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Inhibition of human glutathione transferases by dinitronaphthalene derivatives

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ABSTRACT

Glutathione transferase (GST) enzymes catalyze the conjugation of glutathione with reactive functional groups of endogenous compounds and xenobiotics, including halonitroaromatics. 1-Chloro-2, 4-dinitrobenzene (CDNB) is one of the most commonly used substrates for GST activity assays. We have studied the interactions of dinitronaphthalene analogues of CDNB with recombinant human GST enzymes (Alpha, Mu, and Pi classes) expressed in *Escherichia coli*. Dinitronaphthalene derivatives were found to be GST inhibitors. The highest potency of inhibition was observed towards Mu-class GSTs, M1-1 and M2-2; IC_{50} values for 1-methoxy- and 1-ethoxy-2,4-dinitronaphthalene were in the high nanomolar to low micromolar range. Inhibition accompanies the formation, at the enzyme active site, of very stable Meisenheimer complex intermediates.

Introduction

Glutathione transferase (GST)[3] enzymes catalyze reactions of the endogenous nucleophile glutathione with a wide range of electrophiles, including arene oxides, epoxides, alkyl halides, quinones, and α,β-unsaturated carbonyls [1–3]. The diversity of GST substrates is partly accounted for by the existence of many different GST enzymes: at least 17 cytosolic GSTs are found in humans, in classes Alpha, Mu, Omega, Pi, Sigma, Theta, and Zeta [2]. A second aspect of GST substrate diversity is that each GST enzyme can accept a range of substrates – so-called "catalytic promiscuity" [4–6]. Since the correspondence between GST substrates and enzymes is many-to-many rather than one-to-one, it would be desirable to identify both "universal" (active with any form) and "specific" (active with only one form) GST substrates and GST inhibitors. Specific inhibitors might be used, for example, to counteract the resistance of tumor cells to chemotherapeutic agents that are detoxified by glutathione (GSH) conjugation [7]. GST P1-1, an enzyme that is frequently overexpressed in tumors, has received particular attention as a target [8,9]. Better understanding of the interactions of small molecules with the GST active site will facilitate drug discovery, as has been discussed by Wu and Dong [2].

1-Chloro-2,4-dinitrobenzene (CDNB) is a substrate for most forms of GST; its reaction with glutathione (GSH) yields 2,4-dinitrophenyl glutathione and chloride ion. The use of CDNB as a GST substrate was introduced by Clark and colleagues, who wrote that "it is tempting to regard this compound as a general substrate which may function more or less effectively with any GSH transferase" [10]. CDNB did not prove to be a truly universal substrate (having undetectable activity with human GST T1-1, for example [11]), but it is commonly used for assaying GST activity [12,13].

The mechanism of the reaction of CDNB with GSH is nucleophilic aromatic substitution (S_NAr) via an anionic Meisenheimer complex intermediate [14–16]. When 1,3,5-trinitrobenzene (TNB), a CDNB analogue that lacks a leaving group, is added to rat liver GST 3-3 (M1-1) or GST 4-4 (M2-2) [17] in the presence of GSH, a stable red complex of the enzyme with 1-(S-glutathionyl)-2,4,6-trinitro-cyclohexadienate (a "dead-end" Meisenheimer intermediate) is formed. The complex can be isolated [14] and its crystal structure has been obtained [18] as has that of the corresponding complex with recombinant human GST P1-1 [19]. The TNB-GSH Meisenheimer complex is an inhibitor of GST activity at μM concentrations, as tested with a moth GST [20] and with

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[3] *Abbreviations used:* CDNB, 1-chloro-2,4-dinitrobenzene; CDNN, 1-chloro-2,4-dinitronaphthalene; ESI, electrospray ionization; GSH, glutathione; GST, glutathione transferase; HPLC–MS, high performance liquid chromatography–mass spectrometry; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, lysogeny broth; PMSF, phenylmethanesulfonylfluoride; TNB, 1,3,5-trinitrobenzene.

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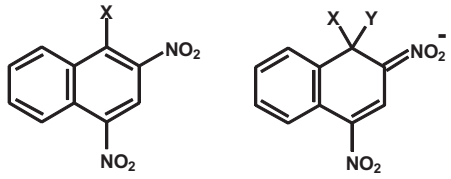

Fig. 1. Left: General structure of the dinitronaphthalene compounds used in this study. Right: general structure of dinitronaphthalene Meisenheimer complexes. In the complexes studied here, Y = glutathione.

affinity-purified human liver and placenta GST preparations [21]. Recombinant human GST P1-1 also forms a Meisenheimer complex with TNB [22].

Naphthalene derivatives form Meisenheimer complexes (Fig. 1) much more readily than do benzene derivatives, due to the greater delocalization of negative charge in the larger ring system [23,24]. For example, the methoxy complex of 1,3-dinitronaphthalene is about 10^4-fold more stable than that of 1,3-dinitrobenzene, as measured by the equilibrium constants for their formation [25] (p. 128). The stabilizing effect of the second aromatic ring in naphthalene is almost as large as that of an additional nitro group (*e.g.*, going from 1,3-dinitrobenzene to TNB) [25]. The facile formation of Meisenheimer intermediates from dinitronaphthalene derivatives suggested to us that these compounds might be good substrates or inhibitors of GST enzymes. 1-Chloro-2,4-dinitronaphthalene (CDNN) is the naphthalene analogue of CDNB [26–28]. The goal of our study was to examine the interactions of CDNN and other dinitronaphthalene derivatives with human cytosolic GST enzymes.

Materials and methods

Sources of chemicals were as follows: glutathione-agarose, IPTG (isopropyl-β-D-1-thiogalactopyranoside), ampicillin sodium salt, tetracycline HCl, β-mercaptoethanol, Martius Yellow, and bovine serum albumin (BSA): Sigma–Aldrich (Oakville, ON); 1-chloro-2,4-dinitrobenzene (CDNB; 98%): Alfa Aesar (Ward Hill, MA); glutathione (99.6%): ChemImpex (Wood Dale, IL); 2,4-dinitronaphthalene (DNN; 100%): Accu-Standard, Inc. (New Haven, CT); Difco agar, Tryptone, Yeast Extract: Becton, Dickson Co. (Sparks, MD); Oxoid Nutrient Broth No. 2: Oxoid, Ltd. (Hampshire, England); lysozyme: Boehringer-Mannheim (Germany).

Recombinant hGST-expressing *Escherichia coli* strains were the kind gift of Dr. Bengt Mannervik (Uppsala University, Sweden).

UV–visible spectra were recorded on a Cary BIO300 spectrophotometer.

Synthesis and characterization of dinitronaphthalene derivatives

CDNN (See Fig. 1 and Table 1 for structures) was synthesized from 1-hydroxy-2,4-dinitrobenzene (Martius Yellow, DNNOH) according to published procedures [26,27]. The methoxy and ethoxy derivatives (DNNOMe and DNNOEt) were also synthesized in good yields (56% and 78%, respectively) from the reactions of CDNN with the corresponding sodium alkoxides, instead of the potassium salts as previously reported [24]. Reaction conditions: DNNOMe: Na(s), dry MeOH, CDNN, 10 °C to RT, 1 h (56%); DNNOEt: Na(s), dry EtOH, CDNN, 10 °C to RT, 1 h (78%). The melting points and ^{1}H NMR data of DNNOMe and DNNOEt were in agreement with literature values [24].

The GSH conjugate of CDNN was synthesized by a modification of the method of Shiotsuki et al. [29]. GSH and CDNN, each 1 mM, were dissolved in EtOH, 1.2 mL. NaOH (2 M, 1 mL) was added dropwise. The color of the solution immediately changed from yellow to red–orange. The reaction was stirred for 30 min at RT and then neutralized with a few drops of HCl. The solid precipitate that formed was collected by vacuum filtration and washed sequentially with cold water, ethanol, and ethyl acetate. Air-dried solid was recrystallized from 5 mL hot ethanol; yield, 22%. No residual CDNN starting material remained, as determined by HPLC analysis. ESI–LC–MS gave an $(M + H)^+$ peak at m/z = 524.0, as expected, and a smaller $(M + Na)^+$ peak at m/z = 546.0.

Table 1
Dinitronaphthalene compounds used in this study.

Dinitronaphthalene derivatives			
X	Abbreviation	CAS	log P
H–	DNN	606-37-1	2.77
Cl–	CDNN	2401-85-6	3.23
HO–	DNNOH	605-69-6	2.90
		55154-12-6*	–
CH_3O–	DNNOMe	13772-69-5	2.83
CH_3CH_2O–	DNNOEt	15352-94-0	3.34
Glutathione–	DNNSG	–	–

The general structure is shown in Fig. 1. The substituents, abbreviations, and chemical abstracts registry numbers are shown. The last column gives the estimated log P (logarithm of octanol–water partition coefficient) values, taken from the American Chemical Society (SciFinder) database (calculated using Advanced Chemistry Development™ Software v. 11.02).
* Martius yellow anion.

Expression and purification of human cytosolic GSTs

Recombinant hGST proteins were expressed in *E. coli* and purified using a modification of the published protocol [30]. All cultures were grown with vigorous shaking at 37 °C. An aliquot of overnight culture grown in LB (lysogeny broth) with ampicillin, 0.1 mg per mL, was diluted 100-fold into 2TY (tryptone yeast extract) medium (1L) with ampicillin, 0.1 mg per mL, in a 2 L flask. When the culture reached OD_{600} = 0.5, IPTG (1 mM) was added, and induction proceeded overnight. Cells were then harvested by centrifugation and resuspended in lysis buffer (50 mM Tris buffer, pH 7.4; 100 mM NaCl; 1 mM EDTA; 1 mM β-mercaptoethanol; 12 mL). Cells were lysed as previously described [30]; phenylmethanesulfonylfluoride (PMSF) protease inhibitor, 0.1 mM, was added after sonication. Lysate enzyme activity was measured with CDNB (see below) and protein concentration was determined with the Bradford assay, using BSA as reference standard [31]. GST purification was performed on a column (2 mL bed volume) of glutathione-agarose affinity resin pre-equilibrated with cold phosphate-buffered saline (PBS). To remove non-specific binding proteins, the column was washed with PBS until no protein was present in the eluate, as measured by absorbance at 280 nm. GST was then eluted with 50 mM Tris, pH 9, containing 50 mM GSH (3 mL). The eluate was dialyzed overnight at 4 °C against 100 mM potassium phosphate buffer, pH 6.5, containing 10% glycerol, 1 mM β-mercaptoethanol; 1 L. Protein samples were frozen on dry ice and stored at −80 °C until use. GST protein homogeneity was confirmed by SDS–PAGE (data not shown).

CDNB enzyme assay; inhibition by dinitronaphthalene and derivatives; enzyme kinetics

GST activity was assayed with CDNB. Formation of the dinitrophenyl-GSH was monitored at 340 nm with a Cary 300 dual-beam spectrophotometer (1.5 mL quartz cuvettes). Enzyme assays were performed at RT in 100 mM potassium phosphate buffer, pH 6.5, with 1 mM GSH and 0.5 mM CDNB [32].

hGST/inhibitor combinations chosen for detailed analysis were identified by screening the effect of 25 μM inhibitor. For those combinations which showed decreased activity, inhibitor IC_{50}