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Original article

Sulphonamide-based bombesin prodrug analogues for glutathione transferase, useful in targeted cancer chemotherapy

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ABSTRACT

Glutathione transferases (GSTs) are enzymes involved in cellular detoxification by catalysing the nucleophilic attack of glutathione (GSH) on the electrophilic centre of a number of toxic compounds and xenobiotics, including certain chemotherapeutic drugs. The encountered chemotherapeutic resistant of tumour cells, thus, has been associated with the increase of total GST expression. GSTs, in addition to GSH-conjugating activity, exhibit sulphonamidase activity, catalyzing the GSH-mediated hydrolysis of sulphonamide bonds. Such reactions are of interest as potential tumour-directed prodrug activation strategies. In the present work we report the design and synthesis of novel chimaeric sulphonamide derivatives of bombesin, able to be activated by the model human isoenzyme GSTA1-1 (hGSTA1-1). These derivatives bear a peptidyl-moiety (analogues of bombesin peptide: R–[Lue^{13}]-bombesin, R–[Phe^{13}]-bombesin and R–[Ser^{3},Arg^{10},Phe^{13}]-bombesin, where R = $C_6H_5SO_2NH$–) as molecular recognition element for targeting the drug selectively to tumour cells. The released *S*-alkyl-glutathione, after hGSTA1-1-mediated cleavage of the sulphonamide bond, provides an inhibitor of varied strength against GSTs from different sources. These prodrugs are envisaged as a plausible means to sensitize drug-resistant tumours that overexpress GSTs.

1. Introduction

Glutathione transferases (GSTs) are enzymes involved in cellular detoxification by catalysing the nucleophilic attack of glutathione (GSH) on the electrophilic centre of a number of toxic compounds and xenobiotics, including certain chemotherapeutic drugs. The GST superfamily can be subdivided into a number of classes on the basis of their amino acid sequence [1]. Within mammals, the following classes have been defined: alpha, mu, pi, sigma, theta, zeta, kappa and omega [2]. In addition, a subfamily of chloride intracellular channel proteins has been shown to be members of the cytosolic GST structural family but have no known enzymatic activity [3]. Several other soluble GST classes have been reported in insects: delta, epsilon [4]; plants: phi, tau, lambda, dehydroascorbate reductase [5]; and bacteria: beta [6] and chi [7]. The cytosolic GSTs are homodimers or heterodimers [8]. Each monomer has two domains, an α/β domain that includes α1–α3, and a large α-helical domain comprised of helices α4–α9. The former contains a GSH-binding site (G-site) on top of the large α domain. A hydrophobic pocket (H-site) lies between the two domains in which a generally hydrophobic substrate binds and reacts with GSH [8].

Cancer remains the second-leading cause of death in the industrialized world and worldwide; nevertheless it continues to be underserved by effective therapeutic agents [9]. Many of the available agents act systemically and therefore have side effects that range from uncomfortable to life threatening. Recently, products have begun to emerge in this market that are specifically targeted to cancer cells or act in collaboration with the body's immune response to combat the disease. This marks a dynamic change in the way cancer is treated, and such innovative therapies will transform the cancer market during the next decade [10].

Although GSTs' detoxifying ability protects cell from certain diseases, unfortunately it also reduces the effectiveness of certain chemotherapeutic drugs against cancer cells. Indeed, one of the classes of electrophilic compounds that are substrates for the GSTs are certain alkylating agents used in antineoplastic therapy [11]. A common problem encountered in cancer chemotherapy is the

Abbreviations: Boc, *t*-butoxycarbonyl group; Bu^t, *t*-butyl group; CDNB, 1-chloro-2,4-dinitrobenzene; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; G-site, glutathione binding site; GSH, glutathione; GST, glutathione transferase; H-site, hydrophobic binding site; HOBt, 1-hydroxybenzotriazole; IPTG, isopropyl-thiogalactoside; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TFA, trifluoroacetic acid; Trt, trityl group.

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appearance of chemotherapeutic resistant tumour cells that no longer respond appropriately to the antineoplastic agents. This phenomenon, referred to as multi-drug resistance, has complicated attempts towards cancer therapy [12]. A possible origin for the problem appears to be an increase in the expression of total GST activity in tumour cells [13,14]. A plausible mechanism by which GSTs could contribute to drug resistance includes GST-dependent prevention of drug-induced apoptosis via direct interaction with signal transduction proteins, as suggested for GSTP1-1 [15,16] which inhibits c-Jun N-terminal kinase. It has been demonstrated that hGSTA1/A2 protein was increased in blast cells (derived from acute myeloid leukemia patients) showing resistance to doxorubicin *in vitro* [13], and a weak correlation was observed between GST alpha in gastric cancer tissues and cisplatin resistance (*in vitro*) [14]. However, more recently, it was shown that homozygous hGSTA1*B breast cancer patients treated with cyclophosphamide (plus other chemotherapeutic drugs) had a reduced death hazard during the first 5 years following diagnosis compared with homozygous hGSTA1*A individuals (hazard ratio, 0.3) [15]. This observation was attributed to the detoxifying role of hepatic hGSTA1 against therapeutic metabolites of cyclophosphamide.

The present work proposes a prodrug-design approach based on the development of novel chimaeric synthetic sulphonamide-derivatives, susceptible to activation by the human isoenzyme GSTA1-1 (hGSTA1-1). These chimaeric prodrugs feature a peptidyl-moiety (bombesin peptide analogues) that is specifically recognised by a tumour cell specific receptor (bombesin receptor), to provide a potential vehicle for selective drug delivery to cancer cells. Followed by GST-mediated cleavage of the sulfonamide bond, the prodrug releases a potent inhibitor for GSTs. During the past decade, extensive knowledge has been accumulated on the involvement of bombesin peptide-analogues in the mitogenesis of various tumour cells, including small cell lung carcinoma (SCLC), cancers of the gastrointestinal tract, such as pancreatic and colon cancer, as well as breast cancer [17]. The putative role of bombesin-like peptides as autocrine growth factors for these tumours [18] prompted us to design and synthesis chimaeric bombesin-analogues which may prove useful in the combat of certain cancers [18].

2. Materials and methods

2.1. Materials

Reduced glutathione, 1-chloro-2,4-dinitrobenzene (CDNB) and human GSTP1-1 isoenzyme were obtained from Sigma–Aldrich Co (USA). Molecular biology reagents, kits and enzymes were obtained from Invitrogen (USA). Other reagents and analytical grade chemicals were obtained from Sigma–Aldrich Co (USA). The 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, Rink Amide MBHA resin and peptide reagents were purchased from CBL (Patras, Greece), Bachem (Bubendorf, Switzerland) and Novabiochem (Läufelfingen, Switzerland). Benzenesulfonyl chloride (C_6H_5–SO_2–Cl) was obtained from Acros Organics (Geel, Belgium). All solvents and reagents used for solid phase synthesis were of analytical quality and used without further purification.

2.2. Methods

2.2.1. Cloning, expression and purification of GSTs

PCR was used to amplify the full-length ORF of hGSTA1-1 from pKKGTB vector (a much appreciated gift from Prof. W.M. Atkins, Department of Medicinal Chemistry, University of Washington) using the oligo primers synthesized to the 5′ region of the gene (5′-CACCATGGCAGAGAAGCCCAAGCTCCAC-3′) and to the 3′ end of the gene finishing at the TAA stop codon (5′-TTAAAACCT-GAAAATCTTCCTTGCTTC-3′). The PCR reaction was carried out in a total volume of 50 μl contained 6 pmol of each primer, 10 ng plasmid DNA, 0.2 mM of each dNTP, 5 μl 10 × *Pfu* buffer and 2 units of *Pfu* DNA polymerase. The PCR procedure comprised 30 cycles of 2 min at 95 °C, 2 min at 55 °C and 2 min at 72 °C. A final extension time at 72 °C for 10 min was performed after the 30 cycles. The resulting PCR amplicon was TOPO ligated into a T7 expression vector (pET101/D-TOPO®). The resulting expression construct pT7hGSTA1-1 was sequenced along both strands and was used to transform competent BL21(DE3) *Escherichia coli* cells. *E. coli* cells, harbouring plasmid pT7hGSTA1-1, were grown at 37 °C in 1 L LB medium containing 100 μg/mL ampicillin. The synthesis of hGSTA1-1 was induced by the addition of 1 mM IPTG when the absorbance at 600 nm was 0.6–0.8. Five hours after induction, cells were harvested by centrifugation at 8000 r.p.m. and 4 °C for 20 min, re-suspended in sodium phosphate buffer (5 mM, pH 7.7), sonicated, and centrifuged at 10,000 g for 20 min. The supernatant was collected and was applied to a column of *S*-hexyl-Sepharose column (2 ml, 1.5 × 1.5 cm I.D.) previously equilibrated with 5 mM sodium phosphate buffer, pH 7.7. Non-adsorbed protein was washed off with 100 ml equilibration buffer. Bound hGSTA1-1 was eluted with potassium phosphate buffer (50 mM, pH 8.0, containing 10 mM GSH). The eluted fractions were dialysed against 50 mM potassium phosphate buffer, pH 7.5 and stored at 4 °C. For long term storage the enzyme solution was stored at −20 °C in glycerol/0.1 M potassium phosphate buffer pH 7.0, 50/50 (v/v). Expression and purification of *Zea mays* GST I were performed according to Ref. [19]. Human spleen haematopoietic prostaglandin D synthase was purified as described in Ref. [20], whereas soybean GSTU4-4 was expressed and purified as described in Ref. [21].

2.2.2. Electrophoresis

Protein purity was judged by SDS polyacrylamide gel electrophoresis using 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel, according to the methods of Laemmli, 1970 [24]. The protein bands were stained with Coomassie Brilliant Blue R-250.

2.2.3. Synthesis, purification and quality assessment of sulphonamide bombesin-analogues (prodrugs)

Sulfonamide bombesin-analogues were synthesized by Fmoc solid phase methodology [22] utilizing Rink Amide MBHA resin [23] as the solid support. Fmoc-protected amino acids were used with the *t*-butyl group (Bu^t) as side-chain protection group for Glu, *t*-butoxycarbonyl group (Boc) for Trp, trityl (Trt) group for Asn, His, Gln and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group for Arg. Stepwise synthesis of the peptide analogues was achieved with diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) in dimethylformamide (DMF) as coupling agents [24]. Couplings were performed with Fmoc-amino acid, DIC and HOBt in DMF in a 3.0, 3.3 and 4.5 molar excess, respectively, for 2.5 h at room temperature. Completeness of the reaction was monitored by the Kaiser test [25], and the chloranil test [26]. The Fmoc groups were removed by treatment with 20% piperidine in DMF for 40 min.

In order to finally synthesis the sulphonamide bombesin-analogues (prodrugs), the pGlu residue from the N-terminal of the native bombesin sequence was replaced for a Glu which has a free amino group available for coupling with another group (e.g. benzylsulfonyl chloride). Coupling of benzenesulfonyl group with the free aminoterminal, and formation of the respective sulfonamide bond, was achieved using *N*-methylmorpholine. Benzenesulfonyl chloride was used in 3 molar excess and *N*-methylmorpholine in 6 molar excess. The pH of the reaction was monitored and adjusted in the range 10–11 with addition of *N*-methylmorpholine. Coupling of the benzenesulfonyl-group was completed within 3.5 h. After completion of the synthesis, the resin was treated with TFA solution (15 ml/g peptide resin) in the presence of scavengers

(TFA/1,2-ethanedithiol/triethylsilane/water/anisole, 95/1/1/1/2 v/v/v/v/v) for 4 h to liberate the fully deprotected crude peptide conjugate. The released peptide prodrug conjugate was precipitated upon solvent concentration and addition of cold ether. The precipitate was collected by filtration, washed twice with cold ether, dried in vacuum over KOH and purified by gel filtration chromatography on Sephadex G-15 using 20% acetic acid as the eluent. Final purification was achieved by preparative high performance liquid chromatography (HPLC, Mod.10 ÄKTA, Amersham Biosciences, Piscataway, USA) on a Lichrosorb RP18 column (C_{18} solid phase, 7 μm particle size, 250 mM × 8 mm) applying a linear gradient 10–70% acetonitrile (0.1% TFA) for 35 min, and 70–100% acetonitrile (0.1% TFA) for 5 min (flow rate 1.5 ml/min, UV detection at 220 nm and 254 nm). The appropriate fractions were pooled and lyophilized. Synthesis and purification of benzylsulfonyl-GSH was carried out by substituting the fluoride atom of benzylsulfonyl fluoride by reduced GSH according to the procedure of Katusz et al. [27]. All prodrug products were analysed by thin layer chromatography (TLC, Merck pre-coated silica gel plates, type G_{60}-F_{254}; solvent system 1-butanol/acetic acid/water/pyridine (4/1/1/2 v/v/v/v)), before were further checked for their purity by analytical HPLC (ÄKTA Purifier) using a Nucleosil 100 C_{18} column (5 μm particle size; 250 × 4.6 mm), and Electron Spray Ionization-Mass Spectrometry (ESI-MS) on a Micromass-Platform LC instrument.

2.2.4. Assay of enzyme activity and protein

GST assays were performed by monitoring the formation of the conjugate of CDNB (1 mM) and GSH (2.5 mM) at 340 nm ($\epsilon = 9.6\ mM^{-1}\ cm^{-1}$) at 30 °C according to a published method [19–21]. Observed reaction velocities were corrected for spontaneous reaction rates when necessary. All initial velocities were determined in triplicate in buffers at constant temperature. One unit of enzyme is defined as the amount of enzyme that gives 1.0 μmole of product per minute at pH 6.5 at 30 °C. Determination of sulphanilamide cleavage was carried out by measuring the released NH_3, using the Nessler's reagent. Determination of sulfonamide bombesin analogues cleavage by hGSTA1-1 was based on the detection of the non-conjugated substrate GSH using Elman's reagent (2-nitro-5mercaptobenzoic acid) at 412 nm. Assays were carried out at 25 °C for 2–16 h in 20 mM potassium phosphate buffer, pH 7, in the presence of 2.88 mM GSH and 2.2 mM peptide. Control incubations in the absence of GSH, peptide or enzyme were also carried out to correct for background rates of spontaneous chemical cleavage. Specific activities were determined as ΔA/h at 412 nm per mg protein. Protein concentration was determined by the method of Bradford [28] using bovine serum albumin (fraction V) as standard.

2.2.5. Kinetic inhibition studies with benzylsulfonyl-GSH

Initial velocities for the hGSTA1-1-catalysed reaction with GSH as variable substrate were measured at 30 °C in a total volume of 1 ml total mixture containing 100 mM potassium phosphate buffer, pH 6.5, 1 mM CDNB, and different concentrations of GSH in the presence or in the absence of 5 or 10 μM benzylsulfonyl-GSH. With CDNB as variable substrate, the reaction mixture contained in a total volume of 1 ml: 100 mM potassium phosphate buffer, pH 6.5, 1 mM GSH and different concentrations of CDNB, again in the presence or in the absence of 2 or 10 μM benzyl sulfonyl-GSH. The apparent kinetic parameters were determined using the GraFit (Erithacus Software, Ltd., UK) computer program.

2.2.6. Docking of the sulphanilamide lead-ligand into the hGSTA1-1 binding site

The high-resolution structure of hGSTA1-1 in complex with the inhibitor ethacrynic acid (PDB identifier 1GSF) [29] was used in the docking studies. The protein was prepared for docking by removing the inhibitor and adding hydrogen atoms to fulfill unsatisfied valencies. The sulphanilamide (Fig. 2B) was used as a ligand. Ligand pdb file was generated using JME Molecular Editor software [30] and energy minimized. Hydrogen atoms were added to the ligand coordinate file prior to docking. The ArgusLab software, version 4.0 [31,32] was employed for predicting the geometry of the ligand bound to the protein. ArgusLab makes use of either AScore or the Lamarckian genetic algorithm [33] scoring functions to find the low-energy binding modes. A distance-dependent dielectric ($\epsilon = 4r$) and an energy cut-off distance of 10 Å were used in evaluating the interaction energy between protein and ligand. Ligand conformational flexibility was explored through torsion angle sampling and minimization. PyMOL [34] was used for the preparation of structure figures. The type of interactions in the complex hGSTA1-1-sulphanilamide were analysed using iMolTalk (http://i.moltalk.org/).

3. Results and discussion

3.1. Sulphonamidase activity of GSTs

Glutathione transferases (GSTs) catalyze the nucleophilic attack of glutathione on the electrophilic centre of a number of compounds. GSTs exhibit wide substrate specificity and act *in vitro* and *in vivo* on several xenobiotics [35]. In addition to GSH conjugating activity, GSTs exhibit sulphonamidase activity and catalyze the GSH-mediated hydrolysis of sulphonamide bonds to form the corresponding amine (Fig. 1) [36,37].

To analyze this sulphonamidase activity, a collection of different GST isoenzymes that belong to different classes were investigated using sulphanilamide (4-aminobenzenesulfonamide) as a model substrate. In particular, GSTs belonging to class alpha [human GSTA1-1, (hGSTA1-1); mouse GSTA4-4, (mGSTA4-4)], pi [human GSTP1-1, (hGSTP1-1)], sigma [*Schistosoma japonicum* GST, (SjGST); human spleen haematopoietic prostaglandin D synthase, (PDS-GST)], tau [*Glycine max* GST, (GmGSTU4-4)] and phi [*Zea mays* GST I (ZmGSTI)] were examined and the results are presented in Table 1. All but tau and phi class isoenzymes (tau and phi are plant specific classes) show sulphonamidase activity towards sulphanilamide. The isoforms examined exhibited different rates of sulphanilamide cleavage, demonstrating isoenzyme selectivity. These variations are consistent with the fact that GST isoenzymes show marked differences in their abilities to catalyze reactions between GSH and various electrophiles. The notable sulphonamidase activity observed for hGSTA1-1 is of particular importance since this isoenzyme is expressed in several tumours and therefore may represent a therapeutic molecular target in cases where tumour-protective effects depend upon hGSTA1-1 activity. It is interesting to note that alpha class GSTs have hardly been explored with respect to chemotherapy response, even though a number of alkylating chemotherapeutic agents in current use are known to be substrates (e.g. busulphan, thiotepa, and the therapeutic metabolites of cyclophosphamide) [38,39].

Although all mammalian cytosolic GSTs share a highly conserved GSH-binding site within their canonical fold, the electrophilic substrate binding site (H-site), varies significantly among different isoforms [2,8], and it is reasonably expected to provide a source of isoform specificity among substrates and inhibitors. Therefore, molecular docking studies were carried out to provide *in silico* structural information and help to locate the sulphanilamide ligand binding site on hGSTA1-1. The model for the binding of sulphanilamide lead-ligand (NH_2SO_2*Benz*) to hGSTA1-1 was constructed based on: (i) the proposed role of the active site Tyr7 in catalysis, (ii) the fact that the GSH sulphur attacks carbon-4 of sulphanilamide in the reaction and (iii) manual fitting to obtain optimum interactions between the sulphanilamide's hydrophobic

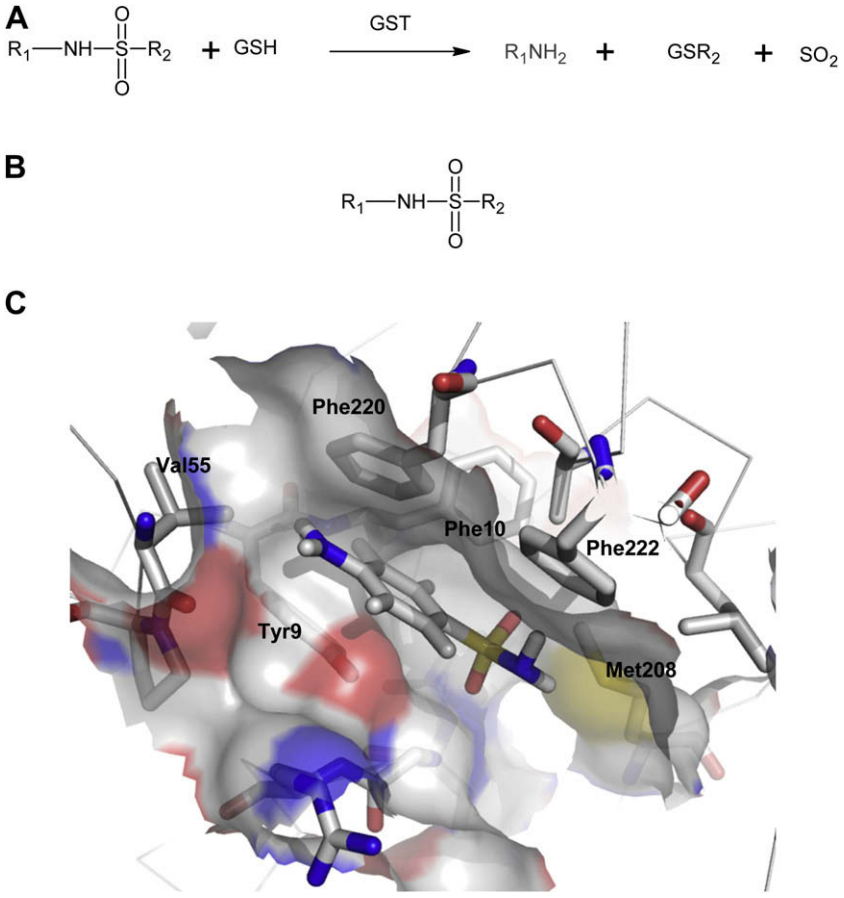


Fig. 1. A. Sulphonamides cleavage by GSTs. B: The general structure of the proposed chimaeric prodrug (e.g. R_1 = bombesin, R_2 = *Benz*). C: Interaction of sulphanilamide lead-ligand with hGSTA1-1 from docking calculations. Side-chains of specific residues contributing to sulphanilamide binding are presented as heavy stick beneath the transparent surface and labelled. The figure was prepared using PyMol [34].

chain and the hydrophobic residues of the H-site. The predicted mode of interaction of the sulphanilamide with hGSTA1-1 is shown in Fig. 1C. The binding of sulphanilamide to hGSTA1-1 may primarily be achieved by hydrophobic interactions that provide the driving force for ligand positioning and recognition. The bulk of the interactions with the enzyme involve mainly hydrophobic residues from the N-terminal domain (Tyr9, Phe10, Val55), and hydrophobic residues from the C-terminal domain Met208, Phe220 and Phe222 (Fig. 5).

Table 1
Sulphonamidase activity of different GST isoenzymes using sulphanilamide as substrate. The isoenzymes used were: human GSTA1-1, (hGSTA1-1); human GSTP1-1, (hGSTP1-1); mouse GSTA4-4, (mGSTA4-4); *Schistosoma japonicum* GST, (SjGST); *Glycine max* GSTU4-4, (GmGSTU4-4); *Zea mays* GST I, (ZmGST I); and human spleen haematopoietic prostaglandin D synthase, (PDS-GST). The enzymatic reactions were corrected for the nonenzymatic hydrolysis. Reported data are the average of three separate experiments.

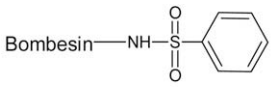
GSTs	Enzyme activity (ΔA/h mg)
SjGST	39.7
hGSTA1-1	35.29
mGSTA4-4	500.0
PDS-GST	245.6
hGSTP1-1	12.9
GmGSTU4-4	nd[a]
ZmGSTI	nd

[a] No detectable.

3.2. Design of sulphonamide prodrugs

The sulphonamidase activity of GSTs suggests that prodrugs activated by GSTs might provide a strategy for targeted cancer chemotherapy. This concept was realized by designing chimaeric sulphonamide derivatives (bombesin analogues, Fig. 2) able to undergo cleavage in tumour cells by intracellular GSTs where certain GST isoenzymes, such as hGSTA1-1, are highly expressed [11,13,14]. Selective targeting of these prodrugs to cancer tissues might be made possible if they bore a chemical moiety (e.g. bombesin analogues) to serve as a ligand for a cancer tissue-specific receptor. Widely variable structures are apparently accommodated by the H-site of GST. For example, sulphonamide derivatized aliphatic or aromatic primary and secondary amines, amides, azides, ureas, and hydrazines have been shown to be substrates for GSTs [37]. Zhao et al. (1999) have shown that GST-mediated sulphonamide cleavage is relatively independent of the nature of the amine derivatized and any amine, in principle, can be derivatized by linkage with sulphonyl moieties, to confer a high or low degree of intracellular liability [36]. This possibility is very significant because it suggests that complex peptides having free amino groups can be transformed to sulphonamides (prodrugs) which are then activated intracellularly upon cleavage by GSTs. The structural element that would target the prodrug selectively to tumour cells may be bombesin peptide analogues which are selectively recognised by tumour cell surface bombesin receptors [17,40,41]. The released S-alkyl-glutathionyl-analogue (GS–R, Fig. 2B) may provide a strong inhibitor against intracellular GSTs, thus facilitating cancer chemotherapy. In this respect, it is likely that the observed strong

A



B

C

	Analogue
Bombesin	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
R—[Leu^{13}]-bombesin	C_6H_5-SO_2-Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
R—[Phe^{13}]-bombesin	C_6H_5-SO_2-Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
R—[Ser^3,Arg^{10},Phe^{13}]-bombesin	C_6H_5-SO_2-Glu-Gln-Ser-Leu-Gly-Asn-Gln-Trp-Ala-Arg-Gly-His-Phe-Met-NH_2

Fig. 2. A: The general structure of the sulphonamide bombesin analogues (prodrugs). B: Sulphonamide cleavage by hGSTA1-1 using GSH as a nucleophile. C: The primary structure of bombesin and its sulphonamide-analogues, R = $C_6H_5SO_2$–.

inhibition of S-alkyl-glutathionyl-analogues against GSTs is associated with a 'product inhibition' mode of action [42]. Product inhibition has been observed with many GS–R conjugates, including, for example, GS–estradiol and GS–aflatoxin conjugates [43,44]. Conjugates such as S-hexyl-GSH and *S*-benzyl-GSH are commonly used *in vitro* as biochemical probes and inhibitors. Among them *S*-benzyl-GSH is usually considered as the strongest inhibitor.

On the basis of the above, bombesin analogues, coupled via a sulphonamide linker to a benzyl group, may provide vehicles for selective delivery of potent GST inhibitors (GS–R) to cancer cells. This approach may be proved effective in overcoming chemotherapeutic drug resistance. In addition, the bombesin analogues have been shown to exhibit antitumour effects on several cell lines such as SW-1990 human pancreatic cancers, nitrosamine-induced pancreatic cancers in hamsters, H69 human SCLC, MKN45 and Hs746T human gastric cancers, HT-29 human colon cancers, PC-82, PC-3, and DU-145 human prostate cancers, androgen independent Dunning R-3327-AT-1 rat prostate cancers, estrogen dependent and independent MXT mouse mammary cancers, MCF-7 MIII human breast cancer, and U-87MG and U-373MG human glioblastomas [17,18].

Table 2
Physicochemical properties of the new sulphonamide bombesin analogues.

Analogue	MW	[M + 1]	Yield (%)	TLC Rf_1	HPLC t_R (min)
R–[Leu^{13}]-bombesin	1778.20	1779.8	78	0.30	17.05R–
R–[Phe^{13}]-bombesin	1812.04	1813.1	79	0.32	17.32
R–[Ser^3,Arg^{10},Phe^{13}]-bombesin	1799.99	1801.8	80	0.35	16.17

3.3. Sulphonamide prodrug synthesis

Three different bombesin analogues [Leu^{13}]-bombesin, [Phe^{13}]-bombesin and [Ser^3,Arg^{10},Phe^{13}]-bombesin (Fig. 2C) were synthesized by Fmoc solid phase methodology [22] utilizing Rink Amide MBHA resin [23] as the solid support. The amino acid substitutions were decided on the basis of their differential potency against the bombesin receptor [17,40,41]. In order to achieve prodrug formation, the pGlu residue from the N-terminal of the native bombesin sequence was replaced for Glu which has a free amino group available for coupling with another group. Coupling of benzenesulfonyl group with the free aminoterminal and formation of the sulfonamide bond, was achieved using *N*-methylmorpholine. Other organic bases such as triethylamine, collidine and

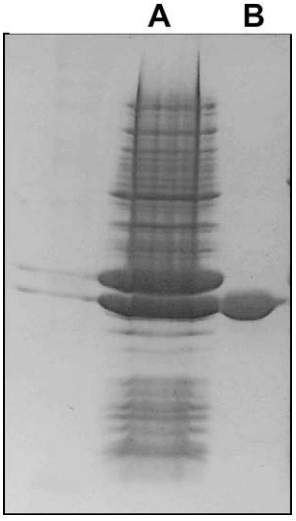


Fig. 3. SDS–polyacrylamide gel electrophoresis of hGSTA1-1 preparations. Protein bands were stained with Coomassie Brilliant Blue R-250. Lane A, *Escherichia coli* crude extract after induction with 1 mM IPTG; Lane B, eluted fraction from *S*-hexyl-GSH-Sepharose.

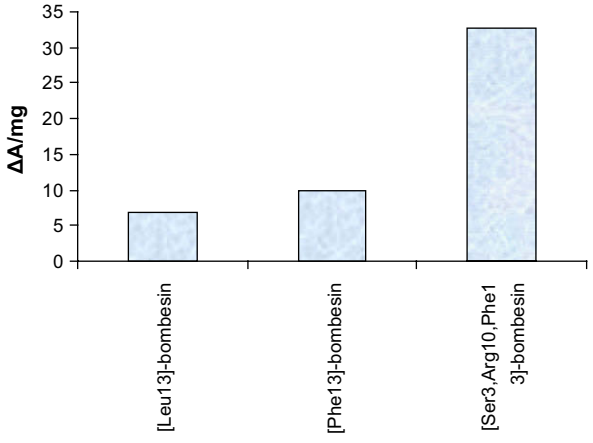


Fig. 4. Schematic graph of the hGSTA1-1 sulphonamidase activity towards the synthetic sulphonamide bombesin analogues (prodrugs). Control incubations in the absence of GSH, peptide or enzyme were carried out to correct for background rates of spontaneous chemical cleavage.

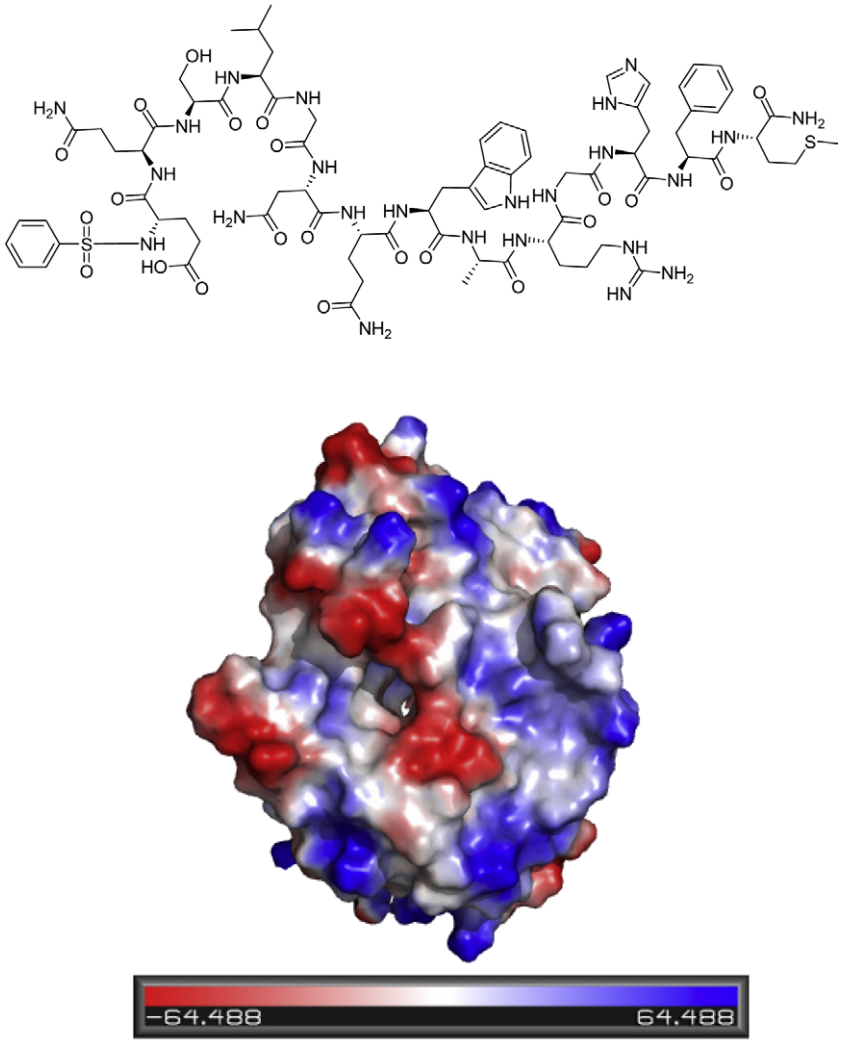


Fig. 5. Analysis of putative binding site of hGSTP1-1. A: The structure of analogue [Ser^{3},Arg^{10},Phe^{13}]-bombesin (sulphonamide bombesin analogue No3). B: Molecular surfaces around the enzyme's binding site were created and coloured by electrostatic potential (red denotes a negative potential, white a neutral potential and blue a positive potential). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

N,*N*-diisopropylethylamine were tested for the coupling reaction, but *N*-methylmorpholine was found to be most effective. The peptide prodrug conjugates finally released from the solid phase were precipitated and purified by GFC (Sephadex G-15, 20% acetic acid liquid phase) followed by preparative HPLC (Lichrosorb RP18). Analysis of prodrugs by TLC resulted in single spots, whereas that by HPLC produced single peaks corresponding to at least 97% of the total peptide peak integrals column. Electron Spray Ionization-Mass Spectrometry (ESI-MS) data were consistent with the expected peptide formulae (Fig. 2C). The physicochemical properties of the new analogues are summarised in Table 2.

3.4. Sulphonamide prodrug cleavage by hGSTA1-1

Before kinetic analysis of the prodrug cleavage reaction, it was necessary to obtain large quantities of hGSTA1-1 isoenzyme. For achieving high expression level of hGSTA1-1 the full-length gene of the enzyme was subcloned into a T7-based expression vector (pET101/D-TOPO®). The resulting recombinant plasmid was used to transform the expression host *E. coli* BL21(DE3). Cell-free extract of the *E. coli* transformants showed high GST activity with a specific activity of 1.6 U/mg protein (Fig. 3), which is approximately 115-fold higher compared to the original expression system based on the pKKGTB expression plasmid. The recombinant enzyme was purified in a single-step procedure, using affinity chromatography on immobilized *S*-hexyl-GSH affinity column (Fig. 3).

The three different bombesin analogues (1: [Leu^{13}]-bombesin, 2: [Phe^{13}]-bombesin and 3: [Ser^{3},Arg^{10},Phe^{13}]-bombesin) were evaluated as potential hGSTA1-1 substrates. Based on previous studies [36,37], the reaction of GST-mediated sulfonamide cleavage occurs as in the scheme shown in Fig. 2B, whereas the catalytic mechanism may be described as follows: binding of GSH at the G-site of hGSTA1-1 results in formation of the thiolate anion GS^{-}. This nucleophile then attacks the (aromatic) carbon atom linked to the sulphonyl moiety of the peptide substrate, which is bound at the H-site of GST. Sulphonamide cleavage results in the formation of the GS-conjugate, the corresponding amine (peptide), and sulphur dioxide. Release of sulphur dioxide and the amine is either concerted or results from subsequent hydrolysis of an intermediate, initially formed sulphur dioxide-amine adduct. All sulphonamide bombesin analogues were cleavaged by the recombinant enzyme (Fig. 4). Among them, [Ser^{3},Arg^{10},Phe^{13}]-bombesin served as a better substrate for hGSTA1-1. This may be due to the presence of a positively charged Arg5 in the analogue, establishing favourable electrostatic interactions with the negative electrostatic potential observed for the enzyme (Fig. 5).

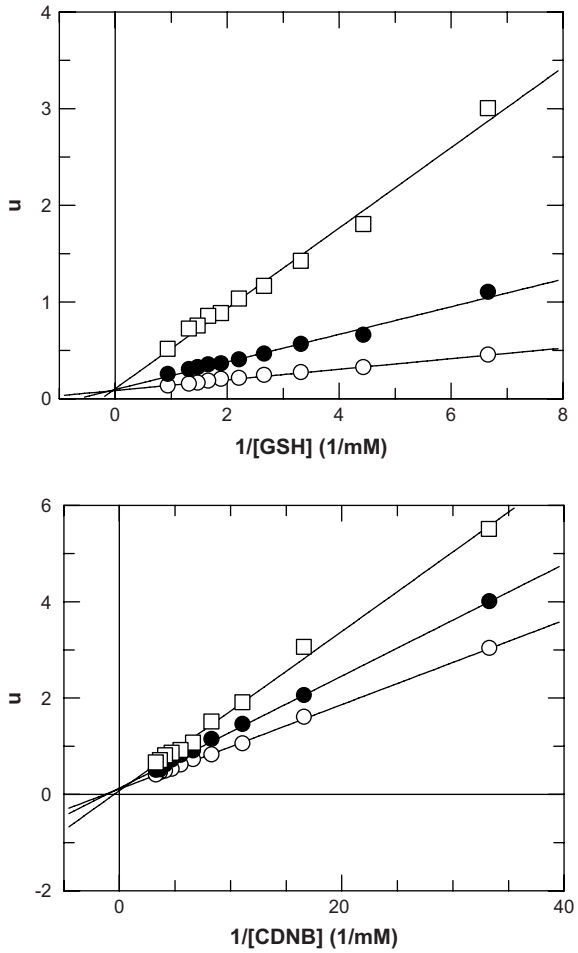


Fig. 6. Kinetic inhibition studies. A: Lineweaver–Burk plots for the inhibition of hGSTA1-1 by benzylsulfonyl-GSH at different GSH concentrations. Enzyme assayed in the absence (○) or in the presence of benzylsulfonyl-GSH (μM): 5 (●); 15 (□). B: Inhibition of hGSTA1-1 by benzylsulfonyl-GSH at different CDNB concentrations. Enzyme assayed in the absence (○) or in the presence of benzylsulfonyl-GSH (μM): 5 (●); 10 (□).

Kinetic inhibition studies were carried out to evaluate the inhibition potency of benzylsulphonyl-GSH. The inhibition patterns are illustrated in Fig. 6. Benzylsulphonyl-GSH exhibited a competitive-type of inhibition with respect to GSH ($K_i = 8.1 \pm 0.7$ μM) and CDNB ($K_i = 1.7 \pm 0.02$ μM). This finding indicates that the released inhibitor binds fairly strongly to the substrate binding site of hGSTA1-1 and is expected to be an effective inhibitor to other GST isoenzymes present in cancer cells.

In the past, modulation by inhibition of GST has been attempted as a means to improve response to cancer drugs. Use of, for example, ethacrynic acid, although effective during its experimental behaviour with various GST isozymes, was not successful enough in the clinic to merit continued development [45]. In another approach the peptidomimetic inhibitor of GSTP1-1, TLK199 [γ-glutamyl-*S*-(benzyl)cysteinyl-R(−)phenyl glycine diethyl ester] was envisaged as a plausible means to sensitize drug-resistant tumours that overexpress GST [46].

More recently GST-activated prodrugs were developed to exploit high hGSTP1-1 levels associated with malignancy, poor prognosis, and the development of drug resistance [11]. For example, in TLK286 (Telcyta), the sulphydryl of a glutathione conjugate has been oxidized to a sulphone [47,48]. The tyrosine-7 in hGSTP1-1 promotes a β-elimination reaction that cleaves the latter compound. The cleavage products are a glutathione analogue and a phosphorodiamidate, which in turn spontaneously forms aziridinium species, the actual alkylating moieties [49]. More recently Saavedra et al., (2004) have designed PABA/NO, a NO-releasing GST-activated prodrug. PABA/NO, after hGSTP1-1-catalyzed conjugation to GSH, releases a diazeniumdiolate ion, with subsequent release of nitric oxide [50]. Encouraging results in both cell and animal models of cancer have suggested that this prodrug exhibits improved cytotoxic selectivity toward cancer cells, most likely due to the high levels of GST in these cells. The above approach exemplifies the one described in the present study, nevertheless it lacks the second feature, namely, a structural element that would target the drug selectively to tumor cells.

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