Designing of Non-hydrolyzing Derivatives for GlxII Inhibitors: Importance of Hydrophobic Moiety in S-site

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Cells contain diverse protective enzymes to get rid of toxic waste compounds produced as byproducts generated via abnormal cellular metabolisms. As such an example, a glyoxalase system plays a crucial role in removal of methylglycol (MG) produced in glycolysis and respiration pathways. It consists of glyoxalase I (GlxI) and glyoxalase II (GlxII).[1] GlxI catalyzes isomerization of hemithioacetals produced as a result of chemical reaction between glutathione (GSH) and MG. In an accompanying step, GlxII hydrolyzes thioester and converts it to non-toxic D-lactate and GSH.

Several attempts based on mechanistic studies have been made to develop a highly selective drug to cure diabetes and malaria disease[2] and various cancers.[3] In tumor cells undergoing higher glycolytic pathways, the level of MG increase and triggers cellular induction of glyoxalase for self-protection as a feedback control. Egyůd and Szent-Grỏrgyi has reported that the proliferating cells exhibits a higher level of GlxI than the quiescent cells.[4] In case of human leukemia HL60 cells, the activity of GlxI decreased and the activity of GlxII markedly increased relative to the level in control HL60.[5] On the other hand, in breast cancer as well as in melanoma cell lines, both GlxI and GlxII activities increased by accumulated MG.[6]

From the fact that even though MG is more cytotoxic to tumor cells, it can be easily degraded by cellular glyoxalase system, it has been envisioned that inhibitors of glyoxalase system could be developed to function as antitumor agents. Vince and Wadd had launched a pioneer investigation about development of inhibitors by introducing substituents into sulfur of GSH.[7] Several GlxI inhibitors, including *p*-bromobenzylglutathione, were developed and proved to be effective anticancer agents.[8]

So far focus has been mainly drawn to GlxI since it is mainly involved in a committed step and the contribution of GlxII becomes trivial since S-D-lactoylglutathione (SLD), the substrate for GlxII, can be easily hydrolyzed (Figure 1A). However, an expansion of our scope to GlxII has become more essential as cDNAs coding for GlxII had been identified in several species including yeasts. The structural studies of active sites have the putative binding mode of glutathione thioester derivatives.[9] GlxII contains conserved domains homologous with metallohydrolases throughout several species. Based on the data obtained with S-(N-hydroxy-N-bromophenylcarbamoyl)glutathione(HBPC-GSH), a slowly hydrolyzing substrate, interaction with GlxII is mainly provided by Gly and Cys components.[10] The hydroxyl group of Tyr-175 forms a hydrogen bonding with the amide nitrogen between Gly and Cys of the GSH moieties. It is still controversal whether there exists a hydrophobic pocket participating in GlxII-substrate interaction as demonstrated in GlxI.[11] Several groups have suggested that there would be such a pocket.[12] However, according to the structural data solved for human GlxII by X-ray crystalligraphy, the bromophenyl group of HBPC-GSH is rather exposed to solvent, not interacting with such a pocket. HBPC instead binds to metal binding sites with its phenyl ring stacking against the imidazole ring of His56.[13]

As an attempt to investigate an importance of such a hydrophobic moiety in binding of GlxII, we have attempted to design non-hydrolyzing derivatives of S-D-lactoylglutathione

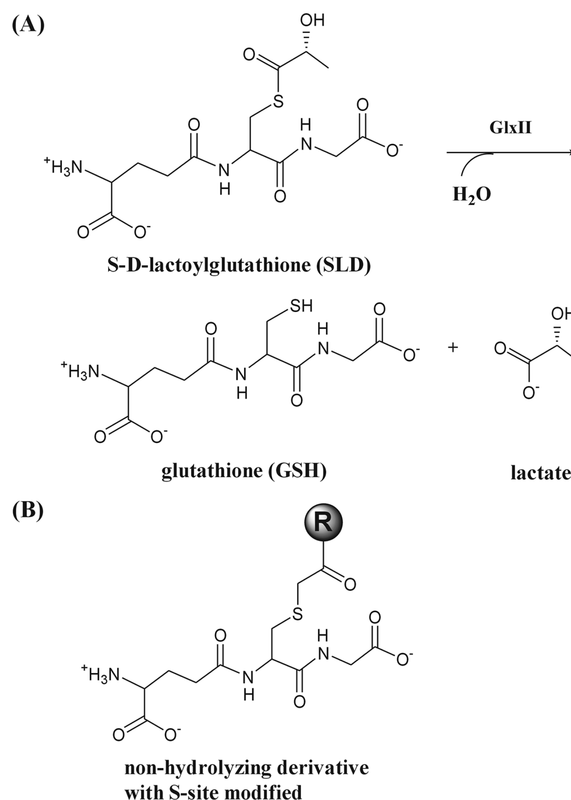

Figure 1. (A) Enzymatic pathway of glyoxalase II (GlxII) (B) Structure of a non-hydrolyzing derivative with a hydrophobic moiety (R).

Table 1. GlxII inhibition assays for compounds **1-6**

	Name of Compounds	R	Yield (%)	Inhibition IC_{50}, μM^a
1	S-(acetophenonyl) GSH	C_6H_5	37.0	239.07 (±10.32)
2	S-(3-methoxyphenacyl) GSH	$3-CH_3O-C_6H_4$	32.4	275.67 (±9.79)
3	S-(4-methoxyphenacyl) GSH	$4-CH_3O-C_6H_4$	39.3	271.93 (±6.21)
4	S-(4-bromophenacyl) GSH	$4-Br-C_6H_4$	40.8	216.27 (±10.83)
5	S-(methyl-2-naphthylketonyl) GSH	$C_{10}H_7$	34.2	141.80 (±1.95)
6	S-(4-phenylaceto-phenonyl) GSH	$4-C_6H_4-C_6H_4$	38.0	182.53 (±5.10)

[a]Values are means of at least three individual experiments.

(Figure 1B). Instead of possessing an easily hydolyzable thioester group, the S-site was modified with a variety of acetophenonyl groups. Various hydrophobic units (R) used in this study are listed in Table 1.

Each acetophenonyl derivative was synthesized by a simple one-step reaction as shown in Figure 2. GSH (Sigma) and each phenacyl bromide (Lancaster) were mixed at 1 : 1.2 ratio in 95% ethanol in water and stirred vigorously for 48 hrs. Reaction was monitored by silica gel TLC (*n*-Buthanol/aceticacid/water = 2 : 1 : 1, v/v) until the starting spot corresponding to GSH disappeared. Reaction mixtures were then filtered under vacuum and solid products were obtained after washing with acetone. The final pure products were fractionated by RP-HPLC (Shodex C-18 semiprep. column) with elution by a linear gradient of water (with 0.1% TFA) and CH_3CN (0.1% TFA) and detection at 214 nm and 254 nm. The final compounds were characterized by ^{1}H-NMR and the yields were between 32-41% as listed in Table 1.

In order to carry out *in vitro* kinetics study of GlxII inhibition, we have used 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) method with a minor modification.[14] 0.2 units/ml of GlxII (Sigma, Bovine liver) was pretreated with 0.03 mM DTNB in 0.1 mM phosphate buffer (pH 6.6) for 10 min and SLG was added as a substrate at a concentration range of 0.15-2.0 mM. The GlxII activity was monitored by UV spectroscopy at 412 nm every 10 sec over 20 min. The Data was evaluated by Grafit 4.0 (Erithacus Software Ltd.) to provide 0.3 mM of K_m and 0.0015 mM/min of V_{max}, which are in good agreement with reported values. For inhibition assays, synthesized compounds (**1-6**) were added at 0.0-0.8 mM concentrations in the presence of SLG fixed at 0.3 mM. Data interpretation by Grafit 4.0 gave us IC_{50} values for each compound to evaluate the concentration needed to inhibit 50% of GlxII activity. The individual IC_{50} values are shown in Table 1.

Among compounds we have developed, the one with

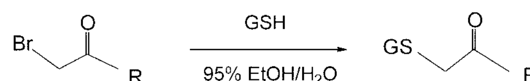

Figure 2. Synthetic scheme for preparation of S-site modified GSH derivatives.

naphthyl group (compound **5**) was more effective than the previously reported inhibitor, S-carbobenzoxy GSH (IC_{50} of 180 μM).[15] Compound **6** was also as effective as the known inhibitor. The hydrophobicity of either naphthyl or phenyl-acetophenonyl group contributes to better binding in S-site, probably by better stacking with the imidazole group of His56. Importance of the hydrophobicity in S-site modification was strongly addressed in our study, implying that the hydrophobic pocket may exist surrounding His56 at least to confer the more stable GlxII-substrate complex. The present study opens a fast and efficient way of preparation of effective inhibitors possessing comparable activities of previously reported inhibitors *via* a one-step process. Oxidation of these hydrophobic inhibitors, especially the one with naphthyl group into sulfoxide derivatives is now in progress with a hope to improve IC_{50} valule for inhibition.

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