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Original Paper

Buthionine Sulphoximine Alone and in Combination With Melphalan (L-PAM) is Highly Cytotoxic For Human Neuroblastoma Cell Lines

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Buthionine sulphoximine (BSO) selectively inhibits glutathione (GSH) synthesis and may enhance the antineuroblastoma activity of melphalan (L-PAM). We determined the cytotoxicity of BSO (dose range 0–1000 μM) alone and in combination with L-PAM (dose range 0–0 μM) in a panel of 18 human neuroblastoma cell lines. BSO alone was highly cytotoxic with 16/18 neuroblastoma cell lines having IC_{90} values (range 2.1–>1000 μM) below the clinically achievable steady-state plasma level of 500 μM BSO. Maximal cell killing correlated with GSH levels decreased to less than 10% baseline, and was partially reversed by the addition of exogenous anti-oxidants (GSH, vitamin E and ascorbate). Fluorocytometric analysis of DNA fragments by the Tunnel method detected 92% of a BSO-sensitive cell line in apoptosis after a 48 h exposure to 500 μM BSO. The combination of L-PAM and BSO synergistically enhanced the cell killing of L-PAM alone by >1–3 logs (combination index <1). We conclude that BSO has significant single-agent cytotoxicity against neuroblastoma and enhances cell killing when combined with L-PAM. © **1997 Published by Elsevier Science Ltd.**

Key words: neuroblastoma, glutathione, buthionine sulfoximine, BSO, melphalan, L-PAM, alkylator resistance

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INTRODUCTION

One of the challenges faced in the treatment of neuroblastoma is that while many children with metastatic disease initially respond to therapy, the tumour recurs and the child dies from progressive disease [1, 2]. The causes for treatment failure are multifactorial, but acquired drug resistance, especially resistance to alkylating agents, probably plays a major role. Acquired resistance to alkylating agents is particularly important since modern neuroblastoma therapy relies heavily upon alkylating agents, especially in the bone marrow transplant (BMT) setting.

Glutathione (GSH) is an intracellular thiol-containing tripeptide that plays a critical role in many aspects of cellular defence, growth and metabolism, and may contribute to alkylator resistance [3–5]. The primary role of GSH is to defend the cell against free radicals, the highly reactive electrophilic species created during normal cellular metabolism, or as a result of chemotherapy or ionising radiation [3]. GSH can also protect tumour cells from nucleophilic agents, such as alkylators, by direct conjugation with glutathione-*S*-transferase (GST) which ultimately transports the alkylating agent from the cell for hydrolysis in the plasma or excretion in the urine or bile [3, 6]. There is also both *in vivo* and *in vitro* evidence that alkylator resistance correlates to increases in total intracellular levels of non-protein sulphydryls, of which GSH is the predominant species [7–10].

Buthionine sulphoximine (BSO), an aminosulphoximine, is a selective inhibitor of γ-glutamylcysteine synthetase (GCS), the rate-limiting step in the *de novo* synthesis of GSH [3]. BSO, as a single agent, has been reported to be minimally toxic to tumour cells in culture at doses of less than 50 μM

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and non-toxic to colony forming unit-granulcyte macrophage (CFU-GM) at doses of 2000 μM [8, 11–14]. A notable exception are melanoma cell lines, where the toxicity of BSO has been correlated to the degree of pigmentation and the production of tyrosinase [15, 16]. Preclinical studies have shown that BSO-mediated depletion of intracellular GSH is effective in increasing the cytotoxicity of alkylating agents [9, 11, 12, 17]. Human phase I trials of BSO combined with melphalan have demonstrated that BSO alone and in combination with chemotherapy is safe and well tolerated, with clinical toxicities limited to moderate, but reversible, bone marrow suppression [18–22].

In this study, we report the *in vitro* effects of BSO alone and in combination with melphalan (L-PAM) for a panel of 18 human neuroblastoma cell lines.

MATERIALS AND METHODS

Human neuroblastoma and cell lines

Human neuroblastoma cell lines (SMS-KAN, SMS-KANR, SMS-KCN, SMS-KCNR, SK-N-BE (1), SK-N-BE (2), SMS-LHN, SMS-SAN, LA-N-5, LA-N-6, SK-N-RA, SK-N-FI, LA-N-1, LA-N-2, SK-N-SH, SK-N-AS, SK-N-DZ, and SMS-MSN) were cultured in RPMI-1640 with 10% heat-inactivated fetal calf serum (FCS) at 37 °C and 5% CO_2 [23]. No antibiotics were used. All cell lines were tested at passage 15–25 with the exception of LA-N-2 which was passage 100.

Chemicals

Melphalan (NSC #14210) was supplied by the National Cancer Institute (Washington, DC, U.S.A.). All other chemicals, including buthionine sulphoximine, were purchased from Sigma Chemical Co., St Louis, Missouri, U.S.A.

Digital image microscopy

Cytotoxicity assays were performed in 96-well plates using a novel Digital Image Microscopy (DIMSCAN) system that has a dynamic range of greater than 4 logs of cell kill [24]. Following incubation with drugs or control medium, fluorescein diacetate (a vital stain) was added to the 96-well plate and incubated for 20 min. Eosin-Y (0.5%) was then added to inhibit background fluorescence in the medium and in non-viable cells. The plates were then analysed by digital image microscopy with an inverted fluorescence microscope to determine the relative fluorescence of each well. Mean relative fluorescence for treated wells was compared to control wells to derive the fractional cytotoxicity.

Dose–response assays

To examine the single-agent activity of BSO, cells were grown to confluence, harvested by Pucks EDTA, resuspended in RPMI-1640 + 10% FCS, and plated in 96-well microtitre FALCON (Beckton Dickinson and Co., Lincoln Park, New Jersey, U.S.A.) plates to a density of 25 000–30 000 viable cells/well. Following plating, cells were exposed to various molar concentrations (0–1000 μM) of BSO (total volume 100 μl) and incubated for 7 days.

To determine the effect of BSO in combination with L-PAM, cells were plated as previously described in 96-well plates and incubated with various concentrations (0–1000 μM) of BSO for 24 h followed by adding melphalan (0–10 μM) and additional BSO (0–1000 μM). After a 7 day incubation, the plates were analysed by DIMSCAN.

Dose effect analysis

Fixed micromolar ratios of BSO and melphalan, alone and in combination, were performed by DIMSCAN as described above. Following a 7 day incubation, the fraction of cells affected was calculated [F_a = 1-(RF condition/RF control)] from relative fluorescence (RF) values obtained from DIMSCAN. Data from BSO dose–response studies were analysed using a single-drug dose response program to calculate inhibitory concentration values [25], and IC_{90} was defined as the concentration of drug required to kill 90% of cells tested. RF data from BSO/melphalan testing of all 18 neuroblastoma cell lines was also transformed into F_a and the non-fixed ratio calculation of the combination index (CI) was calculated by the Chou program. CI > 1 indicates drug antagonism, CI = 1 indicates additive effect and CI < 1 indicates drug synergy.

Apoptosis studies

Cells were cultured as previously described in 75 cm^2 flasks, in the presence or absence of 5×10^{-4} M BSO for 48 h. DNA fragments were identified by two-colour flow cytometry using terminal deoxynucleotidyltransferase (TdT) to incorporate digoxigenin labelled dUTP and dATP. Fluorescein labelled anitidigoxigenin antisera was used to identify the reaction (presence of apoptosis) site and propidium iodide staining was used for DNA staining [26].

Glutathione

Glutathione studies were conducted, in triplicate, in six-well plates containing 0–1000 μM BSO for 24 h and analysed by the *o*-phthalaldehyde method with results normalised to total protein [27].

RESULTS

Cytotoxicity of BSO as a single agent against neuroblastoma

A panel of 18 human neuroblastoma cell lines (12 *MYC*N amplified/6 *MYC*N non-amplified) were treated for 7 days with BSO (0–1000 μM) and analysed for cytotoxicity using the DIMSCAN system. BSO was highly toxic for most neuroblastoma cell lines with 16/18 lines having IC_{90} values below the clinically achievable steady-state level of 500 μM BSO (Table 1).

Table 1. *Concentration of BSO required to kill* 90% *of neuroblastoma cell lines tested (IC_{90})*

Cell line	IC_{90} μM BSO
SMS-KAN	306.0
SMS-KANR	3.7
SMS-KCN	50.7
SMS-KCNR	377.0
SK-N-BE(1)	23.0
SK-N-BE(2)	4.6
LA-N-1	2.1
LA-N-2	7.8
SMS-MSN	7.7
SMS-SAN	5.4
SK-N-DZ	8.9
LA-N-5	140.7
SMS-LHN	9.7
SK-N-RA	823.0
LA-N-6	40.0
SK-N-AS	371.0
SK-N-SH	11.2
SK-N-FI	>1000.0

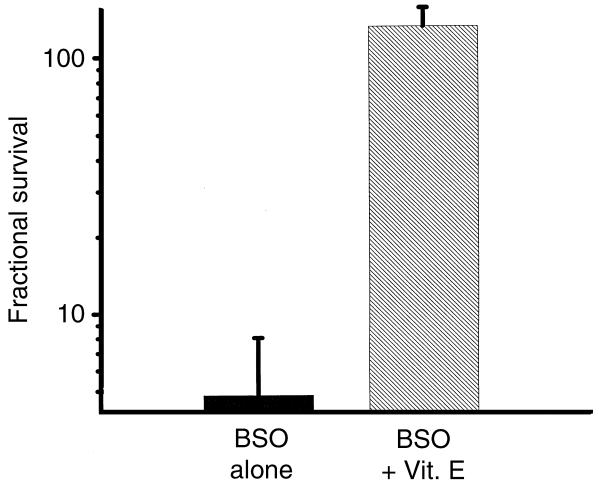

Figure 1. Anti-oxidant mediated reversal of BSO cytotoxicity in the neuroblastoma cell line SK-N-BE(2). 100 μM BSO alone produced 95.2% cytotoxity, whereas 100 μM BSO + 100 ng/ml vitamin E (Vit. E) showed no additional cytotoxicity compared to control.

Glutathione levels following treatment with BSO

As expected, glutathione levels fell following treatment with BSO. Neuroblastoma cell lines were exposed to BSO (0–1000 μM BSO) for 24 h and analysed in triplicate for total GSH content and percentage control GSH fall. The decrease in GSH was dose dependent with 1000 μM BSO producing the greatest decline in GSH concentrations. The percentage decrease in GSH concentration correlated to BSO cytotoxicity, with cell lines having IC_{90} values <20 μM ($n = 6$) showing a greater mean fall in GSH (9.09 ± 4.8%), compared with values those cell lines having $IC_{90} > 20$ μM ($n = 6$) (24.0 ± 10.9%; $P = 0.04$).

Effect of exogenous antioxidants upon BSO toxicity in neuroblastoma

In order to evaluate possible mechanisms of BSO cytotoxicity in neuroblastoma, cell lines were incubated with exogenous antioxidants (ascorbate, α-tocopherol, glutathione) and 100 μM BSO. Cytotoxicity was determined by DIMSCAN. The percentage survival seen in SK-N-BE [2] following 24 h treatment with 100 μM BSO (4.8 ± 3.2% control) was abrogated following the addition of 100 ng/ml α-tocopherol [130.7 ± 23% control ($P < 0.0001$; Figure 1)]. Similar reversal of BSO cytotoxicity occurred when ascorbate or GSH was added to the medium (data not shown).

Apoptosis following treatment with BSO

A BSO sensitive cell line (SMS-KANR) was treated with 500 μM BSO over 48 h and examined for evidence of apoptosis by flow cytometry. SMS-KANR treated with BSO showed 92% apoptotic cells compared to 6% in the untreated control.

Synergism of L-PAM combined with BSO

BSO enhanced the antineuroblastoma effect of L-PAM as shown for representative BSO-sensitive (SMS-LHN) and BSO-resistant (SK-N-FI) cell lines (Figure 2). In SMS-LHN (Figure 2a), 100 μM BSO alone or the synergistic (CI < 1) combination of 10 μM BSO and 1 μM L-PAM produced more cytotoxicity (>3 logs compared to control) than the

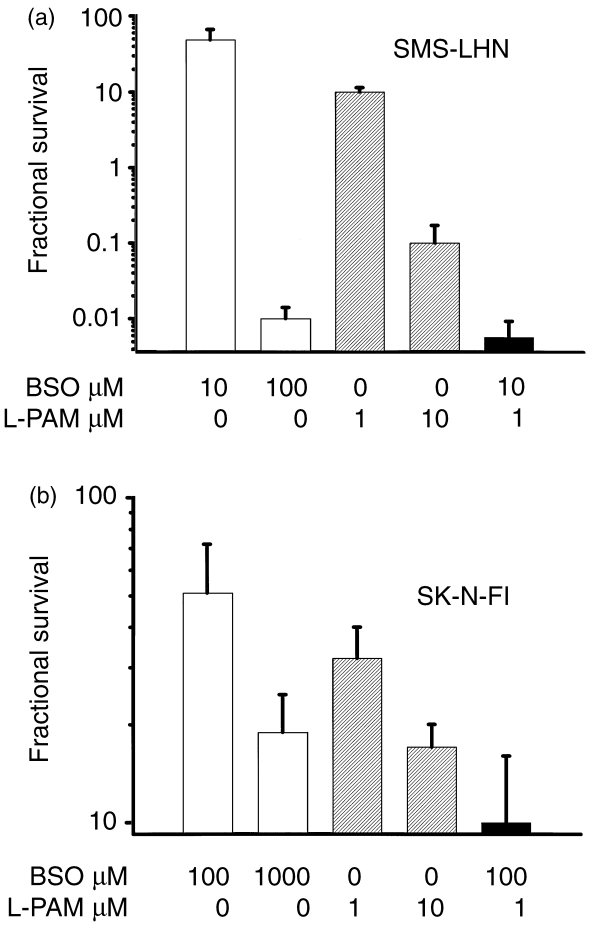

Figure 2. The *in vitro* cytoxicities of BSO alone and in combination with melphalan (L-PM) for the human neuroblastoma cell lines (a) SMS-LHN and (b) SK-N-FI. Note that there is a different scale for (a) and (b).

2 logs of cytotoxicity seen with 10 μM L-PAM as a single agent. Although more resistant to cytotoxic agents, SK-N-FI showed a similar pattern of BSO and L-PAM synergy (Figure 2b). Using single-point analysis of individual cell lines in the presence of 1 μM L-PAM and (depending on BSO sensitivity), either 10 μM BSO (cell lines with $IC_{90} < 20$ μM) or 100 μM BSO (cell lines with $IC_{90} > 20$ μM), 17/18 cell lines showed synergy (CI < 1).

DISCUSSION

Glutathione is a versatile tripeptide that protects cells from oxidative stress by converting peroxides into water [3] and detoxifying nucleophilic xenobiotics into stable, non-toxic conjugates [28]. Changes in GST isoenzyme profiles, an enzyme that catalyses the conjugation of GSH with alkylating agents, have been reported in a variety of tumours and may relate to carcinogenesis and the development of drug resistance [14]. In addition, many tumours show increased levels of γ-glutamyl transpeptidase, an enzyme that maintains high intracellular levels of GSH [29]. A number of *in vitro* studies, including this report, have shown that agents which deplete GSH can restore sensitivity to alkylating agents [8, 9, 11–13].

The majority of neuroblastoma cell lines we tested were highly sensitive to BSO as a single agent. BSO toxicity was prevented by antioxidants other than GSH, implying that the toxicity is related to increases in oxidative stress.

Neuroblastoma cells are known to be highly sensitive to drugs that produce oxidative stress [30]. Therefore, the sensitivity to BSO in neuroblastoma may be related to the unusual amount of free radicals that are generated during catecholamine metabolism.

We have shown that BSO alone kills neuroblastoma by apoptosis via mechanisms that involve free radical stress and that BSO synergistically enhances the *in vitro* cytotoxicity of melphalan for neuroblastoma cell lines. These preclinical data have led to the development of an ongoing clinical trial of melphalan and BSO in children with recurrent neuroblastoma, and it is anticipated that BSO will become a valuable agent for neuroblastoma therapy, especially when used in the setting of myeloablative therapy followed by stem cell support to overcome haematopoietic toxicities.

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