ABCC2-Mediated Biliary Transport of 4-Glutathionylcyclophosphamide and Its Contribution to Elimination of 4-Hydroxycyclophosphamide in Rat

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ABSTRACT

Hematopoietic stem cell transplantation patients conditioned with cyclophosphamide (CY) and total body irradiation have substantially greater risk of nonrelapse mortality when plasma area under the concentration-time curve (AUC) of 4-carboxyethylcyclophosphamide mustard (CEPM) is high. The discovery was paradoxical because CEPM is a nontoxic elimination route of the protoxic CY metabolite hydroxycyclophosphamide (HCY). CY was administered to Wistar and TR rats (a Wistar strain lacking functional ABCC2) at doses of 100 and 200 mg/kg CY, respectively. After either dose, Wistar rats excreted 4-glutathionylcyclophosphamide (GSCY) abundantly in bile; GSCY was absent from bile of TR rats. Liver AUCGSCY was 2- to 2.5-fold greater in TR rats than in Wistar rats after the respective CY doses. Plasma AUCHCEPM of TR rats was approximately twice that of Wistar rats after 100 mg/kg CY, but did not differ between the two strains after 200 mg/kg CY. Conversely, plasma AUCHCY was not different after 100 mg/kg CY, but was 40% greater in TR rats after 200 mg/kg CY. The dose dependence of plasma AUCHCEPM and AUCHCY was explained by the concentrations of HCY attained and the in vitro KM of aldehyde dehydrogenase and inhibition of aldehyde dehydrogenase in TR rats. We conclude that GSCY is a substrate of ABCC2, and plasma AUCHCEPM functions as a reporter of liver exposure to HCY and toxins formed from it when HCY concentration is below the KM of aldehyde dehydrogenase and the activity is not compromised.

Cyclophosphamide (CY) is one of the most frequently used agents to prepare patients for hematopoietic stem cell transplantation (HSCT), the only curative option for several malignant and nonmalignant hematologic diseases (Thomas et al., 1999). Cyclophosphamide’s primary role is to kill mature lymphocytes to allow engraftment, although antitumor effects may also contribute. Hepatic venoocclusive disease (sinusoidal obstruction syndrome, SOS) is one of the leading causes of morbidity and mortality after transplantation (McDonald et al., 1993; DeLeve et al., 2002). The reported incidence of SOS varies by center, ranging from 1 to 54%. The fatality rates vary from 3 to 67% (Bearman, 1995). Inclusion of CY in the preparative regimen is associated with an increased risk of SOS (Brodsky et al., 1990; Dix et al., 1996; Lee et al., 1999).

CY is a prodrug that must be oxidized in the liver to 4-hydroxycyclophosphamide (HCY) by CYP2C9, CYP3A4, and CYP2B6 (Fig. 1) (Chang et al., 1993; Ren et al., 1997). HCY, the proactive metabolite of CY, circulates in the blood and enters cells (as the tautomer aldophosphamide) to fragment by β-elimination, producing phosphoramide mustard and acrolein. Alternatively, HCY is detoxified to 4-carboxyethylcyclophosphoramide mustard (CEPM) by aldehyde dehydrogenase (ALDH)-1A1 and, to much lesser extent, HCY is oxidized by cytochrome P450s to 4-ketocyclophosphoramide mustard (not shown). Disposition products of CY are similar in rats and humans (Sladek, 1994). Phosphoramide mustard is a bifunctional alkylator of DNA and the ultimate cytotoxic metabolite of CY (Struck et al., 1975). Acrolein is a highly reactive aldehyde that covalently binds to cellular macromolecules and, by doing so, may alter...
their functions. It is detoxified by conjugation with GSH (Gurtoo et al., 1981). Acrolein has been shown to damage hepatic sinusoidal endothelial cells (SECs) via GSH depletion when formed by hepatocytes (DeLeve, 1996, 1998). SECs have been shown to be the primary target in the pathogenesis of SOS in an in vivo rat model and in vitro murine SECs in primary culture (DeLeve, 1996; DeLeve et al., 1999).

In a study of 147 patients undergoing HSCT after preparation with CY, 60 mg/kg/day for 2 days in combination with total body irradiation (TBI) 9 to 14.4 Gy in divided doses, we unexpectedly found that patients with AUCCEPM in the highest quartile of those studied had higher incidence of SOS, a 5.9-fold greater risk of nonrelapse mortality at 1 year and an overall survival of 50% less than patients with AUCCEPM in the lowest quartile. No such dependence on AUC CY and AUCHCY was observed (McDonald et al., 2002).

CEPM is nontoxic. Indeed, formation of CEPM from HCY contributes to the relative resistance of early hematopoietic precursors and L1210 leukemia cells from injury after administration of CY (Hilton, 1984; Sladek and Landkamer, 1985; Kohn and Sladek, 1987). On the other hand, CEPM is the only major chemically stable metabolite formed between the activation of CY and the ultimate formation of glutathione conjugates of acrolein and phosphamide mustard. A reasonable hypothesis is that CEPM reports on the intrahepatic (intrahepatic) exposure to reactive toxic species formed from HCY. For this to be true, however, intrahepatic cellular AUCCEPM must be high when intrahepatic cellular AUCHCY is high. Such a relationship requires a substantial route of elimination of HCY from the liver that competes with the formation of CEPM.

Iminocyclophosphamide (Fig. 1) has been reported to undergo reversible conjugation with glutathione chemically and enzymatically (Kwon et al., 1987; Lee, 1991; Dirven et al., 1994a,b). The resulting conjugate, 4-glutathionylcyclophosphamide (GSCY) is a potential substrate of ABCC2, a member of the multidrug resistance protein family (subsequently designated ABCC), localized on the canalicular membrane of hepatocytes. ABCC2 exports organic anions, including glucuronide, glutathione, and sulfate conjugates into bile (Konig et al., 1999; Borst et al., 2000; Ishikawa et al., 2000). We hypothesized that transport of GSCY by ABCC2 from the hepatocyte into bile competes with oxidation of HCY to CEPM and β-elimination to form acrolein. The more efficient the formation and export of GSCY, the less would be the hepatic exposure to HCY, CEPM, and acrolein.

We evaluated this hypothesis by contrasting the elimination of CY metabolites in TR—rats with wild-type Wistar.
rads. The TR—rat, a mutant Wistar, has a 1-base pair deletion in a position corresponding to amino acid 393 of ABCC2. This deletion results in a premature stop codon at amino acid 401 and degradation of the truncated protein product before it localizes in the canalicular membrane (Kusuhara et al., 1998).

The results herein demonstrate that GSCY is a substrate of ABCC2. Biliary excretion of GSCY via ABCC2 resulted in a dose-dependent lower accumulation of HCY in the liver and a lower AUC<sub>CEPM</sub> in plasma, key consequences of the hypothesis. Unfortunately, there is no animal model of CY-induced SOS.

Materials and Methods

Materials. CY was purchased from Sigma-Aldrich (St. Louis, MO). 4-Hydroperoxycyclophosphamide was a generous gift from Dr. Scott Rowley (Fred Hutchinson Cancer Research Center, Seattle, WA); identity was confirmed in our laboratory by NMR. CEPM was purchased from Alexis (San Diego, CA). M<sub>2III-6</sub> purchased from Alexis (Ronkonkoma, NY). All other chemicals and solvents were of reagent grade or better. Parent TR—rats were a generous gift from Dr. Mary Vore (University of Kentucky, Lexington, KY). TR—rats used in this study were inbred (sister-brother matings) in the animal facility at the Fred Hutchinson Cancer Research Center, and phenotype was confirmed by Western blot with monoclonal antibody M<sub>2III-6</sub> purchased from Alexis (San Diego, CA).

In Vivo Rat Studies. Before administration of CY and the collection of blood samples, rats were anesthetized with ketamine (100 mg/kg) plus xylazine (12.5 mg/kg) administered i.p. For the collection of bile, the common bile duct was cannulated with polyethylene-10 tubing. To measure urine excretion of CEPM, ureters were cannulated with polyethylene-50 tubing. CY (100 or 200 mg/kg) in 0.9% saline; two dose levels were studied because of the known nonlinearities of CY pharmacokinetics) was given i.v. as needed for measurement of different chemical species. Blood samples were split and processed for analysis of CY pharmacokinetics (Ren et al., 1998). Inhibition of ALDH-mediated CEPM formation was examined in liver cytosol from TR— and Wistar rat livers and stored at −80°C. A standard containing a fixed amount of GSCY was prepared and used to describe the relative concentration for comparison between TR— and Wistar rats.

Sample Preparation. Blood samples were split and processed for CEPM and HCY separately. For analysis of HCY, 220 μl of blood was transferred to a 0.5-ml Eppendorf tube containing derivatizing solution (150 mM pH 3.5 citrate/pNP, 1:1), sealed, inverted several times, and centrifuged at 5800g for 60 s. Two hundred and fifty microliters of supernatant was transferred to a 1.5-ml Eppendorf tube containing 250 μl of pNP solution and then stored at −70°C until analysis. On the day of analysis, samples and standards (concentration from 5 to 90 μM for the low dose and 18 to 180 μM for the high dose) were thawed at room temperature and extracted according to a previously described method (Slattery et al., 1996). The samples were finally reconstituted in 100 μl of acetoni-trile/water (1:2) and subjected to HPLC analysis.

For analysis of CEPM in plasma, 80 μl of blood was centrifuged at 5800g for 60 s. Plasma was removed and frozen at −70°C until analysis. Standards of CEPM in plasma were prepared by spiking authentic CEPM in blank plasma. Final concentrations of CEPM standard ranged from 1.5 to 68 μM. On the day of analysis, plasma samples and standards were thawed and deproteinized by addition of 3 volumes of acetonitrile/methanol (4:1). Samples were spun at 4°C for 20 min, and the supernatant was transferred to a 200-μl injection vial and subjected to LC-MS. Bile samples were diluted 20-fold with 30 mM ammonium acetate (pH 5.0) and then analyzed by LC-MS.

To measure HCY in livers, approximately 0.2 g of liver was homogenized in 1 ml of 100 mM potassium phosphate buffer (pH 7.4). One-half milliliter of homogenate was derivatized with 1.5 ml of derivatizing solution and then extracted following the procedure described above. Analytes were reconstituted in 100 μl of acetonitrile/water (1:2) and subjected to HPLC analysis. For GSCY, 0.2 g of liver was homogenized in 1 ml of 30 mM ammonium acetate buffer (pH 5.0). The homogenate was deproteinized as described above for plasma samples and analyzed by LC-MS. Urine samples were diluted 100-fold in ammonium acetate buffer (30 mM, pH 4.0). Standards were prepared in blank urine (diluted 100-fold with buffer) to yield final concentrations from 2 to 200 μM for CEPM. Five hundred microliters of the diluted sample or standard was extracted twice with 2.5 ml of ethyl acetate. Two milliliters of ethyl acetate from each extraction was removed, combined, and evaporated under nitrogen. Samples were reconstituted in 1 ml of mobile phase and analyzed by LC-MS.

HPLC Analysis. Final reconstituted HCY blood samples were injected on a Rainin 4.6 × 100-mm microsorb C18 column (Rainin, Woburn, MA) and eluted with conditions described elsewhere (Slattery et al., 1996). The effluent was monitored at 400 nm. Final reconstituted HCY liver samples were injected onto a 4.6 × 250-mm Econosil C8 column (Alltech, Deerfield, IL). The mobile phase consisted of 22% acetonitrile, 25% methanol, and 53% buffer (25 mM ammonium phosphate, 0.15% acetic acid, pH 5.0), delivered at a rate of 1.1 ml/min. The retention time of HCY was 20 min and the total run time was 30 min.

LC-MS Analysis. The LC-MS was a Hewlet Packard 1100 LC system coupled with an MSD-1100 mass selective detector. Samples were injected on a Zorbax R<sub>C</sub> 8 2.1 × 150-mm column. The mobile phase was 40% methanol and 60% buffer (pH 5.0, 30 mM ammonium acetate), delivered at a rate of 0.2 ml/min. Single ion channels m/z 261 and 293 were monitored for CY and CEPM, respectively. GSCY in samples was detected in ion channel m/z 566. Although we did not have authentic GSCY as a standard, the identity of the putative GSCY peak was confirmed by the mass of the parent ion. The base peak at m/z 566 is consistent with (GSCY + H)<sup>+</sup>, where both chlorides are lost. In addition, the relative intensity of the signals at m/z 566, 568, 570, 572, and 576 was consistent with a molecule containing two chlorides. Finally, GSH degraded from GSCY occurring at the same retention time as the GSCY signal was always monitored as a confirmatory peak for GSCY in ion channel m/z 308. The linear range of the GSCY signal with amount injected was established with a lower limit of 10% of the lowest area observed on a sample chromatogram. A standard containing a fixed amount of GSCY was prepared and stored at −80°C. This reference sample was used to correct the interday variation introduced by sample preparation and MSD response. Peak area or peak height of the GSCY peak on LC-MS was used to describe the relative concentration for comparison between TR— and Wistar rats.

In Vitro Enzyme Activity. Activities of several enzymes in either rat liver cytosol or microsomes were examined. Liver cytosol and microsomes were prepared by differential centrifugation from frozen TR— and Wistar rat livers and stored at −70°C (Gibbs et al., 1996). Cytosolic GST activity was measured by using 1 mM chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). Kinetics of ALDH-mediated CEPM formation was examined in liver cytosol according to a method described elsewhere (Ren et al., 1998). Inhibition of ALDH activity after CY treatment was studied ex vivo with indole-3-acetaldehyde as substrate (Ren et al., 1998). Microsomal P<sub>450</sub> oxidation of CY to HCY was examined at 0.1, 0.5, and 2 mM CY, respectively, in an incubation system containing 1 mg/ml liver mi-
crosomal protein and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) (Ren et al., 1997). Protein concentrations in liver homogenate, cytosol, and microsomes were determined with the Bio-Rad (Oakland, CA) protein assay kit with bovine serum albumin as the standard.

**Data Analysis.** All data are represented as mean ± S.D. AUC from 0 to 3 h after administration of CY in plasma and liver was determined by noncompartmental analysis. The cumulative amount of CY, CEPM, and GSCY excreted in bile over time was taken to be the product of the peak area per microliter of bile and bile volume (microliters). Biliary and renal clearance of CEPM were calculated by dividing the total amount excreted in bile or urine over 3 h by plasma AUC_CEPM. Comparison of AUC in plasma between TR− and Wistar rats was done by Student’s t test. Concentration-time curves of HCY and GSCY in liver were compared between TR− and Wistar rats by two-way ANOVA using SPSS 10.0 (SPSS Science, Inc., Chicago, IL) because the data resulted in a single value of AUC for each strain. All other comparisons were performed by Student’s t test. p < 0.05 was considered significant.

**Results**

The time course of cumulative biliary excretion of GSCY and liver GSCY after i.v. administration of CY is shown in Fig. 2. Figure 2, A and C, show that GSCY was abundant in the bile of Wistar rats, but it was not detectable in the bile of TR− rats after both 100- and 200-mg/kg doses. Figure 2, B and D, show that GSCY accumulated in the livers of TR− rats to a much greater extent than in the liver of Wistar rats. AUC_{GSCY} in the liver of TR− rats was 2.03 times the value of Wistar rats after 100 mg/kg CY and 2.45 times the value of Wistar rats after 200 mg/kg (data not shown).

Because bile flow in TR− rats is known to be substantially less than Wistar rats (Jansen et al., 1985), we examined the potential role of bile flow on the results reported in Fig. 2. At 100 mg/kg, bile flow in TR− rats was one-third that of Wistar rats (32 ± 4 μl/min/kg versus 99 ± 12 μl/min/kg, p < 0.001) (Table 1). Total biliary excretion of CY and CEPM over 3 h in TR− rats was proportionally decreased (p < 0.001), whereas that of GSCY was abolished, indicating that diminished bile flow could not account for the lower biliary excretion of GSCY observed in TR− rats. The trend after 200 mg/kg CY was similar to the low dose (Table 1).

Figure 3 shows the time course of HCY in liver and liver AUC_{HCY}. After 100 mg/kg CY, HCY concentrations in liver over 3 h were moderately greater in TR− than in Wistar rats (p = 0.027; Fig. 3A) and liver AUC_{HCY} was 24% greater in

![Fig. 2.](image-url)  
Cumulative biliary excretion of GSCY and GSCY in liver over 3 h after CY administration in TR− and Wistar rats. Wistar rats (squares) and TR− rats (diamonds) received CY via femoral vein at 100 mg/kg (A and B) and 200 mg/kg (C and D) respectively. In A and C, bile was collected serially over 3 h from individual anesthetized rats. Each point represents the mean ± S.D. (n = 5–6). In B and D, rats were killed at various time points after administration of CY. Liver was removed and homogenized for analysis of GSCY. Each point represents the mean ± S.D. (n = 4). GSCY in liver in TR− was greater than that in Wistar rats (two-way ANOVA, p < 0.001 for both 100 and 200 mg/kg).
TR/H11002 rats (Fig. 3B). After 200 mg/kg CY, the concentrations of HCY in liver were substantially greater in TR/H11002 than in Wistar rats \( (p \leq 0.007; \text{Fig. 3C}) \) and liver AUC HCY was 46% greater in TR/H11002 rats (Fig. 3D).

The time course of CEPM and HCY in plasma after administration of 100 and 200 mg/kg CY i.v. is shown in Fig. 4. Plasma concentrations of CEPM were higher in TR rats after 100 mg/kg CY (Fig. 4A), but they were not different from Wistar rats after 200 mg/kg CY (Fig. 4C). In contrast, although the peak blood concentration of HCY was slightly higher in TR rats \( (48 \pm 20 \mu M) \) than in Wistar rats \( (32 \pm 11 \mu M) \), the difference was not statistically significant after 100 mg/kg CY (Fig. 4B); while given 200 mg/kg CY, blood concentrations of HCY were higher in TR rats than in Wistars (Fig. 4D). Plasma AUC CEPM in TR rats was 1.8 times that in Wistar rats after 100 mg/kg CY \( (p < 0.001; \text{Fig. 5A}) \), whereas AUC HCY was not different (Fig. 5B). After 200 mg/kg CY,

<table>
<thead>
<tr>
<th>CY Dose</th>
<th>Bile Flow ( \mu l/min/kg )</th>
<th>GSCY*</th>
<th>CY*</th>
<th>CEPM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg/kg</td>
<td>TR −</td>
<td>32 ± 4</td>
<td>ND</td>
<td>153.2 ± 61.7</td>
</tr>
<tr>
<td>Wistar</td>
<td>99 ± 12</td>
<td>44.5 ± 8.6</td>
<td>ND</td>
<td>415.6 ± 57.6</td>
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<tr>
<td>Ratio (Wistar/TR −)</td>
<td>3.1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>p Value*</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>200 mg/kg</td>
<td>TR −</td>
<td>36 ± 4</td>
<td>ND</td>
<td>392.4 ± 43.3</td>
</tr>
<tr>
<td>Wistar</td>
<td>96 ± 12</td>
<td>128.8 ± 35.5</td>
<td>ND</td>
<td>1223.2 ± 118.3</td>
</tr>
<tr>
<td>Ratio (Wistar/TR −)</td>
<td>2.7</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>p Value*</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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ND, not detectable.

Cumulative amount excreted in bile was calculated by multiplying peak area under LC-MS in 1 \( \mu l \) of bile by volume of bile (microliters) with normalization by rat body weight (grams). The unit is \( \times 10^3 \) peak area per gram of body weight.

\(^{*}p \text{ Value was determined by Student's } t \text{ test between TR− and Wistar rats.}\)
plasma AUC\textsubscript{CEPM} was not different between rat strains (Fig. 5A), whereas plasma AUC\textsubscript{HCY} was approximately 1.4-fold greater in TR\textsuperscript{−}/H11002 rats (p = 0.005; Fig. 5B). There are two potential reasons for the diminished difference between TR\textsuperscript{−} and Wistar rat with regard to CEPM in plasma: 1) a saturation of ALDH attributable to a \( K_m \) in the range of concentrations at the enzyme, and 2) inhibition of ALDH by acrolein (a known inhibitor of ALDH) in TR\textsuperscript{−} rats at 200 mg/kg CY. The value of \( K_m \) of CEPM formation from HCY was determined in cytosol from rat livers. The values of \( K_m \) were 48 ± 4.3 and

Fig. 4. Time course of CEPM and HCY concentration in plasma after i.v. administration of CY at 100 mg/kg (A and B) and 200 mg/kg (C and D) in Wistar rats (squares) and TR\textsuperscript{−} rats (diamonds). Blood samples were drawn over 3 h from anesthetized rats as in Fig. 2. Each point represents the mean ± S.D. (n = 5–8).

Fig. 5. Plasma AUC\textsubscript{CEPM} (A) and AUC\textsubscript{HCY} (B) after administration of CY at 100 and 200 mg/kg in TR\textsuperscript{−} (open columns) and Wistar rat (solid columns). Each column represents the mean ± S.D. (n = 5–8). **, p < 0.005 by \( t \) test.
64 ± 22 μM for TR− and Wistar rats, respectively (data not shown). At 200 mg/kg CY, the peak plasma concentration of HCY in TR− is 145 ± 38 μM, which is greater than that in the Wistar rat, 81 ± 15 μM (data not shown). Because acrolein is a potent inhibitor of ALDH, and more acrolein is formed from 200 mg/kg CY than from 100 mg/kg (an expectation based on the greater abundance of HCY in liver after the higher dose; Fig. 3), we measured the residual ALDH activity using indo-3-acetaldehyde as the substrate in liver cytosol in the respective strains 3 h after the administration of CY. The result is shown in Fig. 6. ALDH activity in TR− was reduced by 40% after 200 mg/kg CY (p < 0.005), whereas the activity was inhibited by 22% after 100 mg/kg CY (p = 0.057). In contrast, there is no significant change of ALDH activity in Wistar rats after both 100 and 200 mg/kg CY.

To evaluate the possible role of interstrain differences in cytochrome P450 activity in the oxidation of CY to HCY, ALDH activity in the oxidation of aldocyclophosphamide to CEPM, and the formation of GSCY from iminocyclophosphamide by GST, we determined enzymatic activities for the formation of HCY and CEPM at several concentrations of CY and HCY, chosen to be relevant to the in vivo studies. GST activity in rat liver cytosol was measured with the probe substrate CDNB. Table 2 shows that critical enzyme activities did not differ between the two strains of rat. Because CEPM is mainly eliminated by excretion (Sladek, 1994; Yule et al., 1995), renal and biliary clearance of CEPM after CY administration was determined. Although the biliary clearance of CEPM was less in TR− rats than in Wistar rats, this route is minor in comparison with renal clearance, which was not different between the strains (Table 2). Total excretory clearance of CEPM (renal clearance plus biliary clearance) was similar in the two strains of rat (Table 2).

**Fig. 6.** ALDH activity remaining in livers of TR− and Wistar rats after CY administration at 100 mg/kg (solid columns) and 200 mg/kg (hatched columns) compared with untreated rats (open columns). Rats were killed 3 h after CY was given intravenously and liver was removed. Liver cytosol was prepared and ALDH activity was measured using indo-3-acetaldehyde as substrate. Activity was expressed as a percentage of control activity in untreated rat. Each column represents mean ± S.D. (n = 4–6). *p < 0.03 by t test.

**Discussion**

Based on the clinical observation of a positive correlation between the plasma AUC of a nontoxic metabolite, CEPM, and both the development of liver toxicity and an approximately 6-fold greater risk of nonrelapse mortality (McDonald et al., 2002), we hypothesized a new route for elimination of HCY from the liver which would compete with CEPM formation and thereby render plasma AUC_{CEPM} a reporter of hepatic exposure to the toxins produced from HCY. The results show that GSCY transport from hepatocytes by ABC2 fulfills this role and results in the pharmacokinetic observations made clinically once the dose dependence of CEPM formation observed in rats is taken into account.

GSCY was abundant in the bile of Wistar rats and absent from the bile of TR− rats, whereas TR− rats actually had more GSCY in liver (Fig. 2). Although the diminished bile flow of the TR− rat could account for lower biliary elimination of CY and CEPM in comparison with Wistar rats, the lower biliary elimination of GSCY in TR− rats was much less than could be accounted for by the lower bile flow (Table 1). Similarly, formation of HCY and CEPM were not different between strains, nor was hepatic GST activity (Table 2). Together, these results strongly implicate GSCY as a substrate of ABC2.

Absence of ABC2 resulted in a higher AUC_{HCY} in the liver, the relative difference being greater at 200 than 100 mg/kg CY (Fig. 3). Thus, the exposure of the liver to HCY (and toxins formed from it) is a function of ABC2 activity, as hypothesized. At 100 mg/kg CY, the higher hepatic AUC_{HCY} was reflected in a higher plasma AUC_{CEPM} (Fig. 5), whereas at 200 mg/kg CY the higher liver AUC_{HCY} was reflected in a higher plasma AUC_{HCY}. The dose dependence of the systemic expression of the hepatic AUC_{HCY} is consistent with the K_{m} of rat liver cytosolic activity of ALDH, 50 to 60 μM (data not shown). At 100 mg/kg CY, peak plasma HCY concentration was 32 μM in Wistar rats and 48 μM in TR− rats. At 200 mg/kg CY, the corresponding peak plasma HCY values were 81 and 146 μM. Thus, after 200 mg/kg CY, it seems that HCY concentrations were sufficient to begin to saturate ALDH, whereas they were less than the K_{m} after 100 mg/kg CY. We also observed a dose dependence in the inhibition of hepatic cytosolic ALDH activity in TR− rats, but an absence of inhibition in Wistar rats. Because acrolein is a potent inhibitor of ALDH in vitro (Mitchell and Petersen, 1993), previously shown to inhibit the enzyme ex vivo under conditions similar to this study (Ren et al., 1998), this result is consistent with greater hepatocellular exposure to acrolein in TR− rats, with exposure increasing as CY dose and ALDH inhibition increased. The dose-dependent inhibition of ALDH would, of course, also diminish the likelihood that higher hepatic AUC_{HCY} would be reflected in higher plasma AUC_{CEPM} after 200 mg/kg CY.

In the patients being treated with 60 mg/kg CY in whom the relationship between AUC_{CEPM} and both enhanced liver toxicity and nonrelapse mortality was discovered, peak HCY

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1 Evaluation of the transport of GSCY was limited to in vivo studies by the complexity of the metabolism required to produce GSCY in cells. ABC2-transfected Madin-Darby canine kidney (a common model for ABC2 transport) is unable to form HCY from CY and have insufficient GST activity to efficiently form GSCY from HCY. Our unpublished data. Because GSCY is too polar to cross the membrane, it cannot be loaded into cells as such.
concentration in plasma was approximately 8 µM on day 1 of therapy and 16 µM on day 2, in comparison with an average \( K_{\text{m}} \) of 13 µM for HCY as a substrate of human liver cytosolic ALDH. In these patients, greater toxicity accompanied higher plasma AUC\text{CEPM}, hypothesized to reflect higher hepatic exposure to HCY and subsequent toxins. Thus, the low-dose observations in rats seem to be most consistent with the observations in human beings. The high-dose results in rats suggest that diminished ALDH activity enhances hepatic exposure to HCY and could compromise the utility of plasma AUC\text{CEPM} as a useful clinical index of potential toxicity. Aldehyde dehydrogenase-1A1, the isoenzyme that oxidizes HCY (as the tautomer aldocyclophosphamide) to CEPM, has been reported to be polymorphic in humans based on ethanol metabolism, but the incidence of genetic deficiency seems to be very low (Vasiliou and Pappa, 2000).

We conclude 1) that GSCY is a substrate of ABCC2; 2) that diminished ABCC2 activity results in greater hepatic exposure to GSCY, and, as a consequence, HCY and its cytotoxic chemical degradation products acrolein and phosphoramide mustard; and 3) that enhanced hepatic exposure to HCY and its degradation products is reflected in higher plasma AUC\text{CEPM} when hepatic ALDH is not saturated by its substrate HCY and is not inhibited (presumably by acrolein) or by greater plasma AUC\text{ICY} under the conditions of ALDH saturation and/or inhibition. Our results strongly suggest that CEPM serves as a chemically stable chemical reporter of hepatic exposure to HCY and its toxic degradation products in human beings undergoing high-dose CY in preparation for HSCT.

It is desirable to correlate the findings in the ABCC2 deficient rat with enhanced liver toxicity in this rat to CY. However, there is no successful animal model of SOS after administration of CY with or without irradiation (Allen et al., 1969; Shulman et al., 1987; DeLeve et al., 1999). High-dose CY in combination with TBI followed by bone marrow transplantation in rats results in dose-limiting toxicities of other systems: hemorrhages in the gastrointestinal tract, the lungs, and the urinary bladder or cardiotoxicity (Hagenbeek and Martens, 1982, 1983). This has hampered the direct evaluation of correlation between CY metabolism and liver toxicity in rats. A clinical study using pharmacokinetically guided dosing for CY to directly test the hypothesis that decreasing exposure of CEPM will reduce the incidence of SOS in transplant patients receiving CY-TBI has been initiated.

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### References


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