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Ligand-induced glutathione transferase degradation as a therapeutic modality: Investigation of a new metal-mediated affinity cleavage strategy for human GSTP1-1

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ABSTRACT

Glutathione transferases (GST, EC. 2.5.1.18) are overexpressed in cancer cell and have been shown to be involved in cancer cell growth, differentiation and the development of multi-drug resistance (MDR) mechanism. Therefore, GST inhibitors are emerging as promising chemosensitizers to manage and reverse MDR. The present work aims to the synthesis, characterization and assessment of a new active-site chimeric inhibitor towards the MDR-involved human GSTP1-1 isoenzyme (hGSTP1-1). The inhibitor [BDA–Fe(III)] was designed to possess two functional groups: the anthraquinone moiety, as recognition element by hGSTP1-1 and a metal chelated complex [iminodiacetic acid-Fe(III)] as a reactive moiety, able to generate reactive oxygen species (ROS), through Fenton reaction. Upon binding of the BDA–Fe(III) to hGSTP1-1 in the presence of hydrogen peroxide, reactive oxygen species (ROS) are generated, which promoted the specific cleavage of hGSTP1-1 in a time and concentration-dependent manner. Electrophoretic analysis showed that each enzyme subunit is cleaved at a single site. Amino acid sequencing as well as molecular modelling studies established that the cleaved peptide bond is located between the amino acids Tyr103 and Ile104. This ligand-induced hGSTP1-1 degradation and inactivation strategy is discussed as a new approach towards chemosensitization of MDR cancer cells.

1. Introduction

The cytosolic glutathione transferases (GSTs, EC. 2.5.1.18) catalyze the conjugation of glutathione (γ-Glu-Cys-Gly, GSH) with a broad range of electrophilic substrates, including alkylating drugs (e.g. bendamustine, busulfan, chlorambucil, cisplatin) that are used in antineoplastic therapy, leading to their detoxification [1–4]. Consequently, GSTs detoxifying ability although it protects and detoxify normal cells from deleterious compounds, unfortunately, also compromises the efficiency of antineoplastic therapy [4–6]. GSTs contribute two distinct roles in the development of cancer cell MDR phenomenon: either participate in direct detoxification of chemotherapeutics or by inhibiting the mitogen-activated protein (MAP) kinase pathway [7,8]. Extensive published work have demonstrated that hGSTP1-1, a particular GST overexpressed in most tumor cell lines and tumor tissues [5,6], forms a tight complex with c-Jun N-terminal kinase (JNK), thus preventing the apoptotic cascade related to the phosphorylation of c-Jun [8–10].

The development of chemotherapeutic resistant tumor cells, during cancer chemotherapy, remains an important problem that restricts the success of the therapeutic protocols and approaches since cancer cells do not respond appropriately to the antineoplastic drugs [6,11]. Hence, one of the proposed strategies to overcome multidrug resistance can be based on the use of specific GST inhibitors [3,11–17], that can function as regulatory agents in those cases where anticancer drugs are substrates for GSTs and therefore are detoxified through GSH catalysis. So far, the majority of GST-based drug design efforts are focused mainly on structure-guided approaches using heterocyclic compounds or GSH analogues as leaders for inhibitor design [3,11–17].

The isoenzyme hGSTP1-1 is an attractive drug target due to its high levels of disease association (target validation) and druggability (target tractability) properties [5,6,17–21]. hGSTP1-1 functions as a homodimer protein [18]. Each monomer has two domains, an α/β domain (N-terminal domain) that includes helices α1-α3 and a large α-helical

Abbreviations: BDA–Fe(III), bromaminic-iminodiacetic acid-Fe(III) complex; CB3GA, Cibacron blue 3GA; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GST, glutathione transferase; G-site, GSH binding site; H-site, hydrophobic site; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MDR, multi-drug resistance; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate.

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domain (C-terminal domain). The N-terminal domain forms the GSH binding site (G-site), whereas the hydrophobic site (H-site), which binds the xenobiotic substrates, is located mainly at the C-terminal domain [18].

Several metal chelate, are effective reagents for cleavage of the polypeptide chains by reactive oxygen species (ROS) [22–26]. Such reagents that composed by a metal-chelating group and a functional group capable of reacting with a specific group on the protein have been synthesized and employed for probing structure-function relationships in proteins [26].

In the present work, in an effort of designing an affinity reagent capable of interacting with hGSTP1-1 we achieved the design, synthesis and evaluation of a chimeric inhibitor composed by an affinity and a reactive moiety. We showed that upon interaction of hGSTP1-1 with the inhibitor, ROS are generated by Fenton chemistry reactions that inactivate and chemically digest the enzyme.

1.1. Materials

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and all other reagents (Cibacron blue 3GA, bromaminic acid) and analytical grade chemicals were obtained from Sigma-Aldrich Co (USA). Molecular biology reagents, enzymes and kits were obtained from Invitrogen (USA).

2. Methods

2.1. Cloning, expression, and purification of hGSTP1-1

Expression of recombinant hGSTP1-1 isoenzyme was achieved in *E. coli* BL21(DE3) cells according to Zompra et al., [14]. Purification of recombinant hGSTP1-1 was achieved by using affinity chromatography on S-hexyl-glutathione-Sepharose, according to Zompra et al., [14]. Before kinetics analysis the purified enzyme was subjected to extensive dialysis against the potassium phosphate buffer (20 mM, pH 6.5).

2.2. Synthesis and purification of inhibitors

Purification of Cibacron blue 3GA (CB3GA) and other analogues was carried out as described in [27,28]. The synthesis of BDA–Fe(III) was achieved using the general protocol described by Baqi et al., 2009 [29], as follows: bromaminic acid (0.5 g), iminodiacetic acid (0.5 g), Na_2CO_3 (0.14 g) and $CuSO_4$ (0.020 g) were dissolved in 12 mL of water. The reaction mixture was stirred for 12 h at 80 °C. After completion of the reaction the mixture was cooled for 12 h at 4 °C, and the formed precipitate was filtered off. The Fe(III) complex was prepared by directly mixing $Fe(NO_3)_3 9H_2O$ with the bromaminic-iminodiacetic acid conjugate in aqueous solution at the iron-to-ligand molar ratio (1:1) with stirring for 1.0 h, as described by Zou et al., 2013 [30]. Purification was achieved by lypophilic Sephadex LH 20 as described in [28].

2.3. Assay of hGSTP1-1 activity and determination of protein concentration

GST assays were performed by monitoring the formation of the conjugate between CDNB and GSH at 340 nm ($\varepsilon = 9.6\ mM^{-1}\cdot cm^{-1}$) according to a published method [14,21]. Half maximal inhibitory concentration (IC_{50}) values were calculated from the initial velocities of hGSTP1-1-dependent conjugate formation in the presence of different concentration of inhibitors. The experimental data (initial velocities) were analysed using the computer program GraphPad Prism 5. All initial velocities were determined in triplicate in buffers equilibrated at constant temperature. One unit of enzyme is defined as the amount of enzyme that produces 1.0 μmole of product per min.

2.4. Enzyme inactivation studies by BDA–Fe(III) inhibitor

Inactivation of hGSTP1-1 was performed in 1 mL of incubation mixture containing MES buffer (pH 6.5, 50 mM), BDA–Fe(III) (5–20 μM), enzyme and hydrogen peroxide (1 mM). The rate of inactivation was

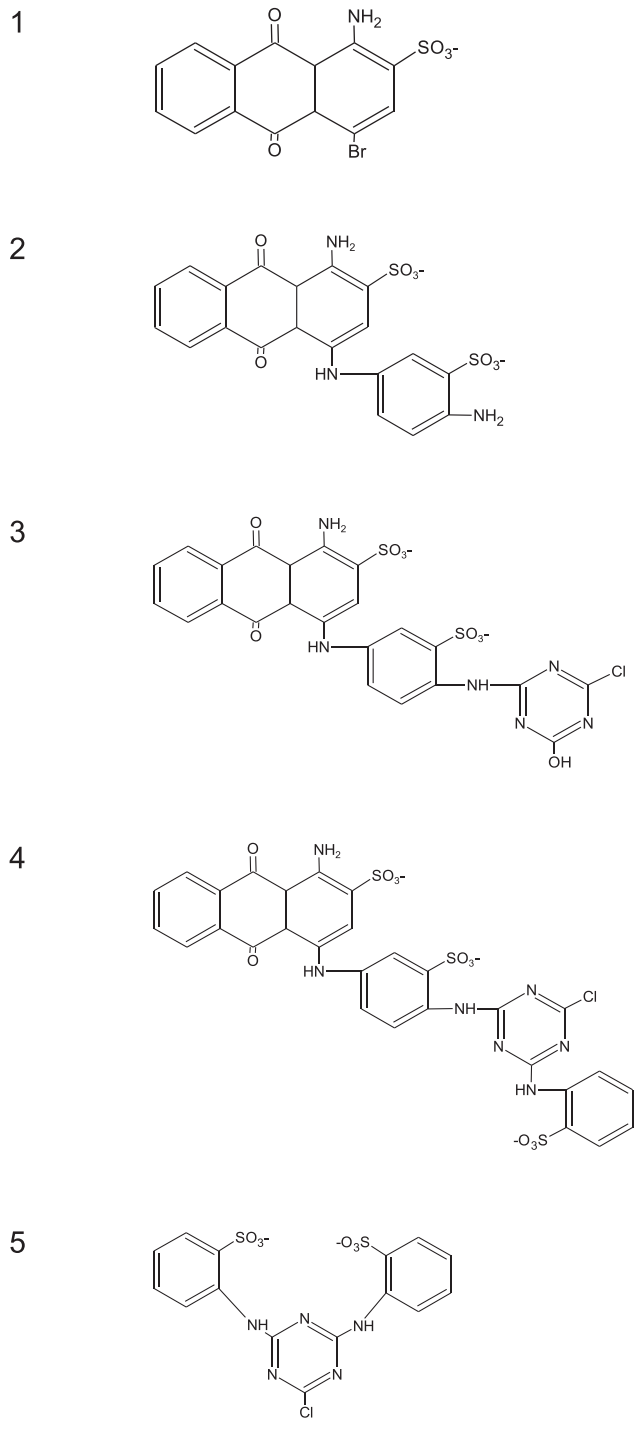

Fig. 1. The structure of CB3GA analogues used in the present study. 1: 1-amino-4-bromo-9,10-dioxo-4a,9,9a,10-tetrahydroanthracene-2-sulphonic acid; 2: 1-amino-4-[(4-amino-3-sulphophenyl)amino]-9,10-dioxo-4a,9,9a,10-tetrahydroanthracene-2-sulphonic acid; 3: hydrolysed 1-amino-4-[3-(4,6-dichlorotriazin-2-ylamino)-4-sulphophenylamino] anthraquinone-2-sulphonic acid, 4: Cibacron blue 3GA; 5: 2-({4-chloro-6-[(2-sulphophenyl)amino]-1,3,5-triazin-2-yl}amino)benzenesulphonic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)