Antagonism of Buthionine Sulfoximine Cytotoxicity for Human Neuroblastoma Cell Lines by Hypoxia Is Reversed by the Bioreductive Agent Tirapazamine

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ABSTRACT

Relapse of neuroblastoma (NB) commonly occurs in hypoxic tissues. Buthionine sulfoximine (BSO), an inhibitor of glutathione (GSH) synthesis, is cytotoxic for NB cell lines in atmospheric oxygen (20% O₂). Tirapazamine (TPZ) is a bioreductive agent that forms a toxic-free radical in hypoxia. We determined in four NB cell lines cytotoxicity using the DIMSCAN digital imaging fluorescence assay, glutathione (GSH) levels by the DTNB-GSSG reductase method, apoptosis, reactive oxygen species (ROS), and mitochondrial membrane potential (ΔΨm) by flow cytometry. Hypoxia (2% O₂) antagonized BSO-mediated ROS, apoptosis, and cytotoxicity but not GSH depletion. TPZ synergistically enhanced BSO cytotoxicity in hypoxia for all four NB cell lines, achieving 2–4 logs of cell kill. BSO depleted GSH (8–42% of controls) in 20 and 2% O₂, whereas TPZ only decreased GSH in hypoxia. Maximal GSH depletion was induced by BSO + TPZ. N-acetylcysteine abrogated GSH depletion caused by TPZ but not by BSO. BSO increased ROS, decreased ΔΨm, and caused apoptosis in 20% O₂ but not in 2% O₂. TPZ elevated ROS in 2% O₂ (but not in 20% O₂), whereas BSO + TPZ increased ROS both in 20 and 2% O₂. In hypoxia, TPZ alone or TPZ + BSO caused an 80% decrease of ΔΨm at 24 h, preceding apoptosis in 74–86% of cells at 48 h. Thus, hypoxia significantly antagonizes BSO-mediated cytotoxicity for NB cell lines, but TPZ reversed the inhibition of BSO-mediated cytotoxicity in hypoxia, causing increased ROS, ΔΨm decrease, GSH depletion, apoptosis, and synergistic cytotoxicity. These data additionally define the role of ROS in BSO-mediated cytotoxicity and suggest that combining BSO with TPZ could have clinical activity against NB in hypoxic sites.

INTRODUCTION

NB is a childhood tumor of the sympathetic nervous system that commonly metastasizes at the time of diagnosis to hypoxic sites such as bone and bone marrow (1–4). Treatment of high-risk NB (stage 4 patients >1-year-old at diagnosis and stage 3 disease with MYCN amplification or high-risk histopathology) with myeloablative, multiagent chemoradiotherapy, supported with purged autologous bone marrow transplantation and followed by 13-cis-retinoic acid, has improved the outcome for high-risk NB (2). However, >50% of high-risk NB patients still ultimately die from progressive disease that is refractory to additional therapy and often associated with acquired loss of p53 function in the recurrent tumor cells (5). In contrast to the metastatic pattern seen at diagnosis, it is more common for high-risk NB recurring after intensive therapy to metastasize to high oxygen tension sites such as the lungs and central nervous system (4). Therefore, hypoxic tissue may serve as a sanctuary site for NB, consistent with the decreased responses to chemotherapy and radiotherapy in hypoxia reported for other tumor types (6), and molecular changes that confer drug resistance may also facilitate growth of NB in well-oxygenated tissues. It is important to define the impact that hypoxia has upon the activity of chemotherapeutic agents for NB and to identify drugs that act both independent of p53 function and retain cytotoxicity in a reduced-oxygen environment.

BSO, a selective inhibitor of γ-glutamylcysteine synthetase, can decrease GSH, an intracellular antioxidant that plays a critical role in many aspects of cellular defense, particularly as a means to detoxify ROS (7, 8). BSO-mediated depletion of GSH is highly cytotoxic for NB cell lines in vitro in standard culture conditions (atmospheric O₂), likely because of unopposed ROS produced during catecholamine synthesis (9), and the protective effect of GSH in NB can be replaced by antioxidants such as NAC (9). When a highly nucleophilic drug such as the alkylating agent L-PAM enters a cell, GSH conjugates with the drug, and the complex is ultimately transported out of the cell for detoxification (7, 10). BSO-mediated GSH depletion has been shown to synergize the activity of alkylating agent cytotoxicity for a variety of tumor cell lines (7), including NB (11–16). Importantly, GSH depletion combined with myeloablative concentrations of L-PAM was active against highly drug-resistant, p53 nonfunctional, NB cell lines (15, 16).

Bioreductive agents such as TPZ are preferentially cytotoxic in hypoxia and show activity against a variety of tumor cell lines (17–20). The toxic species has been inferred to be a hydroxyl radical that is produced by a cofactor-dependent, one-electron reduction of TPZ, catalyzed by various cellular reductases, including the flavoenzyme NADPH reductase (18, 21). This highly reactive radical can be detoxified by oxygen, but in hypoxia, it is converted to a stable two-electron reduction product (known as SR4317) by reaction with cellular constituents. Exposure to TPZ under hypoxic conditions leads to ROS-mediated DNA strand breaks (single and double), chromosome aberrations, and cell death (19, 22). In hypoxia, TPZ enhances the in vitro activity of radiation and alkylating agents (23–28).

In this article, we report that physiological hypoxia (2% O₂) antagonizes the cytotoxicity of BSO but not BSO-mediated GSH depletion. We also show that the bioreductive agent TPZ reverses hypoxia-mediated antagonism of BSO, and in hypoxia, the combination of BSO and TPZ are synergistically cytotoxic for NB cell lines, including a p53 nonfunctional cell line (5) resistant to a variety of chemotherapeutic drugs.

MATERIALS AND METHODS

Human NB Cell Lines. Human NB cell lines SMS-SAN and SK-N-BE(2) were cultured in RPMI 1640 (Invitrogen Scientific, Santa Ana, CA) with 10% fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA); CHLA-15 and CHLA-20 were cultured in Iscove’s modified Dulbecco’s medium (BioWhitaker, Walkersville, MD) supplemented with 3 mM L-glutamine, insulin, and transferrin 5 μg/ml each and 5 ng/ml selenious acid (ITS Culture Supplement;
Collaborative Biomedical Products, Bedford, MA) and 20% fetal bovine serum (complete medium). All cell lines were continuously cultured at 37°C in 5% CO₂. Experiments were carried out at passage 15–30. Cells were detached from culture plates or flasks with the use of a modified Puck’s Solution A plus EDTA (Puck’s EDTA), which contains 140 mM NaCl, 5 mM KCl, 5.5 mM glucose, 4 mM NaHCO₃, 0.8 mM EDTA, 13 μM phenol red, and 9 mM HEPES buffer (pH 7.3); Ref. 29.

Reduced Oxygen Conditions. For hypoxia assays, cells were seeded into plates or flasks and placed into a sealed, humidified, modular incubation chamber (30) that was flushed for 90 s at 10 psi with a mixture of 2% O₂, 5% CO₂, and 93% nitrogen (referred to as 2% O₂ mixture) and then incubated at 37°C. Under these conditions, medium in plates and flasks attains a Po₂ of ~15 mm Hg (31). This level of oxygen is below the degree of hypoxia found in bone marrow (3) and in the range of hypoxia found in tumor tissue (32). BSO and TPZ stock solutions were diluted in whole medium that had been allowed to equilibrate overnight in a loosely capped flask in a modular chamber flushed with 2% O₂ mixture as described above. After drug addition, plates and flasks were refilled with the 2% O₂ mixture for 90 s, and the chamber was sealed at ~5 psi over pressure to reduce atmospheric leaks, incubated at 37°C, and refilled with the 2% O₂ mixture every other day until assayed.

Chemicals. L-BSO was obtained as a 50 mg/ml solution from the Investigational Drug Branch, National Cancer Institute (Rockville MD), and was diluted to various concentrations in complete medium. TPZ was provided by Sanofi-Winthrop, Inc. (Malvern, PA), and was diluted freshly with sterile water to make a 1 mg/ml stock solution (1 mg/ml = 5.6 mM). TPZ concentrations are presented as μg/ml for ease of comparison to previously reported studies, and equivalent concentrations in μM are also provided. Eosin Y and the thiol antioxidant NAC were purchased from Sigma Chemical Co. (St. Louis, MO). NAC was freshly dissolved in medium at final concentration of 800 μM, adjusted pH to 7.4 with NaOH, and sterilized by 0.22-μm filtration. FDA was from the Eastman Kodak Company (Rochester, NY). Carboxy-DCFDA and the fluorescent probe JC-1 were from Molecular Probes (Eugene, OR). Stock solutions of FDA (1 mg/ml), carboxy-DCFDA (10 mM), and JC-1 (2 mg/ml) were dissolved in DMSO and stored at ~20°C (FDA and JC-1) or liquid nitrogen vapor (carboxy-DCFDA).

Cytotoxicity Assay. All the cytotoxicity assays were performed in 96-well plates using a semi-automated Digital Image Microscopy (DIMSCAN) system that has a dynamic range of 4 logs of cell kill (33). CHLA-20, and SK-N-BE(2) cells (fast growing) were plated at 5000 cells/well; SMS-SAN cells (slower growing) were plated at 15,000 cells/well; all cell lines were seeded in 100 μl of complete medium/well. Cells were cultured both in normoxia (20% O₂) and hypoxia (2% O₂) and were allowed to attach 1 day before addition of BSO at 0–750 μM ± TPZ at 0–3 μg/ml (0–16.8 μM) in complete medium (to various final concentrations in 200 μl of complete medium) in replicates of 12 wells/condition. For some assays, NAC was added to a final concentration of 800 μM, 3 h before the addition of BSO ± TPZ, which was then added in 100 μl of complete medium to various final drug concentrations as described above. Plates were assayed at 6 days after initiation of drug exposure.

For measurement of cytotoxicity, FDA was added to the 96-well plate (final concentration = 10 μg/ml) and incubated for 20 min. Afterward, 30 μl of eosin Y (0.5% in normal saline) was added to quench background fluorescence of FDA in the medium and in nonviable cells (34). Total fluorescence/well (after digital thresholding to eliminate background fluorescence) was then measured using a DIMSCAN system, and the results were expressed as the fractional survival cells compared with control cells. The concentration of drug lethal for 99% of cells (LC₉⁹) were calculated using the software Dose-Effect Analysis with Microcomputers (35).

Dose Effect Analysis. Synergistic drug interactions were determined by fixed ratio dose-response assays of the drugs alone and in combination (BSO: TPZ concentration ratio 250 μM:1 μg/ml, which is 45 μM BSO:1 μg M TPZ). DIMSCAN analysis was then performed and the CI calculated using Dose Effect Analysis with Microcomputers software (30, 35, 36). A CI < 1 indicates drug synergy, with higher degrees of synergy associated with lower values. A CI value equal to 1 signifies an additive effect, whereas a CI > 1 indicates antagonism. In addition to determining the CI value, the significance of the increase in cell kill for the drugs in combination versus each individual drug at each dose level tested was calculated by the two-tailed Student’s t test.

GSH. Intracellular GSH was measured in triplicate by culturing cells in 25-cm² tissue culture flasks (2 × 10⁵ cells) with or without BSO (500 μM) and TPZ (2 μg/ml, 11.2 μM), alone or in combination, for 24 h in 20% O₂ or 2% O₂. Cells were analyzed for total GSH content within 48 h by the DTNB-GSSG reductase method as described previously (37).

Apoptosis. To quantify apoptotic cells with sub-G₀ DNA content, cells were cultured in complete medium in 25-cm² flasks, with or without BSO (500 μM), TPZ (2 μg/ml), or BSO (500 μM) + TPZ (2 μg/ml; 11.2 μM), for 24 h in 20% O₂ or 2% O₂. Cells were then harvested, washed in PBS, centrifuged, and resuspended in 1 ml of 0.1% sodium citrate containing PI (0.05 mg) and RNase (50 μg) for 30 min at room temperature in the dark. DNA content was measured on a Coulter Epics Elite flow cytometer using a 488-nm argon laser and a 610 ± 10-nm band pass filter (38). Apoptosis was also detected using a commercial TUNEL assay kit (APO-Direct, Pharmigen, San Diego, CA). After staining, cells were analyzed by flow cytometry, and the percentage of cells showing apoptosis was determined using band pass filters of 525 ± 10 nm for FITC and 610 ± 10 nm for PI (39).

Measuring ROS. CHLA-15 cells (2 × 10⁶ cells in 5 ml of complete medium/25 cm² flask) were incubated with BSO (500 μM) for 24 h and TPZ (2 μg/ml; 11.2 μM) for 4 h, alone or in combination, in 20% O₂ or 2% O₂. Medium was discarded under subdued lighting and replaced with 50 μM carboxy-DCFDA (31) in 2 ml of complete medium for 20 min at 37°C. Cells were harvested with Puck’s EDTA, transferred to foil-wrapped tubes, and analyzed immediately by flow cytometry, using a 525 ± 10 nm band pass filter. As a positive control, cells were loaded with carboxy-DCFDA for 20 min as above with 3 ml of hydrogen peroxide (100 μM in whole medium) added 15 min before harvesting for flow cytometry.

Assessment of Mitochondrial Membrane Potential Transition. Loss of Δψₘ leads to release of cytometry C and factors that trigger apoptosis (40, 41). The Δψₘ was determined (42) in the CHLA-15 cell line after a 24-h treatment with BSO (500 μM) and TPZ (2 μg/ml; 11.2 μM), as single agents or in combination, in 20% O₂ or 2% O₂ conditions. After collection with Puck’s EDTA, cells were resuspended in 1 ml of complete medium containing 10 μg/ml JC-1 for 10 min at 37°C. JC-1 is cationic dye that exhibits potential-dependent accumulation in mitochondria (42), indicated by a fluorescence emission shift from green (525 ± 10 nm) to red (610 ± 10 nm), measured by flow cytometry. Mitochondria depolarization is specifically indicated by a decrease in the red to green fluorescence intensity ratio. The ratio in the control group was considered as 1.

Statistics. Significance (unpaired two-sided Student’s t test) and correlation (Pearson’s coefficient) were determined by Microsoft Excel 2000 software.

RESULTS

Cytotoxicity Assays of BSO and TPZ, Alone or in Combination, under Normoxia and Hypoxia. The dose response of four human NB cell lines to BSO (0–750 μM) or TPZ (0–3 μg/ml) was determined both as single agents and in combination, in normoxia (20% O₂) and hypoxia (2% O₂), as shown in Fig. 1, with the LC₉⁹ and CI values shown in Table 1. In normoxia, BSO was highly toxic, achieving ≥2-log cell kill, and the LC₉⁹ values were lower than the clinically achievable steady state plasma levels (300–500 μM; Ref. 43) in three of four cell lines tested. TPZ required high concentrations to achieve cytotoxicity in normoxia, with LC₉⁹ values surpassing clinically achievable steady-state plasma concentrations (2.88 ± 0.37 μg/ml = 16 ± 2 μM; Ref. 44). The cytotoxicity of BSO for all four NB cell lines was reduced in hypoxia, whereas the cytotoxicity of TPZ was enhanced in hypoxia, achieving >3-log cell kill in three cell lines (SMS-SAN, CHLA-15, and CHLA-20). LC₉⁹ values for TPZ as a single agent in hypoxia were 0.6–3.9 μg/ml (3.3–21.9 μM), which were in the clinically applicable range (≤10 μg/ml).
for most cell lines and conditions tested. CI values in hypoxia (2% O₂) combination was synergistic, with CI/H₁₁₀₂₁
hypoxia.

Concentration tested for BSO and TPZ demonstrated drug synergy (CI calculated for experiments conducted in normoxia.

obtained from 6 days of drug(s) exposure. TPZ conversions (g/ml) are shown. The CI for SAN are for BSO (50 μM)
achievable range, unlike the values of 5.3–33.8 μg/ml (29.8–190 μM) calculated for experiments conducted in normoxia.

As single agents, BSO in hypoxia or TPZ in normoxia showed only modest activity. However, CI values calculated at each drug concentration tested for BSO and TPZ demonstrated drug synergy (CI < 1) for most cell lines and conditions tested. CI values in hypoxia (2% O₂) for BSO + TPZ indicated strong synergy for SMS-SAN, SK-N-BE(2), and CHLA-20. In hypoxia, the CI was ≤0.3 for SMS-SAN at all tested concentrations, and a decreasing CI was seen with increasing drug concentrations in SK-N-BE(2) from 0.27 to 0.005 and in CHLA-20 from 0.7 to 0.34. In hypoxia, the CI for CHLA-15 did not indicate synergy at TPZ concentrations <1 μg/ml (5.6 μM), with the CI = 1.08 and 1.02 but indicated modest synergy (CI = 0.67 and 0.46) at TPZ concentrations ≥2 μg/ml (11.2 μM). In normoxia (20% O₂), BSO + TPZ showed modest synergy in SMS-SAN (CI < 0.72),

strong synergy for CHLA-15 (CI = 0.52–0.02), and synergy only at TPZ concentrations of >1 μg/ml (5.6 μM) for CHLA-20 (CI = 0.57–0.35). The cytotoxicity of each single agent was compared with BSO + TPZ in combination for each drug concentration tested, and there was a statistically significant increase in cell kill for the combination (P < 0.05, usually P < 0.001) for SMS-SAN and CHLA-20 in 2% and 20% O₂, for SK-N-BE(2) in 2% O₂, and for CHLA-15 in 20% O₂. However, consistent with the CI values, BSO + TPZ did not significantly increase the cytotoxicity (P > 0.05) over that of BSO alone for SK-N-BE(2) in 20% O₂ or over that of TPZ alone for CHLA-15 in 2% O₂.

Apoptosis after Treatment with BSO ± TPZ. The SMS-SAN cell line was treated with 500 μM BSO ± 2 μg/ml (11.2 μM) TPZ for 24 h and then examined for apoptosis by PI staining and flow cytometry. Spontaneous apoptosis (control) was seen in 7% of cells in normoxia and 8% in hypoxia (Fig. 2). In normoxia, apoptosis was increased to 33% by BSO, 32% by TPZ, and 49% by BSO + TPZ. In hypoxia, BSO induced little (10%) apoptosis relative to controls, whereas TPZ caused 65% apoptosis, and TPZ + BSO induced 64% of cells to undergo apoptosis. TUNEL assay results (data not shown) demonstrated a similar pattern of induced apoptosis.

Effect of BSO ± TPZ on GSH. Cell lines were incubated for 24 h with 500 μM BSO and 2 μg/ml (11.2 μM) TPZ (alone or in combination) and total GSH was assayed. In normoxia (Fig. 3), BSO induced a significant decrease in GSH to <40% baseline in all cell lines (P < 0.01) with neither basal GSH levels nor the extent of GSH depletion correlating with the cytotoxic response to BSO: LC₉⁹ versus GSH level (r = 0.08) and LC₉⁹ versus percentage of GSH decrease (r = 0.25). Although hypoxia dramatically inhibited BSO-mediated cytotoxicity, hypoxia did not inhibit BSO-mediated GSH depletion (Fig. 3). In hypoxia, TPZ decreased GSH levels in the three p53 functional cell lines (SMS-SAN, CHLA-15, and CHLA-20) but not in the p53 nonfunctional, highly drug-resistant cell line SK-N-BE(2) (5), whereas in normoxia, single agent TPZ decreased GSH only for SMS-SAN and CHLA-15. TPZ slightly enhanced BSO-mediated GSH depletion for all four cell lines in normoxia, but in hypoxia, this effect was only pronounced in the three p53-functional cell lines. GSH depletion (percentage of BSO alone) by TPZ + BSO, in normoxia versus hypoxia, was 67 versus 9% for SMS-SAN, 58 versus 12% for CHLA-15, and 71 versus 37% for CHLA-20 compared with 82 versus 79% for SK-N-BE(2).

Effect of NAC on BSO and TPZ Toxicity. To better explore the role of ROS in the cytotoxicity of BSO and TPZ in NB cell lines, CHLA-20 was treated with the thiol antioxidant NAC (800 μM), BSO (500 μM), and TPZ (2 μg/ml; 11.2 μM), alone or in combination, in normoxia and hypoxia. GSH levels were assayed after 24 h, and cell viability was measured after 6 days (Fig. 4). Relative to controls, NAC treatment alone induced a 1.4-fold (P < 0.05) increase in cell viability (Fig. 4A) and a 1.1-fold (P = 0.18) elevation of GSH in normoxia (Fig. 4B). NAC failed to restore GSH levels depleted by BSO in both normoxia (P = 0.06) and hypoxia (P = 0.85; Fig. 4B) but abrogated

Table 1 Cytotoxicity and CI of BSO and TPZ in normoxia (20% O₂) and hypoxia (2% O₂)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>LC₉⁹BSO (μM)</th>
<th>LC₉⁹TPZ (μM)</th>
<th>CI at BSO (500 μM) + TPZ (2 μg/ml)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>20% O₂</td>
<td>2% O₂</td>
<td>20% O₂</td>
</tr>
<tr>
<td>SMS-SAN</td>
<td>65.3</td>
<td>308.6</td>
<td>5.3</td>
</tr>
<tr>
<td>SK-N-BE(2)</td>
<td>193.5</td>
<td>&gt;1000</td>
<td>33.8</td>
</tr>
<tr>
<td>CHLA-15</td>
<td>161.5</td>
<td>&gt;1000</td>
<td>6.9</td>
</tr>
<tr>
<td>CHLA-20</td>
<td>737.6</td>
<td>&gt;1000</td>
<td>19.0</td>
</tr>
</tbody>
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Fig. 1. TPZ restored BSO-mediated cytotoxicity inhibited by hypoxia. Dose-response of NB cell lines to BSO ( ), TPZ ( ), or BSO plus TPZ ( ) are shown in normoxia (20% O₂) and hypoxia (2% O₂). Drugs were studied over a range of concentrations relevant to clinically achievable levels, BSO at 0–750 μM ± TPZ at 0–3 μg/ml (0–16.8 μM). Cells were treated with drugs for 6 days in the oxygen tensions indicated, and cytotoxicity was analyzed with the use of a fluorescence-based assay using digital imaging microscopy (DIMSCAN). Points represent the mean fractional survival; error bars represent SD. The combination was synergistic, with CI < 1 at many or all concentrations in three of four cell lines (SMS-SAN, CHLA-15, and CHLA-20) in normoxia and in all four cell lines in hypoxia.

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BSO-mediated cytotoxicity in normoxia ($P < 0.01$; Fig. 4A). NAC also decreased cytotoxicity of BSO + TPZ in normoxia and hypoxia ($P < 0.01$ for both; Fig. 4D). GSH levels in BSO + TPZ + NAC-treated cells failed to show a significant difference relative to those of BSO + TPZ treatment in normoxia and in hypoxia ($P = 0.06$ and 0.33, respectively; Fig. 4F). Thus, NAC can decrease BSO- and BSO + TPZ-mediated cytotoxicity but does so without replenishing GSH.

No significant difference in cytotoxicity ($P = 0.81$, Fig. 4C) or GSH depletion ($P = 0.07$; Fig. 4D) was observed between TPZ and TPZ + NAC treatment in normoxia. In hypoxia, basal GSH levels were decreased ($P < 0.01$) compared with GSH levels in normoxia. In hypoxia, NAC alone significantly increased GSH levels relative to control (Fig. 4, B, D, and F; $P < 0.01$), but cell viability was not increased (Fig. 4, A, C, and E; $P = 0.69$). As shown in Fig. 4C, NAC abrogated GSH depletion induced by TPZ in hypoxia ($P < 0.01$) but did not reduce cytotoxicity ($P = 0.81$).

**Effect of BSO and TPZ on ROS Levels.** BSO has been reported to increase ROS levels in BSO-sensitive NB cell lines in normoxia (9). To determine whether BSO can increase ROS in hypoxia and how TPZ affected ROS levels in NB cell lines, we measured ROS by flow cytometry using carboxy-DCFDA (Fig. 5). CHLA-15 cells were exposed to BSO (500 μM; for 24 h) or TPZ (2 μg/ml; 11.2 μM) for 6 h or the combination (BSO, 500 μM alone) for 18 h, then BSO (500 μM) + TPZ (2 μg/ml; 11.2 μM) for 6 h in normoxia and hypoxia before staining with carboxy-DCFDA and flow cytometric analysis.

BSO as a single agent, and in combination with TPZ, increased the mean carboxy-DCFDA fluorescence 3.2- and 3.9-fold, respectively, over controls in 20% O$_2$ (Fig. 5, A and E), TPZ alone did not increase ROS in normoxia (Fig. 5C). TPZ alone increased the mean DCFDA fluorescence 2.4-fold over that of control in hypoxia (Fig. 5D), and the combination of BSO + TPZ in hypoxia also increased carboxy-DCFDA fluorescence 2.7-fold over the control (Fig. 5F) to about the same degree as TPZ alone. In contrast, BSO alone did not increase ROS in hypoxia (Fig. 5B).

**Relationship of Loss of Δψm to Apoptosis.** We determined the temporal relationship between Δψm and apoptosis in the CHLA-15 cell line treated with BSO 500 μM, TPZ (2 μg/ml; 11.2 μM), or in combination, for 24, 48, and 72 h in normoxia and hypoxia, using flow cytometry to measure apoptosis by TUNEL staining and Δψm by JC-1 staining (Fig. 6). Negative control cells provided by the TUNEL kit were used to set flow cytometry gates for positive terminal deoxyribo nucleotidyltransferase-mediated fluorescent staining. The range of spontaneous apoptosis (control of each treatment group) was 1–5%. In normoxia, apoptosis was not detected at 24 h in response to BSO, TPZ, or BSO + TPZ but was increased at 48 h of treatment with BSO (19%), TPZ (51%), and BSO + TPZ (63%); Fig. 6A). After 72 h, the apoptotic fraction of each treatment group increased to 26% (BSO), 57% (TPZ), and 77% (BSO + TPZ). In normoxia, TPZ or BSO + TPZ (but not BSO alone) decreased Δψm at 24 h. A decline of Δψm was seen after a 48-h BSO exposure (Fig. 6B).

In hypoxia, BSO alone failed to induce apoptosis (Fig. 6A). However, TPZ or TPZ+BSO caused apoptosis at 24 h, increasing to >80% at 72 h (Fig. 6A). A decrease in Δψm was seen after 24 h of hypoxic treatment with TPZ or BSO + TPZ, but not with BSO (Fig. 6B). For TPZ, and TPZ + BSO, a decrease in Δψm appeared to precede apoptosis.

**DISCUSSION**

NB initially responds to chemotherapy but recurs in most high-risk patients as a chemotherapy-insensitive disease (1). Tumor hypoxia (found in common sites of NB relapse such as bone and bone marrow) could contribute to enhanced survival of tumor cells, facilitating selection for drug-resistant cells (2–4). Because chemotherapeutic agents are thought to be antagonized by hypoxia (17, 45), identifying regimens that are active in reduced oxygen environments could improve therapy of NB.

GSH is a major intracellular antioxidant that protects DNA by detoxifying alkylating agents and platinum compounds (7, 8, 10). BSO is a specific inhibitor of GSH synthesis (7) and can reverse alkylator resistance in NB cell lines (14–16). Clinical trials in both adults and children have documented that attainable steady-state plasma concentrations during continuous i.v. infusion range from 300 to 500 μM of BSO (43, 46), and responses to BSO combined with L-PAM have been seen in children with recurrent NB (15, 46).

In standard culture conditions (normoxia = 20% O$_2$), BSO alone is highly cytotoxic for most NB cell lines in vitro and causes apoptosis...
by increasing ROS (9). Here, we have shown that hypoxia decreased the single-agent cytotoxicity of BSO for NB cell lines. We used four NB cell lines established at diagnosis before therapy (CHLA-15 and SMS-SAN) or at time of progressive disease after treatment with combination chemotherapy regimens [SK-N-BE(2) and CHLA-20]. As expected from our prior work (9), three of four cell lines were sensitive to BSO in normoxia, with LC50 values below clinically achievable levels. Physiological hypoxia (2% O2) antagonized BSO-mediated apoptosis, generation of ROS, decrease in GSH, and cytotoxicity, with BSO LC50 values being higher in hypoxia relative to normoxia. The thiol antioxidant NAC could also abrogate BSO single-agent cytotoxicity in normoxia, without restoring GSH levels. These data demonstrated that the cytotoxicity of BSO as a single agent resulted not from the GSH depletion alone but was dependent on tumor cell ROS, which is diminished in hypoxia or in the presence of an antioxidant.

TPZ is an anticancer drug that is activated in hypoxic cells to a highly damaging free radical, resulting in selective cytotoxicity of cells grown in hypoxia (21, 47). Preclinical models suggested that TPZ can enhance both irradiation and chemotherapy in hypoxic cancer cells (24–26). In the four NB cell lines we studied, clinically obtainable levels of TPZ achieved 4 logs of cell kill in hypoxia and <1.5 logs of cell kill in normoxia.

Because BSO depleted GSH but did not induce ROS or cytotoxicity in hypoxia, we determined if the free radical of TPZ in hypoxia might restore BSO-mediated cytotoxicity. In hypoxia, TPZ (but not BSO) increased ROS production and synergistically enhanced (CI < 1) BSO cytotoxicity in all four cell lines. TPZ alone significantly decreased GSH in drug-sensitive NB cell lines (CHLA-15 and SMS-SAN), both in normoxia and hypoxia. TPZ also enhanced BSO-mediated GSH depletion (especially in hypoxia), presumably because of increased GSH use. Importantly, TPZ cytotoxicity for NB cell lines could not be abrogated by NAC (but was enhanced by depleting GSH), suggesting that TPZ is directly cytotoxic (rather than acting via ROS) and that the TPZ radical is detoxified by GSH. We observed that although BSO significantly increased the cytotoxicity of TPZ in hypoxia, it did not cause a similar increase in the degree of apoptosis. This suggests that BSO may act via nonapoptotic mechanisms to enhance the cytotoxicity of TPZ. This latter observation is consistent with the ability of BSO to cause p53-dependent cytotoxicity (14–16) when combined with the p53-dependent drug L-PAM (5).

In hypoxia, TPZ induced both GSH-depletion and marked cytotoxicity in three of four cell lines (CHLA-15, CHLA-20, and SMS-SAN). Unlike the other three cell lines, the SK-N-BE(2) cell line did not show depletion of GSH by TPZ, which may at least partially account for the lower cytotoxic response to TPZ in SK-N-BE(2). However, if GSH synthesis was blocked by BSO in hypoxia, TPZ cytotoxicity and GSH depletion was enhanced in all four NB cell lines, and TPZ cytotoxic activity was particularly increased by BSO in SK-N-BE(2) and SMS-SAN.

Of the four cell lines studied, CHLA-15 and CHLA-20 were derived from the same patient at diagnosis and then after progression during induction therapy (cyclophosphamide, doxorubicin, cisplatin, and teniposide; Ref. 34). CHLA-20 showed increased resistance to BSO and TPZ (both in normoxia and hypoxia) when compared with CHLA-15. In both hypoxia and normoxia, basal levels of GSH were ≥1.3 times higher in CHLA-20 compared with CHLA-15. SK-N-BE(2) was derived from a patient treated with vincristine, cyclophosphamide, doxorubicin, and radiation, and it is a highly drug-resistant NB cell line (34). Among the four cell lines we tested, SK-N-BE(2) was the only cell line lacking p53 function (5), and the
only cell line resistant (LC50 value is higher than the average clinically achievable level) to single-agent TPZ in hypoxia. Our data suggest that perhaps a higher basal level of GSH in SK-N-BE(2) and, also in the modestly drug-resistant CHLA-20, might contribute to multidrug resistance. Moreover, the SK-N-BE(2) cell line appears to be able to compensate for the increased GSH utilization (in this case by TPZ), whereas the GSH levels in the other cell lines showed a significant decrease in response to TPZ as a single agent when used in hypoxia. Thus, consistent with data from other tumor systems (10, 48–51), either higher basal levels of GSH and/or an increased ability to generate GSH in response to increased usage by a cytotoxic agent may play a role in NB drug resistance. Additional investigation of the cytotoxic mechanisms for BSO and TPZ and their relationship to p53 function is in progress.

Intracellular redox potential plays a role in the commitment to cell death, and a major determinant of this potential is GSH (52). A low level of GSH could favor a decrease in Δψm (52, 53), which has been demonstrated to play a pivotal role in transducing a variety of pro-apoptotic stimuli (52, 54, 55) and can act as a regulator of intracellular permeability transition pores to finally induce the release of cell death-promoting factors, including cytochrome C and apoptosis-inducing factor (41, 57). Cytochrome C has been demonstrated to be involved in the activation of a caspase cascade (53, 58), whereas apoptosis-inducing factor has been shown to directly trigger apoptosis (59). We showed that BSO induced apoptosis and a loss of Δψm in CHLA-15 cells after 48-h exposure in normoxia. However, in hypoxia, even up to 72 h, BSO still failed to cause apoptosis and Δψm loss. GSH-depletion by BSO required at least 24 h of treatment with BSO (both in normoxia and hypoxia), and ROS was only elevated after a 24-h exposure to BSO in normoxia. These data demonstrate that in NB cells, GSH depletion by BSO results in increased ROS, Δψm loss, apoptosis, and cytotoxicity because of an oxygen-dependent increase in tumor cell ROS.

Treatment with TPZ for 6 h could induce Δψm loss without induction of apoptosis in hypoxic CHLA-15 cells (data not shown). A 24-h exposure to TPZ decreased Δψm dramatically, both in normoxia and hypoxia, whereas induction of apoptosis by TPZ in normoxia required 48 versus 24 h in hypoxia. Thus, Δψm loss preceded TPZ-mediated apoptosis. Previous studies have reported that Fas-mediated apoptosis in NB cells is mitochondria dependent (40) and Bcl-2 (or its homologues) have been shown to act on mitochondria to regulate apoptosis (60, 61). Additional studies will be needed to address whether hypoxia interacts with the Bcl-2-family of proteins to affect apoptosis.

In conclusion, our data confirm our previous observation that in normoxic conditions, endogenous ROS are increased in BSO-treated NB because of GSH depletion (9). Moreover, these experiments demonstrated that: (a) BSO-induced apoptosis and cytotoxicity was reduced by hypoxia because of diminished tumor cell ROS, whereas BSO-induced GSH depletion was not affected by hypoxia. (b) TPZ reversed the hypoxia-induced antagonism of BSO-mediated ROS increase, Δψm decrease, apoptosis, and cytotoxicity. (c) In hypoxia, TPZ may have ROS-independent cytotoxicity because the antioxidant NAC did not diminish TPZ cytotoxicity (d) NAC antagonized BSO-induced cytotoxicity without affecting GSH depletion, supporting an essential role for ROS in the cytotoxic action of BSO for NB cells. (e) The antioxidant NAC could reverse the GSH depletion caused by TPZ, but not by BSO, and TPZ caused GSH depletion and cytotoxicity only when combined with BSO in the multidrug-resistant SK-N-BE(2) cell line. These data emphasize the importance of inhibiting GSH biosynthesis to overcome drug resistance. (f) The enhancement of TPZ cytotoxicity caused by BSO occurred without an increase in apoptosis, suggesting the involvement of nonapoptotic cytotoxic mechanisms in GSH-depleted NB cells. Thus, although BSO is unlikely to be effective as a single agent against NB because of hypoxic conditions in vivo, our data support further study of BSO in combination with drugs such as TPZ.

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