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Synergism of Buthionine Sulfoximine and Melphalan Against Neuroblastoma Cell Lines Derived After Disease Progression

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Background. Despite intensive-alkylator based regimens, >50% of patients with high-risk neuroblastoma (NB) die from recurrent disease that is probably due, in part, to acquired alkylator resistance. ***Procedure.*** Using buthionine sulfoximine (BSO)-mediated, glutathione (GSH) depletion to modulate melphalan (L-PAM) resistance, we examined six NB cell lines established after progressive disease following either standard chemotherapy, BSO/L-PAM therapy, or myeloablative therapy and autologous hematopoietic stem cell transplant (AHSCT). ***Results.*** Four of the six cell lines (three p53-nonfunctional and one p53-functional) showed high-level L-PAM resistance. ***Conclusions.*** Fixed ratio analysis demonstrated BSO/L-PAM synergy (combination index >1) for all cell lines tested. In L-PAM-resistant cell lines, the minimal cytotoxicity observed for BSO combined with nonmyeloablative concentrations of L-PAM was markedly enhanced (>4 logs total cell kill) when BSO was combined with myeloablative concentrations of L-PAM. In alkylator-resistant NB, the optimal use of BSO may require dose escalation of L-PAM to levels requiring AHSCT. Med. Pediatr. Oncol. 35:659–662, 2000. © 2000 Wiley-Liss, Inc.

Key words: synergism; buthionine sulfoximine; melphalan; neuroblastoma cell lines; disease progression

INTRODUCTION

Most patients with stage 4 neuroblastoma (NB) diagnosed at age >1 year or stage 4 <1 year with *MYCN* amplification initially respond to therapy, but many ultimately succumb to recurrent disease that is refractory to chemotherapy [1–5]. Acquired alkylator resistance is one likely mechanism for treatment failure; current therapy of high-risk NB relies heavily on alkylating agents such as cyclophosphamide, carboplatin, cisplatin, and melphalan (L-PAM) [3,6,7]. We have previously shown that sustained resistance (LC_{90} > 40 μM) to the alkylating agent L-PAM occurs in a majority of cell lines established from patients who relapsed after myeloablative therapy that included intensive L-PAM (210 mg/m^2) during the preautologous hematopoietic stem cell transplant (AHSCT) conditioning [8,9]. However, insofar as high-level drug resistance in many NB may be due to a loss of p53 function [10], modulators of alkylator resistance will have to achieve cytotoxicity independent of p53 to be effective against such tumors.

Buthionine sulfoximine (BSO), a specific inhibitor of glutathione synthesis, depletes GSH and can reverse alkylator resistance [11–15]. BSO alone is highly cytotoxic for most NB cell lines in vitro and results in apoptosis following the generation of reactive oxygen species (ROS) [16]. Furthermore, BSO combined with L-PAM is synergistic against NB cell lines [17]. Nonmyeloablative trials of BSO and L-PAM in adults have shown the combination to be well-tolerated with reversible bone marrow suppression as the major clinical toxicity [18–21]. In this report, we show that BSO synergistically reverses L-PAM resistance in a group of highly L-PAM-resistant NB cell lines established after disease progression but only when L-PAM is escalated to levels achievable in the myeloablative setting.

MATERIALS AND METHODS

Cell Lines

Six NB cell lines established from patients who had progressive disease following standard chemotherapy (CHLA-20), BSO/LPAM therapy (CHLA-171), or my-

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TABLE I. Summary of Postprogressive Disease Neuroblastoma Cell Lines

	Post-AHSCT	p53 Functional	L-PAM LC_{90} (μM)	BSO LC_{90} (μM)	CI <1 at [AHSCT][a]
CHLA-20	−	+	6	133	+
CHLA-171	−	−	42	509	+
CHLA-51	+	+	2	5	+
CHLA-79	+	+	4	10	+
CHLA-90	+	−	47	37	+
CHLA-134	+	−	52	939	+

[a][AHSCT], concentration L-PAM achievable in autologous hematopoietic stem cell transplantation.
Values represent mean ± standard error.

eloablative therapy and autologous hematopoietic stem cell transplant (post-AHSCT; CHLA-51, CHLA-79, CHLA-90, CHLA-134) were used for all studies in this report. Cell culture conditions, chemicals, digital image microscopy (DIMSCAN), glutathione levels, p53 function, and statistics were conducted as previously reported [8–10,16,17,22,23]. A single-drug dose-response software program was used to calculate Lethal Concentration (LC) values, with LC_{90} defined as the respective concentration of drug required to kill 90% of cells tested [24–26]. Similarly, synergy between L-PAM and BSO was calculated using fixed ratio (BSO:L-PAM at 1:1 for CHLA-51, at 10:1 for all other cell lines) analysis [25–27], with synergy defined as a combination index (CI) <1. Synergy was also calculated by fixed ratio analysis to generate computer-simulated isobolograms (not shown) at values of LC_{90} [24–27]. Dose combinations (L-PAM/BSO) for synergy testing were as follows: for CHLA-51, 0.6 μM/0.6 μM, 1.25 μM/1.25 μM, 2.5 μM/2.5 μM, 5 μM/5 μM, and 10 μM/10 μM; for all other cell lines, 3 μM/30 μM, 10 μM/100 μM, 20 μM/200 μM, 30 μM/300 μM, and 40 μM/400 μM.

RESULTS

Cytotoxicity Assays of BSO and L-PAM as Single Agents

The results of the cytotoxicity assays by DIMSCAN for L-PAM and BSO as single agents are summarized in Table I for all six cell lines. CHLA-171 and CHLA-134 were moderately resistant (LC_{90} ≥509 μM) to L-(S,R) BSO. Among the three p53 functional cell lines, CHLA-51 was highly sensitive (LC_{90} 2 μM) to L-PAM at nonmyeloablative levels (≤3 μM achieved using 15 mg/m^2), whereas CHLA-20 and CHLA-79 were intermediately L-PAM sensitive (4 μM and 6 μM, respectively). CHLA-171, CHLA-90, and CHLA-134 (all p53 nonfunctional) were highly resistant to L-PAM (LC_{90} 47.0 μM ± 5 μM) at concentrations exceeding plasma levels obtained in the myeloablative setting [28,29].

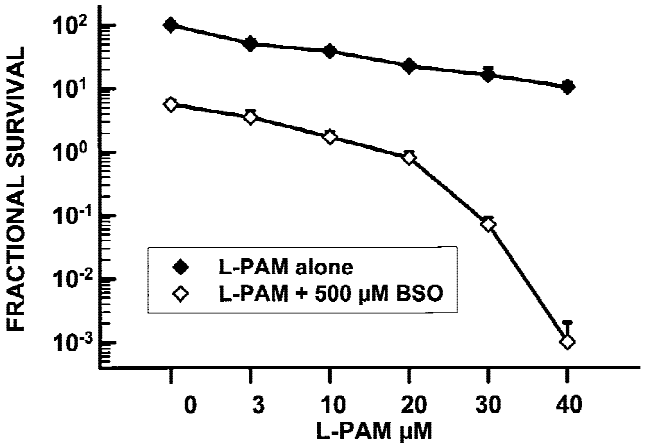

Fig. 1. L-PAM dose response for CHLA-171. L-PAM as a single agent and L-PAM combined with a constant dose of 500 μM L-(S,R) BSO. Values represent mean ± standard error.

Cytotoxicity Assays of BSO Combined With L-PAM

Using a fixed ratio analysis, drug synergy (CI < 1) between BSO and L-PAM was observed for all cell lines at concentrations of L-PAM achievable in the AHSCT setting (Table I). In p53 functional, postprogressive disease cell lines (CHLA-20, CHLA-51, and CHLA-79), the combination of BSO and L-PAM was synergistic at L-PAM concentrations (≤3 μM) achievable in the nonmyeloablative setting. Importantly, in highly L-PAM resistant, p53 nonfunctional cell lines (CHLA-90, CHLA-134, CHLA-171), drug synergy was not seen until the concentration of L-PAM was escalated to levels of L-PAM that are achievable only in the myeloablative setting (≥10 μM). The ability of L-PAM at myeloablative doses to synergize with BSO was particularly striking for CHLA-171 and CHLA-90 when 40 μM L-PAM was combined with 400 μM BSO, resulting in greater than a 4 log increase of cell kill compared to 40 μM of L-PAM alone. The dose response curve of L-PAM (0–40 μM) in combination with a constant level of 500 μM for the representative cell line CHLA-171 is shown in Figure 1.

Glutathione (GSH) Decreased by BSO

All cell lines were incubated in various concentrations of BSO (0–500 μM) for 24 hr, assayed for total GSH, and found to have depletion of GSH to <50% baseline. The concentrations of GSH (both baseline and following a 24 hr incubation with 500 μM BSO) in a representative L-PAM-resistant cell line (CHLA-171) are compared to the mean GSH under the same conditions for 10 previously reported [16] NB cell lines (Fig. 2). The baseline level of GSH in CHLA-171 was 0.49 ± 0.02 nmol/pg protein compared to a mean of 0.59 ± 0.08 nmol/pg

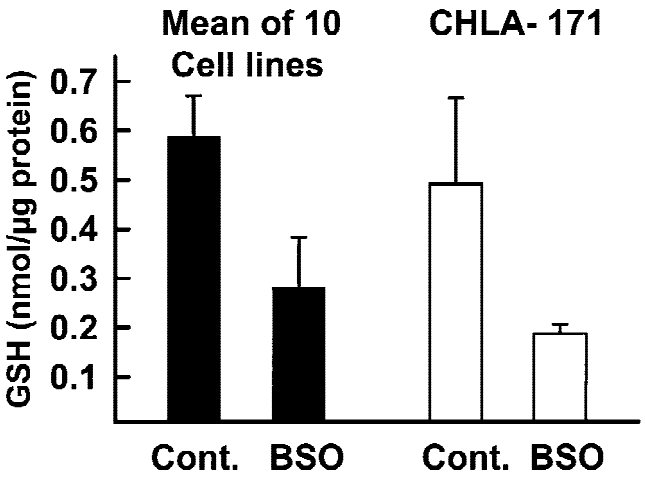

Fig. 2. Glutathione levels following BSO treatment. Values represent mean ± standard error.

protein in the 10 cell lines, and GSH was depleted to 38% (CHLA-171) vs. 47% (mean of 10 cell lines).

DISCUSSION

Alkylating agents are integral to both induction therapy and myeloablative therapy for high-risk NB [1,4,6], and multidrug resistance associated with nonfunctional p53 is frequently found in NB cell lines established after disease progression [10]. Therefore, overcoming drug resistance may require p53-independent modulators of alkylator resistance. We studied six post-progressive disease NB cell lines that had a single-agent LC_{90} for L-PAM ranging from 2 to 52 μM compared to peak L-PAM plasma concentrations of 3.1 ± 0.8 μM reported in our nonmyeloablative trial of BSO + L-PAM [30]. Data from the current report suggest that, for some NB cell lines, escalation of L-PAM to ≥10 μM is needed to achieve multilog drug synergy. This suggests that nonmyeloablative L-PAM, both as a single agent and combined with BSO, would be ineffective for some patients with refractory disease. L-PAM resistance was not due to a failure of BSO to deplete GSH; there was no discernible difference in GSH (baseline and percentage depletion) between CHLA-171 and 10 other neuroblastoma cell lines. However, our data suggest that a multilog tumor cell kill might have occurred had BSO been used with doses of L-PAM achievable only in the myeloablative setting. Despite being obtained from a patient who relapsed after AHSCT, CHLA-51 was highly sensitive to both BSO and L-PAM as single agents. This is in contrast to the L-PAM resistance observed in other post-AHSCT neuroblastoma cell lines [9] and suggests that sensitivity to alkylating agents is retained in some patients who relapse after myeloablative therapy.

This current report shows, for some neuroblastoma cell lines (including those lacking p53 function), a marked and sustained resistance to L-PAM exists, and BSO-mediated GSH depletion will not overcome the alkylator resistance that occurs frequently in NB cell lines established after AHSCT when L-PAM levels obtainable in the nonmyeloablative setting are used. However, the current data suggest that BSO may overcome high levels of L-PAM resistance (even in those cell lines lacking functional p53) if the L-PAM dose can be escalated to levels requiring 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 NB and could improve outcome for NB patients undergoing myeloablative therapy after developing progressive disease. A clinical trial is in progress to determine the maximally tolerated dose of L-PAM given together with BSO and supported with AHSCT.

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