Amino Acids (2005) 29: 255–261
DOI 10.1007/s00726-005-0232-7

Amino Acids
Printed in Austria

Potent isozyme-selective inhibition of human glutathione *S*-transferase A1-1 by a novel glutathione *S*-conjugate

I. Cacciatore[1], **A. M. Caccuri**[2], **A. Cocco**[1], **F. De Maria**[2], **A. Di Stefano**[1], **G. Luisi**[1], **F. Pinnen**[1], **G. Ricci**[2], **P. Sozio**[1], and **P. Turella**[2]

[1] Department of Drug Sciences, "G. D'Annunzio" University, Chieti, Italy
[2] Department of Chemical Science and Technology, "Tor Vergata" University, Rome, Italy

Received April 5, 2005
Accepted May 19, 2005
Published online August 8, 2005;

Summary. Elevated levels of glutathione *S*-transferases (GSTs) are among the factors associated with an increased resistance of tumors to a variety of antineoplastic drugs. Hence a major advancement to overcome GST-mediated detoxification of antineoplastic drugs is the development of GST inhibitors. Two such agents have been synthesized and tested on the human Alpha, Mu and Pi GST classes, which are the most representative targets for inhibitor design. The novel fluorescent glutathione *S*-conjugate L-γ-glutamyl-(*S*-9-fluorenylmethyl)-L-cysteinyl-glycine (**4**) has been found to be a highly potent inhibitor of human GSTA1-1 *in vitro* ($IC_{50} = 0.11 \pm 0.01\ \mu M$). The peptide is also able to inhibit GSTP1-1 and GSTM2-2 isoenzymes efficiently. The backbone-modified analog L-γ-(γ-oxa)glutamyl-(*S*-9-fluorenylmethyl)-L-cysteinyl-glycine (**6**), containing an urethanic junction as isosteric replacement of the γ-glutamyl-cysteine peptide bond, has been developed as γ-glutamyl transpeptidase-resistant mimic of **4** and evaluated in the same inhibition tests. The pseudopeptide **6** was shown to inhibit the GSTA1-1 protein, albeit to a lesser extent than the lead compound, with no effect on the activity of the isoenzymes belonging to the Mu and Pi classes. The comparative loss in biological activity consequent to the isosteric change confirms that the γ-glutamyl moiety plays an important role in modulating the affinity of the ligands addressed to interact with GSH-dependent proteins. The new specific inhibitors may have a potential in counteracting tumor-protective effects depending upon GSTA1-1 activity.

Keywords: Glutathione – Glutathione *S*-transferase inhibitors – Glutathione *S*-conjugate – γ-Glutamyl transpeptidase – Pseudopeptide

Abbreviations follow the recommendations of the IUPAC IUB Commission on Biochemical Nomenclature as given in Eur J Biochem (1984) 138: 9–37. The following additional abbreviations are used: Boc, *tert*-butyloxycarbonyl; *t*-Bu, *tert*-butyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; Fm, fluorenylmethyl; HOBt, *N*-hydroxybenzotriazole; NMM, *N*-methylmorpholine; N*p*, *p*-nitrophenyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran

Introduction

Many different mechanisms are involved in protecting cellular components against cytotoxic and genotoxic compounds (Lang and Pelkonen, 1999). A central role in detoxification and metabolism is played by glutathione (γ-Glu-Cys-Gly), through maintenance of the thiol-disulfide status of cellular proteins, synthesis and transport of endogenous substrates, and protection against electrophilic and oxidizing agents (Pompella et al., 2003; Davies et al., 2001). In cellular detoxification, reduced glutathione (GSH) is conjugated with reactive species through catalysis by the major phase II detoxifying enzymes glutathione *S*-transferases (GSTs, EC 2.5.1.18) (Hayes et al., 2005; Salinas and Wong, 1999; Oakley et al., 1997; Wilce and Parker, 1994), producing the more hydrophilic GSH conjugates.

Consistent with its deactivation function, this GSH-dependent metabolic system is supposed to have a role in reducing the effectiveness of drug treatments, and particularly anticancer chemotherapy. A typical adaptive response to drug-induced cellular stress is *de facto* represented by an alteration in the levels and distribution of GSH and associated proteins, notably GSTs belonging to class Alpha, Mu and Pi (O'Brien and Tew, 1996).

With this premise, the approach of GST inhibition in order to potentiate conventional chemotherapy (Schultz et al., 1997) appears challenging. In that respect, the development of *S*-conjugates of glutathione as natural modulators of these cytoprotective pathways represents a pertinent area of investigation (Burg et al., 2002; Morgan et al., 1996). The expediency of these agents lies in the fact that they target the GST active site with great affinity, despite their therapeutic potential is limited by

sensitivity to γ-glutamyl transpeptidase (γ-GT)-mediated degradation.

In recent years we have been actively pursuing novel strategies for the structural modification of the GSH molecule, addressed to enhance both metabolic stability and affinity to GST binding sites through the attribution of specific chemical and conformational properties (Cacciatore et al., 2003; Paglialunga Paradisi et al., 2003; Calcagni et al., 1996, 1995; Luisi et al., 1993). As an extension in this field, we are presently interested in targeting GST with novel GSH conjugates and their isosteres at the γ-glutamyl-cysteine amide bond, which may have potential as γ-GT resistant drugs.

It came to our attention that comparatively few studies are focused on the Alpha class GSTs, in spite of the many lines of evidence implicating these proteins in multidrug resistance and pressing towards the need for selective Alpha inhibitors. Augmented levels of class Alpha GSTs represent in fact a specific mechanism whereby cells acquire resistance to alkylating agents, which are primary drugs in the treatment of hormone-dependent cancers (Fotouhi-Ardakani et al., 1998; Morel et al., 1994; Lewis et al., 1988). Alkylating agents and particularly polifunctional nitrogen mustards are known to undergo sequencial biotrasformation leading to different cytotoxic species. Quite interestingly, often Alpha isozymes can selectively inactivate these toxicants even up to the ultimate metabolites (Dirven et al., 1994; Bolton et al., 1991).

In order to develop GST inhibitors addressed to the Alpha class, we played on structural features of the hydrophobic binding pocket (H site) of this protein, which is reported to be more lipophilic in character compared to that of Mu or Pi class GSTs (Salinas and Wong, 1999; Koehler et al., 1997). Accordingly, the new GSH conjugate L-γ-glutamyl-(*S*-9-fluorenylmethyl)-L-cysteinyl-glycine (**4**) and its analog L-γ-(γ-oxa)glutamyl-(*S*-9-fluorenylmethyl)-L-cysteinyl-glycine (**6**), both characterized by the presence of a fluorenylmethyl group as cysteine S-derivatizing moiety, were designed as potential GSTA1-1 inhibitors. The insertion of this large aromatic substituent is expected to complement the GSTA1-1 cavity better than the other more polar H sites, drastically increasing the affinity towards this isoform and isoenzymatic selectivity. Again, an attractive feature of this group lies in its fluorescent properties, to profit by for easier monitoring in biological tests. In the backbone-modified analog **6** the original approach of introducing an urethanic OCONH fragment as γ-GT resistant surrogate of the γ-glutamyl CONH junction (Calcagni et al., 1996) has been considered to confer metabolic stability, which is a desirable pre-requisite for *in vivo* application of peptidic inhibitors. From a formal point of view, the new pseudopeptide incorporates an L-γ-oxaglutamic acid (Glo) residue as Glu substitution. This chemical modification deserves investigation since, even though the resulting pseudopeptide backbone of the conjugate still approximates the GSH structure, it is supposed to indroduce unique conformational and biochemical consequences at the level of the γ-Glu subsite, the most demanding recognition zone in most of the GSH-dependent proteins (Burg and Mulder, 2002; Adang et al., 1988).

Synthesis, characterization and biological evaluation of H-Glu[-Cys(Fm)-Gly-OH]-OH (**4**) and H-Glo[-Cys(Fm)-Gly-OH]-OH (**6**) on catalytic activity of the major classes of human GSTs are reported herein.

Materials and methods

Amino acid derivatives were purchased from Sigma-Aldrich and Bachem. Bicinchoninic acid (BCA) Protein Assay Reagent was obtained from Pierce, Rockford, IL. Melting points were determined on a Büchi B-450 apparatus and are uncorrected. TLC was performed on Merck 60 F_{254} plates developed with the following solvents: (A) chloroform/methanol (99:1); (B) chloroform/methanol (98:2); (C) 1-butanol/acetic acid/water (2:1:1). Optical rotations were taken at 20°C with a Perkin-Elmer 241 polarimeter. UV measurements were conducted on a Kontron double-beam Uvikon 940 apparatus thermostated at 25°C. Elemental analyses (C, H, N and S) were performed on a Carlo Erba 1106 Analyzer and were within ±0.4% of the theoretical values. IR spectra were recorded employing a Perkin-Elmer FTIR 1600 spectrophotometer. ^{1}H and ^{13}C NMR experiments were performed on a Varian VXR 300 MHz instrument (δ expressed in ppm). The mass spectra of **4** and **6** were obtained in electrospray (ES) conditions by a LCQ (Finnigan, S. Josè, CA, USA) instrument. A 60 μM solution of the sample in methanol/water (1:1) was directly injected at the flow rate of 5 μl/min.

Peptide synthesis

Boc-Cys(Fm)-Gly-OBu^t (**1**). Boc-Cys(Fm)-OH (2.74 g, 6.87 mmol) was suspended in THF (15 ml) and HOBt (0.93 g, 6.87 mmol) was added with stirring. The solution was cooled to 0°C and an ice-cold solution containing HCl · H-Gly-OBu^t (1.15 g, 6.87 mmol) and NMM (0.69 g, 6.87 mmol) in THF (15 ml) was added, followed by portionwise addition of a solution of DCC (1.41 g, 6.87 mmol) in THF (7 ml). After 5 h at 0°C and 16 h at 5°C, the reaction mixture was filtered and the resulting solution evaporated under *vacuum*. The residue was taken up in AcOEt and the organic layer washed with 1 N $KHSO_4$, saturated aqueous $NaHCO_3$ and H_2O. The residue obtained after drying and evaporation was chromatographed on silica gel using a $CHCl_3$/MeOH (99:1) mixture as eluent, to give **1** (3.31 g, 94%) as a white solid (m.p. 115–116°C, MeOH). R_f(A) = 0.7; $[\alpha]_D = -8.3°$ (c = 1, $CHCl_3$); IR (KBr) ν_{max}: 3375, 3250, 3030, 1750, 1700, 1655, 1570, 1500 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.45 (18H, s, CH_3), 2.8 (1H, m, Cys β-CH_B), 3.0 (1H, m, Cys β-CH_A), 3.15–3.3 (2H, m, Fm CH_2), 3.85 (2H, m, Gly CH_2), 4.15 (1H, m, Fm CH), 4.25 (1H, m, Cys α-CH), 5.4 (1H, br d, Cys NH), 6.65 (1H, br t, Gly NH), 7.25–7.4 (4H, m, ArH), 7.65–7.8 (4H, m, ArH). Anal. ($C_{28}H_{36}N_2O_5S$).

HCl · H-Cys(Fm)-Gly-OBu^t (**2**). To a solution of HCl 4 M in dioxane (100 ml) stirred at ice bath temperature, a solution of protected dipeptide **1** (3.11 g, 6.06 mmol) in dioxane (5 ml) was added under argon. After 1 h

under stirring at room temperature, the solvent was removed and the residue repeatedly washed with diethyl ether to give 2.64 g (97%) of compound **2** as a white solid. The product could be used in the subsequent reaction without further purification.

Boc-Glu[-Cys(Fm)-Gly-OBu^t]-OBu^t (**3**). Boc-Glu-OBu^t (0.46 g, 1.51 mmol) was suspended in THF (4.5 ml) and HOBt (0.20 g, 1.51 mmol) was added with stirring at 0°C. An ice-cold solution of the hydrochloride **2** (0.68 g, 1.51 mmol) and NMM (0.15 g, 1.51 mmol) in THF (3 ml) was added, followed by chilled portions of a solution of DCC (0.31 g, 1.51 mmol) in THF (2 ml). The mixture was stirred 5 h at 0°C and refrigerated overnight. The solid was removed by filtration, the filtrate evaporated to dryness and the residue taken up in AcOEt. The organic layer was washed with 1 N $KHSO_4$, saturated aqueous $NaHCO_3$ and H_2O. Drying and evaporation followed by purification of the resulting crude material on a silica gel column developed with $CHCl_3$/MeOH (98:2) afforded protected tripeptide **3** as a white foam (0.77 g, 73%). R_f(B) = 0.35; $[\alpha]_D = -6.8°$ (c = 1, $CHCl_3$); IR (KBr) ν_{max}: 3320, 3030, 1735, 1720, 1650, 1600, 1525 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.4 (27H, s, CH_3), 1.8 (2H, m, Glu β-CH_2), 1.9 (1H, m, Glu γ-CH_B), 2.2 (1H, m, Glu γ-CH_A), 2.8 (1H, m, Cys β-CH_B), 3.0 (1H, m, Cys β-CH_A), 3.2–3.35 (2H, m, Fm CH_2), 3.8 (2H, m, Gly CH_2), 4.15 (1H, m, Fm CH), 4.2 (1H, m, Cys α-CH), 4.5 (1H, m, Glu α-CH), 5.2 (1H, br d, Cys NH), 6.7 (1H, br t, Gly NH), 6.8 (1H, br d, Glu NH), 7.25–7.4 (4H, m, ArH), 7.65–7.8 (4H, m, ArH). Anal. ($C_{37}H_{51}N_3O_8S$).

H-Glu[-Cys(Fm)-Gly-OH]-OH (**4**). The protected tripeptide **3** (0.61 g, 0.87 mmol) was treated with TFA 50% in DCM (8 ml) at room temperature. After 1 h 30 min the solution was evaporated under reduced pressure below 40°C and the residue triturated with portions of fresh diethyl ether to give the corresponding trifluoroacetate in 75% yield as a white solid.

The salt (0.39 g, 0.65 mmol) was dissolved in a water/methanol (1:1) mixture (2 ml) and the resulting solution treated with 0.1 M sodium phosphate buffer pH 7. The concentrated solution was subjected to column chromatography on Sephadex-LH-20 using H_2O as eluent to afford the tripeptide **4** (0.25 g, 79%) as a white solid (m.p. 181°C dec., MeOH/AcOEt). R_f(C) = 0.45; $[\alpha]_D = -7.0°$ (c = 1, H_2O); IR (KBr) ν_{max}: 3500–3295 br, 1630–1620, 1605, 1525 cm^{-1}; ^{1}H NMR (D_2O): δ 1.85 (2H, m, Glu β-CH_2), 2.2 (2H, m, Glu γ-CH_2), 2.35 (1H, m, Cys β-CH_B), 2.6 (1H, m, Cys β-CH_A), 3.25 (2H, m, Fm CH_2), 3.5 (3H, m, Gly CH_2 and Fm CH), 4.1 (2H, m Cys and Glu α-CH), 7.15–7.3 (4H, m, ArH), 7.4 (2H, m, ArH), 7.65 (2H, m, ArH). ^{13}C NMR (D_2O): δ 26.36 (Glu Cβ), 31.46 (Glu Cγ), 35.25 (Cys Cβ), 36.98 (Fm CH_2), 43.51 (Gly Cα), 46.31 (Fm CH), 53.09 (Glu Cα), 54.18 (Cys Cα), 119.87, 124.70, 127.18, 127.60, 140.79, 145.53 (aromatics), 164.95, 174.08, 174.39 and 176.14 (CO). MS (*m*/*z*): 484 (M–H$^+$). Anal. ($C_{24}H_{27}N_3O_6S$).

Boc-Glo[-Cys(Fm)-Gly-OBu^t]-OBu^t (**5**). To a stirred solution containing the hydrochloride **2** (1.82 g, 4.05 mmol) and NMM (0.41 g, 4.05 mmol) in dioxane (10 ml) Boc-Glo(ON*p*)-OBu^t (Calcagni et al., 1996) (1.73 g, 4.05 mmol) in dioxane (8 ml) was added. The mixture was warmed to 80°C and left to stir for 23 h. The formed precipitate was removed by filtration and the solution evaporated *in vacuo* to give an oily residue which was taken up in $CHCl_3$. The organic layer was washed with 1 N $KHSO_4$, saturated aqueous Na_2CO_3 and H_2O, dried and filtered. The filtrate was concentrated under reduced pressure, and the solution eluted from a silica gel column using a $CHCl_3$/MeOH (98:2) mixture as eluent, to give the protected tripeptide **5** as a white foam (1.67 g, 59%). R_f(B) = 0.4; $[\alpha]_D = +5.6°$ (c = 1, $CHCl_3$); IR (KBr) ν_{max}: 3400 br, 3100, 1735, 1705, 1660, 1540 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.4 (27H, s, CH_3), 2.65 (1H, m, Cys β-CH_B), 2.9 (1H, m, Cys β-CH_A), 3.05–3.2 (2H, m, Fm CH_2), 3.8 (2H, m, Gly CH_2), 4.0–4.4 (5H, m, Fm CH, Cys and Glo α-CH, Glo β-CH_2), 5.3 (1H, br d, Cys NH), 5.65 (1H, br d, Glo NH), 6.6 (1H, br t, Gly NH), 7.2–7.35 (4H, m, ArH), 7.5–7.65 (4H, m, ArH). Anal. ($C_{36}H_{49}N_3O_9S$).

H-Glo[-Cys(Fm)-Gly-OH]-OH (**6**). The tripeptide derivative **5** (1.52 g, 2.17 mmol) was deprotected by treatment with TFA 40% in DCM (21 ml) at room temperature in 2 h 30 min. Solvent removal under *vacuum* below 40°C, followed by trituration of the oily material with anhydrous ethyl ether afforded the trifluoroacetate in 84% yield as a white powder, which was used without further purification.

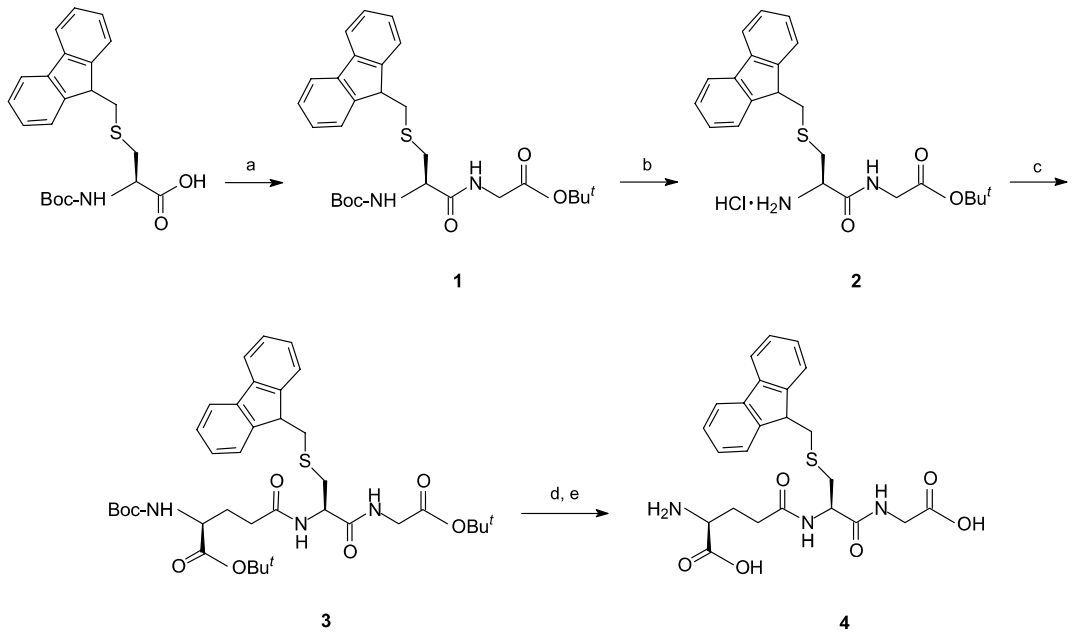
The salt (1.1 g, 1.82 mmol) was dissolved in a water/methanol (1:1) mixture (4 ml) and the pH of the resulting solution adjusted to 7.0 by NMM. The concentrated solution was applied on a Sephadex LH-20 column eluted with a water/methanol (2:1) mixture, and the ninhydrin positive fractions were combined to give the final compound **6** as a pure amorphous solid (0.30 g, 42%). R_f(C) = 0.55; $[\alpha]_D = +13.2°$ (c = 0.25, H_2O); IR (KBr) ν_{max}: 3320 br, 3030, 1700, 1690–1610, 1530 cm^{-1}; ^{1}H NMR (D_2O): δ 2.4 (1H, m, Cys β-CH_B), 2.6 (1H, m, Cys β-CH_A), 3.25 (2H, m, Fm CH_2), 3.5 (3H, m, Gly CH_2 and Fm CH), 3.9 (2H, m, Glo β-CH_2), 4.2 (2H, m, Cys and Glo α-CH), 7.15–7.3 (4H, m, ArH), 7.5 (2H, m, ArH), 7.65 (2H, m, ArH). ^{13}C NMR (D_2O): δ 34.11 (Cys Cβ), 35.11 (Fm CH_2), 43.35 (Gly Cα), 46.55 (Fm CH), 54.02 (Glo Cα), 54.59 (Cys Cα), 67.92 (Glo Cβ), 120.20, 124.81, 127.68, 128.03, 141.18, 145.65 (aromatics), 156.84 (OCONH), 174.08, 176.22 and 176.74 (CO). MS (*m*/*z*): 486 (M–H$^+$). Anal. ($C_{23}H_{25}N_3O_7S$).

Biological assays

Human GSTA1-1, GSTM2-2, and GSTP1-1 were expressed in *Escherichia coli* and purified as previously described (Lo Bello et al., 1995). Enzymatic experiments concerning the *in vitro* inhibitory activity of the synthesized compounds were performed at 25°C in 1 ml (final volume) of 0.1 M potassium phosphate buffer pH 6.5 containing 1 mM GSH, 1 mM 1-chloro-2,5-dinitrobenzene (CDNB) as acceptor substrate and variable amounts of inhibitor **4** or **6**. Enzymatic activities were determined spectrophotometrically by measuring the change in absorbance at 340 nm due to the GS-DNB conjugate formation ($\varepsilon_{340} = 9.6$ mM^{-1} cm^{-1}), and normalized for the rate of reaction obtained in the absence of inhibitor. One unit of GST activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of conjugate per minute at 25°C. Protein concentrations were determined by using the bicinchoninic acid (BCA) method.

Chemistry

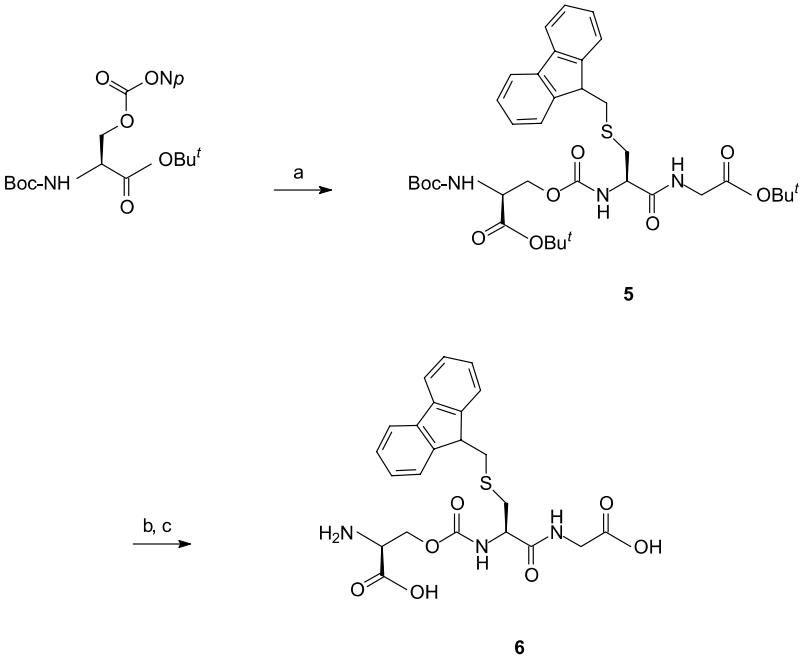
The GSH *S*-derivatives **4** and **6** were synthesized in good overall yields employing solution phase procedures by stepwise elongation of the peptide chain in the *C*-to-*N* direction, as outlined in Schemes 1 and 2. A minor problem associated with the preparation of the target peptides was the choice of the most convenient synthetic intermediates, since Fmoc as well as methyl ester derivatizations had to be avoided to ensure *N*- and *C*-terminal deprotection without affecting the base sensitive fluorenylmethyl *S*-protecting group (Bodanszky and Bednarek, 1982). We took note of the report by Hruby et al. (Han et al., 2001), which describes an efficient method for the selective removal of the Boc amino protecting group in peptide *t*-butyl esters. For the purpose Boc-Cys(Fm)-Gly-OBu^t (**1**) was chosen as precursor to both tripeptides **4** and **6**. Dipeptide **1** was obtained in good yields by DCC/HOBt mediated condensation of commercially available Boc-Cys(Fm)-OH with H-Gly-OBu^t, and then subjected to Boc cleavage by means of HCl 4 M in dioxane at room temperature according to the cited protocol to pro-



Scheme 1. Reagents and conditions: *a*) HCl · H-Gly-OBu^t, DCC, HOBt, NMM, *THF*, 0°C, 5 h and 5°C, 16 h; *b*) HCl 4 M in *dioxane*, r.t., 1 h; *c*) Boc-Glu(OH)-OBu^t, DCC, HOBt, NMM, *THF*, 0°C, 5 h and 5°C, 16 h; *d*) TFA 50% in *DCM*, r.t., 1 h 30 min; *e*) 0.1 M sodium phosphate buffer pH 7, *MeOH*

vide HCl · H-Cys(Fm)-Gly-OBu^t (**2**). The subsequent coupling via DCC/HOBt of the hydrochloride **2** with Boc-Glu-OBu^t to furnish the protected tripeptide Boc-Glu[-Cys(Fm)-Gly-OBu^t]-OBu^t (**3**) proceeded in good yields (Scheme 1). The complete removal of N^{α}-Boc and *t*-butyl ester groups in the key compound **3** by acidolysis with TFA 50% in DCM by following well-established conditions afforded the corresponding trifluoroacetate salt, which was then converted to the fully deprotected tripeptide H-Glu[-Cys(Fm)-Gly-OH]-OH (**4**) by addition of 0.1 M sodium phosphate buffer pH 7.

A simple modification of the aforementioned route gave access to modified tripeptide **6** (Scheme 2). The urethanic junction was built up by convenient β-function-



Scheme 2. Reagents and conditions: *a*) **2**, NMM, *dioxane*, 80°C, 23 h; *b*) TFA 40% in *DCM*, r.t., 2 h 30 min; *c*) NMM, $H_2O/MeOH$

alization of a protected serine residue. Thus acylation of Boc-Ser-OBu^t with *p*-nitrophenyl chloroformate according to the procedure previously reported by us (Calcagni et al., 1996) gave Boc-Glo(ON*p*)-OBu^t as γ-oxa-glutamic acid (Glo) derivative. The active carbonate was coupled with HCl · H-Cys(Fm)-Gly-OBu^t (**2**) in the presence of NMM in dioxane at 80°C for 23 h to afford Boc-Glo[-Cys(Fm)-Gly-OBu^t]-OBu^t (**5**) in satisfactory yields. The cleavage in a single step of all the protecting groups in **5** by TFA 40% in DCM, followed by treatment of the resulting trifluoroacetate with NMM in hydro-alcoholic solution, gave the deblocked pseudotripeptide H-Glo[-Cys(Fm)-Gly-OH]-OH (**6**) in acceptable amounts. The crude peptides **4** and **6** were purified to homogeneity by gel filtration on a Sephadex LH-20 column and fully characterized by optical rotation, NMR and FT-IR spectroscopy. The structures of the final peptides were confirmed by *electrospray ionization* mass spectrometry.

Results and discussion

The GSH conjugate **4** and its isostere **6** were synthesized and assayed for their ability to inhibit the GSH conjugation reaction with CDNB, mediated by human GSTA1-1, GSTP1-1 and GSTM2-2 isoenzymes. In the case of H-Glu[Cys(Fm)-Gly-OH]-OH (**4**) an highly potent inhibitory activity towards GST A1-1 was observed, with an $IC_{50} = 0.11 \pm 0.01\ \mu M$ (Fig. 1, Panel A). The same compound is able to inhibit Mu and Pi isoforms efficiently ($IC_{50} = 6.45 \pm 0.74\ \mu M$ and $32.85 \pm 1.26\ \mu M$, respectively) (Fig. 1, Panels B and C). From the data shown, conjugate **4** appears a far better inhibitor of the Alpha protein in respect with the other isoenzymes, confirming the role of the fluorenylmethyl nucleus in modulating the interaction with the largely hydrophobic cavity typical of this class. Based on these results, conjugate **4** seemed a promising starting compound in the development of GSH-derived selective GST inhibitors.

Unfortunately this derivative cannot be used *in vivo* due to its γ-GT sensitiveness. In the search for a metabolically stable GST inhibitor, we exploited the chemical strategy to remove the major site for metabolic attack with the introduction of a more resistant carbamate junction. This substitution was investigated for the potential to confer stability towards γ-GT, as well as to produce changes in the nature of inhibition. In the inhibitory experiments

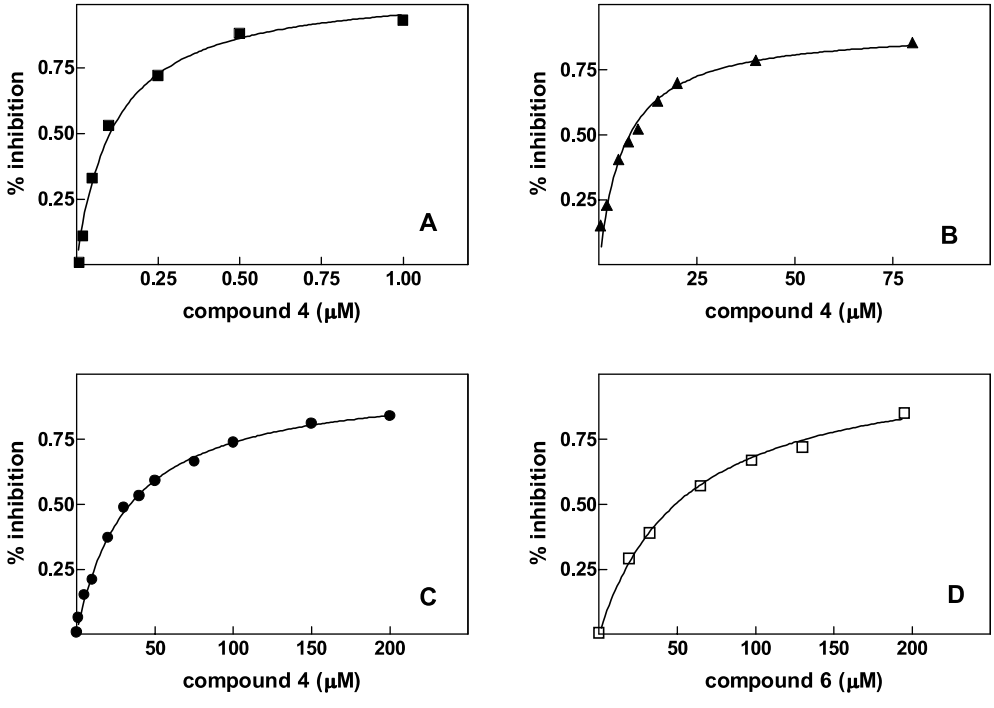


Fig. 1. Inhibition of GST activity by H-Glu[-Cys(Fm)-Gly-OH]-OH (**4**) and H-Glo[-Cys(Fm)-Gly-OH]-OH (**6**). Inhibition experiments were performed at 25°C in 0.1 M potassium phosphate buffer pH 6.5 containing 1 mM GSH, 1 mM CDNB and variable amounts of inhibitor **4** or **6**. Inhibition of GSTA1-1, GSTM2-2 and GSTP1-1 activities by compound **4** is reported in panels **A**–**C**. Solid line is the best fit of experimental data to a hyperbolic binding equation which fulfils the IC_{50} values of $0.11 \pm 0.01\ \mu M$, $6.45 \pm 0.74\ \mu M$ and $32.85 \pm 1.26\ \mu M$ for GSTA1-1 (panel **A**), GSTM2-2 (panel **B**) and GSTP1-1 (panel **C**) respectively. The inhibition of GSTA1-1 by compound **6** is reported in panel **D** with an IC_{50} value of $55.03 \pm 4.79\ \mu M$

conducted with GSTP1-1 and GSTM2-2, pseudopeptide **6** shows no appreciable activity, while a residual affinity for the GSTA1-1 isozyme is conserved ($IC_{50} = 55.03 \pm 4.79\,\mu M$) (Fig. 1, Panel D). This change in biological activity may be reasonably ascribed to a drop in productive interactions at the level of the γ-Glu subsite, connected with the specific conformational and electronic properties of the OCONH surrogate. Further studies on characterization of enzyme inactivation type by compounds **4** and **6** are under way.

Conclusions

The suggestion that the complex detoxifying pathways involving GSH and glutathione-dependent proteins may be concerned with anticancer drug resistance has spurred much interest towards the development of specific modulators of xenobiotic conjugation and transport, with the aim to provide increased understanding of resistance mechanisms and to generate more effective tools for cancer treatment.

In this context a novel GSH conjugate and its γ-oxaglutamyl isostere were synthesized and evaluated as GST inactivators. The results of this study disclose an important lead compound, which is characterized by an extremely potent and selective inhibitory activity towards human GSTA1-1. Further experimentation is necessary to prove the nature of the binding for this strong inhibitor. The incorporation of an OCONH unit as isosteric modification of the native γ-glutamyl moiety furnished a γ-GT resistant analog which maintains inhibitory activity towards the GSTA1-1 isoform, albeit to a weaker extent than the parent compound, while is devoid of effects on the remaining isoenzymes. Since our first introduction on the GSH backbone, this chemical variation at the level of the γ-glutamyl peptide bond has been used extensively in a variety of GSH-derived compounds in an effort to confer stability to the resulting pseudopeptides. Invariably, a comparison of the biological activity profile between the unmodified and the modified peptides has corroborated the conviction that the affinity of ligands for GSH-related proteins is essentially governed by the integrity of the γ-glutamyl moiety. For the case at hand, the OCONH *versus* CONH replacement at the Glu-Cys proteolytic site causes a real reduction of the inhibitory effect towards all the three considered isoforms, but interestingly the activity on the Alpha class is maintained at a satisfactory extent and selectivity is conserved.

These derivatives will be worth studying further to explore their full potential in the treatment of drug resistant tumors, particularly those depending upon GSTA1-1 detoxifying activity.

References

Adang AE, Brussee J, Meyer DJ, Coles B, Ketterer B, van der Gen A, Mulder GJ (1988) Substrate specificity of rat liver glutathione *S*-transferase isoenzymes for a series of glutathione analogues, modified at the gamma-glutamyl moiety. Biochem J 255: 721–724

Bodanszky M, Bednarek MA (1982) Derivatives of *S*-9-fluorenylmethyl-L-cysteine. Int J Pept Protein Res 20: 434–437

Bolton MG, Colvin OM, Hilton J (1991) Specificity of isozymes of murine hepatic glutathione *S*-transferase for the conjugation of glutathione with L-phenylalanine mustard. Cancer Res 51: 2410–2415

Burg D, Mulder GJ (2002) Glutathione conjugates and their synthetic derivatives as inhibitors of glutathione-dependent enzymes involved in cancer and drug resistance. Drug Metab Rev 34: 821–863

Cacciatore I, Caccuri AM, Di Stefano A, Luisi G, Nalli M, Pinnen F, Ricci G, Sozio P (2003) Synthesis and activity of novel glutathione analogues containing an urethane backbone linkage. Il Farmaco 58: 787–793

Calcagni A, Duprè S, Lucente G, Luisi G, Pinnen F, Rossi D (1995) Synthesis and activity of the glutathione analogue γ-(L-γ-azaglutamyl)-L-cysteinyl-glycine. Int J Pept Protein Res 46: 434–439

Calcagni A, Duprè S, Lucente G, Luisi G, Pinnen F, Rossi D, Spirito A (1996) Synthesis and activity of the glutathione analogue γ-(L-γ-oxaglutamyl)-L-cysteinyl-glycine. Arch Pharm Pharm Med Chem 329: 498–502

Davies W Jr, Ronai Z, Tew KD (2001) Cellular thiols and reactive oxygen species in drug-induced apoptosis. J Pharmacol Exp Ther 296: 1–6

Dirven HA, van Ommen B, van Bladeren PJ (1994) Involvement of human glutathione *S*-transferase isoenzymes in the conjugation of cyclophosphamide metabolites with glutathione. Cancer Res 54: 6215–6220

Fotouhi-Ardakani N, Woo A, Lewandowska M, Schecter R, Batist G (1998) Identification of the Yc1 glutathione *S*-transferase mRNA as the overexpressed species in a nitrogen mustard-resistant rat mammary carcinoma cell line. J Biochem Mol Toxicol 12: 11–17

Han G, Tamaki M, Hruby VJ (2001) Fast, efficient and selective deprotection of the *tert*-butoxycarbonyl (Boc) group using HCl/dioxane (4 M). J Peptide Res 58: 338–341

Hayes JD, Flanagan JU, Jowsey IR (2005) Glutathione transferases. Annu Rev Pharmacol Toxicol 45: 51–88

Koehler RT, Villar HO, Bauer KE, Higgins DL (1997) Ligand-based protein alignment and isozyme specificity of glutathione *S*-transferase inhibitors. Proteins: Structure, Function, and Genetics 28: 202–216

Lang M, Pelkonen O (1999) Metabolism of xenobiotics and chemical carcinogenesis. IARC Sc Publ 148: 13–22

Lewis AD, Hickson ID, Robson CN, Harris AL, Hayes JD, Griffiths SA, Manson MM, Hall AE, Moss JE, Wolf CR (1988) Amplification and increased expression of alpha class glutathione *S*-transferase-encoding genes associated with resistance to nitrogen mustards. Proc Natl Acad Sci USA Cell Biol 85: 8511–8515

Lo Bello M, Battistoni A, Mazzetti AP, Board PG, Muramatsu M, Federici G, Ricci G (1995) Site-directed mutagenesis of human glutathione transferase P1-1. Spectral, kinetic, and structural properties of Cys-47 and Lys-54 mutants. J Biol Chem 270: 1249–1253

Luisi G, Calcagni A, Pinnen F (1993) $\Psi(SO_2\text{-NH})$ transition state isosteres of peptides. Synthesis of the glutathione disulfide analogue [γ-Glu-$\psi(SO_2\text{-NH})$-Cys-Gly]$_2$. Tetrahedron Lett 34: 2391–2392

Morel F, Schulz WA, Sies H (1994) Gene structure and regulation of expression of human glutathione *S*-transferases alpha. Biol Chem Hoppe Seyler 375: 641–649

Morgan AS, Ciaccio PJ, Tew KD, Kauvar LM (1996) Isozyme-specific glutathione *S*-transferase inhibitors potenziate drug sensitivity in cultured human tumor cell lines. Cancer Chemother Pharmacol 37: 363–370

Oakley AJ, Lo Bello M, Battistoni A, Ricci G, Rossjohn J, Villar HO, Parker MW (1997) The structures of human glutathione transferase P1-1 in complex with glutathione and various inhibitors at high resolution. J Mol Biol 274: 84–100

O'Brien ML, Tew KD (1996) Glutathione and related enzymes in multidrug resistance. Eur J Cancer 32 A: 967–978

Paglialunga Paradisi M, Mollica A, Cacciatore I, Di Stefano A, Pinnen F, Caccuri AM, Ricci G, Duprè S, Spirito A, Lucente G (2003) Proline-glutamate chimeras in isopeptides. Synthesis and biological evaluation of conformationally restricted glutathione analogues. Bioorg Med Chem 11: 1677–1683

Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF (2003) The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol 66: 1499–1503, and references therein

Salinas AE, Wong MG (1999) Glutathione *S*-transferases – A Review. Curr Med Chem 6: 279–309

Schultz M, Dutta S, Tew KD (1997) Inhibitors of glutathione *S*-transferases as therapeutic agents. Adv Drug Deliver Rev 26: 91–104

Wilce MCJ, Parker MW (1994) Structure and function of glutathione *S*-transferases. Biochim Biophys Acta 1205: 1–18

Authors' address: Dr. Grazia Luisi, Department of Drug Sciences, "G. D'Annunzio" University, Via dei Vestini 31, 66013 Chieti, Italy,
Fax: +39-(0)6-3555 267, E-mail: gluisi@unich.it