The Role of Human Glutathione S-Transferase Isoenzymes in the Formation of Glutathione Conjugates of the Alkylating Cytostatic Drug Thiotepa

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ABSTRACT

Nonenzymatic and glutathione S-transferase (GST) catalyzed glutathione (GSH) conjugation has been postulated as a mechanism by which alkylating cytostatic drugs can be inactivated intracellularly. In this study, we describe studies on the glutathione-dependent biotransformation of thiotepa (tris(l-aziridinyl)phosphine sulfide), a trifunctional alkylating agent.

31P NMR studies showed that thiotepa is stable in 0.07 M phosphate buffer, pH 7.4 ($\tau_{1/2} = 3300$ min). In the presence of glutathione, the rate of disappearance of thiotepa increased greatly ($\tau_{1/2} = 282$ min). Both monogluthationyl thiotepa and digluthationyl thiotepa conjugates were identified by 31P NMR and mass spectrometry. Addition of GST A1-1 (a) to an incubation of thiotepa and GSH further increased the rate of disappearance of thiotepa ($\tau_{1/2} = 100$ min) and increased the rate of formation of monogluthationyl thiotepa. The rate of formation of digluthationyl thiotepa was not altered, suggesting that the formation of digluthationyl thiotepa is not catalyzed by GST A1-1.

The role of purified human GST on the formation of monogluthationyl thiotepa was further studied by HPLC. In incubations with 0.2 mM thiotepa, 1 mM glutathione, and 40 $\mu$M GST, both GST A1-1 and P1-1 enhanced the formation of the monogluthationyl conjugate 30–35-fold above the nonenzymatic formation, while GST A2-2 and M1a-1a did not catalyze the rate of formation of this conjugate. 

The rate of GST on the formation of monogluthationyl conjugates of tepa (tris(l-aziridinyl)phosphine oxide), the major metabolite formed from thiotepa, was also studied. Both GST A1-1 and P1-1 could enhance the formation of the glutathione conjugate 37–46-fold above the spontaneous levels, while GST M1a-1a and A2-2 again did not increase the rate of formation of this conjugate. The results of these studies show that the aziridine moieties in thiotepa/tepa are substrates for both GST A1-1 and P1-1. Thus, GST catalyzed glutathione conjugation of thiotepa might be an important factor in the development of drug resistance towards thiotepa.

INTRODUCTION

Alkylating cytostatic drugs are widely used in the treatment of cancer. Their effectiveness generally lies in their ability to alter DNA replication in rapidly proliferating cells. Thiotepa is an alkylating agent containing a four-coordinated phosphorus atom and three aziridine moieties (Fig. 1). The compound was first synthesized in 1952, and although shown to be active against a variety of tumors, it was not used extensively, possibly because myelosuppression was occasionally severe (1–3). A renewed interest in the use of thiotepa in combination with autologous bone marrow transplantation has developed. Recent Phase I studies showed that thiotepa is active in malignant melanoma, carcinoma of the breast, carcinoma of the colon, germ cell sarcomas, and hematopoietic tumors (4).

The major metabolite formed from thiotepa is tepa (Fig. 1). Rat cytochrome P450 2B1 has been identified as one of the cytochrome P450 enzymes playing a role in this oxidative desulfuration reaction (5). In some studies, tepa was found to be more mutagenic and/or cytotoxic than thiotepa (6, 7), while other studies did not report a difference in the cytotoxicity of these two compounds (5, 8).

Cell death induced by thiotepa might be the result of the formation of cross-links within DNA. Evidence for the formation of cross-links was found in alkaline elution experiments (9, 10). These cross-links are proposed to be formed upon direct nucleophilic ring opening of aziridine groups. An alternative mechanism of thiotepa-induced DNA damage is the formation of DNA adducts from aziridine, like the N2-(2-aminoethyl)guanine adduct (11). This adduct is proposed to be formed after release of aziridine from thiotepa after cleavage of the N-P bond. In this latter model, thiotepa function as a cell-penetrating carrier of aziridine. The biological significance of this model is not clear, since the release of aziridine from thiotepa is strongly pH dependent (11).

The effectiveness of many clinically useful anticancer drugs can be severely limited by the development of drug resistance. A variety of mechanisms can contribute to drug resistance. These include alterations in drug uptake or drug efflux from the cell, changes in drug-metabolizing enzymes, and changes in target enzymes or DNA repair enzymes (reviewed in Ref. 12). An increased conjugation with glutathione, an intracellular cysteine-containing tripeptide present at high concentrations (up to 5–10 mM) in most mammalian cells, has been proposed as a major mechanism in the development of drug resistance towards alkylating agents (reviewed in Refs. 13 and 14). This has been attributed to the ability of GST to compete with DNA for drug binding.

GST can catalyze the conjugation of electrophilic agents with glutathione. A concentration of 30–240 $\mu$M GST has been reported in tumors or tumor cell lines (15–17). An increased concentration of GST is linked to an enhanced detoxification of alkylating agents and, therefore, to the development of drug resistance. For several alkylating agents, i.e., melphalan, chlorambucil, phosphoramide mustard, and ifosfamide mustard, the role of GST in the formation of glutathione conjugates has been demonstrated (18–23). Recently, clinical studies with thiotepa in combination with the GST transferase inhibitor ethacrynic acid were initiated (24). In this study, we describe 31P NMR and HPLC studies on the formation of glutathione conjugates of thiotepa and tepa. Mono- and diglutathionyl conjugates of thiotepa were characterized. The results confirm a catalytic effect of GST A1-1 (a) and P1-1 (a) on the formation of monogluthationyl thiotepa and monogluthationyl tepa.

The rate of formation of these conjugates was increased 30–46-fold above the nonenzymatic levels in the presence of these enzymes.
Materials and Methods

Chemicals. Thiotepa was provided by Lederle Laboratories (Pearl River, NY). Tepa was a gift of the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Glutathione was purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and glutathione reductase and NADPH from Sigma Chemical Co. (St. Louis, MO). [35S]GSH was obtained from Du Pont de Nemours, NEN Division, Den Bosch, the Netherlands. All other chemicals used were of the highest purity obtainable.

GST Purification and Assay. GST isoenzymes were purified from human liver and human placenta using affinity chromatography (5-hexylglutathione-Sepharose 6B) and chromatofocusing as described previously (23). GST activity was assayed using 1-chloro-2,4-dinitrobenzene as a substrate (25). Specific activities (\(\mu\)mol/min/mg protein) were 46, 10, 67, and 109, respectively, for GST A1-1, GST A2-2, GST M1a-1a, and GST P1-1. All enzyme concentrations were expressed as the concentration of the subunit (M, 25,900, 26,700, and 24,800, respectively, for subunits A1, A2, M1a, and P1).

NMR Experiments. 161.9 MHz \(^{31}P\) NMR spectra were collected on a Varian Unity 400 spectrometer. A switchable 5-mm probe was used, which was held at constant temperature (37°C). Data accumulation was initiated using a 40 kHz spectral window, 12,8 k data points, 45° pulse angle, and low power \(^1H\) WALTZ decoupling. Free induction decays were exponentially multiplied, resulting in an additional 1 Hz line broadening in the frequency-domain spectra.

The \(^{31}P\) NMR spectra were referenced against 85% H3PO4 in D2O as external standard. Incubations were carried out in 0.1 M acetate buffer (pH 5.0) or 0.07 M phosphate buffer (pH 7.4) with 5 mM EDTA, 3 mM NADPH, 0.8 units/ml glutathione reductase, 10% D2O, 5 mM thiotepa, and with and without 50 mM GSH. Measurements were started when the lock signal showed that the sample temperature was constant. Ten measurements of 6 minutes were made, followed by measurements of 24 min. Peak intensities were quantified. From these data, rate constants were determined by fitting an appropriate kinetic model to these data, using the curve fit option of SlideWrite Plus, version 5.0 (Advanced Graphics Software, Carlsbad, CA). Rate constants for the disappearance of thiotepa were derived from a least squares fitting of the observed concentrations of thiotepa according to \(A(t) = A_0 \exp(-kt)\). Half-life times were calculated using \(t_{1/2} = (-\ln 2)/k\).

HPLC Experiments. Incubations were carried out in 0.07 M phosphate buffer (pH 7.4) with 5 mM EDTA, 3 mM NADPH, 0.8 units/ml glutathione reductase, 1 mM GSH/[35S]GSH, and with or without 40 \(\mu\)M GST. Reactions were started with the addition of thiotepa/tepa, which was dissolved in phosphate buffer immediately before use. Incubation temperature was 37°C. Incubations were terminated by the addition of N-ethylmaleimide to a final concentration of 10 mM and were stored at -20°C until analysis.

The HPLC system used consisted of a Pharmacia LKB HPLC pump 2248 (Uppsala, Sweden) equipped with a flow-through radioactivity detector (Radiomatic Flo-one A500; Meriden, CT). A 0.5-ml flow cell was used. As scintillation cocktail Flo-Scint A (Packard Instrument, Groningen, the Netherlands) was used with a flow of 2 ml/min. Formation of glutathione conjugates was assayed on a 250 x 4.6 mm Hypersil ODS column (Shandon HPLC, Cheshire, United Kingdom). Eluent A was 1.54 g/liter ammonium acetate in water and eluent B was methanol (flow = 0.8 ml/min). The solvent program started isocratically with 5% B for 5 min, followed by a linear gradient to 70% B in 15 min.

Mass Spectrometry. Peaks containing monogluthationyl thiotepa and monogluthationyl tepa were isolated, lyophilized, and characterized using HPLC-MS. A Spherisorp 5 RP-8S (100 x 2.1 mm) column was used with 0.1% acetic acid as solvent A and acetonitrile as solvent B. Mass spectrometric analyses were performed using ionspray ionization on a Finnigan MAT TSQ 700.

Analysis of Results. Kinetic parameters (\(K_m\) and \(V_{max}\)) were calculated using the Michaelis Menten equation. The curve-fitting program for the analysis of enzyme kinetic data “EZ-fit” was used for this purpose (26).

Results

\(^{31}P\) NMR Studies with Thiotepa. When 5 mM thiotepa was dissolved in 70 mM phosphate buffer (pH 7.4), a signal in the \(^{31}P\) NMR spectra was found at 8 116.4. Derived from first-order reaction kinetics, the rate constant for the disappearance of the thiotepa signal at 37°C was 2.1 \times 10^{-4}/min (\(t_{1/2} = 3300\) min), showing that thiotepa in aqueous solution is quite stable.

When thiotepa was incubated with a 10-fold molar excess of glutathione, two new signals were found in the \(^{31}P\) NMR spectra. The signal at 8 98.9 was assigned to monogluthationyl thiotepa and the signal at 8 81.7 to diglutathionyl thiotepa (Fig. 2). The formation of...
monoglutathionyl thiotepa (m/z 497) and diglutathionyl thiotepa (m/z 804) was confirmed by HPLC-MS. The signal intensities of the three peaks were determined, and the time course for formation of monoglutathionyl thiotepa conjugates as well as the disappearance of thiotepa is shown in Fig. 3A. The rate constant for the disappearance of thiotepa signal at 37°C was $2.46 \times 10^{-3}$/min ($t_{1/2} = 282$ min), showing that in the presence of glutathione, the disappearance of thiotepa is 12-fold faster than without glutathione.

To study the influence of GST on these reactions, 5 mM thiotepa and 50 mM GSH were incubated with 20 nM GST A2-2. The time course for the disappearance of thiotepa and the formation of monoglutathionyl thiotepa conjugates is shown in Fig. 3B. In this experiment, the rate constant for the disappearance of thiotepa was $7.1 \times 10^{-3}$/min ($t_{1/2} = 99.8$ min). GST A2-2 increased the rate of formation of monoglutathionyl thiotepa. However, the rate of formation of diglutathionyl thiotepa was not altered by the presence of GST A2-2.

In addition, the behavior of thiotepa at pH 5.0 was studied by $^{31}$P NMR. Without GSH, the rate of disappearance of thiotepa was $1.94 \times 10^{-3}$/min ($t_{1/2} = 357.8$ min), showing that at pH 5 thiotepa is less stable than at pH 7.4. In the presence of GSH, the rate of disappearance was $2.82 \times 10^{-3}$/min ($t_{1/2} = 245.8$ min). No signals at δ 98.9 and δ 81.7 were found, showing that no glutathione conjugates were formed. Only signals at δ 20 and δ 21 were found; these signals might be assigned to hydrolysis products of thiotepa.

The Conjugation of Thiotepa with Glutathione Studied by HPLC. Upon HPLC-liquid scintillation analysis of an incubation mixture of 0.2 mM thiotepa with 1 mM GSH/$^{35}$S)GSH, two radiolabeled peaks were found in the chromatogram (Fig. 4). The peak at 5.0 min corresponds to glutathione. The peak at 18.5 min was collected and characterized by HPLC-MS. In the mass spectrum (presented in Fig. 4), a fragment with m/z 497 was found corresponding to monoglutathionyl thiotepa. In addition, a fragment at m/z 557 was found corresponding to M$^+$ plus acetate.

To study the influence of purified human GST on the formation of the monoglutathionyl thiotepa conjugate, 0.2 mM thiotepa and 1 mM GSH were incubated at 37°C in the presence of 40 μM GST A1-1, A2-2, M1a-1a, and P1-1. The formation of monoglutathionyl thiotepa was 30–35-fold increased in the presence of GST A1-1 and P1-1. A 2-fold increase was found in the presence of GST M1a-1a, while the formation of monoglutathionyl thiotepa in the presence of GST A2-2 was not altered (Fig. 5A). The formation of monoglutathionyl thiotepa was dependent on both the incubation time as well as on the amount of protein present (Fig. 6).

Based on these experiments, conditions were selected to determine $K_m$ and $V_{max}$ values for the GST A1-1 and GST P1-1 catalyzed formation of monoglutathionyl thiotepa. Using thiotepa concentrations up to 10 mM, apparent $K_m$ and $V_{max}$ values were determined using Michaelis Menten kinetics. The results are presented in Table 1. $K_m$ determined were in the 5–7 mM range.

Since in tumors a large variation in pH values is found (27), the GST P1-1 and A1-1 catalyzed formation of monoglutathionyl thiotepa was also studied in 0.07 M phosphate buffer with a pH range of 5.5–8.5. From the results presented in Fig. 7, it is clear that the pH optimum for the GST P1-1/A1-1 catalyzed formation of monoglutathionyl thiotepa is pH 8, but also at lower pH values, the formation of this conjugate is increased by GST P1-1.

The major metabolite formed from thiotepa is tepa. A monoglutathionyl conjugate of tepa (m/z 483) was characterized by HPLC-MS. The role of purified human GST in the formation of this conjugate was studied. As demonstrated in Fig. 5B, the presence of GST A1-1 and P1-1 in the incubation mixture enhanced the formation of the glutathione conjugate 37–46-fold above the spontaneous levels. GST A2-2 and M1a-1a did not alter the formation of monoglutathionyl tepa.

DISCUSSION

In certain cell lines and human tumor samples, expansion of intracellular glutathione pools and overexpression of glutathione S-transferase activity appear to be major factors that determine resistance to alkylating cytostatic agents. Depletion of GSH by agents such as buthionine sulfoximine or inhibition of GST activity by ethacrynic acid restores sensitivity to alkylating drugs (reviewed in Refs. 13, 14, and 28). To more clearly understand the role of GSH/GST in the development of drug resistance, more information is needed on the glutathione-dependent biotransformation of alkylating drugs and the role played by individual GST isoenzymes in these conjugation reactions.

Using $^{35}$P NMR and HPLC, we have characterized mono- and diglutathionyl conjugates of thiotepa. The presence of glutathione accelerated the disappearance of thiotepa. The effect of GSH on the
disappearance of thiotepa might be attributed to the fact that GSH is a stronger nucleophile than water. In addition, the pKa of glutathione is much lower compared to water, resulting in an increased protonation of the aziridine moieties of thiotepa in the presence of GSH. This conversion of aziridinyl groups of thiotepa to aziridinium ions has been described by 1H NMR (29). The electrophilic aziridinium species formed are reported to be short lived and react rapidly with nucleophiles, like water and glutathione, leading to the formation of ring-opened products. This nucleophilic ring opening mechanism is supported by observations on the formation of DNA cross-links as found in alkaline elution experiments (9, 10) and ring-opened (chloroethyl) phosphoramides generated in the presence of chloride (29, 30, 31).

A second proposed mechanism of activation of thiotepa is the release of aziridine after a nucleophilic attack of water at the phosphorus atom, resulting in cleavage of a N-P bond. NMR data showed the presence of aziridine as a hydrolysis product of thiotepa under acidic conditions (29). The formation of phosphatidylethanolamine in experiments with [14C]thiotepa (32) and the formation of 7-(2-aminoethyl)guanine adducts (11) support this latter mechanism. However, since the release of aziridine is strongly dependent on the pH (11), the biological significance of this mechanism is not clear.

Our 31P NMR experiments showed that the disappearance of thiotepa is much faster at pH 5.0 compared to pH 7.4. In the presence of GSH, the disappearance of thiotepa was accelerated. However, no (spontaneous) formation of glutathionyl conjugates of thiotepa was found at this pH, probably because the concentration of thiolate ions formed from glutathione is lower at pH 5.0 as compared to pH 7.4.

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**Fig. 4.** Separation of monoglutathionyl thiotepa conjugates from glutathione by reversed phase liquid chromatography. Thiotepa (0.2 mM) and 1 mM GSH were incubated for 45 min at 37°C and 50 µl of the reaction mixture were analyzed as described in the text. Peak 1, glutathione; peak 2, monoglutathionyl thiotepa. Inset, LC-MS spectrum of monoglutathionyl thiotepa.

**Fig. 5.** A, formation of monoglutathionyl thiotepa without or with 40 µM GST. Thiotepa (0.2 mM) and 1 mM GSH were incubated at 37°C for 60 min. B, formation of monoglutathionyl tepa without or with 40 µM GST. Tepa (0.2 mM) and 1 mM GSH were incubated at 37°C for 60 min. Bars, SD.

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GST-MEDIATED GLUTATHIONE CONJUGATION OF THIOTEA

Fig. 6. A, the formation of monoglutathionyl thiota in time in the presence of 10 μM GST P1-1. Thiota (0.2 mM) and 1 mM GSH were incubated at pH 7.4 at 37°C.

B, the formation of monoglutathionyl thiota in the presence of varying concentrations of GST P1-1. Thiota (0.2 mM) and 1 mM GSH were incubated for 45 min at pH 7.4 at 37°C. Bars, SD.

Table 1. Apparent Km and Vmax values for the formation of monoglutathionyl thiota by GST A1-1 and GST P1-1

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<th>Km (mM)</th>
<th>Vmax (nmol/min/mg protein)</th>
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<tbody>
<tr>
<td>GST A1-1</td>
<td>4.8 ± 0.7</td>
<td>26.1 ± 1.7</td>
</tr>
<tr>
<td>GST P1-1</td>
<td>6.8 ± 0.8</td>
<td>54.2 ± 3.1</td>
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31P NMR experiments showed that GST A1-1 increased the rate of formation of monoglutathionyl thiota, but not of diglutathionyl thiota, suggesting that only thiota and not its monoglutathionyl conjugate is a substrate for GSTs. The catalytic effects of both GST A1-1 and P1-1 were even more marked in experiments using lower concentrations of both thiota and GSH. The presence of GST M1a-1a and A2-2 had much less effect on the formation of this conjugate. Also, the formation of monoglutathionyl conjugates of thiota, the major metabolite of thiota, was strongly enhanced by GST A1-1 and P1-1.

For both GST A1-1 and P1-1, the Km for the formation of monoglutathionyl thiota was relatively high. However, this is not unusual for hydrophilic substrates like thiota. Since the GST concentration in tumor cells is quite high (30–240 μM) and the GSH concentration is in the 5–10 mM range, it can be assumed that catalytic constants as determined in this study can still have a marked effect on the rate of formation of glutathionyl conjugates of thiota, simply due to the high molar concentration of the reaction components in cells.

The pH in tumors is lower than of normal tissues. The median tumor pH is 7.1, and in 10% of the tumors studied, the pH was 6.6 (27). In the pH range 5.5–7.0, no nonenzymatic formation of monoglutathionyl thiota was observed. At all pHs tested, a GST P1-1/A1-1 catalyzed formation of monoglutathionyl thiota was found, showing that also at lower pH values the GST P1-1/A1-1 catalyzed formation of monoglutathionyl thiota can proceed.

It remains to be explained if formation of monoglutathionyl thiota/tepa represents a real detoxification since this conjugate has still two aziridine moieties left. Theoretically, this conjugate can still form DNA cross-links and split aziridine. In this respect, it is also important to obtain data on the fate of the monoglutathionyl thiota/tepa conjugates in cells. If these conjugates are transported out of the cell by an ATP-dependent GSH-conjugate efflux pump, as described for the glutathione conjugate of cisplatin (33), formation of monoglutathionyl thiota/tepa represents a detoxification pathway. Recently, the multidrug resistance-associated protein was identified as an ATP-dependent transport protein of GSH S-conjugates (34).

The impact of GSTs on the rate of formation of monoglutathionyl thiota/tepa is much larger than for other alkylating agents. A 2-fold increase in the formation of monochloro, monoglutathionyl conjugates of chlorambucil, melphalan, and phosphoramide mustard was found with GST α enzymes (18, 19, 20, 21, 23), while a 3–5-fold increase in the formation of monochloro, monoglutathionyl ifosfamide mustard was found with GST P1-1 enzymes. Rate-limiting events in the conjugation reactions of chlorambucil, melphalan, IM, and phosphoramide mustard are the formation of the aziridinium intermediate (21, 22, 35). The aziridinium intermediate of IM might deprotonate upon formation, leading to the formation of an aziridine group. The aziridinium intermediates of melphalan, chlorambucil,
and phosphoramide mustard cannot undergo these deprotonation re-
actions. The data up to now suggest that (charged) aziridinium ions
formd from melphanal, chlorambucil, and phosphoramide mustard
are substrates for GST α enzymes. The uncharged aziridine or the
charged aziridinium ion of IM seem to be a substrate for GST P1–1.
Thiotepa and tepa possess three (uncharged) aziridine groups and
were found to be good substrates for GST A1–1/P1–1. However, it is
also possible that the protonated aziridinium ions formed from thi-
tepa/tepa are the ultimate substrates for GST A1–1/P1–1. It is tem-
pitting to speculate that charged aziridinium ions are substrates for
GST A1–1 while the noncharged aziridine intermediates are substrates
for GST P1–1. This hypothesis is, however, not supported by the ex-
perimental results in our studies on the effects of the pH on the GST
catalyzed formation of monoglutathionyl thiotaleta conjugates.

A better understanding of GST substrate specificities with antican-
cer alkylating agents may also help explain alkylating agent cross-
resistance patterns and may eventually be useful in detecting cross-
resistant phenotypes in individual patients based on GST assays of
tumor biopsy specimens.

In conclusion, our studies show that thiotepa as well as its major
metabolite tepa are substrates for GST A1–1 and P1–1. The marked
catalytic effects of GST A1–1 and P1–1 were also found at pH values
typically found in tumor cells. Since overexpression of GST P1–1 and
to a lesser extent of GST A1–1 is observed in tumor cells, enhanced
detoxification of thiotaeta might contribute to the development of drug
resistance towards alkylating agents.

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DNA with aziridine produced during hydrolysis of N,N′,N′′-triethylenethio-


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mitomycin C and Adriamycin in Caco-2 human colon adenocarcinoma cells: a


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