In silico design of 2,2'-dihydroxybenzophenones and xanthone analogues to inhibit human glutathione transferase's (hGSTs) involvement in Multiple Drug Resistance.

Perperopoulou Fereniki[1], Zoi Ourania[1], Thireou Trias[2], Pouliou Fotini[1], Tsoungas Petros[3], Rinotas Vangelis[4], Eliopoulos Elias[2], Douni Eleni[2,4], Labrou Nikolaos[1], Clonis Yannis[1]

Introduction

Glutathione S-transferases catalyze the conjugation of glutathione (GSH) to a variety of hydrophobic substrates, rendering them hydrophilic and facilitating their metabolic processing and secretion from the cell. GSTs are involved in major detoxification mechanisms of the cell from several xenobiotics and drugs. On the other hand, on the basis of the same detoxification mechanisms, cancer cells may acquire resistance by overexpressing GST activities, thus hampering the effectiveness of certain chemotherapeutic drugs and leading to chemotherapeutic resistant tumor cells. Several synthetic drugs and prodrugs exhibiting inhibition potency against GSTs have been proposed as strategies to overcoming multiple drug resistance (MDR) attributed to GST overexpression. In the pursuit of identifying new lead compounds as inhibitors against hGSTs involved in MDR we have used Structure Based Ligand Design techniques to generate *in silico* xanthone and benzophenone derivatives and performed extensive molecular docking and binding evaluation on the structure of hGSTA1-1.

Molecular Modeling and Docking

The structure of hGSTA1-1 in complex with ethacrynic acid and its glutathione conjugate (PDB code 1GSE) was prepared with the Protein Preparation Wizard [1] in **Maestro** (Schrodinger, LLC, New York, NY). A grid including the tripeptide substrate glutathione was set up centered on ethacrynic acid. Ligands were docked flexibly using **Glide** SP [2, 3]. In order to study ligand binding in the presence of CDNB, another grid was set up including both the tripeptide substrate glutathione and CDNB, and in silico molecular docking was repeated. Forty different global molecular properties have been predicted for the compounds using **QikProp** (Schrodinger, LLC, New York, NY). Structural similarity of the compounds was studied using OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com. **OMEGA** 2.5.1.4 [4, 5] was used to generate molecular conformations, while **ROCS** 3.2.0.4 [6] and **EON** 2.2.0.5 identified shape and electrostatic similarity, calculating the TanimotoCombo and EON_ET_combo measures respectively.

Experimental Methods

Expression and purification of hGSTA1-1 from recombinant E. coli cells. Enzyme expression was induced from E. coli BL21 (DE3) cells harboring the plasmid pOXO4-GSTA1 by addition of IPTG. The cells were disrupted by sonication and the intracellular enzyme was purified on an affinity chromatography column bearing immobilized glutathione.

Screening of the benzophenone, N-carbonyl and xanthone analogues for hGSTA1-1 inhibition. Screening assays were implemented by mixing potassium phosphate buffer (100 mM, pH 6.5), 0.75 or 2.5 μmol GSH (in water), 25 or 100 nmol test compound (in DMSO) and enzyme. The mixture was incubated at 25°C for 1 min prior to adding 0.3 or 1 μmol CDNB (in ethanol). The observed rate was used to calculate the remaining activity (%), taking as 100% initial activity value the rate observed after replacing the test compound by an equal volume of DMSO.

Kinetic analysis using CDNB and GSH as a variable substrate, in order to determine inhibition modality and kinetic constants. Kinetic inhibition studies against hGSTA1-1 were implemented using CNDB (37.5-980 μM; GSH constant at 2.5 mM) and GSH (100-2500 μM; CDNB constant at 1 mM) as variable substrates in assay conditions as above.

Xanthone Derivatives

18 xanthone derivatives were synthesized and tested as potential inhibitors against the human isoenzyme GSTA1-1.

Derivatives 7, 8, and 17 are by far the most potent inhibitors for hGSTA1-1 (Table 1). In silico modeling analysis indicated that derivatives **7** and **8** place the xanthone moiety in the pocket in parallel with the benzene rings of Phe220, Phe222, and Tyr9, while the bromine is located 3.5 Å (**7**) and 5.1 Å (**8**) away from the sulfhydryl group of GSH and at an even longer distance (4.8 Å for **7** and 6.5 Å for **8**) from the catalytically important Tyr9 (Figure 1). Derivative **7** competes with **CDNB** for the same binding site with a kinetically determined inhibition constant $K_i(7) = 0.76 \pm 0.18$ μM. Molecular modeling analysis indicated that **17** can be accommodated in the substrate site with the aldehyde group positioned in a polar extension pocket formed by the carbonyl oxygens of residues Pro206 and Pro207, so as to form an H-bond interaction (3.2 Å) with the backbone carbonyl group of Pro206 (Figure 1). Derivative **17** competes with **CDNB** for the same binding site with a kinetically determined inhibition constant $K_i(17) = 1.69 \pm 0.08$ μM.

Biological assays with the derivatives 7, 8, and 17 have been implemented using cell lysate derived from human colon adenocarcinoma cells (Caco-2 cell line) and intact Caco-2 cells.

With regard to derivatives **7** and **8**:

- Their enzyme inhibiting ability has been dramatically decreased, indicating low selectivity of **7** and **8** for GST.
- They functioned as cytotoxic agents with intact Caco-2 cells.

With regard to derivative **17** its inhibitory ability was similar to that with purified enzyme, suggesting some degree of selectivity exhibited by **17** and it has shown a substantially lower cytotoxic effect with intact Caco-2 cells compared with derivative **7**.

In conclusion, derivatives **7**, **8**, and **17** have shown a high inhibitory potency toward hGSTA1-1, of which derivative **17** was the only one to readily inhibit the enzyme in colon cancer cell lysate. Furthermore, all three derivatives were cytotoxic to Caco-2 cells, with 17 being the least cytotoxic. Thus, the xanthone scaffold may be regarded as a pharmacophore for hGSTA1-1 and the three derivatives, especially **17**, as potent precursors for the synthesis of new inhibitors and conjugate prodrugs for human GSTs.

Derivative Code	Compound Structure	Molecular formula	Molecular weight	Inhibition against hGSTA1-1[a] (%)	QPlogPo/w[b]	IC_{50} With Purified hGSTA1-1 (μM)	IC_{50} With Caco-2 Cell Lysate (μM)	LC_{50} With Caco-2 Intact Cells (μM)	TanimotoCombo[d]	EON_ET_combo[d]
Entry **1**		$C_{13}H_8O_2$	195.98	0	2.747	-	-	>400	1.756	1.257
Entry **8**		$C_{15}H_{10}Br_2O_2$	381.95	85.5	4.124	5.30 ± 0.30	ND	>20[c]	1.533	1.438
Entry **17**		$C_{14}H_8O_3$	224.11	86.7	1.386	8.56 ± 0.14	10.54 ± 2.41	151.3 ± 16.3	2	2
Entry **7**		$C_{15}H_{11}BrO_2$	303.04	92.6	3.607	1.59 ± 0.25	ND	50.2 ± 0.8	1.635	1.25

Table 1. Xanthone Derivatives 7, 8, and 17 as Human GSTA1-1 Inhibitors (IC_{50} Values) and Cytotoxic Agents for Caco-2 Cells (LC_{50} Values).

Caco-2, human colon adenocarcinoma cell line; ND, not determined due to low enzyme inhibition at a derivative solubility limit in cell lysate (~10% inhibition at 150 μM); -, no enzyme inhibition at 100 μM xanthone. [a]Mean value of three enzyme activity assays (0.1 mM analogue; error ≤ 3%). [b]Predicted octanol/water partition coefficient. [c]For the cell viability assays, the final percentage of DMSO in cultures was a limiting factor. For compound 8, the maximum concentration tested had to be restricted to 20 μM with 2% DMSO in culture. [d]query (reference) molecule: Entry **17**

Figure 1.

Low energy conformations of xanthone derivatives as predicted by in silico molecular docking.

(a) Xanthone derivatives **7** and **8** are shown in light brown and beige, respectively, whereas the cosubstrate glutathione (GSH) is depicted in magenta. The bromine atom of derivative **7** interacts with the sulfhydryl group of GSH (3.5 Å). (b) Xanthone derivative **17** occupies part of the primary binding site of the enzyme. The OH group (in red) of derivative 17 forms an H-bond interaction with the backbone carbonyl group of Pro206 (3.2 Å).

Benzophenones

11 2,2'-dihydroxybenzophenones and N-carbonyl analogues were synthesized and tested as potential inhibitors against the human isoenzyme GSTA1-1.

On the basis of the screening experiments, compounds 6, 8, 14 and 16 were studied further by enzyme inhibition kinetics (Table 2).

When using CDNB as a variable substrate, compounds **6** and **14** displayed purely competitive inhibition kinetics ($K_i(6) = 1.47 \pm 0.15$ μM and $K_i(14) = 0.38 \pm 0.05$ μM), competing with CDNB for the same binding site of the enzyme.

In silico molecular docking analysis predicts that both inhibitors **6** and **14** (Figure 2), clash with CDNB if attempting to accommodate at the catalytic site of hGSTA1-1 where CDNB binds. With GSH as a variable substrate, both compounds **6** and **14** showed mixed inhibition kinetics, suggesting that inhibitors **6** and **14** may interact at a site other than the GSH-binding site of hGSTA1-1, that site being partly the catalytic CDNB-binding site.

With CDNB as a variable substrate, compound **8** has shown mixed inhibition kinetics with $K_i(8) = 0.36 \pm 0.11$ μM.

The predicted complexes enzyme-8 and enzyme-CDNB-8 are unreactive ones. However, at [8] > 0.5 μM a second molecule of **8** binds hGSTA1-1, hence intensifying the inhibitory effect.

The in silico models predicted close proximity and interaction between the substrate CDNB and **8** (two H-bonds, 2.56 and 2.76 Å) when both bound in the enzyme (Figure 3), suggesting interference of **8** with the enzyme's catalytic function involving CDNB.

With CDNB as a variable substrate, inhibitor **16** has shown mixed inhibition kinetics with Ki(16) = 1.75 ± 0.25 μM.

16 binds at the free enzyme and the enzyme-CDNB complex, with a GSH molecule present on the complexes.

In silico molecular docking shows CDNB at the catalytic primary site and **16** at a secondary binding site (Figure 3) in hGSTA1-1, producing a reactive quadruple complex, enzyme-GSH-CDNB-16.

Compound number	Structure	Molecular formula	Molecular weight	Inhibition potency against hGSTA1-1[a] (%)	QPlogPo/w[b]	Modality of inhibition[c]	IC_{50} against hGSTA1-1 (μM)	LC_{50} against Caco2 cells (μM)	TanimotoCombo[d]	EON_ET_combo[d]
5		$C_{13}H_{10}O_3$	214	0	2.982	-	-	> 400	0.917	0.852
11		$C_{13}H_{11}NO_3$	229	40.5	1.341	-	-	315 ± 1.4	1.113	0.87
6		$C_{18}H_{14}O_3$	290	86,1	4.504	Competitive, linear	1,77 ± 0.10	31.4 ± 0.4	1.046	1.045
14		$C_{20}H_{16}N_2O_3$	332	87,4	3.855	Competitive, linear	0.33 ± 0.05	87 ± 1.9	1.842	1.727
8		$C_{13}H_9BrO_3$	293	87,7	3.396	Mixed, linear	0,24 ± 0.04	120 ± 1.9	1.123	0.822
16		$C_{15}H_{13}BrN_2O_3$	349	96,1	3.039	Mixed, hyperbolic	0.18 ± 0.02	> 400	1.545	1.349

Table 2. Behavior of benzophenones selected from screening experiments against hGSTA1-1 activity (IC_{50}) and Caco2 cells (LC_{50}).

[a]Mean value of three enzyme assays (25 μM analogue; error ≤ 3%). [b]Predicted octanol/water partition coefficient. [c]Compounds **6**, **8**, **14** and **16** showed mixed inhibition modality with the co-substrate GSH. [d]query (reference) molecule: **14**

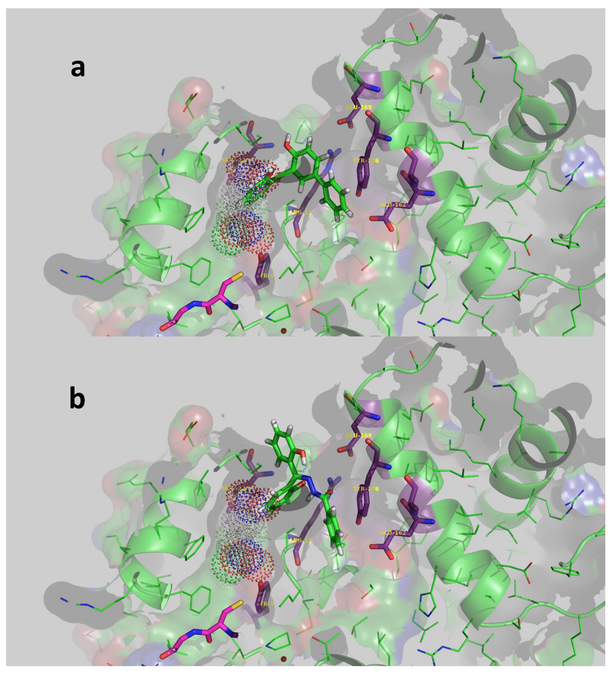

Figure 2.

Low energy conformations of substrates CDNB, GSH and inhibitor **8** (a),(b) and **16** (c), (d) at the most probable binding sites of hGSTA1-1 as predicted by in silico molecular docking in the presence or not of CDNB.

(a) Both inhibitors (green ligands) partly occupy the catalytic site and clash with CDNB (represented by dots) when it is bound at the same site. GSH is depicted in magenta, the S atom in yellow, N atoms in blue and O atoms in red.

Apparently, these locations are not close enough to allow interference of **16** to the catalytic function.

With GSH as a variable substrate, both compounds **8** and **16**, predictably, showed mixed inhibition kinetics

Studying the cytotoxic activity of the inhibitor lead molecules with human colon adenocarcinoma cell line (Caco2).

If low cytotoxicity was a desirable property for a lead compound to qualify for drug design, aiming to eventually produce an enzyme-targeting drug inhibitor, the N-acyl hydrazone analogue **16** appears as a good 'lead structure' since it combines a low cytotoxicity profile (LC50 > 400 μM) with high inhibitory potency against hGSTA1-1 (IC50 = 0.18 ± 0.02 μM).

However, if one were looking for a lead structure conferring both good cytotoxicity and enzyme inhibition, a balanced choice would probably be the other N-acyl hydrazone analogue, **14** (LC50 = 87 ± 1.9 μM and IC50 = 0.33 ± 0.05 μM).

In conclusion, the N-acyl hydrazone analogues, **14** and **16**, appear to be a better choice as leads, compared to the benzophenone analogues **6** and **8**, since the former showed better characteristics as Caco2 cytotoxic agents and hGSTA1-1 inhibitors.

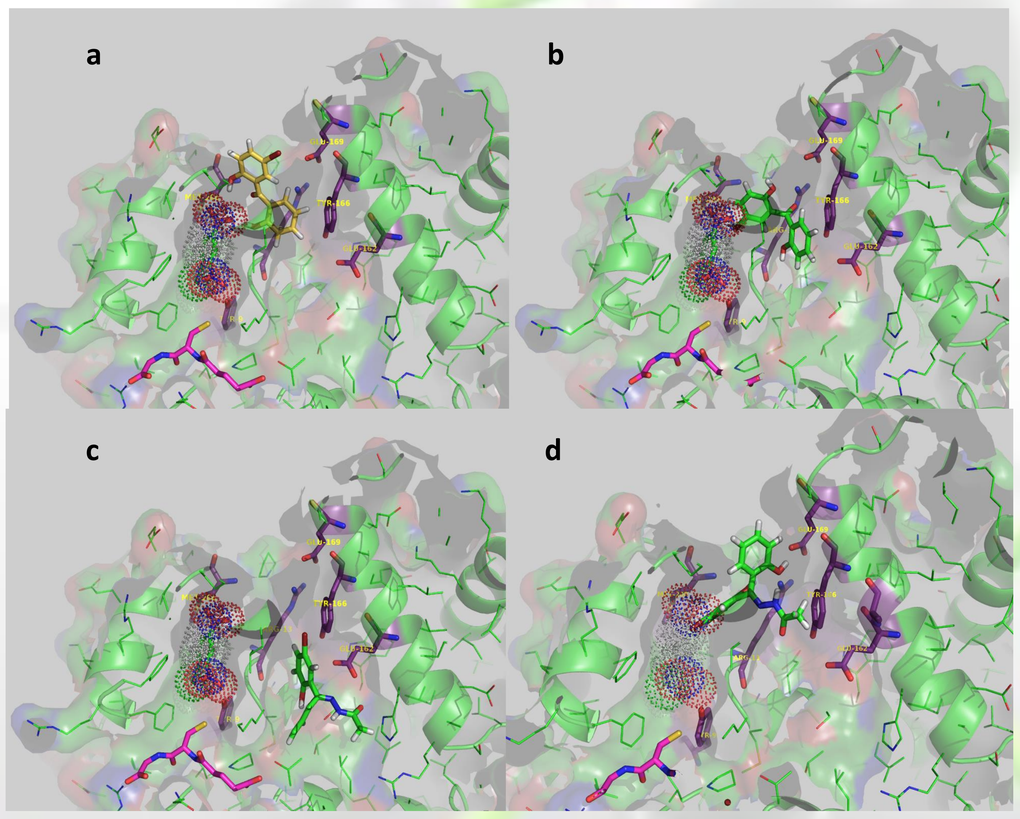

Figure 3.

Low energy conformations of substrates CDNB, GSH and inhibitor **8** (a),(b) and **16** (c), (d) at the most probable binding sites of hGSTA1-1 as predicted by in silico molecular docking in the presence or not of CDNB.

(a) In the presence of CDNB, inhibitor **8** is bound close to CDNB (represented by dots), developing H-bonds (2.56 Å and 2.76 Å). (c) In the presence of CDNB, Inhibitor **16** is bound far enough from CDNB (represented by dots) permitting catalytic function, though at a lower rate. The co-substrate GSH is depicted in magenta, the S atom in yellow, N atoms in blue and O atoms in red.

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[1] *Laboratory of Enzyme Technology, Department of Biotechnology, Agricultural University of Athens, Athens, Greece*
[2] *Laboratory of Genetics, Department of Biotechnology, Agricultural University of Athens, Athens, Greece*
[3] *Department of Biochemistry, Hellenic Pasteur Institute, Athens, Greece*
[4] *Division of Immunology, Biomedical Sciences Research Center "Alexander Fleming", Vari, Greece*