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Bivalent Transition-State Analogue Inhibitors of Human Glyoxalase I

Zhe-Bin Zheng* and Donald J. Creighton*

Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, Maryland 21250

creighto@umbc.edu

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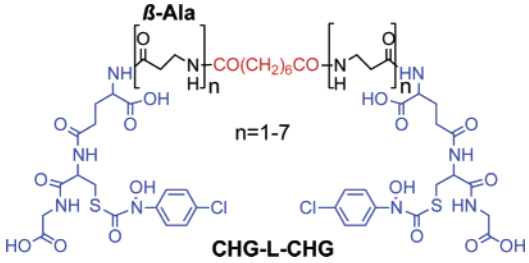
ABSTRACT



A new class of competitive inhibitors of homodimeric human glyoxalase I has been created by cross-linking two molecules of the transition-state analogue *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)glutathione (CHG) through their γ-glutamyl-NH_2 groups with poly-β-alanyl tethers of differing length: [CHG(β-ala)$_n$]$_2$ suberate diamide (n = 1–7). The strongest inhibitors of this antitumor target enzyme likely bind simultaneously to the active site on each subunit to give K_i values as small as 0.96 nM (n = 6).

Human glyoxalase I (hGlxI) is a homodimeric Zn^{2+} metalloisomerase with one active site per monomer.[1] The apparent physiological function of this ubiquitous enzyme is to catalyze the conversion of cytotoxic methylglyoxal (as the GSH thiohemiacetal) to *S*-*D*-lactoylglutathione[2,3] via a proton-transfer mechanism involving a Zn^{2+}-bound enediolate intermediate GSC(OH)=C(O^-)CH_3, where GS equals glutathionyl.[4] Methylglyoxal arises as a normal byproduct of carbohydrate metabolism[5] and is capable of covalently modifying proteins and polynucleic acids critical to cell viability.[6,7] Inhibitors of the enzyme have long been sought as possible anticancer agents, because of their potential ability to induce elevated concentrations of methylglyoxal in tumor cells[8] and the observation that rapidly dividing tumor cells are exceptionally sensitive to the cytotoxic effects of exogenous methylglyoxal, reviewed elsewhere.[9]

In support of this hypothesis, the dialkyl ester prodrugs of the hGlxI inhibitors *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)glutathione (CHG, K_i 46 nM)[10] and *S*-4-bromobenzylglutathione (K_i 170 nM)[11] inhibit the growth of murine and human tumors in laboratory mice at dosing levels of 80 and 100 mg/kg, respectively.

The high affinity of CHG for the enzyme appears to reflect the fact that it is a hydrophobic transition-state analogue of the GlxI reaction.[4,12] Compounds of this type generally bind in the mid-nanomolar concentration range. Nevertheless, more potent inhibitors with higher specificities are needed in order to decrease the dosing levels of inhibitor required

(1) Aronsson, A.-C.; Marmstål, E.; Mannervik, B. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 1235−1240.
(2) Creighton, D. J.; Pourmotabbed, T. In *Molecular Structure and Energetics: Principles of Enzyme Activity*; Liebman, J. F., Greenberg, A., Eds.; VCH Publishers: 1988; Vol. 9, pp 353−386.
(3) Vander Jagt, D. L. In *Coenzymes and Cofactors: Glutathione*; Dolphin, D., Poulson, P. R., Avramovic, O., Eds.; John Wiley and Sons: New York, 1989; Vol. 3, Part A, pp 597−641.
(4) Creighton, D. J.; Hamilton, D. S. *Arch. Biochem. Biophys.* **2001**, *387*, 1−10.
(5) Richard, J. P. *Biochemistry* **1991**, *30*, 4581−4585.
(6) White, J. S.; Rees, K. R. *Chem. Biol. Interact.* **1982**, *38*, 339−347.
(7) Papoulis, A.; Al-Abed, Y.; Bucala, R. *Biochemistry* **1995**, *34*, 648−655.
(8) Vince, R.; Daluge, S. *J. Med. Chem.* **1971**, *14*, 35−37.
(9) Creighton, D. J.; Hamilton, D. S.; Kavarana, M. J.; Sharkey, E. M.; Eiseman, J. L. *Drugs Future* **2000**, *25*, 385−392.
(10) Sharkey, E. M.; O'Neill, H. B.; Kovaleva, E. G.; Kavarana, M. J.; Wang, H.; Creighton, D. J.; Sentz, D. L.; Eiseman, J. L. *Cancer Chemother. Pharmacol.* **2000**, *46*, 156−166.
(11) Sakamoto, H.; Mashima, T.; Sato, S.; Hashimoto, Y.; Yamori, T.; Tsuruo, T. *Clin. Cancer Res.* **2001**, *7*, 2513−2518.

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to inhibit tumor growth in vivo and to reduce the possibility of side effects.[9]

Here, we report for the first time that chemically cross-linking two CHGs with a linker (L) long enough to allow simultaneous binding to each active site of hGlxI dramatically increases binding affinity. This general strategy has been used in the past to increase binding affinities, as the binding energies at each site can potentially contribute to the overall free energy of binding.[13] The X-ray crystal structure of hGlxI in complex with one CHG at each active site (Figure 1, PDB

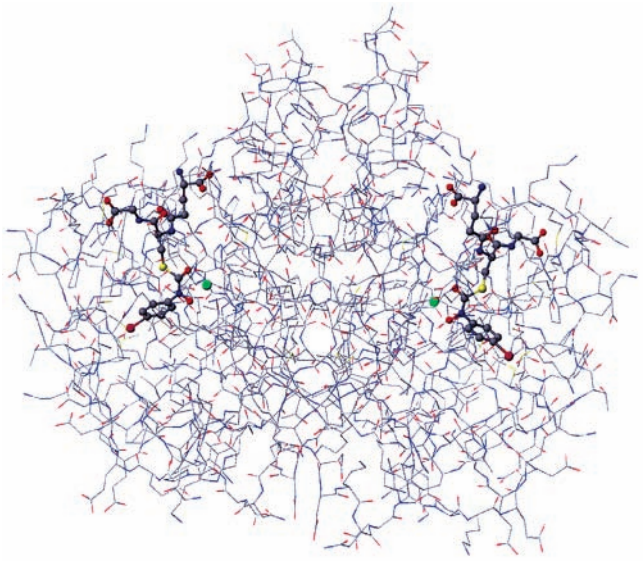


Figure 1. hGlxI in complex with CHG (ball and stick models).

code 1QIN)[14] shows that the γ-glutamyl-NH_2 groups of the bound CHGs are exposed to bulk solvent, are about 30 Å apart, and therefore could be chemically cross-linked.

In Figure 1, green spheres represent catalytically essential Zn^{2+} ions at the active sites, which directly coordinate the cisoid oxygens of the bound CHGs. The dimer interface extends vertically through the center of the figure.

A family of potential bivalent inhibitors was prepared by the successive introduction of n β-alanyl residues at the γ-glutamyl-NH_2 function of CHG, followed by conversion to a suberate diamide to complete cross-linking (Scheme 1).

Synthetic methods were adapted from published procedures,[15] and chemical identities were confirmed by ^{1}H NMR (500 MHz) and HRMS (Supporting Information).

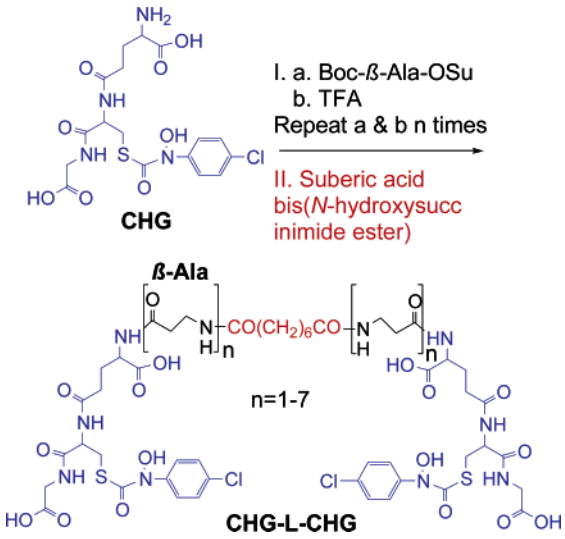
(12) (a) Hamilton, D. S.; Creighton, D. J. *J. Biol. Chem.* **1992**, *267*, 24933−24936. (b) Murthy, N. S. R. K.; Bakeris, T.; Kavarana, M. J.; Hamilton, D. S.; Lan, Y.; Creighton, D. J. *J. Med. Chem.* **1994**, *37*, 2161−2166. (c) Kalsi, A.; Kavarana, M. J.; Lu, T.; Whalen, D. L.; Hamilton, D. S.; Creighton, D. J. *J. Med. Chem.* **2000**, *43*, 3981−3986.

(13) (a) Slon-Usakiewicz, J. J.; Purisima, E.; Tsuda, Y.; Sulea, T.; Pedyczak, A.; Fethuere, M. C.; Yonishi, Y. *Biochemistry* **1997**, *36*, 13494−13502 and references therein. (b) Lyon, R. P.; Hill, J. J.; Atkins, W. M. *Biochemistry* **2003**, *42*, 10418−10428.

(14) Cameron, A. D.; Ridderström, M.; Olin, B.; Kavarana, M. J.; Creighton, D. J.; Mannervik, B. *Biochemistry* **1999**, *38*, 13480−13490.

(15) Zheng, Z.-B.; Nagai, S.; Iwanami, N.; Kobayashi, A.; Natori, S.; Sankawa, U. *Chem. Pharm. Bull.* **1999**, *47*, 777−782.

Scheme 1



The bivalent transition-state analogues shown in Scheme 1 proved to be competitive inhibitors of glyoxalase I from human erythrocytes (hGlxI)[12b] and from yeast (yGlxI); e.g., Figure 2.

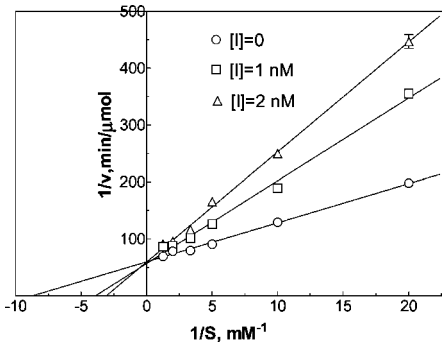


Figure 2. Reciprocal plot of the initial rate of the hGlxI reaction versus concentration of GSH-methylglyoxal thiohemiacetal substrate (S) at different fixed concentrations of [CHG(β-ala)$_6$]$_2$ suberate diamide [I]. Conditions: 50 mM sodium phosphate buffer, pH 7.0, 25 °C.

Model building of the inhibitors into the X-ray structure of hGlxI indicated that analogues with $n \geq 4$ could bridge the active sites, Figure 1. Indeed, a comparison of the competitive inhibition constants with linker length shows a dramatic increase in binding affinity when $n = 6$ or 7, corresponding to linker lengths of 70 and 80 Å, respectively; Table 1, Figure 3.

These results suggest that the longest bivalent inhibitors ($n = 6$, 7) span the active sites on each enzyme molecule. The strongest bivalent inhibitors have K_i values ~500-fold smaller than the monovalent inhibitors CHG(β-ala)$_1$, CHG-(β-ala)$_5$, and CHG(β-ala)$_6$. This corresponds to a ~250-fold difference in affinity per CHG group, given that the bivalent

Table 1. Competitive Inhibition Constants (K_i) of CHG, CHG(β-ala)$_n$, and [CHG(β-ala)$_n$]$_2$ Suberate Diamide with hGlxI, Yeast GlxI (yGlxI), and Bovine Liver GlxII (bGlxII)[a]

compound	linker length (Å)	K_i, hGlxI (nM)[b]	K_i, yGlxI (μM)[c]	K_i, bGlxII (nM)[c]
CHG		46 ± 4[d]	3.6 ± 0.3[d]	1700 ± 17
CHG(β-Ala)$_n$				
n =				
1	4	330 ± 6		1200 ± 12
5	24	744 ± 39		
6	30	583 ± 33		870 ± 60
[CHG(β-Ala)$_n$]$_2$sub.				
n =				
1	21	70.1 ± 1.3	3.0 ± 0.2	35 ± 4
2	31	75.9 ± 7.4		50 ± 5
3	41	84.1 ± 2.4		
4	50	59.6 ± 2.0	9.0 ± 0.3	
5	60	7.5 ± 0.5		
6	70	0.96 ± 0.06	7.2 ± 0.1	142 ± 1
7	80	0.97 ± 0.02	4.1 ± 0.5	79 ± 8

[a] Conditions: sodium phosphate buffer, 50 mM, pH 7, 25 °C. [b] Mean (± SD) for triplicate determinations. [c] Mean (± SD) for duplicate determinations. [d] Taken from ref 12b.

inhibitors contain two CHG groups and the monovalent inhibitors contain one CHG group. Since the three monovalent derivatives bind to the enzyme with about the same affinity, the linker groups appear not to affect binding affinity significantly. The 4-fold higher affinities per CHG group for the shorter bivalent inhibitors (n = 1−4) versus the control compounds such as CHG(β-ala)$_6$ might be indicative of a peripheral site near the active site that can weakly bind a CHG group, or that the average solution conformation of the bivalent inhibitors is nearer to that of the bound species, reducing the unfavorable entropy of binding. The bivalent inhibitor [CHG(β-ala)$_6$]$_2$ suberate diamide (K_i = 0.96 nM) binds about 50-fold more tightly than CHG (K_i = 46 nM) to hGlxI. Binding would be tighter, except that amidation of the γ-glutamyl-NH$_2$ decreases binding affinity by 13-fold,

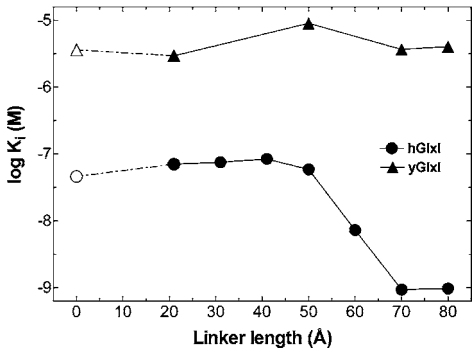
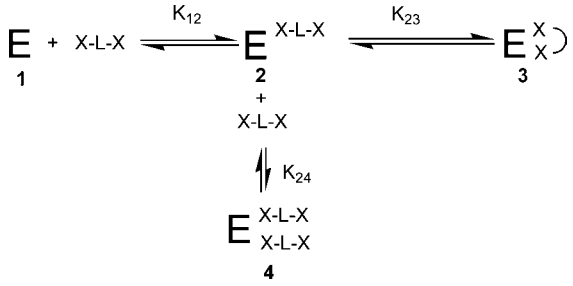


Figure 3. Log plot of K_i versus linker length for the bivalent inhibitors of Table 1 for hGlxI (●) and yGlxII (▲). Open symbols are for CHG alone.

as evidenced by the K_i value of 583 nM for CHG(β-ala)$_6$ versus that for CHG.

Tight binding by the bivalent inhibitors (n = 6, 7) appears to reflect the fact that (a) once the first CHG group binds to the enzyme there is a dramatic increase in the probability of binding of the remaining CHG group and (b) the observed affinity constant for the bivalent inhibitor with the enzyme is the product of the binding constants for each CHG group. This can be understood on the basis of the following system of equilibria, Scheme 2.

Scheme 2



In Scheme 2, E = enzyme, X−L−X is the bivalent inhibitor with identical binding groups X, connected by the linker L, and the Ks are defined as binding constants. Under dilute conditions, where $K_{23} \gg K_{24}$[X−L−X], only the equilibria associated with K_{12} and K_{23} must be considered. Thus, the binding constant of the bivalent inhibitor, where X = CHG (n = 6), for the enzyme (K_i^{-1}) is directly proportional to the product of the two microscopic binding constants (eq 1).

$$(K_i)^{-1} = K_{12}K_{23} = 1 \times 10^9\ \mathrm{M}^{-1} \qquad (1)$$

To a good first approximation,

$$K_{12} \approx 2(K_{ii})^{-1} = 3.4 \times 10^6\ \mathrm{M}^{-1} \qquad (2)$$

where K_{ii} is the observed inhibition constant for CHG(β-ala)$_n$, n = 6. The coefficient of 2 in eq 2 corrects for the fact that the bivalent inhibitor contains two CHG groups available for binding while the monovalent inhibitor contains one available CHG group. Thus, binding of CHG in the intermediate complex **2** is a highly favorable process, on the basis of the calculated value of K_{23}, eq 3.

$$K_{23} = 1 \times 10^9\ \mathrm{M}^{-1}/3.4 \times 10^6\ \mathrm{M}^{-1} = 294 \qquad (3)$$

The concept of effective concentration (EC)[16] can also be used to access the thermodynamic advantage of placing two interacting groups in the same "molecular entity" versus

(16) McNaught, A. D.; Wilkinson, A. *IUPAC Compendium of Chemical Terminology*, 2nd ed.; Blackwell Science, 1997.

having the reacting groups in separate molecules.[17] By replacing the term "molecular entity" with "complex **2**", shown in Scheme 2, the EC for unbound CHG in this complex is obtained by dividing the unimolecular equilibrium constant by the bimolecular equilibrium constant for binding of the monovalent inhibitor to the enzyme (eq 4).

$$\mathrm{EC} = K_{23}/(K_{ii})^{-1} = 174\ \mu\mathrm{M} \qquad (4)$$

The EC significantly exceeds the dissociation constant of CHG(β-ala)$_6$ ($K_i = 0.58\ \mu$M) with the enzyme. Therefore, binding is a cooperative phenomenon in which the initial binding of the first tethered CHG group to the first active site dramatically increases the probability that the second tethered CHG group will bind to the remaining active site.

The maximum EC depends to a significant extent on the entropy lost on binding.[18] In the present case, the magnitude of the EC is determined not only by the increase in the average local concentration of the unbound CHG group with respect to the unoccupied active site in complex **2** over that of the unbound monovalent inhibitor but also by an "orientation effect". This reflects the effect of the linker on the orientation of the unbound CHG group with respect to the unoccupied active site in complex **2**, which either makes binding more favorable by reducing unfavorable degrees of translational and/or rotational freedom or makes binding less favorable by reducing favorable degrees of translational and/or rotational freedom. Therefore, the EC value reported here for $n = 6$ is primarily a property of the linker with hGlxI. This EC can be used to calculate expected K_i values for bivalent inhibitors in which (β-ala)$_n$ suberate diamide chemically cross-links any one of a number of different GSH derivatives reported to be either reversible or irreversible inhibitors of hGlxI.[19]

To assess the specificity of the bivalent inhibitors for hGlxI versus other GSH-dependent enzymes, the inhibition studies were extended to include yGlxI and bGlxII, a thioester hydrolase for *S-D*-lactoylglutathione. These enzymes were of interest because they are both inhibited by CHG[12b] and both are monomeric enzymes, although yGlxI has been argued to contain two active sites.

Yeast GlxI does not show evidence of a dramatic increase in binding affinity with increasing linker length (Table 1, Figure 3). Thus, there is no evidence that binding of the longest bivalent inhibitor to yGlxI involves simultaneous binding to two active sites. This is somewhat surprising in view of sequence comparisons indicating that yGlxI is the result of a gene duplication event, which retained amino acid residues indicative of two active sites per monomer.[20] Indeed, topological mapping of the yGlxI polypeptide on the crystal structure of hGlxI indicates the presence of two active sites that are analogous in structure to that observed in the X-ray crystal structure of the human enzyme.[21] Mutagenesis studies indicate that both active sites are functional. Nevertheless, there must be subtle differences between the active sites on the two enzymes, as CHG binds 78-fold less tightly to yGlxI than to hGlxI (Table 1). Perhaps there are differences in the surface topology of yGlxI not found in hGlxI that preclude simultaneous binding to each active site using the bivalent inhibitors described in this study. Whatever the explanation, cross-linking has increased inhibitor selectivity by almost 100-fold, as CHG binds 78-fold more tightly to hGlxI than to yGlxI, while [CHG(β-ala)$_6$]$_2$ suberate diamide binds about 7500-fold more tightly (Table 1).

For the thioester hydrolase bGlxII, the CHG(β-ala)$_n$ monomers inhibit the enzyme as well as CHG (Table 1), indicating that the γ-glutamyl-NH$_2$ group of the bound inhibitor probably extends into bulk solvent and that substituents at this position do not interfere with binding. In addition, there is no dramatic increase in binding affinity with increasing linker length, fully consistent with a single active site for GlxII. As observed with hGlxI, the affinity of the bivalent inhibitors for bGlxII is somewhat greater per CHG group (7-fold) than for CHG(β-ala)$_6$ monomer. Comparing the inhibition constants of CHG and [CHG(β-ala)$_6$]$_2$ suberate diamide for hGlxI versus bGlxII, cross-linking increases binding selectivity from 37-fold to 148-fold, a 4-fold increase. Finally, CHG and [CHG-(β-ala)$_6$]$_2$ suberate diamide show no inhibitory activity with GSH peroxidase or human placental GSH transferase up to an inhibitor concentration of 20 μM.

In summary, the longest bivalent transition state analogues described here are the strongest competitive inhibitors of hGlxI yet reported. Moreover, they exhibit improved selectivity over several other GSH-dependent enzymes tested. This lays the experimental and conceptual foundation for the development of a new class of powerful inhibitors for this important antitumor target enzyme. The alkylester prodrug strategy[10] could be used to deliver the bivalent inhibitors into tumor cells, as the inhibitors can be readily converted to the tetra-*O*-ethyl esters by incubation in ethanolic/HCl (Zheng and Creighton, unpublished).

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Supporting Information Available: Kinetic and synthetic methods and analytical data on synthetic products. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) (a) Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183−278. (b) Morawetz, H.; Goodman, N. *Macromolecules* **1970**, *3*, 699−700.

(18) (a) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678−1683. (b) Jencks, W. P. *Adv. Enzymol.* **1975**, *43*, 219−410. (c) Page, M. I. *Adv. Chem., Int. Ed. Engl.* **1977**, *16*, 449−459.

(19) Creighton, D. J.; Zheng, Z.-B.; Holewinski, R. J.; Hamilton, D. S.; Eiseman, J. L. *Biochem. Trans.* **2003**, *31*, 1378−1382.

(20) (a) Ridderström, M.; Mannervik, B. *Biochem. J.* **1996**, *316*, 1005−1006. (b) Clugston, S. L.; Daub, E.; Kinach, R.; Miedema, D.; Barnard, J. F. J.; Honek, J. F. *Gene* (Amsterdam) **1997**, *186*, 103−111.

(21) Frickel, E.-M.; Jemth, P.; Widersten, M.; Mannervik, B. J. *Biol. Chem.* **2001**, *276*, 1845−1849.