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A metabolically stable tight-binding transition-state inhibitor of glyoxalase-I

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Abstract—The design, synthesis, and enzyme kinetics evaluation of a transition-state inhibitor of glyoxalase-I is described. The union of the hydroxamic acid zinc-chelator with a urea isostere for the glu–cys amide bond led to a glutathione analog which retained inhibitory potency toward glyoxalase-I while possessing resistance toward γ-glutamyltranspeptidase mediated breakdown. This compound is viewed as a potential lead for the development of second-generation glyoxalase-I inhibitors wherein, the problems pertaining to metabolism and selectivity are overcome.

The aldehyde of pyruvic acid, methylglyoxal, has been the subject of a large number of investigations due to its cytotoxicity.[1] This cytotoxic metabolite is transiently formed in a myriad of metabolic pathways, for example, lipid peroxidation, glycolysis, and DNA metabolism.[2] The removal of this ketoaldehyde from the body occurs through the glyoxalase pathway involving the cofactor glutathione. Two key enzymatic steps are involved in this process (Fig. 1): the isomerization of the hemithioacetal of methylglyoxal and glutathione (GSH) to *S*-D-lactoylglutathione (by glyoxalase-I) and its subsequent glyoxalase-II catalyzed transformation to D-lactate, that is then actively transported out of the cell and oxidized back to pyruvate which enters Kreb's cycle.[3] This cascade limits the use of α-ketoaldehydes as potential antitumor agents.

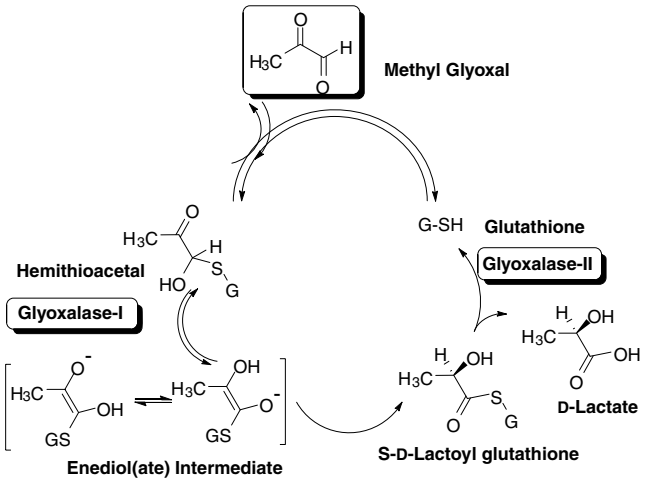

Figure 1. Glyoxalase pathway.

In 1969, our laboratory was the first to propose the obstruction of the glyoxalase pathway as a means to cause accumulation of methylglyoxal in cancer cells, eventually leading to their death.[4] We subsequently reported the competitive nature of *S*-alkylglutathione analogs toward glyoxalase-I (Glx-I)[5] and the discovery of *S*-*p*-bromobenzyl glutathione (PBBG, **1**) (Fig. 2) as a highly potent Glx-I inhibitor.[6] Because of their charged nature, these inhibitors lacked sufficient cell penetration to exhibit antitumor activity. In addition, loss of enzyme inhibition occurred through the rapid hydrolysis of the glu–cys amide bond of the glutathione scaffold by γ-glutamyltranspeptidase[7] (Fig. 2). Years later Lo and Thornalley used ester prodrugs of our PBBG (**1**), which were able to penetrate cell membranes and inhibit the growth of human leukemia 60 (HL-60) cells.[8]

One of the shortcomings of these *S*-alkylglutathiones, otherwise-promising Glx-I inhibitors, was their inability

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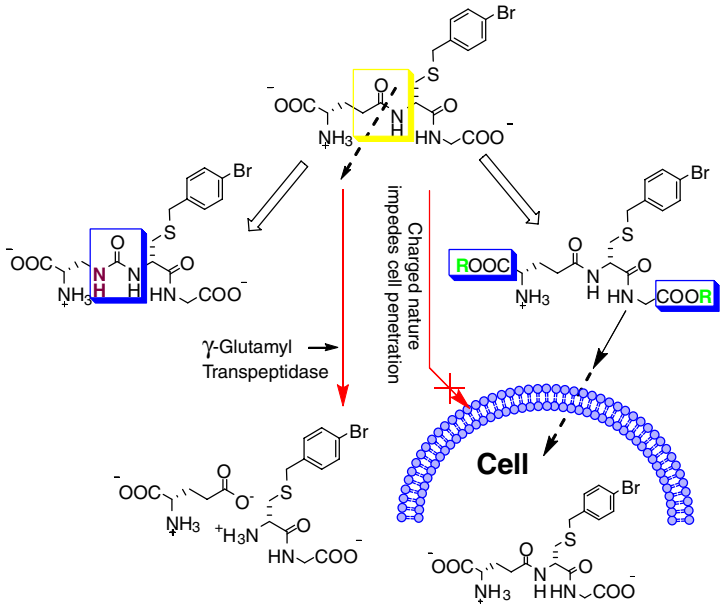


Figure 2. Problems with existing glutathione-based glyoxalase-I inhibitors.

to selectively inhibit tumor cells when compared to normal human cells. This suggested that simple competitive inhibitors of glyoxalase are perhaps inadequate tumor-selective agents. This shortcoming was addressed by Creighton and Murthy[9] through the design of hydroxamic acid-based transition state inhibitors (**3**, for example). The rationale behind this design was thus: the hydroxamic acid moiety acts as a close mimic of the enediol(ate) intermediate[10] of the Glx-I catalytic cycle (Fig. 1), thus conferring upon these molecules a high Glx-I inhibitory potency. Incorporation of a thioester linkage into these compounds caused them to resemble the glyoxalase-II (Glx-II) substrate (*S*-D-lactoylglutathione, Fig. 1). Since the Glx-II activity is abnormally low in certain types of cancer cells[11,12] compared to normal cells, Creighton and Murthy[9,10] suggested that the inactivation of compounds like **3** by Glx-II in tumor cells will be very low, thus rendering the desired tumor-selectivity from their reduced ability to hydrolyze these inhibitors. Although these inhibitors were in fact able to restrict the growth of solid tumors in mice when administered as their ester prodrugs, the problem of their breakdown by γ-glutamyltranspeptidase still remained.

Recently, we initiated a project to revisit the design and synthesis of these inhibitors with an aim to address various pharmacokinetic, metabolic, and synthetic problems that plague the development of this class of molecules into clinically useful antitumor agents.[13] In this report, we wish to describe the application of our urea-isostere strategy to prevent breakdown of the hydroxamic acid inhibitors by γ-glutamyltranspeptidase. Incorporation of the urea linkage and the hydroxamic acid transition-state mimic into **3** gave the tripeptide **4** (Fig. 3). Here, the urea-isostere was expected to provide metabolic stability to the glyoxalase-I

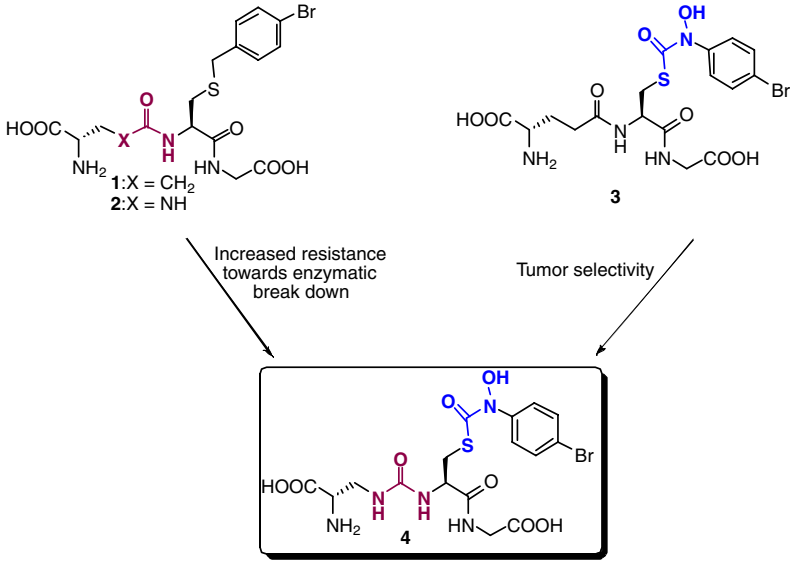


Figure 3. Rationale for the design of **4**.

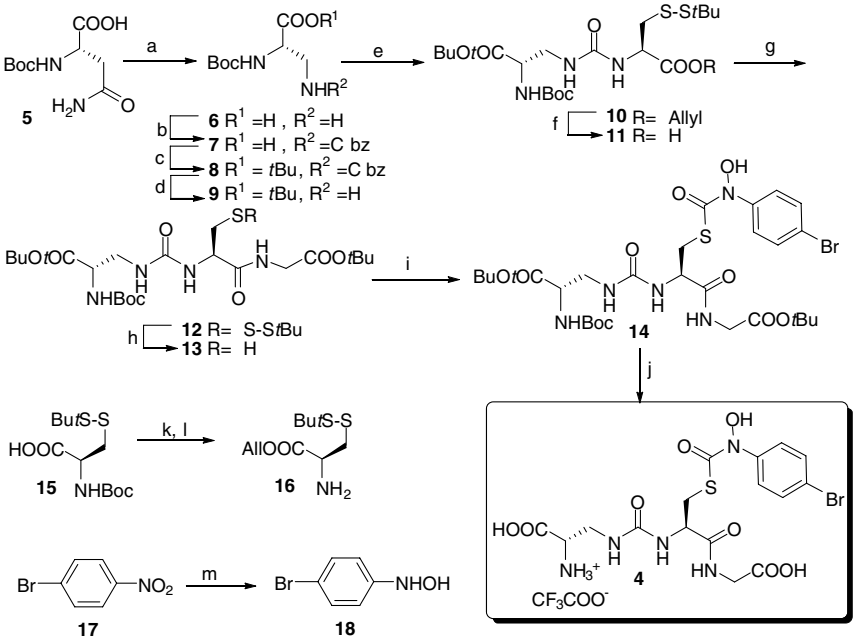
inhibitor **4** along with conservation of glyoxalase-I inhibitory activity.[13] A good body of experimental evidence exists for the presence of a significantly large hydrophobic pocket in the active site of Glx-I, with which the S-*p*-bromobenzyl substituent of PBBG (**1**) interacts.[5,14] This led us to conserve that particular pharmacophore. We then set forth to develop an efficient synthetic route to the tripeptide backbone and the thiocarbamate linkage of compound **4**.

The synthesis of **4** is shown in Scheme 1. The *N*-α-*t*-Boc-L-diaminopropanoic acid-*t*-butyl ester (**9**) fragment was synthesized in four steps from Boc-L-asparagine (**5**). The key step in this synthesis was a Hofmann rearrangement of the terminal carboxamide, which was performed according to the elegant protocol of Zhang[15] that employs iodosobenzene diacetate (PIDA) as the oxidant. The primary amine **6** thus formed was subjected to Cbz protection and the free carboxylic acid was esterified with *t*-BuOH and DCC to give **8**. Hydrogenolysis of the Cbz group afforded amine **9**. Formation of the urea linkage between **9** and L-cysteine(S–S-*t*-Bu) allyl ester hydrochloride (**16**) was accomplished through the use of carbonyl diimidazole (CDI).[16] The S–S-*tert*-butyl-protecting group for cysteine was chosen from the viewpoint of orthogonal deprotection. Pd^0 catalyzed deprotection of the allyl ester in urea dipeptide **10** gave the free acid **11** which was coupled with glycine *tert*-butyl ester hydrochloride in the presence of EDC, HOBt, and *N*-methylmorpholine (NMM), to give the tripeptide **12** in 78% yield. Reductive cleavage of the disulfide protection in **12** by tributylphosphine[17] provided the thiol **13**.

p-Bromophenylhydroxylamine (**18**, an unstable solid) was synthesized by a Rh-catalyzed transfer hydrogenation ('diimide reduction') of 4-bromo nitrobenzene.[18] Insertion of a carbonyl between thiol **13** and *p*-bromophenylhydroxy amine (**18**) was accomplished by sequentially treating **13** with phosgene (to form the thiochloroformate in situ) and then with **18** in dichloromethane. Under these conditions a 51% yield of the thiocarbamate **14** was obtained. Global deprotection of **14** with trifluoroacetic acid afforded the target molecule **4**.[19]

Compound **4** was examined for its ability to inhibit yeast glyoxalase-I. Initial rates were determined by following the increase in UV absorption at 240 nm (0.05 M phosphate buffer, pH 6.6) which corresponds to the isomerization process and formation of *S*-D-lactoyl glutathione (Fig. 1). Methylglyoxal, GSH, **4**, and buffer were added to the cell and allowed to equilibrate at 30 °C for 6 min (to allow formation of hemimercaptal) before addition of the enzyme. The concentrations of hemimercaptal were calculated using the dissociation constant 3.1×10^{-3} M as previously determined for this equilibrium reaction.[6] It has been shown previously that **3** is a tight-binding competitive inhibitor of glyoxalase-I ($K_i = 1.2 \pm 0.2$ μM).[9] Similarly the urea analog **4** was found to be a tight-binding competitive inhibitor with $K_i = 2.19 \pm 0.57$ μM. The K_m for the hemimercaptal of glutathione and methylglyoxal as determined by this assay was 0.35 mM, in agreement with the literature value.[13] Results of these experiments confirm that the urea isostere is well tolerated by glyoxalase-I.

We then tested the stability of the urea linkage in **4** toward γ-glutamyltranspeptidase. Compound **4** was incubated at 37 °C with equine kidney γ-glutamyltranspeptidase (γ-GT, Sigma) in 200 mM AMPD buffer at pH 8.4 in the



Scheme 1. Synthesis of **4**. Reagents and conditions: (a) PIDA, EtOAc/CH_3CN/H_2O (2:2:1), 67%; (b) CbzCl, KOH, K_2CO_3, THF/H_2O (3:1), 94%; (c) *t*-BuOH, DCC, DMAP, CH_2Cl_2, 84%; (d) H2, 10% Pd/C, CH_3OH, 94%; (e) **16**, CDI, NMM, CH_2Cl_2, 67%; (f) $(PPh_3)_4Pd$, morpholine, CH_2Cl_2, 80%; (g) HCl·GlyOtBu, EDC, HOBt, NMM, CH_2Cl_2, 78%; (h) Bu_3P, THF/H_2O, 94%; (i) **18**, $COCl_2$, DIPEA, CH_2Cl_2, 0 °C to rt, 51%; (j) 50% TFA in CH_2Cl_2, 89%; (k) allyl bromide, benzene, reflux, 86%; (l) 4*N* HCl/dioxane, quant.; (m) 5% Rh–C, $NH_2NH_2{\cdot}H_2O$, THF, 71%.

presence of 40 mM of gly–gly as an acceptor peptide for the released glutamic acid. The course of this incubation was monitored by thin-layer chromatography. Excellent separation of the metabolic products was obtained on silica gel TLC plates that were developed with *n*-butanol/acetic acid/water (12:5:3) and sprayed with a solution of fluorescamine in acetone. Glx-I inhibitors PBBG (**1**) and **3** were used as references in this assay. The results of this experiment indicate that PBBG (**1**) and **3** were significantly degraded after 15 min of incubation with γ-GT. TLC of incubation aliquots containing PBBG (**1**) and **3** showed a new higher R_f spot corresponding to complete degradation to the respective dipeptides (cysteinylglycine) and a new lower R_f spot corresponding to γ-glutamylglycylglycine, the other expected product from the transpeptidase reaction.[13] The identities of these spots were confirmed by comparison with authentic samples of these dipeptides. Compound **3** after 1 h showed a further degradation of cysteinylglycine dipeptide by release of the hydroxylamine portion (a higher R_f spot). In contrast to the above, compound **4** did not show any degradation even after incubation for 15 h, thus confirming its stability against γ-GT mediated cleavage.

In summary, we have developed an efficient synthetic route to the required urea-isostere containing hydroxamic acid-based inhibitor **4**. The target molecule, **4**, was found to retain the inhibitory potency of the corresponding carbo-analog **3** against glyoxalase-I while possessing resistance to cleavage by γ-glutamyl transpeptidase. The design of metabolically stable glyoxalase-I inhibitors based on **4** may be useful in potentiating the antitumor activity of α-ketoaldehydes. We are currently designing and synthesizing ester prodrugs of **4** that would potentially possess the ability to penetrate cell membranes and thus render **4** suitable for testing against tumor cells.

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19. Spectral data of compound **4**: ^{1}H NMR (300 MHz, CD_3OD-CF_3COOD) δ ppm 7.59 (d, $J = 9.0$, 2H, Ar), 7.49 (d, $J = 8.7$ Hz, 2H, Ar), 4.60 (q, $J = 4.8$, 8.4 Hz, 1H, α-CH:Cys), 4.48 (q, $J = 4.8$, 7.8 Hz, 1H, α-CH:Dap), 4.17 (m, 2H, CH_2:Gly), 3.68–3.62, 3.41–3.34 (2m, 2H, β-CH_2:Dap), 3.24–2.98 (m, 2H, β-CH_2:Cys); ^{13}C NMR (75 MHz, CD_3OD-CF_3COOD) δ 174.5, 172.6, 170.5, 159.2, 157.1 (C=O), 140.6 (C_{Ar}NOH), 131.5 (C_{Ar}: ortho to Br), 121.1 (C_{Ar}Br), 117.7 (C_{Ar}: ortho to NOH), 52.4 (α-C:Cys), 49.1 (α-*C*:Dap), 44.3 (β-C:Dap), 42.6 (CH_2:Gly), 31.5 (β-C:Cys); ESI-HRMS *m*/*z* 522.0276 $(M+H)^+$; $C_{16}H_{20}BrN_5O_8S + H^+$ requires 522.0294; reverse-phase HPLC was run on Varian Microsorb column (C18, 5 μ, 4.6 × 250 mm) using two solvent systems with 0.5 mL/min flow rate and detected at 254 nm. Solvent system 1: 0.04 M TEAB (triethylammonium bicarbonate) in water/70% acetonitrile in water = 1/1, $t_R = 5.74$ min, purity = 99.4%. Solvent system 2: 0.04 M TEAB in water/70% acetonitrile in water = 10–100% B linear, $t_R = 17.52$ min, purity = 98.07%.