Glutathione analogues as novel inhibitors of rat and human glutathione S-transferase isoenzymes, as well as of glutathione conjugation in isolated rat hepatocytes and in the rat *in vivo*

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Inhibitors of rat and human Alpha- and Mu-class glutathione S-transferases that effectively inhibit the glutathione (GSH) conjugation of bromosulphophthalein in the rat liver cytosolic fraction, isolated rat hepatocytes and in the rat liver *in vivo* have been developed. The GSH analogue (R)-5-carboxy-2-γ-(S)-glutamylamino-N-hexylpentamide [Adang, Brussee, van der Gen and Mulder (1991) *J. Biol. Chem.* 266, 830–836] was used as the lead compound. To obtain more potent inhibitors, it was modified by replacement of the N-hexyl moiety by N-2-heptyl and by esterification of the 5-carboxy group with ethyl and dodecyl groups. In isolated hepatocytes, the branched N-2-heptyl derivatives were stronger inhibitors of GSH conjugation of bromosulphophthalein than the N-hexyl derivatives. The ethyl ester compounds were more efficient than the corresponding unesterified derivatives. The dodecyl ester of the N-2-heptyl analogue was the most effective inhibitor in isolated hepatocytes, but was

relatively toxic *in vivo*. However, the corresponding ethyl ester was a potent *in vivo* inhibitor: GSH conjugation of bromosulphophthalein (as assessed by biliary excretion of the conjugate) was decreased by 70 % after administration of a dose of 200 μmol/kg. The isoenzyme specificity of the inhibitors towards purified rat and human glutathione S-transferases was also examined. The unesterified compounds were more potent than the esterified analogues, and inhibited Alpha- and Mu-class isoenzymes of both rat and human glutathione S-transferase (K_i range 1–40 μM). Other GSH-dependent enzymes, i.e. GSH peroxidase, GSH reductase and γ-glutamyltranspeptidase, were not inhibited. Thus (R)-5-ethyloxycarbonyl-2-γ-(S)-glutamylamino-N-2-heptylpentamide, the *in vivo* inhibitor of GSH conjugation, may be useful in helping to assess the role of the Alpha and Mu classes of glutathione S-transferases in cellular biochemistry, physiology and pathology.

INTRODUCTION

Glutathione conjugation is an important biotransformation reaction in all organisms. Several families of glutathione S-transferase (GST; EC 2.5.1.18) isoenzymes catalyse this reaction [1]. The endogenous tripeptide, glutathione (γ-L-glutamyl-L-cysteinylglycine), is utilized to detoxify (and sometimes to toxify) a wide range of both endogenous and xenobiotic substances [1–3]. In addition, the GSTs are involved in the biosynthesis of, for example, leukotrienes and prostaglandins [4]. GSTs of the Alpha and Pi classes (for nomenclature see [3]) are thought to play a role in the resistance of cancer tissues to chemotherapeutic drugs [5,6]. In order to elucidate the role of GSH conjugation in physiological or pathological processes, effective *in vivo* inhibitors of GSTs are essential. So far, the diuretic drug ethacrynic acid [7] and buthionine sulfoximine [8], an inhibitor of γ-glutamylcysteine synthetase, have been used in *in vivo* studies. Although ethacrynic acid does inhibit some GSTs, both of these compounds prevent GSH conjugation by a depletion of GSH levels, which may have more consequences than inhibition of GSTs alone.

Therefore there is a need for selective inhibitors of GSTs. The aim of the present study was to design such compounds.

The active site of GST contains two adjacent substrate-binding subsites, the G-site for GSH and the hydrophobic H-site where the acceptor substrate binds. In an attempt to design specific inhibitors, several GSH analogues with a high affinity for the G-site were developed in our laboratory by Adang and co-workers [9–11]. Thus several selective inhibitors of the GSTs targeted to the G-site and stabilized against γ-glutamyl transpeptidase (γ-GT) breakdown were developed and shown to inhibit GST isoenzyme 3-3. Lyttle et al. [12] have also synthesized reversible GST inhibitors based on analogues of GSH. So far, these compounds have not been reported to be effective in intact cells or *in vivo*.

One of the inhibitors synthesized by Adang et al. [9], (R)-5-carboxy-2-γ-(S)-glutamylamino-N-hexylpentamide (R-Hex) [named γ-L-glutamyl-α-(D-2-aminoadipyl)hexylamine in that publication], was chosen as the lead compound for the design of *in vivo* active inhibitors. The inhibitory effect of four derivatives and analogues of R-Hex on the GSH conjugation of the substrate

Abbreviations used: GST, glutathione S-transferase; BSP, bromosulphophthalein; R-Hex, (R)-5-carboxy-2-γ-(S)-glutamylamino-N-hexylpentamide; R-Hep, (R)-5-carboxy-2-γ-(S)-glutamylamino-N-2-heptylpentamide; Et-R-Hex, (R)-5-ethyloxycarbonyl-2-γ-(S)-glutamylamino-N-hexylpentamide; Et-R-Hep, (R)-5-ethyloxycarbonyl-2-γ-(S)-glutamylamino-N-2-heptylpentamide; Do-R-Hep, (R)-5-dodecyloxycarbonyl-2-γ-(S)-glutamylamino-N-2-heptylpentamide; D-Aad, D-α-aminoadipic acid; DCC, N,N-dicyclohexylcarbodi-imide; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; γ-GT, γ-glutamyl transpeptidase; CDNB, 1-chloro-2,4-dinitrobenzene; Z, benzyloxycarbonyl; OBzl, benzyloxy; Boc, t-butoxycarbonyl; OSu, N-hydroxysuccinimide ester; OtBu, t-butyl ester; TMS-Cl, chlorotrimethylsilane.

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bromosulphophthalein (BSP) [13–15] in isolated rat hepatocytes, rat liver cytosol and in the rat *in vivo* are described. Their enzyme and isoenzyme specificities were investigated. The results indicate that the derivatives are competitive inhibitors (with respect to GSH) of human and rat GSTs of the Alpha and Mu classes. They inhibit the conjugation of BSP in cytosol and hepatocytes to various extents. (*R*)-5-Ethylloxycarbonyl-2- γ -(*S*)-glutamyl-amino-*N*-2-heptylpentamide (Et-R-Hep) is an effective *in vivo* inhibitor.

MATERIALS AND METHODS

Materials

BSP was obtained from Janssen Chimica (Beerse, Belgium), reduced glutathione from Merck (Darmstadt, Germany), and D- α -aminoadipic acid (D-Aad), L-methionine, BSA (fraction V) and γ -GT (Type 1, from bovine kidney) from Sigma (St. Louis, MO, U.S.A.). Z-Glu-OBzl was purchased from NovaBiochem (Laufelfingen, Switzerland) and Boc-Glu(OSu)-OtBu from Bachem (Bubendorf, Switzerland). *NN'*-Dicyclohexylcarbodi-imide (DCC), *N*-hydroxysuccinimide and benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) were obtained from Fluka (Buchs, Switzerland). BSP–GSH was prepared as described by Whelan et al. [16]. All other chemicals were of reagent grade; dimethyl formamide was of analytical purity, all other solvents were distilled before use.

Animals

Male Wistar rats (SPF; 180–220 g body weight) from the Sylvius Laboratories, Leiden University, were used. The rats were housed in Macrolon cages on standard hardwood bedding. The animals had free access to tap water and standard laboratory chow (SRM-A; Hope Farms, Woerden, The Netherlands).

Analytical methods

^1H -NMR spectra were measured at 200 or 300 Mhz using a JEOL JNM-FX 200 spectrometer or a Bruker WM-300 spectrometer equipped with an ASPECT-2000 computer, operating in the Fourier transform mode, respectively. Tetramethylsilane (TMS) and 4,4'-dimethyl-4-silapentanesulphonate (DSS) were used as internal reference for samples in C_2HCl_3 and $^2\text{H}_2\text{O}$ respectively. Chemical shifts (δ) are given in p.p.m. ^{13}C -NMR spectra were measured at 50.3 MHz using a JEOL JNM-FX 200 spectrometer.

Mass spectra were recorded on a BIO-ION 20 plasma desorption mass spectrometer (Bio-ion Nordic AB) equipped with a short flight-tube.

Peptide synthesis

R-Hex and (*R*)-5-carboxy-2- γ -(*S*)-glutamylamino-*N*-2-heptylpentamide (R-Hep)

D-Aad was first protected at the δ -carboxylate group with benzyl alcohol and chlorotrimethylsilane (TMS-Cl) in an inert atmosphere according to the procedure described by Brook and Chan [17] for L-Glu. Recrystallization from ethanol/pyridine afforded (*R*)-2-amino-5-benzylloxycarbonylpentanoic acid (**1**) in 65 % yield. The γ -*N*-hydroxysuccinimide ester of Z-Glu-OBzl (**2**) was synthesized according to Anderson et al. [18]. DCC was added to a solution of Z-Glu-OBzl and *N*-hydroxysuccinimide in tetrahydrofuran. The reaction was allowed to proceed overnight at 4 °C. The dicyclohexylurea formed was removed by filtration

and the filtrate was concentrated *in vacuo*. Recrystallization from propan-2-ol yielded **2** (76 %).

(*R*)-5-Benzylloxycarbonyl-2- γ -(*S*)-*N*-benzylloxycarbonyl- α -O-benzylglutamylamino]pentanoic acid (**3**) was prepared from **1** and **2** by a modification of the method described by Anderson et al. [18]. **1** was suspended in dimethyl formamide containing diisopropylethylamine and **2**. The reaction was allowed to proceed overnight. When the reaction was complete, the mixture was concentrated, acidified, dissolved in ethyl acetate and washed with water. The ethyl acetate layer was collected, dried over magnesium sulphate and the solvent was evaporated *in vacuo*. The residue was recrystallized from ethyl acetate/light petroleum (b.p. 40–60 °C) (1:1, v/v) to yield 60 % of pure product **3**. To a solution of **3** and *NN*-diisopropylethylamine in tetrahydrofuran was added BOP and 1-hexylamine (for **4**) or 2-heptylamine (for **5**). After 30 min the reaction mixture was concentrated *in vacuo*. The residue was dissolved in ethyl acetate and washed with sodium bicarbonate, water and potassium bisulphate. The organic layer was dried over magnesium sulphate and concentrated *in vacuo* to yield 80 % (*R*)-5-benzylloxycarbonyl-2- γ -(*S*)-*N*-benzylloxycarbonyl- α -O-benzylglutamylamino]-*N*-hexylpentamide (**4**) or (*R*)-5-benzylloxycarbonyl-2- γ -(*S*)-*N*-benzylloxycarbonyl- α -O-benzylglutamylamino]-*N*-2-heptylpentamide (**5**). Deprotection was accomplished in a hydrogen pressurized reaction flask in which **4** or **5** was present, dissolved in absolute ethanol, in the presence of a catalytic amount of 10 % palladium on charcoal. Hydrogenation and intense shaking were continued overnight. The catalyst was removed by filtration and solvent was removed by evaporation *in vacuo*. The residue was suspended in water and lyophilized to yield 92 % of the pure fluffy white powders R-Hex and R-Hep respectively from **4** and **5**. R-Hex: mass spectrum m/e 374 (MH^+); ^1H δ (p.p.m.) (MeO^3H) 0.90 [3 H, t, J 6.8 Hz, $\text{C}_6\text{H}_2(\text{CH}_2)_4\text{CH}_3$], 1.31 [6 H, m, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2(\text{CH}_2)_3\text{CH}_3$], 1.49 [2 H, m, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2(\text{CH}_2)_3\text{CH}_3$], 1.69 [4 H, m, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$], 2.11 [2 H, m, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$], 2.31 [2 H, t, J 6.9 Hz, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$], 2.49 [2 H, t, J 7.2 Hz, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$], 3.15 [2 H, m, $\text{C}_6\text{H}_2(\text{CH}_2)_4\text{CH}_3$], 3.62 [1 H, t, J 5.8 Hz, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$], 4.27 [1 H, m, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$]; ^{13}C (p.p.m.) (MeO^3H): 14.37 [$\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2(\text{CH}_2)_3\text{CH}_3$], 30.71, 31.54, 35.52, 36.68, 38.49, 40.48, 41.15, 41.42, 43.30, 48.02 (10 CH_2), 62.09, 63.23 (2 CH), 179.63, 180.93, 181.39, 183.96 (2 CONH, 2 CO_2^-). R-Hep: ^1H δ (p.p.m.) (MeO^3H): 0.89 [3 H, t, J 2.1 Hz, $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 1.09 and 1.12 [3 H, dd, J 6.6 Hz, $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 1.38 [8 H, m, $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 1.65 (4 H, m, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$), 2.12 [2 H, m, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$], 2.31 [2 H, t, J 6.9 Hz, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$], 2.48 [2 H, t, J 7.2 Hz, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{CO}$], 3.61 [1 H, t, J 5.8 Hz, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$], 3.84 [1 H, m, $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 4.26 [1 H, m, $\text{C}_6\text{H}_2\text{C}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$]; ^{13}C (p.p.m.) (MeO^3H): 14.41 [$\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 21.02 [$\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 22.48, 23.64, 26.97, 27.89, 32.76, 32.81, 32.96, 34.60, 37.41 (9 CH_2), 46.47 [$\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 54.76, 55.55 (2 CH), 173.35, 173.99, 174.87, 177.32 (2 CONH, 2 CO_2^-).

(*R*)-5-Ethylloxycarbonyl-2- γ -(*S*)-glutamylamino-*N*-hexylpentamide (Et-R-Hex) and Et-R-Hep

D-Aad was protected at the δ -carboxylate group with ethyl alcohol and TMS-Cl as described above for **1** to yield (*R*)-2-amino-5-ethylloxycarbonylpentanoic acid (**6**) in 80 % yield.

(*R*)-5-Ethylloxycarbonyl-2- γ -(*S*)-*N*-benzylloxycarbonyl- α -O-benzylglutamylamino]pentanoic acid (**7**) was synthesized from **2** and **6** as described for the synthesis of **3**. (*R*)-5-ethylloxycarbonyl-2- γ -(*S*)-*N*-benzylloxycarbonyl- α -O-benzylglutamylamino]-*N*-hexylpentamide (**8**) and (*R*)-5-ethylloxycarbonyl-2- γ -(*S*)-*N*-

benzyloxycarbonyl- α -*O*-benzylglutamylamino]-*N*-2-heptylpentamide (**9**) were synthesized from **7** similarly to the synthesis of **4** and **5** from **3**. The overall yield of **8** and **9** from **6** was 78 %. Deprotection was carried out as described for **4** and **5** to yield Et-R-Hex and Et-R-Hep from **8** and **9** respectively in a yield of 85 %. Et-R-Hex: ^1H δ (p.p.m.) (acetone- d_6 , $^2\text{H}_2\text{O}$): 0.86 [3 H, t, J 6.4 Hz, $\text{C}_6\text{H}_5\text{C}_6\text{H}_4(\text{CH}_2)_3\text{CH}_3$], 1.22 [9 H, m, $\text{C}_6\text{H}_5\text{C}_6\text{H}_4(\text{CH}_2)_3\text{CH}_3$ and $\text{COOCH}_2\text{CH}_3$], 1.45 [2 H, m, $\text{C}_6\text{H}_5\text{C}_6\text{H}_4(\text{CH}_2)_3\text{CH}_3$], 1.61 (4 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$), 2.16 (6 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$ and $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$), 3.17 [2 H, t, J 6.3 Hz, $\text{C}_6\text{H}_5\text{C}_6\text{H}_4(\text{CH}_2)_3\text{CH}_3$], 4.09 (2 H, q, J 7.1 Hz, $\text{COOCH}_2\text{CH}_3$), 4.36 (2 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$ and $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$); ^{13}C (p.p.m.) (acetone- d_6 / $^2\text{H}_2\text{O}$): 13.9 and 14.05 [$\text{COOCH}_2\text{CH}_3$ and $\text{C}_6\text{H}_5\text{C}_6\text{H}_4(\text{CH}_2)_3\text{CH}_3$], 20.65, 22.40, 26.43, 28.03, 29.14, 31.30, 31.62, 33.41, 39.48 (9 CH_2), 52.85, 53.26 (2 CH), 60.36 ($\text{COOCH}_2\text{CH}_3$), 171.29, 171.81, 171.99, 173.48 (2 CONH, 2 CO_2). Et-R-Hep: mass spectrum m/e 416 (MH^+); ^1H δ (p.p.m.) (acetone- d_6 / $^2\text{H}_2\text{O}$): 0.87 [3 H, t, J 6.8 Hz, $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 1.12 and 1.14 [3 H, d, J 6.6 Hz, $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 1.3 [9 H, m, $\text{CH}_3\text{C}_\alpha\text{HC}_\beta\text{H}_2(\text{CH}_2)_3\text{CH}_3$ and $\text{COOCH}_2\text{CH}_3$], 1.44 [2 H, m, $\text{CH}_3\text{C}_\alpha\text{HC}_\beta\text{H}_2(\text{CH}_2)_3\text{CH}_3$], 1.72 (4 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}_2^-$), 2.26 (2 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$), 2.35 (2 H, t, J 6.8 Hz, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$), 2.53 (2 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$), 3.88 [2 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$ and $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 4.11 (2 H, q, J 7.1 Hz, $\text{COOCH}_2\text{CH}_3$), 4.27 (1 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$); ^{13}C (p.p.m.) (acetone- d_6 / $^2\text{H}_2\text{O}$): 16.64 and 16.76 [$\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$ and $\text{COOCH}_2\text{CH}_3$], 23.13 and 23.24 [$\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 24.09, 25.40, 28.65, 29.40, 34.43, 34.51, 36.44, 38.98, 39.10 (9 CH_2), 48.30 [$\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 56.56 (2 CH), 63.60 ($\text{COOCH}_2\text{CH}_3$), 174.91, 175.41, 176.40, 176.49 (2 CONH, CO_2^- , COOC_2H_5).

(*R*)-5-Dodecyloxycarbonyl-2- γ -(*S*)-glutamylamino-*N*-2-heptylpentamide (Do-R-Hep)

Boc-Glu-(OSu)-OtBu and **1** were reacted to form (*R*)-5-benzyloxycarbonyl-2-[γ -(*S*)-*N*-t-butyloxycarbonyl- α -*O*-t-butylglutamylamino]pentanoic acid (**10**), similarly to the synthesis of **3** from **1** and **2**. Then **10** was reacted with 2-heptylamine and BOP as described for the synthesis of **4** to yield (*R*)-5-benzyloxycarbonyl-2-[γ -(*S*)-*N*-t-butyloxycarbonyl- α -*O*-t-butylglutamylamino]-*N*-2-heptylpentanoic acid (**11**) in an overall yield of 80 %. Removal of the benzyloxycarbonyl group of **11** was performed as described for the deprotection of **5** to obtain (*R*)-5-carboxy-2-[γ -(*S*)-*N*-t-butyloxycarbonyl- α -*O*-t-butylglutamylamino]-*N*-2-heptylpentanoic acid (**12**). The free 5-carboxy group of **12** was esterified as follows: **12** was dissolved in tetrahydrofuran containing 4-dimethylaminopyridine and DCC. Dodecyl alcohol was added and the reaction was allowed to proceed overnight. Dicyclohexylurea formed was filtered off and the solvent was evaporated *in vacuo* to yield 60 % of the product (*R*)-5-dodecyloxycarbonyl-2-[γ -(*S*)-*N*-t-butyloxycarbonyl- α -*O*-t-butylglutamylamino]-*N*-2-heptylpentanoic acid (**13**). Deprotection was carried out as described by Kappeler [19], then **13** was dissolved in anhydrous dichloromethane and trifluoroacetic acid (1:1, v/v). After 5 h the deprotection was complete. The solvent was evaporated *in vacuo* and lyophilized from 1,4-dioxane to yield (70 %) Do-R-Hep as a fluffy white powder. The structure of the Do-R-Hep is shown in Figure 1. Mass spectrum m/e 556 (MH^+); ^1H δ (p.p.m.) (C^2HCl_3): 0.87 [6 H, m, $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$ and $\text{COO}(\text{CH}_2)_{11}\text{CH}_3$], 1.08 [3 H, m, $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 1.26, 1.53 and 2.32 [38 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$, $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$ and $\text{COOCH}_2(\text{CH}_2)_{10}\text{CH}_3$], 3.85 [2 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$ and $\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 4.02

Figure 1 Structure of Do-R-Hep

[2 H, t, J 6.4 Hz, $\text{COOCH}_2(\text{CH}_2)_{10}\text{CH}_3$], 4.45 (1 H, m, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$); ^{13}C (p.p.m.) (C^2HCl_3) 13.93, 14.02 [$\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$ and $\text{COOCH}_2(\text{CH}_2)_{10}\text{CH}_3$], 20.24 [$\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 19.51, 21.00, 22.48, 22.60, 24.88, 25.70, 25.84, 28.56, 29.06, 29.26, 29.56, 31.60, 31.83, 31.97, 33.52, 36.29 (16 CH_2), 45.35 [$\text{CH}_3\text{C}_\alpha\text{H}(\text{CH}_2)_4\text{CH}_3$], 53.20 ($\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{CO}$ and $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{C}_\delta\text{H}_2\text{CO}_2^-$), 64.56 [$\text{COOCH}_2(\text{CH}_2)_{10}\text{CH}_3$], 172.31, 172.69, 173.48, 173.54 (2 CONH, COO^- , COOC_2H_5).

Incubations with isolated rat hepatocytes

Hepatocytes were isolated by collagenase perfusion according to Seglen [20], as described by Nagelkerke et al. [21]. The inhibitors R-Hex and R-Hep were dissolved in methanol, and Et-R-Hex, Et-R-Hep and Do-R-Hep were dissolved in chloroform. The required amount of inhibitor solution was added to the incubation vials and the solvent was removed under a stream of nitrogen. Incubations were performed in a rotary shaker (200 rev./min) under an atmosphere of 95 % O_2 /5 % CO_2 . Cells [diluted to 3×10^6 cells/ml with Hanks/Hepes buffer containing 1.5 % (w/v) BSA] were preincubated at 37 °C for 30 min with 1 mM L-methionine to stimulate GSH synthesis in the vials. The incubation was initiated by addition of 0.3 ml of BSP solution (final concentration 0.5 mM) to 2.7 ml of cell suspension. The viability of the cells before and after the incubations was assessed by Trypan Blue exclusion, and was always > 85 %. Samples of the incubation mixture (200 μl) were added to 200 μl of a saturated aqueous urea solution to release the BSP-GSH conjugate from its tight binding to proteins [22]. Protein was then precipitated by addition of 600 μl of acetonitrile on ice. Samples were stored at -20 °C until HPLC analysis.

Incubations with rat liver cytosolic GSTs

A 25 % (w/v) homogenate of rat liver in ice-cold KCl was prepared with a Potter-Elvehjem homogenizer. After centrifugation at 9000 g for 20 min, the supernatant was centrifuged for 60 min at 100000 g . The resulting supernatant (cytosol) was collected. All procedures were performed at 0-4 °C.

Cytosol (1 mg/ml) was incubated with inhibitor dissolved in DMSO, 1 mM GSH and 0.5 mM BSP at 37 °C in an incubation volume of 2 ml. The final concentration of DMSO in the incubations was 1 % (v/v). Control incubations contained 1 % (v/v) DMSO but no inhibitor. Aliquots of 200 μl of incubation mixture were taken at various time points and treated as described above.

Experiments *in vivo*

The rats were anaesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal). The bile duct and the external jugular veins were catheterized as described by Mulder et al. [23].

During the experiment, the animals were kept on a heating pad to maintain the body temperature at 38 °C. When the condition of the animals had stabilized, an infusion of a solution containing BSP (1.5 $\mu\text{mol/min}$ per kg) and taurocholic acid sodium salt (6 $\mu\text{mol/min}$ per kg) in saline was commenced through the cannulated jugular vein at a rate of 1.9 ml/h. Taurocholic acid was added to the infusion solution to counteract any cholestatic action of BSP [15]. After 45 min a steady state of BSP-GSH excretion in the bile was reached. Then a bolus injection of Et-R-Hep (100 or 200 $\mu\text{mol/kg}$) dissolved in 200 μl of 0.1 M HCl was administered over a period of 5 min through the remaining cannulated jugular vein. Control rats received a bolus of 200 μl of 0.1 M HCl containing no inhibitor. Bile samples were collected in 15 min fractions throughout the experiment. Samples were stored at -20 °C until HPLC analysis.

Another group of rats received an infusion of BSP-GSH (1 $\mu\text{mol/min}$ per kg BSP-GSH and 6 $\mu\text{mol/min}$ per kg taurocholic acid) instead of BSP as described above.

HPLC analysis

Samples containing BSP and BSP-GSH were diluted with HPLC buffer and were analysed according to the method described by Snel et al. [22] with slight modifications. The chromatographic system consisted of a Separations Promis II autoinjector coupled to a Sykam S1000 solvent delivery system, an S110 low pressure gradient mixer and an S2000 HPLC controller, a Waters model 440 absorbance detector (at 254 nm) and a Kipp and Zonen BD40 pen recorder. Chromatography was performed with a Chrompack Spherisorb ODS-2 glass reverse-phase column (0.3 cm $\times$ 10 cm; particle size 5 μm) and a Chrompack ODS-2 guard column (1 cm $\times$ 0.2 cm). Gradient elution was carried out at a flow rate of 0.5 ml/min with a mobile phase consisting of a 10 mM sodium phosphate buffer, pH 6.0 (solvent A) and acetonitrile (solvent B). The initial conditions (0 % B) were maintained for 2.5 min after injection of the sample (volume 20 μl); acetonitrile was then linearly increased to 30 % in 15 min. The column was brought back to the initial conditions in 2.5 min and maintained at 0 % B for 5 min before injection of the next sample.

Preparation of rat GSTs

Rat GST isoenzymes 1-1, 2-2, 3-3, 4-4 and 7-7 were isolated from liver and placenta as previously described by Vos et al. [24], and purified as described by Bogaards et al. [25]. Rat Theta enzyme 5-5 was prepared as described by Meyer et al. [26].

Purification of human GSTs

GSTs M1a-1a, A1-1 and A2-2 were prepared from human liver cytosol. The GST pool was prepared by affinity chromatography according to Vander Jagt et al. [27]. The Alpha enzymes were then separated from GST M1a-1a according to Hussey et al. [28]. GST M1a-1a was purified by anion-exchange FPLC at pH 7.8, while Alpha enzymes were similarly separated at pH 9.5 [29]. GST M3-3 was purified from human testis by the same method used for GST M1a-1a above. GST P1-1 was prepared from human kidney cytosol by affinity chromatography [27] and purified by anion-exchange FPLC at pH 7.8.

Kinetics and inhibition of GST isoenzymes

Inhibition experiments were conducted at 37 °C in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA. The electrophilic substrate for the assay with the Alpha-, Pi- and Mu-class GSTs was 1-chloro-2,4-dinitrobenzene (CDNB) at a fixed

concentration of 1 mM. Inhibition kinetics were performed at 10 concentrations of GSH (50–500 μM) and with or without inhibitor; the inhibitors were dissolved in DMSO [final concentration of DMSO did not exceed 3 % (v/v)]. Reactions were initiated by addition of CDNB and were monitored for 2 min at 340 nm [30]. The reaction rate was corrected for the non-enzymic reaction. The results were analysed using the computer program Kinenort kindly provided by Dr. A. G. Clark.

The assays for GST 5-5 were performed with 0.5 mM 1,2-epoxy-3-(*p*-nitrophenoxy)propane as the electrophilic substrate and 1–10 mM GSH, as described by Habig et al. [30].

Stability of the peptides against β -GT

The rate of degradation of the inhibitors and GSH by γ -GT was determined by incubating 250 μM of the inhibitors or GSH with an appropriate amount of enzyme at 37 °C in a 0.1 M Tris/HCl buffer, pH 7.4, supplemented with 1 mM dithiothreitol and 1 mM EDTA. At selected time intervals (0, 5, 10, 15 min), 350 μl aliquots of the incubation mixture were heat-inactivated for 2 min at 90 °C. The sample was then cooled to 0 °C. The amount of inhibitor or GSH present in the samples was analysed by HPLC according to a modification of the method described by Reed et al. [31]. The sample was treated with 35 μl of a fresh aqueous solution of iodoacetic acid (80 mM) and neutralized with an excess of NaHCO_3 powder. After 60 min in the dark at room temperature, 350 μl of an alcoholic solution of 1-fluoro-2,4-dinitrobenzene (1.5 % in absolute ethanol) was added and the samples were stored in the dark overnight.

The HPLC system used was the same as described above for the analysis of BSP. A 20 μl aliquot of the sample was injected into the HPLC column (Altech 5 micron Kromasil NH_2 column; 250 mm $\times$ 4.6 mm). Solvent C was methanol/water (4:1, v/v) and solvent D was prepared by adding 200 ml of a solution of 272 g of sodium acetate trihydrate, 122 ml of water and 378 ml of acetic acid to 800 ml of solvent C. The gradient used for GSH was linear, from 10 to 99 % D in 14 min, followed by a 3 min isocratic period at 99 % D. Thereafter the column was equilibrated at 10 % D for the next injection. The gradient was modified for the analysis of the inhibitors: the column was maintained at 10 % D for the first 10 min after injection, followed by a linear increase to 20 % D in 10 min. The eluent flow rate was 1 ml/min and the absorbance at 340 nm was monitored. The retention time of GSH was 9.2 min, and for R-Hex, R-Hep, Et-R-Hex, Et-R-Hep and Do-R-Hep it was 7.4, 7.0, 3.2, 3.2 and 3.0 min respectively.

Inhibition of γ -GT, glutathione reductase and glutathione peroxidase

The inhibition of the activity of other GSH-dependent enzymes by 500 μM of the inhibitors (50 μM Do-R-Hep due to limited solubility) was measured. The inhibition of γ -GT activity was determined using purified enzyme, whereas those of GSH peroxidase and GSH reductase were determined by measuring the enzyme activity in rat liver cytosol.

γ -GT activity was measured as described by Meister et al. [32] at pH 7.4. The γ -L-glutamyl-*p*-nitroanilide concentration was varied between 100 and 500 μM and inhibition was measured at pH 7.4. The GSH reductase assay was performed as described by Bilzer et al. [33]. The GSSG concentration was varied between 0.2 and 1 mM. The coupled assay [34] with GSH reductase was used to determine GSH peroxidase activity with 0.5 mM H_2O_2 , 0.2–1 mM GSH and inhibitors at pH 7.0.

RESULTS

In the rat, BSP is metabolized exclusively by the Mu-class GSTs 3-3 and 3-4 to yield BSP-GSH [3]. The extent of inhibition of BSP-GSH formation in three systems (cytosolic GSTs, hepatocytes and the rat *in vivo*) was chosen in order to assess the inhibitory activity of several GSH analogues on GSH conjugation. In addition, the enzyme and GST isoenzyme specificities of the inhibitors were determined.

Hepatocyte and cytosol incubations with BSP as substrate

The lead compound, R-Hex, weakly inhibited BSP conjugation by rat hepatocytes (Table 1). When R-Hex was slightly modified by replacing the *N*-hexyl moiety by *N*-2-heptyl, the resulting branched-side-chain analogue R-Hep exhibited a considerably increased inhibitory activity (Table 1; Figure 2).

Reports in the literature indicate that the monoethyl ester of GSH penetrates the cell much better than the unesterified tripeptide [35]. R-Hex and R-Hep were therefore esterified with an ethyl group at the free 5-carboxy function of the pentamide (Et-R-Hex and Et-R-Hep respectively). Esterification indeed greatly increased the inhibitory effects of both analogues in hepatocytes, with the branched Et-R-Hep still being the more effective. In order to examine the effect of the introduction of a

Table 1 Inhibition of GSH conjugation of BSP in isolated rat hepatocytes and in rat liver cytosol

BSP (0.5 mM) was incubated with 1 mM (hepatocytes) or 0.5 mM (cytosol) inhibitor for 30 min. Values are means $\pm$ S.D. of three experiments.

Inhibitor	Inhibition (%)	
	Hepatocytes	Rat liver cytosol
R-Hex	14 $\pm$ 4	39 $\pm$ 1
R-Hep	50 $\pm$ 4	57 $\pm$ 2
Et-R-Hex	68 $\pm$ 4	61 $\pm$ 2
Et-R-Hep	83 $\pm$ 4	79 $\pm$ 2
Do-R-Hep	91 $\pm$ 2	85 $\pm$ 1

Figure 2 Time course of BSP-GSH conjugation in hepatocytes

Hepatocyte incubations were performed with 0.5 mM BSP and 1 mM inhibitor for 90 min. The figure shows controls (+), and incubations with R-Hex (□), R-Hep (○), Et-R-Hex (■), Et-R-Hep (●) and Do-R-Hep (▲). A typical experiment is shown.

Figure 3 Inhibition of GSH conjugation by Do-R-Hep

Various concentrations of Do-R-Hep were incubated with 0.5 mM BSP and cytosol (Δ) or isolated hepatocytes (▲) for 30 min. A typical experiment is shown.

much longer alkyl chain, a dodecyl ester derivative (Do-R-Hep) was synthesized. Do-R-Hep was the most potent inhibitor, resulting in virtually complete inhibition of GSH conjugation of BSP at 1 mM (Figure 2).

In order to evaluate the effectiveness of the inhibitors without the possibly complicating factor of cell penetration, the inhibition of GSH conjugation of BSP by rat liver cytosolic GST was evaluated (Table 1). Although the concentration of inhibitor used in the cytosol experiments was half that in the hepatocyte incubations, the extent of inhibition by the analogues was similar in both experiments; however, R-Hex was only effective in cytosol.

A concentration range of the strongest inhibitor, Do-R-Hep, was tested in both systems. Figure 3 indicates that Do-R-Hep was more potent in cytosol than in intact cells at all concentrations. There was no indication of cytotoxicity at the concentrations used.

Experiments *in vivo* with BSP as substrate

The strongest *in vitro* inhibitor, Do-R-Hep, was administered in the rat *in vivo*. Preliminary experiments indicated that the compound was an effective inhibitor of GSH conjugation. However, due to acute toxicity of the compound (two of the five rats died immediately after administration of 100 μ mol/kg Do-R-Hep), no further *in vivo* studies were conducted with Do-R-Hep.

Et-R-Hep was well tolerated, with no obvious toxicity in the rat. To examine the inhibitory effect of the compound on the conjugation of BSP, an infusion of BSP was administered. After 45 min, a bolus injection of a low (100 μ mol/kg) or high (200 μ mol/kg) dose of Et-R-Hep was administered, and the effect on the rate of excretion of BSP-GSH conjugate in the bile was followed. The excretion rate of the conjugate decreased markedly and in a dose-dependent manner (Figure 4a). The low dose resulted in 40% inhibition and the high dose inhibited the BSP-GSH excretion rate by 66% compared with the steady-state level in control rats. The rate of excretion of BSP-GSH in the bile was maximally inhibited about 30 min after administration of Et-R-Hep. Thereafter the inhibition steadily decreased, resulting in steady-state BSP-GSH excretion rates of 75 and 60% of control values for the low and high doses respectively.

The rate of excretion of unconjugated BSP in the bile (Figure

Figure 4 Inhibition of conjugation of BSP *in vivo* and effect on the excretion of BSP-GSH in the bile

Anaesthetized rats were administered an infusion containing BSP ($1.5 \mu\text{mol}/\text{min per kg}$) (a and b) or BSP-GSH ($1 \mu\text{mol}/\text{min per kg}$) (c) and taurocholic acid ($6 \mu\text{mol}/\text{min per kg}$). After 45 min, a bolus injection of 100 (○) or 200 (●) $\mu\text{mol}/\text{kg}$ Et-R-Hep was administered (I). Controls received only solvent (+). The excretion rates of BSP-GSH (a, c) and BSP (b) in bile are shown (means $\pm$ S.D., $n = 3$).

4b) remained unchanged after administration of $100 \mu\text{mol}/\text{kg}$ Et-R-Hep, but after the $200 \mu\text{mol}/\text{kg}$ dose a significant increase from 425 to $650 \text{ nmol}/\text{min per kg}$ BSP was observed (Figure 4b).

Inhibition of the excretion of the GSH conjugate from the hepatocyte into the bile would also cause a decrease in the biliary excretion of BSP-GSH. Therefore BSP-GSH was infused into the rat and the effect of Et-R-Hep on the biliary excretion of BSP-GSH was examined (Figure 4c). Et-R-Hep had no inhibitory effect on the excretion rate of BSP-GSH into the bile.

Table 2 Inhibition of rat and human GST isoenzymes

The inhibition was determined with 1 mM CDNB and $50\text{--}500 \mu\text{M}$ GSH. The mechanism of inhibition in all cases was competitive towards GSH, with K_i values given as means $\pm$ S.E.M. n.d. = not detectable, as the inhibitor was not soluble at the high concentrations required to establish the K_i value.

Isoenzyme	K_i (μM)				
	R-Hex	R-Hep	Et-R-Hex	Et-R-Hep	Do-R-Hep
Rat					
1-1	54 ± 5	22 ± 2	93 ± 7	39 ± 8	0.2 ± 0.05
2-2	1.5 ± 0.2	1.3 ± 0.1	10 ± 1	10 ± 1	n.d.
3-3	115 ± 15	20 ± 2	n.d.	n.d.	n.d.
4-4	24 ± 3	10 ± 1	n.d.	n.d.	n.d.
Human					
A1-1	7 ± 1	9 ± 1	37 ± 7	17 ± 4	n.d.
A2-2	5.3 ± 0.5	2.4 ± 0.3	211 ± 24	45 ± 7	n.d.
M1a-1a	11.3 ± 0.6	4.3 ± 0.2	360 ± 50	125 ± 19	n.d.
M3-3	133 ± 28	40 ± 7	n.d.	n.d.	n.d.

The rate of bile flow remained constant at all times during the experiment.

Inhibition of pure rat and human GST isoenzymes

To characterize the nature of the inhibition by the five synthetic peptides, their effect on the activity of purified rat and human GST isoenzymes was ascertained. The compounds (at $500 \mu\text{M}$) had no effect on the activity of isoenzymes of the Pi (P1, 7-7) and Theta (5-5) classes. However, R-Hex and R-Hep were good inhibitors of the Alpha (A1-1, A2-2, 1-1, 2-2) and Mu (M1a-1a, M3-3, 3-3, 4-4) enzymes, with R-Hep being more potent (Table 2). In all cases competitive inhibition towards GSH was observed.

All three esterified compounds (at $500 \mu\text{M}$) were ineffective towards the rat Mu isoenzymes. The Alpha-family isoenzymes were inhibited moderately by the ethyl esters, whereas the dodecyl ester was a powerful, rather selective, inhibitor of the 1-1 isoenzyme (K_i $0.2 \mu\text{M}$). All inhibitors, with the exception of Do-R-Hep, inhibited enzyme 2-2 most effectively. R-Hep was the most effective inhibitor, affecting all isoenzymes of the Alpha and Mu families.

The inhibition of human isoenzymes was similar to that of the rat isoenzymes. R-Hex and R-Hep inhibited all the isoenzymes of the Alpha and Mu classes, R-Hep being more potent than R-Hex. The isoenzyme A2-2 was the most sensitive, with K_i s of 5.3 and $2.4 \mu\text{M}$ respectively for R-Hex and R-Hep. The esterified compounds were much less effective than their unesterified forms. Do-R-Hep was ineffective against all human isoenzymes (at $100 \mu\text{M}$).

None of the compounds inhibited the other GSH-dependent enzymes (γ -GT, GSH reductase and GSH peroxidase) at $250 \mu\text{M}$.

Degradation of the inhibitors by γ -GT

The stability of the inhibitors against hydrolysis by γ -GT was examined under conditions resulting in 90% degradation of GSH in 15 min. R-Hex and R-Hep were degraded by 40 and 30% respectively, whereas the esterified compounds were not sensitive to degradation by γ -GT.

DISCUSSION

Inhibition of the GSH conjugation of BSP in rat liver cytosol, isolated hepatocytes and in the rat *in vivo*

Four of the compounds studied effectively inhibited the GSH conjugation of BSP in isolated hepatocytes (Table 1). The branched *N*-2-heptyl derivatives were more potent than their straight-chain *N*-hexyl counterparts both in isolated hepatocytes and towards (purified) GSTs (Tables 1 and 2). Possibly these analogues interact more extensively with the hydrophobic binding site of GST, resulting in a stronger inhibition by the branched than the straight-chain compounds.

The relatively weak inhibitory effects of R-Hex and R-Hep might have been due to slow uptake into the cell as a consequence of the presence of two free carboxylic groups, the α -carboxyl of the glutamic acid and the 5-carboxyl group of the pentamide. However, towards BSP, the esterified compounds were also more effective than their parent compounds in incubations with rat liver cytosol. This suggests that the increase in inhibitory activity in hepatocytes is not merely a result of increased cellular uptake of these compounds. It is possible that in particular the dodecyl moiety in Do-R-Hep interacts with a site on GST that is involved in BSP binding, thereby contributing to increased inhibition in both hepatocytes and cytosol. Studies on the inhibition of BSP conjugation at various concentrations of Do-R-Hep (Figure 3) indicate that, at the same concentration, the inhibitor is more effective in cytosol than in hepatocytes. A concentration of 75 μ M was required for 50% inhibition of BSP conjugation in hepatocytes, whereas in cytosol a concentration of 10 μ M resulted in a similar effect. This difference may be attributed to sub-optimal cell penetration. However, accumulation in the lipophilic membranes or limited solubility of the inhibitor in the incubation mixture may also have reduced the effect in the hepatocyte.

Based on the *in vitro* studies, Do-R-Hep seemed a promising compound for use in the rat *in vivo*. Unfortunately, due to acute toxicity, this derivative could not be used for further investigations. The other effective inhibitor in hepatocyte studies, Et-R-Hep, was not (acutely) toxic, and therefore its effectiveness as an *in vivo* inhibitor of GSH conjugation could be assessed in the rat.

Et-R-Hep was a potent inhibitor *in vivo*, resulting in marked inhibition of conjugation within 30 min of administration (Figure 4a). This was followed by a period during which the biliary excretion of BSP-GSH increased slightly, but it remained significantly below control levels throughout the experiment. With the high dose of Et-R-Hep, inhibition of BSP-GSH conjugation was compensated by an increase in the excretion of BSP in the bile (Figure 4b), indicating that the decreased biliary excretion of BSP-GSH is not due to inhibition at the level of transport of BSP from the blood into the hepatocyte. Infusion of BSP-GSH indicated that the inhibitory effect of Et-R-Hep was not on the excretion of the conjugate from the hepatocyte into the bile.

Thus we could confirm that the inhibitory effect of Et-R-Hep on the excretion of the GSH conjugate of BSP in the bile of the rat is indeed caused by an inhibition of GST activity in the rat *in vivo*.

Selectivity of the GSH analogues towards rat and human GST isoenzymes and other GSH-dependent enzymes

BSP is conjugated in the rat by the Mu family of isoenzymes [3]. Our GSH analogues inhibited the Alpha and Mu families of both rat and human GSTs (as assessed with CDNB as substrate), while Pi and Theta enzyme activities were not affected. The inhibition of human GST isoenzymes was also determined to assess the clinical relevance of the results. Alpha-class GSTs are

thought to play a role in the resistance of cancer cells to alkylating agents such as chlorambucil and other nitrogen mustards [36]. Smokers lacking transferase Mu seem to have a higher risk of developing lung cancer than those with GST Mu activity [37]. GST Mu is also involved in the detoxification of mutagenic and carcinogenic epoxides [styrene 7,8-oxide and benzo(a)pyrene 4,5-oxide] [38,39]. Inhibitors of both these families of isoenzymes thus have interesting potential applications in the study of the role of GST in toxicity. Unfortunately, none of our GSH analogues inhibited GST Pi, which is overexpressed in numerous human tumours [40,41].

The δ -carboxylate terminus of D-Aad interacts with the binding site of the GST in a similar way to the glycine in GSH [9]. This may explain why the esterified compounds are much less effective inhibitors of purified isoenzyme than the corresponding unesterified compounds. It is clear, however, that this does not apply to BSP as substrate (Table 1): in rat liver cytosol, the esterified compounds were more effective towards BSP conjugation than the unesterified inhibitors. This discrepancy may be due to differences in binding of the substrates CDNB and BSP to the hydrophobic binding site in the GST so that their conjugation is differently influenced by the various inhibitors (for a similar situation see [42]).

Some other GSH-dependent enzymes were not affected by the inhibitors, indicating that they are rather selective for the GST enzymes. This is an important prerequisite for an inhibitor with *in vivo* applications.

General aspects

All the inhibitors used have been designed to be stabilized against degradation by γ -GT, the major metabolic degradation pathway for GSH and GSH conjugates *in vivo*. They are indeed less sensitive to γ -GT degradation because the amino acid coupled to γ -Glu has the D-configuration. Esterification clearly increased their resistance to breakdown by γ -GT.

Studies by Anderson et al. [35] indicated that the monoesters of GSH in which the glycine carboxyl group is esterified are readily transported into cells and are de-esterified intracellularly. In contrast, GSH itself is not effectively transported into cells. The ethyl esters Et-R-Hep and Et-R-Hex are esterified at the 5-carboxyl group of D-Aad, which corresponds to the glycine carboxyl group in GSH. Therefore, in analogy to GSH, the esterified inhibitors could be taken up into the cell much more efficiently than the unesterified compounds, to release the more potent inhibitors R-Hex and R-Hep. Thus, although the esterified derivatives are less effective inhibitors than the unesterified compounds with purified isoenzymes, the *in vivo* effects of the former analogues could be much greater.

Et-R-Hep represents, the first of a new class of inhibitors of GSH conjugation, based on analogues of GSH that are effective *in vivo* in the rat. The role of GSH conjugation in *in vivo* toxification and detoxification reactions can be investigated using this inhibitor. Do-R-Hep could be valuable as an effective inhibitor of GSH conjugation in studies using cytosol and isolated hepatocytes.

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