Molecular Modeling, Synthesis, and Preliminary Biological Evaluation of Glutathione-*S*-Transferase Inhibitors as Potential Therapeutic Agents

Antonio Procopio,*,† Stefano Alcaro,† Sante Cundari,§ Antonio De Nino,§ Francesco Ortuso,† Paolo Sacchetta,‖ Alfonso Pennelli,‖ and Giovanni Sindona§

Dipartimento di Scienze Farmaco-Biologiche, Università della Magna Graecia, Complesso Ninì Barbieri, 88021 Roccelletta di Borgia (Cz), Italy, Dipartimento di Chimica, Università della Calabria, Ponte Bucci, cubo 12C, 87036 Arcavacata di Rende (Cs), Italy, R&D Department, Sigma Tau i.f.r. SpA, via Pontina km 30.400, 00040 Pomezia (Rome), Italy, and Dipartimento di Scienze Biomediche, Università "G. d'Annunzio" Chieti Via dei Vestini, 65100 Chieti, Italy

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Changes in the GSH/GST system have been found to correlate with resistance to anticancer alkylating agents, presumably through accelerated detoxification of these drugs since some GSTs have been shown to catalyze the conjugation of GSH to specific antineoplastic agents. GSH-alkyl derivatives were designed by molecular modeling, synthesized, and tested as inhibitors of human GST-Pi.

Alkylating agents are a class of clinically useful anticancer drugs whose efficacy lies in altering DNA replication by causing single- or double-stranded DNA breaks and/or cross-linking.[1] Despite their considerable clinical success, significant problems remain that limit the utility of this modality for cancer treatment, notably the development of drug resistance.[1,2] Generally, resistance may be one of two types: intrinsic or acquired. The latter is conferred by cellular alterations that occur as a result of drug exposure, providing cells with selective survival advantages.

In the case of alkylating agents, resistance has been attributed to multiple factors including impaired cellular uptake of drug and altered levels of γ-L-glutamyl-L-cysteinylglycine (glutathione, GSH) and glutathione-*S*-transferases (GSTs). Glutathione is the most abundant nonprotein thiol in the cell with concentrations ranging from 30 μM in plasma to 3.0 mM in the kidney proximal tubules and levels which may reach 10 mM in tumors of various organs.[3] The GSTs are a family of multifunctional isoenzymes that are widely distributed throughout mammalian systems. At least eight different types of human GST have been identified from isozyme families (Alpha, Mu, Pi, Sigma, Theta, Zeta, Omega, Kappa).

GSTs catalyze the conjugation of a wide variety of carcinogenic, mutagenic, toxic, and pharmacologically active electrophiles to the cellular nucleophile GSH, producing metabolites that are generally less toxic and more readily excreted.[5]

Alkylating agents are very often potent electrophiles, and many reports have characterized these drugs as substrates for GSTs. Changes in the GSH/GST system have been found to correlate with resistance to anticancer alkylating agents, presumably through accelerated detoxification of these drugs. Some GSTs have been shown to catalyze the conjugation of GSH to specific antineoplastic agents. (e.g. melphalan, chlorambucil, cyclophosphamide, BCNU, mechloretamine, etc.).[6]

Overexpression of GST isozymes has been reported in a number of different human malignancies, including cancers of the lung,[7] colon,[7b,8] kidney,[7b,9] ovary,[8b,9b] esophagus,[10] and stomach.[7a,10] This phenomenon can be considered as a typical adaptive cellular response to protect vital cellular nucleophiles from drug-induced damage,[11] leading to increased survival and enhanced resistance to chemotherapy. The link between drug response and GST expression suggests there is potential for the use of GST inhibitors in modulating the efficacy of electrophilic cancer drugs. Several glutathione analogues that target this system have been developed and have been used both experimentally and clinically in an attempt to improve efficacy (glutathione-S-transferase inhibitors and prodrugs, glyoxalase I inhibitors, and *S*-nitrosoglutathione).

Concomitant examination of the crystal structure of the GST isozymes and putative GSH binding, together with the synthesis of glutathione analogues, provided the basis for the development of intracellular competitors.[12] A broad class of both competitive and noncompetitive inhibitors of GST already exists (ethacrinic acid, BSO, GSH analogues, etc.)[12c,13]

The EA−GSH conjugate was found to be an enzyme inhibitor, affecting the GST α-catalyzed reaction between chlorambucil and GSH.[14] This supported the prediction that GST inhibitors interfere with GST-mediated conjugation of alkylating agents, modulating the efficacy of these drugs.[15]

Adang et al.[16] synthesized a series of glutathione analogues containing a sulfydryl group in which the C-terminal glycine was replaced by different amino acids. These compounds were then used as cosubstrates in reactions catalyzed by rat α and μ GSTs with 1-chloro-2,4-dinitrobenzene (CDNB) as electrophilic substrate. Also, the conjugation of aromatic compounds to GSH gives products which can function as cosubstrates for GST,[13a,17] and GST-π specific inhibitors were also proposed.[13,18] However, therapeutic limitations including toxicity, carcinogenicity, or unsuitability as a drug

* To whom correspondence should be addressed. Tel. +390961391131; Fax: +390961391270. E-mail: procopio@unicz.it.
† Università della Magna Graecia.
‡ Università della Calabria.
§ Sigma Tau i.f.r. SpA.
‖ Università "G. d'Annunzio" Chieti Via dei Vestini.

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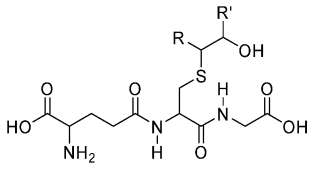


1: R=H, R'=H; **2**: R=Ph, R'=H;
3: R=H, R'=Ph; **4**: R=Ph, R'=O

Figure 1.

for human use have inspired the design and synthesis of new inhibitors, and the best results were obtained by way of GSH analogues.The technique of developing enzyme inhibitors based on substrates is widely used in medicinal chemistry via the principle of microscopic reversibility in the Michaelis−Menten scheme, which states that, in a system at equilibrium, any molecular process and the reverse of that process occur, on the average, at the same rate.[13a,e] Many inhibitors are substances that structurally resemble the enzyme substrate, but either do not react or react very slowly in comparison. A competitive inhibitor acts by reducing the concentration of free enzyme available for substrate binding. Since several studies have described alkyl and aryl epoxides conjugated to GSH in the presence of GST,[19] we designed our original series of analogues by using an empirically based method and functionalizing glutathione as *S*-alkyl derivatives. These can be considered GSH-conjugated substituted oxiranes (Figure 1). These molecules were modeled and synthesized, and their ability to inhibit human GST-π, elevated in many tumor types,[20] was evaluated in vitro.

Molecular Modeling. Molecular modeling studies were performed to investigate the interaction between GST and compounds **1**−**4** (Figure 1).

The identification of the enzyme model to be used in our simulation was conducted using the 45 models of human GST crystallographic structures deposited into the Protein Data Bank (PDB).[21] We focused our attention onto the GST-Pi isoform complexes with GSH or its analogues founding 17 structures. The comparison of the 17 PDB models confirmed a high degree of conservation in the 3D structures of residues involved in the catalytic site. The recognition of all ligands was almost identical, as were intermolecular contributions, hydrogen bonding, and electrostatic and hydrophobic contacts.

For our simulations, we decided to adopt the 6GSS model. [22] This choice was justified by structural comparisons among 6GSS, 8GSS, and 19GS that were the lower resolution GST-Pi GSH complexes. 8GSS was discarded because it showed the interaction of three GST monomers complexing GSH. 6GSS was preferred to 19GS since this last model reported not only GSH recognition but also other small organic molecules, such as the bromosulfalein, located close to the binding site interacting with both GSH and Phe8.

To analyze the differences among the ligands complexed of all models reported in Figure 1S (see Supporting Information), the root-mean-square deviation (RMSd) was computed considering only the atomic coordinates of the substructure defined by the GSH common scaffold (Figure 2 in Supporting Information).

The PDB analysis indicated a common enzyme interaction pathway for all ligands driven by the GSH common moiety (compound **5**). Discrepancies in the affinity were attributed to the different thiol substituents. To rationalize the effect of chemical modification, the computational work was carried out using two different approaches. The first step was dedicated to the evaluation of the GSH common skeleton enzyme recognition.

The interactions of each isolated substituent with the GST were studied by means of the program GRID v. 20 [23] using four different probes mimicking the nature of thiol substituent (**1**−**4**): C3 for the sp3 carbon atoms, C1= for the sp^2 carbon atoms (i.e. phenyl groups), O1 for the alcoholic oxygen atoms, and O for the sp^2 oxygen atoms. These calculations were performed with no ligand in the GST active site, using a resolution equal to 1 Å (directive NPLA = 1). GRID results were considered with two purposes: first to verify the overlap of the original PDB ligand (GSH, compound **5**) in its crystallographic positioning, and second to analyze areas, close to **5**, suggesting other productive interactions. As depicted in Figure 2, where C3 maps were not displayed due to the high similarity to those obtained with the C1= probe, the GRID calculation accuracy was confirmed, identifying the position of several GSH groups pertinent to each probe. Areas close to compound **5**, where new moieties could be located, particularly suggested modification of the thiol moiety. Actually, the region around this group was indicated both by hydrophilic (N1 and O1) and hydrophobic (C3 and C1=) probes. Moreover, this region proved sterically able to locate new substituents. The second step considered the recognition of compounds **1**−**5** within the GST catalytic site using the 6GSS original PDB dimer as receptor. Docking studies started building our compounds into the 6GSS chain A active site by introducing the appropriate chemical moieties onto the thiol group of **5** located at its crystallographic positioning. For all compounds, both carboxylic groups of the GSH common skeleton were considered to be in the ionized form. In compound **4**, taking into account physiological pH and the presence of two ionized groups, the carboxylic terminus of the substituent chain was considered to be in its neutral form. For compounds **2**, **3** and **4**, both the *R* and *S* configurations of the thiol substituent were modeled and reported, with **r** and **s** suffixes, respectively (i.e. **2r**, **2s**, **3r**, **3s**, **4r**, and **4s**). **5** was additionally included for validation purposes. All complexes were energy minimized using 2000 steps of the Polak-Ribiere Conjugated Gradient algorithm with the AMBER* force field [24] and the implicit model of solvation GB/SA water [25] as implemented in MacroModel v. 7.2. [26] During these optimization procedures, our molecules were allowed to fully relax their internal degree of freedom, but protein atom coordinates were kept constrained with a constant force equal to 100 kJ/mol·Å.

To study the interaction of compounds **1**−**5** with GST, 5000 conformations of the ligand, starting from the optimized one, were generated into the enzyme catalytic site for each complex, using the Monte Carlo method (applied to all thiol substituent rotatable bonds). The exclusion of the dihedral angles belonging to the GSH common skeleton from the Monte Carlo search was implemented after the RMSd analysis computed onto the PDB model, reported in Figure 2, that revealed a remarkable rigidity of this substructure.

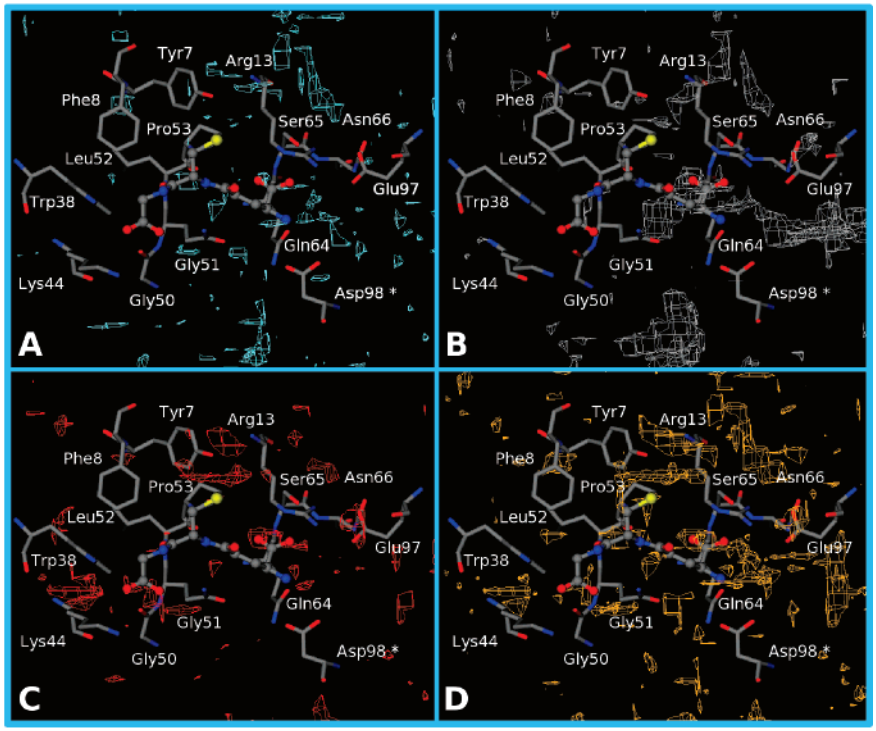


Figure 2. GRID maps computed onto 6GSS model (A: N1 at −6.5 kcal/mol; B: C1 = at −2.5 kcal/mol; C: O at −5.0 kcal/mol; D: O1 at −6.5 kcal/mol). For clarity only residues within 5Å from the GSH (reported in ball and stick notation) have been displayed. *From chain B.

Table 1. Docking Results[a,b]

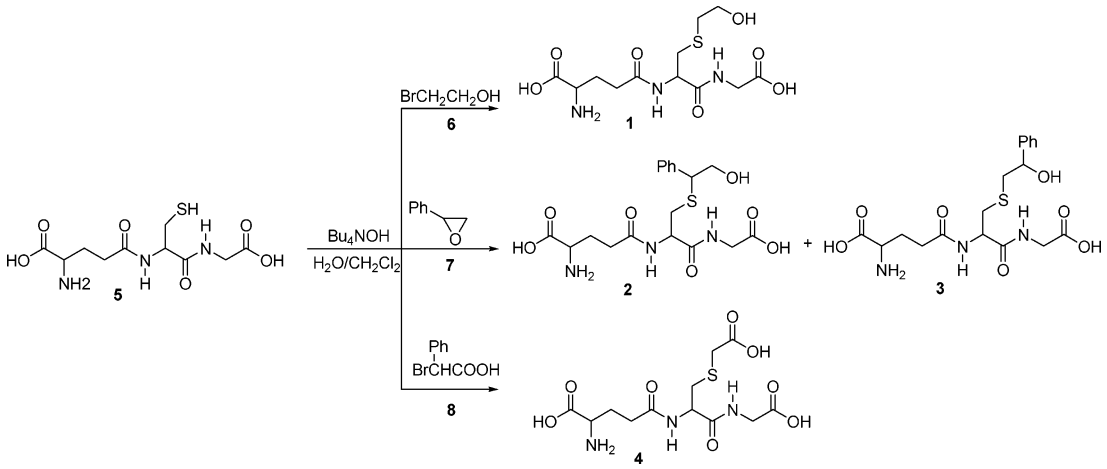
compound	RB	NCONF	AND	GM%	ΔG
1	7	1038	3.03	99.18	−63.32
2r	8	633	4.91	76.52	−69.23
2s	8	868	3.51	92.59	−68.28
2rs	- -	- -	- -	- -	−68.76
3r	8	656	4.66	99.74	−72.71
3s	8	1165	2.85	90.76	−72.62
3rs	- -	- -	- -	- -	−72.67
4rq	7	511	8.81	99.77	−69.71
4s	7	325	5.27	82.10	−68.08
4rs	- -	- -	- -	- -	−68.90
5	2	3	2.00	98.89	−57.52

[a] RB = number of rotatable bonds analyzed by Monte Carlo; NCONF = total number of configuration; AND = average number of duplicates; GM% = Boltzmann population of the global minimum energy structure; ΔG = estimated free binding energy in kcal/mol. [b] Data of compounds **rs** were computed as the average of the **r** and **s**.

All generated structures were submitted for energy multiminimization using the same force field and solvent environment previously described. In this case, to take into account the induced fit phenomena, the positional constant force constrain was applied only to the GST backbone atoms. A preliminary docking evaluation was carried out considering the total number of generated structures within 50 kJ/mol above the global minimum energy (NCONF), their distribution, and the average number of duplicate structures (AND). In our experience, an AND value equal or higher than 2 indicates a good exploration of the conformational space.[27] According to the MOLINE method,[28] final geometries were used for estimation of the free energy of binding ΔG (Table 1).

AND values revealed a sufficiently complete exploration of the conformational space for all compounds. Moreover, theoretical thermodynamic data revealed a good agreement with experimental K_i values (see Table 1), allowing a preliminary validation of our docking approach. Interestingly, ΔGs indicated, for chiral substituted compounds **2**−**4**, weak effects of their thiol side chain configurations on GST affinity. The docking structural analysis was extended to the comparison of the **5** Monte Carlo results versus the crystallographic model 6GSS by computing the RMSd between theoretical and crystallographic models.[29] Remarkably, we observed an almost complete reproduction of the experimental geometry as reported by the RMSd value equal to 0.147 Å. Such a result, taking into account the exhaustive exploration of compounds **1**−**5** GST recognition, reported by the AND data, and the good agreement between theoretical and experimental affinity data, indicated satisfactory quality of our docking approach. After the validation steps, with the aim to investigate the different binding modes and GST affinities of compound **1**−**5**, we analyzed the global minimum energy configuration generated by docking studies considering their Boltzmann population. The GSH common skeleton of **1**−**5** compounds revealed the same kind of interaction. Specifically, we observed in all cases at least two intramolecular hydrogen bonds between the terminal carboxylic moieties the their α-located amide groups. Compounds **1**, **2r**, **2s**, and **3r** showed a third intramolecular hydrogen bond between the hydroxyl group positioned onto the thiol substituent and the GSH glutamyl γ sp^2 oxygen atom. All ligands displayed three intermolecular hydrogen bonds among the GSH Gly carboxyl terminus and Trp38, Gln51, and Lys44. This last residue also gave a significant electrostatic contribution to the complex stabilization. Another intermolecular hydrogen bond was observed between the GSH Cys carbonyl group and Leu52 backbone amide. Such a residue, through its carbonyl group, was also involved

Scheme 1



in a second hydrogen bond with the GSH Cys amide. These important interactions were also reported between the GSH amine terminus and Gln64. A highly productive contribution was revealed for the γ glutamyl carboxyl group, which accepted one hydrogen bond from Ser65 and a second from Arg13—also involved from an electrostatic point of view—and two with the backbone and terminus amides of Asn66. Finally, the highly functionalized GSH common skeleton also showed hydrophobic contacts between its methylene γ glutamyl side chain and Pro53. The most populated geometric analysis followed with the interaction between thiol substituents and the GST binding site. Compound **1** revealed an intermolecular hydrogen bond and van der Waals contact, respectively, between the alcoholic and methylene groups and Arg13. **2r** and **2s**, due to a rotation of the thiol bond, demonstrated large binding mode similarity. In particular, both molecules showed the same interactions reported for **1** plus a $\pi-\pi$ contact between their aromatic ring and Tyr108. Compounds **3r** and **3s** reported a different recognition. **3r** showed binding mode interaction similar to that indicated for compounds **2r** and **2s** but limiting the phenyl ring exposure to the solvent. Actually, such a moiety was located in a hydrophobic pocket defined by Tyr103, Tyr108, Ile104, and the Arg100 side chain. Conversely **3s** showed its phenyl ring out of this pocket, in a position similar to that seen for **2r** and **2s**. The better GST interaction can be justified also in terms of the intermolecular hydrogen bond observed between the **3s** alcoholic group and Tyr108. **4r** and **4s** reported a binding mode similar to **3s**; in this case, the hydrogen bond involved the carboxylic group and, respectively, Arg13 and Tyr108. No specific interactions were reported for the free thiol group of compound **5**. In conclusion, the interaction analysis performed on the common skeleton of compounds **1**—**5** compared favorably to the energy affinity data with the thiol substituent structures. Such a moiety showed in all cases a similar binding GST recognition. Variations in the affinity can be addressed only by the different number and kind of interactions that the thiol substituent can establish with GST. It was not a surprise to observe that compound **5** reported the highest binding free energy, followed by compound **1**, which showed the smaller and less functionalized thiol substituent. **2r**, **2s**, **4r**, and **4s** reported a similar binding mode and similar interaction according to their ΔGs. Finally, the highest GST affinity of **3r** and **3s** can be attrinuted to additional hydrophobic effects.

Synthesis. With the aim to synthesize the modeled molecules **1**, **2**, **3**, and **4** (Figure 1), the relevant electrophilic precursors were selected, namely 2-bromoethanol (**6**) for molecule **1**, styrene oxide (**7**) for molecules **2** and **3**, and 2-bromophenylacetic acid (**8**) for **4**.

We have previously developed a mild method for the one-pot synthesis of *S*-conjugated cysteines by using a NaOEt/EtOH system,[30] but the poor solubility of GSH in most organic solvents prevented us from applying the reported method. We were forced to employ a binary system with a basic phase-transfer reaction using tetrabutylammonium hydroxide (TBAH) which is a strong base and possesses good solubility in water and in many organic solvents.[31] GSH (in the aqueous phase) reacts with TBAH to form a salt which subsequently transfers to organic phase for the intrinsic reaction. For all cases reported here, we used the commercially available TBAH 40% aqueous solution. Chloroform was the organic cosolvent selected, based on the reported higher activity of TBAH in this solvent.[32]

In a typical reaction, glutathione was dissolved in a 10% aqueous solution of tetrabutylammonium hydroxide and allowed to stir at r.t. with an equal volume of a 4 M solution of the electrophile in chloroform (Scheme 1). Reaction progress was monitored by RP-HPLC analysis.

It is already known that opening of styrene oxide **7** by means of a nucleophile will produce two different products.[4a] In fact, the reaction between GSH and styrene oxide in basic media gives two regioisomers, **2** and **3**, obtained in the ratio 48:52, with a combined overall yield of 68%. It was possible to separate these isomers by means semipreparative RP-HPLC as diasteromeric mixtures, but efforts to obtain pure diastereoisomers failed. In the case of 2-bromophenylacetic acid (**8**), the product **4** was obtained as diastereomeric mixture with a yield of 65% after semipreparative RP-HPLC separation. The separation of the pure diastereoisomeric form of compounds **2**—**4** does not seem a substantial problem since the ΔG's evaluation for chiral substituted compounds **2**—**4** indicated the configuration of the thiol side chains to have only weak effects on GST affinity (Table 2).

Table 2. Yield Obtained after RP-HPLC Separation of Compounds **1**−**4**

compound	yield %
1	75
2	31
3	34
4	65

Table 3. Enzymatic Kinetics in Vitro[a]

compound	IC_{50} (μM)	K_i (μM)	mechanism
1	280	187	mix
2	9.0	1.11	pure competitive
3	4.7	0.49	pure competitive
4	5.5	0.66	pure competitive

[a] Kinetic parameters: $K_M^{GSH} = 250$ μM; $K_M^{CDNB} = 1.4$ mM.

h-GSTP1-1 activity was assayed spectrophotometrically at 340 nm, according to procedure of Habig and Jakoby. Inhibition experiments were performed using 1 cm cuvettes with 1 mL (final volume) of 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, GSH (from 0.1 to 3 mM), 1 mM CDNB (1-chloro-2,4-dinitrobenzene), suitable amounts of enzyme, and in the presence of fixed inhibitor (compounds **1**−**4**) concentrations ranging from 0 to 1 mM.[33]

The assays were performed using the enzyme hGSTP1−1 >sp|P09211|GTP-human glutathione S-transferase P (EC 2.5.1.18) (GST class-pi) (GSTP1-1), *Homo sapiens* (human).

The results reported in Table 3 show that **2**, **3**, and **4** inhibit GST catalytic action through a competitive mechanism with low IC_{50} and K_i values, as compared to many other GST inhibitors proposed. Compound **1**, in contrast, seems to be the only poor GST inhibitor. Actually, such an observation was previously highlighted by molecular modeling simulation that indicated hydrophobic GRID maps in the area where the thiol-substituted chain was located by docking experiments. Therefore, the interaction between GST and compound **1**, due to its small hydrophilic side chain, lacks, with respect to **2**−**4**, a relevant hydrophobic contribution; however, this result may not reflect other GST classes.

Experimental Section

In a typical reaction, 500 mg (1.63 mmol) of glutathione in 7.0 mL of aqueous *n*-Bu_4NOH 10% and 7.0 mL of a 4 M chloroform solution of the electrophile were stirred at r.t. The progress of reaction was followed bu RP-HPLC analysis [Phenomenex Jupter C18, 250 × 4.6 mm, 10 μm, UV 272 nm, 1.0 mL/min, (H_2O (1‰ TFA/methanol 80/20]. The reaction was completed in almost 36 h. Acidification with trifluoroacetic acid (TFA) and evaporation under vacuum gave the product which was purified by semipreparative RP-HPLC chromatography [Phenomenex Jupter C18, 250 × 10 mm, 10 μm, UV 272 nm, 4.0 mL/min, (H_2O (1‰ TFA/methanol 80/20] (see Supporting Information).

Supporting Information Available: Code list of the 45 GST human isoform models found into PDB citied in Table 1 and Figure 2; Figure 2 reporting ligand structures and their RMSd in angstoms computed, with respect to the 6GSS, onto the GSH common skeleton in the conformation bound to the active site of each PDB models; Table listing yield, elemental analyses, ^1H NMR, and MS data of target compounds **1**−**4**, and sequence of hGSTP1 used in the biological assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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