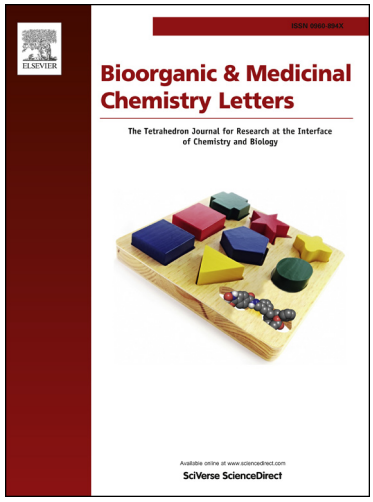

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ABSTRACT

The zinc metalloenzyme glyoxalase I (GlxI) catalyzes the glutathione-dependent inactivation of cytotoxic methylglyoxal. Two competitive bivalent GlxI inhibitors, polyBHG2-62 (K_i = 1.0 nM) and polyBHG2-54 (K_i = 0.3 nM), were synthesized based on the transition-state analog *S*-(*N*-bromophenyl-*N*-hydroxycarbamoyl) glutathione (BHG). The most effective inhibitor, polyBHG2-54, is the first subnanomolar inhibitor of GlxI, and is over 50-fold more potent than BHG itself.

Human glyoxalase I (GlxI) is a 42 kDa[1] dimeric Zn^{2+} metalloenzyme that detoxifies methylglyoxal *in vivo* by converting it into *S*-D-lactoylglutathione, which is then converted to D-lactate by glyoxalase II.[2,3] Since high activities of GlxI are present in tumor tissues, inhibitors of GlxI increase the accumulation of cytotoxic methylglyoxal, which results in significant anti-tumor activity both *in vitro* and *in vivo*.[4] A potent and selective GlxI inhibitor could therefore potentially result in an adjuvant to restore chemotherapy sensitivity in tumor cells.[5,6]

Linking two identical binding groups by a spacer unit has been proposed[7] to improve both the selectivity and the activity of inhibitors compared with the corresponding univalent ligands, and this approach has been verified experimentally.[8-10] However, optimization of the spacer unit between the binding groups remains a challenge, and is crucial in fragment-based drug design.[11]In this paper, we extend our previous work on bivalent transition-state analog inhibitors of human glyoxalase I (hGlxI)[10] to include two new competitive bivalent GlxI inhibitors in which symmetric ligands are linked by linkers that differ in flexibility, length, and water solubility.

We previously developed a new class of competitive inhibitors of homodimeric human glyoxalase I by cross-linking two molecules of the transition state analog *S*-(*N*-chlorophenyl-*N*-hydroxycarbamoyl)glutathione (CHG) through their γ-glutamyl-NH_2 groups with poly-β-alanyl tethers of different length: [CHG(β-ala)$_n$] suberate diamide (n = 1–7). The strongest inhibitors of this antitumor target enzyme likely bind simultaneously to the active site on each subunit, and give K_i values as low as 0.96 nM (n = 6), a 50-fold tighter binding than the monomer inhibitor CHG (K_i = 46 nM).[5,12] Cross-linking not only improves the binding affinity, but also improves the selectivity by almost 100-fold for human GlxI (hGlxI) relative to yeast GlxI (yGlxI). In the X-ray crystal structure of the hGlxI complex with CHG, the γ-glutamyl-NH_2 groups are exposed to solvent, and are about 30 Å apart. However, a 70–80 Å tether length was found to give the best inhibition.

Nevertheless, these inhibitors have two drawbacks that need to be addressed: amidation of the γ-glutamyl-NH_2 groups decreases the binding affinity by 7 to 13-fold, and the bivalent inhibitors have low solubility, making them unable to cross the cell membrane. In this work, we modify the linker by replacing the amide groups with ethylcarbonyl groups, which do not affect the binding affinity of CHG. Two new inhibitors based on *S*-(*N*-bromophenyl-*N*-hydroxycarbamoyl) glutathione (BHG)[5,12] exhibit inhibition constants of 1 nM or less, and one of these

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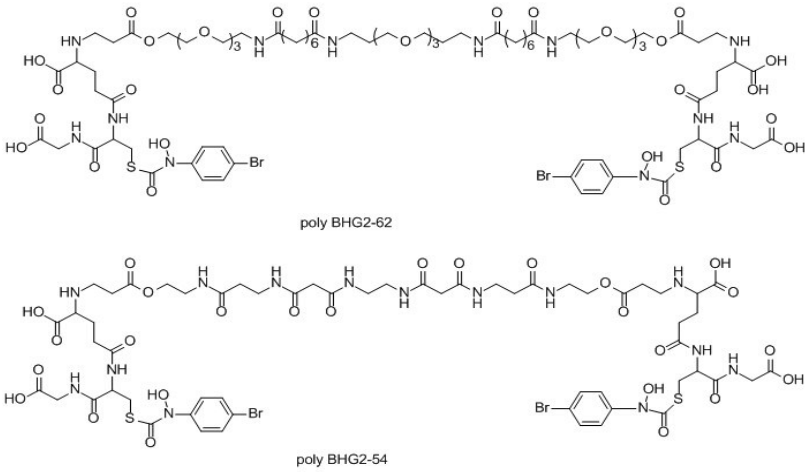


Figure 1.The chemical structures of newly synthesized bivalent inhibitors.

compounds has a PEG linker, which increases water solubility and should result in an enhanced ability to cross cell membranes.[13-15]

The two competitive bivalent GlxI inhibitors polyBHG2-62 (K_i = 1 nM) and polyBHG2-54 (K_i = 0.3 nM) (Figure 1) were designed based on the transition-state analog *S*-(*N*-bromophenyl-*N*-hydroxycarbamoyl) glutathione (BHG) by an examination of the X-ray crystal structure of human GlxI in complex with one CHG at each active site (Figure 2, PDB code 1QIN).[10] CHG is a stable transition-state analog that chelates the active site zinc and mimics the transition-state. We chose a central scaffold of poly β-ala and suberic acid bis(*N*-hydroxysuccinimide ester) for the linkage of the two binding sites.

In our previous work, amide groups were used to link the CHG units to the spacer groups. However, amidation of CHG decreases the affinity of the inhibitor, as illustrated in Figure 3. Both compounds A (K_i = 330 nM) and B (K_i = 130 nM) have inhibition constants that are significantly higher than that of CHG itself (46 nM). Addition of an ethylcarbonyl substituent to give compound C (K_i = 46 nM), however, does not decrease the inbibition constant relative to CHG itself. These results indicate that the carbonyl group on the substituent is crucial. On the other hand, the unfavorable effect of a carbonyl group near the nitrogen may be rationalized by the weakened hydrogen bonds/electrostatic interactions observed in the X-ray structure (PDB code 1QIN).[10]

The bivalent transition state analogs incorporate a symmetric scaffold based on **1** and **3** (Scheme 1), in which the terminal carbonyls are protected by pyrrolidine-2,5-dione. The intermediate **1** was used as a flexible linker with O,O-(dipropylamine)diethyleneglycol, and suberic acid bis-(*N*-hydroxysuccinimide ester) was used to adjust the linker spacing.

To synthesize two inhibitors with different distances between the active groups, a building block strategy was used (Schemes 2 and 3). PolyBHG2-62[17] was synthesized in five steps, as outlined in Scheme 2, and synthesis of polyBHG2-54[18] followed a similar strategy (Scheme 3). In Scheme 3, O,O-(dipropylamine) diethyleneglycol was replaced by poly β-ala to generate the more rigid intermediate **3**, which was used to prepare polyBHG2-54, in which the length of the linker is different from PolyBHG2-62. For these inhibitors *S*-(*N*-bromophenyl-*N*-hydrox

y-carbamoyl) glutathione (BHG, K_i = 14 nM)[5,12] is used instead of CHG because of its better binding to GlxI.

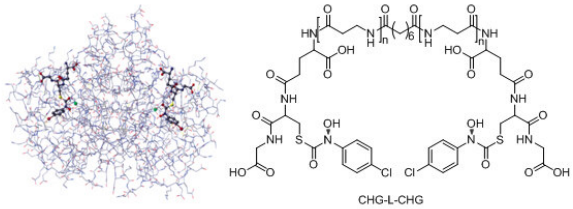


Figure 2. The structure of human GlxI in complex with CHG (ball and stick model)

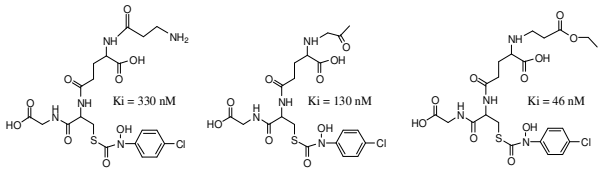


Figure 3. Variation of *Ki* with substituents on CHG

The bivalent inhibitors PolyBHG2-62 and polyBHG2-54 exhibit much tighter binding ability than BHG itself, with K_i values of 1.0 nM and 0.3 nM, respectively. The tightest binding compound (polyBHG2-54) is more than 50-fold more potent than BHG, and three-fold more potent than the best bivalent inhibitor we obtained previously.[6] Modification of the linker by using ethylcarbonyl groups instead of amide groups on the BHG results in the strongest inhibitor yet reported for hGlxI. Importantly, use of the PEG linker in polyBHG2-62 retains most of the inhibition toward GlxI, while providing enhanced water

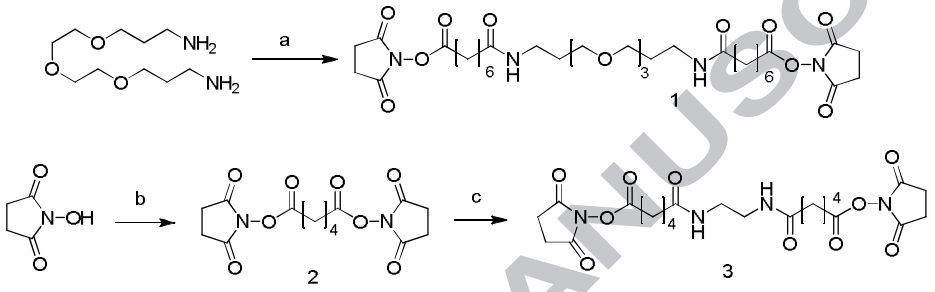
solubility. It should be noted that, compounds polyBHG2-62 and polyBHG2-54 have similar solubilities (26 mg/mL and 32 mg/mL, respectively). This combination of enhanced water solubility and strong binding is an important step toward the development of inhibitors for GlxI that can cross cell membranes.

Table 1, shows the selectivities of the newly synthesized compounds with different glyoxalase I enzymes, including hGlxI, yeast GlxI (yGlxI), and bovine liver GlxII (bGlxII). CHG was used as a positive control. Surprisingly, the two target compounds exhibited higher levels of inhibition than CHG in the hGlxI control. Poly BHG2-62 (K*i* 1.0 nM) and poly BHG2-54 (K*i* 0.3 nM) are almost 50-fold more active and 156-fold more active than CHG, respectively. In addition, CHG binds 78-fold less tightly to yGlxI than to hGlx1. Thus, cross-linking increases the inhibitor selectivity by approximately 158-fold, as CHG binds 78-fold more tightly to hGlxI than to yGlx, while poly BHG2-54 binds about 12,300-fold more tightly. A comparison of the inhibition constants of CHG and poly BHG2-54 for hGlxI versus bGlxII shows that cross-linking increases binding selectivity 8.6-fold, from 37-fold to 320-fold.

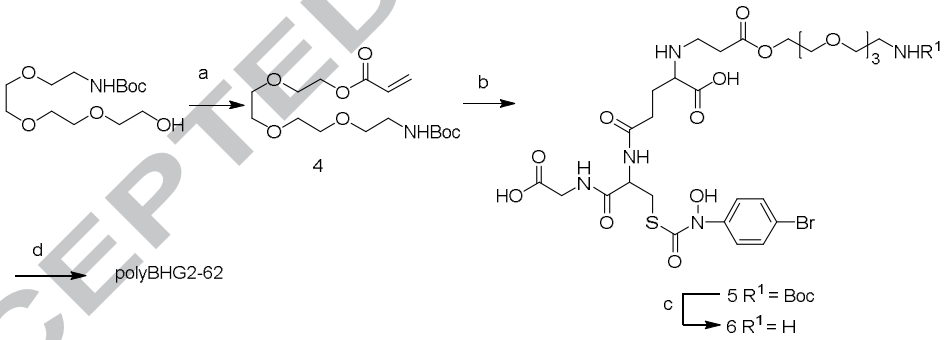
Table 1
Competitive inhibition constant (*Ki*) of CHG with hGlxI, yeast GlxI (yGlxI), and bovine liver GlxII (bGlxII)[a]

Compound	*Ki*, hGlxI (nM)	*Ki*, yGlxI (μM)	*Ki*, bGlxI (nM)
CHG	46 ± 4[d]	3.6 ± 0.3[d]	1700 ± 17
poly BHG2-62	1.0 ± 0.02	6.9 ± 0.2	138 ± 2
poly BHG2-54	0.3 ± 0.02	3.7 ± 0.1	81 ± 5

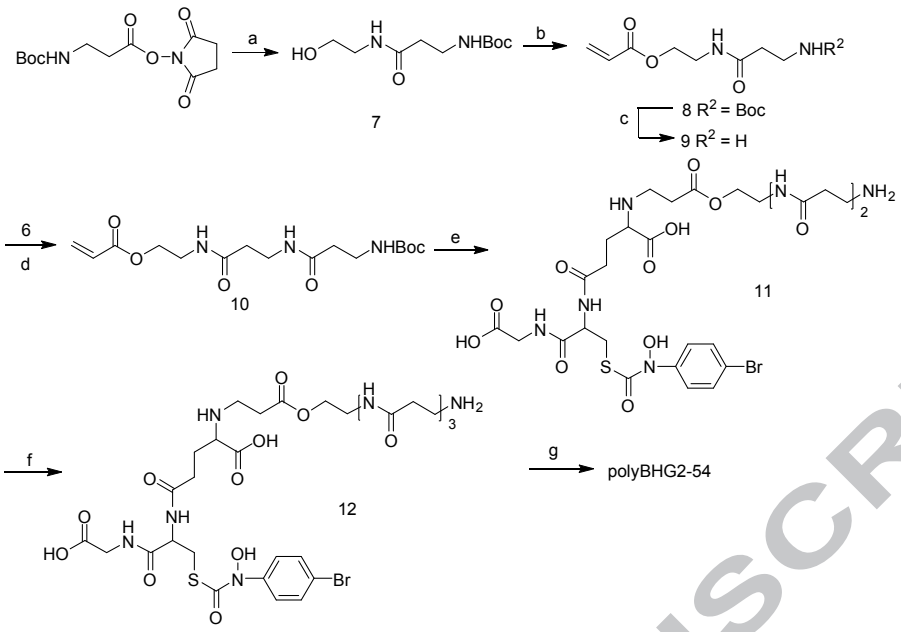
[a]Conditions: sodium phosphate buffer, 50 mM, PH 7, 25 °C.
[b]Mean (± SD) for triplicate determinations.
[c]Mean (± SD) for duplicate determinations.
[d] Ref 12.



Scheme 1. Preparation of symmetric scaffold **1** and **3**: Reagents and conditions: (a) suberic acid bis (*N*-hydroxysuccinimide ester), DMF, 0 °C, 57%; (b) adipoyl chloride, diisopropylethylamine (DIPEA), CH_2Cl_2, 0 °C, 48%; (c) ethylenediamine, DMF, 0 °C, 6%.



Scheme 2. Preparation of poly BHG2-62. Reagents and conditions: (a) acryloyl chloride, DIPEA, DMF, 0 °C, 35%; (b) BHG, DIPEA, DMF, 21%; (c) TFA, 53%; (d) **1**, DIPEA, DMF, 72%.



Scheme 3. Preparation of polyBHG2-54. Reagents and conditions: (a) ethanolamine, DMF; (b) acryloyl chloride, DIPEA, DMF, 0 ℃, 53%; (c) TFA; (d) *N*-*t*-Boc-*β*-ala-*N*-hydroxysuccinimide ester, DIPEA, DMF, 55%; (e) 1) BHG, DIPEA, DMF; 2) TFA, 5.2%; (f) 1) *N*-*t*-Boc-*β*-ala-*N*-hydroxysuccinimide ester , DIPEA, DMF; 2) TFA, 25%; (g) **3**, DIPEA, DMF, 11%.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found in the online version.

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17. Spectral data of PolyBHG2-62: ^{1}H NMR (500 MHz, D_2O Tris buffer, δ), 7.67 (*d*, *J* =8.3 Hz, 4H), 7.54 (*d*, *J* =8.3 Hz, 4H), 4.53 (*m*, 2H), 3.76 (*m*, 41H), 3.64 (*m*, 9H), 3.45 (*t*, *J* =5.2 Hz, 4H), 3.22 (*t*, *J* = 6.7 Hz, 6H), 3.18 (*m*, 5H), 2.60 (*m*, 6H), 2.32 (*m*, 10H), 2.24 (*m*, 2H). **ESI-HRMS** *m/z* 2031.6582 $(M+H)^+$, $C_{82}H_{128}Br_2N_{12}O_{33}S_2+H^+$ requires 2031.6588.
18. Spectral data of PolyBHG2-54: ^{1}H NMR (500 MHz, D_2O Tris buffer, δ), 7.69 (*d*, *J* = 8.7 Hz, 4H), 7.55 (*d*, *J* = 8.7 Hz, 4H), 4.75 (*dd*, *J* = 7.9, 4.12 Hz, 2H), 4.27 (*m*, 2H), 3.86 (*d*, *J* = 17.3Hz, 2H), 3.85 (*d*, *J* = 17.3Hz, 2H), 3.72 (*m*, 7H), 3.49 (*m*, 12H), 3.38 (*m*, 5H), 3.22 (*m*, 8H), 2.49 (*m*, 19H), 2.30 (*m*, 9H), 2.19 (*m*, 4H), 1.38 (*m*, 4H), 1.23 (*m*, 2H), 0.96 (*m*, 2H). **ESI-HRMS** *m/z* 1977.5521 $(M+H)^+$, $C_{76}H_{110}Br_2N_{18}O_{30}S_2+H^+$ requires 1977.5516.

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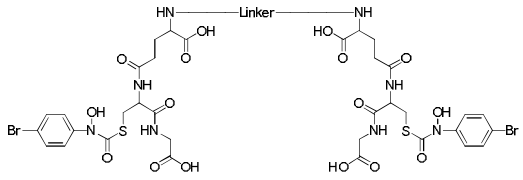

BHG-L-BHG