Supporting Information

Bivalent Transition State Analog Inhibitors of Human Glyoxalase I

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Materials and methods. Oxidized and reduced glutathione, suberic acid bis(*N*-hydroxysuccinimide ester), human and yeast glyoxalase I, bovine liver glyoxalase II, human placenta glutathione tranferase and yeast glutathione reductase were purchased from Sigma Chem. Co. Boc-*β*-Ala-OSu was purchased from Novabiochem. All other reagents were of the highest purity commercially available.

NMR spectra were taken on a GE QE-500 NMR spectrometer. Mass spectral data were obtained at the Center for Biomedical and Bio-organic Mass Spectrometry, Washington University. UV spectra were recorded using a Beckman DU 640 spectrophotometer. HPLC was carried out using a Waters high-performance liquid chromatography system composed of a 600 Controller, Delta 600 Pumps, and 996 Photodiode Array Detector. Analytical HPLC was performed using a Nova-Pak C_{18}, 4 μm, 3.9 x 150 mm column. Preparative HPLC was performed using a SymmetryPrep C_{18}, 7 μm, 19 x 150mm column.

The synthetic route used to cross-link the transition state analog *S*-(4-chlorophenyl-*N*-hydroxyphenyl)glutathione (CHG) is summarized in Scheme I in the main text:

CHG(*β*-Ala) To a solution of CHG (1g, 2.1 mmol) in a mixture of 15 ml of DMF and 3 ml of diisopropylethylamine was added Boc-*β*-Ala-OSu (900 mg, 3.6 mmol). The reaction mixture was stirred at room temperature and the progress of the reaction was monitored by following the disappearance of the CHG peak obtained by RP-HPLC of aliquots of the reaction mixture, using 25% acetonitrile in water containing 0.1% trifluoroacetic acid as an eluting solvent. After the reaction had gone to 99% completion (~1.5 h), solvent was removed in vacuo, the residue dissolved in 10 ml TFA and stirred for 1h at room temperature. Diethyl ether (100 ml) was added to the reaction mixture and the precipitate was collected by filtration. The crude product was fractionated by reverse-phase HPLC (Waters Symmetry prep C_{18}, 7 μm, 19 x 150 mm) using the same running solvent. The product peaks were collected, evaporated to dryness and maintained under vacuum overnight to give the final product as a white solid: yield 89% (1.15 g) ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.49 (d, J=8.9 Hz, 2H), 7.44 (d, J=8.9 Hz, 2H), 4.60 (dd, J=8.8, 4.4 Hz, 1H), 4.14 (dd, J=8.8, 4.6 Hz, 1H), 3.78 (d, J=17.3 Hz, 1H), 3.74 (d, J=17.3 Hz, 1H), 3.35 (dd, J=14.5, 4.5 Hz, 1H), 3.21 (m, 2H), 3.12 (dd, J=14.5, 8.8 Hz, 1H), 2.65 (m, 2H), 2.38 (m, 2H), 2.11 (m, 1H), 1.94 (m, 1H). HRMS (ESI) *m/z* 548.1216 $[M+H]^+$ (calc'd for $C_{20}H_{27}N_5O_9SCl$: 548.1218).

CHG(β-Ala)$_2$ through CHG(β-Ala)$_7$. These compounds were prepared by the successive addition of Boc-β-Ala-OSu to CHG(β-Ala) using the procedure described above: CHG(β-Ala)$_2$. Yield: 75% (0.88g). ^{1}H NMR (500 MHz, D_2O Tris buffer, pD 8.7) δ 7.48 (d, J=8.8 Hz, 2H), 7.44 (d, J=8.8 Hz, 2H), 4.62 (dd, J=8.8, 4.4 Hz, 1H), 4.15 (dd, J=8.4, 4.6 Hz, 1H), 3.77 (d, J=17.3 Hz, 1H), 3.74 (d, J=17.3 Hz, 1H), 3.41 (t, J=6.2 Hz, 2H), 3.37 (dd, J=14.5, 4.5 Hz, 1H), 3.19 (t, J=6.7 Hz, 2H), 3.12 (dd, J=14.5, 8.9 Hz, 1H), 2.60 (t, J=6.7 Hz, 2H), 2.44 (t, J=6.2 Hz, 2H), 2.36 (m, 2H), 2.10 (m, 1H), 1.93 (m, 1H). HRMS (ESI) *m/z* 619.1579 $[M+H]^+$ (calc'd for $C_{23}H_{32}N_6O_{10}SCl$: 619.1589); CHG(β-Ala)$_3$. Yield: 75% (0.88g). ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.49 (d, J=8.9 Hz, 2H), 7.44 (d, J=8.9 Hz, 2H), 4.61 (dd, J=8.8, 4.4 Hz, 1H), 4.14 (dd, J=8.5, 4.8 Hz, 1H), 3.78 (d, J=17.1 Hz, 1H), 3.75 (d, J=17.1 Hz, 1H), 3.40 (m, 4H), 3.33 (dd, J=14.5, 4.6 Hz, 1H), 3.18 (t, J=6.7 Hz, 2H), 3.11 (dd, J=14.6, 8.9 Hz, 1H), 2.59 (t, J=6.7 Hz, 2H), 2.44 (t, J=6.2 Hz, 2H), 2.39 (t, J=6.6 Hz, 2H), 2.35 (m, 2H), 2.10 (m, 1H), 1.93 (m, 1H). HRMS (ESI) *m/z* 690.1944 $[M+H]^+$ (calc'd for $C_{26}H_{37}N_7O_{11}SCl$: 690.1960); CHG(β-Ala)$_4$. Yield: 54% (0.37g). ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.49 (d, J=8.9 Hz, 2H), 7.44 (d, J=8.9 Hz, 2H), 4.61 (dd, J=8.6, 4.4 Hz, 1H), 4.15 (dd, J=8.1, 4.6 Hz, 1H), 3.78 (d, J=17.2 Hz, 1H), 3.75 (d, J=17.2 Hz, 1H), 3.41 (m, 6H), 3.35 (dd, J=14.4, 4.4 Hz, 1H), 3.19 (t, J=6.7 Hz, 2H), 3.11 (dd, J=14.6, 8.9 Hz, 1H), 2.58 (t, J=6.7 Hz, 2H), 2.45 (t, J=6.2 Hz, 2H), 2.40 (t, J=6.6 Hz, 2H), 2.10 (m, 1H), 1.93 (m, 1H). HRMS (ESI) *m/z* 761.2321 $[M+H]^+$ (calc'd for $C_{29}H_{42}N_8O_{12}SCl$: 761.2331); CHG(β-Ala)$_5$. Yield: 50% (0.2g). ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.49 (d, J=8.9 Hz, 2H), 7.44 (d, J=8.9 Hz, 2H), 4.60 (dd, J=8.8, 4.4 Hz, 1H), 4.15 (dd, J=8.5, 4.8 Hz, 1H), 3.78 (d, J=17.2 Hz, 1H), 3.75 (d, J=17.2 Hz, 1H), 3.39 (m, 8H), 3.35 (dd, J=14.6, 4.4 Hz, 1H), 3.17 (t, J=6.7 Hz, 2H), 3.13 (dd, J=14.4, 8.9 Hz, 1H), 2.59 (t, J=6.7 Hz, 2H), 2.45 (t, J=6.4 Hz, 2H), 2.39 (m, 6H), 2.35 (m, 2H), 2.10 (m, 1H), 1.93 (m, 1H). HRMS (ESI) *m/z* 832.2715 $[M+H]^+$ (calc'd for $C_{32}H_{47}N_9O_{13}SCl$: 832.2703); CHG(β-Ala)$_6$ Yield: 55% (0.12g). ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.49 (d, J=8.9 Hz, 2H), 7.44 (d, J=8.9 Hz, 2H), 4.59 (dd, J=8.8, 4.4 Hz, 1H), 4.15 (dd, J=8.5, 4.8 Hz, 1H), 3.78 (d, J=17.2 Hz, 1H), 3.75 (d, J=17.2 Hz, 1H), 3.41 (m, 10H), 3.30 (dd, J=14.5, 4.6 Hz, 1H), 3.10 (t, J=6.6 Hz, 2H), 2.95 (dd, J=14.4, 8.9 Hz, 1H), 2.55 (t, J=6.7 Hz, 2H), 2.41 (m, 8H), 2.35 (m, 2H), 2.10 (m, 1H), 1.93 (m, 1H). HRMS (ESI) *m/z* 903.3074 $[M+H]^+$ (calc'd for $C_{35}H_{52}N_{10}O_{14}SCl$: 903.3074); CHG($\beta$-Ala)$_7$. Yield: 55% (0.12g). ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.49 (d, J=8.9 Hz, 2H), 7.43 (d, J=8.9 Hz, 2H), 4.59 (dd, J=8.7, 4.4 Hz, 1H), 4.15 (dd, J=8.2, 4.6 Hz, 1H), 3.78 (d, J=17.4 Hz, 1H), 3.75 (d, J=17.4 Hz, 1H), 3.39 (m, 12H), 3.34 (dd, J=14.6, 4.5 Hz, 1H), 3.13 (t, J=6.5 Hz, 2H), 3.11 (dd, J=14.4, 8.9 Hz, 1H), 2.56 (t, J=6.7 Hz, 2H), 2.44 (t, J=6.5 Hz, 2H), 2.40 (m, 10H), 2.35 (m, 2H), 2.10 (m, 1H), 1.93 (m, 1H). HRMS (ESI) *m/z* 974.3434 $[M+H]^+$ (calc'd for $C_{38}H_{57}N_{11}O_{15}SCl$: 974.3445)

[CHG(β-Ala)]$_2$ suberate diamide. To a solution of CHG(β-Ala) (14 mg, 0.026 mmol) in a mixture of 0.3 ml DMF and 0.1 ml diisopropylethylamine was added suberic acid bis(*N*-hydroxysuccinimideester) (4.7 mg, 0.013 mmol). The reaction mixture was stirring at room temperature overnight. The solvent was removed in vacuo and the crude product was fractionated by preparative reverse-phase HPLC using a linear gradient of 25-40% acetonitrile in water containing 0.1% trifluoroacetic acid. The product peaks were collected, evaporated to dryness and maintained under vacuum overnight to give the final product as a white solid: yield 25% (4.1 mg). ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD

8.7) δ 7.47 (d, J=8.8 Hz, 4H), 7.41 (d, J=8.8 Hz, 4H), 4.60 (dd, J=8.8, 4.3 Hz, 2H), 4.16 (dd, J=8.1, 4.7 Hz, 2H), 3.77 (d, J=17.2 Hz, 2H), 3.75 (d, J=17.2 Hz, 2H), 3.37 (m, 4H), 3.35 (dd, J=14.5, 4.2 Hz, 2H), 3.10 (dd, J=14.5, 9.0 Hz, 2H), 2.43 (m, 4H), 2.34 (m, 4H), 2.15 (t, J=7.4 Hz, 4H), 2.10 (m, 2H), 1.93 (m, 2H), 1.49 (m, 4H), 1.22 (m, 4H). HRMS (ESI) *m/z* 1255.2902 $[M+Na]^+$ (calc'd for $C_{48}H_{62}N_{10}O_{20}S_2Cl_2Na$: 1255.2858).

[CHG(*β*-Ala)$_2$]$_2$suberate diamide through [CHG(*β*-Ala)$_7$]$_2$suberate diamide. These compounds were prepared by crosslinking CHG(*β*-Ala)$_2$-CHG(*β*-Ala)$_7$ using the procedure described above for the preparation of [CHG(*β*-Ala)]$_2$suberate diamide: [CHG(*β*-Ala)$_2$]$_2$suberate diamide. ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.48 (d, J=8.8 Hz, 4H), 7.41 (d, J=8.8 Hz, 4H), 4.61 (dd, J=8.7, 4.4 Hz, 2H), 4.16 (dd, J=8.2, 4.7 Hz, 2H), 3.77 (d, J=17.1 Hz, 2H), 3.75 (d, J=17.1 Hz, 2H), 3.38 (m, 8H), 3.34 (dd, J=14.5, 4.3 Hz, 2H), 3.11 (dd, J=14.5, 8.9 Hz, 2H), 2.43 (t, J=6.6 Hz, 4H), 2.38 (t, J=6.6 Hz, 4H), 2.34 (m, 4H), 2.16 (t, J=7.4 Hz, 4H), 2.10 (m, 2H), 1.93 (m, 2H), 1.50 (m, 4H), 1.23 (m, 4H). HRMS (ESI) *m/z* 1397.3572 $[M+Na]^+$ (calc'd for $C_{54}H_{72}N_{12}O_{22}S_2Cl_2$: 1397.3600); [CHG(*β*-Ala)$_3$]$_2$suberate diamide. Yield 29% ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.49 (d, J=8.8 Hz, 4H), 7.42 (d, J=8.8 Hz, 4H), 4.60 (dd, J=8.8, 4.5 Hz, 2H), 4.15 (dd, J=8.2, 4.6 Hz, 2H), 3.77 (d, J=17.4 Hz, 2H), 3.75 (d, J=17.4 Hz, 2H), 3.38 (m, 12H), 3.34 (dd, J=14.5, 4.6 Hz, 2H), 3.11 (dd, J=14.6, 9.0 Hz, 2H), 2.39 (m, 12H), 2.34 (m, 4H), 2.17 (t, J=7.2 Hz, 4H), 2.10 (m, 2H), 1.93 (m, 2H), 1.50 (m, 4H), 1.24 (m, 4H). HRMS (ESI) *m/z* 759.2294 $[M+2H]^{2+}$ (calc'd for $C_{60}H_{84}N_{14}O_{24}S_2Cl_2$: 759.230); [CHG(*β*-Ala)$_4$]$_2$suberate diamide. Yield 14% ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.44 (d, J=8.5 Hz, 4H), 7.37 (d, J=8.5 Hz, 4H), 4.54 (dd, J=8.7, 4.4 Hz, 2H), 4.10 (dd, J=8.2, 4.8 Hz, 2H), 3.73 (d, J=17.3 Hz, 2H), 3.71 (d, J=17.3 Hz, 2H), 3.34 (m, 16H), 3.29 (dd, J=14.5, 4.6 Hz, 2H), 3.06 (dd, J=14.5, 8.9 Hz, 2H), 2.33 (m, 16H), 2.30 (m, 4H), 2.13 (t, J=7.2 Hz, 4H), 2.10 (m, 2H), 1.90 (m, 2H), 1.49 (m, 4H), 1.20 (m, 4H). HRMS (ESI) *m/z* 852.2449 $[M+2Na]^{2+}$ (calc'd for $C_{66}H_{92}N_{16}O_{26}S_2Cl_2Na_2$: 852.249); [CHG(*β*-Ala)$_5$]$_2$suberate diamide. Yield 18% ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.49 (d, J=8.4 Hz, 4H), 7.42 (d, J=8.4 Hz, 4H), 4.59 (dd, J=8.8, 4.4 Hz, 2H), 4.15 (dd, J=8.3, 4.8 Hz, 2H), 3.78 (d, J=17.3 Hz, 2H), 3.75 (d, J=17.3 Hz, 2H), 3.39 (m, 20H), 3.33 (dd, J=14.5, 4.4 Hz, 2H), 3.11 (dd, J=14.6, 8.9 Hz, 2H), 2.38 (m, 20H), 2.34 (m, 4H), 2.18 (t, J=7.3 Hz, 4H), 2.10 (m, 2H), 1.93 (m, 2H), 1.52 (m, 4H), 1.24 (m, 4H). HRMS (ESI) *m/z* 912.2955 $[M+H+Na]^{2+}$ (calc'd for $C_{72}H_{103}N_{18}O_{28}S_2Cl_2Na$: 912.295); [CHG(*β*-Ala)$_6$]$_2$suberate diamide. Yield 10% ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7)) δ 7.50 (d, J=8.7 Hz, 4H), 7.42 (d, J=8.7 Hz, 4H), 4.61 (dd, J=8.7, 4.4 Hz, 2H), 4.16 (dd, J=8.3, 4.7 Hz, 2H), 3.77 (d, J=17.1 Hz, 2H), 3.75 (d, J=17.1 Hz, 2H), 3.39 (m, 24H), 3.35 (dd, J=14.5, 4.5 Hz, 2H), 3.11 (dd, J=14.5, 8.9 Hz, 2H), 2.40 (m, 24H), 2.36 (m, 4H), 2.18 (t, J=7.3 Hz, 4H), 2.11 (m, 2H), 1.93 (m, 2H), 1.52 (m, 4H), 1.24 (m, 4H). HRMS (ESI) *m/z* 912.2955 $[M+H+Na]^{2+}$ (calc'd for $C_{78}H_{113}N_{20}O_{30}S_2Cl_2Na$: 912.295); [CHG(*β*-Ala)$_7$]$_2$suberate diamide. Yield 19% ^{1}H NMR (500 MHz, D_2O TRIS buffer, pD 8.7) δ 7.49 (d, J=8.7 Hz, 4H), 7.42 (d, J=8.7 Hz, 4H), 4.59 (dd, J=8.8, 4.4 Hz, 2H), 4.15 (dd, J=8.2, 4.6 Hz, 2H), 3.78 (d, J=17.2 Hz, 2H), 3.75 (d, J=17.2 Hz, 2H), 3.39 (m, 28H), 3.33 (dd, J=14.4, 4.4 Hz, 2H), 3.11 (dd, J=14.4, 8.9 Hz, 2H), 2.40 (m, 28H), 2.34 (m, 4H), 2.18 (t, J=7.3 Hz, 4H), 2.09 (m, 2H), 1.93 (m, 2H), 1.53 (m, 4H), 1.24 (m, 4H). A good quality MS of this compound could not be obtained. However, a high quality HRMS (ESI) of the [glycyl, glutamyl] tetra-O-ethyl ester of this compound (*m/z* 1099.9457 $[M+2H]^{2+}$ (calc'd for $C_{92}H_{140}N_{22}O_{32}S_2Cl_2$: 1099.9420) indirectly confirmed

the identity of [CHG(β-Ala)$_7$]$_2$suberate diamide. The tetra O-ethyl ester was prepared by incubating [CHG(β-Ala)$_7$]$_2$suberate diamide in ethanolic HCl (Zheng and Creighton, unpublished).

Inhibition kinetics. The inhibition constants (K_is) of the CHG derivatives with human and yeast GlxI were calculated from the change in the slopes of reciprocal plots of [GSH-methylglyoxal thiohemiacetal] versus the initial rates (ΔOD_{240}) of product formation (*S-D*-lactoylglutathione) at different fixed concentrations of inhibitor. For the different kinetic runs, the concentration of substrate thiohemiacetal was varied while the concentration of free glutathione was maintained at 0.2 mM, by changing the total concentrations of methylglyoxal and glutathione on the basis of the dissociation constant for the thiohemiacetal (K_d = 2.2 mM). Conditions: 50 mM sodium phosphate buffer, pH 7.0, 25 ºC. Apparent K_m values at different concentrations of inhibitor, from which the K_i values were calculated, were obtained by computer fitting the initial rate data to the Michaelis-Menten equation.

The inhibition constants for the remaining enzymes described in this study employed kinetic assays previously reported in the literature: bovine liver glyoxalase II with *S-D*-lactoylglutathione,[1] glutathione transferase with ethacrynic acid,[2] glutathione reductase with oxidized glutathione and NADPH[3].

References:

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