INHIBITION OF GLUTATHIONE CONJUGATION BY GLUTATHIONE ANALOGUES IN THE PERFUSED RAT LIVER

Effect of Esterification on the Potency of $\gamma$-L-GLUTAMYL-$\alpha$-(D-2-AMINO ADIPYL)-N-2-HEPTYLAMINE

SIVI OUWERKERK-MAHADEVAN, ROMMEL G. TIRONA, RICHARD A. RIPPING, JAN H. T. M. PLOEMEN, PETER J. VAN BLADEREN, K. SANDY PANG, JACQUES H. VAN BOOM, AND GERARD J. MULDER

Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University (S.O.-M., R.A.R., G.J.M.); Division of Organic Chemistry, Leiden University, Leiden (R.A.R., J.H.B.); Division of Toxicology, TNO Nutrition and Food Research (J.H.T.M.P., P.J.B.); and Faculty of Pharmacy, University of Toronto (R.G.T., K.S.P.)

ABSTRACT:

To assess the role of GST's (glutathione S-transferases) in the (de)toxicification of their substrates, an in vivo active inhibitor based on the structure of glutathione (GSH), $\gamma$-L-glutamyl-$\alpha$-(D-2-amino adipyl)-N-2-heptylamino monoethyl ester (Et-R-Hep), was developed. To increase its effectiveness, analogues esterified with alkyl chains of varying lengths and one diesterified derivative (DiEt-R-Hep) were synthesized. The unesterified analogue, R-Hep, was also tested. Their isoenzyme selectivity was characterized using purified rat GST isoenzymes. Furthermore, the extent of inhibition of the GSH conjugation of (RS)-2-bromoisovalerylurea (BIU) was evaluated in rat liver cytosol, isolated hepatocytes, and in liver perfusions. All compounds inhibited Alpha- (1-1 and 2-2) more effectively than Mu (3-3 and 4-4) class GSTs; Pi-(5-5) and Theta (7-7) classes were minimally inhibited. The unesterified R-Hep was the most effective inhibitor towards purified isoenzymes; its $K_i$ value towards GST 3-3 (S-BIU as substrate) was 27 $\mu$M. The monoethyl ester derivative, Et-R-Hep (K, 270 $\mu$M for 3-3), was the most potent inhibitor in hepatocytes and in the perfused liver: 50 $\mu$M inhibited the conjugation of (S)-BIU by 50%. Longer ester chains of diesterification did not increase the inhibitory potency; R-Hep had less inhibitory activity. In all systems, only the (S)-enantiomer of BIU, which is conjugated mainly by Alpha class GSTs, was inhibited, confirming Alpha isoenzyme selective inhibition.

GSTs1 (glutathione S-transferases; EC 2.5.1.18) are a family of phase II biotransformation enzymes that provide protection against a wide range of electrophilic compounds. Four major classes of cytosolic GST can be distinguished: the Alpha, Mu, Pi, and Theta class (2, 3) which have different but overlapping substrate specificities. GSTs catalyze the conjugation of the endogenous tripeptide glutathione (GSH; $\gamma$-L-glutamyl-$\alpha$-cysteinylglycine) to compounds containing an electrophilic center. These may be either xenobiotics, like drugs, or endogenous compounds, such as products of lipid peroxidation (4–6). Recent studies indicate a role of GSTs in cancer chemotherapy; resistance to alkylating agents is frequently associated with increases in the levels of GSH and GST (7).

In vivo inhibitors of GST are valuable tools for characterizing the role of GSTs in drug resistance and toxicity of xenobiotes. However, most of the existing inhibitors are either too toxic to be used in vivo or are only effective in vitro (7). Moreover, none of these inhibitors is specific towards a particular GST isoenzyme. Recently, the diuretic drug ethacrynic acid has been used as an inhibitor of GST in vivo (8). However, because it is a substrate for GSH conjugation, it causes GSH depletion, which by itself may have toxic consequences (7). Therefore, there is a need for developing more selective in vivo effective GST inhibitors that would leave GSH levels unaffected.

We have recently shown that the GSH analogue Et-R-Hep ($\gamma$-L-glutamyl-$\alpha$-(D-2-amino adipyl)-N-2-heptylamino monoethyl ester; systematic name: (R)-5-ethylxycarbonyl-2-$\gamma$-(S)-glutamylamino-N-2-heptylpentamide (fig. 1A)), is an effective inhibitor of GST isoenzymes of the Alpha and Mu class in the rat in vivo (9, 10). In this compound, the $\gamma$-cysteinylglycine moiety of GSH was replaced by D-Aad (D-amino adipic acid), to obtain a metabolically more stable analogue. To increase its cellular uptake, the lipophilicity of the compound was increased by introducing an ethyl ester on the $\delta$-carboxyl group on D-Aad, corresponding to the carboxyl group of glycine in GSH (see figs. 1 A and C). Et-R-Hep and a few other analogues inhibited the Alpha GSTs more effectively than the Mu isoenzymes (9). This was confirmed by experiments in which the GSH conjugation of (R,S)-2-bromoisovalerylurea (BIU) was measured in rat liver cytosol and hepatocytes: the conjugation of only the (S)-BIU enantiomer, which is preferentially conjugated by the Alpha GSTs, was inhibited. The conjugation of (R)-BIU, which is mainly catalyzed by the Mu GSTs, was unaffected (10).

We report on the effect of esterification of the R-Hep backbone (see fig. 1) on the potency of the resulting esters as GST inhibitors in the

1 Abbreviations used are: GST, glutathione S-transferase; GSH, glutathione; Et-R-Hep, (R)-(S)-ethylxycarbonyl-2-$\gamma$-(S)-glutamylamino-N-2-heptylpentamide; D-Aad, ($\alpha$-amino adipic acid; BIU, 2-bromoisovalerylurea; (R)- and (S)-I-U-G, GSH conjugates of (S) and (R)-BIU, respectively; R-Hep, (R)-5-carboxy-2-$\gamma$-(S)-glutamylamino-N-2-heptylpentamide; Glu-Oct-Hep, 2-$\gamma$-(S)-glutamylamino-N-2-heptyloctamide; BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane; Do-R-Hep, (R)-5-dodecylxycarbonyl-2-$\gamma$-(S)-glutamylamino-N-2-heptylpentamide; Pt-R-Hep, (R)-5-pentyloxycarbonyl-2-$\gamma$-(S)-glutamylamino-N-2-heptylpentamide; DiEt-R-Hep, (R)-5-ethylxycarbonyl-2-$\gamma$-ethylene oxycarbonyl-(S)-glutamylamino-N-2-heptylpentamide; (S)-Bl, (S)-bromoisovaleric acid; (R)-I-G, GSH conjugate of (S)-Bl.

The work was supported by a grant from the Netherlands Organization for Scientific Research (NWO; reg. no. 900-21-142).

Send reprint requests to: G. J. Mulder, Ph.D., Division of Toxicology, LACDR, Leiden University, P. O. Box 9503, 2300 RA Leiden, The Netherlands.

Send reprint requests to:

group of the glutamic acid moiety to form the diester (fig. 1A). A derivative of Et-R-Hep in which the ethyl ester was incorporated in the D-Aad backbone structure (i.e. the COO-C₂H₅ replaced by a C₃H₇, as in Glu-Oct-Hep) was also tested (fig. 1B). This was based on the earlier finding (14) that the glycine-carboxyl group of GSH was not required for effective conjugation by GSTs; glycine could be replaced by ethylamine. Therefore, the Glu-Oct backbone might be a simple alternative to the esterified Glu-Aad backbone, the more so because it was expected to be at least equally lipid soluble as the latter esterified compound.

The effect of these modifications on the isoenzyme specificity of the compounds towards purified rat GST isoenzymes was characterized, as well as their inhibitory potency in isolated hepatocytes and in rat liver cytosol using (R,S)-BIU as substrate for conjugation. In addition, we have studied their effect on GSH conjugation in the recirculating in situ rat liver perfusion.

Materials and Methods

Materials. Racemic BIU was purchased from Brocacef (Maarsen, The Netherlands). The (R) and (S) enantiomers of BIU were prepared as described earlier (15). The HPLC standard (RS)-IU-G was prepared as described elsewhere (16). D-Aad, L-methionine, and BSA (Fraction V) were from Sigma (St. Louis, MO). CDNB was from Janssen Chimica (Beere, Belgium). EPNP was purchased from Eastman Kodak Co. (Rochester, NY). The inhibitors R-Hep, Et-R-Hep, and Do-R-Hep were prepared as described earlier (9). The other inhibitors were synthesized by similar methods published in detail in (17).

Animals. Male Wistar rats (CRL: WI/WU BR; SPF quality; 280–350 g body weight; liver weight 9–16.7 g) obtained from Charles River (Sulzfeld, Germany) were used for the study. They had free access to tap water and a commercial diet (SRM-A; Hope Farms, Woerden, The Netherlands).

Inhibition of rat GST Isoenzymes. Rat GST isoenzymes were isolated from liver and kidney as described by Vos et al. (18) and Mannervik et al. (19). Rat Theta enzyme was prepared as described by Meyer et al. (21).

The rate of enzymatic conjugation of CDNB (1 mM) with GSH (1 mM) in the presence of 20–50 nM GST, was spectrophotometrically monitored at 37°C in 0.1 M potassium phosphate buffer pH 7.4 containing 1 mM EDTA and corrected for nonenzymatic reaction rate (22). The reaction rate was determined in the presence and absence of 100 and 500 μM of the inhibitors, which were added just before CDNB. Since Do-R-Hep was insoluble at 500 μM, measurements were only performed at 100 μM. All inhibitors were dissolved in DMSO (final concentration < 3% (v/v)). The assay for GST 5–5 was performed using 0.5 mM EPNP as electrophilic substrate and 10 mM GSH (21).

Incubations with Rat Liver Cytosolic GSTs. Rat liver cytosol (4 mg/ml (prepared as described in (9)) was incubated with 50 μM of the inhibitors (in DMSO) or solvent (final concn of DMSO 1.7%v/v), 1 mM GSH and 250 μM (R)- or (S)-BIU in 50 mM sodium phosphate buffer, pH 7.4 at 37°C for 3 hr. To an aliquot (250 μl) of the incubation mixture 500 μl ice-cold HPLC buffer A containing 60% (v/v) methanol was added. This mixture was centrifuged and the resulting supernatant was stored at -20°C until analysis by HPLC within 7 days.

Incubations with Isolated Hepatocytes. Hepatocytes were isolated by collagenase perfusion as described earlier (23). The inhibitors were dissolved in methanol/chloroform = 1/4 (v/v) and added to empty plastic incubation vials such that their final concentration in the incubations would be 50 μM; then the solvent was evaporated under a stream of nitrogen. Incubations were performed as described previously (9): Cells (diluted to 3×10⁶ cells/ml with Hanks/Hepes buffer, pH 7.4, containing 1.5% (w/v) BSA) were added to the vials and preincubated with 1 mM L-methionine for 30 min. The experiment was initiated by adding a solution containing either (R)- or (S)-BIU to the cell suspension such that the final concentration of substrate was 250 μM in an incubation volume of 3 ml. The incubations were performed in a rotary shaker (205 rpm under an atmosphere of 95% O₂/5% CO₂). Samples of the incubation mixture (250 μl) were taken at 180 min and added to 500 μl of ice-cold HPLC buffer A containing 60% (v/v) methanol to precipitate proteins.
These were removed by centrifugation prior to HPLC analysis. The viability of the cells was assessed before and after the experiment by Trypan Blue exclusion and was always > 85%. The GSH content of these cells, as determined by Ellman’s reagent (26), was always between 3.3 and 4.0 µmol/ml packed hepatocytes.

Recirculating in Situ Rat Liver Perfusions. Male rats were used as liver donors. The surgery was conducted under pentobarbital anesthesia (60 mg/kg) as described previously (25) with a few modifications. The perfusion medium consisted of 20% (v/v) washed bovine red blood cells (courtesy of Slachthuis Leiden b.v., Leiden, The Netherlands), 1% (w/v) BSA, 0.3% (w/v) glucose in Krebs-Henseleit bicarbonate solution, buffered to pH 7.4. Perfusate was oxygenated with 95% oxygen: 5% carbon dioxide and kept at 37° in a 500 ml rotating reservoir (MX International, Aurora, CO). Liver viability during the perfusion was assessed on the basis of gross appearance, bile flow rate, and oxygen consumption rate. The GSH content of the liver at the end of the perfusion ranged from 2.9 to 4.8 µmol/g of liver (mean of 3.7 ± 0.6) as determined by Ellman’s reagent (26).

After surgery, the livers were perfused in single pass fashion for 10 min to stabilize the liver. Thereafter, perfusion was conducted for 105 min under a constant flow (10 ml/min) in a recirculating fashion: the venous outflow from the liver was returned to the reservoir (total perfusate volume was 100 ml). Immediately after onset of recirculation, the inhibitor in 1 ml of 0.1 M HCl/saline (controls received only saline) was added to the reservoir, to yield a final concentration of 50 µM; for Et-R-Hep, an additional concentration of 500 µM was used. The pH of the perfusion medium was not affected by the addition of the acidic inhibitor solution. Ten min later, 25 µmol (RS)-BIU, dissolved in 10 ml Krebs-Henseleit bicarbonate solution, was added to the reservoir, to result in the initial BIU concentration of 250 µM.

Bile was collected in fractions after addition of BIU. At the end of the experiment, perfusate was sampled and analyzed for the presence unchanged BIU and GSH conjugates. Bile and perfusate samples were stored at −30° until analysis.

Quantitation of the GSH Conjugates of BIU. The GSH conjugates of BIU were analyzed according to the HPLC method with electrochemical detection as described by Te Koppele et al. (27) with minor modifications. The separation of the diastereomeric GSH conjugates of BIU was performed using a glass Inertsil ODS-2 column (100 × 3 mm i.d.) from Chrompack (Bergen op Zoom, The Netherlands), fitted with a Chrompack ODS-2 guard column (10 × 2 mm). For the analysis, a Spectroflow model 400 pump (Kratos Analytical, Ramsey, NJ) equipped with a Promis II autoinjector (Spark Holland, Emmen, The Netherlands) was used. The eluent used consisted of Buffer A/methanol = 82/18 (v/v) at a flow rate of 0.45 ml/min. Buffer A contained 0.1 M NaNO₃, 0.01 M KBr, 0.01 M citric acid, 0.1 mM EDTA, and 0.1 mM 1-decanesulfonic acid.

Samples of the cytosol and hepatocyte incubations were diluted five times before analysis; bile samples were diluted 25 times. Of the diluted sample, 25 µl was injected onto the HPLC system and the amount of GSH conjugates present was quantified by using a solution of (RS)-IU-G as an external standard.

Determination of Octanol/Buffer Partition Coefficients

Partition coefficients were determined by dissolving R-Hep and Et-R-Hep in perfusion medium, pH 7.4 (without red blood cells and BSA), and Pt-R-Hep, Do-R-Hep, and DiEt-R-Hep in 1-octanol to a concentration of 200 µM. One ml of this solution was intensively vortexed for 1 min with one ml of 1-octanol (for R-Hep and Et-R-Hep) or perfusion buffer (the other compounds). The phases were separated by centrifugation. The amount of compound present in both phases was determined by HPLC.

Of each phase, 20 µl was injected onto a Chrompack Inertsil ODS-2 column equipped with a guard column, as described above for the analysis of BIU. The HPLC system consisted of a Promis autoinjector coupled to two Kratos Spectroflow 400 pumps controlled by a Kratos Spectroflow 450 solvent programmer. Detection was at 214 nm using a Spectroflow 783 programmable absorbance detector. The mobile phase consisted of 25 mM ammonium acetate buffer, pH 4.5 and acetonitrile. Elution was isocratic, the amount of acetonitrile varying per inhibitor, as shown in Table 1. Each compound yielded two peaks on HPLC, corresponding to two diastereomers.

Protein Binding of the Inhibitors

The extent of protein binding of the compounds in BSA containing perfusate (without red blood cells) was determined by ultracentrifugation. Perfusion (with or without BSA) was spiked with 200 µM of the inhibitors (dissolved in 0.1 M HCl/saline) and incubated for 1 hr at 37°. The perfusate (400 µl) was then centrifuged for 10 min at 1090 × g at 37° through Amicon YM1 membrane discs fitted in a MPS-1 Micropartition system (Amicon, Dronten, The Netherlands). The amount of compound present in the ultrafiltrate was determined by HPLC as described above for the determination of partition coefficients.

Statistical Analysis. The statistical significance of the results presented in figs. 2, 3, and 4 was determined using (a) an ANOVA (indicating significant differences at p < 0.05) followed by (b) Dunnett’s test (SAS system for Windows, version 6.12, PROC GLM). The significance levels are indicated by * (p < 0.05).

The statistical significance of the results presented in fig. 5 was determined using Shapiro-Wilk’s test which indicated that the data followed the normal distribution and ANOVA (which indicated overall significant differences at p < 0.05) followed by Tukey’s multiple comparison set at α = 0.05 (SAS system for Windows, version 6.12, PROC GLM). Significant differences are indicated at p = 0.05 (*).

Results

Inhibition of Rat GST Isoenzymes. To characterize their isoenzyme selectivity, the inhibitors were tested with purified rat GST isoenzymes (table 2). The analogues were most effective towards the Alpha class isoenzymes, 1–1 and 2–2; the Mu class isoenzymes 3–3 and 4–4 were inhibited to a lesser extent. Although R-Hep was the most potent inhibitor, towards both Alpha and Mu class isoenzymes (confirming earlier results (9)), the esterified analogues were quite as active as well: except for the diethylester DiEt-R-Hep, they were almost equally inhibitory towards 1–1 and 2–2 as R-Hep. However, towards the Mu isoenzymes 4–4 and, especially, 3–3 they were considerably less active than R-Hep: at 500 µM they had only 50% of the inhibitory potency of R-Hep (table 2).

Introduction of an ethyl ester at the δ-carboxyl group of D-Aad (Et-R-Hep) resulted in a slightly decreased potency towards the Alpha isoenzymes (71% to 68% inhibition for 1–1, and 100% to 62% inhibition for 2–2 at 100 µM inhibitor) but a much more pronounced decrease towards the Mu enzymes (from 51% to 4% inhibition for 3–3, and 67% to 28% for 4–4). Introduction of a second ethyl ester group at the α-carboxyl group of the glutamic acid moiety (producing DiEt-R-Hep) yielded an even less effective inhibitor, giving no more than 37% inhibition of Alpha GSTs and only 5 and 23% inhibition of Mu GSTs 3–3 and 4–4, respectively.

Glu-Oct-Hep, in which the COOC₃H₈ moiety of the D-Aad group of Et-R-Hep was replaced by a C₃H₈ straight alky chain (fig. 1), was equally potent at Et-R-Hep towards Alpha class GSTs and more effective towards GSTs of the Mu class.

## Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acetonitrile (% v/v)</th>
<th>Retention Time (min) of the Diastereomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-Hep</td>
<td>20</td>
<td>5.0</td>
</tr>
<tr>
<td>Et-R-Hep</td>
<td>25</td>
<td>11.5</td>
</tr>
<tr>
<td>Pt-R-Hep</td>
<td>40</td>
<td>4.25</td>
</tr>
<tr>
<td>Do-R-Hep</td>
<td>45</td>
<td>5.9</td>
</tr>
<tr>
<td>DiEt-R-Hep</td>
<td>35</td>
<td>4.0</td>
</tr>
</tbody>
</table>

The concentration of acetonitrile used for isocratic elution and the retention times of two peaks, corresponding to the two diastereomers of each compound, is shown.

The extent of protein binding of the compounds in BSA containing perfusate (without red blood cells) was determined by ultracentrifugation. Perfusion (with or without BSA) was spiked with 200 µM of the inhibitors (dissolved in 0.1 M HCl/saline) and incubated for 1 hr at 37°. The perfusate (400 µl) was then centrifuged for 10 min at 1090 × g at 37° through Amicon YM1 membrane discs fitted in a MPS-1 Micropartition system (Amicon, Dronten, The Netherlands). The amount of compound present in the ultrafiltrate was determined by HPLC as described above for the determination of partition coefficients.
None of the compounds inhibited GSTs 5–5 and 7–7 strongly. At 500 μM, DiEt-R-Hep inhibited the rate of conjugation of CDNB by GST 7–7 by 29%. Et-R-Hep, Pt-R-Hep, and Glu-Oct-Hep inhibited isoenzyme 5–5 by 20–27% at that concentration; since in the 5–5 assay the GSH concentration was 10 mM (in stead of the 1 mM GSH concentration in the assays for the other isoenzymes) the degree of inhibition of GST 5–5 may actually be underestimated as compared to the other isoenzymes.

The conjugation of the two BIU enantiomers was studied with pure GST 3–3. The $K_m$ values for the (R)- and (S)-BIU enantiomers were 1.5 and 5.2 mM, respectively, whereas the $K_m$ for GSH was 50 μM towards both substrates. Both R-Hep and Et-R-Hep inhibited the enzyme in a competitive fashion with both GSH and the acceptor substrates. With GSH as variable substrate the $K_i$ of R-Hep was 44 ± 6 μM (R-BIU; mean ± SD) and 27 ± 2 μM (S-BIU), whereas the $K_i$ of Et-R-Hep was 967 ± 163 μM (R-BIU) and 273 ± 19 μM (S-BIU).

Inhibition of BIU GSH Conjugation by Rat Liver Cytosol and Isolated Rat Hepatocytes. The GSH conjugation of (R)-BIU was not inhibited by any of the compounds, neither in hepatocyte incubations nor in rat liver cytosol. In contrast, all the analogues inhibited (S)-BIU conjugation (fig. 5). R-Hep and the esterified analogues, Pt-R-Hep and Do-R-Hep, were the most potent compounds in cytosol, causing concentration in the assays for the other isoenzymes) the degree of inhibition of GST 5–5 may actually be underestimated as compared to the other isoenzymes.

Livers were perfused in situ with (RS)-BIU (25 μmol) and solvent, R-Hep, Et-R-Hep or DiEt-R-Hep. The cumulative excretion of the GSH conjugates of (S)-BIU, (R)-BIU and (S)-BI in 90 min is shown as means ± SD of three experiments. * indicates values significantly different from control ($p \leq 0.05$)
were tested to determine the extent of inhibition of BIU conjugation when conjugated by GSTs to form (R)- and (S)-BIU conjugates (GSH). Intact liver was assessed in a recirculating liver perfusion model, again and hepatocytes.

Oct-Hep was by far the least effective towards BIU, in both cytosol and hepatocytes. In the perfused liver, these are excreted either into bile or via the HPLC column. In cytosol and hepatocyte experiments, in liver perfusions also, Et-R-Hep had no effect on the conjugation of (R)-BIU (fig. 2B). None of the other inhibitors had any effect on the conjugation of (R)-BIU (figs. 3 and 4).

The effect of esterification of the two carboxylate groups was assessed by comparing the effect of the unesterified compound (R-Hep), the monoethyl ester (Et-R-Hep), and the diethyl ester (DiEt-R-Hep) (fig. 3). The unesterified R-Hep caused a statistically nonsignificant decrease of 22% of the biliary excretion of the (S)-BIU conjugate. Surprisingly, the diethyl ester showed only a 13% statistically nonsignificant decrease.

The extent of inhibition of (S)-BIU conjugation by monoesters and diesters of varying chain lengths was approximately equal: a 50–60% inhibition was observed. Et-R-Hep was only slightly more effective than Pt-R-Hep and Do-R-Hep (fig. 4). Again, (R)-BIU conjugation was not affected. Glu-Oct-Hep was not evaluated in the rat liver perfusion because it was only slightly effective towards BIU in cytosol and hepatocytes.

The compounds had no statistically significant effect on the excretion of (R)-I-G, the minor metabolite of (S)-BIU (figs. 2–4). After 90 min, 42%–63% of the dose administered was recovered in bile in the form of GSH conjugates and 12–21% in the perfusate, mainly as unconjugated BIU.

**Octanol-Buffer Partition Coefficients and Protein Binding Experiments.** Octanol-buffer partition coefficients and the extent of protein binding of the compounds used in the perfusion experiments were determined (table 3). As could be expected, the unesterified R-Hep was highly water soluble, compared with the monoesterified compounds, which had higher log p values with increasing chain lengths. DiEt-R-Hep was of intermediate lipophilicity between Et-R-Hep and Pt-R-Hep.

R-Hep and its mono- and diethyl ester analogues were bound to protein to similar extents. Introduction of a longer alky chain, as in Pt-R-Hep, resulted in considerably higher protein binding. Protein binding of Do-R-Hep could not be measured because it formed aggregates that could not pass through the ultrafiltration membrane even in the absence of BSA. These aggregates had a diameter of 1000–5000 nm, as determined by photon correlation spectroscopy, using a Malvern 4700 C system (Malvern Instruments, Malvern, Worcs., UK), at 27° and a 90° angle between laser and detector.

The extent of protein binding of all compounds was stereoselective, in the perfused liver. Based on earlier in vivo studies with Et-R-Hep (10), we estimated that 500 μM would be required for sufficient inhibition. However, a 50 μM concentration produced an almost equally strong inhibition: the cumulative excretion of (R)-I-G was inhibited by approximately 50% (fig. 2A). As in cytosol and hepatocyte experiments, in liver perfusions also, Et-R-Hep had no effect on the (R)-BIU conjugation (fig. 2B). None of the other inhibitors had any effect on the conjugation of (R)-BIU (figs. 3 and 4).

**TABLE 3**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Octanol (%)</th>
<th>Buffer (%)</th>
<th>Log P</th>
<th>Protein Bound Fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1–1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2–2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3–3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4–4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>R-Hep</td>
<td>71</td>
<td>100</td>
<td>51</td>
<td>67</td>
</tr>
<tr>
<td>Et-R-Hep</td>
<td>68</td>
<td>62</td>
<td>4</td>
<td>28</td>
</tr>
<tr>
<td>Pt-R-Hep</td>
<td>76</td>
<td>74</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Do-R-Hep</td>
<td>74</td>
<td>56</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>DiEt-R-Hep</td>
<td>38</td>
<td>36</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>Glu-Oct-Hep</td>
<td>66</td>
<td>76</td>
<td>24</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>79</td>
<td>100</td>
</tr>
<tr>
<td>Et-R-Hep</td>
<td>78</td>
<td>100</td>
<td>29</td>
<td>37</td>
</tr>
<tr>
<td>Pt-R-Hep</td>
<td>100</td>
<td>100</td>
<td>38</td>
<td>39</td>
</tr>
<tr>
<td>DiEt-R-Hep</td>
<td>91</td>
<td>75</td>
<td>22</td>
<td>42</td>
</tr>
<tr>
<td>Glu-Oct-Hep</td>
<td>97</td>
<td>100</td>
<td>54</td>
<td>67</td>
</tr>
</tbody>
</table>

<sup>a</sup> Peaks 1 and 2 correspond to the diastereomers eluting in that order from the HPLC column.

<sup>b</sup> Protein binding could not be determined, because the compound formed a suspension.

<sup>c</sup> Not determined.

some 65–70% inhibition. The mono- and diethyl ester compounds Et-R-Hep and DiEt-R-Hep were somewhat less effective (45–55% inhibition).

In hepatocytes, the mono- and diester analogues were equally potent, but the unesterified, less lipophilic R-Hep, was less inhibitory (24% versus 40–45% inhibition by the esterified compounds). Glu-Oct-Hep was by far the least effective towards BIU, in both cytosol and hepatocytes.

**Rat Liver Perfusion.** The effectiveness of the inhibitors in the intact liver was assessed in a recirculating liver perfusion model, again using (R5)-BIU as substrate. (R) and (S)-BIU undergo stereoconversion when conjugated by GSTs to form (S)-I-G and (R)-I-G, respectively. In the perfused liver, these are excreted either into bile or perfusate (25, 26). (S)-BIU can also undergo amidase catalyzed hydrolysis to (S)-2-bromoisovaleric acid ((S)-BI) which can then be conjugated to its GSH conjugate (R)-I-G and excreted in bile.

Two concentrations of Et-R-Hep as added to the perfusion medium were tested to determine the extent of inhibition of BIU conjugation in the perfused liver. Based on earlier in vivo studies with Et-R-Hep (10), we estimated that 500 μM would be required for sufficient inhibition. However, a 50 μM concentration produced an almost equally strong inhibition: the cumulative excretion of (R)-I-G was inhibited by approximately 50% (fig. 2A). As in cytosol and hepatocyte experiments, in liver perfusions also, Et-R-Hep had no effect on the (R)-BIU conjugation (fig. 2B). None of the other inhibitors had any effect on the conjugation of (R)-BIU (figs. 3 and 4).

The extent of esterification of the two carboxylate groups was assessed by comparing the effect of the unesterified compound (R-Hep), the monoethyl ester (Et-R-Hep), and the diethyl ester (DiEt-R-Hep) (fig. 3). The unesterified R-Hep caused a statistically nonsignificant decrease of 22% of the biliary excretion of the (S)-BIU conjugate. Surprisingly, the diethyl ester showed only a 13% statistically nonsignificant decrease.

The extent of inhibition of (S)-BIU conjugation by monoesters and diesters of varying chain lengths was approximately equal: a 50–60% inhibition was observed. Et-R-Hep was only slightly more effective than Pt-R-Hep and Do-R-Hep (fig. 4). Again, (R)-BIU conjugation was not affected. Glu-Oct-Hep was not evaluated in the rat liver perfusion because it was only slightly effective towards BIU in cytosol and hepatocytes.

The compounds had no statistically significant effect on the excretion of (R)-I-G, the minor metabolite of (S)-BIU (figs. 2–4). After 90 min, 42%–63% of the dose administered was recovered in bile in the form of GSH conjugates and 12–21% in the perfusate, mainly as unconjugated BIU.

**Octanol-Buffer Partition Coefficients and Protein Binding Experiments.** Octanol-buffer partition coefficients and the extent of protein binding of the compounds used in the perfusion experiments were determined (table 3). As could be expected, the unesterified R-Hep was highly water soluble, compared with the monoesterified compounds, which had higher log p values with increasing chain lengths. DiEt-R-Hep was of intermediate lipophilicity between Et-R-Hep and Pt-R-Hep.

R-Hep and its mono- and diethyl ester analogues were bound to protein to similar extents. Introduction of a longer alky chain, as in Pt-R-Hep, resulted in considerably higher protein binding. Protein binding of Do-R-Hep could not be measured because it formed aggregates that could not pass through the ultrafiltration membrane even in the absence of BSA. These aggregates had a diameter of 1000–5000 nm, as determined by photon correlation spectroscopy, using a Malvern 4700 C system (Malvern Instruments, Malvern, Worcs., UK), at 27° and a 90° angle between laser and detector.

The extent of protein binding of all compounds was stereoselective,
the diastereomer eluting from the HPLC column first being bound to protein much more strongly than the second. This effect was most pronounced for Pt-R-Hep.

**Discussion**

These results indicate that against the GST isoenzymes themselves, the unesterified R-Hep is the most effective inhibitor, while in cellular systems esterified derivatives are more effective because they penetrated the cells more readily.

Esterification presumably decreases the affinity of the inhibitor for binding to the active site of GSTs. This is confirmed by the $K_i$ values of R-Hep and Et-R-Hep as determined for isoenzyme 3–3 towards (S)- and (R)-BIU: the values for R-Hep were one order of magnitude lower than those for Et-R-Hep. The DiEt-R-Hep diester would be expected to have an even lower affinity because the $\gamma$-carboxyl group of the $\gamma$-Glu moiety (which is esterified in DiEt-R-Hep) engages into an interaction with groups in the G-site of the enzyme (14). In the monoester the ethyl group presumably decreases affinity because the carboxyl group of the glycine moiety also interacts with the G-site (14). That may be the reason why Glu-Oct-Hep, the analogue of Et-R-Hep in which the ester function was replaced by a carbon-carbon bond (see fig. 1B), was approximately as potent as the monoester compounds toward GST 1–1 and 2–2. Yet, Et-R-Hep is still a potent inhibitor, especially of the Alpha family members 1–1 and 2–2; this seems in contrast to the results of Morgan et al. (unpublished data quoted in (30)) who reported that monoester derivatives of their GSH analogues were very poor GST inhibitors.

Very few detailed studies are available on the kinetic mechanism of the reaction catalyzed by the GSTs. The most extensive studies have been performed with GST 3–3, which was also used in the present study. Jakoby et al. (31) proposed a hybrid mechanism: a combination of ordered sequential and ping-pong mechanism. Later work indicated that the mechanism was most likely random sequential (32, 33). In intact cells, where the intracellular GSH concentration is high, the mechanism is probably ordered, with GSH adding first (34). Our results are in agreement with a random-order model: competitive inhibition patterns towards both GSH and acceptor substrate indicates that both substrates and inhibitor compete for the free enzyme. If the mechanism were ordered, with GSH adding first, competition would be observed only towards GSH as the variable substrate (35).

Esterification at the $\delta$-carboxyl group of D-Aad, regardless of the alkyl chain length strongly decreased the inhibitory potency towards Mu class GST's, and to a lesser extent towards Alpha GST's. This difference presumably is related to differences in the G-site between the two isoenzyme families.

The preferential inhibition of Alpha as compared to Mu class GST's may explain the results with the racemic substrate BIU. (S)-BIU is conjugated more effectively by Alpha class GST and (R)-BIU by Mu class GST (36). Indeed, the predominantly Alpha GST substrate (S)-BIU was inhibited by all compounds, but (R)-BIU was not inhibited by any of the derivatives. R-Hep was again the most potent inhibitor; the monoesters were slightly less effective, and the diethylster was much less potent. Surprisingly, Glu-Oct-Hep, which was as effective as the monoesters with isoenzymes, was much less effective towards BIU in rat liver cytosol. This most likely is because of the different substrates used: CDNB was the electrophilic substrate in the isoenzyme studies, whereas BIU was the substrate used in cytosol.

**Inhibition of GST in Hepatocytes and Rat Liver Perfusion.** The inhibitory potency of the compounds in intact cells is a result of three factors: the extent of cell penetration (dependent largely on lipophilicity of the compound), the rate of hydrolysis of the ester (yielding the most effective inhibitor, R-Hep), and the intrinsic inhibitory potency of the compounds (esters versus R-Hep) towards the various GSTs.

The lipophilicity of a compound is reflected by its octanol-buffer partition coefficient. Mono- and diesterification of the charged, polar carboxylic acid groups in R-Hep yielded more lipophilic compounds; especially the compounds with long alkyl side chains, Pt-R-Hep and Do-R-Hep. Thus they would be expected to penetrate into the cells most efficiently.

Furthermore, usually only the fraction of the compound unbound to protein (BSA) is available for uptake into cells. The more lipophilic compounds are more tightly bound to BSA. Although the extent of protein binding could not be measured for Do-R-Hep, we estimate that it is even more strongly protein bound, because of the presence of an even longer alkyl side chain. Because there is a complex interaction between the factors lipophilicity and protein binding, a simple correlation between the magnitude of these parameters and the extent of cell penetration cannot be made.

Only (S)-BIU conjugation was inhibited in both isolated hepatocytes and the perfused liver. In isolated hepatocytes, R-Hep was much less effective than the esterified compounds, most likely because of its lower lipophilicity, resulting in a slower uptake into the cell. The monoester compounds were roughly equally effective in hepatocytes; once they pass the cell membrane, they may be hydrolyzed by esterases, to yield the same unesterified residue R-Hep, which is the most effective inhibitor of Alpha class GST, or the esterified compounds as such may inhibit GST (table 2).

Previously, the conjugation of BIU has been studied in detail in a single-pass perfused rat liver system (29). Owing to limited availability of the inhibitors, we have characterized the effect of the inhibitors on the conjugation of (RS)-BIU in a recirculating rat liver perfusion system to assess their relative potency towards GST conjugation in the intact liver. In accordance with the results in hepatocytes and cytosol, (S)-BIU but not (R)-BIU conjugation was inhibited by all inhibitors, indicating isoenzyme selective inhibition of Alpha GSTs. For Et-R-Hep, we had already observed this in the rat in vivo (9).

The three monoesters were equipotent in the inhibition of (S)-BIU conjugation; apparently, difference in lipophilicity, protein binding, rate of hydrolysis, and their intrinsic potency compensate so that the end result is equipotency. All of them were more active than unesterified R-Hep, presumably owing to the low lipophilicity of R-Hep. The same has been demonstrated for GSH itself; monoethyl GSH is transported into the cell much more efficiently than unesterified GSH (12). It has been reported, however, that for some GSH conjugates and GSH analogues monooenestification does not enhance cellular uptake (30, 37).

Diesterification of GSH, certain GSH conjugates, and GSH analogues (12, 30, 37) yields compounds that penetrate the cell even better than the monoesters. Surprisingly, however, the diester of Et-R-Hep was less effective in liver perfusions, although it was as effective as the monoesters in hepatocytes.

In conclusion, Et-R-Hep is the most effective GST inhibitor in isolated hepatocytes and liver perfusion; derivatives with longer alkyl chains are slightly less potent. Towards purified isoenzymes or in rat liver cytosol however, unesterified R-Hep is the most potent compound; it may be the active form of the inhibitors inside the cell. These compounds are useful tools to study the role of GSTs in biotransformation, toxicity, and in physiological processes in vivo.

**Acknowledgments.** The authors thank Irma Meijerma and Marty Blom for their generous supply of isolated hepatocytes and Mathieu Ouwerkerk for his assistance with the statistical analysis of the results.
INHIBITION OF GSTs IN PERFUSED LIVER

References


