In Vitro Studies on the Mechanism of Action of Hepsulfam in Chronic Myelogenous Leukemia Patients

Jeffrey R. Hincks, Arun Adlakha, Cheryl A. Cook, Candace S. Johnson, Philip Furmanski, and Neil W. Gibson

ABSTRACT

In the present study we have characterized the cytotoxicity and DNA damage induced by hepsulfam and busulfan in cells isolated from both chronic myelogenous leukemia (CML) patients and normal donors. Hepsulfam inhibited colony-forming units-granulocyte, macrophage to a greater extent than busulfan in peripheral blood cells (PBCs) isolated from CML patients. Normal PBCs were equally sensitive to both agents and were more sensitive than the cells isolated from CML patients. Hepsulfam induced DNA interstrand cross-links in PBCs and bone marrow from both CML and normal volunteers, whereas busulfan produced few or no DNA interstrand cross-links. In addition, hepsulfam induced higher levels of DNA interstrand cross-linking than busulfan in three samples isolated from CML patients in blast crisis. Busulfan did however cause a small number of DNA strand breaks to be formed in human cells. Both agents produced similar levels of DNA-protein cross-links in PBCs from CML patients. These results suggest that the mechanism of DNA reactivity of hepsulfam and busulfan differ and that hepsulfam may prove useful in the treatment of CML.

INTRODUCTION

For the last 20 to 30 years the major use of the alkylating agent busulfan has been to treat chronic myelogenous leukemia (1). Clinically, CML can be divided into two phases: chronic and acute (1). 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 this disease, and no longer respond to the antitumor effects of this drug. There is, therefore, an urgent need to develop or identify new compounds, with an improved therapeutic index, to treat patients with CML and in particular to find agents which may have activity in the acute phase of this disease.

We have previously reported that hepsulfam (1,7-heptanediol disulfamate, NSC 329680) was more toxic to two human leukemia cell lines (HL-60 and K562) and one mouse leukemia cell line (L1210) than busulfan (2, 3), a drug which has been used extensively over the last 30 years to treat CML. We also found that hepsulfam induced DNA interstrand cross-links in these leukemic cells to a much higher degree than busulfan. In contrast to this difference in DNA interstrand cross-linking both agents were found to induce DNA-protein cross-linking.

These results highlighted the fact that the mechanisms by which hepsulfam and busulfan react with DNA differ and suggested that hepsulfam might be a useful agent for the treatment of leukemia.

Since cell lines are not always reflective of tumor cells in vivo and since there are no normal passaged counterparts to CML cell lines, the present studies were undertaken to examine cytotoxicity and DNA damage by hepsulfam and busulfan to freshly isolated CML and normal hematopoietic cells. We found that hepsulfam was more toxic to cells obtained from CML patients when compared with busulfan. In contrast, cells obtained from normal volunteers were equally sensitive to both agents and showed a greater degree of sensitivity than cells from CML patients. Hepsulfam was found to induce a higher level of DNA interstrand cross-links in PBCs isolated from both CML patients and normal volunteers when compared to busulfan.

MATERIALS AND METHODS

Cell Specimens and Preparation. PBCs were isolated from eight normal healthy volunteers (A–H) and from 10 CML patients (1–10). BM samples were collected from one healthy normal subject and two CML patients. Informed consent was obtained from all donors.

All of the CML patients were Philadelphia chromosome positive and patients 1–6 and 9 were in the chronic phase with WBC counts exceeding 26,000/μl. Patients 7, 8, and 10 were in blast crisis at the time of sample collection with WBC counts exceeding 200,000/μl. Patient 7 had 86% blast cells, while patient 8 had 11% blast cells with 60% neutrophils. Patients 5, 6, and 9 had received no previous treatment, while the other CML patients had received hydroxyurea and alpha interferon.

Peripheral blood cells were collected by centrifuging heparinized blood or bone marrow over Ficoll-Hypaque (specific gravity, 1.077 g/ml; Pharmacia, Piscataway, NJ). The isolated cells were washed two times and resuspended in RPMI 1640 with 10% fetal bovine serum (HyClone, Logan, UT).

Drug Exposures. Hepsulfam (NSC 329680) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute; busulfan was purchased from Sigma Chemical Co. (St. Louis, MO). Both drugs were dissolved in sterile dimethyl sulfoxide just prior to treatment of the cells. PBC and BM cells (2 x 10⁶ cells/ml for alkaline elution assays and 0.2 x 10⁶ cells/ml for CFU-GM assays) were exposed to the indicated concentrations of drug for 2 h except where noted. The concentration of dimethyl sulfoxide in either control or treated cells was never greater than 0.1% (v/v). Cells used in the CFU-GM assay were plated immediately, while cells used in the alkaline elution experiments were incubated for additional periods in drug-free medium.

CFU-GM Assays. After the drug treatments, the cells were washed and resuspended in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, and the CFU-GM assay was carried out as described previously (4). A total of 10⁵ CML cells or 2 x 10⁵ normal cells were plated in triplicate, in medium containing 1% methylenecellulose (Fisher Scientific, Fair Lawn, NJ) and 10% MO (human T cell leukemia) (American Type Culture Collection CRL 8066) conditioned medium as a source of colony-stimulating factor (5). The cells were cultured for 2 weeks at 37°C in a humidified atmosphere of 5% CO₂
and 95% air. The number of colonies or clusters (>10 cells) were counted using an inverted microscope. The average number of colonies (clusters) obtained from CML PBCs ranged from 130-420/10^6 cells plated, whereas the range obtained with normal PBCs was 8-15 colonies/10^6 cells plated. In these and previous experiments there was a linear relationship between the number of cells plated and the number of colonies formed.

Alkaline Elution Experiments. Gravity-flow alkaline elution experiments were performed as previously described (6, 7). The DNA content in the fractions collected during alkaline elution was quantitated by a fluorometric technique, as previously described (8, 9). Results were then quantified as previously described (6–9).

RESULTS

PBCs from CML patients and normal volunteers were exposed to hepsulfam or busulfan for 2 h and plated in the CFU-GM assay. Typical results from individual donors are shown in Table 1. The data are presented as a surviving fraction relative to control for two concentrations of drug. Hepsulfam decreased the surviving fraction of CFU-GM to a greater extent than busulfan in the majority of CML samples. In general, there was a 4- to 5-fold variation in the sensitivity of PBCs from CML patients to hepsulfam with less variation being observed for busulfan. In contrast there was little difference in the sensitivity of PBCs from normal volunteers to either agent. Inhibition of colony formation was also tested with other drug concentrations; the data shown in Fig. 1 are representative of all CML patients and normal volunteers. In general, a rapid decrease in the surviving fractions of CFU-GM from CML patients was seen at the lower concentrations of both drugs which slowed at the higher concentrations, producing a biphasic response. At these higher drug concentrations PBCs from CML patients were more sensitive to hepsulfam than busulfan. The normal cells were equally sensitive to hepsulfam and busulfan, producing a rapid reduction in surviving CFU-GM (monophasic curve) with few if any CFU-GM detected at concentrations ≥125 μM (Fig. 1). The normal cells tended to be more sensitive to both hepsulfam and busulfan treatment than the CML cells (Fig. 1; Table 1).

Alkaline elution experiments were performed on cells isolated from one patient (CML patient 6) and treated with drug for 2 h followed by a 0-, 6-, or 12-h postincubation in drug-free medium. Busulfan produced a time- and concentration-dependent induction of DNA interstrand cross-links with the maximum effect occurring at 500 μM drug and a 12-h postincubation (1.616 Gy equivalents; Fig. 2A). Busulfan did not induce an appreciable amount of DNA interstrand cross-linking in any of the treatment exposure conditions. Both hepsulfam and busulfan induced DNA-protein cross-links after a 6- or 12-h postincubation (Fig. 2B).

Based on the results from this time-course experiment, the high cell numbers required for the assays, and the limited availability of cells, the major portion of the subsequent experiments focused on the detection of drug-induced DNA interstrand cross-links after a 2-h drug treatment and a 12-h postincubation. Fig. 3 shows the DNA interstrand cross-link indices obtained in the same patient samples as those shown in the representative toxicity experiment (Fig. 1). Hepsulfam induced DNA interstrand cross-links in CML cells and also in normal cells, with a higher level detected in the normal samples (Fig. 3). Negligible or low levels of DNA interstrand cross-links were detected in the CML or normal cells after busulfan exposure (Fig. 3).

Fig. 4 shows DNA interstrand cross-link indices determined after PBCs from CML patients (left) or normal subjects (right) were exposed to hepsulfam or busulfan. These data can be directly compared to the cytotoxicity data presented in Table 1 for the same individuals. The results shown in Fig. 4 illustrate that hepsulfam induced DNA interstrand cross-links in all CML patients and normal subjects, while busulfan induced minimal levels or no interstrand cross-linking. Interstrand cross-linking due to hepsulfam exposure appears to be higher in the normal cells than the leukemic cells.

The results of additional DNA interstrand cross-linking experiments are presented in Table 2. These results can be summarized as follows. Hepsulfam produced a higher level of DNA interstrand cross-linking than busulfan in the two CML patients who were in blast crisis. The cross-link indices of 1.250 (CML patient 7) and 2.830 (CML patient 8) were detected in PBCs from patients in blast crisis after a 2-h exposure to 500 μM hepsulfam, whereas 1000 μM busulfan produced interstrand cross-link indices of 0.347 (CML patient 7) and 0.090 (CML patient 8). Cells isolated from BM showed a similar trend as seen for PBCs, i.e., hepsulfam induced DNA interstrand cross-links in both CML and normal samples and busulfan induced little or no DNA interstrand cross-linking (Table 2). In addition, there was more drug-induced DNA interstrand cross-linking in PBC than BM cells in the one patient from whom both cell sources were tested (CML patient 5, Table 2). In these experiments we observed that busulfan induced negative DNA interstrand cross-link indices. This may indicate that in these cells busulfan induced DNA strand breaks. DNA strand breaks were only detected in cells exposed to 1000 μM busulfan after a 6- or 12-h postincubation (data not shown).

As in our previous study we also analyzed the cytotoxicity and DNA interstrand cross-linking induced by longer times of drug exposure and lower concentrations of hepsulfam and busulfan (2). These conditions more accurately reflect those that may occur when patients are treated with either agent in vivo (2). A similar trend was observed (Fig. 5) to that seen with

<table>
<thead>
<tr>
<th>Patient description</th>
<th>Exposure time (h)</th>
<th>Hepsulfam</th>
<th>Busulfan</th>
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<tr>
<td></td>
<td>125 μM</td>
<td>500 μM</td>
<td>125 μM</td>
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<tr>
<td>CML 2</td>
<td>2</td>
<td>0.337 ± 0.024&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.091 ± 0.100</td>
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<tr>
<td>CML 3</td>
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<td>0.569 ± 0.050</td>
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<td>CML 4</td>
<td>2</td>
<td>0.128 ± 0.001</td>
<td>0.109 ± 0.022</td>
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<tr>
<td>CML 5</td>
<td>2</td>
<td>0.213 ± 0.025</td>
<td>0.087 ± 0.015</td>
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<tr>
<td>CML 6</td>
<td>2</td>
<td>0.426 ± 0.066</td>
<td>0.285 ± 0.014</td>
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<td>Normal A</td>
<td>2</td>
<td>&lt;0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal B</td>
<td>2</td>
<td>0.031 ± 0.040</td>
<td>&lt;0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal C</td>
<td>2</td>
<td>0.161 ± 0.033</td>
<td>0.023 ± 0.033</td>
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<sup>a</sup>PBCs after isolation were exposed to the appropriate drug for either 2 or 4 h.
<sup>b</sup>Mean ± SD, n = 3.
<sup>c</sup>No colonies formed. The value shown is the surviving fraction expected if 1 colony had formed.

Table 1 The surviving fraction of CFU-GM from CML and normal PBCs exposed to hepsulfam or busulfan
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DNA interstrand cross-links hepsulfam induced these lesions in both CML patients and normal volunteers, while busulfan did not (Fig. 6).

DISCUSSION

We have previously shown that hepsulfam induced greater cytotoxicity than busulfan in L1210 murine leukemia cells (3), K562 and HL-60 human leukemia cells (2), and BE and HT-29 human colon carcinoma cells (2). We now report that hepsulfam is also more toxic to freshly isolated PBCs from CML patients than busulfan, as measured by a CFU-GM assay. The survival curves detected in this assay were biphasic, suggesting that some clonogenic cells within the PBCs from CML patients are more sensitive than others. The slope of the initial section of the survival curve showed equal sensitivity to both drugs with the resistant component being more sensitive to hepsulfam than busulfan. We have seen a similar biphasic response when either HL-60 or K562 cells were exposed to busulfan (2). This result may explain the fact that busulfan can only control the progress of CML for a limited time. After busulfan exposure there is still at least 1% of the cells which were not sensitive. It is these resistant cells, which are presumably still able to proliferate, that may be responsible for the progression of CML from the chronic to the acute phase of the disease. The results presented in this manuscript may hint at the fact that hepsulfam may delay the progression of CML from the chronic to the acute phase of the disease to a greater degree than that observed with busulfan and, in addition, suggest that hepsulfam may be active in the acute phase of CML.

In contrast, the survival curves detected with PBCs from normal volunteers was monophasic, suggesting a single class of clonogenic cell. In addition, PBCs from normal volunteers were more sensitive to hepsulfam or busulfan than CFU-GM from CML PBCs. The increased sensitivity of PBCs from normal volunteers when compared to the sensitivity of PBCs from CML patients has also been reported with a wide range of cytotoxic chemotherapeutic agents (10, 11). The explanation for such results appears to lie in the fact that the clonogenic cells contained within PBCs of normal volunteers are not the same as those that exist in PBCs from CML patients (12, 13).

In an attempt to explain these differences in the sensitivity of PBCs we have analyzed the DNA damage induced by hepsulfam and busulfan using the technique of alkaline elution. The time-course experiments illustrate that hepsulfam induced the short times of drug exposure and high concentrations of drug; i.e., hepsulfam was more toxic to PBCs from CML patients than busulfan as measured by the CFU-GM assay, whereas PBCs from normal volunteers showed no preferential sensitivity (Fig. 5, A and D). Fig. 5 also shows that in one experiment busulfan at concentrations up to 10 \( \mu \text{M} \) was 3- to 5-fold more cytotoxic than hepsulfam, but a biphasic response for busulfan meant that at higher doses hepsulfam was more cytotoxic (Fig. 5B). As was seen with our earlier experiments, PBCs from normal volunteers were more sensitive to the toxicity of both agents than were the PBCs from CML patients.

When cells were exposed to drug for 12 h and then assayed for...
surviving fraction from 3 plates; ears, ± SD.

(•) for 6 h (A. CML 5: D, normal volunteer E), 12 h (fi, CML 5; E, normal volunteer F), and 18 h (C, CML 5; F, normal volunteer H). Points, means of three normal volunteers (D-F) after exposure to hepsulfam (O) or busulfan (O). In one experiment busulfan induced only DNA-protein cross-links compared with our previous studies which were carried out in various conditions.

These results are in agreement with our previous studies is the fact that hepsulfam is more cytotoxic than busulfan, particularly to leukemic cells, and that the increase in toxicity appears to be associated with an increase in DNA interstrand cross-linking. An exception to this trend is seen with the results from PBCs obtained from normal volunteers. In these cells there is no preferential sensitivity to hepsulfam when compared with busulfan, yet hepsulfam does induce DNA interstrand cross-links and busulfan does not.

In certain experiments throughout this study we observed that busulfan induced negative DNA interstrand cross-links. This may indicate that in these cells busulfan actually induced DNA strand breaks and that these breaks masked the detection of DNA interstrand cross-links. In one experiment busulfan induced DNA strand breaks after a 6- or 12-h postincubation, while no DNA strand breaks were detected in cells exposed to hepsulfam. The induction of DNA strand breaks, however, may be an important contributor to the cytotoxic effect observed with busulfan or may actually reflect the repair of busulfan induced damage.

It is important to consider the cellular differences which exist within our different samples in order to understand why the results must be interpreted with caution. First, as previously mentioned the CFU-GM assays only determine the toxicity to progenitor cells capable of forming colonies or clusters. CFU-GM were used because they are presumed to be the cells that give rise to both the CML cells and their normal progenitors and thus that toxicity to this compartment was most relevant to therapeutic efficacy. In contrast, the alkaline elution experiments assay for DNA damage within the total cell population. This is because it is impractical to isolate sufficient numbers of purified CFU-GM to carry out such biochemical determinations. The PBCs from normal volunteers consist mainly of lymphocytes (80–87%), granulocytes (3–10%), and monocytes (8–15%). The PBCs from CML patients (n = 10), however, consist of a large number of immature myelocytes including blasts (2–93%), promyelocytes (1–16%), myelocytes (1–9%), metamyelocytes (8–26%), neutrophils (3–72%), eosinophils (1–4%), and basophils (1–21%) with very few lymphocytes (1–10%). The proportion of each individual cell type can drastically vary in different patients. Our previous study showed that in one cell line representative of promyelocytes (HL-60) and in another representing blasts (K562) the levels of hepsulfam induced DNA interstrand cross-linking and cytotoxicity were very similar (2). Consistent with these observations were the findings that PBCs from patients in blast crisis showed similar results to PBCs obtained from patients in the chronic phase of the disease. In addition, there is a general trend within the cell populations tested that the levels of DNA interstrand cross-
linking detected are dependent upon the proportion of neutrophils present. This may indicate that the DNA interstrand cross-links are being formed to a greater extent within neutrophils in preference to other cells present within the total cell population. The significance of these observations remains unclear at this time.

In conclusion, this and the previous study have shown that the mechanisms by which hepsulfam and busulfan react with DNA differ. Hepsulfam appears to be a more potent cytotoxic agent for CML and could prove useful in the therapy. Phase I clinical trials with hepsulfam are currently being performed and the results are awaited with interest.

REFERENCES

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