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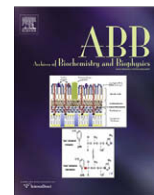

Molecular evolution of Theta-class glutathione transferase for enhanced activity with the anticancer drug 1,3-bis-(2-chloroethyl)-1-nitrosourea and other alkylating agents ☆

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ABSTRACT

Glutathione transferase (GST) displaying enhanced activity with the cytostatic drug 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and structurally related alkylating agents was obtained by molecular evolution. Mutant libraries created by recursive recombination of cDNA coding for human and rodent Theta-class GSTs were heterologously expressed in *Escherichia coli* and screened with the surrogate substrate 4-nitrophenethyl bromide (NPB) for enhanced alkyltransferase activity. A mutant with a 70-fold increased catalytic efficiency with NPB, compared to human GST T1-1, was isolated. The efficiency in degrading BCNU had improved 170-fold, significantly more than with the model substrate NPB. The enhanced catalytic activity of the mutant GST was also 2-fold higher with BCNU than wild-type mouse GST T1-1, which is 80-fold more efficient than wild-type human GST T1-1. We propose that GSTs catalyzing inactivation of anticancer drugs may find clinical use in protecting sensitive normal tissues to toxic side-effects in treated patients, and as selectable markers in gene therapy.

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Introduction

Chloroethylnitrosoureas (CENUs)[1] are chemotherapeutic agents used in clinical treatment of different types of cancers [1]. In order to become potent cytotoxic compounds CENUs must be converted into electrophilic products that can alkylate macromolecules [2–4]. This occurs by spontaneous decomposition of the compounds in aqueous media. Due to their high lipophilicity CENUs penetrate the blood–brain barrier and are frequently used in treatment of brain tumors and recent attention has been directed to localized chemotherapy based on CENU implants [5]. In general, the usefulness of antitumor agents is severely limited by their toxicity to normal tissues in patients undergoing treatment. Toxic effects of CENUs include bone marrow suppression, loss of hair, and gastrointestinal dysfunction with nausea and vomiting. In addition to adverse side-effects a common clinical problem is the appearance of resistant tumor cells that no longer respond to chemotherapy. Many attempts have been made to synthesize CENUs with enhanced antineoplastic activity in combination with reduced toxicity to normal cells. For example, proline analogs of CENUs have been synthesized to obtain compounds whose action on neoplastic cells is characterized by higher selectivity [6]. A complement to creating new CENUs could be to administer an enzyme that provides protection to the rapidly proliferating normal cells that are most seriously affected by the toxic effects of the drug, in particular the hematopoietic cells of the bone marrow.

One of the most widely used CENUs is 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU, also called carmustine), a cytostatic drug employed in the treatment of brain tumors, lymphomas, multiple myeloma, and malignant melanoma [1]. The biotransformation of BCNU involves conjugation with the intracellular nucleophile glutathione (GSH). The reaction between BCNU and GSH is catalyzed by glutathione transferases (GSTs), as first demonstrated with cytosol fractions from rodent livers [7,8], and is considered to be a drug resistance mechanism. The chemical mechanism involves an alkyltransferase reaction resulting in detoxication. The ubiquitous GSTs have a central role in detoxication reactions occurring in many biological species including man [9,10]. GSTs catalyze the attack of GSH on a variety of electrophilic substrates, thereby usually decreasing their reactivity with cellular macromolecules [11]. Our early research involving rat and human cell lines suggested that Mu-class GSTs were major contributors to resistance against BCNU [12,13]. However, an extensive study involving 12 different GSTs in purified form, demonstrated that human Theta-class GST T1-1 (hGST T1-1) was more active than the Mu class GSTs

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[1] *Abbreviations used:* CENUs, chloroethylnitrosoureas; GSH, glutathione; GST, glutathione transferase; hGST T1-1, human glutathione transferase T1-1; rGST T2-2, rat GST T2-2; mGST T1-1, mouse GST T1-1; rGST T1-1, rat GST T1-1; NPB, 4-nitrophenethyl bromide; EPNP, 1,2-epoxy-3-(4-nitrophenoxy)-propane; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; CCNU, 3-cyclohexyl-1-(2-chloroethyl)-1-nitrosourea.

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by at least an order of magnitude [14]. Nevertheless, naturally occurring GSTs are characterized by relatively modest activity with anticancer drugs, suggesting that their catalytic efficiencies could be enhanced by directed evolution.

The development of GST variants that display higher efficiency in catalyzing the conjugation of GSH with BCNU and other alkylating agents could provide a means for protection of normal cells that are susceptible to toxic side-effects of the cytostatic drug. Administration of a protective enzyme with improved catalytic activity towards the anticancer drug to a patient's bone marrow cells, lung tissues, or gastrointestinal tract could counter the undesirable toxicity of chemotherapy [15,16]. This auxiliary treatment with GSTs or other protective enzymes may allow dose escalation of the cytostatic drug and improve efficacy in the attempt to eradicate the tumor cells. Besides chemotherapy, GSTs with enhanced catalytic activity with cytostatic compounds could serve as selectable markers in gene therapy [17]. Development of such treatment modalities inspired the protein engineering presented here.

Based on the discovery that wild-type hGST T1-1 is the most efficient enzyme of all GSTs tested with BCNU [14], a mutant library was constructed by recombination of DNA coding for Theta-class GST T1/T2 (F1 generation) [18] with DNA encoding wild-type mouse and rat GST T1-1 to generate the F2 generation [19]. The F2 generation was chosen for screening of enzymes with enhanced alkyltransferase activity. To make the experimental procedure more efficient and to limit possible health hazards of the work with BCNU, the library was screened for mutants active with the surrogate substrates NPB and EPNP, which are both electrophilic alkylating agents of similar size as BCNU (Fig. 1).

Materials and methods

Materials

Wizard® Plus Minipreps DNA Purification System was purchased from Promega (Madison, WI, USA). Restriction enzymes, DNase I, and T4 DNA ligase were from Roche (Mannheim, Germany). *Taq* DNA Polymerase was from Fermentas (Vilnius, Lithuania), *Pfu* DNA Polymerase from Stratagene (La Jolla, CA, USA), and QIAquick from Qiagen (Hilden, Germany).

Amplification and digestion of DNA from the F1 generation

DNA encoding five mutants with high alkyltransferase activity selected from the Theta-class GST T1/T2-library [18] was recombined by DNA shuffling [20] with DNA encoding wild-type mouse GST T1-1 (mGST T1-1). DNA containing nucleotides 243–723 of the coding sequence of rat GST T1-1 (rGST T1-1) was also included as starting material for a new generation of mutants, i.e. the F2 library [19]. DNA preparations from the five selected mutants were separately amplified by PCR. The PCR primers used for amplification were pKK for (5′-AAT TGT GAG CGG ATA ACA AT-3′) and Eco RGNB (5′-AAG CTG AAA ATC TTC-3′). The DNA obtained was pooled, a total of ~1 mg, and was digested at room temperature with 0.2–0.8 unit DNase I (Roche, Mannheim, Germany) in 20 mM Tris–HCl pH 8.0, 1 mM $MgCl_2$. The digestion was performed intermittently in several rounds of 2–3 min each. After each round the digestion mixture was frozen on dry ice while a small sample of the reaction mixture was analyzed on a 2% (w/v) agarose gel. Freezing the solution stopped the digestion during the analysis. When the size of the fragments was ⩽100 bp, DNase I was inactivated by heating the reaction mixture to 70 °C for 10 min. After phenol and chisam extractions the DNA was separated by electrophoresis on a 2% (w/v) agarose gel and all fragments smaller than 100 bp were recovered from the gel. DNase digestions of *mGSTT1* and *rGSTT1* cDNA were performed separately in the same way as for the pooled mutant DNA. The two PCR products were mixed, precipitated, run on a 1% (w/v) gel and recovered from the gel with QIAquick. The amplified DNA was subjected to DNase digestion in a reaction with 0.5–2 units of DNase I (Roche) in 20 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$. The digestion was performed stepwise, and after each step a small sample of the reaction mixture was analyzed on a 2% (w/v) gel. Freezing the sample on dry ice stopped the digestion in the rest of the reaction mixture. When the fragments had decrease in size to ⩽100 bp the DNase was inactivated by heating at 70 °C for 10 min followed by phenol and chisam extraction. The DNA was run on a 2% (w/v) gel and all fragments ⩽100 bp were recovered from the gel, precipitated, and dissolved in water.

Reassembly of DNA fragments

The recovered fragments from the DNase digestion, including fragments of T1/T2 mutants, *mGSTT1*, and *rGSTT1*, were mixed together and reassembled in the presence of 0.2 mM dNTPs, 40 U/ml of *Pfu* DNA polymerase, and buffer as recommended by the manufacturer (Stratagene). The PCR conditions were 3 min at 95 °C, followed by 40 cycles of 1 min at 94 °C, 2 min at 50 °C and 2 min at 72 °C, completed by 10 min at 72 °C. A small fraction of the final product was analyzed on a 1% (w/v) gel in order to verify that DNA had been reassembled to the expected size.

Amplification of full-length GST coding sequences and construction of the F2 library

The product from the reassembly reaction was used as template in a PCR to amplify full-length coding sequences. The PCR mixture contained 0.8 mM each of the flanking primers pKK for and Eco

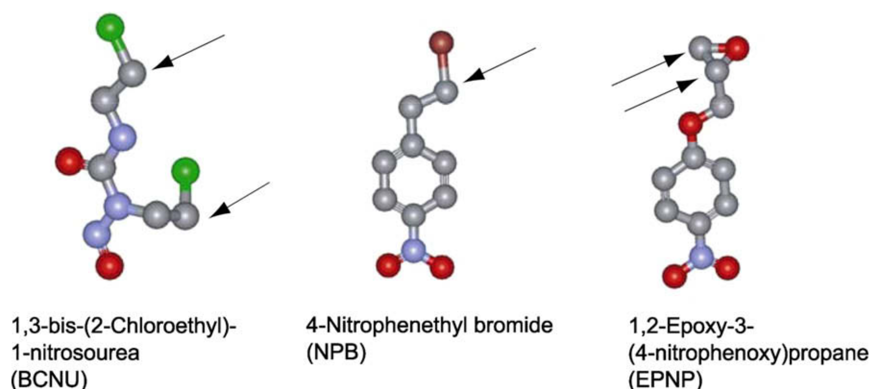

Fig. 1. Structures of three alternative alkylating agents used as electrophilic GST substrates. NPB was adopted as a surrogate substrate for BCNU. EPNP and NPB were used in the screening of libraries for mutants with improved GST activity. Arrows indicate sites of attack by GSH on the substrates.

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