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Synthesis and Biological Evaluation of 2-Substituted-5-(4-nitrophenylsulfonamido)benzoxazoles as Human GST P1-1 Inhibitors, and Description of the Binding Site Features

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Glutathione-*S*-transferases (GSTs) are enzymes involved in cellular detoxification by catalyzing the nucleophilic attack of glutathione (GSH) on the electrophilic center of numerous of toxic compounds and xenobiotics, including chemotherapeutic drugs. Human GST P1-1, which is known as the most prevalent isoform of the mammalian cytosolic GSTs, is overexpressed in many cancers and contributes to multidrug resistance by directly conjugating to chemotherapeutics. It is suggested that this resistance is related to the high expression of GST P1-1 in cancers, thereby contributing to resistance to chemotherapy. In addition, GSTs exhibit sulfonamidase activity, thereby catalyzing the GSH-mediated hydrolysis of sulfonamide bonds. Such reactions are of interest as potential tumor-directed prodrug activation strategies. Herein we report the design and synthesis of some novel sulfonamide-containing benzoxazoles, which are able to inhibit human GST P1-1. Among the tested compounds, 2-(4-chlorobenzyl)-5-(4-nitrophenylsulfonamido)-benzoxazole (**5 f**) was found as the most active hGST P1-1 inhibitor, with an IC_{50} value of 10.2 μM, showing potency similar to that of the reference drug ethacrynic acid. Molecular docking studies performed with CDocker revealed that the newly synthesized 2-substituted-5-(4-nitrophenylsulfonamido)benzoxazoles act as catalytic inhibitors of hGST P1-1 by binding to the H-site and generating conjugates with GSH to form *S*-(4-nitrophenyl)GSH (GS–BN complex) via nucleophilic aromatic substitution reaction. The 4-nitrobenzenesulfonamido moiety at position 5 of the benzoxazole ring is essential for binding to the H-site and for the formation of the GST-mediated GSH conjugate.

Introduction

Glutathione-*S*-transferases (GSTs) are a family of dimeric multifunctional enzymes that play an important role in metabolism and detoxification of numerous xenobiotics, electrophilic chemicals (including drugs), environmental carcinogens, and products of oxidative stress in living organisms.[1] Aside from their detoxifying reaction (typically a conjugation reaction), GSTs catalyze many other types of reaction, such as isomerization and reduction involved in many biological processes, e.g., steroid and prostaglandin biosynthesis, tyrosine catabolism, and cell apoptosis.[2]

For the GST-mediated detoxification reactions, a substrate–GSH conjugation (complex formation) is required. GSTs are a family of phase II detoxification enzymes that catalyze the conjugation of GSH to a wide variety of electrophilic compounds. GSTs catalyze the conjugation of the reduced form of glutathione (GSH) to electrophilic centers of endogenous and exogenous hydrophobic compounds.[1] For GST-mediated catalytic reactions, the activation of the sulfur atom of the G-site-bound glutathione (GSH) to the thiolate anion (GS^-, a strong nucleophile) is required to generate a GS–substrate conjugate by attack of the electrophilic center of substrates bound to the H-site. Furthermore, the GS-conjugated compounds may be actively extruded from the cell through specialized pumps; principally, the multidrug-resistance proteins MRP-1 and MRP-2.[3]

GSTs are found in almost all organisms, from mammals to plants, and even in some prokaryotes. Human GSTs consist of three families: cytosolic GSTs, mitochondrial GSTs, and microsomal GSTs. In mammalian cells cytosolic GSTs are further categorized into seven major classes according to their amino acid sequence: alpha, mu, pi, theta, zeta, omega, and sigma subfamilies, which have been identified in dimeric forms.[4]

Human glutathione-*S*-transferase pi1-1 (hGST P1-1) is a member of the pi class subfamily of cytosolic GSTs and is composed of two homodimer GST P1 subunits. Structural analysis indicates that hGST P1-1 is a soluble protein of 209 amino acid residues with a relative molecular mass of 23.224 kDa.[5] The crystal structures show that each GST subunit of the protein dimer contains an independent catalytic site composed of two components. The first is a binding site specific for GSH or a closely related homologue (the G-site) formed from a conserved group of residues in the N-terminal domain of the polypeptide. The second component is a site that binds the hydrophobic substrate (the H-site), which is structurally variable

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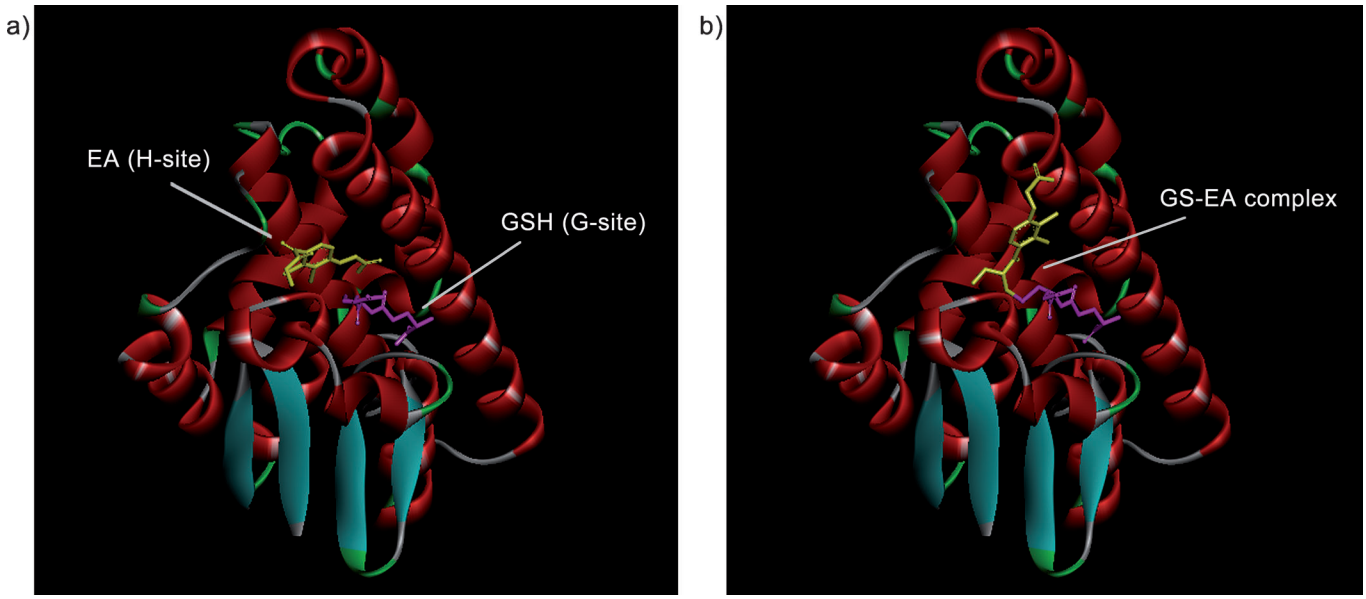
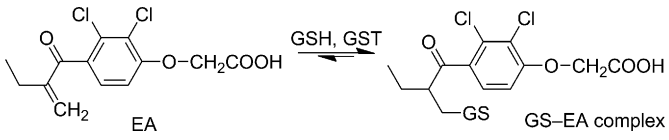


Figure 1. a) Binding sites of hGST P1-1 (PDB ID: 2GSS), GSH (shown in purple, taken from PDB ID: 6GSS), and the specific substrate (EA) (shown in yellow, taken from PDB ID: 2GSS), shown as bound separately in H- and G-sites. b) EA and GSH conjugate (GS–EA complex) in hGST P1-1 enzyme (PDB ID: 3GSS).

(Figure 1). Residue variations in the H-site across the different GST classes determine substrate specificity.[6]

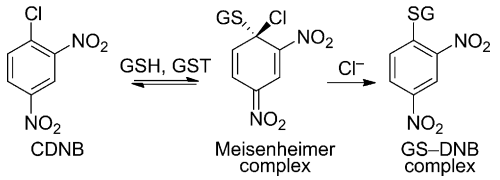
hGST P1-1 is the most prevalent isoform of the mammalian cytosolic GSTs. It is known that hGST P1-1 participates in a particular role in one of the mechanisms of the development of resistance in cancer cells toward the administration of anticancer agents in chemotherapy. Several synthetic drugs and prodrugs that show inhibitory potency against GSTs have been proposed as strategies to overcoming multiple drug resistance (MDR) attributed to GST overexpression. Ethacrynic acid (EA) is one of the first specific inhibitors to be used to sensitize cancer cells to the cytotoxic effect of several chemotherapeutic drugs.[7–9] As shown in Figure 1, EA acts as a substrate of GST, forming a conjugate with GSH (GS–EA complex) via Michael addition (Scheme 1) by GST-driven catalysis.[10] The use of this



Scheme 1. Michael-type addition of EA and GS–EA complex.

inhibitor as a means of overcoming acquired multidrug resistance has been thoroughly explored, reaching phase II clinical trials. Unfortunately, due to unwanted toxic side effects, EA was withdrawn from further trials.

One of the other well-known diagnostic substrates that forms a GST-mediated conjugate with GSH is 1-chloro-2,4-dinitrobenzene (CDNB), which undergoes a nucleophilic aromatic substitution (S_NAr) reaction that forms a GS–DNB complex via an addition–elimination sequence involving a short-lived



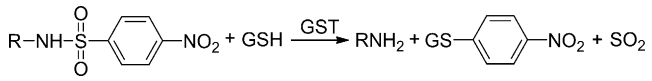
Scheme 2. GST-mediated nucleophilic aromatic substitution of CDNB with GSH to form the GS–DNB complex.

σ-complex intermediate, the Meisenheimer complex (Scheme 2).[4]

hGST P1-1 is overexpressed in many cancers and contributes to multidrug resistance by directly conjugating to chemotherapeutic agents including cisplatin, adriamycin, etoposide, thiotepa, and chlorambucil. It is suggested that this resistance is related to the high expression of hGST P1-1 in cancers such as breast, lung, colon, pancreas, and cervix, thereby contributing to resistance to chemotherapy.[11] Studies have shown that hGST P1-1 levels correlate with resistance to standard chemotherapy and are elevated in biopsies of tumor tissues that have become resistant to therapy after administration of anticancer agents.[12,13] Consequently, inhibitors of human GST P1-1 catalytic activity remain a potential therapeutic tool in combating cancer cell resistance to drugs. To overcome this resistance, specific hGST P1-1 inhibitors are in demand.

hGST P1-1 appears also to regulate a c-Jun N-terminal kinase (JNK) pathway that is part of the apoptosis control system. Thus, hGST P1-1 defends tumor cells by direct detoxification of anticancer drugs and by blocking apoptosis through its effects on JNK.[7,14] Therefore, hGST P1-1 is considered a promising target for inhibition in cancer treatment, and considerable efforts have been undertaken to find specific inhibitors for this enzyme. Furthermore, a variety of sulfonamido-containing

compounds are apparently accommodated by the H-site of GST and are found as substrates of GSTs. Published data clearly show that GSTs display sulfonamidase activity by catalyzing the GSH-mediated hydrolysis of sulfonamide bonds (Scheme 3).[15,16] Groups capable of withdrawing sufficient elec-



Scheme 3. GST/GSH-mediated hydrolysis of *para*-nitrobenzenesulfonamides.

tron density from the α-carbon atom to the sulfonyl group are an absolute requirement in this enzymatic process.[15] GST-mediated sulfonamide cleavage results in the formation of the GS conjugate, the corresponding amine, and sulfur dioxide. The released GS conjugate may provide a strong inhibitor against intracellular GSTs, thus facilitating cancer chemotherapy.[17] The sulfonamidase activity of GSTs also suggests that prodrugs activated by GSTs might provide a strategy for targeted cancer chemotherapy. The design of new sulfonamide derivatives, which are able to undergo cleavage in tumor cells by highly expressed intracellular GSTs such as hGST P1-1, could be a viable approach.[17,18]

The benzoxazoles have been the aim of much research for many years because they constitute an important class of heterocyclic compounds exhibiting substantial chemotherapeutic activities.[19–35] Recently, we reported some 2,5,6-trisubstituted benzoxazole derivatives, which exhibit antimicrobial,[27–29] antiviral,[30,31] antitumor activities, inhibiting eukaryotic topoisomerase II in a cell-free system.[32–35] The aim of this research was to develop new and effective hGST P1-1 enzyme inhibitors from sulfonamido-containing benzoxazole derivatives. In this study we synthesized some novel 2-substituted-5-(4-nitro/aminophenylsulfonamido)benzoxazole derivatives and investigated their inhibitory activities toward hGST P1-1. To describe the binding site features of these benzoxazoles on hGST P1-1, molecular docking studies were performed with CDocker.

Results and Discussion

Chemistry

5-(4-Nitro/aminophenylsulfonamido)benzoxazole derivatives were synthesized as new structures via a three-step route. Firstly, 5-amino-2-(4-substituted phenyl/benzyl)benzoxazole derivatives **3a–f** were obtained by heating the appropriate acid with 2,4-diaminophenol in polyphosphoric acid (PPA).[28,36,37] Second, 5-amino-2-(4-substituted phenyl/benzyl)benzoxazole derivatives **3a–f** and 4-nitrobenzenesulfonyl chloride (**4**) were treated in pyridine and dichloromethane to get 5-(4-nitrophenylsulfonamido)benzoxazole derivatives **5a–f**.[38] Finally, 2-(4-ethylphenyl)-5-(4-aminophenylsulfonamido)benzoxazole (**6c**) was obtained by reduction of the NO_2 group to NH_2[39] by using Pd-C/H_2, as shown in Scheme 4.

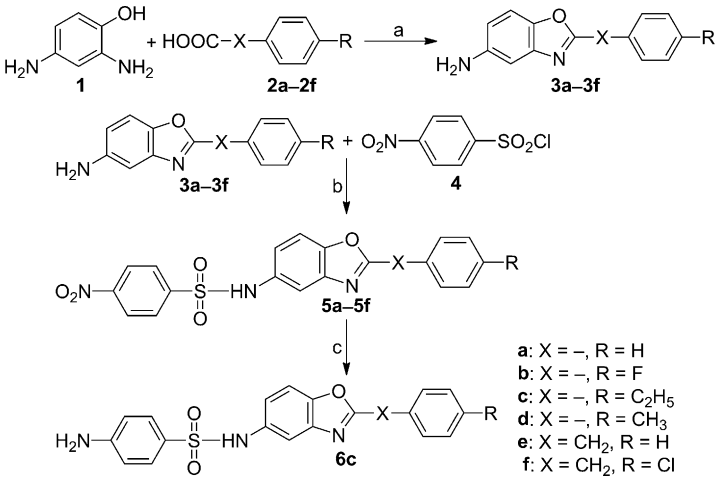
hGST P1–1 in vitro inhibitory activity

Each GST subunit of the protein dimer contains an independent catalytic site composed of two components. GSH binding site (G-site) forms from a conserved group of amino acid residues in the N-terminal domain of the polypeptide; the hydrophobic substrate binding site (H-site) is much more structurally variable and is formed from residues in the C-terminal domain (Figure 1).[6] The H-site of the pi-class enzyme is half hydrophobic and half hydrophilic.[40] It is reported that GSTs catalyze the GSH-mediated hydrolysis of sulfonamide bonds to form the corresponding amine (Figure 3 below).[15,16] Atoms and/or atom groups positioned *ortho* and/or *para* on the phenyl ring such as nitro, capable of withdrawing sufficient electron density from the α-carbon atom, to which the sulfonyl group is attached, are shown to activate the corresponding GST-mediated GSH hydrolysis of sulfonamides.[15]

In this study, newly synthesized 2-substituted-5-(4-nitro/aminophenylsulfonamido)benzoxazole derivatives were screened for in vitro inhibition of hGST P1-1. Among all of the tested compounds, 2-(4-chlorobenzyl)-5-(4-nitrophenyl-sulfonamido)-benzoxazole (**5f**) exhibited the most potent inhibitory activity for hGST P1-1, with an IC_{50} value of 10.2 μM).[41] According to inhibitor activity results given in Table 1, compound 2-(4-ethylbenzyl)-5-(4-aminophenylsulfonamido) benzoxazole (**6c**) displayed no inhibitory activity toward hGST P1-1 due to the lack of electron-withdrawing effect of the amino group at the α-carbon atom of the phenyl ring attached to the sulfonyl group.

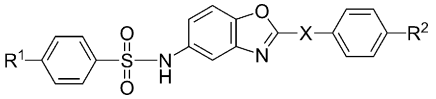
Description of the binding site features

Rational approaches for finding new leads for therapeutic targets are increasingly based on three-dimensional information about receptors. One can predict the binding conformation of a ligand in its receptor and the affinity between the ligand and the protein with the correct poses of ligands in the binding



Scheme 4. Synthetic pathway of 5-(4-nitro/aminophenylsulfonamido)benzoxazole derivatives. *Reagents and conditions*: a) PPA, 170–200 °C, 1.5–2.5 h; b) pyridine and CH_2Cl_2, RT, 16 h; c) Pd-C/H_2, RT, 30 min, 60.35%.

Table 1. The structures of the tested sulfonamide-containing benzoxazoles and their in vitro hGST P1-1 inhibitory effects.



Compd	R^1	R^2	X	IC_{50} [μM]
5a	NO_2	H	–	16.67
5b	NO_2	F	–	16.67
5c	NO_2	C_2H_5	–	28.57
5d	NO_2	CH_3	–	12.5
5e	NO_2	H	CH_2	33.33
5f	NO_2	Cl	CH_2	10.2
6c	NH_2	C_2H_5	–	NI[a]
EA				10.37

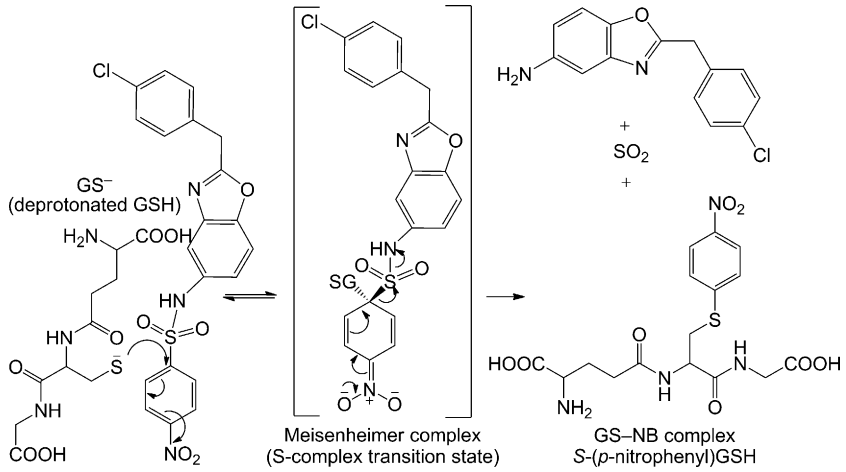
[a] No inhibition.

pocket of a protein. A process is described by which two molecules fit together in 3D space.

It is reported that the active site residues Tyr7 and Tyr108 play important roles for the activity of hGST P1-1.[10,42] Different models have been proposed for the activation of GST, all highlighting the key role of active site residues Tyr7 and Tyr108. In the mechanism for activation of GSH, Tyr7 acts as a general base, promotes proton abstraction from the GSH thiol, and creates a thiolate anion with high nucleophilic reactivity.[42] Additionally, the hydroxy group of Tyr108 appears to contribute to the catalytic mechanism as a general acid in the conjugation reaction of GSH with EA.[10]

In this study, newly synthesized 5-(4-nitro/aminophenylsulfonamido)benzoxazole derivatives, CDNB, and EA were docked into the H-site of hGST P1-1 by using CDocker in order to explore the mechanism for inhibition of hGST P1-1. EA, which is the known specific inhibitor of hGST P1-1, showed hydrogen bonds with Arg13 and a H_2O molecule, and a π–π interaction with Tyr108; these are in accordance with the X-ray structure binding features.[43] One of the well-known diagnostic substrates of hGST P1-1, CDNB revealed hydrogen bonds with Tyr108 and Trp38; a π–π interaction with Phe8, and a π–cation interaction with Tyr108. The most potent hGST P1-1 inhibitor, benzoxazole derivative **5f**, revealed hydrogen bonds with Gln51 and Tyr108 and a π–cation interaction with Lys44 (Figure 2). The docking scores showed that the binding energies of EA, CDNB, and **5f** are −19.8691, −19.2405, −18.2525 kcal mol^{-1}, respectively (Table 2).

As shown in Figure 3, the van der Waals contact distance between the sulfur atom of GSH and the electrophilic chlorine-bound carbon atom of CDNB is 3.295 Å. Similarly, the van der Waals contact distance between the sulfur atom of GSH and the electrophilic carbon atom of the 4-nitrophenyl moiety attached to the sulfonyl group is 3.322 Å. Notably, the van der Waals contact distances are quite similar to each other. Therefore, we consider that **5f** can be involved in the S_NAr reaction, like CDNB. It is also known that GSTs catalyze the GSH-mediated cleavage of sulfonamide bonds to form the corresponding amine.[15,16] As shown in Scheme 5, the thiolate anion of GSH can attack the electrophilic carbon atom of 4-nitrobenzene to which the sulfonyl group is attached to start the S_NAr reaction. As shown in Scheme 5, an S-complex transition state—a Meisenheimer complex—initially forms, and then the *S*-(4-nitrophenyl)GSH conjugate (GS–NB complex), sulfur dioxide, and 2-(4-chlorobenzyl)-5-aminobenzoxazole are yielded as the GST/GSH-mediated hydrolysis products of the compound **5f**.



Scheme 5. hGST-P1-1-mediated *S*-(4-nitrophenyl)GSH conjugate (GS–NB complex) formation of **5f** via nucleophilic aromatic substitution reaction.

Table 2. Docking results.

Compd	E_{bind} [kcal mol^{-1}]	Interacting Residues[a]
5a	−11.2459	Tyr7, Phe8, Val10, **Arg13** (2.33 Å), Trp38, Gln51, Leu52, Ile104, **Tyr108** (2.34 Å), Thr109, Gly205
5b	−7.9622	Tyr7, Phe8, Val10, Arg13,[c] Val35, Trp38, **Gln39** (2.57 Å), Gln51, Leu52, Gln64, Ile104, Tyr108,[b] Gly205
5d	−13.0223	Tyr7,[c] Phe8,[b,c] Val10, **Trp38** (2.23 Å), Gln39, Lys44, Gln51 (2.03 Å), **Tyr108** (2.42 Å), Gly205
5f	−18.2525	Tyr7, Phe8, Val10, Arg13, Val35, Trp38, Gln39, Gly41, Lys44,[c] **Gln51** (2.29 Å), Ile104, **Tyr108** (2.29 Å), Gly205
EA	−19.8691	Tyr7, Phe8, Val10, Gly12, **Arg13** (2.09 Å), Val35, Trp38, Ile104, Tyr108,[b] Gly205, **H_2O** (water mediated H bond with Arg13)
CDNB	−19.2405	Tyr7, Phe8,[b] Val10, **Trp38** (2.32 Å), Gln51, Ile104, **Tyr108**[c] (2.38 Å), Gly205

[a] van der Waals contact distance: < 4 Å; **H-bonds** indicated in bold text. [b] π–π interactions. [c] π-cation interactions.

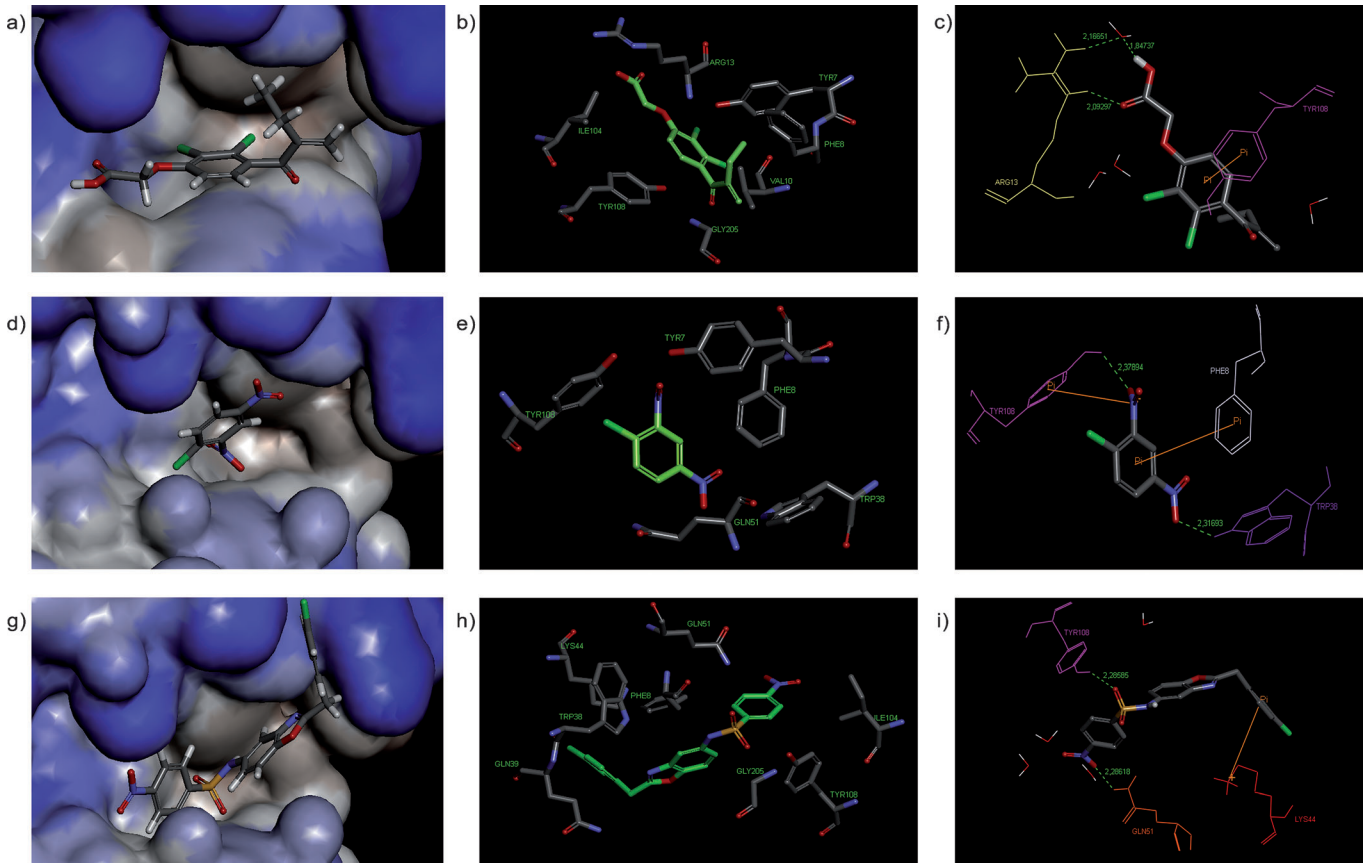


Figure 2. a) Docking overlay of EA in hydrophobicity-based surface area. b) Docked position of EA with interacting residues within 3.2 Å. c) Docked position of EA: the carboxyl group hydrogen bonds with Arg13 (yellow) and a H_2O molecule, the phenyl ring undergoes π–π interactions with Tyr108 (pink). d) Docking overlay of CDNB in hydrophobicity-based surface area. e) Docked position of CDNB with interacting residues within 3 Å. f) Docked position of CDNB: *ortho*-nitro group hydrogen bonds with Tyr108 (pink), *para*-nitro group hydrogen bonds with Trp38 (purple), the phenyl ring undergoes π–π interactions with Phe8 (white), the N atom of the *ortho*-nitro group has π–cation interactions with Tyr108 (pink). g) Docking overlay of **5 f** in hydrophobicity-based surface area. h) Docked position of **5 f** with interacting residues within 3 Å. i) Docked position of **5 f**: the sulfonyl group hydrogen bonds with Tyr108 (pink), the *para*-nitro group hydrogen bonds with Gln51 (orange), the phenyl ring at position 2 of the benzoxazole ring undergoes π–cation interactions with Lys44 (red).

Conclusions

The X-ray crystallographic results published by Oakley et al. in 1997 confirmed that the well-known hGST P1-1-specific inhibitor EA binds to the ligand binding site (H-site) of hGST P1-1, while GSH binds the G-site, forming a conjugate with GSH via conjugation reaction. These observations clearly demonstrate that EA acts as a substrate of hGST P1-1, forming a conjugate with GSH, where the EA–GSH conjugate also acts as an inhibitor. Therefore, EA is a known inhibitor of hGST P1-1, but also acts as a substrate.[43]

hGST P1-1 has been shown to catalyze the conjugation of GSH with the alkylating agents chlorambucil and thiotepa, suggesting that overexpression of hGST P1-1 in cells exposed to these drugs would confer resistance. Elevated cellular levels of hGST P1-1 have been shown to accompany resistance to various common anticancer drugs, and the addition of the GST inhibitors such as EA restored sensitivity to alkylating agents in drug-resistant cells.[1] EA has been reported to potentiate the cytotoxic effects of chlorambucil in human colon carcinoma cell lines and melphalan in human colon tumors.[1] A variety of GST inhibitors were shown to modulate drug resistance by sensitizing tumor cells to anticancer drugs.[1] Consequently, it is accepted that hGST P1-1 contributes directly to drug resistance in some cell types via their catalytic activity; therefore, inhibitors of hGST P1-1 remain as potential therapeutic tools. This therapeutic strategy requires the GST-targeted agent to inhibit catalytic function.

The development of chemotherapy-resistant tumor cells is a significant problem encountered in cancer treatment. The overexpression of hGST P1-1 in many cancer tissues and in drug-resistant cell lines suggests that elevated hGST P1-1 expression may be of direct relevance not only to acquired resistance, but also in natural resistance.[6] It is possible that hGST P1-1 confers drug resistance by two distinct means: 1) direct inactivation (detoxification) of chemotherapeutic drugs and 2) inhibition of the mitogen-activated protein (MAP) kinase pathway.[7] Furthermore, hGST P1-1 was identified as capable of blocking the stress-activated kinase pathway, thereby protecting cells against apoptosis in response to cellular stress from reactive oxygen species. Therefore, hGST P1-1 is considered as a promising target for inactivation in cancer treatment, and numerous groups have spent considerable effort finding potent inhibitors of this enzyme.[44]

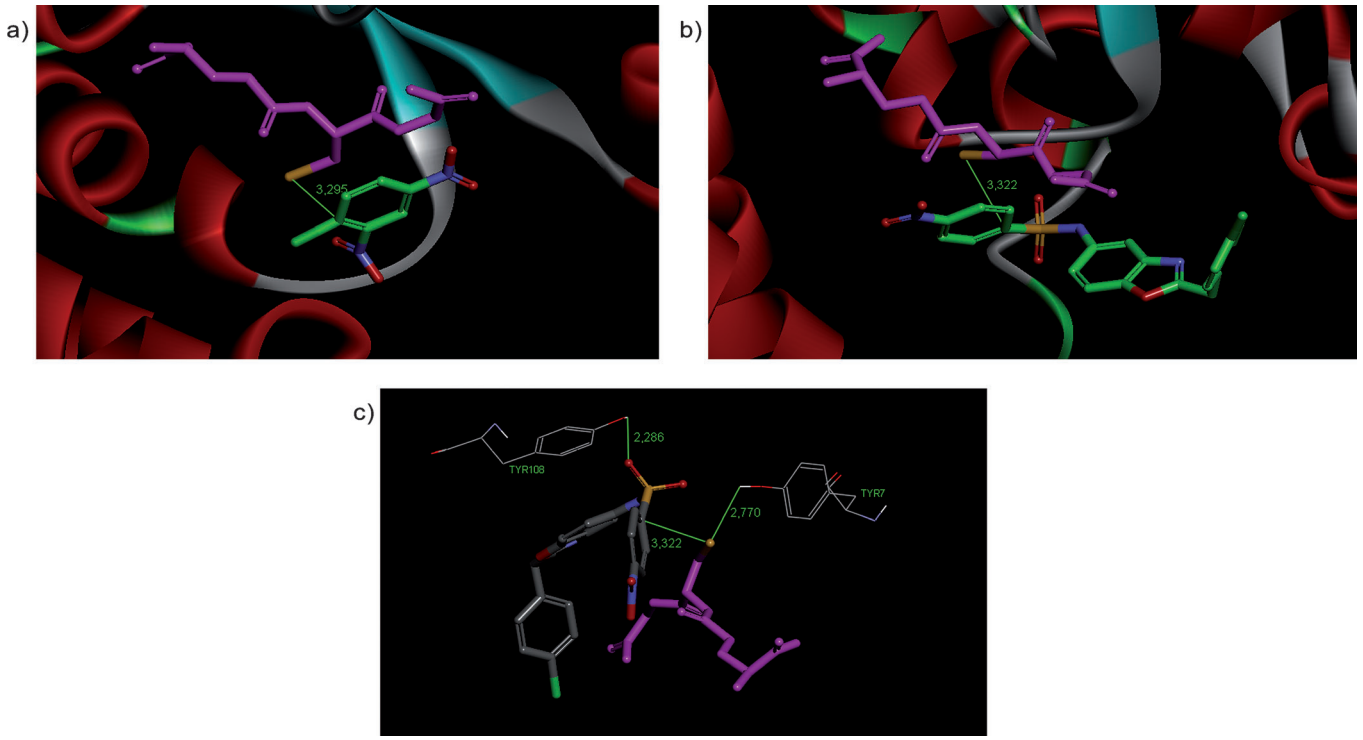


Figure 3. All C atoms are green for CDNB and **5 f**. a) van der Waals contact distance between electrophilic carbon atom of CDNB and the S atom of GSH (purple) is 3.295 Å. b) van der Waals contact distance between the carbon atom of **5 f** and the S atom of GSH (purple) is 3.322 Å. c) van der Waals contact distance between the nucleophilic C atom of **5 f** and the S atom of GSH (purple), and the H-bond distances with residues Tyr7 and Tyr108 in hGST P1-1 are indicated.

In this work, we attempted to observe novel lead compounds that act as human GST P1-1 inhibitors, available for use as chemotherapeutic agents. For this purpose, we synthesized some novel 2-substituted-5-(4-nitro/aminophenylsulfonamido)benzoxazole derivatives to investigate their hGST P1-1 inhibitory activities. Among the tested sulfonamido-containing benzoxazole derivatives, 2-(4-chlorobenzyl)-5-(4-nitrophenylsulfonamido)benzoxazole (**5 f**) was found to be the most potent inhibitor showing a similar potency with the reference drug EA.

Based on previous studies by Zhao et al.[15] and Koeplinger et al.,[16] both in 1999, the reaction of GST-mediated GSH-dependent sulfonamide cleavage occurs by binding of GSH at the G-site of GST, resulting in the formation of the thiolate anion GS^-. This nucleophile then attacks the (aromatic) carbon atom linked to the sulfonyl moiety of the sulfonamide lead substrate, which binds at the H-site of GST. Sulfonamide cleavage results in the formation of the GS conjugate, the corresponding amine, and sulfur dioxide. The released GS conjugate provides a strong inhibitor against intracellular GSTs, thus facilitating cancer chemotherapy.[15,16] In accordance with these findings, our molecular docking results demonstrate that the 4-nitrophenylsulfonamido-containing benzoxazole derivative **5 f** bind to the H-site of the enzyme and forms the *S*-(4-nitrophenyl)GSH conjugate (GS–NB complex) via S_NAr reaction by GST-mediated hydrolysis, as shown in Scheme 5.

In contrast, the synthesized compound 2-(4-ethylbenzyl)-5-(4-aminophenylsulfonamido)benzoxazole (**6 c**) did not exhibit any inhibitory activity toward hGST P1-1, as it was unable to activate the corresponding GST-mediated GSH hydrolysis to effect the GSH conjugation reaction, because of the electron-donating effect of the *p*-amino group toward the α-carbon atom attached to the sulfonyl group. Consequently, it was found that groups capable of withdrawing sufficient electron density from the α-carbon atom to the sulfonyl group are an absolute requirement. The electrophilic substructure of the sulfonyl group was solely responsible for activation of the sulfonamide bond toward cleavage. On the other hand, the amine portion had little or no impact on the cleavability of sulfonamide substrates, but influenced the catalytic rate.[15,16]

To analyze the binding site features of the sulfonamido-containing benzoxazole derivatives, molecular docking studies were performed by using CDocker, and the model for the binding of the sulfonamide lead ligand to hGST P1-1 was constructed based on: 1) the fact that the GSH thiolate anion attacks the α-carbon of the sulfonamide in the reaction and 2) manual fitting to obtain optimum interactions between the sulfonamide's hydrophobic chain and the hydrophobic residues of the H-site. The predicted modes of interaction of the sulfonamido-containing benzoxazoles with hGST P1-1 are shown in Figure 2 g,h,i and 3 b,c. It was found that the new synthesized 2-substituted-5-(4-nitrophenylsulfonamido)benzoxazoles bind to the H-site of hGST P1-1 and form conjugates with GSH, generating the *S*-(4-nitrophenyl)GSH conjugate (GS–BN complex) via S_NAr reaction. A 4-nitrophenylsulfonamido moiety substituted at position 5 of the benzoxazole ring is es-

sential for binding to the H-site and formation of the GS–NB complex for inhibitory activity.

According to the observed in vitro hGST P1-1 inhibitory activity results, the tested compound **5 f** exhibited strong hGST P1-1 inhibition, making it a promising lead compound for further in vitro and in vivo studies to develop new chemotherapeutic agents.

Experimental Section

Chemistry

All chemicals and solvents were purchased from commercial venders and were used without purification. The progress of the reaction was monitored on ready-made silica gel plates (Merck). The melting points were measured with a capillary melting point apparatus (Büchi B540) and are uncorrected. Yields were calculated after recrystallization. IR spectra were recorded on a Jasco FT/IR-420 spectrometer as KBr discs. The ^{1}H NMR spectra were recorded with a Varian Mercury 400 MHz FT spectrometer, chemical shifts (δ) are reported in ppm relative to TMS, and coupling constants (J) are reported in Hz. Mass spectra were taken on a Waters Micromass ZQ instrument using the ESI method. Elemental analyses were performed with a Leco CHNS-932 CHNS-O analyzer; results (C, H, N, S) were within $\pm$0.4% of the calculated amounts.

General procedure for the preparation of 2-(4-substituted phenyl/benzyl)-5-aminobenzoxazoles 3 a–f: The derivatives were synthesized by heating 2,4-diaminophenol dihydrochloride (0.01 mol) with suitable acid (0.01 mol) in polyphosphoric acid (PPA; 24 g) with stirring at 170–200 °C for 1.5–2.5 h. At the end of the reaction period, the residue was poured into an ice–water mixture and neutralized with excess NaOH (10% solution), and the residue was filtered and boiled with charcoal (200 mg) in ethanol and filtered. After the evaporation of solvent in vacuo, the crude product was obtained and recrystallized from an ethanol/water (1:3) mixture.[28,36,37]

General procedure for the preparation of 5-(4-nitrophenylsulfonamido)benzoxazole derivatives 5 a–f: To a solution of 2-(4-substituted phenyl)-5-amino benzoxazole **3 a–f** (0.048 mmol), in CH_2Cl_2 (2 mL), 4-nitrobenzenesulfonyl chloride (0.52 mmol) was added. The reaction mixture was stirred at room temperature for 16 h. At the end of the reaction the residue was filtered and washed with a saturated solution of $CuSO_4$ and $NaHCO_3$ in water,[38] then recrystallized with a mixture of ethyl acetate/*n*-hexane (1:4). The crystals were dried in vacuo. All of the compounds are new.

2-Phenyl-5-(4-nitrophenylsulfonamido)benzoxazole (5 a): To a solution of 2-phenyl-5-aminobenzoxazole (**3 a**) (0.048 mmol) in CH_2Cl_2 (2 mL), pyridine (0.95 mmol) and 4-nitrobenzenesulfonyl chloride (0.52 mmol) were added to give the compound **5 a** (56.96%): mp: 247–250 °C; IR (KBr): $\tilde{\nu}$ = 3264, 3105, 1618–1554, 1606, 1532, 1481, 1346, 1326, 1157, 1108–1054, 857–668 cm^{-1}; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.08 (dd, J = 9.2 Hz, J = 2.0 Hz, 1H), 7.44 (d, J = 1.6 Hz, 1H), 7.60–7.64 (m, 4H), 7.98 (d, J = 8.8 Hz, 2H), 8.15 (dd, J = 7.6 Hz, J = 1.6 Hz, 2H), 8.33 ppm (d, J = 8.8 Hz, 2H); MS (ESI): m/z (%): 394.25 (100) $[M-H]^+$ calcd for $C_{19}H_{13}N_3O_5S$: C 57.72, H 3.31, N 10.63, S 8.11, found: C 57.84, H 3.50, N 10.81, S 8.14.

2-(4-Fluorophenyl)-5-(4-nitrophenylsulfonamido)benzoxazole (5 b): To a solution of 2-(4-fluorophenyl)-5-aminobenzoxazole (**3 b**) (0.048 mmol) in CH_2Cl_2 (2 mL), pyridine (0.95 mmol) and 4-nitrobenzenesulfonyl chloride (0.52 mmol) were added to give compound **5 b** (46.01%): mp: 230–232 °C; IR (KBr): $\tilde{\nu}$ = 3246, 3118, 1684–1559, 1603, 1524, 1476, 1342, 1318, 1223, 1163, 1143–1063, 857–672 cm^{-1}; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.14 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H), 7.43–7.51 (m, 3H), 7.70 (d, J = 8.4 Hz, 1H), 8.00 (d, J = 9.2 Hz, 2H), 8.21 (q, 2H), 8.37 (d, J = 8.8 Hz, 2H), 10.71 ppm (s, 1H); MS (ESI): m/z (%): 412.30 (100) $[M-H]^+$ calcd for $C_{19}H_{12}FN_3O_5S$: C 55.21, H 2.93, N 10.17, S 7.76, found: C 55.14, H 3.22, N 10.29, S 7.82.

2-(4-Ethylphenyl)-5-(4-nitrophenylsulfonamido)benzoxazole (5 c): To a solution of 2-(4-ethylphenyl)-5-amino benzoxazole (**3 c**) (0.048 mmol) in CH_2Cl_2 (2 mL), pyridine (0.95 mmol) and 4-nitrobenzenesulfonyl chloride were added to give compound **5 c** (53.19%): mp: 217–219 °C; IR (KBr): $\tilde{\nu}$ = 3270, 3125, 2968, 1673–1498, 1607, 1527, 1476, 1346, 1310, 1162, 1139–1086, 854–675 cm^{-1}; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.22 (t, 3H), 2.70 (q, 2H), 7.13 (dd, J = 8.8 Hz, J = 2.0 Hz, 1H), 7.44 (d, J = 8.0 Hz, 2H), 7.50 (d, J = 2.4 Hz, 1H), 7.69 (d, J = 8.4 Hz, 1H), 8.01 (d, J = 8.8 Hz, 2H), 8.06 (d, J = 8.0 Hz, 2H), 8.37 (d, J = 9.2 Hz, 2H), 10.70 ppm (s, 1H); MS (ESI): m/z (%): 422.25 (100) $[M-H]^+$ calcd for $C_{21}H_{17}N_3O_5S$: C 59.57, H 4.05, N 9.92, S 7.57, found: C 59.50, H 4.30, N 9.95, S 7.60.

2-(4-Methylphenyl)-5-(4-nitrophenylsulfonamido)benzoxazole (5 d): To a solution of 2-(4-methylphenyl)-5-aminobenzoxazole (**3 d**) (0.048 mmol) in CH_2Cl_2 (2 mL), pyridine (0.95 mmol) and 4-nitrobenzenesulfonyl chloride (0.52 mmol) were added to give compound **5 d** (42.79%): mp: 244–245 °C; IR (KBr): $\tilde{\nu}$ = 3281, 3126, 1646, 1497, 1608, 1529, 1480, 1348, 1313, 1161, 1138–1086, 853–669 cm^{-1}; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.41 (s, 3H), 7.12 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H), 7.42 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 2.0 Hz, 1H), 7.68 (d, J = 8.8 Hz, 1H), 7.98 (d, J = 8.8 Hz, 2H), 8.04 (d, J = 8.4 Hz, 2H), 8.36 (d, J = 8.4 Hz, 2H), 10.69 ppm (s, 1H); MS (ESI): m/z (%): 410.96 (100) $[M+H]^+$ calcd for $C_{20}H_{15}N_3O_5S$: C 58.67, H 3.69, N 10.26, S 7.83, found: C 58.58, H 3.79, N 10.26, S 7.85.

2-Benzyl-5-(4-nitrophenylsulfonamido)benzoxazole (5 e): To a solution of 2-benzyl-5-aminobenzoxazole (**3 e**) (0.048 mmol) in CH_2Cl_2 (2 mL), pyridine (0.95 mmol) and 4-nitrobenzenesulfonyl chloride (0.52 mmol) were added to give compound **5 e** (55.01%): mp: 145–146 °C; IR (KBr): $\tilde{\nu}$ = 3063, 2861, 1568–1497, 1607, 1526, 1476, 1349, 1310, 1166, 1114–1088, 852–668 cm^{-1}; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 4.30 (s, 2H), 7.06 (dd, J = 8.8 Hz, J = 2.0 Hz, 1H), 7.28–7.40 (m, 5H), 7.40 (d, J = 1.6 Hz, 1H), 7.58 (d, J = 8.8 Hz, 1H), 7.96 (d, J = 9.2 Hz, 2H), 8.35 (d, J = 8.4 Hz, 2H), 10.66 ppm (s, 1H); MS (ESI): m/z (%): 410.22 (100) $[M+H]^+$ calcd for $C_{20}H_{15}N_3O_5S$: C 58.67, H 3.69, N 10.26, S 7.83, found: C 58.32, H 3.96, N 10.26, S 7.90.

2-(4-Chlorobenzyl)-5-(4-nitrophenylsulfonamido)benzoxazole (5 f): To a solution of 2-(4-chlorobenzyl)-5-aminobenzoxazole (**3 f**) (0.048 mmol) in CH_2Cl_2 (2 mL), pyridine (0.95 mmol) and 4-nitrobenzenesulfonyl chloride (0.52 mmol) were added to give the compound **5 f** (40.06%): mp: 179–181 °C; IR (KBr): $\tilde{\nu}$ = 3115, 3053, 2860–2791, 1685–1493, 1606, 1526, 1472, 1350, 1312, 1167, 1115–1059, 1085, 847–670 cm^{-1}; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 4.32 (s, 2H), 7.06 (dd, J = 8.4 Hz, J = 1.6 Hz, 1H), 7.38–7.43 (m, 5H), 7.58 (d, J = 9.2 Hz, 1H), 7.95 (d, J = 9.2 Hz, 2H), 8.35 (d, J = 8.8 Hz, 2H), 10.66 ppm (s, 1H); MS (ESI): m/z (%): 444.18 (100) $[M+H]^+$; 446.05 (40) $[M+H+2]^+$ calcd for $C_{20}H_{14}ClN_3O_5S$: C 54.12, H 3.18, N 9.47, S 7.22, found: C 54.07, H 3.31, N 9.43, S 7.20.

General procedure for the preparation of 2-(4-ethylphenyl)-5-(4-aminophenylsulfonamido)benzoxazole (6 c): 2-(4-Ethylphenyl)-5-(4-nitrophenylsulfonamido)benzoxazole (**5 c**) (0.5 mmol) in ethanol (50 mL) was reduced by hydrogenation using H_2 (40 psi) and Pd-C (40 mg, 10%) until uptake of H_2 ceased.[40] The catalyst was filtered

on a bed of Celite, washed with ethanol, and the filtrate was concentrated in vacuo. The crude product was purified by recrystallization with a mixture of ethyl acetate/*n*-hexane (1:4) to obtain **6c**. The crystals were dried in vacuo. The compound is new (60.35%): mp: 251–254 °C; IR (KBr): $\tilde{\nu}$ = 3407–3339, 3188, 2963, 1596–1499, 1578, 1462, 1310, 1158, 1144–1092, 831–682 cm^{-1}; ^1H NMR (400 MHz, [D_6]DMSO): δ = 1.22 (t, 3H), 2.70 (q, 2H), 5.96 (s, 2H), 6.52 (dd, J = 7.2, J = 1.6, 2H), 7.10 (dd, J = 8.8, J = 2.0, 1H), 7.38–7.45 (m, 5H), 7.62 (d, J = 9.2, 1H), 8.06 (d, J = 8.0, 2H), 9.93 ppm (s, 1H); MS (ESI): *m/z* (%): 394.43 (100) $[M+H]^+$ calcd for $C_{21}H_{19}N_3O_3S$: C 64.11, H 4.87, N 10.68, S 8.15, found: C 63.92, H 5.16, N 10.70, S 7.93.

Expression and purification of human GST P1-1

Recombinant hGST P1–1 was expressed in *Escherichia coli* strain XL-1 Blue at 37 °C and purified by using *S*-hexylglutathione–Sepharose 6B.[45] *E. coli* XL-1 cells containing pKXHP1 plasmid were grown overnight in 50 mL 2YT media (16 g tryptone, 10 g yeast extract, 5 g NaCl, and 100 mg L^{-1} ampicillin) and then transferred to 500 mL 2YT media and incubated at 37 °C in a shake incubator. Incubation continued until the absorbance of the culture at 600 nm was 0.2–0.4. Then 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the expression of hGST P1-1. The cells were incubated for 16 h and then centrifuged at 7000 rpm (1500 *g*) for 7 min. The pellets were kept at −80 °C for 30 min. The pellets were resuspended in lysis buffer (10 mM Tris·HCl, 1 mM EDTA, 0.2 mM dithiothreitol (DTT) pH 7.0 and protease inhibitor cocktail, 0.2 mg mL^{-1} lysozyme) and mixed gently on ice for 30 min and then disrupted by sonication (5×20 s pulses, amplitude 80%, pulse on: 20; off: 40). Phenylmethanesulfonyl fluoride (170 μM) was added, and the supernatant fraction was obtained by centrifugation at 15000 rpm (15000 *g*) at 5 °C for 1 h.

Epoxy-activated *S*-hexylglutathione–Sepharose 6B affinity matrix was used for purification of hGST P1-1. The supernatant fraction was applied to the matrix equilibrated with binding buffer and stirred gently for 40 min on ice. The matrix containing bound hGST P1-1 was washed with Buffer A (10 mM Tris·HCl, pH 7.8, 1 mM EDTA, 0.2 M NaCl, 0.2 mM DTT) to eliminate nonbound proteins. The matrix was packed on top of a Sephadex G-25 column equilibrated with Buffer A in the cold room at 4 °C. The enzyme was then eluted with Buffer B (10 mM Tris·HCl, pH 7.8, 1 mM EDTA, 0.2 M NaCl, 0.2 mM DTT, 5 mM *S*-hexylglutathione). The fractions containing GST activity were concentrated on ice and then dialyzed with Buffer A without NaCl. The purity of the enzyme was determined by SDS-PAGE, applying both optimal and excessive protein amounts for analysis to visualize possible impurities.

hGST P1-1 in vitro inhibition assay

hGST P1-1 in vitro inhibitory activity of the synthesized sulfonamido-containing benzoxazoles was measured on a Shimadzu UV-2501 PC spectrophotometer by measuring the initial rate of absorbance change at 340 nm with CDNB. Standard enzymatic assay conditions consisted of 0.1 M phosphate buffer (pH 6.5) containing 1 mM EDTA, 1 mM GSH, and 1 mM CDNB at 30 °C. The reaction system contained 5% ethanol (from the CDNB stock solution), but the solvent had a negligible inhibitory effect on enzyme activity. The enzymatic reaction was obtained by subtracting the nonenzymatic rate from the rate measured in the presence of enzyme.[41]

The most potent compounds identified with CDNB were also tested spectrophotometrically with PEITC as substrate in a standard assay.[46] The molar absorption coefficient used for CDNB was $\Delta\varepsilon_{340}$ = 9.6 $mM^{-1}cm^{-1}$ and for PEITC $\Delta\varepsilon_{274}$ = 8.89 $mM^{-1}cm^{-1}$. The IC_{50} value was determined as the inhibitor concentration that gives 50% inhibition of enzyme activity. For determining the inhibition type effected by the test compounds, varied concentrations of GSH and CDNB were used. The compounds tested as inhibitors were prepared freshly in 2 mM stock solutions in 96% ethanol because of their sensitivity to light.

Data analysis

All measurements were made in triplicate, and each point on the graphs was given with standard deviation of the mean value. IC_{50} values of the compounds were determined by regression analysis using GraphPad Prism software version 4.0. Among the all tested compounds, **5f** 2-(4-chlorobenzyl)-5-(4-nitrophenylsulfonamido)-benzoxazole was found as the most potent inhibitor for hGST P1–1, with an IC_{50} value of ~10 μM[41] (Table 1).

Computational methods

Molecular docking

Preparation of the enzyme: The crystal structure of hGST P1-1 complexed with ethacrynic acid was retrieved from the Protein Data Bank (PDB ID: 2GSS).[43] Accelrys Discovery Studio 3.5[47] software was used for preparation of protein and ligands. The target protein was taken, the ligand was extracted, hydrogen atoms were added, and their positions were optimized using the all-atom CHARMm force-field and the Adopted Basis set Newton Raphson (ABNR) method available in Discovery Studio 3.5 protocol until the RMSD gradient was <0.05 kcal mol^{-1} $Å^{-2}$. The minimized protein was defined as the receptor using the binding site module. The binding site was defined from the cavity-finding method, which was modified to accommodate all the important interacting residues in the active site of GST (H-site). The binding sphere for 2GSS (6.64, 3.67, 26.88, 9.312) was selected from the active site using the binding site tools.

Preparation of ligands: Novel synthesized benzoxazole derivatives, CDNB, and standard drug ethacrynic acid were sketched, all-atom CHARMm force-field parameterization was assigned and then minimized using the ABNR method as described above. Conformational searches of the ligands were carried out using a simulated annealing molecular dynamics (MD) approach. The ligands were heated at a temperature of 700 K and then annealed to 200 K.

Docking

The CDocker[48] method was carried out with Discovery Studio 3.5. The protein is held rigid while the ligands are allowed to be flexible during refinement. The docking parameters were as follows: Top Hits: 10; Random Conformations: 10; Random Conformations Dynamics Step: 20000; Grid Extension: 8.0; Random Dynamics Time Step: 0.002. The docking and scoring methodology was first validated by docking of the known inhibitor, ethacrynic acid (EA). The docked position of EA overlaps well with the crystal structure position, with an RMSD of 0.905 Å. Afterward, molecular docking studies were performed on the new synthesized compounds.

Analysis of results

Finally, all docked poses were scored by applying Analyze Ligand Poses sub-protocol, and binding energies were calculated by applying Calculate Binding Energy sub-protocol in Discovery Studio 3.5 by using in situ ligand minimization step (ABNR method) and using implicit solvent model (GBMV). The lowest binding energy was taken as the best-docked conformation of the compound for the macromolecule.

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Keywords: benzoxazoles · glutathione · glutathione-*S*-transferase · molecular modeling · sulfonamides

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