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Overcoming the Drug Resistance in Breast Cancer Cells by Rational Design of Efficient Glutathione *S*-Transferase Inhibitors

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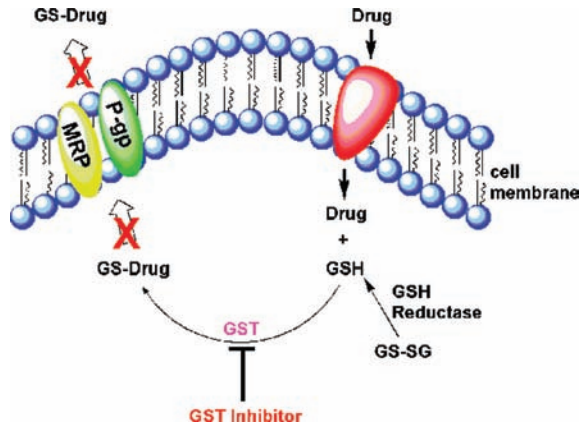
ABSTRACT



A new type of competitive human GST inhibitors has been developed via the bioisostere and structure activity profile strategies; we report their discovery, preparation, inhibitory activity, and synergetic effect in combination with chemotherapy drugs against breast cancer cells.

Drug resistance plays an important role in the success or failure of anticancer therapies. The development of drug resistance is a major disadvantage for the use of chemotherapeutic drugs (e.g., cisplatin, thiotepa, chlorambucil, doxorubicin) in cancer treatments.[1] Many approaches have been tried to elucidate the possible mechanisms involved in drug resistance. A key proposed mechanism relates to the participation of mutidrug-resistance-related proteins (MRP) and P-glycoprotein (P-gp), which effectively remove a wide range of endogenous electropliles and exogenous compounds (e.g., anticancer drugs) out of the cytosol to reduce the intracellular toxicity.[2] In addition, glutathione *S*-transferases

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(GSTs, E.C.2.5.1.18), a family of GSH-dependent enzymes, contribute importantly to drug resistance by catalyzing adduct formation between glutathione (GSH) and anticancer drugs (Figure 1).[3] GSTs in mammals were originally divided into

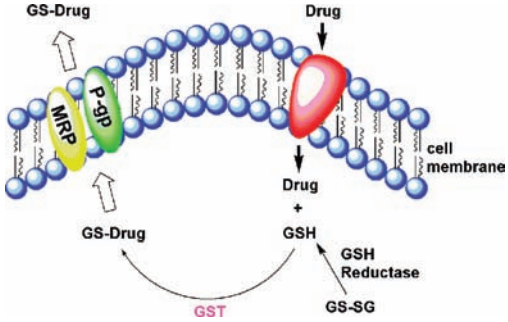


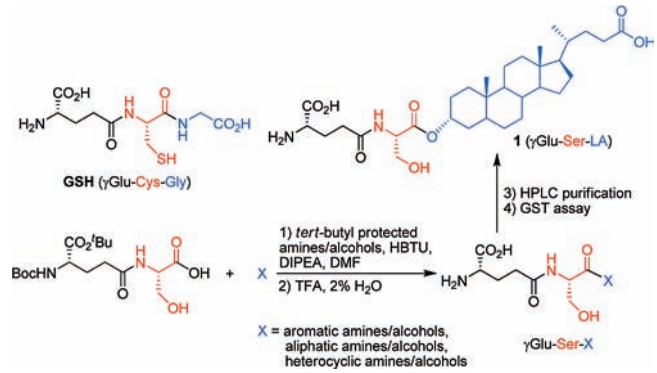
Figure 1. Possible GST-mediated activation of anticancer drug resistance in cancer cells.

eight different classes on the basis of their biofunctional properties and sequence identities.[4] In particular, it has been shown that different GST isoenzymes such as GST P1-1, GST A2, and GST M1 are overexpressed in many cancer cell lines including breast cancer.[5] Therefore, GST inhibition has been recognized as an important strategy for suppression of GST-mediated anticancer drug resistance and regulation of cell signaling processes.

To date, two types of inhibitors of GSTs, non-GSH compounds[6] and GSH analogues,[7] have been reported, and some of them have been demonstrated to enhance the cytostatic effect of numerous anticancer drugs. Distinctive strategies have been utilized to identify GST inhibitors, including a convenient NMR-based screening,[8] dynamic combinatorial chemistry,[9] peptidomimetic GSH-conjugates,[10] and Pt(IV) carboxylate framework.[3d] Although several successful inhibitor candidates of GSH analogues have been developed from these methods, they are typically restricted to GSH-ethacrynic acid derivatives[7c,e] or prepared through lengthy chemical procedures. Furthermore, some of the GSH analogues display poor permeability across the plasma membrane, and their clinical applications for inhibiting drug resistance are relatively limited. Herein we report the first rapid discovery of GST inhibitors via the bioisostere strategy and discover the capability of lithocholic acid as one of elements in GSH-type inhibitor design to enhance cell permeability. In addition, we demonstrated that the lead compound **3** shows synergetic effect with chemotherapy drugs against breast cancer cells, and our approaches should pave the way for the design of effective GST inhibitors.

In the initial design of GSH-based analogues, we chose γGlu-Ser-X as the framework (Scheme 1), which is capable

Scheme 1. Compound **1** Composed of Lithocholic Acid (LA) Moiety Is the Lead Selected by GST Assay



of searching bioactive component to target GST enzymes. With the oxygen in place for sulfur atom, the serine residue should be in a position to mimic the cysteine of GSH with the absence of ability as a real substrate. The synthesis of the γGlu-Ser-X series was started with condensation of Boc-Glu(O*t*-Bu)-Ser-OH and X (aromatic, aliphatic, and heterocyclic amines/alcohols), followed by the removal of Boc and *t*-Bu group. Individual components from the library were purified by HPLC and tested as possible inhibitors for GSTA2, GSTM1, and GSTP1-1, representative of enzymes of corresponding human α, μ, and π classes. Analyses of the screening results lead to a rapid identification of a new optimal binding component, lithocholic acid (LA). As is evident from Table 1, compound **1** (γGlu-Ser-LA) displayed low inhibition ability

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Table 1. Inhibitory Constants of Glutathione Mimic with LA Moiety against Different GSTs[a]

compound	inhibitory activity, IC_{50} (μM)		
	GSTA2	GSTM1	GSTP1-1
GSH	440.0 ± 32.0[b]	120.0 ± 20.0[c]	125.0 ± 6.0[d]
1	>100 (22)[e]	15.9 ± 0.6	>100 (0)[e]
2	10.4 ± 1.1	3.8 ± 0.6	>100 (0)[e]
3	3.6 ± 0.3 (0.6 ± 0.1)[f]	16.3 ± 2.1 (4.6 ± 1.6)[f]	1.4 ± 0.2
4	>100 (9)[e]	11.6 ± 1.0	>100 (0)[e]
5	>300 (5)[g]	6.1 ± 0.3	>300 (0)[g]
6	188.5 ± 14.8	37.2 ± 7.4	
7	>200 (27)[h]	87.7 ± 2.7	<200 (52)[h]
8	>200 (0)[h]	<200 (55)[h]	>200 (20)[h]
9	>50 (25)[i]	>50 (46)[i]	>200 (0)[h]

[a] Inhibitor concentration at which half-maximal enzyme activity is obtained (IC_{50}, mean ± SEM, $n = 3$). [b] Value (K_m) is from ref 11. [c] Value (K_m) is from ref 12. [d] Value (K_m) is from ref 13. [e] The % inhibition, in parentheses, at 100 μM is expressed as the % inhibition of enzyme activity. [f] Values reported are K_i. [g] The % inhibition, in parentheses, at 300 μM is expressed as the % inhibition of enzyme activity. [h] The % inhibition, in parentheses, at 200 μM is expressed as the % inhibition of enzyme activity. [i] The % inhibition, in parentheses, at 50 μM is expressed as the % inhibition of enzyme activity.

against GSTA2, and no inhibition was detected against GSTP1-1 at concentrations as high as 100 μM. By contrast, this molecule is a highly selective inhibitor against GSTM1 with $IC_{50} = 15.9 \pm 0.6$ μM, suggesting that compound **1** might be an acceptable probe in the study of GSTM1-mediated drug-resistant malignancies. For comparison, Table 1 lists the substrate K_m values of GSH for different GST isoenzymes.

Because of the overexpression of different GSTs in tumor cells, discovery of the compounds inhibiting multiple GST isozymes is therefore a high priority. Since compound **1** represents an effective core structure, we used it as the lead compound for the development of structure activity profile of GST inhibitors. To enhance the potency, we explored the serine residue of compound **1** with alterations of a series of amino acids, i.e., phenylalanine, aspartic acid, alanine, and methionine (Figure 2). As a representative example, synthesis

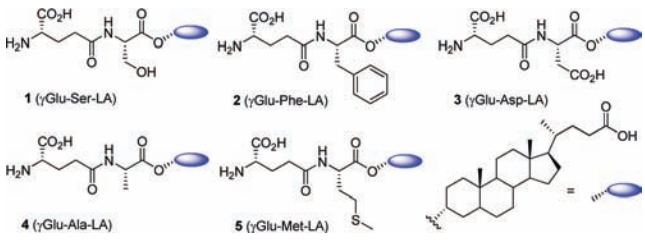
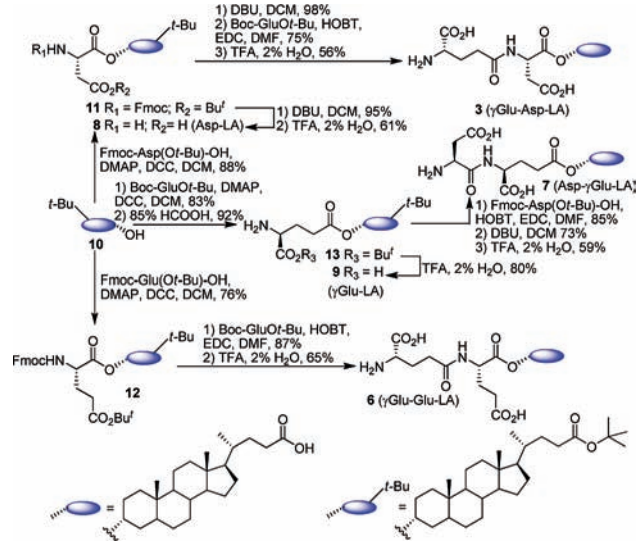


Figure 2. Lead optimization process for discovery of **3**.

of compound **3** was performed by Fmoc solution-phase peptide synthesis approach on **10** using Fmoc-Asp(O*t*-Bu)-OH and Boc-Glu(O*t*-Bu)-OH to give protected **3** followed by removal of the remaining Boc and *t*-Bu groups (Scheme 2, compounds **2**, **4**, and **5**; see Supporting Information).

Scheme 2. Synthesis of SAR Probes for Human GSTs



Among all these compounds, compound **3** was the most active compound with an IC_{50} of 3.6 and 1.4 μM toward GSTA2 and GSTP1-1, respectively. In the case of GSTM1 inhibition, compounds **2** and **5** exhibit a 3- to 4-fold potency increase over **3**, while **4** exhibits a slight 1.4-fold increase, suggesting that adding hydrophobicity is important for promoting affinity. Interestingly, the results reveal that compound **5** is at least 2.5-fold more potent than **1** to serve as a selective GSTM1 inhibitor. Moreover, compound **5** was 50-fold more selective and active against GSTM1 ($IC_{50} = 6.1$ μM) than against GSTA2 (5% inhibition in the presence of 300 μM of **5**) and GSTP1-1 (0% inhibition in the presence of 300 μM of **5**).

The inhibition characteristics of compound **3** were evaluated using steady-state kinetic analysis in the presence of different concentrations of GSH. As shown by Lineweaver−Burk plots (Figure 3), compound **3** inhibits GSTA2 and GSTM1 in a competitive manner with $K_i = 0.6 \pm 0.1$ and 4.6 ± 1.6 μM, respectively (Table 1). The type of inhibition we observed for compound **3** indicates that substrate (GSH) and inhibitor compete for the same active site (G-site) simultaneously. Together, the results serve to explain why LA-based analogues, γGlu-aa (amino acid)-LA, are a new alternative use for GSH in GST inhibitor design.

To further explore structure−activity relationships (SAR), four derivatives (**6**−**9**) of compound **3** (γGlu-Asp-LA) with systematic variations were prepared as depicted in Scheme

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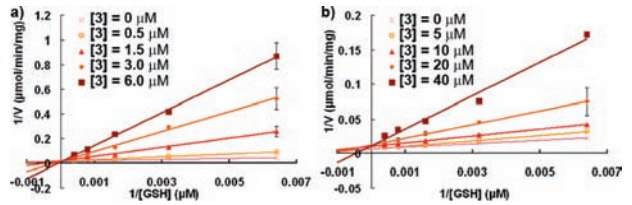

Figure 3. Lineweaver−Burk inhibitory analysis of (a) GSTA2 and (b) GSTM1 steady-state kinetics by compound **3**. (a) Reciprocal velocity versus reciprocal GSH concentration with inhibitor **3** (0, 0.5, 1.5, 3.0, 6.0 μM). CDNB concentration was held constant at 1 mM. (b) Reciprocal velocity versus reciprocal GSH concentration with inhibitor **3** (0, 5, 10, 20, 40 μM). CDNB concentration was held constant at 1 mM.

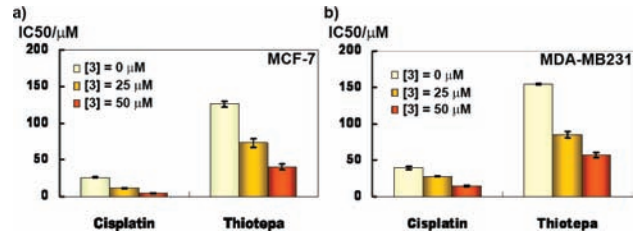

Figure 4. Synergetic effects of compound **3** on cell viability of human breast cancer cells MCF-7 and MDA-MB231.

2. Surprisingly, replacement of the aspartic acid with glutamic acid **6** (γGlu-Glu-LA), gave a 2- to 52-fold less potent inhibitor (Table 1) than **3** (γGlu-Asp-LA), indicating that adding a methylene group can diminish affinity. Moreover, modification and removal of the γGlu-postion of **3** dramatically decreased potency (**7**−**9**). This result is in line with the previous reports that the γglutamyl moiety of GSH is highly important in GSTs recognition.

Reversal of drug resistance by inhibiting GST isozymes has been extensively studied for therapeutic use. To further demonstrate this specific impact of the GST inhibitor on anticancer drug cytotoxicity, we investigated compound **3**-mediated regulation of cytostatic effect *in vitro*. Inhibition of cell viability by cisplatin and thiotepa in the presence of GST inhibitor **3** (0, 25, and 50 μM) against two breast cancer cells, MCF-7 and MDA-MB-231, was studied using MTT assays. A dose-dependent inhibitory effect on cell viability was observed, and respective IC_{50} values of cisplatin or thiotepa were determined after 48 h (Figure 4, see Supporting InformationTable S1). The maximal enhancement of cisplatin-induced inhibition of cell viability was observed at 50 μM compound **3**, up to 640% (i.e., decrease in IC_{50} value by 6.4-fold) against MCF-7 and up to 270% (i.e., decrease in IC_{50} value by 2.7-fold) against MDA-MB-231. Next, viability inhibition of thiotepa was enhanced by compound **3** (25 and 50 μM), up to 170−320% against MCF-7 and up to 180−270% against MDA-MB-231. Thus, the MCF-7 cell line was favorably resistant to cisplatin and, to lesser extent, to thiotepa, when compared to MDA-MB-231 cell line. For each cell line, compound **3** was found to have negligible effect on proliferative inhibition in the range 0−50 μM. These observations strongly suggest that compound **3** enhances the antitumor efficacy of cisplatin/thiotepa in breast cancer cells by blocking the pathway of GST-mediated drug resistance rather than involving a direct antiproliferative/cytotoxic effect.

We utilized two complementary strategies, bioisostere design and structure−activity profile, to identify new and effective GST inhibitors with acceptable cell permeability. More importantly, the lead compound **3** shows synergetic effect with chemotherapy drugs against two breast cancer cell lines through the inactivation of GST isozymes. Extension of this approach is designed to explore high-affinity GST inhibitors, which is of great value in pharmacological and clinical applications.

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Supporting Information Available: Human GST assay, IC_{50} values, general experimental procedures, and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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