

# Buthionine Sulfoximine and Myeloablative Concentrations of Melphalan Overcome Resistance in a Melphalan-Resistant Neuroblastoma Cell Line

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**Background:** Alkylator resistance contributes to treatment failure in high-risk neuroblastoma. Buthionine sulfoximine (BSO) can deplete glutathione and synergistically enhance in vitro sensitivity to the alkylating agent melphalan (L-PAM) for many neuroblastoma cell lines, but optimal use of this combination needs to be defined because clinical responses have been less frequent and not durable.

**Patients and Methods:** The authors established and characterized a neuroblastoma cell line (CHLA-171) from a patient who died of progressive disease after treatment with BSO and low-dose L-PAM.

**Results:** CHLA-171 lacks *MYCN* amplification, expresses PGP (P-glycoprotein) 9.5 RNA, and shows cell surface antigen expression (human leukocyte antigen class I weakly positive, but HSAN 1.2 (hybridoma, SAN 1.2) and anti-GD2 (anti-ganglioside GD2 antibody) strongly positive) characteristic of neuroblastoma cell lines. Twenty-four hours of BSO treatment (0–1,000  $\mu\text{mol/L}$ ) maximally depleted CHLA-171 glutathione to 36% of baseline. The cytotoxic response of CHLA-171 to BSO and L-PAM, alone and in combination, was measured by digital image microscopy (DIMSCAN) over a range of drug concentrations and compared with drug levels obtained in the patient during BSO/L-PAM therapy. As single agents, CHLA-171 was highly resistant to L-PAM ( $\text{LD}_{90} = 42 \mu\text{mol/L}$ ; peak plasma concentration in the patient equals 3.9  $\mu\text{mol/L}$ ) and moderately resistant to BSO ( $\text{LD}_{90} = 509 \mu\text{mol/L}$ ; steady-state concentration in the patient equals 397  $\mu\text{mol/L}$ ). Treatment with a 10:1 (BSO:L-PAM) fixed ratio combination synergistically overcame resistance (3–4 logs of cell kill, combination index <1) at clinically achievable levels of BSO

(100–400  $\mu\text{mol/L}$ ) and levels of L-PAM (10–40  $\mu\text{mol/L}$ ) clinically achievable only with hematopoietic stem cell support.

**Conclusions:** The in vitro results obtained for CHLA-171 suggest that BSO/L-PAM therapy may be optimally effective for drug-resistant neuroblastoma using myeloablative doses of L-PAM.

**Key Words:** Neuroblastoma—Buthionine sulfoximine—Melphalan—Alkylator resistance—Myeloablative therapy—P-glycoprotein.

Neuroblastoma, a pediatric malignancy of the sympathetic nervous system, continues to be a therapeutic challenge (1). Most patients with stage 4 disease diagnosed at age older than 1 year (or younger than 1 y with *MYCN* amplification) initially respond to therapy, but many ultimately die of recurrent disease that is refractory to chemotherapy (1–5). Acquired alkylator resistance is one likely mechanism for treatment failure because current treatment of high-risk neuroblastoma relies heavily on alkylating agents such as cyclophosphamide and melphalan (L-PAM), often in combination with carboplatin or cisplatin (1,6–8). Before disease progression, myeloablative therapy supported by purged autologous hematopoietic stem cell transplantation has shown improved survival for children with high-risk neuroblastoma (6,9), especially if followed-up by treatment with high-dose, pulse 13-*cis*-retinoic acid (6). The authors have previously shown that sustained resistance (mean  $\text{LD}_{90} = 84 \mu\text{mol/L}$ ) to the alkylating agent L-PAM occurs in most cell lines established from patients who experienced relapse after myeloablative therapy that included intensive L-PAM (210  $\text{mg/m}^2$ ) during the preautologous hematopoietic stem cell transplantation conditioning (10,11). Therefore, modulation of alkylator resistance is one novel strategy that may improve the efficacy of autologous hematopoietic stem cell transplantation and ultimately increase the rate of survival for children with stage 4 neuroblastoma.

Glutathione is a ubiquitous intracellular tripeptide that protects cells from oxidative stress and has been shown to detoxify alkylating agents (12–14). Buthionine sulfoximine (BSO), a specific inhibitor of  $\gamma$ -glutamyl cysteine synthetase (the rate-limiting enzyme in glutathione synthesis), depletes glutathione and can reverse alkylator resistance (12,15–18). The authors have previously reported that BSO, as a single agent, is highly cytotoxic for neuroblastoma cell

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Submitted for publication August 22, 2000; accepted October 17, 2000.

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Supported in part by the Neil Bogart Memorial Laboratories of the T.J. Martell Foundation for Leukemia, Cancer, and AIDS Research, by National Cancer Institute Grants CA82830 and CA60104, and by a National Research Service Award 2 T32 CA 09659 for training in basic research in oncology. Also supported in part by the NIH GCRC Grant 3MO1RO043–38. Performed at the GCRC at Children's Hospital Los Angeles.

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lines in vitro and results in apoptosis because of increased generation of reactive oxygen species (19,20). Combining BSO with L-PAM at clinically achievable (nonmyeloablative) concentrations is synergistic in neuroblastoma cell lines (20). Nonmyeloablative trials of BSO and L-PAM in adults have shown the combination to be safe and well tolerated, with reversible bone marrow suppression as the major clinical toxicity (21–24). The authors recently completed a pilot clinical trial (NCI #T95–0092) of BSO and low-dose L-PAM in children with recurrent neuroblastoma (25,26). A neuroblastoma cell line (CHLA-171) was established from a postmortem blood specimen of a patient treated on this regimen who ultimately died of recurrent disease. The authors show here that CHLA-171 is resistant to BSO and L-PAM as single agents and that BSO can synergistically reverse L-PAM resistance, but only at high levels of L-PAM that are achievable in the myeloablative setting.

## MATERIALS AND METHODS

### Patient History and Cell Line Characteristics

The patient was an 8-year-old girl with a stage 4 *MYCN* nonamplified neuroblastoma. Histology was classified as unfavorable by the criteria of Shimada et al. (27) (maturing ganglioneuromatous stroma with nodules of poorly differentiated neuroblastoma); and urine catecholamine and serum ferritin levels were increased. The patient achieved a very good partial response (28,29) on the Children's Cancer Group high-risk neuroblastoma protocol CCG-3891, randomized to chemotherapy consolidation and 13-*cis*-retinoic acid (6,30).

The tumor recurred and the patient received two courses of vincristine, doxorubicin, and cyclophosphamide (31), with no response. The patient was then treated with two courses of BSO/L-PAM (NCI #T95–0092; BSO 3 g/m<sup>2</sup> bolus followed-up by continuous infusion of 0.75 mg/m<sup>2</sup> per hr × 72 hr; L-PAM 15 mg/m<sup>2</sup> bolus at hr 48 of therapy) (25,26).

Pharmacokinetics performed on this patient showed a peak plasma concentration of 3.90 μmol/L L-PAM and a steady-state concentration of 397 μmol/L L-(S,R) BSO. The disease recurred after the second course of BSO/L-PAM and bone pain required palliative radiotherapy. On the third day of radiation, a severe headache developed. Magnetic resonance imaging of the brain showed extensive metastatic disease in the dura. The patient subsequently received a complete course of irradiation to whole brain (2,340 cGy), total pelvis (3,000 cGy), and lower extremities (1,800 cGy), and ultimately died 21 months after the initial diagnosis.

The postmortem examination was conducted 3 hours after death, and metastatic tumor tissue from the lung parenchyma and 50 mL heparinized blood (mononuclear cells isolated by Lymphocyte Separation Medium, Organon Teknika, Durham, NC, U.S.A.) from the left ventricle were cultured in Iscove-modified Dulbecco medium (Bio Whit-

taker, Walkersville, MD, U.S.A.) supplemented with 2 mmol/L L-glutamine (Gemini Bioproducts, Calabasas, CA, U.S.A.), 16.6 mg/mL each of insulin and transferrin, 16.6 μg/mL selenious acid (ITS culture supplement; Collaborative Biomedical Products, Bedford, MA, U.S.A.), and 20% heat-inactivated fetal bovine serum at 37°C in a 5% CO<sub>2</sub> incubator (STERI-CULT 200; Forma Scientific, Marietta, OH, U.S.A.). Gentamicin (100 μg/mL) was used only during the first week of establishing the cell line; thereafter, no further antibiotics were used to facilitate detection of mycoplasma. No cell line resulted from tumor taken from the lung parenchyma. The cell line (CHLA-171) grew from the postmortem blood sample and was used at passage 10 to 20 for all experiments in this report.

### Chemicals

The L-PAM (NSC #14210) and L-(S,R) BSO (NSC #326321) were provided by the National Cancer Institute. A 3.3-mmol/L stock solution of L-PAM was made in 0.1 N NaCl/ethanol (unfiltered). A 10-mmol/L stock solution of BSO was made, dissolved in distilled water, and passed through a 0.22-μm filter. Stock solutions were made fresh for each experiment. Final dilutions of both drugs were made in Iscove medium with 10% heat-inactivated fetal bovine serum and, as required, adjusted to pH 7.4 with 0.1 N HCl or NaOH. All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

### Cytotoxicity Assays

Cell viability after drug treatment was performed in 96-well plates using a novel digital image microscopy (DIMSCAN) system that has a dynamic range of 4 logs of cell kill (32). Comparison studies have shown viable cells measured by DIMSCAN exclude trypan blue and are clonogenic (33), and drug sensitivity/resistance as measured by DIMSCAN correlates with clinical drug exposure (10, 11,34).

After incubation with drugs or control medium, a vital stain, fluorescein diacetate (10 μg/mL), was added to the 96-well plate and incubated for 20 minutes. Eosin-Y (800 μg/mL) was then added to quench background fluorescence in the medium and in nonviable cells. The plates were then analyzed on an inverted microscope, with the relative fluorescence of each well quantified by the digital imaging system software designed for the DIMSCAN system. The mean fluorescence for treated wells was compared with control wells to derive the surviving fraction.

### Dose-Response Assays

After plating, cells were exposed to various concentrations of L-(S,R) BSO (0–1,000 μmol/L) and incubated for 24 hours before the addition of L-PAM (0–40 μmol/L). Cytotoxicity was evaluated by DIMSCAN after an additional 7-day incubation. In vitro exposure to L-PAM without drug washout is an appropriate model for clinical application because of the short half-life of L-PAM in tissue

culture conditions (35). The dose ranges for both BSO and L-PAM were based on peak (L-PAM) or steady-state (BSO) plasma concentrations from standard and myeloablative human trials (Table 1).

### Dose Effect Analysis

Cytotoxicity assays were performed by DIMSCAN as described previously. After incubation, the fraction of cells affected ( $F_a$ ) was calculated [ $F_a = 1 - (\text{relative fluorescence condition/relative fluorescence control})$ ] from relative fluorescence values obtained using DIMSCAN. Data from individual dose-response studies were analyzed using a single-drug dose-response software program to calculate lethal concentration (LC) values (36), with  $LC_{50}$ ,  $LC_{75}$ , and  $LC_{90}$  defined as the respective concentration of drug required to kill 50%, 75%, or 90% of cells tested. Similarly, synergy between L-PAM and BSO was calculated using fixed ratio (BSO:L-PAM at 10:1) analysis (36–39), with synergy defined as a combination index  $<1$ . Synergy was also calculated by fixed-ratio analysis (BSO:L-PAM at 10:1) to generate computer-simulated isobolograms at values of  $LC_{50}$ ,  $LC_{75}$ , and  $LC_{90}$  (36–39). Dose combinations (L-PAM and BSO, respectively) for synergy testing were 3 and 30, 10 and 100, 20 and 200, 30 and 300, and 40 and 400  $\mu\text{mol/L}$ .

### Glutathione

Glutathione studies were conducted in 12.5-cm tissue culture flasks ( $2 \times 10^6$  cells/flask) containing 0, 5, 50, 500, and 1,000  $\mu\text{mol/L}$  BSO for 24 hours. Cells were then detached with Pucks Saline A plus 1  $\mu\text{mol/L}$  edetic acid, harvested with a Pasteur pipette (Fisher Scientific, Pittsburgh, PA, U.S.A.), washed in isotonic (6.3 mmol/L edetic acid, pH 7.4) phosphate-buffered saline, and centrifuged for 3 minutes at 16,000 g, and the resulting pellet was acidified by 200  $\mu\text{L}$  5% sulfosalicylic acid. After recentrifugation for 3 minutes at 16,000 g, the supernatant was flash-frozen in liquid nitrogen and analyzed for total glutathione content within 48 hours by the DTNB (5,5'-dithiobis-2-nitrobenzoic

acid-GSSG) glutathione reductase method using 96-well plates and a Thermomax microplate reader for 2 minutes at 405 nm, with results normalized to total protein (40). Glutathione data previously reported (20) for 10 neuroblastoma cell lines (SMS-KANR, SMS-KAN, SK-N-BE(2), SMS-KCNR, SMS-LHN, SK-N-SH, SMS-SAN, SK-N-FI, SK-N-RA, and SK-N-AS) were used to compare baseline and BSO-depleted glutathione levels in CHLA-171.

### Analysis of Plasma Buthionine Sulfoximine and Melphalan Levels

Plasma L-(S,R) BSO was measured by a modified high-performance liquid chromatography technique for  $\alpha$ -difluoromethylornithine, as previously described (21). This method uses a precolumn derivatization with ophthalaldehyde, followed-up by gradient elution with phosphate buffer (pH 7.5) and methanol. Plasma L-PAM was measured by high-performance liquid chromatography per the method previously described (21).

### Polymerase Chain Reaction and Reverse Transcription Polymerase Chain Reaction

As previously described (10), quantitative fluorescent polymerase chain reaction was used to determine *MYCN* genomic amplification. Tyrosine hydroxylase expression and P-glycoprotein 9.5 expression were determined by reverse transcription polymerase chain reaction (10). Tyrosine hydroxylase expression in a cell line from a childhood small round cell tumor is specific for neuroblastoma, whereas strong P-glycoprotein 9.5 expression is restricted to neural tumors (41).

### Detection of Surface Antigens

To confirm neuroblastoma origin, flow cytometry was used to determine the binding of the neuroblastoma-associated monoclonal antibodies HSAN 1.2 (42) and anti-GD2 (14G2A) (43). In addition, binding of antihuman leukocyte antigen class I antibody W6-32 (42) was analyzed by flow cytometry, as previously described (10).

TABLE 1. Plasma levels of L-PAM and L-(S,R) BSO

Study	Drug	Peak plasma concentration	Css (mean)	Type of therapy	Reference
Lazarus	L-PAM	16–40 $\times \mu\text{mol/L}$	N/A	Myeloablative	46
Pinguet	L-PAM	5–48 $\times \mu\text{mol/L}$	N/A	Myeloablative	47
Hersh	L-PAM	17.7–54.0 $\times \mu\text{mol/L}$	N/A	Myeloablative	48
Anderson	L-PAM	2.3–3.9 $\times \mu\text{mol/L}^*$	N/A	Standard	25
Anderson	BSO	N/A	398 $\times \text{mol/L}$ ( $\pm 194$ )*	Standard	25
Bailey	BSO	1,530 $\times \mu\text{mol/L}$	465 $\times \text{mol/L}^\ddagger$ ( $\pm 189$ )	Standard	21
Bailey	BSO	4.6–8.0 $\times \mu\text{mmol/L}^\ddagger$	N/A	Standard	22

Summary of plasma levels of both L-PAM and L-(S,R) BSO. Css defined as plasma concentration at steady state.

\*Indicates preliminary results of recently completed BSO/L-PAM trial in pediatric patients (NCI T95-0092) and are part of a manuscript in preparation.

†Not applicable signified by N/A.

‡Indicates levels at BSO dosing (0.75 gm/m<sup>2</sup>  $\times$  72 hrs) equivalent to the NCI T95-0092 pediatric trial.

§Indicates levels reported for multiple dose levels.

## RESULTS

### Pathology Results and Characterization of CHLA-171

The autopsy revealed extensive multiorgan metastasis of neuroblastoma, including marked infiltration of lung parenchyma, pleura, and epidural space. Histologic analyses showed a stroma-poor, undifferentiated tumor without neuroblastic maturation in lung parenchyma, liver, bone marrow, and epidural space. Mononuclear cells isolated from postmortem left ventricular blood grew attached colonies of tumor cells that proliferated continuously and were designated as CHLA-171.

Cells from CHLA-171 and frozen samples of postmortem tumor were analyzed for genomic amplification of *MYCN* by polymerase chain reaction, and no amplification was detected. Reverse transcription polymerase chain reaction showed that both CHLA-171 and postmortem tumor tissue were positive for expression of the neural tumor marker PGP 9.5. Tyrosine hydroxylase expression by reverse transcription polymerase chain reaction was negative in CHLA-171 and postmortem tumor but positive in tumor obtained at the time of original diagnosis. Flow cytometry demonstrated that CHLA-171 had strong positive binding of neuroblastoma-associated antibodies (HSAN 1.2 and anti-GD2) and weak binding of the antihuman leukocyte antigen class I antibody W6/32. Small, blue, round cell tumors consistent with neuroblastoma developed in athymic (nu/nu) mice injected with CHLA-171. Taken together, the molecular and immunologic markers, combined with the histologic appearance in an immune-deficient murine model, confirmed the neuroblastoma origin of CHLA-171.

### Cytotoxicity Assays of Buthionine Sulfoximine and Melphalan as Single Agents and in Combination

The results of the cytotoxicity assays by DIMSCAN for L-PAM and BSO as single agents are shown in Figure 1. CHLA-171 was moderately resistant ( $LC_{50}$  42.0  $\mu\text{mol/L}$ ,  $LC_{90}$  509  $\mu\text{mol/L}$ ) to L-(S,R) BSO at concentrations cor-

responding to plasma levels in nonmyeloablative clinical trials (Table 1), with 1,000  $\mu\text{mol/L}$  producing 1.4 logs of cytotoxicity. Similarly, when exposed to a range of concentrations corresponding to plasma L-PAM levels reported for both standard and myeloablative therapy, CHLA-171 was highly resistant to L-PAM ( $LC_{50}$  4.1  $\mu\text{mol/L}$ ,  $LC_{90}$  42.3  $\mu\text{mol/L}$ ), with 40  $\mu\text{mol/L}$  L-PAM resulting in 0.98 logs of cytotoxicity.

### Cytotoxicity Assays of Buthionine Sulfoximine Combined With Melphalan

The dose-response curve of L-PAM in combination with a constant level of 500  $\mu\text{mol/L}$  is shown in Figure 1. Throughout all doses ranges of L-PAM, cytotoxicity was enhanced by the addition of 500  $\mu\text{mol/L}$  BSO. However, despite being combined with 500  $\mu\text{mol/L}$  BSO, the nonmyeloablative concentration of 3  $\mu\text{mol/L}$  L-PAM resulted in only 1.45 logs of cytotoxicity. To determine whether BSO and L-PAM achieved synergistic cytotoxicity for CHLA-171, a fixed-ratio (10:1) analysis was performed to determine the combination index for each drug mixture (Fig. 2). When BSO at 30  $\mu\text{mol/L}$  was coincubated with the nonmyeloablative L-PAM concentration of 3  $\mu\text{mol/L}$ , no evidence of synergy (combination index = 1.38) was observed. At all other combinations tested, especially with concentrations of L-PAM that clinically can be achieved only in the myeloablative setting, L-PAM and BSO showed a high degree (combination index <0.3) of drug synergy. The highest concentrations of L-PAM and BSO tested caused significant cytotoxicity for CHLA-171, with 400  $\mu\text{mol/L}$  BSO and 40  $\mu\text{mol/L}$  L-PAM producing 4.3 logs of cell kill. Synergy was also observed by generating isobolograms for  $LC_{50}$ ,  $LC_{75}$ , and  $LC_{90}$  (not shown).

### Glutathione Decreased by Buthionine Sulfoximine

CHLA-171 was incubated in various concentrations of BSO (0–1,000  $\mu\text{mol/L}$ ) for 24 hours and then assayed for

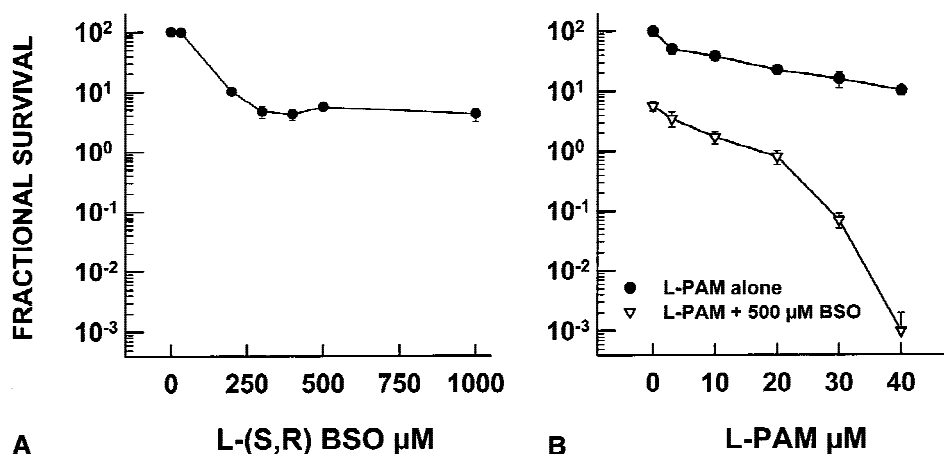
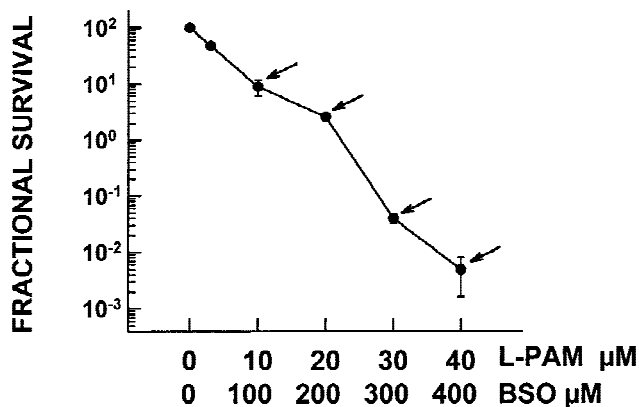


FIG. 1. (A) Dose-response curve of L-(S,R) BSO as a single agent. (B) Dose-response curves of L-PAM as a single agent (filled circle), and L-PAM combined with a constant dose of 500  $\mu\text{mol/L}$  L-(S,R) BSO (open triangle). Values represent mean + standard error.



**FIG. 2.** Dose-response curve of L-(S,R) BSO combined with L-PAM at a 10:1 fixed ratio. The combination index (CI) for each experimental point was calculated, with synergy (CI < 1) observed for data points marked by an arrow. The CI values for the actual 10:1 (BSO to L-PAM) drug mixtures were 30:3 μmol/L, CI = 1.3; 100:10 μmol/L, CI = 0.35; 200:20 μmol/L, CI = 0.16; 300:30 μmol/L, CI = 0.0025; 400:40 μmol/L, CI = 0.00034. Values represent mean + standard error.

total glutathione. The decrease in glutathione after BSO treatment was dose-dependent, with 1,000 μmol/L BSO depleting glutathione to 36% of baseline (not shown). The concentration of glutathione (both baseline and after a 24-hour incubation with 500 μmol/L BSO) in CHLA-171 was compared with the mean glutathione concentration undergoing the same conditions for 10 previously reported (20) neuroblastoma cell lines. The baseline level of glutathione in CHLA-171 was  $0.49 \pm 0.02$  nmol/pg protein compared with a mean of  $0.59 \pm 0.08$  nmol/pg protein in the 10 cell lines. Treatment of CHLA-171 with 500 μmol/L BSO for 24 hours depleted glutathione to 38%, compared with an average reduction of glutathione to 47% in 10 other (20) neuroblastoma cell lines.

## DISCUSSION

CHLA-171 represents a novel neuroblastoma cell line established from a patient who experienced relapse after BSO/L-PAM therapy. Clinically, the tumor metastasized to locations (e.g., lung parenchyma, pleural, dura) rarely seen in newly diagnosed neuroblastoma (5). Tumor recurrence in such sites is more common after myeloablative therapy than at diagnosis (central nervous system, 5% vs. 0.6%; lung, 7% vs. 3%) (5,44,45), suggesting that changes conferring drug resistance in neuroblastoma may enhance tumor survival in certain tissues.

CHLA-171 showed a marked resistance to L-PAM that is consistent with our previous report of L-PAM resistance in neuroblastoma cell lines established at the time of recurrence after myeloablative therapy with L-PAM (10). The authors have recently shown that unlike drug-sensitive neuroblastoma cell lines (which have functional *p53*), most of the neuroblastoma cell lines (including CHLA-171) that

show a high level of multidrug resistance (including L-PAM) do not have functional *p53* (44). Thus, L-PAM resistance in CHLA-171 may be mediated, at least in part, by a loss of *p53* function. Moreover, the authors have shown here that depletion of glutathione with BSO can be combined with high levels of L-PAM to overcome drug resistance in the CHLA-171 cell line, suggesting that such an approach may be active against tumors lacking functional *p53*.

Melphalan as a single agent had an LC<sub>90</sub> of 42 μmol/L for CHLA-171, compared with our previous report showing that neuroblastoma cell lines established from patients at the time of diagnosis are sensitive to L-PAM, with a mean LC<sub>90</sub> of  $5.2 \pm 6$  μmol/L (10). In the same study, neuroblastoma cell lines established from patients who had relapse after standard chemotherapy were moderately resistant to L-PAM (LC<sub>90</sub>  $27.3 \pm 30$  μmol/L), and neuroblastoma cell lines obtained from patients who had relapse after high-dose L-PAM (210 mg/m<sup>2</sup>) supported by bone marrow transplant were extremely resistant to L-PAM (LC<sub>90</sub>  $273 \pm 530$  μmol/L) (10,11). In our recently completed nonmyeloablative phase I trial of L-PAM and BSO, we observed peak plasma concentrations for L-PAM of 2.3 to 3.9 μmol/L (25). The data presented in this report show that for the patient from whom CHLA-171 was established, L-PAM, both as a single agent and combined with BSO, would be ineffective at the doses used. This resistance was not caused by a failure of BSO to deplete glutathione in CHLA-171 because there was no discernible difference in glutathione levels (baseline and percentage depletion) between CHLA-171 and 10 other neuroblastoma cell lines. However, the current data suggest that a multilog tumor cell kill may have occurred if BSO had been used with doses of L-PAM achievable only in the myeloablative setting.

Our current clinical data in neuroblastoma for BSO/L-PAM (25), and the results from studying the CHLA-171 cell line, suggest that neuroblastoma can develop a marked and sustained resistance to L-PAM, and that glutathione depletion with BSO will not overcome that resistance at L-PAM levels that can be obtained in the nonmyeloablative setting. However, the data from CHLA-171 suggest that BSO can overcome L-PAM resistance if the L-PAM dose can be increased with hematopoietic stem cell support. Thus, if BSO/L-PAM is tolerable in the myeloablative setting, BSO may enhance the activity of L-PAM against drug-resistant neuroblastoma and could improve the outcome for patients with neuroblastoma undergoing myeloablative therapy after progressive disease develops. A clinical trial to determine the maximally tolerated dose of L-PAM administered together with BSO and supported with autologous hematopoietic stem cell transplantation is underway.

**Acknowledgments:** The authors thank Diane Thompson, Juan-Juan Zuo, William Meek, and Paul Alfaro for technical assistance and Michelle Solorzano for assistance in manuscript preparation.

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