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Rosemary H. Aaron · Gertrude B. Elion
O. Michael Colvin · Michael Graham · Steven Keir
Darell D. Bigner · Henry S. Friedman

Busulfan therapy of central nervous system xenografts in athymic mice

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Abstract We evaluated the antitumor activity of busulfan against a panel of tumor cell lines and xenografts in athymic nude mice derived from childhood high-grade glioma, adult high-grade glioma, ependymoma, and medulloblastoma. Busulfan displayed similar activity against a panel of four medulloblastoma cell lines (D283 Med, Daoy, D341 Med, and D425 Med) and four corresponding sublines with laboratory-generated or clinically acquired resistance to 4-hydroperoxycyclophosphamide [D283 Med (4-HCR), Daoy (4-HCR), D341 Med (4-HCR), and D458 Med] and cross-resistance to melphalan. This is consistent with a nearly total lack of cross-resistance of busulfan to 4-hydroperoxycyclophosphamide. Busulfan was active in the therapy of all but one of the subcutaneous xenografts tested, with growth delays ranging from 14.3 days in D612 EP to 58.4 days in D528 EP. Busulfan produced statistically significant increases in the median survival of mice bearing intracranial D456 MG (66%–90%), D612 EP (18%–33%), and D528 EP (89%) xenografts. These studies suggest that busulfan may be active against medulloblastomas, high-grade gliomas, and ependymomas as well as against cyclophosphamide-resistant neoplasms.

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R. H. Aaron · M. Graham · S. Keir · H. S. Friedman (✉)
Department of Pediatrics, Duke University Medical Center, Durham, NC 27710, USA

D. D. Bigner · H. S. Friedman
Department of Pathology, Duke University Medical Center, Durham, NC 27710, USA

G. B. Elion
Department of Pharmacology, Duke University Medical Center, Durham, NC 27710, USA

D. D. Bigner · H. S. Friedman
Preuss Laboratory for Brain Tumor Research, Duke University Medical Center, Durham NC 27710, USA

M. Colvin
The Johns Hopkins Oncology Center, The Johns Hopkins University, Baltimore, MD 21205, USA

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Introduction

The most common solid tumors in children under 15 years of age are central nervous system (CNS) tumors [6]. The limited success with conventional treatment consisting of surgery and/or irradiation for brain tumors suggests that new therapeutic strategies using chemotherapy need to be explored. Busulfan (1,4-dimethanesulfonoxybutane) is a bifunctional alkylating agent that is the treatment of choice for chronic myelogenous leukemia due to its marked cytotoxic effect on granulocytic cells [20]. It is also being used in high doses in combination chemotherapy as a preparative regimen for allogeneic bone marrow transplantation [28, 35]. Evaluation of busulfan against CNS tumors was suggested by the activity of other alkylating agents such as cyclophosphamide and melphalan against CNS tumors [12, 15, 17], the lack of significant cross-resistance found between other alkylating agents and busulfan [11, 37], and the excellent penetration of busulfan across the blood-brain barrier with a mean cerebrospinal fluid-to-plasma ratio of at least 0.95 [23, 39]. The goal of the present study was to define the antitumor activity of busulfan against a spectrum of childhood CNS tumor cell lines in vitro and xenografts growing in athymic nude mice.

Materials and methods

Cell lines

The human medulloblastoma cell lines D283 Med [14], D341 Med [16], Daoy [24], D425 Med [5], and D458 Med [5] and the medulloblastoma sublines with laboratory-generated resistance to 4-hydroperoxycyclophosphamide (4-HC), i.e., D283 Med (4-HCR), D341 Med (4-HCR), and Daoy (4-HCR) [18] were grown as previously described. D425 Med [5] was derived from a medulloblastoma originating in the posterior fossa of a 5-year-old boy; D458 Med [5] was derived from medulloblastoma recurring in his spinal fluid following treatment with radiotherapy, cyclophosphamide, and cisplatin. None of the glioma or

ependymoma xenografts described below grow reliably following initiation into culture.

Animals

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

Xenografts

A panel of human CNS tumor-derived xenografts were used for in vivo studies. D456 MG and D212 MG were derived from childhood high-grade gliomas as described elsewhere [4, 19]. D528 EP and D612 EP were derived from posterior fossa ependymomas in children aged 2 and 3 years, respectively. D245 MG was derived from an adult high-grade glioma as described previously [36]. D317 MG and D409 MG were derived from glioblastomas multiforme in two adults. D425 Med was derived from a childhood medulloblastoma [5].

Drugs

Busulfan and melphalan were provided by Burroughs Wellcome Co. (Research Triangle Park, N.C.).

Limiting dilution analysis

Cells were harvested in exponential growth and mixed at a density of 10^6 cells/ml with busulfan (concentrations: 0, 2.5, 5, 25, 50, and 125 μM). Additional studies wih busulfan or melphlan used the drug exposure previously described for 4-Hc [18]. Serial 5-fold dilutions were made from each original tube, and 100 µl was plated per well in a 96-well flat-bottomed tissue culture plate (6 wells/dilution, 8 dilutions/original tube). The plates were incubated at 37° C in a humidified atmosphere containing 5% CO_2 for 12 days, and the wells were examined for colony formation (>30 cells) using an inverted microscope. Each well was scored as positive (± 1 colony) or negative for the presence of colonies. The number of colonies counted per treatment group versus the control value (drug vehicle alone) were analyzed by Spearman analysis to estimate the log kill in each treatment group [25, 31]. Drug concentrations producing 1- and 2-log kills were calculated by linear regression analysis.

Subcutaneous xenograft transplantation

Subcutaneous (s.c.) tumor transplantation into the right flank was performed as described previously with inoculation volumes of 30–100 µl [15].

Intracranial xenograft transplantation

Intracranial (i.c.) tumor transplantation into the right cerebrum was performed as described previously with inoculation volumes of 5 µl [15].

Tumor measurements

Tumors were measured every 3–4 days with vernier calipers (Scientific Products, McGraw, Ill.). The tumor volume was calculated according to the following formula:

$$\frac{(\text{width})^2 \times (\text{length})}{2}.$$

Xenograft therapy

Busulfan was given to mice in a single i.p. injection in a volume of 90 ml/m^2 using 10% dimethylsulfoxide in saline at a dose of 60.3 mg/m^2, which represents the dose lethal to 10% of the treated mice [15]. For s.c. tumor studies, groups of seven to ten randomly selected mice were treated when the median tumor volume exceeded 200 mm^3. Control animals were treated with 10% dimethylsulfoxide in saline. For i.c. tumor studies, groups of 10–15 randomly selected mice were treated with busulfan or vehicle as described above on day 18 (D-458 MG), day 24 (D528 EP), or day 29 (D612 EP), which are the days that represent 50% of the time elapsing between the initial tumor inoculation and the first death as previously defined in i.c. tumor-bearing mice receiving no therapy.

Assessment of response

The response of s.c. 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 5 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 [15]. The response of i.c. xenografts was assessed by the percentage of increase in median survival. Statistical analysis was performed using the Wilcoxon rank-order test as described previously [15].

Results

Limiting dilution assay

The dose of busulfan (continuous exposure) or melphalan (1 h of exposure) that produced 1 log kill in each of the eight medulloblastoma cell lines is indicated in Table 1. Studies using 1 h of drug exposure to busulfan revealed no cytotoxicity. A comparison with previously defined doses of 4-HC producing 1 log kill in these lines [18] reveals the absence of cross-resistance to busulfan, with the possible exception of Daoy (4-HCR), which displays limited cross-resistance.

Table 1 Limiting-dilution of busulfan and 4-HC cytotoxicity against human medulloblastoma cell lines

Cell line	Busulfan dose producing 1 log kill (μM)[a]	Melphalan dose producing 1 log kill (μM)[b]	4-HC dose producing 1 log kill (μM)[b]
D283 Med	1.4	3.8	4.7
D283 Med (4-HCR)	1.9	17.0	37.3
Daoy	1.9	3.2	6.1
Daoy (4-HCR)	4.4	10.1	35.6
D341 Med	2.4	10.0	6.6
D341 Med (4-HCR)	0.8	18.6	9.2
D425 Med	4.4	2.7	6.0
D458 Med	3.1	4.3	7.2

[a] Cells were exposed to busulfan continuously for 12 days
[b] Cells were exposed to melphalan or 4-HC for 1 h. Values were reproduced for comparative analysis [18]

Table 2 Treatment of s.c. human CNS xenografts with busulfan

Xenografts	Histology	Experiment number	Number of animals treated with busulfan[a]	Median time to 5 × initial tumor volume (days)	T–C[b]	Regressions[c]
D-212 MG	Childhood high-grade glioma	1	8	29.9	0.45	0/8
D-456 MG	Childhood high-grade glioma	2	10	22.6	15.8*	8/9*
		3	10	27.5	16.1*	8/10* (1)
		4	8	54.3	18.5*	5/7*
		5	8	31.1	15.4*	5/8*
		6	7	34.3	20.5*	7/7*
D-245 MG	Adult high-grade glioma	7	9	31.8	22.5*	9/9* (3)
		8	8	27.6	21.7	8/8*
D-317 MG	Adult high-grade glioma	9	7	20.2	6.9*	0/7
D-409 MG	Adult high-grade glioma	10	8	47.4	39.7*	8/8* (2)
D612 EP	Ependymoma	11	7	50.8	14.3*	1/7 (1)
		12	9	41.5	17.8*	0/9
D528 EP	Ependymoma	13	9	61.3	58.4*	9/9* (1)
		14	7	53.5	55.4*	7/7* (1)
		15	7	72.7	58.1*	6/7* (2)
		16	7	64.0	50.8*	6/7* (1)
D425 Med	Medulloblastoma	17	8	28.0	15.5*	6/8* (1)

Busulfan was given in a single i.p. injection in a volume of 90 ml/m² using 10% dimethylsulfoxide in saline at a dose of 60.3 mg/m²

* Statistically significant value ($P < 0.01$)

[a] All control arms contained 10 animals each

[b] T–C, growth delay in days, is defined as the difference between the median time required for tumors in treated (T) and control (C) animals to reach 5 times the volume measured at the initation of treatment

[c] Regression is defined as a decrease in tumor volume over two successive measurements. Numbers in parentheses indicate the number of complete regressions

Table 3 Treatment of i.c. human CNS xenografts with busulfan

Xenograft	Histology	Experiment number[a]	Median day of death[b]		Increase in median survival (%)
			Treated	Control	
D-456 MG	Childhood high-grade glioma	1	59 (44–75)	31 (24–36)	90*
		2	63 (50–69)	38 (29–40)	66*
		3	64 (54–76)	38 (32–40)	68*
D612 EP	Ependymoma	4	72 (55–86)	61 (51–64)	18*
		5	96 (90–111)	72 (52–83)	33*
D528 EP	Ependymoma	6	116 (98–133)	62 (43–84)	89*

Busulfan was given in a single i.p. injection in a volume of 90 ml/m² using 10% dimethylsulfoxide in saline at a dose of 60.3 mg/m²

* Statistically significant value ($P < 0.01$)

[a] All experiments used 10 animals each in the control and treatment arms

[b] Calculated as the median day of death of drug-treated mice minus the median day of death of saline-treated mice divided by the median day of death of saline-treated mice. Numbers in parentheses denote the range

Subcutaneous xenograft therapy

Toxicity

In all, 2 deaths among the 127 animals treated were attributable to drug toxicity. The median nadir weight loss was 1.9%. No neurologic toxicity, including seizures, was noted.

Activity.

Busulfan was active in the therapy of virtually all xenografts tested, with growth delays ranging from 14.3 days in D612 EP to 58.4 days in D528 EP (Table 2). Tumor regressions were seen in D456 MG (glioma), D245 MG (glioma), D409 MG (glioma), D612 EP (ependymoma), and D528 EP (ependymoma).

Intracranial xenograft therapy.

Busulfan produced statistically significant increases of 18%–90% in the median survival of mice bearing i.c. D456 MG, D612 EP, and D528 EP xenografts (Table 3). All mice displayed enlarged craniums at death, consistent with expanding intracranial masses, and were autopsied for confirmation of gross tumor.

Discussion

Alkylating agents, including cyclophosphamide, melphalan, and cisplatin, are the most active antineoplastic drugs currently employed in the treatment of CNS tumors [12, 15, 17]. Nevertheless, with rare exception, these agents have not substantially increased the survival of patients with these tumors. This failure has been ascribed to restricted access of the drug to the i.c. site due to the reduced permeability of the blood-brain barrier and has led numerous investigators to design trials that focus exclusively on agents with physiochemical features favoring i.c. access rather than cellular antineoplastic activity [13]. This is an oversimplification of the problem, however, and avoids the main issue, which is selection of highly active compounds that achieve pharmacologically relevant levels in the target tumors [13]. Partial i.c. delivery of a very active agent will no doubt prove superior to superb delivery of a relatively inactive agent.

Busulfan is a dimethanesulfonyloxyalkane that was first noted to have activity against murine ascites carcinoma. Since the demonstration of its antitumor activity against chronic myelogenous leukemia [20], busulfan has been the treatment of choice (in the absence of bone marrow transplantation) for this disease. Busulfan has not demonstrated appreciable activity against solid tumors [2, 27, 30]. We chose to evaluate the activity of busulfan against a panel of human CNS cell lines and xenografts due to a combination of factors, including the activity of other alkylating agents against CNS tumors [12, 15, 17], the lack of cross-resistance between busulfan and cyclophosphamide [11, 37], and the extraordinary penetration of this compound to the cerebrospinal fluid [23, 28]. Our studies revealed prodigious activity of busulfan against a heterogeneous panel of CNS s.c. and i.c. xenografts, including tumors derived from childhood high-grade glioma, adult high-grade glioma, medulloblastoma, and ependymoma. Although our i.c. model uses implanted and not endogenous CNS tumors and, accordingly, may have more permeable tumors than do models using carcinogen-induced i.c. tumors [21], the implanted tumor model has identified compounds subsequently shown to be active in patients with brain tumors [15, 17]. Furthermore, clinical studies of CNS tumors in patients have demonstrated that these tumors have a degree of permeability similar to that seen in several of our implantable tumor models [22]. Minimal evidence, if any, for cross resistance to 4-HC-resistant (and melphalan cross-resistant) medulloblastoma cell lines was seen in vitro, confirming earlier observations [11, 37] and suggesting a role for busulfan against cyclophosphamide-resistant neoplasms.

The precise mechanism of the cytotoxic action of busulfan remains unclear. Initial studies demonstrated that busulfan did not cause significant DNA cross-linking, unlike other alkylating agents [1, 29, 40]. Subsequent investigations found evidence of busulfan-induced cross-linking such as the formation of diguanyl compounds containing a four-carbon bridge [7, 38]. Using newer alkaline-elution techniques, investigators have found dose-dependent DNA interstrand cross-linking in rodent Yoshida sarcoma [3]. It has also been suggested that the cytotoxic mechanism of action of busulfan is linkage of DNA to protein through a thiol group [9, 10]. This suggestion is supported by the work of Roberts and Warwick [32–34], who extensively investigated the reactions of busulfan with thiol groups and busulfan metabolism in the rat. The reaction of busulfan with thiol compounds in vitro and in vivo creates a positively charged sulfonium ion, which could further alkylate sulfur groups and would permit cross-linking of DNA to protein. Further studies may prove helpful in improving our understanding of the mechanisms of resistance to alkylators such as cyclophosphamide in tumors that retain sensitivity to busulfan [18].

Studies evaluating the role of busulfan in the clinical therapy of brain tumors have been initiated. Kalifa et al. [26] have shown activity in very heavily pretreated patients with recurrent medulloblastoma (three of six patients), recurrent ependymoma (one of five patients), and recurrent primitive neuroectodermal tumors (one of two patients) using busulfan (600 mg/m^2) plus thiotepa (350 mg/m^2 × 3) with autologous bone marrow rescue. Our preclinical studies suggest activity for busulfan against high-grade gliomas, medulloblastomas, and ependymomas, and we are now evaluating busulfan-containing regimens in patients with these histologies as well as in those with cyclophosphamide-resistant medulloblastoma.

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