Mechanisms of Toxicity of Hepsulfam in Human Tumor Cell Lines

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ABSTRACT

1,7-Heptanediol disulfamate (hepsulfam, NSC 329680) is a new anticancer agent which is currently undergoing phase I clinical trials. The mechanism of action of this compound is not clear at this time. We have recently shown that hepsulfam was more toxic to L1210 leukemia cells than was busulfan. Consistent with the difference in toxicity, we found that hepsulfam induced DNA interstrand cross-links in L1210 mouse leukemia cells, whereas busulfan did not. In the present study, we have found that hepsulfam was more cytotoxic to two human leukemia cell lines (HL-60 and K562) and to two human colon carcinoma cell lines (BE and HT-29) than was busulfan. As in L1210 cells, hepsulfam induced a higher level of DNA interstrand cross-links than busulfan. Both compounds induced DNA-protein cross-links. Hepsulfam was also more cytotoxic to the human leukemia cell lines when the concentrations were reduced 10-fold and the duration of drug exposure was increased to 12 h. This more accurately reflects the drug exposures that human leukemia cells may encounter in vivo. Under these 12-h drug exposures, hepsulfam was still able to form DNA interstrand and DNA-protein cross-links, whereas busulfan was only able to form DNA-protein cross-links. These results show that busulfan and hepsulfam react with DNA differently and that hepsulfam is a more potent cytotoxic agent.

INTRODUCTION

1,7-Heptanediol disulfamate (hepsulfam, NSC 329680) is a new anticancer agent which is currently undergoing phase I clinical trials. Hepsulfam, which has close structural similarity to busulfan [a comparison of structures has previously been published (1)], was found to have excellent antileukemic activity against both the P388 and L1210 mouse leukaemias in vivo as well as good activity against some solid tumors including a human mammary xenograft (2). In contrast, busulfan was found to have minimal activity when tested against these in vivo tumor models (2).

Recently, we found that hepsulfam was more cytotoxic to the L1210 leukemia cell line in vitro than was busulfan (1). In agreement with the cytotoxicity data was the finding that hepsulfam induced DNA interstrand cross-links in L1210 leukemia cells, whereas busulfan did not (1). The induction of DNA interstrand cross-links by hepsulfam occurred in a delayed manner and required 12 h to reach peak levels. It is not clear whether these DNA interstrand cross-links are the cause of cytotoxicity because both agents were capable of forming DNA-protein cross-links, also in a delayed manner. At equimolar concentrations, hepsulfam formed a greater quantity of DNA-protein cross-links than did busulfan. At equitoxic concentrations, however, busulfan appeared to form a greater number of DNA-protein cross-links. The importance of either DNA interstrand cross-linking or DNA-protein cross-linking to the cytotoxicity of hepsulfam remains unresolved at this time.

In this study we have compared the in vitro cytotoxicity and DNA reactivity of both hepsulfam and busulfan to two human leukemia cell lines (K562 and HL-60) as well as to two human colon carcinoma cell lines (BE and HT-29). The following questions were addressed: Will hepsulfam be more cytotoxic to human leukemia cell lines when compared to busulfan? If so, will this preferential sensitivity of leukemia cell lines to hepsulfam extend to cell lines representative of solid tumors? Finally, which DNA lesion correlates best with this profile of cytotoxicity?

MATERIALS AND METHODS

Cell Culture. HL-60 human leukemia cells were grown in culture in RPMI 1640 medium that was supplemented with 20% heat-inactivated (56°C, 30 min) fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, and 0.1 mg/ml kanamycin. K562 human leukemia cells were grown in RPMI 1640 medium that was supplemented with 10% bovine calf serum (HyClone). Stock cultures were maintained in exponential phase at a density of 0.3-1.8 x 10^6 cells/ml. Colony-forming ability was determined by the soft-agar technique described by Chu and Fisher (3). Briefly, following drug treatment at 37°C in RPMI 1640, cells were seeded into polystyrene culture tubes (Falcon Plastics, Oxnard, CA) that contained the appropriate RPMI medium and 0.2% agar (Difco Laboratories, Detroit, MI). The efficiency of untreated control cells in forming colonies was 70% for K562 cells and 1% for HL-60 cells. BE and HT-29 colon carcinoma cell lines were grown in culture following the conditions previously used (4). Inhibition of colony-forming assays was also done as previously described (4).

The DNA of K562 or HL-60 cells used in alkaline elution assays was radioactively labeled by growing 2.5 x 10^6 cells for 24 h in [3H]thymidine (0.1μCi/ml; specific activity, 32 Ci/mmol). [3H]thymidine stock solutions contained 10 μCi/ml in 10^-4 M thymidine. Internal standard cells were labeled by growing in the presence of [2-14C] thymidine (0.02 μCi/ml; specific activity, 52.2 mCi/mmol).

Drug Treatment. Hepsulfam (NSC 329680) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, and busulfan was purchased from Sigma Chemical Co. (St. Louis, MO). Each drug was dissolved in sterile dimethyl sulfoxide immediately before treatment of cell cultures. HL-60 or K562 leukemia cells (1 x 10^6/ml) were treated with various concentrations of drug for 2, 3, 6, 9, or 12 h at 37°C. The concentration of dimethyl sulfoxide in either control or treated cells was never greater than 2% (v/v). Following drug exposure, the cells were washed by centrifugation in RPMI 1640 medium and resuspended in fresh medium. Following this wash, cells were either assayed immediately for DNA damage by alkaline elution or incubated at 37°C for various periods before assay. BE and HT-29 human colon carcinoma cells were processed for alkaline elution analysis or cytotoxicity assays as previously described (4). Drug treatments in the cytotoxicity assays were for 2 h.

Alkaline Elution Experiments. Alkaline elution experiments were carried out as previously described (1, 4). For analysis of interstrand cross-links, cells were lysed on 0.8-μm pore size polycarbonate filters with 2% sodium dodecyl sulfate, 0.025 M EDTA, and 0.1 M glycine (pH 10.0), which was allowed to flow through the filter by gravity. Following lysis, 2 ml of 2% sodium dodecyl sulfate, 0.02 M EDTA, and 0.1 M glycine (pH 10.0) containing 0.5 mg proteinase K/ml were added to a reservoir over the filter and pumped through the filter for approximately 1 h at 2 ml/h. DNA was eluted from filters by pumping 0.02 M EDTA solution adjusted to pH 12.2 with tetraethølammonium.
hydroxide, which contained 0.1% sodium dodecyl sulfate.

For assay of DNA-protein cross-links, cells were lysed on 2-μm pore size polystyrene filters with lysis solution as described above. Filters were then washed with 5 ml of 0.02 mM EDTA (pH 10.0) prior to elution with tetraethylammonium hydroxide and EDTA (pH 12.2). In these assays, no proteinase K digestions were performed. In the DNA interstrand cross-link experiments, both drug-treated and untreated standard and drug-treated cells received 3 Gy γ-irradiation from a 137Cs source. The results were then quantified as previously described (5, 6).

RESULTS

The in vitro cytotoxicity of hepsulfam and busulfan to HL-60 and K562 human leukemia cells as well as to BE and HT-29 human colon carcinoma cells is shown in Fig. 1. When all cell lines were exposed to drug for 2 h at 37°C, hepsulfam was more cytotoxic than busulfan. The concentration of busulfan required to produce a 1 log cell kill in the K562, HL-60, and BE cell lines, and a 70% cell kill in the HT-29 cell line, was generally 2- to 3-fold greater than that of hepsulfam.

In an attempt to understand these differences in toxicity we have examined the ability of hepsulfam and busulfan to induce DNA interstrand cross-links in all four cell lines by the technique of alkaline elution. Previous results in L1210 leukemia cells showed that peak levels of DNA interstrand cross-links were induced by busulfan 12 h after a 2-h drug treatment (2).

In this study, therefore, we initially measured DNA interstrand cross-link formation at this same time point. Table 1 shows that hepsulfam induces DNA interstrand cross-links in a dose-dependent manner in all four cell lines. In contrast, busulfan failed to induce an appreciable level of DNA interstrand cross-links in any of the four cell lines tested. These results are consistent with the data obtained in L1210 leukemia cells; i.e., hepsulfam induced DNA interstrand cross-links, whereas the levels of lesions formed by busulfan were minimal.

Table 1 also shows the levels of DNA-protein cross-links formed in the four cell lines after a 2-h exposure to either hepsulfam or busulfan at 37°C followed by a 12-h drug-free incubation. In this instance, both hepsulfam and busulfan induced DNA-protein cross-links. At equimolar concentrations hepsulfam induced a 2- to 4-fold greater quantity of DNA-protein cross-links in the human leukemic HL-60 and K562 cell lines than did busulfan. For instance in the K562 leukemia cell line 500 μM hepsulfam and 500 μM busulfan gave DNA-protein cross-link indices of 1.6 and 0.56 Gy equivalents, respectively. At equitoxic concentrations, however, hepsulfam and busulfan gave similar DNA-protein cross-link indices of 0.72 and 0.56 Gy equivalents, respectively. At equimolar concentrations, hepsulfam induced a 2- to 3-fold higher level of DNA-protein cross-links in the BE and HT-29 cell lines than did busulfan.

In the experiments described above we measured cytotoxicity and DNA reactivity using high concentrations of drug and short durations of drug exposure. Because this situation certainly overestimates the pharmacological parameters that would be obtained in vivo we also exposed the K562 and HL-60 leukemia cells to lower concentrations of drug for longer durations of drug exposure, similar to those that would be encountered in vivo (7). Fig. 2 shows the cytotoxicity of hepsulfam and busulfan to the K562 cell line when the times of drug exposure were varied from 3–12 h. Hepsulfam was more cytotoxic than busulfan to this cell line at all times of drug exposure. As was seen in Fig. 1, the K562 cell line displays an initial shoulder region in the survival curve.

Surprisingly, at these lower concentrations, the sensitivity of the HL-60 cell line to hepsulfam and busulfan was similar at most durations of drug exposure (Fig. 3). In agreement with the data obtained in Fig. 1, no shoulder region was observed with hepsulfam. The sensitivity of the HL-60 cell line to busulfan, however, appears to be biphasic; i.e., as the concentration of busulfan is increased beyond 50 μM, no increase in toxicity is observed. At these higher concentrations of drug HL-60 cells are more sensitive to hepsulfam than busulfan. This suggests that the HL-60 cell line may consist of two subpopulations of cells: one that is sensitive to busulfan and one that is resistant. In contrast, no biphasic response was observed with hepsulfam, suggesting that, if two subpopulations of cells exist, they are equally sensitive to hepsulfam. In addition, at these longer durations of drug exposure, HL-60 cells appear to be more sensitive than K562 cells to both hepsulfam and busulfan.

Using a 12-h drug exposure, we have determined the levels of DNA interstrand and DNA-protein cross-links that are induced in the HL-60 and K562 leukemia cell lines by either hepsulfam or busulfan. Hepsulfam induced DNA interstrand cross-links in both cell lines, in a dose-dependent manner (Table 2). Busulfan, however, failed to induce any appreciable DNA interstrand cross-links under these conditions of drug treatment. Both hepsulfam and busulfan were able to form DNA-protein cross-links during the 12-h drug exposure. Indeed, the levels of DNA-protein cross-linking induced by hepsulfam or
Table 1 DNA interstrand and DNA-protein cross-link indices obtained in four human cell lines after exposure to hepsulfam or busulfan

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Hepsulfam</th>
<th>Busulfan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125 μM</td>
<td>250 μM</td>
</tr>
<tr>
<td>HL-60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISC*</td>
<td>0.18 ± 0.05</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>DPC</td>
<td>0.32 ± 0.13</td>
<td>0.72 ± 0.11</td>
</tr>
<tr>
<td>K562</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISC</td>
<td>0.18 ± 0.07</td>
<td>0.36 ± 0.12</td>
</tr>
<tr>
<td>DPC</td>
<td>0.38 ± 0.22</td>
<td>0.72 ± 0.22</td>
</tr>
<tr>
<td>BE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISC</td>
<td>0.08 ± 0.01</td>
<td>0.17 ± 0.06</td>
</tr>
<tr>
<td>DPC</td>
<td>0.53 ± 0.36</td>
<td>0.68 ± 0.17</td>
</tr>
<tr>
<td>HT-29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISC</td>
<td>0.06 ± 0.02</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>DPC</td>
<td>0.32 ± 0.12</td>
<td>0.62 ± 0.24</td>
</tr>
</tbody>
</table>

* ISC, DNA interstrand cross-link; DPC, DNA-protein cross-link.

* Mean ± SD of at least 3 independent experiments.

**DISCUSSION**

We have previously shown that hepsulfam was a more potent cytotoxic agent to mouse L1210 leukemia cells than was busulfan (1). Consistent with this increased sensitivity of L1210 cells was the finding that hepsulfam was able to induce DNA interstrand cross-links in these cells, whereas busulfan was not. Both compounds were able to form DNA-protein cross-links, although busulfan formed a greater quantity of these lesions when compared to hepsulfam at equitoxic concentrations (1). In this study, we have found that hepsulfam was more cytotoxic to four human cell lines than busulfan. Again, hepsulfam was able to induce DNA interstrand cross-links in these cells, with minimal levels being induced by busulfan. However, the levels of DNA-protein cross-links that both agents formed were similar when compared at equitoxic concentrations. It would thus appear that the preferential sensitivity of these cell lines to hepsulfam is more closely associated with DNA interstrand cross-linking than with DNA-protein cross-linking. The mechanism of action of busulfan in these cell lines is unclear at this time. The possibility always exists that each agent acts by a different mechanism: hepsulfam via DNA interstrand cross-linking and busulfan via DNA-protein cross-linking. We also cannot yet exclude the involvement of other DNA effects such as DNA intrastrand cross-links.

There are a number of differences between hepsulfam and busulfan, both structurally and in terms of their chemical reactivity, which could explain the observed differences in DNA reactivity (for structures see Ref. 1). First, there are distinct differences in the hydrolytic pattern of decomposition observed between busulfan and hepsulfam. Busulfan undergoes nucleophilic attack by water to give 4-methane-sulfonyloxybutanol and this reaction intermediate undergoes an intramolecular displacement to render the final decomposition products, tetrahydrofuran and methanesulfonic acid (8). In contrast, no evidence of such an intramolecular mechanism was observed with hepsulfam. This compound forms the intermediate 1,7-heptane-monosulfamic acid ester prior to complete hydrolysis to 1,7-heptanediol (9). Second, busulfan is capable, in the presence of glutathione transferases, of reacting with glutathione to form a sulfonium ion metabolite (10). In contrast, hepsulfam is unable to react with glutathione in either the presence or absence of glutathione transferases (7).

Structurally, hepsulfam and busulfan differ both in terms of the length of their carbon chain between their electrophilic centers and also in the strength of the leaving group. These differences may be extremely important in determining the types and quantities of DNA lesions that each agent is capable of forming and, ultimately, their therapeutic effect. Previous
controlled for a number of years by busulfan, but ultimately the chronic and acute (14). The chronic phase of CML can be cally alter the structural flexibility of DNA and in doing so may contrast to the work of Bedford and Fox (11) who reported that this occurs only 6–12 h after drug treatment. The mechanism by which a DNA-protein cross-link can display antitumor activity is not readily apparent. It has been documented, however, that UV-induced DNA-protein cross-links may be related to cytotoxicity (13). It could be envisioned that covalent DNA-protein linkages (in essence, bulky DNA adducts) will drastically alter the structural flexibility of DNA and in doing so may have a profound effect on cell replication, gene transcription, and possibly DNA repair.

For a number of years busulfan was the drug of choice to treat CML³ (14). CML has at least two distinct clinical phases, chronic and acute (14). The chronic phase of CML can be controlled for a number of years by busulfan, but ultimately the majority of patients undergo blastic transformation, enter the acute phase of the disease, and no longer respond to the antitumor effects of this drug. It would be of interest, therefore, to compare the sensitivities of acute and chronic CML cells to hepsulfam and busulfan and to examine resultant damage to DNA in these cells. However, there are no cell lines available which are representative of this chronic phase of the disease. The HL-60 cell line is a promyeloid leukemia (15). The K562 cell line was established from a patient in the acute phase of CML and can be considered representative of it (16). Furthermore, this patient's prior chemotherapy included busulfan. The data obtained in this study tend to support the following conclusions. Hepsulfam, which is more toxic to the K562 cell lines than busulfan, may be a useful drug with which to treat CML patients both in the chronic and acute phases. The increased toxicity of hepsulfam to both leukemic and colon carcinoma cells also suggests that this agent may have a broader spectrum of activity than just leukemias. Indeed, the antitumor activity observed with hepsulfam in the National Cancer Institute antitumor screens, including solid tumors, would support this conclusion (2). Initially, however, hepsulfam would appear to be an ideal candidate for clinical trial in CML patients. To this end we report in an accompanying paper the cytoxicity and DNA reactivity of hepsulfam and busulfan to peripheral blood cells and bone marrow cells from normal individuals and CML patients.

## REFERENCES


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* The abbreviation used is: CML, chronic myelogenous leukemia.

### Table 2 DNA interstrand and DNA-protein cross-link indices obtained in HL-60 or K562 leukemia cells after exposure to hepsulfam or busulfan

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Hepsulfam</th>
<th>Busulfan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 µM</td>
<td>50 µM</td>
</tr>
<tr>
<td>HL-60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISC*</td>
<td>0.29 ± 0.06</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td>DPC</td>
<td>0.21 ± 0.02</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td>K562</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISC</td>
<td>0.29 ± 0.03</td>
<td>0.59 ± 0.17</td>
</tr>
<tr>
<td>DPC</td>
<td>0.15 ± 0.15</td>
<td>0.41 ± 0.01</td>
</tr>
</tbody>
</table>

* ISC, DNA interstrand cross-link; DPC, DNA-protein cross-link.

* Mean ± SD of at least 3 independent experiments.
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