Anti-Cancer Drugs, 3, pp. 531–539

Preclinical activity of hepsulfam and busulfan in solid human tumor xenografts and human bone marrow

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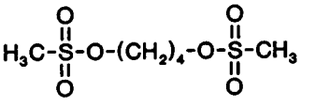
Hepsulfam (1,7-heptanediol disulfamate, NSC 329680) is a new antineoplastic alkanesulfonate agent which has demonstrated a broader preclinical activity than busulfan. The compound is currently undergoing clinical trials. We have studied the activity of hepsulfam and busulfan simultaneously in human tumor xenografts *in vitro* in a clonogenic assay and *in vivo* in tumor-bearing animals in order to assess the activity of both compounds in model systems of slowly growing malignancies. In a total of 37 different tumors of various histologies, both agents demonstrated broad spectrum *in vitro* activity. The median IC_{50} of hepsulfam and busulfan was determined as 0.93 and 3.31 μg/ml, respectively. At a concentration of 1.0 μg/ml, hepsulfam was active in eight of 37 tumors (22%) in the clonogenic assay, whereas busulfan effected inhibition of colony formation in one of 37 lines (3%). At the same concentration, however, hepsulfam demonstrated a clear *in vitro* toxicity to human bone marrow cells (CFU-GM) from healthy donors, whereas busulfan did not reveal a myelosuppressive effect. Evaluation of equitoxic concentrations *in vitro* revealed a higher activity of hepsulfam, especially in non-small cell lung cancer. In tumor-bearing nude mice, the approximate LD_{10} dose was determined as 150 mg/kg single bolus injection given i.p. on day 1 for both compounds. Hepsulfam demonstrated superior *in vivo* activity in a large cell lung cancer xenograft and a gastric carcinoma model. The preclinical activity of hepsulfam suggests a possible role of this compound in the treatment of solid human malignancies. However, the increased bone marrow toxicity of hepsulfam as compared with busulfan might be critical for further clinical application. Non-small cell carcinomas of the lung might be target tumors for further clinical studies.

Key words: Bone marrow toxicity, busulfan, clonogenic assay, hepsulfam, nude mouse studies.

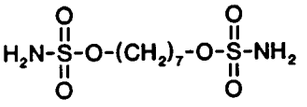
Introduction

The development of new cytotoxic agents with activity in systemic neoplastic disease has been a major aspect of oncological research throughout the last 30 years. One of the first drugs to be successfully introduced to clinical cancer chemotherapy was busulfan, a bifunctional alkanesulfonate with alkylating properties. This compound showed activity in slowly proliferating cells of the hematopoietic system and is still used mainly in treatment of chronic myelogenous leukemia (CML) and polycythemia vera.[1] Solid human tumors of various histologies did not respond to busulfan therapy in clinical studies.

The 1,7-heptanediol disulfamate (hepsulfam, NSC 329680) is a new antineoplastic alkanesulfonate agent with close structural similarity to busulfan (Figure 1). It was developed in an attempt to improve the antineoplastic efficacy of busulfan through the introduction of a more polar leaving



busulfan



hepsulfam

Figure 1. Chemical structures of busulfan (NSC 750) and hepsulfam (NSC 329680).

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group and showed a broader preclinical activity than busulfan in the NCI *in vivo* screening systems. Busulfan demonstrated good activity in the s.c. implanted CD8F1 mammary carcinoma model only, whereas for hepsulfam the spectrum of activity included the P388 and L1210 murine leukemias as well as the B16 melanoma, M5076 sarcoma, CD8F1 mammary carcinoma, colon adenocarcinoma 38 and the MX-1 mammary xenograft models. Schedule dependency studies determined a single i.p. bolus injection as the most effective mode of application. Cross-resistance of melphalan- and cisplatin-resistant P388 sublines to hepsulfam was observed *in vitro*.[2] In view of these data, the compound has been selected by the NCI for further development to clinical trial.

We have studied the antineoplastic effect of hepsulfam in comparison with busulfan simultaneously in human tumor xenografts established in thymusaplastic nude mice, in order to assess the activity of both compounds in model systems of slowly growing malignancies with a close resemblance to patient tumors. Studies were performed *in vitro* in a clonogenic assay and *in vivo* in tumor-bearing animals.

Materials and methods

Test procedure

In recent years, we have developed a four-step test procedure allowing for preclinical *in vitro* and *in vivo* testing of experimental agents for antineoplastic efficacy.[3,4] In the first testing stage primary screening is done *in vitro* in a modified clonogenic assay in six human tumors which are well characterized for chemosensitivity to standard anticancer agents (four sensitive and two resistant xenograft lines). Secondary *in vitro* screening is performed in a total of 14 responsive and six resistant human tumor xenografts. In stage III the toxicity on two to four human bone marrow specimens from healthy donors is studied. Compounds with a greater or similar effect on tumor cells compared with human bone marrow are subsequently studied *in vivo* in the most sensitive xenografts transplanted s.c. into nude mice. The comparison of *in vitro* and *in vivo* activity allows an assessment of the relevant *in vitro* dose based on the *in vivo* pharmacological behavior of a compound. If a remission or at least no change is observed *in vivo*, a compound undergoes disease-oriented testing usually in up to 40–60 xenografts. Since the tumors selected for stage IV have a chemosensitivity profile similar to the clinic, target tumors for clinical studies can be identified.

Nude mice and tumors

For all experiments, athymic nude mice (outbred NMRI *nu/nu* strain) grown in our own breeding facilities were used. The animals were housed in macrolon cages under laminar air flow conditions with free access to food and acidified water. Room temperature was controlled at 24 ± 1°C and relative humidity was above 70%.[5]

Human solid tumors established in serial passage in thymusaplastic nude mice were used as tumor material. The human origin of the tumors was confirmed by isoenzymatic and immunohistochemical methods. Tumor models were selected with regard to the drugs to be tested from a panel of 220 regularly growing xenografts. All tumors were derived from different patients. Characterization of these models included histology, growth behavior, chemosensitivity to standard anticancer drugs *in vitro* and *in vivo*, isoenzyme phenotype analysis, hormone receptor analysis, and DNA histogram as generated by flow cytometry.[6–10]

Clonogenic assay

In vitro activity of experimental agents was studied in a modification of the two-layer soft agar culture system introduced by Hamburger and Salmon.[11–14]

Preparation of a single cell suspension. Solid human tumor xenografts were mechanically disaggregated and subsequently incubated with an enzyme cocktail consisting of collagenase 0.05%, DNAse 0.07% and hyaluronidase 0.1% in RPMI 1640 at 37°C for 30 min. The cells were washed twice and passed through sieves of 200 and 50 μm mesh size. The percentage of viable cells was determined in a hemocytometer using trypan blue exclusion.

Human bone marrow. Human bone marrow cells were aspirated from the iliac crest of healthy volunteers into preservative-free heparinized syringes. Mononuclear cells with a density of less than 1.007 g/ml were separated by density centrifugation in Ficoll-paque, washed and plated as described below.

Culture methods. Culture plates consisted of 35 mm petri dishes with two layers of soft agar. The bottom layer contained 1.0 ml Iscoves's modified

Dulbecco's medium with 20% fetal calf serum and 0.5% agar. For the top layer, a total of 0.2–1 × 10^6 tumor or bone marrow cells was added to 1.0 ml of the same culture medium and 0.3% agar and plated onto the base layer. Cytostatic drugs were applied by continuous exposure in 0.2 ml medium onto the top layer (drug overlay). In each assay, six control plates received the vehicle only; drug treated groups were plated in triplicate. Five groups received hepsulfam at concentrations between 0.01 and 100 μg/ml, and five groups were treated with busulfan at the same concentrations. In previous experiments, the sensitivity of cells to hepsulfam and busulfan has been shown to increase as the drug exposure time is increased. In order to allow for a valid comparison of both drugs, the compounds were tested simultaneously with identical exposure times in each tumor line.

Human bone marrow cells required 0.03 ml of a placenta-conditioned medium which stimulates growth of human granulocyte progenitor cells.[15] For xenograft lines showing a low plating efficiency *in vitro* (12% of the tumors tested), 0.06 ml of a pooled human serum of healthy donors was added in order to increase colony growth.[16]

Cultures were incubated at 37°C and 7% CO_2 in a humidified atmosphere for 6–21 days and monitored closely for colony growth using an inverted microscope. Within this period, *in vitro* tumor growth led to formation of colonies with a diameter of 60 μm or greater in the top soft agar layer. At the time of maximum colony formation (after 5–12 days of incubation), counts were performed with an automatic image analysis system (Bausch & Lomb Omnicon FAS IV). At 24 h prior to evaluation, vital colonies were stained using a tetrazoliumchloride dye.[17] Drug effects were expressed in terms of the percentage of survival, obtained by comparison of the mean number of colonies in the treated plates with the mean colony count of the untreated controls (test-versus-control-group value, $T/C = \text{colony count}_{\text{treated group}} \times 100/\text{colony-count}_{\text{control group}}$). A compound was considered active if it reduced colony formation to 30% or less of the control group value ($T/C \leq 30\%$). The majority of clinically established anticancer agents are active at a concentration of 1 μg/ml or less (continuous exposure). Some drugs, e.g. nitrosoureas or DTIC, require dose levels between 6 and 30 μg/ml.

An assay was considered fully evaluable if the following quality control criteria were fulfilled:[14]

- The mean number of colonies in the control group dishes was ≥100 for a colony diameter of 60 μm (≥50 for an 80 μm diameter).
- The initial plate counts on day 0 or 2 were <20% of the final control group count.
- The coefficient of variation in the control group was ≤50%.
- The positive reference compound 5-fluorouracil (5-FU; at the toxic dose of 100 μg/ml) must affect a colony survival of <20% of the controls.

Nude mouse tests: experimental set-up

Female athymic nude mice (6–8 weeks old) of NMRI genetic background were used for tumor implantation. Tumor slices averaging 3 × 3 × 0.5–1 mm were transferred s.c. into both flanks of the animals. At the beginning of therapy, the xenografted malignancies had grown to a median tumor diameter of 7 mm. Mice were randomly assigned to treatment groups and untreated controls. Each group consisted of five or six mice bearing six to ten evaluable tumors.

Tumor growth was recorded weekly by two-dimensional measurement with calipers. For rapidly growing tumor lines (doubling times less than 5 days) measurements were done twice weekly. Tumor size (TS) was calculated according to the formula length × width ($TS = a \times b$). The antitumoral effect of hepsulfam and busulfan (single i.p. bolus injections on day 1 of the experiments) was evaluated following maximal tumor regression, in resistant tumors after 3–4 weeks. Toxicity was assessed by weekly measurement (twice weekly in rapidly growing xenograft lines) of the median body weight of the animals. At the maximum tolerated dose (MTD) level the mice were allowed approximately an LD_{10} or a median body weight loss of 10–15% in the 2 weeks following the last injection.

Data evaluation was performed using specifically designed software. Relative tumor size (RTS) values were calculated for each single tumor by dividing the TS day X by the TS day 0 at the time of randomization ($RTS = TS_X \times 100/TS_0$). Median RTS values were used for further evaluation. Tumor doubling time (DT) of test and control groups was defined as the period required to reach a RTS of 200%. The effect of treatment was classified as complete remission (RTS on day 21 or 28 of 10% or less of the initial value), partial remission (11–50%), minimal regression (51–75%), no change (76–124%) or progression (125% or above). A tumor was considered to be sensitive if at least a minimal regression was achieved.

Additionally, the RTS of treated groups and controls was compared (T/C value). The specific growth delay (SGD) was calculated with regard to DT as described by Steel.[18]

$$SGD = \frac{DT_{\text{treated group}} - DT_{\text{control group}}}{DT_{\text{control group}}}$$

Drugs

Hepsulfam and busulfan were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, USA). In nude mouse experiments, both compounds were applied as solution in a small volume (less than 90 μl per mouse) of pure dimethylsulfoxide (DMSO) i.p. For *in vitro* studies, stock solutions of both drugs were prepared in DMSO and physiological saline (0.1/2.9, v/v), frozen immediately at −20°C in small aliquots for a maximum of 3 months and protected from light. Final solutions containing 0.1% DMSO were prepared freshly for each experiment.

Results

In vitro activity of hepsulfam and busulfan

Hepsulfam and busulfan were studied simultaneously in 37 different human tumor xenografts in the clonogenic assay. Drug concentrations ranged between 0.01 and 10.0 μg/ml (Tables 1 and 2). Clear dose–response effects were observed for both compounds, with response rates between 0 (0.01 μg/ml) and 51% (10 μg/ml) for hepsulfam, and between 0 (0.01 μg/ml) and 32% (10 μg/ml) for busulfan, respectively. The median IC_{50} of hepsulfam and busulfan was calculated to be 0.93 and 3.31 μg/ml, respectively.

At a concentration of 1.0 μg/ml, hepsulfam reduced colony formation to less than 30% of the control group value in eight of 37 xenografts (22%). Differential cytotoxicity was observed, with activity in cancers of the lung, stomach (tumor line GXF 209) and melanomas (MEXF 276). At the same concentration, a cytotoxic effect was observed for busulfan in one of 37 lines studied (3%), one human melanoma xenograft (MEXF 276) was responsive *in vitro*. Tumors of other histologies did not display *in vitro* sensitivity to busulfan.

Comparison of the cytotoxic effect of hepsulfam and busulfan at a concentration of 10.0 μg/ml revealed a similar response profile of human tumor xenografts of different histiotypes. Tumors of the gastrointestinal tract, lung, breast and testes as well as melanomas were responsive to both agents. Renal cell carcinomas as well as human tumor xenografts of the head and neck, pancreas and uterine body were equally resistant to hepsulfam and busulfan. At this high concentration, hepsulfam

Table 1. *In vitro* effect of hepsulfam (NSC 329680) on human tumor xenografts

Tumor histology	Hepsulfam (μg/ml)			
	0.01	0.1	1	10
Colon	0/5[a]	0/5	0/5	1/5
Gastric	0/3	0/3	1/3	2/3
Lung NSCLC[b]	0/11	0/11	5/11	7/11
SCLC[c]	0/3	1/3	1/3	3/3
Breast	0/4	0/4	0/4	2/4
Ovarian	0/1	0/1	0/1	1/1
Testicular	0/2	0/2	0/2	2/2
Melanoma	0/3	1/3	1/3	1/3
Renal	0/2	0/2	0/2	0/2
Head and neck	0/1	0/1	0/1	0/1
Pancreatic	0/1	0/1	0/1	0/1
Uterine body	0/1	0/1	0/1	0/1
Active/total	0/37	2/37	8/37	19/37
%	0%	5%	22%	51%

[a] Responsive (T/C ≤ 30%)/total.
[b] Non-small cell lung cancer.
[c] Small cell lung cancer.

Table 2. *In vitro* effect of busulfan (NSC 750) on human tumor xenografts

Tumor histology	Busulfan (μg/ml)			
	0.01	0.1	1	10
Colon	0/5[a]	0/5	0/5	1/5
Gastric	0/3	0/3	0/3	2/3
Lung NSCLC[b]	0/11	0/11	0/11	2/11
SCLC[c]	0/3	0/3	0/3	2/3
Breast	0/4	0/4	0/4	2/4
Ovarian	0/1	0/1	0/1	0/1
Testicular	0/2	0/2	0/2	2/2
Melanoma	0/3	0/3	1/3	1/3
Renal	0/2	0/2	0/2	0/2
Head and neck	0/1	0/1	0/1	0/1
Pancreatic	0/1	0/1	0/1	0/1
Uterine body	0/1	0/1	0/1	0/1
Active/total	0/37	0/37	1/37	12/37
%	0%	0%	3%	32%

[a] Responsive (T/C ≤ 30%)/total.
[b] Non-small cell lung cancer.
[c] Small cell lung cancer.

was active in 19 of 37 human tumor xenografts (51%), whereas busulfan inhibited tumor cell growth in the clonogenic assay in 12 of 37 lines (32%).

In vitro bone marrow toxicity

The bone marrow toxicity of hepsulfam and busulfan was assessed using marrow specimens from healthy donors (Figure 2, mean values ±SD, $n = 3$). At a concentration of 0.01 μg/ml, hepsulfam and busulfan did not significantly inhibit colony formation of human bone marrow cells in the clonogenic assay. Higher concentrations revealed an increased bone marrow toxicity of hepsulfam as compared with busulfan. At 1 μg/ml, colony formation of hepsulfam treated groups was suppressed to 26% of the control group value, whereas busulfan effected a T/C value of 70%. If a concentration of 10 μg/ml was used, treatment with hepsulfam or busulfan resulted in a decrease of bone marrow colony counts to 3 or 31% of the untreated controls, respectively. At 100 μg/ml both compounds effected a nearly complete suppression of colony formation.

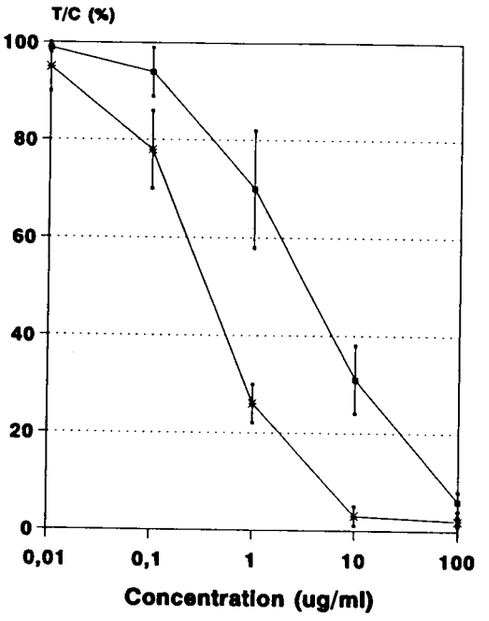

Figure 2. *In vitro* toxicity of hepsulfam (*) and busulfan (■) on human bone marrow (CFU-GM). Mean values ±SD, $n = 3$.

In vivo toxicity of hepsulfam and busulfan

The MTDs of hepsulfam and busulfan *in vivo* were determined in tumor-bearing nude mice. As the MTD of a given drug may vary with age, strain and sex of nude mice, both compounds were applied at dose levels of 300, 150 and 75 mg/kg/day given i.p. as a single bolus injection.

For hepsulfam, application of 300 mg/kg/day resulted in clear toxicity (Table 3). Within the first 2 weeks after treatment, seven of 11 animals (64%) died, the median relative body weight loss was 14%. Injection of 150 mg/kg/day, day 1 i.p., appeared to be the approximate LD_{10} dose which could be applied to tumor-bearing nude mice. After 14 days, a lethality of 12% (two out of 17 animals died) and a median drug-induced body weight loss of 4% were observed. The lowest dose of 75 mg/kg/day was well tolerated.

Table 3. *In vivo* toxicity of hepsulfam and busulfan in tumor-bearing nude mice

Drug	Dose (mg/kg)	Schedule[a]	Route	Day 14[a]		Day 21	
				BWC[b]	LD[c]	BWC	LD
Control				+0.4	0/17	+1.5	0/17
Hepsulfam	300	day 1	i.p.	−14.4	7/11	−2.3	9/11
	150	day 1	i.p.	−4.0	2/17	−0.1	3/17
	75	day 1	i.p.	−0.7	1/17	+3.5	2/17
Busulfan	300	day 1	i.p.	NE	11/11	NE	11/11
	150	say 1	i.p.	−6.3	2/17	−2.1	3/17
	75	day 1	i.p.	−2.2	1/17	+0.7	2/17

[a] Single bolus injections.
[b] Median relative body weight change of animals (%); NE, not evaluable.
[c] Lethal/total no. of animals treated.

Table 4. Characteristics of human tumor xenograft models selected for *in vivo* studies of hepsulfam and busulfan

Tumor designation	Origin	Histology	Established (month/year)	Doubling time (days)[a]	Chemo-sensitivity[b]
GXF 209	stomach	adenocarcinoma, poorly differentiated	8/80	9–12	4/11
LXFL 529	lung	large cell lung cancer, undifferentiated	4/84	5–6	8/16
MEXF 276	skin	malignant melanoma	7/81	8–10	0/12

[a] Range, in serial passage.
[b] Sensitivity to standard anticancer agents: no. active drugs/total no. of drugs tested.

Overall, the *in vivo* toxicity of busulfan was comparable to hepsulfam (Table 3). The highest dose of 300 mg/kg/day was clearly toxic, all animals treated died within 14 days after drug application. The approximate LD_{10} dose was determined as 150 mg/kg/day given day 1 i.p.; application of 75 mg/kg/day was well tolerated.

In vivo effect of hepsulfam and busulfan

The antitumor activity of hepsulfam and busulfan, given as single i.p. bolus injections on day 1 in all experiments, was studied simultaneously in three human tumor xenograft lines growing s.c. in nude mice. All tumors selected were initially shown to be responsive to both agents at a concentration of 10 μg/ml in the clonogenic assay. Characteristics of the tumor models used for *in vivo* studies are given in Table 4.

In the large cell lung cancer xenograft LXFL 529 (Figure 3), busulfan therapy at a single dose of 150 mg/kg injected i.p. on day 1 of the experiment resulted in a short tumor regression, and no change between day 21 and 35. On day 42, progressive growth was observed. Hepsulfam treated tumors regressed completely (RTS 0%) and did not regrow within the observation period of 105 days.

The gastric cancer xenograft GXF 209 (Figure 4) showed progressive tumor growth of untreated

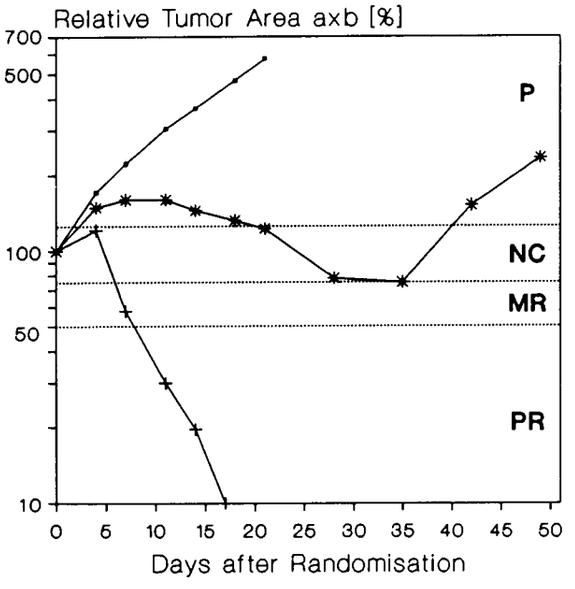

Figure 3. *In vivo* effect of hepsulfam (+) and busulfan (*) at equitoxic dose levels (single bolus of 150 mg/kg given day 1 i.p. for both compounds) in the large cell lung cancer xenograft LXFL 529. P, progression; NC, no change; MR, minor remission; PR, partial remission. (■) Control.

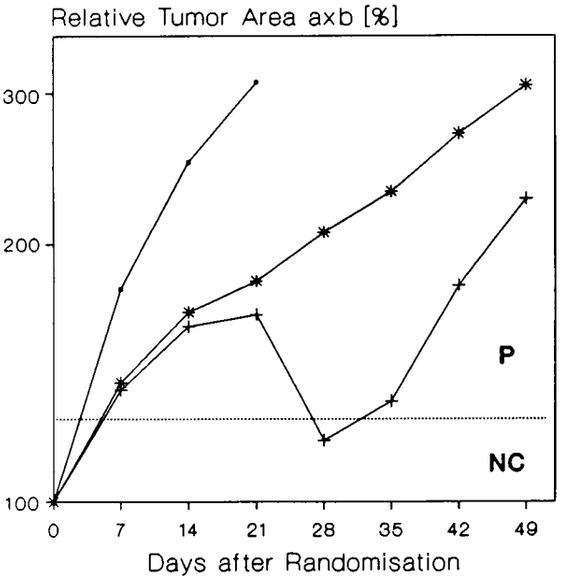

Figure 4. *In vivo* effect of hepsulfam (+) and busulfan (*) at equitoxic dose levels (single bolus of 150 mg/kg given day 1 i.p. for both compounds) in the gastric cancer xenograft GXF 209. P, progression; NC, no change. (■) Control.

control tumors with a median tumor doubling time of 9.2 days. Application of busulfan (single dose of 150 mg/kg given day 1 i.p.) effected inhibition of tumor growth with a DT of 27.2 days and a SGD of 1.95; however, all tumors treated grew progressively. In contrast, *in vivo* therapy with hepsulfam at a dose level of 150 mg/kg/day given day 1 i.p. effected no change on day 28 (relative tumor size 118%). Progressive growth was noted on day 35 leading to a relative tumor size of 200% (tumor doubling) at day 45 and an SGD of 3.90.

In the malignant melanoma xenograft MEXF 276, both compounds did not exert an antineoplastic effect. Growth curves of tumors treated with either hepsulfam or busulfan did not differ from the untreated controls, showing progressive growth with a DT of 9.5 days.

Discussion

Hepsulfam and busulfan were tested simultaneously for antineoplastic efficacy in human tumor xenografts established in serial passage in athymic nude mice.

In vitro, both compounds were evaluated in a total of 37 different solid human tumors of various histiotypes at drug concentrations between 0.01 and 10 μg/ml. Overall, hepsulfam showed a higher cytotoxic efficacy than busulfan in the clonogenic assay, as indicated by a lower median IC_{50} and a higher response rate of the novel compound. The *in vitro* cytotoxicity profiles of both drugs at high concentrations were similar, including tumors of the gastrointestinal tract, lung, breast, testes as well as melanomas. However, only a small number of xenografts were studied for each histology. Our data are in good accordance with preclinical results obtained by other groups.[2] L1210 leukemia cells were found to be 7-fold more sensitive to hepsulfam than to busulfan *in vitro*.[19] The same authors[20] found hepsulfam to be 2- to 3-fold more cytotoxic than busulfan against two human leukemia (HL-60 and K562) and two human colon (BE and HT-29) carcinoma cell lines after treatment for 2–12 h. Additionally, hepsulfam demonstrated antineoplastic efficacy in a human tumor cloning system. At a concentration of 77 μg/ml, the compound was active in ovarian, breast, colon, lung and gastric tumors as well as melanoma.[21]

Assessment of the effect of both compounds on human bone marrow cells from healthy donors *in vitro* indicated possible differences with regard to the spectrum of toxicity of the drugs. Significant inhibition of colony formation of human bone marrow cells (CFU-GM) was observed for hepsulfam at a concentration of 1.0 μg/ml (effecting a T/C of 26%). For a comparable *in vitro* marrow toxicity, busulfan had to be used at a 10-fold higher concentration (T/C of 31% at 10 μg/ml). These data are in accordance to results published recently[22] showing that hepsulfam was superior to busulfan in inhibiting colony-forming units, both granulocyte and macrophage, in freshly isolated peripheral mononuclear blood cells from patients with chronic myelogenous leukemia. However, cytotoxicity data with bone marrow cells from normal individuals were not presented by this group. In contrast, our data were obtained using bone marrow cell populations from healthy donors, giving an indication of the possible myelosuppressive effect of both compounds. Furthermore, evaluation of the *in vitro* toxicity of a compound in human bone marrow allows for the determination of the 'relevant' *in vitro* concentration of the drug.[13,14] In general, concentrations toxic to human bone marrow (T/C $\leq$10%) tend to overestimate the antineoplastic potential of a given drug, as myelosuppression may limit the application of the agent at higher dose levels in an *in vivo* setting. Comparison of the *in vitro* antineoplastic activity of hepsulfam and busulfan at approximately equitoxic concentrations (i.e. hepsulfam 1 μg/ml and busulfan 10 μg/ml) reveals a similar response profile for most solid tumor types and a selectively higher *in vitro* activity of hepsulfam in non-small cell lung cancer.

The difference in cytotoxicity of both compounds might be explained by the difference in the ability to produce DNA-interstrand cross-links. At equimolar concentrations, only hepsulfam was able to induce DNA-interstrand cross-links in L1210 cells, whereas busulfan did not exert comparable DNA damage.[19] In human leukemia and colon carcinoma cell lines, hepsulfam induced a higher level of DNA-interstrand cross-links than busulfan. Both compounds induced DNA–protein cross-links.[20] These results suggest that mechanisms of DNA reactivity of hepsulfam and busulfan differ. Furthermore, busulfan and hepsulfam show different hydrolysis reactions in aqueous solution,[23,24] which may contribute to the lower *in vitro* cytotoxicity of busulfan. Recently, sensitivity of human breast cancer cell lines to hepsulfam was shown to correlate with total cytoplasmic glutathione S-transferase (GST) activity as well as levels of the GST-π isoenzyme. Glutathione depletion with buthionine sulfoximine increased the

sensitivity of breast cancer cell lines in a dose-dependent manner. In conclusion, the GST/glutathione detoxification system seems to play a role in hepsulfam resistance.[25]

In order to validate the *in vitro* data, hepsulfam and busulfan were studied simultaneously at equitoxic dose levels in tumor-bearing nude mice. For both compounds, the approximate LD_{10} dose was determined as 150 mg/kg given i.p. as a single bolus injection on day 1 of the experiments. At this dose level, application of hepsulfam or busulfan to tumor-bearing animals resulted in a mortality rate of 12% and a median relative drug-induced body weight loss of less than 10% within 14 days after drug application. Toxicity of hepsulfam was sub-acute with deaths between day 7 and 12, whereas busulfan demonstrated acute toxicity with lethality at days 1 and 2 after drug application. Control group animals did not show indications of toxic effects.

All tumors selected for *in vivo* studies had demonstrated *in vitro* sensitivity to both compounds at a concentration of 10 μg/ml. However, tumor regressions in nude mice were observed only for the large cell lung cancer LXFL 529 and the gastric cancer GXF 209, with a higher degree of sensitivity to hepsulfam. These data indicate that the high concentrations required for the broad activity of busulfan *in vitro* cannot be achieved *in vivo*. The lower IC_{50} and higher cytotoxicity of hepsulfam at low concentrations in the clonogenic assay might result in an effective use of the compound *in vivo*. The selectively higher activity of hepsulfam in non-small cell lung cancer *in vitro* was confirmed *in vivo* in nude mice bearing the large cell lung cancer model LXFL 529. The differences in activity were less pronounced in the gastric cancer xenograft GXF 209. In the chemoresistant malignant melanoma model MEXF 276, both drugs were equally ineffective in the *in vivo* setting. In the clonogenic assay, this melanoma line was shown to be sensitive to both compounds (Tables 1 and 2). This discrepancy between *in vitro* sensitivity and *in vivo* resistance might be explained by differences in the *in vitro* and *in vivo* pharmacology of a given drug as well as by various theoretical aspects limiting the predictive value of the clonogenic assay, especially with regard to the estimation of tumor response.[14] These data indicate the importance of *in vivo* studies for the final assessment of the antineoplastic potential of experimental anticancer agents. Overall, the superior activity of hepsulfam as compared with busulfan in the clonogenic assay was confirmed in tumor-bearing animals *in vivo*.

Conclusion

The broad spectrum of antitumor activity of hepsulfam suggests a possible role for this compound, not only in treatment of CML, but also in therapy of solid human tumor types. Non-small cell carcinomas of the lung might be target tumors for clinical phase II studies. However, the increased bone marrow toxicity of hepsulfam as compared with busulfan might be critical for clinical application of this compound. So far, several clinical phase I and pharmacokinetic studies have been published,[26–28] mainly exploring repeated 30 min i.v. infusions of hepsulfam q21–35 days. In all trials, dose-limiting toxicity was delayed myelosuppression with leukopenia and thrombocytopenia. Further studies are necessary in order to determine whether there is a therapeutic window for the clinical use of hepsulfam in cancer chemotherapy.

Acknowledgments

The authors wish to thank Jackie Plowman, PhD, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland, USA, for fruitful discussions and support of this work. We thank our co-workers, Eva Wallbrecher, Verena Haberstroh and Manuela Groß for technical assistance.

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(Received 30 July 1992; accepted 12 August 1992)