European Journal of Medicinal Chemistry 44 (2009) 2009–2016

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

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

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ARTICLE INFO

Article history:
Received 1 June 2008
Received in revised form
26 September 2008
Accepted 2 October 2008
Available online 17 October 2008

Keywords:
Alkylating agents
Antineoplastic therapy
Drug resistance
Detoxification
Enzyme-activated prodrug
Glutathione transferase

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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doi:10.1016/j.ejmech.2008.10.009

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