Structure-based design of anticancer prodrug PABA/NO

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Abstract: Glutathione S-transferase (GST) is a superfamily of detoxification enzymes, represented by GSTα, GSTμ, GSTπ, etc. GSTα is the predominant isoform of GST in human liver, playing important roles for our well being. GSTπ is overexpressed in many forms of cancer, thus presenting an opportunity for selective targeting of cancer cells. Our structure-based design of prodrugs intended to release cytotoxic levels of nitric oxide in GSTπ-overexpressing cancer cells yielded PABA/NO, which exhibited anticancer activity both *in vitro* and *in vivo* with a potency similar to that of cisplatin. Here, we present the details on structural modification, molecular modeling, and enzymatic characterization for the design of PABA/NO. The design was efficient because it was on the basis of the reaction mechanism and the structures of related GST isozymes at both the ground state and the transition state. The ground-state structures outlined the shape and property of the substrate-binding site in different isozymes, and the structural information at the transition-state indicated distinct conformations of the Meisenheimer complex of prodrugs in the active site of different isozymes, providing guidance for the modifications of the molecular structure of the prodrug molecules. Two key alterations of a GSTα-selective compound led to the GSTπ-selective PABA/NO.

Keywords: structure-based, drug design, anticancer, prodrug, PABA/NO

Introduction

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a superfamily of enzymes derived from distinct gene classes that have been designated α, μ, π, etc. GSTs catalyze the conjugation of the sulfur atom of glutathione (GSH) to an electrophilic center of endogenous and exogenous compounds, thereby increasing their aqueous solubility for subsequent excretion. Being ubiquitous and quite abundant in mammalian tissues, they initiate the metabolism of a broad range of alkylating agents and therefore play a central role in the detoxification of many carcinogens as well as anticancer chemotherapeutic agents (Jakoby and Habig 1980; Mannervik 1985; Pickett and Lu 1989; Armstrong 1991, 1994, 1997; Hayes and Pulford 1995; Sheehan et al 2001; Dixon et al 2002). Of the superfamily, GSTπ is especially important in cancer therapy because it is often expressed at significantly higher levels in preneoplastic and neoplastic cells (Sato et al 1984; Sugioka et al 1985; Suguoka et al 1985; Sato 1988, 1989; Muramatsu et al 1995). It has also been shown that elevated levels of total GST and overexpression of GSTπ often accompany the development of drug resistance in tumors of patients undergoing chemotherapy (Morgan et al 1996; O'Brien and Tew 1996; Townsend and Tew 2003). Such factors have stimulated recent efforts to target GSTs as a primary objective in the discovery of anticancer agents (Flatgaard et al 1993; Lyttle et al 1994; Kauvar 1996; Rosario et al 2000; Townsend et al 2002).

We have been trying to turn the GSTπ-overexpression to the tumor's disadvantage by developing PABA/NO [O^2-{2,4-dinitro-5-[4-(*N*-methylamino)benzoyloxy]phenyl}1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate] that releases the established cytolytic

agent nitric oxide (NO) upon metabolism by GSTπ (Findlay et al 2004). PABA/NO belongs to a new family of anticancer prodrugs, the O^2-aryl diazeniumdiolates (O^2ADs), electrophilic species shown to transfer their aryl groups to attacking nucleophiles with cogeneration of ions that spontaneously release NO at physiological pH (Saavedra et al 2001). The GST-catalyzed GSH addition of PABA/NO proceeds with the formation of a Meisenheimer-complex intermediate (Figure 1a), and subsequently the leaving group of the reaction releases two moles of NO (Figure 1b). Therefore, within the GST-overexpressing cancer cells, the intracellular GSH is irreversibly consumed, and the NO thus generated could contribute to chemotherapy by inhibiting DNA synthesis,

forming toxic reactive nitrogen/oxygen intermediates, and inhibiting enzymes capable of preventing or repairing cellular damage. PABA/NO produces antitumor effects comparable with cisplatin in a human ovarian cancer model grown in SCID mice and is also potent against proliferation of the OVCAR-3 cell line (Findlay et al 2004; Saavedra et al 2006). PABA/NO is GSTπ-selective, ie, it is more efficiently metabolized by GSTπ. It was designed on the basis of the mechanism of GST-catalyzed reaction and the structures of GSTs, especially the structural information of GSTs at the transition state.

The attack of GSH on 1-chloro-2,4-dinitrobenzene (CDNB) is the basis for the most widely used spectrophotometric assay for measurement of GST activity (Habig et al 1974).
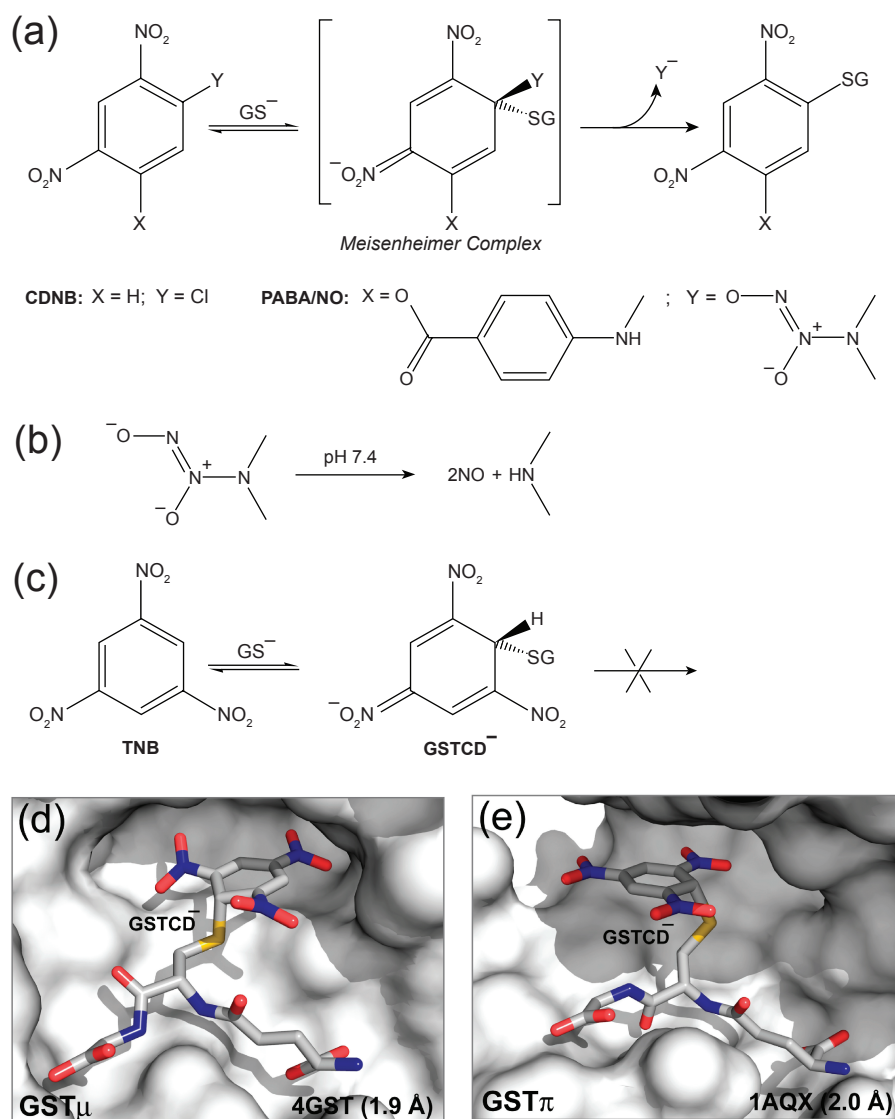

Figure 1 The Meisenheimer complex. (**a**) The mechanism of GST-catalyzed reaction of GSH with PABA/NO and with CDNB, showing the formation of the Meisenheimer complex as the reaction intermediate. (**b**) The diazeniumdiolate ion releases two moles of NO at neutral pH. (**c**) A relatively stable model for the Meisenheimer complex is provided by the GSTCD$^-$ ion. (**d**) The GSTμ•GSTCD$^-$ structure (PDB entry 4GST). (**e**) The GSTπ•GSTCD$^-$ structure (PDB entry 1AQX). In panels d and e, the protein is shown as a molecular surface while the ligand as a stick model in atomic color scheme (carbon in gray, nitrogen in blue, oxygen in red, and sulfur in orange).

As shown in Figure 1a, the reaction proceeds in solution via the formation of the Meisenheimer-complex intermediate (Miller 1968). Therefore, GST would stabilize the Meisenheimer-complex intermediate, at least to the extent that the intermediate resembles the transition state for its formation. However, the instability of the Meisenheimer complex has thus far precluded its structural elucidation. In contrast, the reversible reaction of GSH with 1,3,5-trinitro-benzene (TNB) to form the 1-(glutathion-S-yl)-2,4,6-trinitro-cyclohexadienate anion (GSTCD$^-$), a potent inhibitor of GST, provides a relatively stable model for this intermediate (Clark and Sinclair 1988; Graminski et al 1989) as shown in Figure 1c. To date, crystal structures of the GSTμ•GSTCD$^-$ complex (PDB entry 4GST; Ji et al 1993) shown in Figure 1d and the GSTπ•GSTCD$^-$ complex (PDB entry 1AQX; Prade et al 1997) shown in Figure 1e have been determined, which have guided us to model the GSTα•GSTCD$^-$ complex. Together, the structures and the model played an essential role in our structure-based development of PABA/NO.

Structure-based drug design is a growing field in which remarkable advances have been made in recent years (Anderson 2003; Scapin 2006). Although the development of PABA/NO was briefly mentioned previously (Findlay et al 2004), the details, involving structural modification, molecular modeling, and enzymatic characterization of two key intermediate compounds, have not been reported. Besides, the structure of PABA/NO in the previous report was not correct (Saavedra et al 2006). Here, we present the structure-based design of PABA/NO in detail, during which the structures of different classes of GSTs, especially the structural information for the transition state of GST-catalyzed reaction of GSH and CDNB, played an essential role. With this unique example, we demonstrate again the importance of structure-based approach in drug development and the value of structural information for the transition state of an enzyme-driven reaction in structure-based drug design.

Materials and methods
Structural modifications of O^2AD molecules
Figure 2 depicts our structural modifications starting from GSTα-selective compounds **1** and resulting in GSTπ-selective compound **4** (PABA/NO). Compounds **1**, **2**, and **3** were prepared as previously described (Saavedra et al 2001), as was compound **4** (Findlay et al 2004).

Modeling the GSTα•GSTCD$^-$ complex
Molecular modeling studies were carried out on an SGI Fuel workstation with program packages CNS and O (Jones and Kjeldgaard 1997; Brünger et al 1998). The initial model of GSTCD$^-$ bound to GSTα was built on the basis of the crystal structures of the GSTCD$^-$ found in the active sites of GSTμ (PDB entry 4GST), and GSTπ (PDB entry 1AQX), and then it was docked into the active site of GSTα in complex with the GSH adduct of ethacrynic acid (PDB entry 1GSE; Cameron et al 1995). The model complex, built in dimeric form because GSH interacts with the side chains from both subunits, was subject to geometry optimization using the conjugate gradient method by Powell embedded in CNS (Powell 1977; Brünger et al 1998). The Engh and Huber geometric parameters were used as the basis of the force field (Engh and Huber 1991). The coordinates of the GSTα•GSTCD$^-$ model (Figure 3a) are available from the corresponding author upon request.

Modeling GST with bound Meisenheimer complex of O^2AD
The initial models of the Meisenheimer complex of compounds 1 and 4 (GS1$^-$ and GS4$^-$) bound to the three GST isozymes were

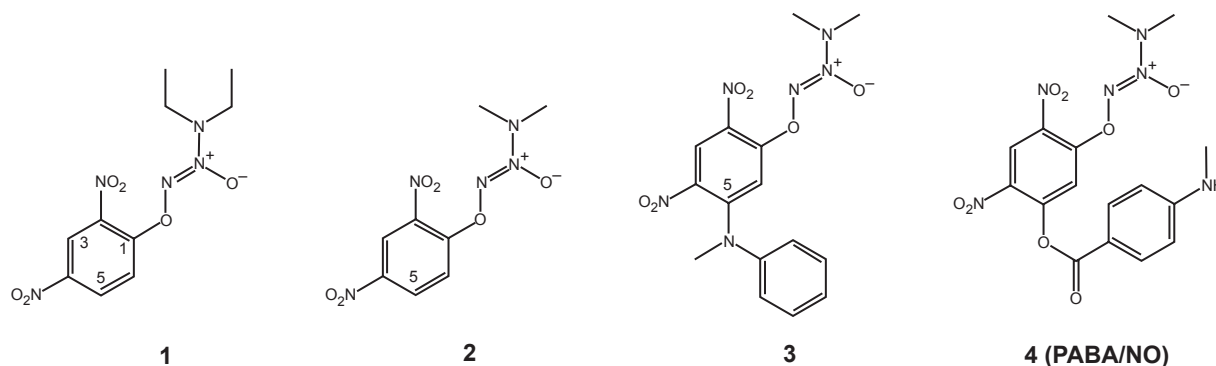**Figure 2** Structure-based design of PABA/NO. Structural modifications of the GSTα-selective compounds 1 and 2 have led to the GSTπ-selective compound 4 (PABA/NO).
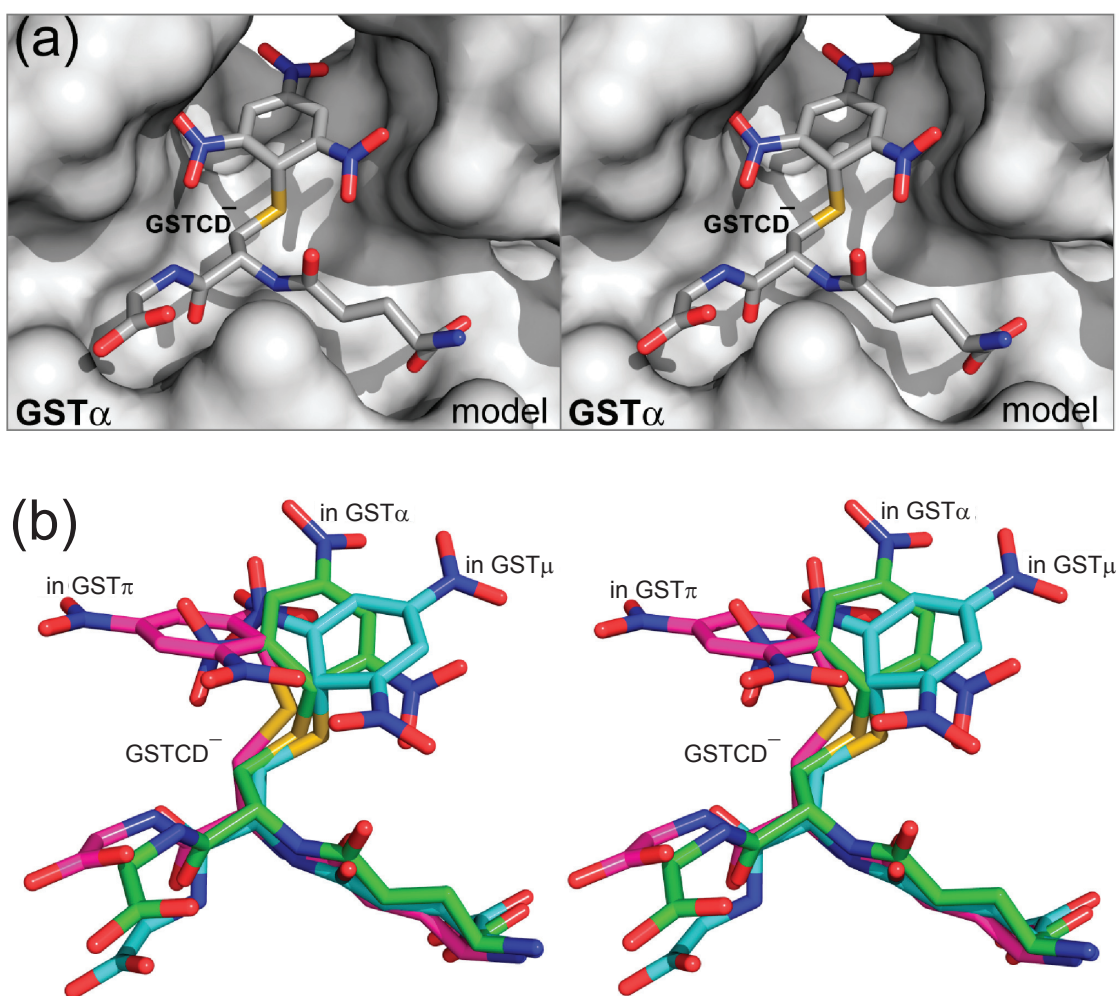
Figure 3 The GSTα•GSTCD⁻ model. (**a**) Stereoview showing the GSTα•GSTCD⁻ model, constructed on the basis of the crystal structure of GSTα in complex with the GSH conjugate of ethacrynic acid (PDB entry 1GSE), the GSTμ•GSTCD⁻ structure (PDB entry 4GST), and the GSTπ•GSTCD⁻ structure (PDB entry 1AQX). The protein is shown as a molecular surface while the ligand as a stick model in atomic color scheme (carbon in gray, nitrogen in blue, oxygen in red, and sulfur in orange). (**b**) Stereoview showing the alignment of the GSTCD⁻ ions in the GSTα•GSTCD⁻ model, in the GSTμ•GSTCD⁻ structure (PDB entry 4GST), and in the GSTπ•GSTCD⁻ structure (PDB entry 1AQX). The GSTCD⁻ ion is shown as a stick model in atomic color scheme (nitrogen in blue, oxygen in red, and sulfur in orange; carbon in green when bound in GSTα, cyan in GSTμ, and magenta in GSTπ).

built on the basis of the crystal structures of compounds **1** and **4** (Saavedra et al 2001, 2006) and the GSTCD⁻ structures in the GSTα•GSTCD⁻ model, the GSTμ•GSTCD⁻ structure (PDB entry **4**GST), and the GSTπ•GSTCD⁻ structure (PDB entry 1AQX). The initial models of GS**2**⁻ were derived from those of GS**4**⁻ by removing the 5-position bulky group, and then they were docked into the active sites of the corresponding GSTs. The dimeric model complexes, except for GSTπ•GS**1**⁻ (Figure 4c) and GSTα•GS**4**⁻ (Figure 4g), were subject to geometry optimization in the same manner as for the GSTα•GSTCD⁻ complex.

Kinetic studies

The recombinant GST isoenzymes were either purchased from Panvera or purified as described previously (Zimniak et al 1994). The reaction mixture, in a final volume of 1 mL,

contained 0.1 M phosphate buffer (pH 6.5–7.0), 1–5 μg/mL of the desired GST protein, 1–2 mM GSH and varying concentrations of compound **1** or compound **2** (20–200 μM). Change in absorbance was monitored spectrophotometrically at 380 nm. The enzyme activity was calculated using an extinction coefficient of 3.58 mM⁻¹ cm⁻¹. Kinetic parameters were calculated using the Michaelis-Menten equation by software KiNET (Kinexus, Vancouver, Canada). The experiments were repeated three times and the results were averaged.

The same set of kinetic studies was carried out in two independent laboratory settings and the results were consistent.

Illustrations

The schematic illustrations for the crystal structures and the models were generated with PyMOL (DeLano 2002).

Results and discussion

Structure of a transition-state analog in complex with GSTα

GSTα is the predominant isoform of GST in human liver, whereas GSTπ is most abundant in extrahepatic tissues and is overexpressed in many types of tumors. In order to target tumor cells while avoiding toxicity to the liver, structural information is needed for GSTα in complex with the transition-state analog GSTCD⁻. On the basis of available structural information, we have built a model of the GSTα•GSTCD⁻ complex (Figure 3a). Together, the GSTμ•GSTCD⁻ structure (PDB entry 4GST), the GSTπ•GSTCD⁻ structure (PDB entry 1AQX), and the GSTα•GSTCD⁻ model have shown that the conformation of the GSTCD⁻ ion exhibits such dramatic differences when it is bound in the active site of different GSTs that the aromatic ring of the GSTCD⁻ ion stretches out in distinct directions (Figure 3b), providing the guidance for our structure-based modification of O^2AD molecules toward the GSTπ-selective PABA/NO.

Compound 1 is GSTα-selective

Compound 1 (Figure 2) is the first O^2AD molecule we have ever synthesized (Saavedra et al 2001). All three classes of GSTs catalyze the NO release of **1**. However, it is GSTα-selective, 2-fold over GSTμ and 145-fold over GSTπ (Table 1). To understand the structural basis for the different catalytic efficiencies, we have constructed model complexes of the three GSTs with GS**1**⁻, the Meisenheimer complex of **1**. The models of GSTα•GS**1**⁻ (Figure 4a) and GSTμ•GS**1**⁻ (Figure 4b) suggest favorable interactions between the protein and the reaction intermediate. The GSTπ•GS**1**⁻ model, however, suggests unfavorable interaction between the diethyl group of the diazenium-diolate moiety and the protein (Figure 4c). Therefore, the first modification of **1** was to replace the diethylamino group of the diazeniumdiolate moiety with a dimethyl-amino group, which was predicted to improve the fit of the reaction intermediate derived from the resulting compound to GSTπ.

Compound 2 indicates that our design is on the right track

As expected, compound **2** (Figure 2) is indeed a better substrate for GSTπ than compound **1**. The catalytic efficiency of GSTπ toward **2** exhibits a noticeable increase (Table 2), which is likely due to the reduced steric interactions between the diazeniumdiolate moiety of the reaction intermediate and the protein (Figure 4c). Moreover, the two other GSTs are less active toward 2, with a 4-fold reduction in the catalytic efficiency for GSTα and a 3-fold reduction for GSTμ (Table 2). Although it is not as obvious, the reduction of the hydrophobic interactions between the protein and the diazeniumdiolate moiety of GS**2**⁻, the Meisenheimer complex of 2, may have played a role. Although 2 is still GSTα-selective, 1.5-fold over GSTμ and 24-fold over GSTπ, the improvement has indicated that our structure-based design is on the right track. The next modification is to further reduce the GSTα selectivity while increasing the GSTπ selectively.

We have constructed model complexes of GS**2**⁻ with GSTα (Figure 4d), GSTμ (Figure 4e), and GSTπ (Figure 4f). The models suggest that the addition of a sterically demanding constituent at position 5 of the aryl ring should increase its accommodation in the active site of GSTπ (Figure 4f) while decreasing its accommodation in the active site of GSTα (Figure 4d). Thus, compound **3** was designed and synthesized (Figure 2). Unfortunately, the solubility of **3** was too low, rendering its enzymatic characterization impossible. Further modification aimed to improve the solubility has led to the synthesis of compound **4**, ie, the PABA/NO (Figure 2).

PABA/NO (Compound 4) is GSTπ-selective

As expected, PABA/NO (compound 4) is a better substrate for GSTπ compared to GSTα (Findlay et al 2004). The model of GSTα in complex with the Meisenheimer complex of 4 (GSTα•GS4⁻) suggests unfavorable interaction between the bulky group at position 5 of the aryl ring system (Figure 4g). In contrast, both GSTμ and GSTπ can accommodate this

Table 1 Kinetic data for human GST isoenzymes with Compound 1 as the variable substrate

Enzyme[a]	K_m (μM)	V_{max} (nmol min⁻¹ mg⁻¹)	k_{cat} (s⁻¹)	k_{cat}/K_m [b] (mM⁻¹ s⁻¹)
GSTα	196 ± 63	200898 ± 37633	171	872
GSTμ	171 ± 51	85508 ± 14322	73	427
GSTπ	523 ± 143	4017 ± 848	3.1	6

Notes: [a]Molecular weight used for calculation of k_{cat}: GSTα, 51000 Da; GSTμ, 51160 Da; GSTπ, 46000 Da; [b]Catalytic efficiency.

Table 2 Kinetic data for human GST isoenzymes with Compound **2** as the variable substrate

Enzyme[a]	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m[b] (mM^{-1} s^{-1})
GSTα	136 ± 27	37932 ± 3793	32	237
GSTμ	1910 ± 327	337169 ± 53003	287	150
GSTπ	237 ± 404	2997 ± 404	2.2	9.7

Notes: [a]Molecular weight used for calculation of k_{cat}: GSTα, 51000 Da; GSTμ, 51160 Da; GSTπ, 46000 Da; [b]Catalytic efficiency.

group (Figure 4, panels h and i). Thus, the structure-based modifications of 1 have reversed the GST isoenzyme selectively of O^2AD.

GSTμ activity toward O^2AD

The expression of GSTμ is null in 50% of the human population due to a gene deletion (Rowe et al 1997). Therefore, our current effort has been focused on GSTα and GSTπ while keeping GSTμ in the scope of our investigation for better understanding the reaction mechanism. Strategies have been developed to avoid GSTμ for the development of next-generation O^2AD anticancer prodrugs.

The Meisenheimer complex of O^2AD

We have previously shown that the transient Meisenheimer complex is generated in the reaction of compound 1 (Saavedra et al 2001). On the basis of this mechanism, during which the Meisenheimer complex of O^2AD is formed (Figure 1a), we have used the GSTμ•GSTCD$^-$ structure (Figure 1d), the GSTπ•GSTCD$^-$ structure (Figure 1e), and the GSTα•GSTCD$^-$ model (Figure 3a) to guide the construction of the three GSTs in complex with the Meisenheimer complex of O^2ADs (Figure 4).

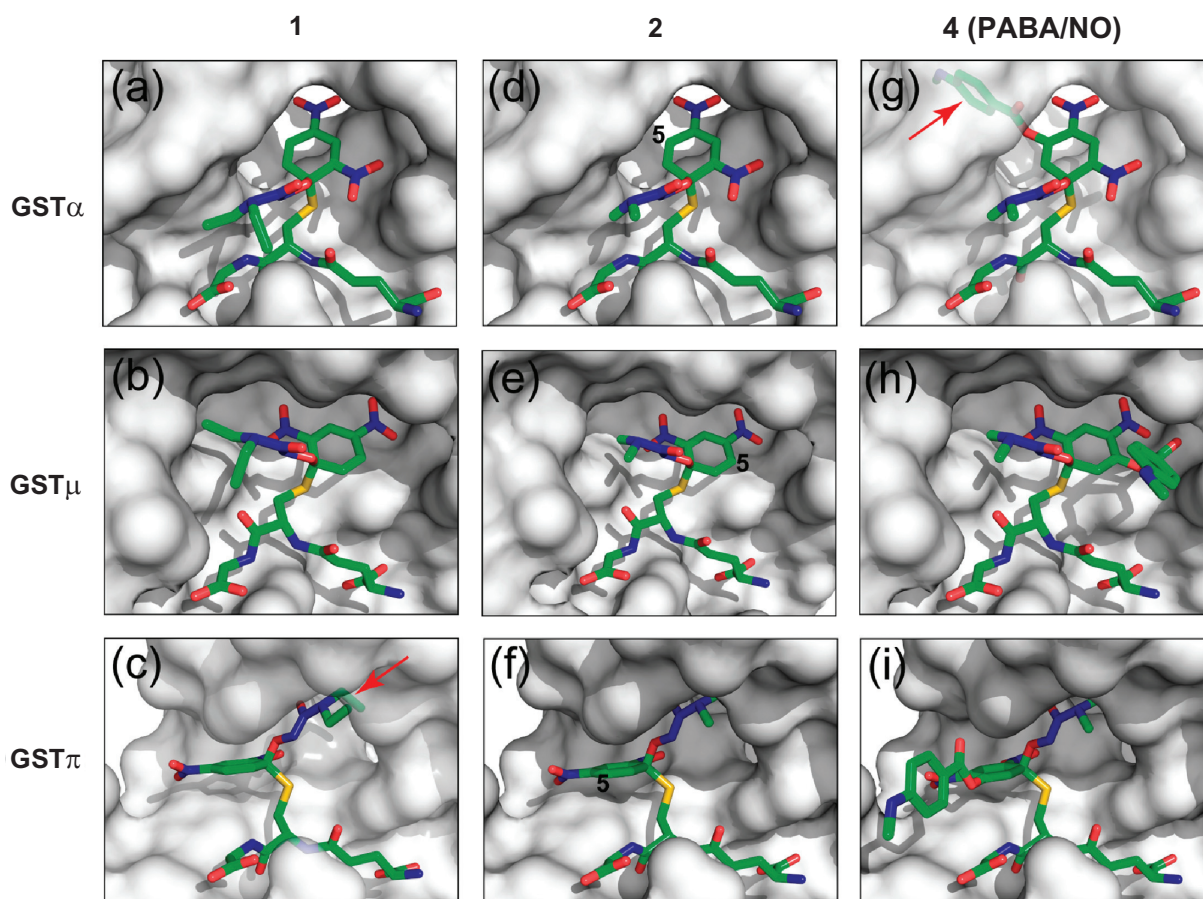The model complexes of GST with the Meisenheimer complexes of O^2AD have played a crucial role in guiding

Figure 4 Models of the three GST isozymes with bound Meisenheimer complex of compound **1**, **2**, and **4**. The GST active sites are illustrated as non-transparent surfaces, except for a transparent surface in panels c and g to show the unfavorable interaction between the ligand and the protein (indicated with red arrows), and the ligands as stick models in atomic color scheme (carbon in green, nitrogen in blue, oxygen in red, and sulfur in orange).

the modification of compounds **1** and **2**, resulting in the development of compound **4**, the GSTπ-selective PABA/NO as an anticancer drug lead. The key factors in this design include (1) understanding the difference in the shape and property of the substrate-binding site of different GSTs and (2) using the structures of GSTCD⁻ in different GST complexes to guide the molecular modeling. For example, the substrate-binding site is broad in both GSTμ and GSTπ (Figure1 panels d and e). However, it is virtually a hydrophobic cavity in GSTμ, but is approximately half hydrophobic and half hydrophilic in GSTπ (Ji et al 1997). It is mainly the property of the binding site that dictates the orientation of the trinitrophenyl ring system of GSTCD⁻ in these two isoenzymes. In support of this notion, the ring system of the GSH adduct of phenanthrene 9,10-oxide also points into opposite directions in these two isoenzymes (Ji et al 1994, 1997). Unlike GSTμ and GSTπ, GSTα has a narrower substrate-binding site, which limits the conformational freedom of the trinitrophenyl moiety of GSTCD⁻ (Figure 3a).

Conclusions

This paper describes the details on structural modification, molecular modeling, and enzymatic characterization for structure-based design of PABA/NO. The design was efficient because it was on the basis of the reaction mechanism and the structures of related GST isozymes at both the ground state and the transition state. The ground-state structures outlined the shape and property of the substrate-binding site in different isozymes, and the known transition-state structures helped us build a model of GSTα at the transition state of which the structural information was not available. The structural information thus derived for the transition state, showing distinct conformations of the Meisenheimer complex of O^2ADs in the active site of different isozymes, guided the modifications of the molecular structure of O^2AD molecules. Two key alterations of a GSTα-selective compound, the reduction of the size of the amino group and the introduction of the bulky group, led to the GSTπ-selective PABA/NO.

Acknowledgments

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