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Characterization of the mechanisms of busulfan resistance in a human glioblastoma multiforme xenograft

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Abstract Busulfan is an alkylating agent commonly used in the treatment of chronic myelogenous leukemia and in combination with cyclophosphamide in preparation for allogeneic bone marrow transplantation. Serial treatment of a childhood high-grade glioma xenograft (D-456 MG) with busulfan resulted in a busulfan-resistant xenograft, D-456 MG(BR). Cross-resistance to 1,3-bis(2-chloroethyl)-1-nitrosourea was seen but not resistance to cyclophosphamide or CPT-11. Cytoplasmic levels of glutathione in D-456 MG(BR) were approximately one-half those found in D-456 MG. This depletion could not be explained by levels of glutathione-*S*-transferase, or by amplification, rearrangement, or increased levels of transcript of γ-glutamylcysteine synthetase. Furthermore, depletion of glutathione in D-456 MG did not alter busulfan activity. Quantitation of busulfan levels in D-456 MG and D-456 MG(BR) xenografts following treatment of mice at the dose lethal to 10% of the animals demonstrated that significantly lower levels of drug were achieved in D-456 MG(BR). These studies suggest that alterations in drug transport or metabolism of busulfan may play a role in the resistance of D-456 MG(BR) to this alkylator.

Key words Busulfan · Glioblastoma multiforme · Xenograft · Drug resistance

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Introduction

Busulfan (1,4-dimethanesulfonoxybutane) is a bifunctional alkylating agent that has a marked cytotoxic effect on granulocytic cells, making it the treatment of choice for chronic myelogenous leukemia [6, 7, 15, 21, 28]. High-dose busulfan in combination with cyclophosphamide is an effective preparative regimen for patients undergoing allogeneic or autologous bone marrow transplantation for leukemias, solid tumors, and hemoglobinopathies [7, 33]. Busulfan has also been shown to be effective against xenografts of solid tumors derived from central nervous system tumors [1].

Resistance to chemotherapeutic agents is the major problem for treatment of human malignancies. Emergence of drug-resistant cell populations invariably leads to tumor progression and subsequent patient death, warranting efforts to elucidate mechanisms responsible for drug resistance and modulations effective in bypassing or reversing this resistance. Resistance to alkylating agents, including busulfan, is multifactorial and includes increased aldehyde dehydrogenase activity [18, 29], elevated levels of glutathione (GSH) [2, 10, 13, 18, 35, 37], increased activity of glutathione *S*-transferase (GST) [4, 9, 32, 34, 38], alterations in drug transport, delivery and metabolism [17] and enhanced DNA repair [3, 11, 14, 20].

We now report the generation of a busulfan-resistant xenograft D-456 MG(BR) derived from a childhood high-grade glioma, characterized by substantially lower busulfan levels compared to the busulfan-sensitive parent, D-456 MG.

Materials and methods

Animals

Male and female athymic BALB/c nude mice (*nu*/*nu* genotype, 6 weeks or older) were used for all studies and were maintained as described previously [5, 8].

Xenografts

Xenografts derived from a busulfan-sensitive childhood high-grade glioma, D-456 MG, were maintained in the same fashion as previously described [19]. Resistance to busulfan was generated by treatment of D-456 MG xenografts growing subcutaneously in nude mice with busulfan at a dose of 60.3 mg/m^2 (20.1 mg/kg) which is the LD_{10}. Upon tumor regression and regrowth, xenografts were passed into new mice. Subsequent to tumor growth these mice were treated with busulfan. This sequence was repeated on every xenograft generation. Xenografts of this serially treated line were tested every three passages for response to busulfan.

Subcutaneous xenograft transplantation

A 50 μl tumor homogenate was inoculated into the right flank of recipient mice as described previously [16].

Drug treatment

Busulfan, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and cyclophosphamide were supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, Md.). Buthionine *S*-*R*-sulfoximine (BSO) was synthesized as previously described [25]. CPT-11 was provided by Pharmacia and UpJohn (Kalamazoo, Mich.). All antineoplastic agents were administered intraperitoneally at a dose equivalent to the LD_{10}. Cyclophosphamide was dispensed at 1391 mg/m^2 in 0.9% saline on day 1. Busulfan was administered at a dose of 60.3 mg/m^2 (20.1 mg/kg) in 10% dimethyl sulfoxide using 0.9% saline on day 1. CPT-11 was given daily at 120 mg/m^2 (40 mg/kg) in 10% dimethyl sulfoxide in 0.9% saline on days 1–5 and 8–12. Drug vehicles have been previously described [1, 16, 18, 27].

Tumor measurements

Tumors were measured twice weekly with hand-held vernier calipers (Scientific Products, McGraw, Ill.). Tumor volume was calculated according to the following formula: $[(\text{width})^2 \times (\text{length})]/2$.

Assessment of tumor response

The response of subcutaneous xenografts was assessed by delay in tumor growth and by tumor regression. Growth delay, expressed as T–C, is defined as the difference in days between the median time required for the tumors of treated (T) and control (C) animals to reach a volume five times greater than that measured at the time of original treatment. Tumor regression is defined as a decrease in tumor volume over two successive measurements. Statistical analysis was performed using the Wilcoxon rank order test for growth delay and Fisher's exact test for tumor regressions as described previously [16].

GSH levels

GSH was measured by the method of Tietze [39] using the modification of Griffith [24] as previously described [17].

GST activity

Activity of GST was measured by the method of Habig et al. [26] as previously described [17].

BSO depletion of GSH

BSO was given to animals both in drinking water at 20 m*M* and via intraperitoneal injection at 0.56 mg/g body weight every 12 h for 84 h. Busulfan was given after 54 h of BSO pretreatment.

Analysis of γ-GC-S gene for amplification, rearrangements and basal transcript levels

A 222-bp γ-GC-S cDNA probe covering the region +393 to +614 of the human liver γ-GC-S heavy subunit cDNA [22] was amplified by reverse transcription-polymerase chain reaction using RNA from a human glioblastoma cell line as previously described [4]. Control probes were a 1.1-kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and a 2.0-kb human β-actin cDNA, both from Clontech (Palo Alto, Calif.). The cDNAs were ^{32}P-labeled by random priming and purified on Bio-Spin 6 columns (BioRad, Richmond, Calif.). Amplification and rearrangements in the γ-GC-S gene were examined by Southern analysis and basal transcript levels by Northern analyses, as we recently described [4].

Briefly, for the southern blotting, genomic DNA was isolated from the tumor cells using standard phenol/chloroform techniques [36]. DNA (10 μg) was digested with *Eco*R1 or other selected restriction enzymes or enzyme combination. Following electrophoresis in a 0.75% agarose gel, denaturation, neutralization and capillary transfer onto nylon, the membranes were hybridized overnight with a ^{32}P-labeled γ-GC-S probe, washed, and autoradiographed on Kodak XAR-5 X-ray film. The γ-GC-S probe was then stripped off and rehybridized with a β-actin probe.

Northern blotting was performed with total RNA extracted from tumor cells using the acid guanidinium thiocyanate phenol-chloroform method [12]. Total RNA was loaded 10 μg per lane), electrophoresed in a 1.3% agarose–formaldehyde gel, and the fractionated RNA was capillary transferred to nylon membranes and hybridized with the ^{32}P-labeled γ-GC-S cDNA probe, as described above. After autoradiography, the members were stripped and rehybridized with a ^{32}P-labeled GAPDH probe.

Busulfan levels in tumor tissue

Animals bearing D-456 MG or D-456 MG(BR) xenografts were sacrificed at 0, 15, 30, 60, 90, and 120 min after treatment with busulfan at the LD_{10}. Busulfan concentrations were determined in triplicate from 0.1 g aliquots of treated and untreated tumor or normal murine brain tissue by a modification of a previously published HPLC method [31]. Briefly, homogenized tissue samples were derivatized with diethyldithiocarbamate and extracted with methanol/ethyl acetate followed by a solid phase separation using C18 Sep-Pak columns (Waters, Milford, Mass.). Methanol eluents were injected onto a Waters reverse phase, isocratic HPLC system with UV detection at 278 n*M*. The externally standardized assay curve had a range of 0.0 μg/0.1 g to 10 μg/0.1 g, a lower limit of quantitation of 1 μg/0.1 g tumor sample and an r^2 of 0.9996. Spiked high- and low-quality control samples were within 10% of expected values.

Results

Xenograft response to chemotherapy

Busulfan produced growth delays of 13.3 days and 10.9 days and 10/10 and 10/10 regressions, respectively, in the

Table 1 Chemotherapy of D-456 MG and D-456 MG(BR) xenografts in athymic nude mice. Cyclophosphamide was dispensed at 1391 mg/m^2 in 0.9% saline on day 1. Busulfan was administered at a dose of 60.3 mg/m^2 (20.1 mg/kg) in 10% dimethyl sulfoxide using 0.9% saline on day 1. CPT-11 was given daily at 120 mg/m^2 (40 mg/kg) in 10% dimethyl sulfoxide in 0.9% saline on days 1–5 and 8–12

Drug	D-456 MG		D-456 MG(BR)	
	T–C[a] (days)	Regression[b]	T–C[a] (days)	Regression[b]
Busulfan	13.3*	10/10	5.1*	2/10
	10.9*	10/10	8.3*	0/8
BCNU	6.6*	6/9	2.3	0/10
	6.9*	6/10	5.8*	0/8
Cyclophosphamide	26.5*	9/9	22.8*	7/7*
CPT-11	90+*	9/9	90+*	9/9*

*$P < 0.001$ vs controls
[a]T–C, growth delay in days, was defined as difference in time for tumors of treated (T) and control (C) animals to reach five times the volume measured at the initiation of treatment
[b]Regression was defined as a decrease in volume over two successive tumor measurements

parent line D-456 MG compared with growth delays of 5.1 days and 8.3 days and 2/10 and 0/8 regressions, respectively, in the resistant line D-456 MG(BR). Partial cross-resistance was demonstrated to BCNU in D-456 MG(BR). No cross-resistance was seen to cyclophosphamide or CPT-11 (Table 1). D-456 MG(BR) xenografts were sequentially transplanted for 11 passages without serial busulfan treatment. The response to busulfan at that time was unchanged, demonstrating that this was stable resistance in the absence of continued busulfan treatment.

GSH levels and GST activity

GSH levels in D-456 MG were twofold higher compared to D-456 MG(BR). The GST activity was virtually identical in D-456 MG and D-456 MG(BR).

BSO-mediated GSH depletion

BSO effectively depleted GSH levels in the parent line and in D-456 MG(BR) (Table 2). Busulfan plus BSO produced growth delays and tumor regressions against D-456 MG or D-456 MG(BR) which were not significantly different from the results produced with busulfan alone (Table 3).

Analysis of the γ-glutamylcysteine synthetase gene

The results of the Southern analysis for amplification and/or rearrangement of the γ-GC-S heavy-chain gene in the two xenografts are summarized in Fig. 1 and 2. Using the enzymes *Bam*HI, *Eco*RI, and *Hind*III, we found no differences in restriction patterns or band intensities. These results indicate no obvious amplification or rearrangement of the γ-GC-S gene in D-456 MG or

Table 2 Glutathione levels and glutathione-*S*-transferase activity in D-456 MG and D-456 MG(BR) xenografts. Values are means ± SD

	D-456 MG	D-456 MG (BR)	Parent : resistant cell line
GSH (nmol/mg)	3.1 ± 0.3	1.5 ± 0.5	2.0
	3.4 ± 0.2	1.52 ± 0.3	2.2
GST (nmol/min/mg protein)	116.4 ± 18.6	134.7 ± 17.3	0.86

Table 3 Treatment of D-456 MG and D-456 MG(BR) xenografts with busulfan ± BSO. The GSH values are means ± SD

Treatment		D-456 MG			D-456 MG(BR)		
		GSH(nmol/mg)	T–C (days)[a]	Regressions[b]	GSH(nmol/mg)	T–C (days)	Regressions
Busulfan	Experiment 1	3.1 ± 0.3*	13.3	10/10*	1.51 ± 0.5	5.1*	2/10
	Experiment 2	3.4 ± 0.2*	10.9	10/10*	1.52 ± 0.3	8.3*	0/8
Busulfan + BSO	Experiment 3	0.2 ± 0.3*	11.2	7/10*	0.17 ± 0.3	3.3	0/10
	Experiment 4	0.2 ± 0.3*	10.4	7/10*			

*$P < 0.001$ vs controls
[a]T–C, growth delay in days, was defined as the difference between the median time required for tumors in treated (T) and control (C) animals to reach five times the volume at the initiation of treatment
[b]Regression was defined as a decrease in tumor volume over two successive measurements

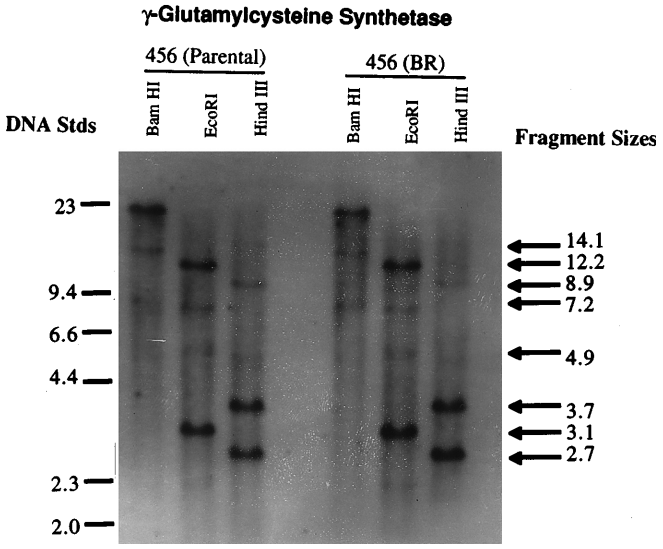

Fig. 1 Southern analyses of human glioma xenografts D-456 MG and D-456 MG(BR)

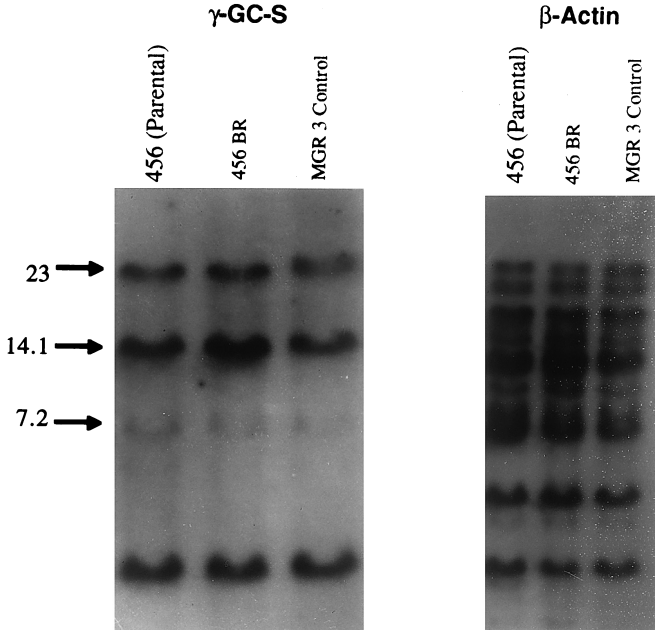

Fig. 2 Southern analyses of human glioma xenografts D-456 MG parental and busulphan-resistant cell lines

D-456 MG(BR) xenografts to account for the observed differences in GSH levels.

γ-GC-S gene transcripts

Densitometric quantitation of Northern hybridization bands revealed no obvious difference in the basal levels of transcripts of the heavy chain γ-GC-S gene between D-456 MG and D-456 Mg(BR) xenografts. γ-GC-S:GAPDH ratios of the hybridization bands were 0.42 for D-456 MG compared with 0.44 for D-456 MG(BR) (Fig. 3).

Drug levels in the xenograft

Levels of busulfan achieved in D-456 MG(BR) xenografts were consistently lower than those achieved in D-456 MG xenografts (Table 4).

Discussion

Busulfan is a bifunctional alkylating agent commonly employed in the treatment of chronic myelogenous leukemia [6, 7, 15, 21, 28], and as part of a preparative regimen for allogeneic or autologous bone marrow transplantation [7, 33]. Recent laboratory studies have suggested that busulfan is also active against xenografts derived from adult and childhood CNS tumors [1]. Several clinical trials are currently evaluating the role of autologous bone marrow transplantation following busulfan ablation in children with recurrent or newly diagnosed high-risk brain tumors [23, 30]. The expanding role busulfan may play in the treatment of CNS

Fig. 3 Northern analyses of human glioma xenografts D-456 MG and D-456 MG(BR)

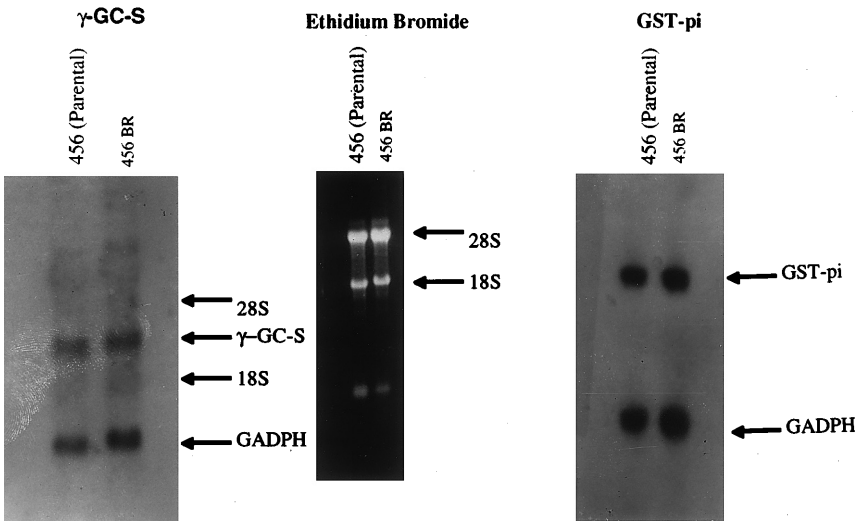

Table 4 Busulfan levels (μg/0.1 g) in D-456 MG and D-456 MG(BR) xenografts following treatment. Values are the means ± SD of three tumors in individual mice

Time (min)	D-456 MG (μg/0.1 g)	D-456 MG(BR) (μg/0.1 g)
0	0.00	0.00
15	3.1 ± 0.6	1.5 ± 0.3
30	2.4 ± 0.4	1.5 ± 0.4
60	2.7 ± 0.2	2.1 ± 0.4
90	2.5 ± 0.3	1.8 ± 0.3
120	2.2 ± 0.5	1.8 ± 0.3

tumors warrants studies to define potential mechanisms of resistance to busulfan. Alkylator resistance is multifactorial, and may include mechanisms such as increased aldehyde dehydrogenase activity [18, 29], elevations of GSH [10, 13, 18, 35, 37], increased activity of GST [4, 9, 32, 34, 38], altered drug delivery and/or metabolism [16, 18] and enhanced DNA repair [3, 11, 14, 20]. The only specific mechanism of resistance to busulfan reported to date is elevated GST activity in a Yoshida cell line [32].

The current studies were conducted to generate a malignant glioma-derived xenograft with resistance to busulfan and to define potential mechanisms of resistance as well as the presence of cross-resistance and/or collateral sensitivity to other agents. D-456 MG(BR) was partially resistant to busulfan with a moderate decrease in tumor growth delay and virtually no tumor regressions seen following treatment. D-456 MG(BR) was partially cross-resistant to BCNU but demonstrated sensitivity to cyclophosphamide and CPT-11 equivalent to the busulfan-sensitive parent xenograft D-456 MG. The cross-resistance to BCNU suggested a potential role of O^6-alkylguanine-DNA alkyltransferase (AGAT)-mediated resistance to busulfan, since D-456 MG has been shown to be a Mer^+ xenograft in which BCNU sensitivity is restored by inhibition of AGAT [19]. However, treatment of D-456 MG(BR) with BCNU plus O^6-benzylguanine, a potent inhibitor of AGAT, did not enhance busulfan activity (data not shown), suggesting that busulfan activity is not modulated by AGAT activity. The current studies indicate that busulfan resistance is not accompanied by cross-resistance to cyclophosphamide, a finding similar to the lack of busulfan resistance in cyclophosphamide-resistant cell lines cross-resistant to melphalan [1].

The marked decrease in GSH levels in D-456 MG(BR) was surprising and unanticipated since one would have predicted an elevated level consistent with that seen with other cell lines and xenografts resistant to alkylating agents, albeit not busulfan. Depletion of tumor GSH levels with BSO did not reduce busulfan activity against the parent xenograft D-456 MG. We currently have no explanation for the reduced GSH levels in D-456 MG(BR). The modest increase in GST activity in D-456 MG(BR) suggests that GSH may be utilized at a higher level in this xenograft, thus leading to the lower intracellular levels.

The major distinction between D-456 MG and D-456 MG(BR) was the differences in tumor busulfan levels following treatment with this alkylator. Tumor busulfan levels were consistently and significantly lower in D-456 MG(BR) compared to D-456 MG, suggesting that alteration of busulfan metabolism and/or transport may be part of the mechanism involved in the resistant xenograft. The possibility that decreased accumulation and/or altered metabolism may underlie the busulfan resistance of these tumors warrants further studies designed to define the precise nature of these alterations operational in D-456 MG(BR).

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