Comparison of the Mechanism of Action of Busulfan with Hepsulfam, a New Antileukemic Agent, in the L1210 Cell Line1

Diane Y. Pacheco, Nancy K. Stratton, and Neil W. Gibson2

Laboratory of Pharmacology, AMC Cancer Research Center, Denver, Colorado 80214 [D. Y. P., N. K. S., N. W. G.], and School of Pharmacy, University of Colorado, Boulder, Colorado 80309 [N. W. G.]

ABSTRACT

1,7-Heptanediol disulfamate (hepsulfam, NSC 329680) is a new antileukemic agent with close structural similarity to busulfan. The mechanism of action of hepsulfam is not known and it has recently been entered into Phase I clinical trials by the National Cancer Institute. Waud et al. have recently shown that hepsulfam has good antitumor activity against mouse L1210 leukemia in vitro (Waud et al., Proc. Am. Assoc. Cancer Res., 29:333, 1988). In contrast, busulfan was inactive against this model tumor system. In the present study, we have compared the in vitro cytotoxicity of hepsulfam with that of busulfan and we also examined the ability of these compounds to induce DNA damage in the L1210 leukemia cell line. Our results show that L1210 leukemia cells were 7-fold more sensitive to hepsulfam than busulfan. Only hepsulfam produced an appreciable quantity of DNA interstrand cross-linking in L1210 cells, with the peak of cross-link formation being delayed 12 h following a 2-h drug treatment. In contrast, both compounds also produced DNA-protein cross-linking, again with the formation of peak levels being delayed 6-12 h after drug treatment. At equimolar concentrations, hepsulfam produced a greater quantity of DNA interstrand cross-links and DNA-protein cross-links than busulfan. In contrast, busulfan produced a greater quantity of DNA-protein cross-links, when compared to hepsulfam at equitoxic concentrations.

INTRODUCTION

For the last 20 to 30 years, the major use of the alkylating agent busulfan has been to treat CML1 (1). It has been observed clinically that 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 and no longer respond to the antitumor effects of this drug. There is, therefore, an urgent need to develop new antileukemic compounds, with an improved therapeutic index, to treat patients diagnosed with CML and in particular to find agents which may be active in the acute phase of this disease.

Recently, 1,7-heptanediol disulfamate (hepsulfam, NSC 329680), an alkylating agent with close structural similarity to busulfan (see Fig. 1), was shown to inhibit the growth of L1210 and P388 mouse leukemias (2). In these antitumor tests, hepsulfam showed a far greater spectrum of activity than busulfan and, as a result, has been entered into Phase I clinical trials by the NCI (2). Despite this, the mechanism of action of hepsulfam is unknown. In this study, we wished to compare the in vitro cytotoxicity of hepsulfam with that of busulfan and also to examine the ability of these compounds to induce DNA damage in the L1210 leukemia cell line. We chose this leukemia cell line because hepsulfam is active against this tumor in vivo, whereas busulfan is inactive.

MATERIALS AND METHODS

Cell Culture. Mouse L1210 leukemia cells were grown in culture in RPMI 1640 medium that was supplemented with 10% heat inactivated (56°C, 30 min) bovine calf serum (Hyclone, Logan, UT), 1 mm L-glutamine, and 0.1 mg/ml kanamycin. Stock cultures were maintained in exponential phase at a density of 0.3-1.8 x 10⁶ cells/ml. Colony-forming ability was determined by the soft-agar technique described by Chu and Fisher (3). Following a 2-h drug treatment at 37°C in RPMI 1640, cells were seeded into polystyrene culture tubes (Falcon Plastics, Oxnard, CA) that contained RPMI 1640 medium and 0.1% agar (Difco Laboratories, Detroit, MI). The efficiency of untreated control cells in forming colonies was approximately 60% in this system.

The DNA of L1210 cells used in alkaline elution assays was radioactively labeled by growing 2.5 x 10⁶ cells for 24 h in [methyl-²¹H]-thymidine (0.1 µCi/ml; specific activity 32 Ci/mmol). [²¹H]Thymidine stock solutions contained 10 µCi/ml in 10⁻² M thymidine. Internal standard cells were labeled by growing in the presence of [²¹H]-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, NCI, and busulfan was purchased from Sigma (St. Louis, MO). Each drug was dissolved in sterile dimethyl sulfoxide immediately before treatment of cell cultures. Cells (1 x 10⁶/ml) were treated with various concentrations of drug for 2 h at 37°C. The concentration of dimethyl sulfoxide in either control or treated cells was never greater than 3% (v/v). Following drug exposure, the cells were washed by centrifugation in RPMI 1640 and were 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 of time before assay.

Alkaline Elution Experiments. Alkaline elution experiments were carried out as previously described (4, 5). For analysis of interstrand cross-links and DNA strand breaks, cells were lysed on 0.8-µm pore size polycarbonate filters with 2% sodium dodecyl sulfate, 0.025 M EDTA, 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-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 0.2 M EDTA solution that was adjusted to pH 12.2 with tetraethylammonium hydroxide, which contained 0.1% sodium dodecyl sulfate.

For assay of total DNA cross-links and DNA-protein cross-links, cells were lysed on 2-µm pore size polycarbonate filters with lysis solution, as described above. Filters were then washed with 5 ml of 0.02 M EDTA, pH 10.0 prior to elution with tetraethylammonium hydroxide, EDTA, pH 12.2. In these assays, no proteinase K digestions were performed. In the strand break, DNA interstrand, and total cross-link experiments, internal standard [³¹C]thymidine L1210 cells were irradiated with 6 Gy of X-ray (G.E. Maximar 250-II) in the cold. In the DNA interstrand and DNA total cross-link assays, control and drug-treated cells were irradiated with 30 Gy of X-ray. In the DNA-protein cross-link experiments, both the internal standard and drug-treated cells were irradiated with 30 Gy of X-ray. Results were then quantified as previously described (4, 5).

RESULTS

Cell killing assays, based upon reduced colony formation, showed L1210 leukemia cells at a 1 log cell kill to be much more sensitive to hepsulfam than busulfan (Fig. 2). The dose-reduction factor for killing of L1210 leukemia cells by busulfan,

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CML, chronic myelogenous leukemia.
both drugs were compared at equitoxic concentrations (Fig. 4). Cross-links than busulfan. The total DNA cross-link index, with the DNA interstrand cross-link data, hepsulfam, at equi-
potent concentration of hepsulfam. At equitoxic concentrations, hepsulfam, which is approximately 7, i.e., a 7-fold higher concentration of hepsulfam had to be applied to LI210 leukemia cells to reduce their survival to the same degree as a given concentration of busulfan.

In an attempt to understand this difference in toxicity, we have examined the ability of both hepsulfam and busulfan to induce DNA interstrand cross-links in LI210 leukemia cells by the technique of alkaline elution. Only hepsulfam produced an appreciable quantity of DNA interstrand cross-links in LI210 cells with the peak of cross-link formation being delayed 12 h following a 2-h drug treatment (Fig. 3). There was evidence of removal of these hepsulfam induced DNA interstrand cross-links between 12 and 24 h after treatment with drug. In contrast to those results obtained with hepsulfam, busulfan failed to induce DNA interstrand cross-links in LI210 cells 12 h after a 2-h drug treatment (Fig. 3). These data are in good agreement with the preferential sensitivity of LI210 leukemia cells to hepsulfam.

Total DNA cross-links (which reflects both DNA interstrand and DNA-protein cross-links) were also measured in LI210 leukemia cells. These lesions were formed by both drugs and peaked 12 h after a 2-h drug treatment (Fig. 4). In agreement with the DNA interstrand cross-link data, hepsulfam, at equi-molar concentrations, produced a greater quantity of total DNA cross-links than busulfan. The total DNA cross-link index, however, was higher for busulfan than for hepsulfam, when both drugs were compared at equitoxic concentrations (Fig. 4). Again, the meaning of such results is unclear.

The difference between the total cross-link index and the DNA interstrand cross-link index can be taken to represent DNA-protein cross-links (compare Fig. 3 with Fig. 4). To confirm this fact, we determined the quantity of DNA-protein cross-links induced by both drugs when analyzed by the conventional alkaline elution method. We found that DNA-protein cross-links were formed (Fig. 5) and that 750 μM busulfan and 500 μM hepsulfam gave a DNA-protein cross-link index of 1.56 and 1.95 Gy equivalents, respectively. This is consistent with the observed differences between total and interstrand cross-link indices, which at the same concentrations were 1.3 and 1.1, respectively. These data tend to strengthen the assumption that the difference between total DNA cross-links and DNA interstrand cross-links closely reflects the level of DNA-protein cross-links.

**DISCUSSION**

Our in vitro cytotoxicity results are in complete agreement with those obtained by the NCI in the in vivo antitumor tests. That is that LI210 leukemia cells, which are relatively resistant to busulfan, are sensitive to hepsulfam. The clinical implications of this difference in activity will only become apparent when similar studies are performed in human leukemia cell lines. One may speculate, however, that hepsulfam has the potential to become a useful drug in the treatment of CML and possibly even the busulfan-resistant phase of CML.

At this point, it is important to discuss the impact that the minor structural differences between hepsulfam and busulfan may have upon their chemical reactivity and probably, therefore, their therapeutic effect (6–9). Busulfan, upon hydrolysis, undergoes nucleophilic attack by water to give 4-methanesulfonyloxybutanol, which subsequently undergoes an intramolecular displacement to render the final decomposition product, tetrahydrofuran and methanesulfonic acid (6). In contrast, no evidence of such an intramolecular mechanism was observed with hepsulfam (7). Instead, this compound formed the intermediate 1,7-heptanemonoylsulfamic acid ester prior to complete hydrolysis to 1,7-heptanediol (7). Furthermore, busulfan, in the presence of glutathione transferases, was found to react with glutathione to form a sulfonium ion metabolite (8). The glutathione transferases are a family of enzymes which are...
believed to be responsible for catalyzing the conjugation of glutathione to electrophilic metabolites formed from alkylating agents (10). The resultant decrease in the intracellular concentration of reactive drug spares critical targets such as DNA from levels of alkylation and cross-linking that cannot be repaired effectively (11). Hepsulfam, however, is unable to react with glutathione in either the absence or the presence of glutathione transferases (9). This latter aspect, which would explain our results, may also suggest that cells that develop resistance to busulfan via the glutathione transferase detoxification mechanism would not be cross-resistant to hepsulfam. Such a phenomenon would support the hypothesis that hepsulfam may become an extremely useful drug with which to treat CML patients who have developed resistance to busulfan.

As previously mentioned, we found that, at equimolar concentrations, hepsulfam produced a far greater quantity of DNA cross-links than busulfan. In contrast, at equitoxic concentrations, busulfan produced more cross-links in L1210 leukemia cells than hepsulfam. These results suggest to us that the lack of uptake of busulfan relative to hepsulfam is not the reason for the lack of formation of DNA interstrand cross-links. Indeed, the levels of DNA-protein cross-linking observed with busulfan relative to hepsulfam, even at equimolar concentrations, suggest that the concentration of reactive drug obtained intracellularly with busulfan may be similar to that obtained with hepsulfam.

Structural considerations also suggest that the difference in levels of DNA interstrand cross-linking may have a chemical explanation. In this regard, Tong and Ludlum (12), were able to identify a DNA-cross-linked busulfan adduct but were unable to distinguish whether this adduct was of interstrand or intrastrand origin. The structural differences between hepsulfam and busulfan, in terms of the length of their carbon chains between electrophilic centers, may be an important determinant of their abilities to form DNA interstrand or intrastrand cross-links. For instance, it has been recognized for some time that the relative position of the two alkylating centers within bifunctional alkylating agents, as well as their ability to span selected target nucleophile distances, is crucial to the type and quantity of DNA adducts that are formed (13, 14). It would be extremely interesting if, despite their close structural similarity, hepsulfam were able to form DNA interstrand cross-links but not intrastrand cross-links and the opposite were the case for busulfan (that is, it could form DNA intra- but not interstrand cross-links). Of course, both drugs may form both lesions although at different ratios. Such differences in their reactivity with DNA would be consistent with those differences already observed in their chemical reactivity with other nucleophiles and may be the reason for the observed differences in toxicity.

In contrast to the results shown here, Bedford and Fox (13) reported that busulfan failed to induce DNA-protein cross-links in a rat lymphosarcoma cell line. These authors, however, only assayed immediately to 4 h after a 1-h drug treatment. Our data show that, although no evidence of DNA-protein cross-linking was found immediately after a 2-h drug treatment, these lesions did start to form 6–12 h after this treatment. This is a very surprising result, because other bifunctional alkylating agents form DNA-protein cross-links immediately after drug treatment (15, 16). The explanation for the immediate formation of DNA-protein cross-links by bifunctional alkylating agents has been that the initial reaction of the cross-linking agent is most likely alklylation of protein amino and/or sulphydryl groups. The alkylated protein then reacts rapidly with the most nucleophile sites on DNA, usually with the displacement of small leaving groups such as the chlorine atom. In this instance, however, the initial alkylated protein adduct formed by busulfan or hepsulfam may react with DNA in a much slower manner, due to the steric hindrance caused by the bulky sulfonic acid leaving group. Also, the initial rate of reaction with the protein may be slower than for other alkylating agents. The possibility that a DNA-protein cross-link may be formed by an initial alkylation of DNA followed by a second reaction with a protein cannot be ruled out. In this scenario, an explanation similar to that described above would also explain the kinetic differences that we observe. The importance of this difference in the kinetics of the formation of DNA-protein cross-links by busulfan or hepsulfam, compared with other bifunctional alkylating agents, is not clear at this time.

In conclusion, we have shown that hepsulfam is a more cytotoxic antileukemic agent in vitro than busulfan. This is in agreement with data obtained in vivo against the same leukemia cell type (2). The difference in the ability of both compounds to produce DNA interstrand cross-links correlates well with the differential cytotoxicity that we observe. In contrast, the formation of DNA-protein cross-links would not appear to be related to the antileukemic mechanism of these compounds. The excellent activity of hepsulfam against the L1210 leukemia both in vitro and in vivo suggests that it may have some potential in treating forms of leukemia other than CML. Indeed, the broad spectrum of antitumor activity observed with hepsulfam in the NCI antitumor screens would support this contention (2). Hepsulfam, however, would initially appear to be an ideal candidate for clinical trial in leukemic patients.

**REFERENCES**

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