Hepsulfam Sensitivity in Human Breast Cancer Cell Lines: The Role of Glutathione and Glutathione S-Transferase in Resistance


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ABSTRACT

Hepsulfam (NSC 329680, 1,7-heptanediol disulfamate) is an alkylating agent that showed excellent activity against mouse and human mammary carcinoma in preclinical studies. We therefore studied the cytotoxicity of this drug in six human breast cancer cell lines (AdrMCF7, WTMCF7, Hs578T, MDA-MB-231, T47D, and MDA-MB-468). Clonogenic assays of these cell lines showed a range of sensitivity with the 90% inhibitory concentration ranging from 3.1 μM hepsulfam (MDA-MB-468) to 32.3 μM hepsulfam (AdrMCF7) after 24-h exposure to the drug. To evaluate possible mechanisms responsible for this observed variation in sensitivity to hepsulfam, we have studied glutathione S-transferase (GST) activity and glutathione (GSH) in these cell lines. Total cytoplasmic GST activity correlated with sensitivity; the most sensitive cell lines had the lowest GST activity, while the two most resistant cell lines, AdrMCF7 and Hs578T, had the highest GST levels of the six cell lines. Western blot analysis showed that the only detectable isoenzyme was GST-α. The amount of GST-α isofrom correlated with hepsulfam sensitivity in the three most resistant cell lines and was undetectable in the three most sensitive cell lines. Cellular concentrations of GSH did not correlate with hepsulfam sensitivity. However, GSH depletion with buthionine sulfoximine increased sensitivity to hepsulfam in a dose-dependent fashion in all six cell lines. Evaluation by mass spectrometry revealed that glutathione can form conjugates with hepsulfam. We conclude that the GST/GSH detoxication system plays a role in the sensitivity of these breast cancer cell lines to hepsulfam.

INTRODUCTION

Alkylating agents are a mainstay in the therapy of both early and advanced breast cancer. The dose-response characteristics of these drugs in vitro make them particularly well suited for use in autologous bone marrow transplantation and, as such, they have become key elements of high-dose therapy utilizing bone marrow rescue (1). Unfortunately, the emergence of clinical drug resistance frequently limits the efficacy of these agents. It is therefore important to develop both new alkylating agents and better methods to detect and, ultimately, circumvent drug resistance.

Hepsulfam (NSC 329680, 1,7-heptanediol disulfamate) (Fig. 1) is a derivative of the alkylating agent, busulfan. It was synthesized in an attempt to improve the antitumor efficacy of the parent compound by the introduction of a more polar leaving group. Preclinical studies with hepsulfam showed excellent activity against busulfan-resistant murine and human mammary tumors in vivo. In addition, P388 leukemia sublines with acquired resistance to both cyclophosphamide and N,N'-bis(2-chloroethyl)-N-nitrosourea lacked cross-resistance to hepsulfam (2). The cytotoxicity of hepsulfam has been investigated by alkaline elution studies which have shown that the drug induces a higher level of DNA intrstrand cross-links than busulfan in normal and malignant hematopoietic cells as well as in solid tumor cell lines (3, 4). Thus, hepsulfam appears, both in vivo and in vitro, to be a more potent alkylating agent and to have a broader spectrum of activity than busulfan.

The development of resistance to alkylating agents is a major factor in treatment failure of many cancers. The mechanisms responsible for chemotherapeutic drug resistance are multifactorial and include altered drug delivery and uptake, decreased drug activation, increased metabolic inactivation, active drug efflux, and enhanced repair of DNA (1). While many of these factors are operative in alkylating agent resistance, the major mechanism of cytotoxicity of the clinically effective alkylating agents appears to be the formation of DNA inter- and intrastrand cross-links resulting in cessation of DNA synthesis (1, 5, 6). This interaction between DNA and drug is dependent upon a number of variables including the localized concentration of cellular nucleophilic targets, the energy state of these targets, the mechanism of alkylation, and the reactivity of the particular alkylating agent (7, 8). One potential nucleophilic target is the intracellular thiol pool. GSH3 represents the major intracellular nonprotein thiol. As such, it is notable that elevated GSH levels have been found in tumor cells resistant to alkylating agents (9–12) and that DNA interstrand cross-linking is reduced in alkylating agent-resistant cells that have elevated levels of intracellular nonprotein thiols (13). Additionally, experimental cellular GSH depletion with BSO, which irreversibly binds the enzyme γ-glutamylcysteine synthetase to block GSH synthesis, results in enhanced cytotoxicity of a number of alkylating agents (14, 15). Conjugation of GSH to either endogenous substrates or xenobiotics is catalyzed by the GSTs which comprise a family of detoxicating cytoplasmic enzymes grouped into three classes based on biochemical, immunological, and structural properties (Classes α, μ, and π) (16). Thus, it is not surprising that increased levels of these enzymes have been associated with resistance to alkylating agents (17–20).

In the current study we have examined the de novo sensitivity of a panel of human breast cancer cell lines to hepsulfam. We have further studied these cell lines for GSH content, GST activity, and GST isoenzyme composition to examine the role that these factors might play in defining cellular response to this drug. Additionally, we have utilized BSO-mediated depletion of GSH to characterize further the cellular mechanisms responsible for sensitivity and resistance to this drug. Finally, we have studied the reaction of hepsulfam with GSH by mass spectrometry.

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3 The abbreviations used are: GSH, glutathione; BSO, L-buthionine sulfoximine; GST, glutathione S-transferase; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; IC50, concentration required for 90% inhibition of colony formation; CDNB, 1-chloro-2,4-dinitrobenzene; FCS, fetal calf serum; AdrMCF7, Adriamycin-resistant cell line; WT, wild-type cell line.

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MATERIALS AND METHODS

Reagents. Hepsulfam (NSC 329680) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Hepsulfam was dissolved in DMSO, and aliquots were stored at -20°C and thawed immediately before use. Previous studies of hepsulfam stability have shown that there is <20% breakdown of the drug at 37°C in fresh human plasma or normal saline solution over 24 h (21). Primary anti-GST antibodies (limits of detection) were stored at -20°C and thawed immediately before use. Preliminary anti-GST antibodies (limits of detection) were purchased from Amersham Corporation (Arlington Heights, IL).

Cells and Cell Culture. Human breast cancer cells Hs578T, T47D, MDA-MB-231, and MDA-MB-468 were obtained from the laboratory of Dr. M. Lipman (Vincent T. Lombardi Cancer Center, Georgetown University, Washington, DC), while WTMCF7 and Adr^MCF7 cells were the gift of Dr. K. Cowan (National Cancer Institute, Bethesda, MD). The Adr^MCF7 cell line was selected by serial passage of WTMCF7 in the presence of increasing concentrations of doxorubicin and was maintained in 10 µM doxorubicin (22). Adr^MCF7, WTMCF7, MDA-MB-468 cells were maintained in minimal essential medium (Biofluids, Rockville, MD) containing 5% FCS, while Hs578T, T47D, and MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with 5% FCS. All cell lines were grown at 37°C in 5% CO_2 and passed weekly. Adr^MCF7 cells were passaged in doxorubicin-free medium for at least four passages before testing.

Clonogenic Assay. Cells (1.0 to 2.5 x 10^3) were plated onto 6-well dishes on Day 0. Twenty-four h later triplicate wells were treated with various concentrations of hepsulfam in medium with 5% FCS. The DMSO vehicle concentration was constant. After 24 h of drug treatment, cells were rinsed 3 times with PBS, and fresh medium with 10% FCS was added. Hs578T and MDA-MB-231 cells required the addition of 5000 feeder cells of the same cell type per well. Feeder cells were lethally irradiated with 3000 cGy from a cobalt 60 irradiator. For glutathione depletion studies, cells were pretreated with 25 or 50 µM BSO for 24 h prior to hepsulfam treatment, and BSO therapy was continued through the 24-h period of hepsulfam exposure. Cells were incubated for 7 to 14 days, fixed with 25% methanol, and stained with crystal violet. Colonies of >50 cells were scored visually by microscopy. Results represent the mean ± SEM of 4 experiments. Cloning efficiency by this method was 4 to 11%.

GST Enzyme Assay. Cells were plated overnight at 20,000 cells/well in 96-well plates. Plates were washed in PBS, and cells were lysed with 0.1% digitonin. GST activity was assayed spectrophotometrically using CDNB as a substrate by the method of Habig et al. (23) using an automated plate reader. Protein was measured by the Bradford assay (24). Results are expressed in nmol of product/min/mg of protein and represent the mean ± SEM from 4 experiments.

Immunoblot Analysis. Immunoblotting was performed according to the procedure of Towbin et al. (25). Fifty-µg protein samples derived from log-phase cells were resolved on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes. After preincubation with bovine serum albumin, filters were incubated for 4 h at 23°C with a 1:2000 dilution of primary antibody (rabbit antiserum directed against either human α, human γ, or rat μ class GST). Following washing, membranes were incubated for 90 min at 23°C with 5 µCi of ^125I-labeled donkey anti-rabbit immunoglobulin G secondary antibody. After further washing, filters were exposed to Kodak XAR-5 film.

Glutathione S-Transferase Activity and Isoenzymes Expression. As increased levels of GST activity have been correlated with hepsulfam resistance from the most resistant cell line on the left to the most sensitive cell lines on the right. The enzymatic activities of the two most resistant cell lines, Adr^MCF7 and Hs578T, were 123 ± 9 and 121 ± 10 nmol of product/min/mg of protein, respectively. These values are at least 3 times greater than the activity of the more sensitive cell lines. In general, higher GST activity was correlated with hepsulfam resistance.

RESULTS

Hephsulfam Sensitivity in Human Breast Cancer Cell Lines. The in vitro cytotoxicity of hepsulfam as measured by clonogenic assay in six human breast cancer cell lines is shown in Fig. 2. This reveals a wide range of sensitivity among these cell lines with IC50 ranging from 32.3 µM hepsulfam for the most resistant cell line, Adr^MCF7, to 3.1 µM hepsulfam for the most sensitive cell line, MDA-MB-468. It is notable that even the most resistant of these breast cancer cell lines exhibited a greater sensitivity to hepsulfam than a variety of leukemic cell lines (5, 27). This spectrum of sensitivity and resistance prompted further study of these cell lines in an attempt to understand their differential sensitivity.

Glutathione S-Transferase Activity and Isoenzymes Expression. As increased levels of GST activity have been associated with resistance to alkylating agents, the total GST activity of these cell lines using CDNB as a substrate was examined as shown in Fig. 3. Values are arranged in order of hepsulfam resistance from the most resistant cell line on the left to the most sensitive cell lines on the right. The enzymatic activities of the two most resistant cell lines, Adr^MCF7 and Hs578T, were 123 ± 9 and 121 ± 10 nmol of product/min/mg of protein, respectively. These values are at least 3 times greater than the activity of the more sensitive cell lines. In general, higher GST activity was correlated with hepsulfam resistance.
We next tested whether variable sensitivity of human breast cancer cells to hepsulfam could be correlated with expression of specific GST isoenzyme(s). The immunoblot analysis in Fig. 4 showed that the only detectable GST isoenzyme in the cell lines tested was the ι isoenzyme; no GST-α or GST-μ protein was detected in any cell line. Levels of the GST-ι isoenzyme by Western blot again correlated with sensitivity to hepsulfam. The two most resistant cell lines, Adr-R MCF7 and Hs578T, had the highest levels while the MDA-MB-231 cell line had an intermediate level, and the three most sensitive cell lines had low or undetectable GST-ι isoenzyme.

Glutathione Levels in Human Breast Cancer Cell Lines and Effects of BSO on Glutathione and Hepsulfam Sensitivity. We then examined the relationship between glutathione level and hepsulfam sensitivity. Baseline GSH levels in the six cell lines tested varied by less than 2-fold, ranging from 24.5 nmol/mg of protein (Adr-R MCF7) to 46.4 nmol/mg of protein (MDA-MB-231) and did not correlate with hepsulfam sensitivity. Treatment with BSO for 24 or 48 h resulted in a time- and dose-dependent decrease in GSH level such that 50 μM BSO for 48 h resulted in a >95% reduction in GSH level in all six cell lines (data not shown).

The effect of glutathione depletion on hepsulfam sensitivity in the six breast cancer cell lines was measured by clonogenic assay after exposure of cells to 25 or 50 μM BSO. Neither dose of BSO resulted in any detectable cytotoxicity as cloning efficiency was not discernibly affected by BSO treatment (data not shown). Fig. 5 shows that GSH depletion by BSO resulted in an increase in hepsulfam sensitivity in all six human breast cancer cell lines. This increased sensitivity was BSO dose dependent and correlated with the degree of GSH depletion. Fig. 6 shows the IC₅₀ in μM hepsulfam of the 6 human breast cancer cell lines after exposure to various concentrations of BSO. This bar graph shows that those cell lines with the greatest initial resistance to hepsulfam had the greatest percentage of increase in sensitivity to the drug after GSH depletion.

Reaction of Hepsulfam with Glutathione. Incubation of glutathione with hepsulfam at 40°C for 2 h produced a monoglutathionyl conjugate of hepsulfam as shown in Fig. 7 demonstrating that this chemical reaction can occur spontaneously under near physiological conditions.

DISCUSSION

We have used six human breast cancer cell lines to evaluate the cytotoxicity of the new alkylating agent, hepsulfam. Our study shows that there are wide variations in hepsulfam sensitivity in the cell lines tested with an approximate 10-fold range as measured by IC₅₀. We have found that resistance to hepsulfam can be correlated with total cellular GST activity as well as levels of the GST-ι isoenzyme. Although there was no correlation between basal glutathione level and hepsulfam sensitivity, all six human breast cancer cell lines tested exhibited a dose-dependent increase in hepsulfam sensitivity with BSO-mediated GSH depletion. This effect was independent of the original sensitivity to the drug or basal GSH level. We have additionally demonstrated the formation of a monoglutathionyl conjugate of hepsulfam by mass spectrometry.

The GSH/GST detoxication system is an important element of cellular defense against a large array of injurious agents. The GST-mediated conjugation of toxic electrophiles to GSH results in the formation of thiocysteine intermediates that are more rapidly inactivated and eliminated. This diversion of toxic compounds, such as alkylating agents, away from their potential nucleophilic targets on DNA reduces their effective cellular concentration, thereby decreasing cytotoxicity. GST enzymes have also been shown to have peroxidase activity leading to detoxication of peroxides that may be generated via oxidation-reduction recycling of chemotherapeutic agents, again resulting in decreased cytotoxicity (28).

The data presented here suggest that cellular resistance to hepsulfam may be mediated, at least in part, by the GSH/GST system. Although Brodfuehrer et al. (29) reported that hepsul-
Fig. 5. Survival of six human breast cancer cell lines exposed to various concentrations of hepsulfam for 24 h in the presence of 0, 25, or 50 μM BSO. Survival in each cell line was measured after 24 h of pretreatment with 0 (•), 25 (○) or 50 (△) μM BSO. Results are expressed on a log scale as the fraction of treated to control colonies. Points, mean from 4 separate experiments; bars, SEM.

HepSulfam did not react with GSH, we report here the detection of a monoglutathionyl conjugate of hepsulfam by mass spectrometry. This apparent discrepancy may be explained by the fact that Brodfuehrer et al. measured GSH disappearance from buffer and blood for only 1 h and from rat liver cytosol for only 2 h. While the reaction rate of hepsulfam with GSH is not known, we were able to detect conjugation by mass spectrometry at 2 h. Pacheco et al. (27) have shown that peak hepsulfam-induced DNA interstrand and DNA-protein cross-links are found 12 h after a 2-h drug treatment, suggesting that longer time periods may be necessary to detect hepsulfam-GSH adducts, particularly when using the less specific technique of gas chromatography. Additionally, Brodfuehrer et al. used rat liver cytosol protein to investigate the role of enzyme-catalyzed conjugation of GSH with hepsulfam. Rat liver normally contains very low levels of GST 7-7, the rat equivalent of human GST-7T (30), the only isoenzyme detected in the breast cancer cells in our study. Since GST isoenzymes have been shown to have substrate specificity (16, 28), it is possible that low levels of this isoenzyme in rat cytosol and the short time span studied account for the lack of evidence for the GST-catalyzed reaction in that paper.

In this study, we detected no correlation between basal GSH levels and hepsulfam sensitivity. Our results may indicate that the spontaneous reaction between hepsulfam and GSH is very slow and must be enzymatically mediated under physiological conditions. Predominance of the enzyme-catalyzed reaction would explain our findings with regard to both GST content and BSO depletion of GSH, since depletion of GSH would decrease the rate of an enzymatic reaction as well as a spontaneous reaction.

There are numerous examples of GST elevation in association with alkylating agent resistance (17-19). Ciaccio et al. (31,
32) have shown that all three GST isoenzymes, \( \pi \), \( \alpha \), and \( \mu \), significantly increase the formation of glutathione-chlorambucil conjugates although the \( \alpha \) form is the most potent isozyme for this reaction. Additionally, transfection studies have shown that human GST-\( \pi \), rat GST Ya (Class \( \alpha \)), or rat GST Yb (Class \( \mu \)) can confer alkylating agent resistance when expressed in cultured mammalian cells (20). It has been suggested that resistance to different classes of alkylating agents may be associated with distinct GST isoenzyme classes, and the role of individual GST isoenzymes has been investigated in recent studies. Bolton et al. (33) have shown that only Class \( \alpha \) GST isozyme catalyzes the conjugation of L-phenylalanine mustard with glutathione, and Smith et al. (34) have demonstrated the specificity of Class \( \mu \) GST isoenzyme for denitrosation of 1,3-bis(2-chloroethyl)-1-nitrosourea. In the present study we have correlated hepsulfam cytotoxicity with expression of GST-\( \pi \). The role of GST isoenzymes in the conjugation of hepsulfam to GSH is the topic of ongoing studies.

Our results on GST activity and isoenzyme expression in human breast cancer cell lines find parallel in surveys of human breast cancer specimens. A recent study of 45 primary human breast carcinomas showed a wide range of GST activities from 5 to 208 nmol of product/min/mg of protein (35). GST-\( \pi \) isoenzyme protein was detected in 44 of 45 specimens and was the predominant isoenzyme found in these tumors as in our cell lines. Class \( \mu \) GST protein was noted in about half of the samples, and the absence of GST-\( \mu \) protein was found to be due to deletion of the GST-\( \mu \) gene in those tumors. Our failure to detect GST-\( \mu \) may reflect a similar deletion of the gene in our cell lines, a possibility we have not examined. None of the tumors expressed GST-\( \alpha \), a finding consistent with our inability to detect GST-\( \alpha \) in the six cell lines. Finally, an inverse relationship between expression of GST and estrogen receptor has been suggested in some (36, 37) but not all surveys (35) of human breast tumor specimens. As the cell lines in our study with both the highest (Adr\#MCF7) and lowest (MDA-MB-468) GST activities are estrogen receptor negative, our limited study supports the findings of Shea et al. (35) who were unable to show a strong link between total GST activity and steroid receptor status in human breast tumors.

Finally, the studies of Teicher, Frei, and coworkers (38–40) have added considerably to our understanding of alkylating agent sensitivity and resistance. The notion that this resistance is relative was supported by their observation that alkylating agents generally maintained a log-linear dose-response relationship through several logs of cell kill, while that linearity was lost at about 2 logs of cell kill for other classes of agents. In addition, even with strong \textit{in vitro} selection pressure, these studies showed that alkylating agent resistance was difficult to generate and that selected cell lines generally exhibited little cross-resistance to other alkylators. We have noted that sensitivity to hepsulfam remains log-linear in all six breast cancer cell lines tested and that this phenomenon continues under the pressure of GSH depletion. These observations provide a sound rationale for the use of alkylating agents such as hepsulfam, either singly or in combination, in the clinical setting of high-dose chemotherapy with bone marrow support where resistance can reasonably be expected to be overcome by dose escalation.

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REFERENCES

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