

## Cytotoxic agents

# Synergistic cytotoxicity of buthionine sulfoximine (BSO) and intensive melphalan (L-PAM) for neuroblastoma cell lines established at relapse after myeloablative therapy

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### Summary:

Patients with high-risk neuroblastoma (NB) initially respond to aggressive, alkylator-based therapy only to die from recurrent disease that is refractory to chemotherapy, including alkylating agents. We examined the ability of buthionine sulfoximine (BSO)-mediated glutathione (GSH) depletion to modulate melphalan (L-PAM) resistance in five NB cell lines established after progressive disease following myeloablative therapy (high-dose melphalan, carboplatin, etoposide and total body irradiation) supported by autologous hematopoietic stem cell transplant (AH SCT), and in 15 NB cell lines established at diagnosis or after non-myeloablative therapy (pre-AH SCT). Four of five post-AH SCT NB cell lines and 10 of 15 pre-AH SCT NB cell lines were sensitive to single agent BSO ( $LC_{90} < 300 \mu M$  BSO), while two of five post-AH SCT lines and one of 15 pre-AH SCT lines showed high-level resistance to L-PAM ( $LC_{90} > 30 \mu M$ ). Fixed ratio analysis demonstrated BSO/L-PAM synergy (combination index  $< 1$ ) for all five post-AH SCT and for all 15 pre-AH SCT cell lines tested. Multi-log cytotoxicity (often exceeding four logs of cell kill) was observed in post-AH SCT L-PAM-resistant cell lines (including p53 non-functional lines) only when clinically achievable concentrations of BSO were combined with myeloablative concentrations of L-PAM. We conclude that most neuroblastoma cell lines, including post-AH SCT NB cell lines that are highly resistant to myeloablative levels of L-PAM and lack p53 function, are sensitive to clinically achievable concentrations of L-PAM and BSO. However, some L-PAM-resistant NB cell lines (especially those lacking p53 function) require dose escalation of L-PAM to myeloablative concentrations in order to demonstrate significant synergistic cytotoxicity. Thus, optimal clinical application of BSO/L-PAM may require AH SCT.

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Neuroblastoma, a childhood malignancy arising from the sympathetic nervous system, remains a therapeutic challenge. The majority of patients diagnosed after 1 year of age with stage 4 disease typically respond to intensive, alkylator-based therapy, only to relapse and die from refractory disease.<sup>1–5</sup> In addition, there is a cohort of extremely high-risk children who have persistent tumor in bone marrow after induction chemotherapy ( $> 100/10^5$  tumor cells in bone marrow by immunocytology after 12 weeks induction therapy) that are at high risk for relapse after consolidation therapy.<sup>6</sup> Dose intensification and myeloablative therapy (including melphalan  $210 \text{ mg/m}^2$ ) followed by autologous hematopoietic stem cell transplantation (AH SCT) and high-dose, pulse 13-*cis*-retinoic acid, have made a positive impact on event-free survival for high-risk neuroblastoma patients,<sup>7</sup> but further intensification of standard alkylating agents will be limited by extramedullary toxicities.

As neuroblastoma therapy is heavily based upon alkylating agents, including melphalan (L-PAM), treatment failure is likely to be related to acquired alkylator resistance. In order to restore sensitivity to alkylating agents and improve response in children failing standard high-risk therapy, it will be necessary to identify methods to overcome alkylator resistance without adding unacceptable toxicity. In addition, unlike neuroblastoma cell lines that are sensitive to L-PAM, most drug-resistant neuroblastoma cell lines do not have functional p53 (often due to mutation), and abrogation of p53 function confers high-level, multi-log resistance to drug-sensitive neuroblastoma cell lines.<sup>8</sup> Therefore, as high-level drug resistance in neuroblastoma may be due to a loss of p53 function, overcoming alkylator resistance may require approaches that are independent of p53 function.

Glutathione (GSH) is a ubiquitous intracellular tri-peptide that protects cells from oxidative stress and has been shown to detoxify alkylating agents.<sup>9–11</sup> Buthionine sulfoxi-

mine (BSO), a specific inhibitor of  $\gamma$ -glutamyl cysteine synthetase (the rate-limiting enzyme in GSH synthesis), depletes GSH, and can reverse alkylator resistance.<sup>9,12–15</sup> We have previously reported that BSO, as a single agent, is highly cytotoxic for neuroblastoma cell lines *in vitro* and results in apoptosis due to increased generation of reactive oxygen species (ROS).<sup>16,17</sup> The combination of BSO with clinically achievable (non-myeloablative) concentrations of L-PAM was synergistic for neuroblastoma cell lines established at diagnosis or following non-myeloablative therapy.<sup>16</sup> Non-myeloablative trials of BSO and L-PAM in adults and children have achieved BSO levels of  $\sim 500 \mu\text{M}$  and L-PAM levels of  $\sim 3 \mu\text{M}$  and have shown that the combination causes reversible bone marrow suppression as the major clinical toxicity.<sup>18–22</sup> In this report, we show for a group of post-AHSCT neuroblastoma cell lines established at relapse after myeloablative therapy, that BSO synergistically reverses L-PAM resistance, but only when L-PAM is escalated to levels achievable in the myeloablative setting.

## Materials and methods

### Human cell lines

Five human neuroblastoma cell lines (CHLA-51, CHLA-79, CHLA-90, CHLA-134, and CHLA-136) established from patients who had recurrent disease following myeloablative therapy and autologous hematopoietic stem cell transplantation (post-AHSCT)<sup>23</sup> were cultured in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Walkersville, MD, USA) supplemented with 2 mM L-glutamine (Gemini Bioproducts, Calabasas, CA, USA), 16.6 mg/ml each of insulin and transferrin, 16.6  $\mu\text{g/ml}$  of selenous acid (ITS culture supplement; Collaborative Biomedical Products, Bedford, MA, USA), and 20% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO<sub>2</sub> incubator. Fifteen human neuroblastoma cell lines (SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, SK-N-BE(1), SK-N-DZ, SMS-LHN, LA-N-5, LA-N-6, SK-N-RA, SK-N-FI, LA-N-1, SK-N-SH, SK-N-AS and SMS-MSN) obtained from patients prior to myeloablative therapy (pre-AHSCT)<sup>23–25</sup> were cultured in RPMI-1640 with 10% heat-inactivated FBS at 37°C in 5% CO<sub>2</sub>. All neuroblastoma cell lines were grown without antibiotics to facilitate detection of mycoplasma. Experiments were conducted at cell line passage 15–25.

### Chemicals

L-PAM (NSC 14210) and L-(S,R) BSO (NSC 326321) were provided by the National Cancer Institute (NCI, Bethesda, MD, USA). A 3.3 mM stock solution of L-PAM was made in 0.1 N HCl/ethanol and left unfiltered. A 10 mM stock solution of BSO was made, dissolved in distilled water, and filtered using a 0.22  $\mu$  filter. Stock solutions were made fresh for each experiment. Final dilutions of both drugs were made in IMDM for post-AHSCT cell lines or RPMI-1640 medium for pre-AHSCT cell lines, with 10% heat-inactivated FBS, adjusted as required) to pH 7.4 with 0.1 N HCl or NaOH. All other chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA).

### 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 greater than four logs of cell kill.<sup>26</sup> Following incubation with drugs or control medium, a vital stain, fluorescein diacetate (10 mg/ml) was added to the 96-well plate and incubated for 20 min. Eosin-Y (800 mg/ml) was then added to quench background fluorescence in the medium and in non-viable cells. The plates were then analyzed on an inverted microscope with the relative fluorescence (RF) of each well quantitated by the DIMSCAN digital imaging system software, using digital thresholding to eliminate background fluorescence.<sup>26</sup> The mean fluorescence for treated wells was compared to control wells to derive the surviving fraction. DIMSCAN is a new method developed in our laboratory that is superior to other cytotoxicity assays in 96-well plates by virtue of its ability to measure greater than four logs of cell killing. Comparison studies have shown that viable cells measured by DIMSCAN exclude trypan blue, and are clonogenic.<sup>26,27</sup> Furthermore, drug sensitivity/resistance measured by DIMSCAN correlates with clinical drug exposure.<sup>28,29</sup>

### Dose–response assays

Cells were grown to confluence, harvested by washing with Puck's saline A + 1 mM EDTA and 10 mM Hepes<sup>30</sup> resuspended in complete medium (IMDM + 20% FBS for post-AHSCT; RPMI-1640 + 10% FBS for pre-AHSCT), and plated in 96-well microtiter FALCON tissue culture plates (Becton Dickinson, Lincoln Park, NJ, USA) at a density of 25 000–30 000 viable cells/well with 16 replicate wells/condition. Following plating, cells were exposed to control medium or various concentrations of L-(S,R) BSO (0–400  $\mu\text{M}$  for post-AHSCT; 0–1000 for pre-AHSCT) and incubated for 24 h. After the 24 h pre-incubation with BSO, L-PAM (dose range 0–40  $\mu\text{M}$ ) was added (total volume/well 100  $\mu\text{l}$ ) and then incubated for an additional 6 days after which cell viability was determined by DIMSCAN.

### Dose–effect analysis

Cytotoxicity assays were performed by DIMSCAN as described above. Following a 7 day incubation, the fraction of cells affected ( $F_a$ ) was calculated ( $F_a = 1 - \text{RF condition} / \text{RF control}$ ) from relative fluorescence (RF) values obtained from DIMSCAN. Data from dose–response studies were analyzed using a single-drug dose–response program to calculate lethal concentration (LC) values.<sup>31–35</sup> LC<sub>90</sub> and LC<sub>50</sub> were defined as the respective concentrations of drug required to kill 90% or 50% of cells tested.

### Drug synergy

Evidence of drug synergy between BSO and L-PAM was determined by multiple drug effect analysis (MDEA) software.<sup>31</sup> The combination index (CI) was the calculated affect (fraction affected) of the two drugs in combination

with the CI value ultimately defined as additive (CI = 1), antagonistic (CI > 1), or synergistic (CI < 1). Experiments for post-AHSCT cell lines were conducted as a fixed ratio analysis (concentration BSO: concentration L-PAM, 10:1, except for CHLA-51 where BSO:L-PAM ratio was 1:1) in order to mathematically determine the expected CI of the two drugs over all ranges of  $F_a$ . Fifteen pre-AHSCT cell lines were also analyzed by non-fixed ratio analysis from an earlier and separate experiment, and therefore the CI for these cell lines only applies to the effect of 1  $\mu\text{M}$  L-PAM in the presence of either 10  $\mu\text{M}$  BSO (those cell lines with  $\text{LC}_{90}$  BSO < 20  $\mu\text{M}$  BSO) or 100  $\mu\text{M}$  BSO (those cell lines with  $\text{LC}_{90}$  BSO > 20  $\mu\text{M}$  BSO). For the same 15 pre-AHSCT cell lines, a dose response of L-PAM (0–10  $\mu\text{M}$ ) was also conducted in the presence of a constant dose of BSO (either 10  $\mu\text{M}$  BSO for those cell lines with  $\text{LC}_{90}$  BSO < 20  $\mu\text{M}$  BSO, or 100  $\mu\text{M}$  BSO for those cell lines with  $\text{LC}_{90}$  BSO > 20  $\mu\text{M}$  BSO) in order to calculate the effect of BSO upon the  $\text{LC}_{90}$  of L-PAM.

*Measurement of intracellular glutathione*

Intracellular glutathione (GSH) was measured in cells seeded into 25  $\text{cm}^2$  tissue culture flasks ( $2 \times 10^6$  cells) and treated with 500  $\mu\text{M}$  BSO for 24 h. Cells were then mechanically harvested with Puck's saline A + 1 mM EDTA and 10 mM HEPES, washed in PBS, centrifuged, and the pellet was acidified with 200  $\mu\text{M}$  5% sulfasalicylic acid, flash frozen in liquid nitrogen vapor, and analyzed for total glutathione content within 48 h by the DTNB-GSSB reductase method, with results normalized to total protein as previously described,<sup>17</sup> except that the GSH assay was adapted to employ a Thermomax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

*Statistics*

Significance between various groups was estimated by unpaired Student's *t*-test performed using Microsoft Excel software.

**Results**

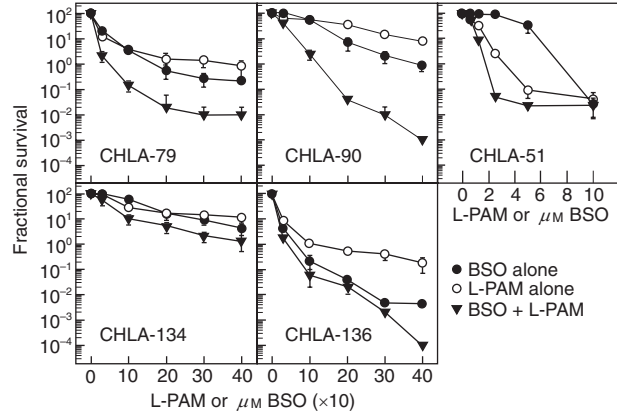
*BSO as a single agent*

The five post-AHSCT cell lines were variably sensitive to BSO as a single agent (Table 1; Figure 1). In comparison

**Table 1** Post-AHSCT neuroblastoma cell lines

Cell line	p53 function	L-PAM	BSO	Decrease in GSH by 500 $\mu\text{M}$ BSO (% control)	CI at L-PAM	
		$\text{LC}_{90}$ ( $\mu\text{M}$ )	$\text{LC}_{90}$ ( $\mu\text{M}$ )		<3 $\mu\text{M}$	$\geq 10 \mu\text{M}$
CHLA-51	+	0.9	7.8	20.3	0.02	0.048
CHLA-79	+	3.5	15.7	31.7	0.61	0.124
CHLA-90	-	60.5	276.0	51.0	1.2	0.103
CHLA-134	-	46.5	936.0	34.9	1.2	0.313
CHLA-136	+	1.2	1.3	26.9	5.4	0.75

The CI reflects concentrations of L-PAM ( $\geq 10 \mu\text{M}$ ) found in myeloablative therapy in combination with BSO. Determination of cell line p53 function was previously reported.<sup>8</sup>



**Figure 1** Dose-response of post-AHSCT neuroblastoma cell lines CHLA-51, CHLA-79, CHLA-90, CHLA-134 and CHLA-136 to L-PAM, BSO and the combination of BSO + L-PAM. Cytotoxicity was determined by DIMSCAN assay. BSO + L-PAM were combined at 10:1 ratio, except for CHLA-51 where the ratio was 1:1. Values represent mean  $\pm$  standard error of the fractional survival.

to clinically achievable levels of BSO (mean  $524 \pm 209 \mu\text{M}$  for a continuous infusion of  $1 \text{ g/m}^2/\text{h}$ ),<sup>20,37</sup> CHLA-51, CHLA-79, CHLA-90, and CHLA-136 had a  $\text{LC}_{90}$  for BSO less than 300  $\mu\text{M}$  (range 0.9–276  $\mu\text{M}$ ), whereas the  $\text{LC}_{90}$  for CHLA-134 was 936  $\mu\text{M}$  BSO. However, there was no significant difference between the mean  $\text{LC}_{90}$  for BSO of the five post-AHSCT cell lines ( $247 \pm 402 \mu\text{M}$ ) compared to the mean  $\text{LC}_{90}$  for BSO previously reported<sup>17</sup> for a panel of neuroblastoma cell lines established prior to intensive myeloablative chemotherapy ( $211 \pm 316 \mu\text{M}$ ;  $P = 0.86$ ).

*L-PAM as a single agent*

The five post-AHSCT cell lines were also variably sensitive to L-PAM as a single agent (Table 1 and Figure 1). In comparison to clinically achievable peak levels of L-PAM in the non-myeloablative setting (mean  $3.2 \pm 0.9 \mu\text{M}$  for  $15 \text{ mg/m}^2$  given as single intravenous bolus), the p53-functional post-AHSCT neuroblastoma cell lines CHLA-51, CHLA-79, and CHLA-136 had a  $\text{LC}_{90}$  for L-PAM less than 4  $\mu\text{M}$  (range 0.9–3.5  $\mu\text{M}$ ), whereas the  $\text{LC}_{90}$  values obtained for the p53 non-functional neuroblastoma cell lines CHLA-90 and CHLA-134 were 60  $\mu\text{M}$  and 46  $\mu\text{M}$  L-PAM, respectively. In contrast, the mean  $\text{LC}_{90}$  for a panel of 15 pre-AHSCT neuroblastoma cell lines (Table 2) was  $7.6 \pm 16.4 \mu\text{M}$  (range 0.1–63.9  $\mu\text{M}$ ). Only one of the pre-AHSCT cell lines had high-level resistance to L-PAM (SMS-MSN), but four pre-AHSCT cell lines (SK-N-DZ, LA-N-6, SK-N-FI and SK-N-RA) showed some degree of L-PAM resistance ( $\text{LC}_{90} > 5 \mu\text{M}$ ). Thus, some post-AHSCT neuroblastoma cell lines have a single-agent sensitivity to L-PAM similar to that seen with cell lines established prior to AHSCT.

*Cytotoxicity of BSO combined with L-PAM*

Using a fixed ratio analysis, drug synergy (CI < 1) between BSO and L-PAM was observed for all post-AHSCT neuroblastoma cell lines at concentrations of L-PAM ( $\geq 10 \mu\text{M}$ ) achievable in the autologous hematopoietic stem cell trans-

**Table 2** Pre-AHSCT neuroblastoma cell lines

Cell line	LC <sub>90</sub> BSO (μM) <sup>17</sup>	LC <sub>90</sub> L-PAM (μM)	LC <sub>90</sub> L-PAM (μM) + 10 μM BSO	LC <sub>90</sub> L-PAM (μM) + 100 μM BSO	CI
LA-N-1	2.1	1.4	0.3		0.70
SK-N-DZ	8.9	16.3	0.2		0.65
SK-N-SH	11.2	0.2	0.1		0.18
SMS-KANR	3.7	0.6	0.1		0.21
SMS-LHN	9.7	0.4	0.1		0.04
SMS-MSN	7.7	63.9	0.1		0.12
SK-N-BE(1)	23.8	0.1		0.1	0.11
LA-N-5	140.7	2.3		0.2	0.61
LA-N-6	40.0	6.4		3.9	0.24
SK-N-AS	371.0	0.8		0.3	0.28
SK-N-FI	>1000.0	9.0		8.2	0.03
SK-N-RA	823.0	11.3		2.6	0.28
SMS-KAN	306.0	0.1		0.1	0.17
SMS-KCN	50.7	0.3		0.1	0.21
SMS-KCNR	377.0	0.8		0.1	0.22

The dose–response for L-PAM in those cell lines with a LC<sub>90</sub> for BSO <20 μM was conducted in the presence of a fixed concentration of 10 μM BSO and the dose–response for L-PAM in those cell lines with a LC<sub>90</sub> for BSO ≥20 μM was conducted in the presence of a fixed concentration of 100 μM BSO. Combination Index (CI) ≤1 indicates synergy between BSO and L-PAM.

plant (AHSCT) setting (Table 1). Although CHLA-51 and CHLA-79 demonstrated synergy (CI <1) between BSO and L-PAM at the non-myeloablative concentration of 3 μM L-PAM (and at higher concentrations), BSO/L-PAM synergy was only observed in the remaining cell lines (CHLA-90, CHLA-134, and CHLA-136) with escalation of L-PAM to concentrations (≥10 μM), which are only achieved clinically in the myeloablative setting (Table 1). Strikingly, 40 μM L-PAM combined with 400 μM BSO, resulted in up to 3.8 log increase (range 1.92–3.8 log) in cell kill for the post-AHSCT lines compared to 40 μM of L-PAM alone. For all 15 pre-AHSCT neuroblastoma cell lines, the addition of BSO not only decreased the LC<sub>90</sub> for L-PAM to submyeloablative levels (1.1 ± 2.3 μM L-PAM) but also resulted in synergy (CI <1) when 1 μM L-PAM was combined with either 10 μM or 100 μM BSO (Table 2).

#### Glutathione (GSH) was decreased by BSO

All post-AHSCT cell lines were incubated in BSO (500 μM) for 24 h and assayed for total GSH. The percentage of control GSH in response to 24 h of 500 μM BSO for the post-AHSCT cell lines is shown in Table 1. The mean percent of control GSH for the five post-AHSCT neuroblastoma cell lines was 33.0 ± 11.5%, and this was comparable to the mean percent of control GSH (46.7% ± 11.8%) under the same conditions for 10 pre-AHSCT neuroblastoma cell lines which we previously reported.<sup>16</sup>

#### Discussion

Selecting therapy for neuroblastoma patients who relapse after intensive, alkylator-based, myeloablative therapy supported by autologous hematopoietic stem cell transplant (AHSCT) poses a major clinical dilemma. As most non-infant stage 4 neuroblastoma patients are now given myeloablative therapy/AHSCT prior to relapse, post-AHSCT

relapse patients will comprise the majority of future patients with disease progression. There is also a cohort of children that either respond poorly to induction therapy, have ≥100/10<sup>5</sup> tumor cells in marrow by immunocytology at week 12 of induction therapy, or develop progressive disease during induction therapy that are at very high risk of relapse post-AHSCT.<sup>6</sup> As alkylating agents are integral to both induction therapy and AHSCT, a possible mechanism for treatment failure in relapsed and poorly responding patients is acquired resistance to alkylating agents. This is supported by our previous reports showing a sustained multi-drug resistance (including to the alkylating agent L-PAM) occurs in some cell lines established from patients who relapsed after myeloablative therapy which included 210 mg/m<sup>2</sup> of L-PAM.<sup>28,29</sup> We have also shown that high-level multi-drug resistance in neuroblastoma is associated with loss of p53 function (apparently acquired and often due to mutation) that is frequently found in neuroblastoma cell lines established after disease progression.<sup>8</sup> Modulation of alkylator resistance in a p53-independent manner is one novel strategy that may provide an effective salvage treatment for patients with recurrent neuroblastoma, and could ultimately improve the efficacy of ‘up-front’ AHSCT for stage 4 neuroblastoma.

We studied five post-AHSCT neuroblastoma cell lines that had a single-agent LC<sub>90</sub> for L-PAM ranging from 0.9 to 60 μM compared to peak plasma concentrations of 3.2 ± 0.9 μM L-PAM reported in our non-myeloablative trial of BSO + L-PAM<sup>20,37</sup> and peak plasma concentrations ranging between 5 and 54 μM in myeloablative trials of L-PAM.<sup>38–40</sup> Data from the current report suggest that for some neuroblastoma cell lines (p53 non-functional and post-AHSCT), escalation of L-PAM to ≥10 μM is needed to achieve multi-log drug synergy in the presence of BSO. This observation is similar to what we have observed in a cell line (CHLA-171) established from a neuroblastoma patient who failed to respond to BSO and non-myeloablative L-PAM,<sup>25</sup> and these results imply that non-myeloablative L-PAM, both as a single agent or combined with

BSO, would be ineffective for some patients with refractory disease. This is in contrast to the synergy observed ( $CI < 1$ ) between low concentrations of BSO (10 or 100  $\mu M$ ) combined with the non-myeloablative concentration of 1  $\mu M$  L-PAM for many NB cell lines established prior to AHSCT. In addition, L-PAM resistance that was not overcome by BSO (ie in the CHLA-134 cell line) was not due to a failure of BSO to deplete GSH, as the % depletion of GSH in CHLA-134 was not significantly different to that seen in the other four post-AHSCT neuroblastoma cell lines or in 10 previously reported neuroblastoma cell lines.<sup>16</sup>

The lack of a high level of L-PAM resistance in CHLA-51 and CHLA-136, despite being established from patients who relapsed after myeloablative therapy employing 210 mg/m<sup>2</sup> L-PAM during pre-AHSCT conditioning, is beyond the scope of this report. This does, however, suggest that sensitivity to bi-functional alkylating agents may be retained in some patients who relapse after myeloablative therapy.

This current report shows for some neuroblastoma cell lines (including those lacking p53 function), that BSO-mediated GSH depletion will not overcome resistance at L-PAM levels obtainable in the non-myeloablative setting. BSO may overcome this high-level of L-PAM resistance (ie achieving  $>3$  logs of cell kill) if the L-PAM dose can be escalated with hematopoietic stem cell support. Thus, if BSO/L-PAM is tolerable in the myeloablative setting, then BSO may enhance the activity of L-PAM against drug-resistant neuroblastoma and could improve outcome for neuroblastoma patients with progressive disease or poor response to standard high-risk induction therapy. A recently completed pilot clinical trial (NCI T95-0092) of BSO and low-dose (15 mg/m<sup>2</sup>) L-PAM in children with recurrent neuroblastoma,<sup>20,37</sup> together with the data presented in this report, led to an ongoing follow-up phase I trial that seeks to define the maximal tolerated dose of L-PAM given with BSO and AHSCT (NCI 68).

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