Jpn. J. Cancer Res. (Gann), 79, 1048–1053 ; September, 1988

Antitumor Activity and Neutrophil-selective Hematopoietic Toxicity of Busulfan Analogs in Mice

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The antitumor activity and hematopoietic toxicity of two busulfan analogs were evaluated in comparison with those of busulfan. Although a program of five daily ip treatments with busulfan was not effective in treating sarcoma 180-bearing mice, a fluorine-containing busulfan analog, 1,4-butanediol di-2,2,2-trifluoroethanesulfonate (BFS), and a water-soluble analog, 1,4-butanediol diisethionate (BIT), were significantly effective when given on the same schedule. Busulfan did not appreciably prolong the life span of either P388- or Meth A-bearing mice, whereas BFS and BIT produced significant increases in the life span. It is worth noting that both the analogs were definitely less toxic to the host mice than busulfan. All the drugs examined exhibited suppressive effects on the counts of total WBCs, neutrophils, and lymphocytes. Relative toxicity toward neutrophils versus lymphocytes was increased significantly in the BFS and BIT treatments compared with busulfan treatment. It seems that the toxicity of busulfan in host mice might be due to unidentified side effects other than bone marrow suppression. These results suggest that BFS and BIT could be improved substitutes for busulfan.

Key words: Busulfan analog — Fluorinated busulfan — Isethionic acid ester — Neutrophil-selective toxicity — Chronic myeloid leukemia

Busulfan (1,4-butanediol dimethanesulfonate, Myleran) has been widely used as the drug of preference for the treatment of myeloproliferative disorders such as chronic myeloid leukemia (CML), especially in the chronic phase.[1] However, aggravation of the blastic crisis of leukemia cells in busulfan-controlled CML patients often occurs, leading to the death of patients. In addition, in busulfan therapy, delayed deleterious side effects have been occasionally documented; pulmonary fibrosis ("busulfan lung" disease), Addison-like disease, and hyperpigmentation, in addition to excessive bone marrow depression.[1] Therefore substitutes for busulfan in the treatment of CML have been extensively studied. Alpha interferon may be one promising candidate.[2] Taking into account that busulfan exerts a fairly selective toxicity toward CML, we have been looking for superior substitutes among busulfan analogs. This paper describes the antitumor activities of two novel busulfan analogs in comparison with that of busulfan in several experimental assay systems, and the characteristics of their hematopoietic toxicity. The first analog is a fluorine-containing compound, 1,4-butanediol di - 2,2,2 - trifluoroethanesulfonate (BFS) which has been selected as the most promising candidate among 12 kinds of mono- and difunctional fluorine-containing aklyl alkanesulfonates.[3] The selection was based on preliminary screening by the total packed cell volume (TPCV) method using sarcoma 180 cells inoculated ip in ICR mice (unpublished data). The second analog is 1,4-butanediol diisethionate (BIT) which has been selected as an improved analogue of water-soluble busulfan derivatives.[4]

CH_3SO_2-O-$CH_2CH_2CH_2CH_2$-O-SO_2CH_3
(busulfan)
$CF_3CH_2SO_2$-O-$CH_2CH_2CH_2CH_2$-O-$SO_2CH_2CF_3$
(BFS)
$HOCH_2CH_2SO_2$-O-$CH_2CH_2CH_2CH_2$-O-$SO_2CH_2CH_2OH$ (BIT)

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The present study revealed that, in comparison with busulfan, both BFS and BIT are less toxic in host mice and more effective in remitting the murine tumors examined. It may be worth noting that both drugs exhibited highly selective toxicity toward myeloid cells at their respective non-toxic doses.

Materials and Methods

Materials The drugs were synthesized according to the preparative methods previously described.[3,4] Experimental animals were purchased from Shizuoka Agriculture Cooperative Association, Hamamatsu. The ICR mice used were 5-week-old females weighing 22–26 g, and the $CD2F_1$ mice (F_1 of BALB/c × DBA/2) used were 7-week-old females, weighing 18–22 g.

Cytotoxicity in Cultured Leukemia L1210 Cells L1210 cells seeded at 1×10^5 cells/ml were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. This cell culture was preincubated for 15 hr, then the drug dissolved in dimethyl sulfoxide (1% final concentration) was added. After further incubation for 2 days in the presence of the drug, the cells which excluded trypan blue were counted.

Antitumor Activity in Mice

Sarcoma S180 in ICR Mice: Sarcoma 180 cells (6×10^6 cells/mouse), maintained in female ICR mice by ip passages, were ip inoculated into ICR mice (6–8 mice in each experimental group). The therapy was initiated 24 hr after the tumor inoculation by ip injection of the drug suspended in 0.1 ml of 0.5% carboxymethylcellulose (for busulfan and BFS) or saline (for BIT). This therapy was continued daily for 5 days. The antitumor activity was evaluated according to the total packed cell volume method[5] on the 7th day after the tumor inoculation.

Leukemia P388 in BDF_1 Mice: Leukemia P388 cells (10^6 cells/mouse), maintained in female DBA/2 mice by ip passages, were ip inoculated into female BDF_1 mice. The therapy was initiated 24 hr after the tumor inoculation by ip injection of 0.2 ml of the drug suspended in 0.5% CMC (for busulfan and BFS) or saline (for BIT). This therapy was continued daily for 5 days. Evaluation of the antitumor activity was made in terms of the percent increase in the mean life span (ILS) with reference to the control.

Sarcoma Meth A in $CD2F_1$ Mice: Meth A cells (10^6 cells/mouse), maintained in female BALB/c mice by ip passages, were ip inoculated into female $CD2F_1$ mice. The therapy was initiated 24 hr after the tumor inoculation by ip injection of 0.2 ml of the drug suspended in 0.5% CMC (for busulfan and BFS) or saline (for BIT). This therapy was continued daily for 5 days. Evaluation of the antitumor activity was made in terms of the percent ILS with reference to the control.

Blood Cell Counts $CD2F_1$ mice were subjected to daily ip injections of 0.2 ml of the drug suspended in 0.5% CMC for 5 days. Each experimental group included 5 mice. The blood was taken from the tail vein of the mice with a glass capillary tube. The WBCs were stained with Turk solution and counted using a hemocytometer. Differentiated leucocyte counts were performed on smear preparations stained with May-Grünwald-Giemsa.

Results

Cytotoxicity of Two Busulfan Analogs, BFS and BIT, in Cultured Leukemia L1210 Cells As shown in Fig. 1, BFS and BIT showed more potent cytotoxicities than busulfan against cultured L1210 cells in the exponential growth phase. Each derivative exhibited dose-dependent cytotoxicity; the IC_{50} values of busulfan, BFS, and BIT were 100, 25, and 60 μM, respectively (averages of 3 separate experiments).

Antitumor Effects of BFS and BIT in Tumor-bearing Mice The antitumor effects of BFS and BIT were compared with those of busulfan toward sarcoma S180 in ICR mice, leukemia P388 in BDF_1 mice, and sarcoma Meth A in $CD2F_1$ mice.

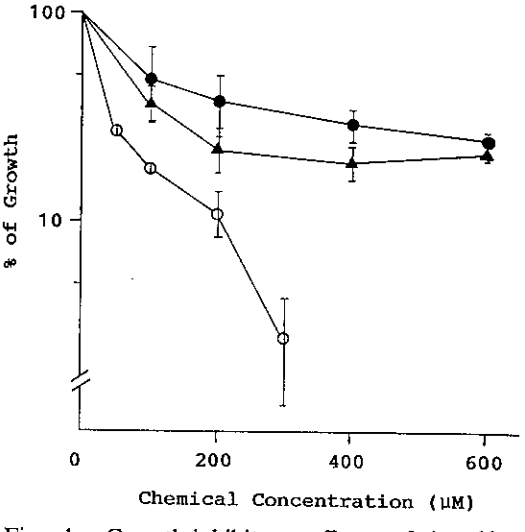

Fig. 1. Growth-inhibitory effects of busulfan analogs on cultured leukemia L1210 cells. ●, Busulfan; ▲, BIT; ○, BFS.

Table I. Antitumor Effect of Busulfan Analogs ($X\text{-}SO_2OCH_2CH_2\text{-})_2$ on Sarcoma S180-bearing ICR Mice

Agent (X-)	Dose[a] (mg/kg/day)	BWC[b] (g)	Tumor volume[c]	T/C[d] (%)	CR[e]
Control		4.4	2.3±1.1	(100)	0/8
CH_3- (busulfan)	100×5	−3.2	0.08±0.1	35	1/3(3)[f]
	30×5	5.1	1.6±0.3	71	0/6
	10×5	3.9	3.4±0.4	150	0/6
CF_3CH_2- (BFS)	100×5	−4.3	0.01±0.02	0.4	5/6
	30×5	1.1	0.02±0.04	0.7	5/6
	10×5	4.8	1.4±1.0	60	1/6
$HOCH_2CH_2$-[g] (BIT)	300×5	−6.3		0	6/6
	100×5	−1.1		0	6/6
	30×5	2.5		31	1/6
	10×5	1.7		104	0/6

a) ICR mice were each inoculated ip with 6×10^6 S180 cells on day 0. The drug was given ip daily for 5 days.
b) Body weight change on day 7 after the peritoneal fluid was removed.
c) Packed tumor cell volume(cm^3)±SD.
d) Percent ratio of the total packed cell volume in the peritoneal cavity with reference to the control.
e) Mice showing complete regression/surviving mice.
f) The number in parentheses indicates the number of mice which succumbed before day 7.
g) Data taken from ref. 4.

Table II. Antitumor Effect of Busulfan Analogs ($X\text{-}SO_2OCH_2CH_2\text{-})_2$ on Leukemia P388-bearing BDF_1 Mice

Agent (X-)	Dose[a] (mg/kg/day)	MST±SD[b] (days)	ILS(%)
Control		9.3±0.7	(0.0)
CH_3- (busulfan)	100×5	3.0±0.8	−67.7
	50×5	6.5±3.5	−30.1
	25×5	11.5±0.7	20.4
	12.5×5	10.0±1.3	7.5
CF_3CH_2- (BFS)	100×5	6.8±2.9	−26.9
	50×5	21.7±1.7	133.3
	25×5	18.2±3.6	95.7
	12.5×5	15.7±1.8	68.8
$HOCH_2CH_2$- (BIT)	100×5	13.0±0.8	39.8
	50×5	17.8±1.9	91.4
	25×5	17.0±2.2	82.8
	12.5×5	15.3±1.4	64.5

a) BDF_1 mice were each inoculated ip with 1×10^6 P388 cells on day 0. The drug was given ip daily on days 1 to 5.
b) Mean survival time±standard deviation.

Against Sarcoma S180 in ICR Mice: Table I shows the antitumor effects of BFS, BIT, and busulfan evaluated by the TPCV method on the 7th day after ip inoculation of S180 cells into ICR mice. Busulfan showed no inhibition at all of tumor growth, and was severely toxic to the host mice at a dose of 100 mg/kg/day (3 out of the 6 mice succumbed to the toxicity), whereas BFS was significantly effective at doses of 30–100 mg/kg/day and none of the mice succumbed even after a dosage of 100 mg/kg/day. BIT was also effective at doses of 30–300 mg/kg/day as previously reported.[4] These results indicate that both BFS and BIT are less toxic than busulfan in ICR mice and more effective in killing intraperitoneally inoculated S-180 cells.

Against Leukemia P388 in BDF_1 Mice: Table II shows the ILS induced by these analogs in P388-bearing BDF_1 mice. Busulfan did not show any appreciable antitumor activity over the total dose range examined, whereas BFS and BIT at doses from 12.5 to 50 mg/kg/day induced significant increases in the life span. BFS and BIT at their optimal doses resulted in 133% and 91% ILS, respectively.

Against Sarcoma Meth A in $CD2F_1$ Mice: Table III shows the ILS induced by these analogs in Meth A-bearing $CD2F_1$ mice. Busulfan did not show any antitumor activity

Table III. Antitumor Effect of Busulfan Analogs ($X\text{-}SO_2OCH_2CH_2\text{-})_2$ on Meth A-bearing $CD2F_1$ Mice

Agent (X-)	Dose[a] (mg/kg/day)	BWC[b] (g)	MST±SD[c] (days)	ILS (%)
Control		0.7	15.2±1.2	(0.0)
CH_3- (busulfan)	100×5	−3.3	3.2±0.7	−78.9
	50×5	−1.3	10.8±0.7	−28.5
	25×5	−0.3	11.7±0.5	−23.0
	12.5×5	0.5	12.3±0.5	−19.1
CF_3CH_2- (BFS)	200×5		2.7±0.5	−84.9
	100×5	−1.3	5.8±1.6	−61.8
	50×5	−0.3	37.7±0.3	148.0
	25×5	0.3	16.2±1.3	13.2
	12.5×5	0.3	14.7±1.3	−3.3
$HOCH_2CH_2$- (BIT)	100×5	−0.3	11.0±0.8	−27.6
	50×5	−0.5	22.5±8.6	48.0
	25×5	0.1	26.8±2.7	76.5
	12.5×5	0.5	21.7±3.7	42.5

a) $CD2F_1$ mice were each inoculated ip with 1×10^6 Meth A cells on day 0. The drug was given ip daily on days 1 to 5.
b) Body weight change on day 3.
c) Mean survival time±standard deviation.

Table IV. Effect on Total WBC, Neutrophil, and Lymphocyte Counts after Treatment of $CD2F_1$ Mice with Busulfan Analogs $(X\text{-}SO_2OCH_2CH_2\text{-})_2$[a]

Days	Cell count ratio referred to the control: Total WBC			Neutrophil			Relative reduction rate neutrophil/lymphocyte[b]		
	Busulfan 25 mg	BFS 50 mg	BIT 25 mg	Busulfan 25 mg	BFS 50 mg	BIT 25 mg	Busulfan 25 mg	BFS 50 mg	BIT 25 mg
0	1.00(0.06)	1.00(0.06)	1.00(0.06)	1.00(0.31)	1.00(0.31)	1.00(0.31)	1.00	1.00	1.00
1	0.96(0.13)	0.71(0.06)	0.55(0.21)	1.52(0.32)	0.77(0.39)	0.54(0.16)	1.80	1.13	1.01
3	0.78(0.18)	0.36(0.09)	0.65(0.22)	1.16(0.58)	0.46(0.20)	0.54(0.33)	1.66	1.35	0.82
5	0.78(0.19)	0.56(0.09)	0.86(0.23)	0.95(0.28)	0.28(0.12)	0.61(0.24)	1.20	0.47	0.69
7	0.53(0.10)	0.31(0.10)	0.68(0.23)	0.42(0.11)	0.06(0.04)	0.12(0.03)	0.74	0.16	0.15
9	0.53(0.06)	0.30(0.08)	0.48(0.07)	0.55(0.09)	0.15(0.08)	0.17(0.02)	1.02	0.44	0.32
11	0.61(0.10)	0.26(0.04)	0.50(0.11)	0.51(0.14)	0.07(0.04)	0.26(0.18)	0.73	0.20	0.43
14	0.86(0.14)	0.18(0.05)	0.45(0.13)	0.49(0.09)	0.07(0.03)	0.13(0.17)	0.47	0.32	0.25
16	0.57(0.11)	0.26(0.07)	0.34(0.05)	0.25(0.13)	0.29(0.03)	0.11(0.04)	0.40	1.07	0.29
18	0.52(0.08)	0.50(0.05)	0.35(0.09)	0.27(0.14)	0.40(0.05)	0.06(0.05)	0.44	0.74	0.14
21	0.79(0.22)	0.50(0.08)	0.44(0.13)	0.36(0.14)	0.82(0.18)	0.04(0.05)	0.40	1.82	0.07
24	0.56(0.09)	0.62(0.03)	0.55(0.23)	0.52(0.23)	1.74(0.45)	0.35(0.13)	0.91	3.53	0.60
28	0.76(0.16)	0.77(0.06)	0.44(0.09)	0.76(0.27)	1.34(0.30)	0.58(0.04)	0.98	2.03	1.41
31	0.88(0.19)	0.82(0.15)	0.79(0.30)	0.83(0.37)	1.35(0.71)	0.89(0.48)	0.93	1.84	1.41

a) The drug was ip administered to 5 $CD2F_1$ mice in each group daily on day 0 to day 4. Each value in the table is the average from 5 mice treated in each group with reference to the averaged control count. The standard deviation is given in parentheses. The control counts on day 0 were 6530(394) for total WBC, 1488(459) for neutrophils, and 4925(810) for lymphocytes. Busulfan (X: CH_3-), 25 mg/kg×5 as a minimal toxic dose; BFS (X: CF_3CH_2-), 50 mg/kg×5 as an optimal therapeutic dose; BIT (X: $HOCH_2CH_2$-), 25 mg/kg×5 as an optimal therapeutic dose.
b) Relative neutrophil count compared to lymphocyte count, i.e., a measure of selective toxicity toward myeloid tissue compared with lymphoid tissue.
c) Each neutrophil count enclosed in a box is less than 30% of the control count.
d) Each relative count enclosed in a box indicates that the relative neutrocyte count is less than 50% of the relative lymphocyte count.

over the total dose range examined, whereas BFS and BIT at optimal doses (50 and 25 mg /kg/day, respectively) induced significant increases in the life span (148 and 77% ILS, respectively). Although the antitumor activity of BIT was less than that of BFS, the effective dose range of BIT was broader than that of BFS.

Effects of BFS and BIT on Total White Blood Cell (WBC), Neutrophil, and Lymphocyte Counts Based on the toxicity toward $CD2F_1$ mice indicated by the treatment of the Meth A-bearing mice described above, the following doses were chosen: BFS, 5 daily treatments with 50 mg/kg/day for the optimal dose; BIT, treatment with 25 mg/kg/day for the optimal dose; and busulfan, treatment with 25 mg/kg/day for a slightly toxic dose. Busulfan treatment at 50 mg/kg/day was also attempted but all the mice succumbed to the toxicity within 18 days after the initiation of treatment. The counts of total WBCs neutrophils, and lymphocytes were measured and normalized with respect to the counts of untreated animals on each day of measurement. The relative counts of WBCs and neutrophils are shown in Table IV. The relative reduction rates of neutrophils vs. lymphocytes are also shown in the same table (the relative counts of lymphocytes are not shown). All the drugs exhibited suppressive effects on total and differentiated WBC counts. In addition, there were several apparent trends of suppression mode depending on the drug used. With regard to the total WBC count, BFS induced the most severe reduction of cells, followed by BIT and then busulfan, although the doses of the former two analogs were not toxic while that of busulfan was toxic. The same trend was found in reductions of neutrophil and lymphocyte counts. These results may suggest that lethal toxicity of busulfan in mice is not mainly due to bone marrow suppression, but to some other unidentified side effects. With regard to the relative rate of reduction of neutrophils vs. lymphocytes, BFS and BIT were found to be more selective toward neutrophils. Thus, the myeloid-targeting characteristics of BFS and BIT might be stronger than that of busulfan. A comparison of the effects of BFS and BIT suggested that the suppressive effect of BFS is manifested at a somewhat earlier stage than that of BIT and that recovery from the neutropenia induced by BFS is faster.

Discussion

The two busulfan analogs examined in the present study are distinguished from busulfan in their partition properties; BFS is more lipophilic, whereas BIT is much more hydrophilic than busulfan. With regard to alkylating ability, it is assumed that BFS might be more reactive and much shorter-lived than busulfan and BIT. Thus, the nucleofugality of the leaving groups of these analogs (2,2,2-trifluoroethanesulfonyl group in BFS, 2-hydroxyethanesulfonyl group in BIT, and methanesulfonyl group in busulfan), was estimated from the rates of hydrolysis of the ethyl esters; their half-lives were 0.12 hr, 2.45 hr, and 6.63 hr, respectively, in 0.25*M* phosphate buffer (pH 7.4) at 37° (experimental data not shown).

As regards the antitumor activity in experimental tumor-bearing mice, BFS and BIT are significantly less toxic in host mice than busulfan and are more effective in remitting inoculated tumors. The result of the treatment of Meth A-bearing mice shows that busulfan is toxic even at 12.5 mg/kg/day, whereas toxicity was not manifested in the treatment with either BFS or BIT at 50 mg/kg/day. Severer decreases in the total WBC count were thereby induced with non-toxic doses of BFS and BIT than that induced with a toxic dose of busulfan. It is, therefore, suggested that BFS and BIT might be bone marrow-targeting agents in less lethal dose ranges as compared with busulfan. In addition, since the relative cytotoxicity toward myeloid cells vs. lymphoid cells was increased significantly in the BFS and BIT treatments compared with the busulfan treatment, it is possible that these analogs might be superior to busulfan in the control of CML. In other words, busulfan might exert unidentified lethal side effects other than the myeloid leukemia cell-targeting therapeutic effects. Although it is difficult to predict the therapeutic superiority of these novel analogs to busulfan from the present experiments, the results provide indications of possible therapeutic benefit in the treatment of chronic myeloid leukemia.

Acknowledgments

The authors are indebted to Dr. Takashi Okano of the Government Industrial Research Institute, Nagoya, for his cooperation in synthesizing fluorine-containing compounds. Part of this work was financially supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

(Received Jan. 23, 1988/Accepted July 27, 1988)

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