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Design, Synthesis and Biological Evaluation of Potent Human Glyoxalase I Inhibitors

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Several glutathione derivatives bearing the S-(N-aryl-N-hydroxycarbamoyl) or S-(C-aryl-N-hydroxycarbamoyl) moieties (10, 10', 13–15) were synthesized, characterized, and their human glyoxalase I (hGLO1) inhibitory activity was evaluated. Compound 10 was proved to be the effective hGLO1 inhibitor with a Ki value of 1.0 nM and the inhibition effect of compound 10 on hGLO1 was nearly ten-fold higher than that of the strongest inhibitor 2 (Ki=10.0 nM) which has been reported in the field of glutathione-type hGLO1 inhibitors. Its diethyl ester prodrug 10' was able to penetrate cell membrane and had good inhibitory effect on the growth of NCI-H522 cell xenograft tumor model.

Key words synthesis; glyoxalase I inhibitor; inhibition constant; cell membrane permeability study; NCI-H522 cell line

Glyoxalase system catalyzes the conversion of cytotoxic methylglyoxal (MG) to non toxic d-lactate via the intermediate S-d-lactoylglutathione.11 It is consisting of glyoxalase I (GLO1), glyoxalase II (GLO2), and reduced glutathione (GSH) (Fig. 1). Glyoxalase system is ubiquitously present in the cytosol of most all mammalian cells.2 A number of studies have provided strong evidence that the GLO1 inhibitor is a potent therapeutic target for many conditions, including diabetes and diabetic complications, cardiovascular disease, chronic renal disease, obesity, as well as multidrug resistant cancer chemotherapy.3

The past decades have witnessed tremendous advances in the discovery and development of GLO1 inhibitors.4–16 One of the most employed compounds are derivatives of S-(N-aryl-N-hydroxycarbamoyl) glutathione originally prepared by Creighton and colleagues.4–5 For example, compounds 1–3 are good inhibitors of human GLO1 (hGLO1), with reported comparable Ki value of 46, 10, and 14 nM, respectively (Fig. 2). Later, More and Vince7 described the first low-nanomolar tight-binding inhibitor 4 via replacement of sulfur atom by methylene. To our delight, compound 4 exhibited Ki value of 8 nM against hGLO1, which is determined by us recently. Some other important natural products have also been widely utilized as a hGLO1 inhibitor, such as delphinidin,9 anthocyanidin,9 methyl gerfelin (M-GFN),10 curcumin,11 and flavonoids.12 A few other promising hGLO1 inhibitors worth mentioning are novel 7-azaindole substituted N-hydroxyypyridones,13 4-bromoacetoxy-l-(S-glutathionyl)-acetoxy butane14 (4BAB) and chiral unnatural azide carboxylate.15 Another recent report has described potent GLO1 inhibitor based on derivatives of 2,4-diaminopyrimidine.16

Although significant achievement has taken place since Creighton’s S-(N-aryl-N-hydroxycarbamoyl) and Vince’s C-(N-aryl-N-hydroxycarbamoyl) glutathione compounds, such type molecules still remains hallmark in this area. Thus, from a synthetic point of view, development of this series molecule for hGLO1 inhibitors, albeit challenging, is still necessary. Herein, we disclose further modifications that we have made to molecule 10, 10' and 13–15. These changes were focused on preserving the N-hydroxycarbamoyl moiety and replacing the S atom by methylene or maintaining original form for S atom while simultaneously introducing different substituents into the phenyl ring in target compounds.

Results and Discussion

Chemistry The target molecule 10 was synthesized as illustrated in Chart 1. Reaction of compounds 5 with 6, employing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) in CH2Cl2 afforded intermediate 8, which subjected to deprotection to provide 10. According to the similar approach, 10' was prepared (Chart 1). Compound 6 has previously been reported17 (Fig. 2). On the other hand, intermediate 7 was synthesized from tert-butoxycarbonyl (Boc)-l-Glu(OBn)-OH using modified procedure17 (Fig. 3).

The S-(N-aryl-N-hydroxycarbamoyl) glutathione derivatives 13–15 were synthesized in three steps as depicted in Chart 2.
Different substituted hydroxylamines were treated with S-propylcarbonochloridithioate, followed by oxidation to give the 12, which were subsequently converted to the desired 13–15. The identities of all of the synthesized compounds were confirmed by 1H-NMR and high resolution (HR)-MS.

**Biological Activities** All of synthesized compounds were evaluated for *in vitro* enzyme activity against hGLO1 except 10’. Compound 2 was reported as a strongest hGLO1 inhibi-
tor with low inhibition constant \( K_i = 10.0 \text{ nM} \) in the field of glutathione-type hGLO1, thus it was used as a positive control in the current assay.

Compound 10 showed the most potent inhibitory activity against hGLO1, with a \( K_i \) value of 1.0 nM, which is 10-fold more potent than inhibitor 2 (Table 1). It should be noted that, to the best of our knowledge, no meta-substituted in the phenyl ring to hGLO1 has been reported in the area of glutathione-type hGLO1. Compound 14 displayed a little weaker hGLO1 inhibitory activity than 10, with a \( K_i \) value of 7.0 nM. Compound 13 exhibited moderate activity, with a \( K_i \) value of 19.2 nM. The \( K_i \) value of 15 is greater than 100 nM.

As shown in Fig. 4, compound 10 proved to be competitive inhibitor of GLO1 from human erythrocyte.

In order to explore the ability to penetrate the cell membrane, diethyl ester prodrug 10′ was synthesized as depicted in Chart 1. When the 10′ is incubated in KBM5 cell, hydrolysis occurs, leading to a mixture of the diester, monoester and free 10 (Figs. 5–7). These results indicated that 10′ can penetrate the cell membrane in as quickly as 2 min and reach a concentration of approximately 300 µM (Fig. 7), which is the concentration of inhibitor that should be effective against GLO1.

As shown in Fig. 8, compound 10′ was administered after 10d at a dose of 30, 80 and 160 mg/kg in NCI-H522 cell line, the tumor growth inhibition (TGI) values were obtained with 24, 28 and 32%, respectively. After 14d, the TGI values were observed with 34, 38 and 37%, which indicated that 10′ has

| Compound | \( K_i \) (nM) |
|-----------|-------------|
| 10        | 1.0±0.5     |
| 13        | 19.2±0.3    |
| 14        | 7.0±0.5     |
| 15        | 138±0.1     |
| 2         | 10.0        |

*a) Conditions: sodium phosphate buffer, 50 mM, pH=7.0, 25°C. *b) Positive control.
good inhibitory effect on the NCI-H522 cell xenograft model.

On the other hand, 10' was evaluated for tumor cell (KBMI7R) proliferation in vitro. Danusertib (IC_{50}=13.9 \mu M) was used as positive control in the assay. The result indicated that, 10' showed good inhibitory activity (IC_{50}=20.4 \mu M) at the level of the enzyme. However, with 20.4 \mu M of IC_{50}, there is still need for further improvement.

The crystal structure of mouse glyoxalase I (mGLO1) complexed with 10 has been deposited in the Protein Data Bank and assigned entry code 4OPN [For details see: Supplementary materials]. In Fig. 9, the binding mode of 10 with mGLO1 is closely similar to that of inhibitor 2 with hGLO1 (PDB code: 1QIN).

The only notable differences are at the phenylacetylene group of 10 and the iodophenyl ring of inhibitor 2. Although both groups insert into the hydrophobic pocket (Leu61A, Phe63A, Phe68A, Leu70A, Phe93A, Leu161B, Ile180B in mGLO1; Phe62A, Leu69A, Phe71A, Leu92A, Met179B, Met183B in hGLO1) of the active site, there is a 30 degree rotation of the phenylacetylene ring against the iodophenyl ring of inhibitor 2. With this rotation, the conjugated phenylacetylene group forms a strong offset π−σ interaction with mPhe93A (ca. 3.8 Å). In hGLOI, mPhe93A is replaced by hLeu92A, but it is very reasonable to assume that the side chain of hLeu92A forms a similar strong offset σ−π interaction with the phenylacetylene ring. Apparently, the interaction between hLeu92A (or mPhe93A) and the iodophenyl ring of inhibitor 2 is attenuated because of the direction of the iodophenyl ring.

**Experimental**

**Chemistry Materials and Methods** Commercial reagents and solvents were purchased from Tokyo Chemical Industry Co., Ltd., Japan, Energy Chemical, China and Adams-beta, China. All moisture or oxygen-sensitive reactions were carried out under an argon or nitrogen atmosphere in oven or heat-dried flasks. Reaction courses were monitored by TLC on silica gel-precoated F254 Merck plates. The TLC spots were visualized in UV (254 nm) and by treatment with alcoholic solution of phosphomolybdic acid, aqueous solution of KMnO₄ or with iodine cylinder. The products were purified by reverse-phase HPLC. The methods are as follows. Reverse-phase HPLC (Agilent technologies 1200 series) was run on Waters column (Xbridge@Prep C18, 5µm, OBDTM, 19×150 mm) using solvent system of water containing 0.1% trifluoroacetic acid–acetonitrile containing 0.1% trifluoroacetic acid=70:30 with 20 mL/min flow rate and detected at 254 nm. The ¹H- and ¹³C-NMR spectra were recorded in CD₃OD or D₂O solution on a Bruker AV 400 MHz spectrometer. Chemical shifts were denoted in ppm (δ), and calibrated by using residual undeterated solvent CH₃OD (3.31 ppm), H₂O (4.80 ppm) or tetramethylsilane (0.00 ppm) as internal reference for ¹H-NMR and the deuterated solvent CD₃OD (48.80 ppm) or tetramethylsilane (0.00 ppm) as internal standard for ¹³C-NMR. The HR-MS were measured on a Waters SYNAPT G2 mass spectrometer by means of the electrospray ionization (ESI) technique. The following abbreviations were used to explain the multiplicities: s=singlet, d=doublet, t=triplet, q=quartet, br s=broad singlet, dd=double doublet, m=multiplet.

**Inhibition Kinetics** The hGLO1 was provided by the School of Pharmaceutical Sciences, Sun Yat-Sen University, and the substrate methylglyoxal was purchased from Sigma-Aldrich Co., U.S.A. Commercial 40% methylglyoxal solution was distilled to remove polymerized contaminants, and then diluted with distilled water. Fresh solutions of glutathione (GSH) were prepared on the day of the assays with distilled, deionized water. The inhibition constants (K_{i}s) of the 10 and 13–15 with human GLO1 were obtained from the change in the initial rates of product formation at various concentrations of the substrate in the presence or absence of the inhibitor. Approximately 0.02 U of enzyme was added to a 1 mL cuvette containing 50 mM sodium phosphate buffer, pH 7.0, 25°C, and different concentrations of thiohemiacetal substrates with fixed concentrations of inhibitor. The initial rates of product formation were followed on the basis of the increase in absorbancy at 240 nm. Inhibition constants were obtained by fitting the initial rate data to the equation for competitive inhibition.

**Cell Permeability Studies** Compound 10' (0.10 mM) was added to separate suspensions of KBM5 cell (1.7×10⁶ cells/mL) in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C. Aliquots (1 mL) were re-

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**Fig. 9. Overlap of mGLO1-Inhibitor 10 (Cyan) and hGLO1-Inhibitor 2 (Blue) Structures**
moved from the cell suspensions as a function of time and each was overlayed onto 0.4 mL of silicone oil contained in an Eppendorf centrifuge tube and centrifuged at 13000×g (15 min) at 4°C. The supernatant and silicone oil were decanted from the cell pellet, and residual oil was removed from the inside of the centrifuge-tube with a cotton swab. One milliliter of 70% ethanol in water was added to the pellet, and the suspension was sonicated for 5 min. Denatured protein was removed by centrifugation (13000×g, 15 min), and the clear supernatant was brought to dryness under a stream of clean air. The resulting residue was fractionated by reverse-phase HPLC [Instrument: UPC10MSMS-003 (API-4000), Column: Waters BEH C18, 2.1×50 mm, 1.7µm, Oven temperature: 60°C, Flow rate: 0.60 mL/min, Retention time: 10, 1.07 min; 10′, 1.11 min; monoester of 10, 1.07 min, λ=259 nm], using a mobile phase composed of 45% methanol in water containing 0.1% formic acid. The concentrations of these species were interpolated from standard curves of integrated peak intensity, obtained from reverse-phase fractionation of KBM5 cell, versus the known amounts of 10, 10′ or monoester of 10 introduced into the cells prior to fractionation.

Measurement of Cell Growth Inhibition BALB/c nude mice were obtained from the Shanghai Sipper–BK Laboratory Animals Co., Ltd., China. Mice were used when 8 weeks old and were maintained in a laminar flow cabinet under specific-pathogen-free conditions. The NCI-H522 cell line was maintained in vitro at 37°C in a humidified 5% CO2 atmosphere in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal calf serum (FCS). Tumor volumes were calculated using longitudinal (L) and transverse (W) diameters using V=(L×W×L)/2, and tumor growth inhibition (TGI) percent values were calculated using the formula TGI=(1−TVsimple/TVcontrol)×100%, where TV is tumor volume. A value of p<0.05 was considered statistically significant.

Crystallization, Data Collection, and Model Building The expression, purification, and crystallization of mouse glyoxalase I (mGLO1) have been described previously. mGLO1 expression plasmid was a kind gift from Dr. Hideo Okumura, Advanced Science Institute, RIKEN, Japan. All compounds and reagents were purchased from Sigma-Aldrich Co. except 10. To obtain mGLO1–10 complex structure, mGLO1 crystals were soaked in a saturated solution of 10 containing 5% dimethyl sulfoxide, 50 mM 2-(N-morpholino)ethanesulfonic acid (MES, pH 5.8), 0.1 M NaCl, and 32% polyethylene glycol (PEG) 2000. The concentration of the 10 was about 10mM. Restrictions of 10 for structure refinement were prepared using Grade Web Server (http://grade.globalphasing.org). Coordinates and structure factors have been deposited in the RCSB Protein Data Bank (PDB) with entry code 4OPN.

General Procedure I for the Preparation of Target Compound 10 CF3-COOH (2 mL) was added to a stirred solution of compound 8 (0.5 mmol) in an ice cold CH3Cl (5 mL). The reaction mixture was stirred at room temperature for 2h, until the completion of the reaction as evidenced by TLC. The resulting reaction mixture was concentrated to dryness, purified by HPLC to give 10 as colorless oil. Yield: 82%, 1H-NMR (400 MHz, CD3OD) δ ppm: 7.74 (s, 1H), 7.64 (d, J=8.0 Hz, 1H), 7.34 (t, J=8.0 Hz, 1H), 7.27 (d, J=7.2 Hz, 1H), 4.47 (t, J=8.0 Hz, 1H), 4.02 (t, J=6.4 Hz, 1H), 3.93 (q, J=17.6 Hz, 2H), 3.51 (s, 1H), 2.81 (brs, 2H), 2.56 (t, J=8.0 Hz, 2H), 2.50−1.99 (m, 4H). HR-MS (ESI): Calcd for [C9H14N2O6+H]+: 449.1667. Found: 449.1665.

Using general procedure I, compound 10′ was prepared. The yield and spectra data of compound 10′ was given below.

Ethyl N′-(5)−{(2-(Ethoxy-2-oxoethyl)-5-((3-ethynylphenyl)(hydroxy)amino)-1,5-dioxopentan-2-yl)-l-glutamine (10′)

Yield: 55%; 1H-NMR (CD3OD, 400 MHz) δ ppm: 7.76 (s, 1H), 7.65 (d, J=6.8 Hz, 1H), 7.35 (d, J=8.0 Hz, 1H), 7.29 (d, J=5.6 Hz, 1H), 4.43−4.49 (m, 1H), 4.26−4.32 (m, 2H), 4.17 (q, J=7.2 Hz, 2H), 4.09 (t, J=6.4 Hz, 1H), 3.95 (q, J=17.6 Hz, 2H), 3.53 (s, 1H), 2.83 (brs, 2H), 2.54 (t, J=7.2 Hz, 2H), 2.11−2.27 (m, 3H), 1.98−2.07 (m, 1H), 1.31 (t, J=7.2 Hz, 3H), 1.26 (t, J=7.2 Hz, 3H). HR-MS (ESI): Calcd for [C29H24N4O8S]+: 505.2293. Found: 505.2296.

General Procedure II for the Preparation of Target Compound 13 Water (20 mL) solution of GSH (3.0 mmol) was added to a stirred solution of 12 (1.5 mmol) in MeOH−H2O (1:1, 20 mL). The reaction mixture was then neutralized to pH 8−9 with NaHCO3 and stirred for 2h at room temperature. The solution was adjusted to pH 3−4 by trifluoroacetic acid and purified by HPLC to give 13 as colorless oil. Yield: 39%; 1H-NMR (D2O, 400 MHz) δ ppm: 7.581−7.589 (m, 1H), 7.46−7.49 (m, 1H), 7.31−7.33 (m, 2H), 4.58 (dd, J=8.0, 5.2 Hz, 1H), 4.00 (t, J=6.4 Hz, 1H), 3.92 (s, 2H), 3.45 (s, 1H), 3.30 (dd, J=14.4, 4.8 Hz, 1H), 3.08 (dd, J=14.4, 8.0 Hz, 1H), 2.46−2.49 (m, 2H), 2.05−2.21 (m, 2H). HR-MS (ESI): Calcd for [C18H21N5O8S]+: 489.1051. Found: 489.1048.

Using general procedure II, compounds 14 and 15 were prepared. The yield and spectra data of each compound are given below.

N'-((R)−1-((Carboxymethyl)amino)-3-((4-ethylphenyl)-(hydroxy)carbamoyl)thio)-1-oxopropan-2-yl)-l-glutamine (14)

Yield: 20%; 1H-NMR (CD3OD, 400 MHz) δ ppm: 7.65 (d, J=8.8 Hz, 2H), 7.45 (d, J=8.8 Hz, 2H), 4.65 (dd, J=8.4, 4.8 Hz, 1H), 4.01 (t, J=6.4 Hz, 1H), 3.93 (s, 2H), 3.47 (s, 1H), 3.40 (dd, J=14.0, 4.8 Hz, 1H), 3.11 (dd, J=14.0, 8.8 Hz, 1H), 2.54 (t, J=7.2 Hz, 2H), 2.11−2.25 (m, 2H). HR-MS (ESI): Calcd for [C23H23N4O8S]+: 489.1051. Found: 489.1040.

N′-((R)−1-((Carboxymethyl)amino)-3-((4-cyanophenyl)-(hydroxy)carbamoyl)thio)-1-oxopropan-2-yl)-l-glutamine (15)

Yield: 48%; 1H-NMR (CD3OD, 400 MHz) δ ppm: 7.87 (d, J=9.2 Hz, 2H), 7.73 (d, J=9.2 Hz, 2H), 4.68 (dd, J=8.4, 5.2 Hz, 1H), 4.00 (t, J=6.4 Hz, 1H), 3.94 (s, 2H), 3.43 (dd, J=14.0, 4.8 Hz, 1H), 3.12 (dd, J=14.4, 8.4 Hz, 1H), 2.55 (t, J=6.8 Hz, 2H), 2.15−2.23 (m, 2H). HR-MS (ESI): Calcd for [C18H21N4O8S+Na]+: 468.1184. Found: 468.1191.

Conclusion

We have synthesized several S-(N-aryl-N-hydroxycarbamoyl) and C-(N-aryl-N-hydroxycarbamoyl) glutathione derivatives and evaluated their biological activity. Compounds 13 and 14 possessed 19.2 and 7.0 µM inhibitory activities against hGLO1, respectively. Compound 10 was identified as an optimal development of this series, and had excellent potency (K_i=1.0 nM) toward hGLO1, which was lower than that of inhibitor 2. In addition, its diethyl ester produg 10′ was able to penetrate cell membranes and had good inhibitory effect on the growth of NCI-H522 cell xenograft tumor model.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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