the antiapoptotic action of bcl-2 to the prevention of cytochrome C release from mitochondria [30, 31], but whether this mechanism is responsible for all instances of protection from apoptosis by bcl-2 is unclear. Although bcl-2 may inhibit the generation or facilitate the detoxification of reactive oxygen species in some paradigms [21, 22], bcl-2 shows antiapoptotic actions under anaerobic conditions as well [28]. Unlike neural cells [21], ectopic expression of bcl-2 in human glioma cells did not increase basal glutathione levels. However, bcl-2 attenuated glutathione depletion induced by BSO moderately (Fig. 5), consistent with the effects of bcl-2 gene transfer in leukemia cells challenged with cytarabine [32]. We conclude that glutathione depletion plays a role in the synergy of treosulfan and BSO observed here, but that neither reactive oxygen species nor bcl-2 family proteins are crucially involved. Other authors have suggested that BSO mediates chemosensitization via mechanisms distinct from glutathione depletion, e.g. antagonism of multidrug transport and inhibition of amino acid uptake [8, 33]. While glutathione depletion evolved at lower concentrations of BSO in LN-229 than in T98G cells (Fig. 1C), this difference probably does not account for the prominent difference in susceptibility to antiproliferative effects of BSO (Fig. 1A,B).

It is at present unknown whether cotreatment with treosulfan and BSO is selectively toxic to neoplastic cells. Untransformed glial cells are not likely to be at risk from enhanced toxicity since astrocytes of the normal brain are largely nonmitotic. However, careful studies in laboratory animals are required to elucidate whether normal proliferative tissues with rapid cell turnover are at increased risk of toxicity with this treatment. The clinical implications of the present study depend on the tolerance of human patients to the BSO and treosulfan concentrations required to induce sensitivity of glioma cells. The concentrations of treosulfan and treosulfan metabolites required here to induce cytotoxicity and limit clonogenicity in the presence of BSO (Fig. 3) can probably be achieved *in vivo* since peak plasma levels of 1–2 mM have been reported for treosulfan [34]. Phase I clinical studies in cancer patients have shown that BSO is tolerated at dosing schedules which induce prominent depletion of glutathione levels in peripheral blood lymphocytes to less than 20–40% [35, 36]. BSO may deplete glutathione more effectively in experimental tumor tissue than in bone marrow or normal brain [37, 38]. In the case of malignant glioma, a locoregionary approach to lower glutathione levels—or to induce whatever biochemical change is crucial for the action of BSO—is also possible, e.g. using intraventricular application [39] or, more likely, infusion of BSO into postoperative tumor cysts via catheters, combined with systemic treosulfan.

This study was supported by grants from the German Research Foundation (We 1502/5-1) to MW.

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