Involvement of Human Glutathione S-Transferase Isoenzymes in the Conjugation of Cyclophosphamide Metabolites with Glutathione

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ABSTRACT

Alkylation agents can be detoxified by conjugation with glutathione (GSH). One of the physiological significances of this lies in the observation that cancer cells resistant to the cytotoxic effects of alkylation agents have higher levels of GSH and high glutathione S-transferase (GST) activity. However, little is known about the GSH/GST-dependent biotransformation of alkylation agents, including cyclophosphamide. Cyclophosphamide becomes cytostatic after the enzymatic formation of 4-hydroxycyclophosphamide. The ultimate alkylation species formed from cyclophosphamide is phosphoramide mustard. In this paper we describe the involvement of purified human glutathione S-transferase isoenzymes GST A1-1, A2-2, M1a-1a, and P1-1 in the formation of two types of glutathionyl conjugates of cyclophosphamide, i.e., 4-glutathionylcyclophosphamide (4-GSCP) and monochloromonoglutathionylphosphoramide mustard.

When 0.1 mM 4-hydroxycyclophosphamide and 1 mM GSH was incubated in the presence of 10 μM GST A1-1, A2-2, M1a-1a, and P1-1 the formation of 4-GSCP was 2-4-fold increased above the spontaneous level. Enzyme kinetic analysis demonstrated the lowest K_m (0.35 mM) for GST A1-1. K_m values for the other GST enzymes ranged from 1.0 to 1.9 mM.

Glutathione S-transferase A1-1 (40 μM) also increased the conjugation of phosphoramide mustard and GSH (both 1 mM) 2-fold, while the other major human isoenzymes, A2-2, M1a-1a, and P1-1, did not influence the formation of monochloromonoglutathionylphosphoramide mustard. These results indicate that only one enzyme within the class of human GST α enzymes was able to catalyze the reaction of the aziridinium ion of phosphoramide mustard with glutathione.

Thus increased levels of GST A1-1 in tumor cells can contribute to an enhanced detoxification of phosphoramide mustard and hence to the development of drug resistance. Since all of the human GSTs tested did catalyze the formation of 4-GSCP, the role of 4-GSCP either as a transport form of activated cyclophosphamide or as a detoxification product is discussed.

INTRODUCTION

Resistance to anticancer chemotherapeutic drugs remains a major obstacle in cancer chemotherapy. Recent studies suggest that GSH, an intracellular cysteine-containing tripeptide present at high concentrations (up to 5–10 mM) in most mammalian cells, is a critical determinant in tumor cell resistance to alkylation cytostatic agents (for review see Ref. 1). This has been attributed to the ability of GSH to compete with DNA for drug binding. The actual structures of these drug-GSH conjugates have been characterized for a number of these agents, i.e., melphalan, chlorambucil, and cisplatin (2–4).

In addition to higher intracellular concentrations of GSH a higher GST activity also has been observed in tumor cells resistant to alkylation agents. A concentration of 4–40 μg GST/mg cytosolic protein has been reported in tumors or tumor cell lines (5–7). According to our calculations this corresponds to a 30–240 μM concentration of GST.

GSTs might catalyze the formation of drug-GSH conjugates. However, little is known about the role of GSTs in the formation of these glutathionyl conjugates. Ciaccio et al. (8), reported that human GST α and π enzymes enhanced the formation of monochloro, monoglutathionylchlorambucil. Meyer et al. (9) reported that only GST α enzymes catalyzed this reaction and not GST μ and π. For melphalan the formation of monochloromonoglutathionylmelphalan was increased by mouse liver GST α isoenzymes but not by GST μ and π (10). Knowledge on the specificity of GSTs for the conjugation reactions of alkylation agents is necessary to provide insight in the role of GSTs in the development of tumor cell resistance since in tumor cells GST α enzymes are expressed (for review see Ref. 11).

CP, an orally active alkylation agent, has a wide spectrum of clinical uses and is an essential component of numerous combination chemotherapeutic regimens. Cyclophosphamide becomes cytostatic after oxidation by hepatic microsomal enzymes to form 4-OHCP (Fig. 1). Three specific human cytochrome P-450 enzymes, i.e., CYP2B6, CYP2C8, and CYP2C9, have been identified as major catalysts of cyclophosphamide activation (12). 4-OHCP equilibrates with the ring-opened aldophosphamide which undergoes spontaneous decomposition to yield PM and acrolein. It is proposed that 4-OHCP can, while PM cannot, enter cells so 4-OHCP might be considered as the carrier molecule of PM across cell membranes (13). Phosphoramide mustard possesses DNA-alkylating activity and is generally considered to be the therapeutically significant cytotoxic metabolite of cyclophosphamide. Phosphoramide mustard alkylates DNA through a positively charged reactive intermediate, i.e., an aziridinium ion formed by the loss of a chloride atom. Phosphoramide mustard has two chloroethyl groups and is able to form alkylated products at two separate nucleophilic sites.

Two types of glutathionyl conjugates of cyclophosphamide have been described, i.e., diglutathionyl-PM and 4-GSCP (14, 15), and the kinetics of the nonenzymatic formation of both conjugates has been studied using 31P NMR spectroscopy (16). Four stereoisomeric 4-GSCP conjugates were found. The formation of 4-GSCP was found to be reversible, and at hydrolysis, PM was being formed. 4-GSCP can therefore be considered as a stabilized reservoir for the production of PM. Conjugation of PM was found to be preceded by the formation of PMGS and subsequently diglutathionyl-PM.

Whether these reactions are catalyzed by the isoenzymes of human GSTs is unknown. We describe in this paper the influence of purified human GSTs on the rate of formation of 4-GSCP and PMGS in vitro. The results confirm a catalytic effect of all three classes of human GSTs tested on the formation of 4-GSCP as well as an effect of GST A1-1 on the rate of formation of PMGS. The impact of these findings on the role of GSTs in drug resistance toward CP is discussed.

MATERIALS AND METHODS

Chemicals. 4-OHCP and phosphoramide mustard [N,N-bis-(2-chloroethyl)phosphorodiamidic acid] were kindly provided by Dr. J. Pohl from ASTA...
4.6-mm Zorbax ODS column (Rockland Technologies, Chadds Ford, PA). A 2-ml flow cell was used. As scintillation cocktail Flo-Scint with a flow through radioactivity detector (Radiomatic Ho-one A500; Men Consisted of a Pharmacia LKB HPLC pump 2248 (Uppsala, Sweden) equipped until analysis.

2 mllmin. A (Packard Instrument, Groningen, the Netherlands) was used with a flow of mustards was assayed on a Mono Q HR 5/5 anion exchange column (Pharmacia LKB, Uppsala, Sweden). Eluent A was 0.02 m Tris buffer (pH 6.8), and eluent B was 0.02 m Tris buffer (pH 6.8) with 0.15 m NaCl. Flow was 1 ml/min. The solvent program started isocratically with 100% solvent A for 5 min followed by a linear gradient to 15% solvent B in 10 min.

**Mass Spectrometry.** In HPLC-MS experiments with glutathionyl conjugates of phosphoramide mustards a Superspher RP select B (200 x 4 mm) column was used with 10 mm ammonium trifluoroacetate with 0.5% TFA (solvent A) and acetonitrile (solvent B). The gradient applied was 5–30% solvent B in 15 min (flow, 1 ml/min). Mass spectrometric analyses were conducted using ion spray ionization at an API III triple-quadruple mass spectrometer (Sciex, Thornhill, Ontario, Canada).

In HPLC-MS experiments with 4-glutathionylcyclophosphamide a Spheri-sorb SS ODS-2 (200x 0.32-mm) column was used. Mobile phase A was 0.1% TFA in water, and mobile phase B was 0.1% TFA in acetonitrile. The solvent program started with 100% solvent A for 5 min, followed by a linear gradient to 20% solvent B in 15 min. Mass spectrometric analyses were performed using electrospray ionization (Analytica) on a Finnigan MAT TSQ 700. Peaks used for quantification of 4-GSCP and mono- and diglutathionyl conjugates of PM were isolated, lyophilized, and characterized with fast atom bombardment mass spectrometry using a Finnigan-MAT900 instrument as described elsewhere (16).

**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 (22).

**RESULTS**

**Conjugation of 4-Hydroxycyclophosphamide and Glutathione.** Incubation of 6 mM 4-OHC and 60 mM glutathione resulted in liquid chromatography-mass spectroscopy detection of a peak with mass fragments $m/z$ 566 and $m/z$ 568. The mass spectrum of this peak, corresponding to 4-GSCP, is presented in Fig. 2. When this incubation mixture was analyzed with reversed-phase HPLC with radiochemical detection one major radioactive peak was found at 26.5 min (Fig. 2). In Fig. 3A the nonenzymatic rate of formation of 4-GSCP at 25°C and 37°C is shown with time. The conjugate was found to be relatively stable at 25°C but less stable at a temperature of 37°C.

All further incubations were performed at a temperature of 25°C. When 0.1 mM 4-OHC and 1 mM GSH were incubated with increasing concentrations of GST A1-1, an enzyme concentration-dependent increase in the formation of 4-GSCP was found (Fig. 3B). In Fig. 4 the formation of 4-GSCP in the presence of 10 μM GST A1-1, M1a-1a, and P-1 is shown. All 3 isoenzymes tested had enhanced the rate of formation of the 4-GSCP conjugate. When these purified GST enzymes were incubated for 15 min with concentrations of 4-OHC up to 2000 μM a concentration-dependent increase in the formation of 4-GSCP was found. From the data of these experiments apparent $K_m$ and $V_{max}$ values were calculated using Lineweaver-Burk plots (Table 1). $K_m$ and $V_{max}$ values were relatively high. The lowest $K_m$ value was found for GST A1-1 (0.35 mM). $K_m$ values for the other GST enzymes ranged from 1.0 to 1.9 mM.

**Conjugation of Phosphoramide Mustard and Glutathione.** Upon incubation of 6 mM PM with 60 mM GSH two peaks were found in the chromatogram (Fig. 5). With liquid chromatography-mass spectrometry it was shown that both $m/z$ 493 (corresponding to monocholoromoglutathionylphosphoramide mustard) and $m/z$ 762 (corresponding to diglutathionylphosphoramide mustard) were present in the incubation mixture (Fig. 5). Also mass fragment $m/z$ 473 (corresponding to monohydroxymonoglutathionylphosphoramide mustard) was found.

The formation of glutathionyl conjugates with time was studied using 6 mM PM and 60 mM GSH at 25°C and 37°C, respectively (Fig. 6A). Within 60 min, peaks characterized as monocholoromoglutathionyl-PM and diglutathionylphosphoramide mustard were found in the HPLC chromatogram. The pattern of formation of these two
Fig. 2. Separation of 4-glutathionylcyclophosphamide from glutathione by reverse phase liquid chromatography. 4-Hydroxycyclophosphamide (6 mM) and GSH (60 mM) were incubated for 5 h at 37°C; 40 µl of the reaction mixture were analyzed as described in the text. The peak with a retention time of 6 min is glutathione, and the peak with a retention time of 25.6 min is 4-glutathionylcyclophosphamide. Inset, LC-MS spectrum of 4-glutathionylcyclophosphamide.

Fig. 3. A, time course of formation of 4-glutathionylcyclophosphamide in an incubation mixture of 6 mM 4-hydroxycyclophosphamide and 60 mM GSH at 25°C (○) and 37°C (●). B, formation of 4-glutathionylcyclophosphamide in the presence of varying concentrations of GST A1-1. The nonenzymatic formation of 4-glutathionylcyclophosphamide is subtracted from all values.
Fig. 4. Time course of formation of 4-glutathionylcyclophosphamide from 0.1 mM 4-hydroxycyclophosphamide and 1 mM GSH at 25°C without enzyme (□) and in the presence of 10 μM GST A1-1 (●), GST M1a-1a (○), and GST P1-1 (+). By means of linear regression analysis it was found that the slope of the lines of GST A1-1, M1a-1a, and P1-1 were statistically significant different (P < 0.05) from the slope of the line without enzyme.

Table 1 Apparent Vmax and Km values for human glutathione S-transferase catalyzed formation of 4-glutathionylcyclophosphamide

<table>
<thead>
<tr>
<th></th>
<th>Km (mM)</th>
<th>Vmax (nmol/min/ng protein)</th>
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<tbody>
<tr>
<td>GST A1-1</td>
<td>0.35 ± 0.09</td>
<td>10.0 ± 0.8</td>
</tr>
<tr>
<td>GST A2-2</td>
<td>1.5 ± 0.4</td>
<td>15.1 ± 2.2</td>
</tr>
<tr>
<td>GST M1a-1a</td>
<td>1.04 ± 0.3</td>
<td>10.4 ± 1.6</td>
</tr>
<tr>
<td>GST P1-1</td>
<td>1.9 ± 0.4</td>
<td>35.1 ± 4.8</td>
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DISCUSSION

If tumor cells have the capacity to inactivate alkylating agents, the amount of DNA damage caused by these agents may be less than in cells in which these mechanisms are not present. Inactivation by both nonenzymatic and GST-catalyzed GSH conjugation has been postulated to be related to the development of such drug resistance in tumor cells.

The question addressed here is whether cyclophosphamide metabolites are substrates for human GST isoenzyme. Previous studies on the spontaneous reactions of activated cyclophosphamide with glutathione showed that mainly two types of conjugates were formed, i.e., 4-GSCP and mono- and diglutathionylphosphoramide mustard (16).

All GSTs tested enhanced the formation of 4-glutathionylcyclophosphamide 2–4 times above the nonenzymatic rate. Km values for the human GSTs were comparable with that described in an earlier study in which these reactions were monitored by 31P NMR spectroscopy (16). Diglutathionyl-PM appeared to be a relatively stable conjugate, at both 25° and 37°C (Fig. 6A).

When 1 mM PM and 1 mM GSH were incubated at 37°C with varying concentrations of GST A1-1, an enzyme concentration-dependent increase in the formation of monochloromonoglutathionylphosphoramide mustard was found (Fig. 6B). Using the same experimental conditions no increase in the formation of monochloromonoglutathionylphosphoramide mustard was found using GST A2-2 (results not shown).

When 40 μM GST A1-1, A2-2, M1a-1a, and P1-1 was incubated for 24 min with 1 mM PM and 1 mM GSH the formation of the monoglutathionyl conjugate was 2-fold increased above the spontaneous rate in incubations with GST A1-1 but not with GST M1a-1a, A2-2, or P1-1 (Fig. 7).
concentrations of GST A1-i at 37°C. The nonenzymatic formation of monochloromonoglutathionylphosphoramide mustard is subtracted from all values.

The formation of the conjugate with enzyme was compared to the spontaneous formation by means of the paired t test. P values were 0.006, 0.02, 0.06, and 0.04, respectively, for GST A1-1, A2-2, M1a-1a, and P1-1. For GST A1-1, A2-2, M1a-1a, and P1-1 and 0.3 mM for GST A1-1. K_m values in the mM range are also observed for other substrates for GSTs, e.g., 1-chloro-2,4-dinitrobenzene, a substrate used in the quantification of GST activity (20, 23).

In contrast to the formation of 4-glutathionylcyclophosphamide the formation of monochloromonoglutathionyl-PM was catalyzed only by GST A1-1 but not by GST A2-2, M1a-1a, or P1-1. In the presence of 40 μM GST A1-1 the rate of formation of the monochloromonoglutathionylphosphoramide conjugate was 2-fold increased above the spontaneous level. The formation of 4-thio derivatives of cyclophosphamide as a "stabilized reservoir of alkylating activity" was suggested by Draeger et al. (24). This study and a previous study (16) showed that formation of 4-glutathionylcyclophosphamide is reversible and therefore that 4-GSCP might be considered as a stabilized form of 4-OHCP.

Kwon et al. (25) described two mechanisms for the formation of 4-thiocyclophosphamide conjugates. One mechanism involved the formation of iminocyclophosphamide and, the other mechanism involved the formation of a hemithioacetal from aldophosphamide that subsequently cyclized to 4-thiocyclophosphamide.

Conjugation of PM with GSH was shown to proceed through the formation of aziridinium ions and not by direct replacement of chloro-

From Fig. 6, it is observed that the formation of monochloromonoglutathionyl mustard and diglutathionylphosphoramide mustard in an incubation mixture of 6 mM phosphoramide mustard and 60 mM GSH at 25°C and 37°C. ○, monochloromonoglutathionylphosphoramide mustard, 25°C; ●, diglutathionylphosphoramide mustard, 25°C; □, monochloromonoglutathionylphosphoramide mustard, 37°C; ■, diglutathionylphosphoramide mustard, 37°C. B, formation of monochloromonoglutathionylphosphoramide mustard in the presence of varying concentrations of GST A1-1 at 37°C. The nonenzymatic formation of monochloromonoglutathionylphosphoramide mustard is subtracted from all values.

In previous 31P NMR experiments we found that the PM signal in incubations with glutathione disappeared at the same rate as in incubations without glutathione. These findings indicate that the rate-limiting step in the conjugation of PM with GSH is the formation of the aziridinium ion. Also the reaction of the nitrogen mustard agents melphalan and chlorambucil with glutathione is believed to proceed via the aziridinium intermediate (26).

The results of the studies with melphalan, chlorambucil (8–10, 26), and phosphoramide mustard (present study) all indicate that mainly GST A enzymes catalyze the reaction of the aziridinium intermediate with glutathione. Increased levels of GST A enzymes in tumors might therefore be related to an enhanced formation of drug-GSH conjugates and hence to the development of drug resistance.

A number of findings suggest a role of GST A enzymes in the development of drug resistance toward alkylating agents. Several alkylator-resistant sublines have been shown to overexpress GST A enzymes (27–30). The introduction of rat or human GST A genes into lines of cultured cells conferred resistance toward alkylating agents (31–33). In addition, a number of studies showed that GST protects against the cytotoxicity of CP (34, 35). Peters et al. (7) showed a 25-fold increase in the concentration of GST A enzymes in esophageal tumors compared to normal esophageal tissue.

Thus, we have identified two types of glutathione conjugates of cyclophosphamide. GSTs were able to catalyze both conjugation reactions. It is tempting to speculate about the role of the formation of these products in the biotransformation of cyclophosphamide and their role in the development of drug resistance. Our findings have led to the following model. Upon hydroxylation of CP by cytochrome P-450 in the liver, 4-OHCP is formed, which can conjugate with GSH, leading to the formation of 4-GSCP. This conjugation might occur nonenzymatically, but it is also catalyzed by all three major classes of cytosolic GSTs, of which the α- and μ-class enzymes are abundantly present in human liver (36). We postulate that 4-GSCP acts as a transport form of 4-OHCP in blood. Uptake of activated cyclophosphamide in cells is likely to occur by conversion of 4-GSCP to 4-OHCP by a number of trans-mercaptalization reactions. Intracellularly, 4-OHCP can conjugate with GSH. This reaction might occur nonenzymatically but is also catalyzed by GST α, μ, and π enzymes. 4-GSCP is possibly transported out of the cell by an ATP-dependent glutathione S-conjugate export pump (4). 4-GSCP would also
equilibrate with 4-OHCP/aldophosphamide giving rise to PM and acrolein. PM can conjugate with glutathione either nonenzymatically or catalyzed by GST A1-1. Monoglutathionyl PM cannot form DNA cross-links, while diglutathionyl PM cannot interact with DNA at all. The proposed model indicates that both glutathione levels and the presence of GSTs, including GST P1-i, can influence the number of DNA alkylations and hence the cytotoxicity of cyclophosphamide. Therefore it is likely that a relationship exists between increased levels of GSH and GSTs in tumor cells and the development of drug resistance. In future studies we will study the formation of these glutathione conjugates in vivo.

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