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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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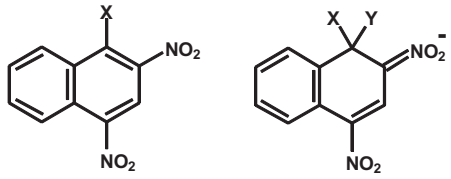


**Fig. 1.** Left: General structure of the dinitronaphthalene compounds used in this study. Right: general structure of dinitronaphthalene Meisenheimer complexes. In the complexes studied here, Y = glutathione.

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 $IC_{50}$

values were measured as follows. Recombinant hGST was pre-incubated with inhibitor (dissolved in MeCN) and GSH, 1 mM, for 5 min at 37 °C; the final concentration of MeCN was 1%. The reaction was initiated by addition of CDNB, 0.5 mM, in ethanol (1% final concentration).

The amount of enzyme per incubation was 15 μg, except for the following cases, where less enzyme was used, to maintain the condition [I] > [E]: M2-2, all inhibitors except DNNOH, 1 μg; M1-1 and DNN, 1 μg; A1-1 and DNNOH, 5 μg. All activity assays were conducted in triplicate. The $IC_{50}$ values were measured by fitting the four-parameter logistic function [33] to each data set, using SigmaPlot™ software.

### *Mechanism of inhibition*

For selected combinations of enzyme and potent inhibitor, double-reciprocal plots were constructed by varying the concentrations of both the inhibitor and CDNB. Enzyme assays were carried out as described above, with [GSH] = 1 mM, and [inhibitor] and [CDNB] as indicated.

### *Reversibility of inhibition/dialysis*

To test reversibility of inhibition, the inhibitor was removed by dialysis. Incubations (1 mL) containing GST A1-1 (25 μg), CDNN (25 μM), and GSH (1 mM) in potassium phosphate buffer, pH 6.5, were dialyzed (molecular weight cut-off, 12–14 kDa) against 1 L of buffer for 25 h at 4 °C. Control incubations lacked CDNN.

### *Meisenheimer complex formation: spectrophotometric experiments*

Meisenheimer complex spectra were recorded as follows [14]. hGST preparations were dialyzed overnight against 100 mM potassium phosphate buffer, pH 6.5, to remove β-mercaptoethanol. Inhibitors (12.5 μM) were added to 100 mM potassium phosphate buffer, pH 6.5, containing 50 μM hGST active sites (*i.e.*, 25 μM dimeric enzyme) and 1 mM GSH. The solvent (MeCN, except MeOH in the case of DNNOH) concentration was 2%.

### *Mutagenicity assays*

The mutagenicity assay (Ames test) [34] was performed with *Salmonella* strain YG1024, a derivative of TA98 which expresses high levels of arylamine *N*-acetyltransferase/*N*-hydroxyarylamine *O*-acetyltransferase and is highly sensitive to the mutagenicity of nitroaromatic compounds [35,36]. Bacteria were grown overnight in Oxoid Nutrient Broth No. 2 containing ampicillin 25 μg/mL and tetracycline 6.25 μg/mL. Mutagen stocks were prepared in MeCN, except for DNNOH, which was prepared in MeOH. Mutagen stock (10 μL) was added to an aliquot of culture (100 μL) and 0.1 M sodium phosphate buffer, pH 7.4, 0.5 mL, for 30 min at 37 °C, with shaking, prior to plating. Assays were done in triplicate on at least two different days. Colony counting was performed using NIST Integrated Colony Enumerator (NICE) software [37]. The plate was photographed with a digital camera; colonies were counted in the inscribed square region of the circular 90 mm-diameter dish and the counts were corrected by multiplication by the ratio of the areas ($\pi/2$).

## Results

### *GST expression*

Six recombinant human cytosolic GST enzymes, A1-1, A2-2, A4-4, M1-1, M2-2, and P1-1, were prepared by expression in *E. coli* [30]. These enzymes are representative of three major classes of xenobiotic-conjugating GST enzymes, Alpha, Mu, and Pi [38,39], and each is a catalyst of CDNB conjugation [40,41]. Theta class enzymes were not tested, since their CDNB conjugation activity is very low.

### *CDNN glutathione adduct*

CDNN reacted with GSH under the conditions used for synthesis of the CDNB adduct: addition of NaOH to a solution of GSH and the electrophile in aqueous ethanol solution [42]. The product 2,4-dinitronaphthylglutathione was isolated and characterized by HPLC–ESI–MS ($M + H^+$, m/z = 524; $M + Na^+$, m/z = 546). To our surprise, however, no reaction was observed in enzyme incubations of GST (15 μg/mL) with GSH (1 mM) and CDNN (*e.g.*, 10 μM) at pH values ranging from 6.5 to 7.4; no change in the CDNN absorbance spectrum was seen; all six GSTs were tested.

### *Inhibition of GST activity by dinitronaphthalene derivatives*

Finding no evidence that CDNN is a substrate for GST-catalyzed GSH conjugation, we then tested the compound as a possible inhibitor of the GST-catalyzed GSH conjugation of CDNB [32]. Indeed, CDNN proved to be a potent inhibitor. Inhibition curves were measured for all six dinitronaphthalene derivatives and all six enzymes, although in several cases (*e.g.*, GST A2-2, A4-4, P1-1) inhibition was weak or undetectable. A typical inhibition curve is shown in Fig. 2 and the $IC_{50}$ values are summarized in Table 2. The strongest inhibitory effects were observed with GST M2-2 and the *O*-alkyl compounds 1-methoxy- and 1-ethoxy-2, 4-dinitronaphthalene, with $IC_{50}$ values below 1 μM. There was no clear relationship between predicted partition coefficient and inhibitory potency (Fig. 3).

CDNN-treated GST A1-1 was subjected to dialysis (see Materials and Methods). Following dialysis, enzyme activity was equal to that of enzyme incubated without the inhibitor (data not shown), indicating that the inhibition was fully reversible. Double-reciprocal plot analysis was consistent with a competitive mode of inhibition (Fig. 4).

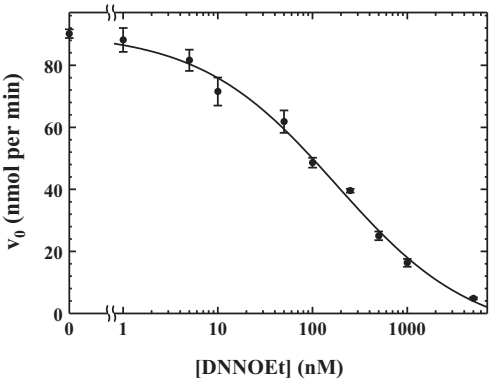


**Fig. 2.** Inhibition of GST M2-2 activity by DNNOEt. GST M2-2 (1 μg) was incubated with the inhibitor (dissolved in acetonitrile) and GSH, 1 mM, at 37 °C for 5 min, in 100 mM potassium phosphate buffer, pH 6.5. CDNB (dissolved in ethanol) was added to initiate the reaction (final concentration, 0.5 mM), and product formation was monitored at 340 nm. Final concentrations of acetonitrile and ethanol were 1% each. Data points represent the mean ± s.e. of three independent experiments. $IC_{50}$ values were determined by curve-fitting, as described in Materials and methods. The corresponding experiment was carried out with the other inhibitors and enzymes, as summarized in Table 2.

**Table 2**
$IC_{50}$ values (μM) of inhibitors towards recombinant human GST enzymes.

| | A1-1 | A2-2 | A4-4 | M1-1 | M2-2 | P1-1 |
|---|---|---|---|---|---|---|
| Sp. Act. | 27.4 | 5.1 | 6.1 | 41.8 | 47.5 | 39.2 |
| CDNN | 3.6 | * | * | 35.7 | 0.7 | 91.2 |
| DNNOH | 0.9 | 76.2 | * | 4.1 | 97.6 | 89.7 |
| DNNSG | 9.8 | * | * | 7.6 | 3.4 | * |
| DNNOMe | * | * | * | 2.9 | 0.12 | * |
| DNNOEt | * | * | * | 2.1 | 0.18 | * |
| DNN | * | * | * | 1.3 | 1.3 | * |

* Indicates no effect at inhibitor concentrations up to 25 μM. The IC50 values were determined as shown in Fig. 3.

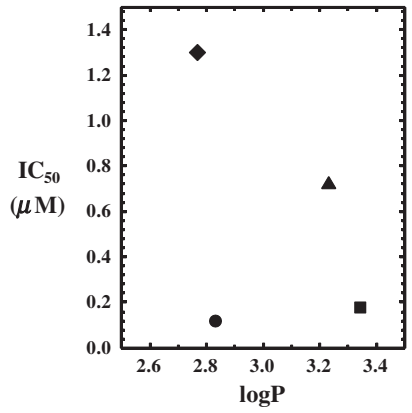


**Fig. 3.** Correlation analysis of $IC_{50}$ values (GST M2-2, Table 2) vs. logP values (Table 1) for the four potent dinitronaphthalene inhibitors for which calculated logP values are available. Symbols: diamond, DNN; circle, DNNOMe; triangle, CDNN; square, DNNOEt.

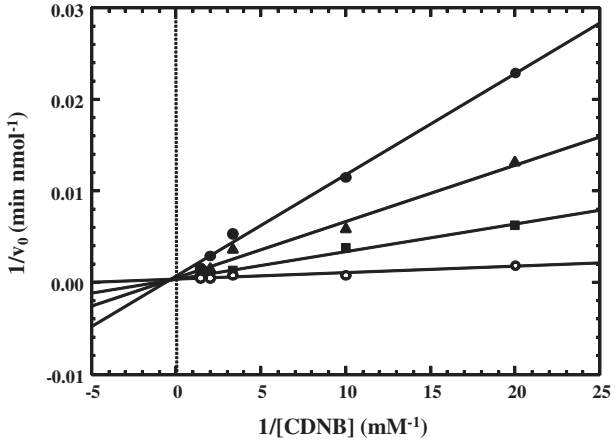


**Fig. 4.** Double-reciprocal plots of GST M2-2 inhibition by DNNOEt. DNNOEt concentrations were 1.2 μM (filled circles), 0.6 μM (triangles), 0.3 μM (squares); no inhibitor, open circles. CDNB concentrations were 50 $\mu M^{-1}$ mM. Assay conditions were as indicated for Fig. 2.

### *Formation of dinitronaphthalene Meisenheimer complexes at the GST active site*

Immediately upon addition of CDNN to an incubation containing a high concentration of GST (approx. 650 μg/mL) and GSH, the characteristic red color of a Meisenheimer complex appeared (Fig. 5 and Table 3). This complex formation had not been seen at low (catalytic) concentrations of enzyme. Meisenheimer complex formation was reversible: the red color was lost after overnight dialysis against buffer as verified with DNN, CDNN,

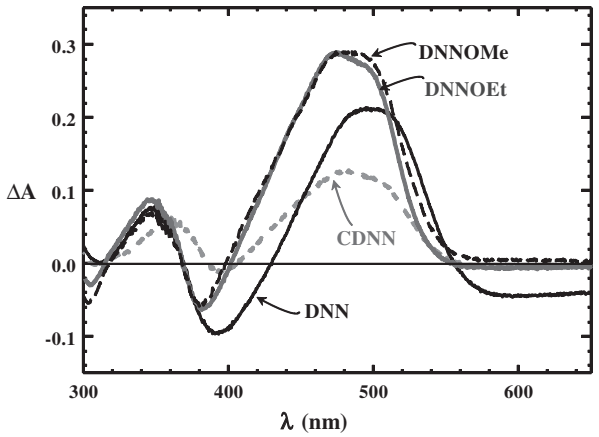


**Fig. 5.** Absorption difference spectra of Meisenheimer complexes. Yankeelov (tandem) cuvettes were used, to facilitate comparison of pre- and post-mixing spectra. Final concentrations were: dinitronaphthalene (12.5 μM), GSH (1 mM), and GST M2-2 (25 μM; concentration of active sites; each enzyme molecule is a homodimer with two identical active sites) in 100 mM potassium phosphate buffer, pH 6.5. Final concentration of acetonitrile (solvent for DNN) was 1%. One compartment of the tandem sample cuvette was filled with enzyme and GSH in buffer; the other compartment contained dinitronaphthalene solution in buffer. The reference tandem cuvette was prepared identically, except that dinitronaphthalene and enzyme were omitted. Spectra (sample – reference) were recorded before and immediately after inverting and mixing both sample and reference cuvettes, at room temperature. The plots show the difference spectra obtained by numerically subtracting the pre-mixing spectrum from the corresponding post-mixing spectrum, for each dinitronaphthalene derivative. Heavy line: DNNOEt; thin line: DNN; short-dashed line: CDNN; long-dashed line, DNNOMe.

**Table 3**
UV–visible spectra of inhibitors and Meisenheimer complexes. The inhibitors (first column) are listed with the $\lambda_{max}$ values of the free dinitronaphthalene compound (second column) and of the corresponding Meisenheimer complex with GST M2-2 and GSH (third column) (by comparison, the $\lambda_{max}$ value for the dimethoxy complex (Fig. 2, *X* = *Y* = -OMe) was given as 495 nm [24]).

| | $\lambda_{max}$ (nm) | |
|---|---|---|
| | Inhibitor | Complex |
| CDNN | 372 | 478 |
| DNNOH | 393, 436 | – |
| DNNSG | 512 | – |
| DNNOMe | 295, 360 | 480 |
| DNNOEt | 295, 360 | 474 |
| DNN | 288, 371 | 495 |

DNNOMe, and DNNOEt (Dissociation constants were not determined, because the inhibitors were generally so potent that, under conditions where [E] ≪ [I], the absorbance spectra were too weak for accurate measurement.).

Once formed, the Meisenheimer complexes were very stable. No significant loss of red color was seen when an incubation of GSTM2-2 (25 μM), GSH (1 mM), and CDNN (12.5 μM) was held at room temperature over a period of hours. Identical incubations held at 37C gradually decolorized over the same length of time. Subsequent addition of a second aliquot of GSH (1 mM) restored the color, indicating that the decolorization is due to the gradual autoxidation of GSH.

### *Mutagenicity assays*

CDNB is a direct-acting frameshift mutagen in the Ames test [43]. Both DNN and DNNOH are direct-acting mutagens in Ames test strains TA100 and TA98, indicating both base-substitution and frameshift mutagenic activity [44]. We tested each of the dinitronaphthalene derivatives (except DNNSG) in strain YG1024. The

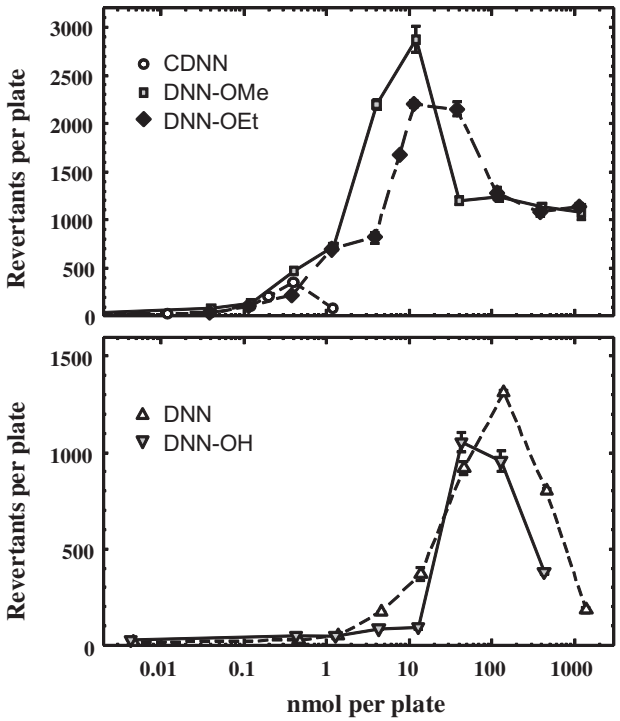


**Fig. 6.** Ames test mutagenicity ($His^+$ revertants per plate) for dinitronaphthalene derivatives; strain YG1024. Data points represent mean ± s.e. of at least six plates from two independent experiments. The declines in revertant yield at higher doses were, in each case, due to toxicity, as evident from thinning of the background lawn of auxotrophic colonies.

results are shown in Fig. 6. All of the tested compounds were direct-acting frameshift mutagens and all showed bacterial toxicity at higher doses, as indicated by a decrease in yield of revertant colonies as well as by the thinning of the background auxotrophic lawn, characteristic of toxicity in the Ames test [45]. CDNN was much more toxic than the other compounds, with toxicity evident above 0.3 nmol per plate, although it was nevertheless possible to observe a mutagenic response at lower doses.

## Discussion

Among the three GST Alpha class enzymes examined, we found that GST A1-1 is sensitive to inhibition by certain dinitronaphthalenes, notably Martius yellow (DNNOH), whereas forms A2-2 and A4-4 are much more resistant (Table 2). This result is consistent with previous studies of these enzymes, which have characterized GST A1-1 as "promiscuous" and GST A4-4 as "highly selective" [46]. GST A2-2 is best known for its GSH peroxidase activity [46] and has much lower activity than GST A1-1 with respect to the GSH conjugation of (for example) polycyclic aromatic hydrocarbon dihydrodiol epoxides [47]. The Mu class enzyme GST M2-2 was the most dinitronaphthalene-sensitive enzyme. As noted by Wu and Dong, "mu-class GSTs have a larger and more open active site than Alpha GSTs. This is consistent with the fact that Mu-class GST substrates include many bulkier electrophilic agents such as aflatoxin B1-epoxides and benzpyrene diols." [2]. GST M2-2 is usually referred to as a muscle-specific enzyme [48], but it is also expressed in brain [49], kidney, heart, testis, and other organs [50]. The BioGPS gene portal [51] database, surprisingly, shows liver as the human cell type with much the highest level of *GSTM2* mRNA expression (Possibly, DNA sequence homology with other Mu-class GSTs led to spurious results in some of these expression analyses.).

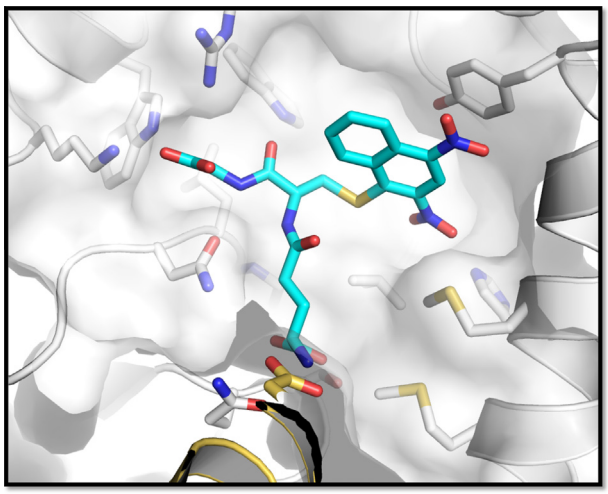

**Fig. 7.** Model of the structure of human GST M1A-1A complexed with glutathionyl dinitronaphthalene. The figure shows the result of adding a second aromatic ring to the structure of the bound glutathionyl-*S*-dinitrobenzene ligand in structure 1XWK.pdb [15], without further optimization.

Kunze and Heps synthesized several GST inhibitors, GSH analogues bearing dialkoxyphosphinyl (O═P—$(OR)_2$) groups replacing the side-chain of the GSH cysteine residue [52]. These compounds were tested against several recombinants human and purified porcine GSTs. The $IC_{50}$ values for recombinant human GST M1-1 were in the low to high micromolar range, and class Mu enzymes were more sensitive than classes Alpha or Pi. In contrast, a lipophilic GSH conjugate, γ-glutamyl-(S-9-fluorenylmethyl)-L-cysteinyl-glycine, showed sub-micromolar potency with human GST A1-1, but weaker inhibition of human GSTs M2-2 and P1-1 [53]. Therefore, the presence of a hydrophobic aryl substituent on the S atom does not guarantee specificity for Mu class GSTs.

A crystal structure is available of GST M1-1 complexed with glutathionyl-*S*-dinitrobenzene [15]. We simply replaced the benzene ring in the structure with a naphthalene ring, without any further modeling of possible favorable interactions between the added aromatic ring and the protein (Fig. 7), and it can be seen that the larger substrate is accommodated without steric clashes. This is consistent with the observed formation of stable complexes with the dinitronaphthalene derivatives.

All of the dinitronaphthalene derivatives tested were direct-acting frameshift mutagens. This result was expected, in view of previous studies of these and related nitroaromatic compounds [44,54]. The increased mutagenicity observed in strain YG1024 relative to strain TA98 is consistent with the view that the compounds are activated by the well-established mechanism of nitroreductase-mediated reduction to *N*-hydroxyarylamine derivatives, followed by *O*-acetylation, ultimately generating a DNA-reactive nitrenium ion [55]. Without doubt, the mutagenicity of nitroaromatics is a major liability to their possible therapeutic application. For this reason, it will be of interest to test whether naphthalene derivatives bearing other electron-withdrawing functional groups (*e.g.*, cyano) are also active as GST inhibitors.

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