Role of Human Glutathione Transferases in Biotransformation of the Nitric Oxide Prodrug JS-K

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Abstract

Nitric oxide (NO) plays a prominent physiological role as a low-molecular-mass signal molecule involved in diverse biological functions. Great attention has been directed to pharmacologically modulating the release of NO for various therapeutic applications. We have focused on O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K) as an example of diazeniumdiolate prodrugs with potential for cancer chemotherapy. JS-K is reportedly activated by glutathione conjugation by glutathione transferase (GST), but the scope of activities among the numerous members of the GSTome is unknown. We demonstrate that all human GSTs tested except GST T1-1 are active with JS-K as a substrate, but their specific activities are notably spanning a 100-fold range. The most effective enzyme was the mu class member GST M2-2 with a specific activity of 273 ± 5 μmol min^{-1} mg^{-1} and the kinetic parameters Km 48 ± 4 μM, k_{cat} 501 ± 29 s^{-1}, k_{cat}/Km 10 x10^6 M^{-1} s^{-1}. The abundance of the GSTs as an ensemble and their high catalytic efficiency indicate that release of NO occurs rapidly in normal tissues such that other mechanisms play a major role in the tumor-killing effect of JS-K.

Introduction

The significant physiological role of gasotransmitters, such as nitric oxide (NO), hydrogen sulfide (H_2S), and carbon monoxide (CO), has been unraveled by numerous investigations [1]. NO, in particular, has been shown to serve as a mediator in various biological processes. The antiproliferative activity of NO has prompted development of chemical derivatives that could serve as molecular vehicles for delivery of NO to targeted sites [2,3]. Among several prodrugs of this kind O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K) has undergone trials in several animals models and provided promise as a novel prodrug in cancer chemotherapy [4]. Common to JS-K and several related NO-releasing agents is their activation by glutathione (Fig. 1), which is promoted by glutathione transferases (GSTs) [5]. A few GSTs available from commercial sources have been assayed with JS-K [4,5], but a more complete investigation with relevant human GSTs has not been performed. In the present study the majority of the members of the human GSTome [6] is tested for activity with JS-K, and their role in the antitumoral activity of the prodrug discussed.

Materials And Methods

JS-K, glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and all other biochemicals were purchased from Sigma-Aldrich, except for the protein assay purchased from Bio-Rad. Expression clones of human GSTs were available in the laboratory as previously described [7].

Protein expression

Isolated colonies of *Escherichia coli* BL21 (DE3) containing recombinant protein (GST M4-4, P1-1 and S1-1) were grown overnight in LB with 100 μg ml^{-1} ampicillin, in a shaking incubator at 200 rpm, 37°C. The

culture was diluted 100-fold and grown to A_{600} = 0.4, before induction with isopropyl-β-d-thiogalactoside (IPTG) to a final concentration of 0.2 mM. The cells were harvested after 3 h by 15 min centrifugation at 7000*g*.

Bacterial cells containing GST M5-5 were cultivated in the same manner as described above with the exception that they were grown overnight without addition of an inducer.

Single colonies of *E.* coli XL1-blue containing recombinant protein (GST A1-1, A2-2, A3-3, M1-1 and M2-2)) were grown overnight in 2TY containing 100 μg ml^{-1} ampicillin at 37°C with shaking. Overnight cultures were then diluted 100-fold into 2TY containing 100 μg ml^{-1} ampicillin and allowed to grow at 37°C with shaking until reaching exponential growth (OD_{600} = 0.3). Expression was then induced by addition of 0.2 mM IPTG and the cells were further grown overnight. Cells were harvested by centrifugation at 5000g for 5 min and the bacterial pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.02% (w/v) sodium azide.

Protein purification

Harvested bacterial cells were lysed on ice for 60 min by treatment with lysozyme (0.2 mg/ml) and Protease Inhibitor Cocktail Tablets, EDTA-Free (Sigma-Aldrich) followed by sonication 5 times 20 s and centrifuged at 30,000*g* for 30 min.

Proteins with an N-terminal his tag (GSTs A1-1, A2-2, A3-3, P1-1 and S1-1) were purified as follows. The bacterial cells were harvested, resuspended in binding buffer (20 mM sodium phosphate, pH 7.4, containing 500 mM NaCl and 20 mM imidazole), and lysed by treatment as above followed by sonication. After centrifugation at 30,000 g for 30 min and 4°C, the supernatant fraction was loaded onto an Ni–IMAC (nickel–immobilized metal ion affinity chromatography) column (His GraviTrap, GE Healthcare). Unbound protein was washed out with 10 ml of binding buffer. The recombinant GSTs were eluted with 3 ml of elution buffer containing 500 mM Imidazole. The purified enzyme was dialyzed against 100 mM sodium phosphate buffer (pH 7.4). GST T1-1 was also his tagged and was purified as previous reported [8].

Proteins without his tag were purified by affinity chromatography either on glutathione (GSTs M1-1, M2-2 and M5-5) or on S-hexyl-glutathione (GSTs A4-4, M4-4) linked to epoxy-activated Sepharose 6B (GE Healthcare). The procedure was as follows: Equilibration of the affinity gel as a batch using a filter glass funnel with binding/washing buffer (10 mM Tris-HCl pH 7.8, 0.2 M NaCl, 0.2 mM DTT and 1.0 mM EDTA). Binding of the protein to the affinity gel by adding the lysate and incubating for 1 hour at 4°C on a tilting board. The affinity gel was washed repeatedly with the same buffer before the proteins were eluted with 20 mM GSH or 20 mM S-hexyl-glutathione in binding/washing buffer and dialyzed against 10 mM Tris-HCl pH 7.8, 0.2 mM DTT, 1.0 mM EDTA and 0.02% (w/v) sodium azide.

The protein content was determined with the Bio-Rad protein assay. The purity of the dialyzed protein was checked with SDS-PAGE.

Standard assays of GST activity

The activities of the GST stocks were determined with the standard substrates, 1 mM CDNB dissolved in ethanol and 1 mM GSH in 0.1 M sodium phosphate buffer pH 6.5, containing 1 mM EDTA, and the formation of the product S-2,4-dinitrophenyl-glutathione (GS-DNP) was followed spectrophotometrically at 340 nm. Their measured activity was compared with formerly determined specific activity values in order to assess the concentration of active enzyme in cases of divergence between the values.

GSTs activities with the prodrug were measured at 30°C with 50 µM JS-K and 1 mM GSH in10 mM Tris-HCl pH 7.5, 1.0 mM EDTA and 0.1% (w/v) bovine serum albumin. The JS-K stock solution was made in acetonitrile and the concentration of the solvent in the enzyme assay was 5% (v/v). The reaction was monitored by GS-DNP formation followed spectrophotometrically at 340 nm, as the product is the same as with CDNB above. All measurements were performed on a Multiscan GO spectrophotometer (Themo Fisher).

Michaelis-Menten kinetics

Initial rate measurements with JS-K dissolved in acetonitrile and 1 mM GSH were carried out for each GST enzyme at 30ºC in 10 mM Tris-HCl pH 7.5, 1.0 mM EDTA and 0.1% (w/v) BSA. Because of the limited aqueous solubility of JS-K no concentration higher than 50 µM was used in the reactions. The data were corrected for the non-enzymatic reaction rates and analyzed by non-linear regression with use of GraphPad Prism 8 software.

Results And Discussion

A spectrophotometric assay of the reaction of glutathione with JS-K (Fig. 1) was based on the formation of DNP-SG measurable at 340 nm, as in the reaction with 1-chloro-2,4-dinitrobenzene (CDNB) described by Clark et al. and commonly used to monitor GST activity [9]. The leaving group in the conjugation of JS-K is 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate, which rapidly decomposes into 4-ethoxycarbonylpiperazine and the projected two active molecules of NO [5]. The human genome encodes up to 17 genes of cytosolic (soluble) GSTs, but two of them are frequently represented by null alleles, *GSTM1*0* and *GSTT1*0*, such that the affected individuals do not express the corresponding enzymes [10]. Five of the seven classes of cytosolic GSTs are primarily responsible for detoxication of drugs and other xenobiotics: alpha (A), mu (M), pi (P), sigma (S), and theta (T). In the present investigation the classes were represented by human GST A1-1, GST A2-2, GST A3-3, GST A4-4, GST M1-1, GST M2-2, GST M4-4, GST M5-5, GST P1-1, GST S1-1, and GST T1-1.

Table 1 shows the activities with JS-K expressed per mg GST protein. For comparison, the specific activities of the enzymes with CDNB as substrate are shown. All enzyme tested except GST T1-1 demonstrated measurable activity with JS-K. Notably, human GST T1-1 also has negligible catalytic activity with CDNB [8]. The JS-K activities of the various GSTs span an approximately 400-fold range.

GST M2-2 displayed the highest specific activity of the tested enzymes: 273 μmol min^{-1} mg^{-1} (Table 1). Two other mu class members, GST M5-5 and the polymorphic GST M1-1, gave specific activities of 58 and 57.8 μmol min^{-1} mg^{-1}, respectively. GST M4-4, which generally has low catalytic activities [11], showed the lowest value of all in the class.

Among the alpha class members, GST A1-1 had the highest specific activity: 42.5 μmol min^{-1} mg^{-1} followed by GST A2-2 and GST A3-3 with 37.2 and 18.3 μmol min^{-1} mg^{-1}, respectively. The lowest activity in the alpha class was shown by GST A4-4, which is also known to have modest CDNB activity [12].

The sigma class enzyme GST S1-1 presented a specific activity of 12.9, twice the GST A4-4 value of 6.3 μmol min^{-1} mg^{-1}, and likewise has low CDNB activity [13]. GST S1-1 is distinguished as the hematopoietic prostaglandin D_2 synthase (HPGDS).

The pi class member GST P1-1 ranks among the least active GSTs with a specific activity of 1.2 μmol min^{-1} mg^{-1}, which is significant because this enzyme is present in most tissues [19] (but not in the hepatocytes of the liver[14]).

Specific activities have previously been reported for commercial enzyme preparations of GST M1-1 (3.7 μmol min^{-1} mg^{-1}), GST A1-1 (14.9 μmol min^{-1} mg^{-1}), and GST P1-1 (0.15 μmol min^{-1} mg^{-1}) at pH 6.5 and 25°C [5], but later redetermined with somewhat altered conditions of pH 7.4 and 37°C to the higher values of 66.3, 126, and 1.36 μmol min^{-1} mg^{-1}, respectively [15] The latter values are in general agreement with the values in Table 1 considering differences in reaction temperature and other conditions. On the other hand, the kcat/Km of 20 mM-1s-1 for GST A1-1 reported by the same group is two orders of magnitude lower than our value of 2000 mM-1s-1 in Fig. 2. Our value of 10,000 mM-1s-1 for GST M2-2 is even higher. The mu class GST M1-1 was reported earlier as 63 mM-1s-1[15].

In general, the activities with JS-K were highly correlated with the activities with CDNB (Fig. 3). This relationship can be rationalized by the similar chemical mechanism, involving an aromatic nucleophilic attack by the sulfur of glutathione to form a Meisenheimer σ-complex, which is followed by the release of the common conjugate S-2,4-dinitrophenylglutathione. A notable exception was the low JS-K activity of GST P1-1 in contrast to the high CDNB activity of the same enzyme. Apparently, the second product moiety of JS-K suppresses the release of S-2,4-dinitrophenylglutathione. In the opposite direction, GST S1-1 deviates somewhat from the general correlation, by being somewhat more active with JS-K than with CDNB (Fig. 3).

The antiproliferative property of JS-K was assumed to be based on NO as the effective agent released from the prodrug [5]. An alternative possibility involving S-2,4-dinitrophenylation of thiols or other cellular nucleophiles was excluded based on the lack of an equal effect of CDNB, which could produce a similar arylation without release of NO. However, the finding that pretreatment of HL-60 human myeloid leukemia

cells with buthionine sulfoxide, an inhibitor of glutathione biosynthesis, did not suppress the antineoplastic effect of JS-K suggests that other mechanisms than the glutathione-activated NO discharge are involved [5]. Accumulating evidence point to the role of signaling through mitogen-activated protein kinases, cell factor β-catenin/T, and ubiquitin-proteasome pathways in cancer cell apoptosis [16].

Even though the molecular mechanisms underlying the therapeutic potential if JS-K remain unresolved, GSTs are unmistakably potent activators of NO release from the compound. The specific activity of 273 μmol min^{-1} mg^{-1} displayed by GST M2-2 falls in the domain of the highest activities with any of a wide variety of alternative chemical reactions catalyzed by the enzyme [17]. The catalytic efficiency k_{cat}/K_m of $1.0x10^7$ M^{-1} s^{-1} is approaching the efficiencies of the most efficient enzymes [18] . Two additional mu class enzymes, GST M1-1 and GST M5-5, show 20% of the GST M2-2 activity, and the alpha class members GST A1-1 and GST A2-2 are almost as active (Table 1).

The GSTs as an ensemble of enzymes occur abundantly in all tissues [19-21], and it would appear that the GSTs collectively provide the means for efficient metabolism of JS-K. GST M2-2 is not expressed in liver, but the highly active GSTs A1-1 and A2-2 and GST M1-1 (except in subjects genetically homozygously *GSTM1* null) are highly abundant hepatic enzymes such that the tissue has high capacity for the biotransformation of JS-K. Particularly high activity can be expected also in testis, small intestine, kidney, adrenal based on the high expression levels of the latter enzymes. By contrast, uterus reportedly expresses only GST P1-1 and GST T1-1, and like erythrocytes, therefore has comparatively low activity with JS-K.

Overall, it would appear that the abundance of GSTs and their predominantly high activity with JS-K would cause the rapid biotransformation of the prodrug in most tissues. Even though many tumors overexpress GST P1-1 [22-24] it is not obvious that the elevation is sufficient to compensate for the relatively low activity of this enzyme in comparison with other GSTs and make the neoplastic cells selectively vulnerable to release of NO from JS-K. In some tumors elevated levels of alpha and mu class GST have also been reported, but neither in these instances can the antineoplastic activity of JS-K be unambiguously attributed to GST-mediated release of NO.

We conclude that the antitumoral effect of JS-K must involve mechanisms more than NO release, and that the interplay between them and the unmistakable degradation of JS-K catalyzed by GSTs requires further incisive investigation.

Table 1. Specific activities of human GSTs with JS-K as substrate.

Assays were made spectrophotometrically at 30°C with 50 μM JS-K and 1 mM GSH in 10 mM Tris-HCl pH 7.5, 1.0 mM EDTA and 0.1% bovine serum albumin. All measurements were made in triplicate and corrected for background reaction. S.D. (standard deviation), Nd (not detectable).

GST	Specific activity ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	±S.D.
A1-1	42.5	±3.5
A2-2	37.2	±0.6
A3-3	18.3	±0.5
A4-4	6.3	±0.2
M1-1	57.8	±2.3
M2-2	273.0	±4.7
M4-4	0.89	±0.02
M5-5	58.0	±0.6
P1-1	1.2	±0.05
S1-1	12.9	±0.01
T1-1	Nd	

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Figures

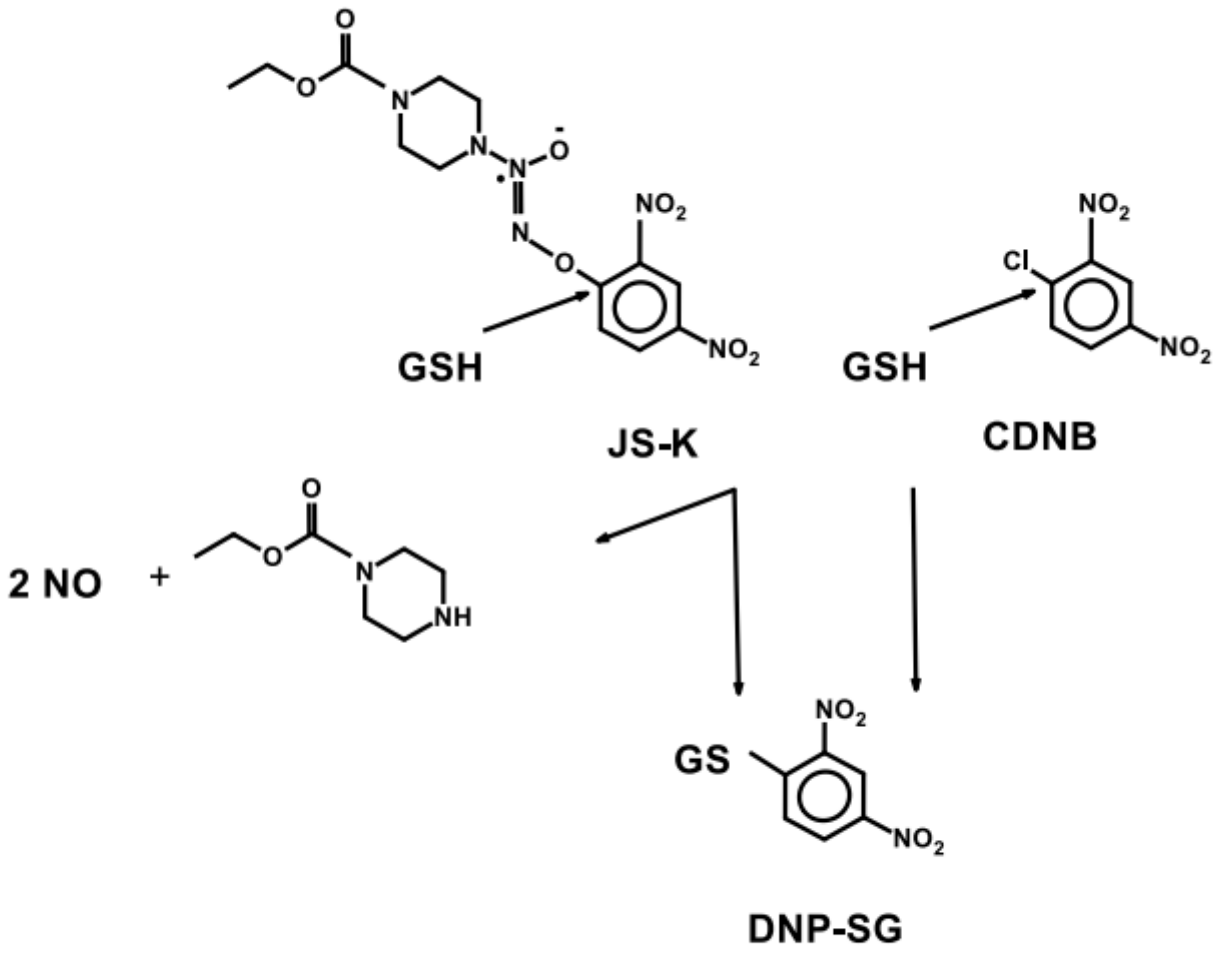

Figure 1

Structural similarities between JS-K (O2-(2,4-dinitrophenyl) 1-[(4 ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate) and CDNB (1-chloro-2,4-dinitrobenzene) and their glutathione (GSH) conjugation. The arrows show the site of nucleophilic attack by GSH leading to the product S-2,4-dinitrophenylglutathione (DNP-SG), which can be monitored spectrophotometrically at 340 nm. JS-K yields also a second product (not shown), which decomposes into nitric oxide and 4-carboethylpiperazine. Arrows show the site of attack by GSH.

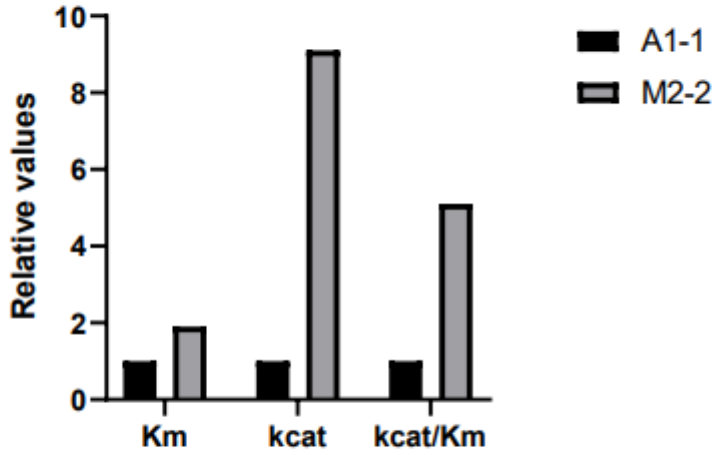

Figure 2

Comparison of kinetic parameters in the conjugation JS-K by GST A1-1 and GST M2-2. Relative values are shown normalized against the parameters of GST A1-1. Original values were: GSTA1-1: Km 27 ±6.2 μM, kcat 55 ±6.2 s-1, kcat/Km 2x106 M-1 s-1; GST M2-2: Km 48 ±4.2 μM, kcat 501 ±29.3 s-1, kcat/Km 10 x106 M-1 s-1.

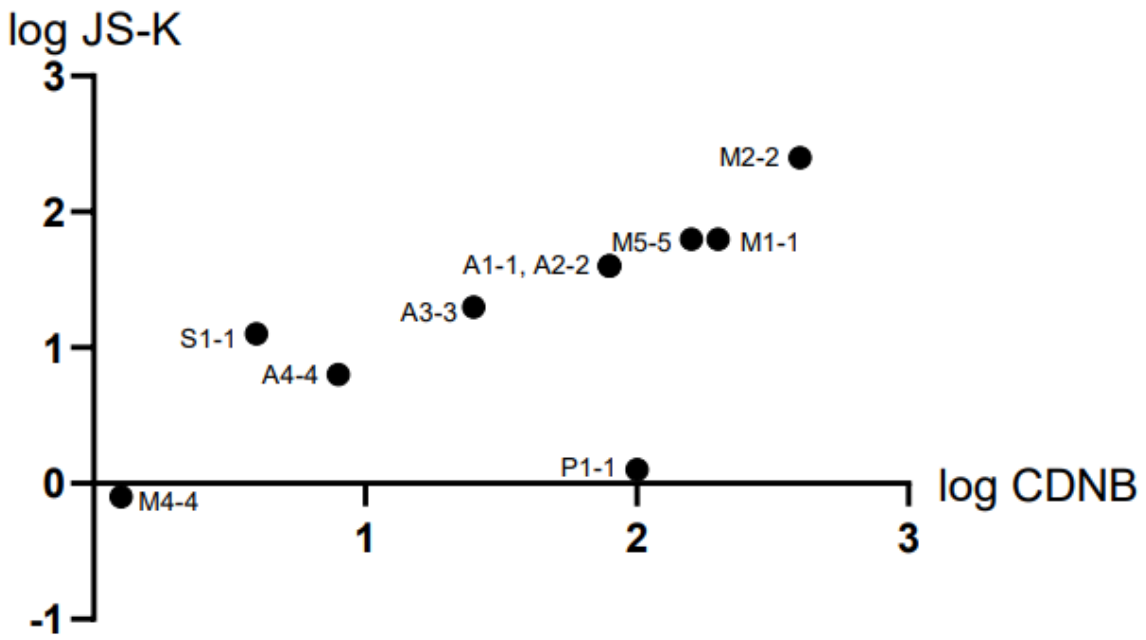

Figure 3

Correlation between specific activities of human GSTs with JS-K and CDNB as substrates. The data point for GST P1-1, with low JS-K and high CDNB activity, is a prominent outlier not included in the correlation analysis yielding a slope of 1.0 and R2 = 0.96.