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Report

Preliminary *In Vitro* Biological Evaluation of a Strategy for Passive Intracellular Glutathione-Derived Glyoxalase I Inhibitor Delivery: A Novel Approach

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Derivatives of glutathione (γ-Glu-Cys-Gly) and glutathione-derived inhibitors of enzymes involved in glutathione-related metabolism can be used as therapeutic agents against various diseases. However, their intracellular delivery is difficult because of poor cell membrane permeability due to high hydrophilicity. Conventional design strategies to enhance cell membrane permeability include the esterification of carboxy groups in γ-Glu and Gly residues. However, our design strategy involves replacing a carboxy group in the Gly residue with a structurally similar but more lipophilic counterpart; the hydrolysis rate of a Gly ester residue in glutathione is slow. Herein, to preliminarily validate our new design strategy, we investigated the cellular uptake and intracellular metabolism of carboxy group-modified glyoxalase I inhibitors derived from glutathione, which were radiolabeled for tracing their behaviors. We synthesized and evaluated three radioiodinated model compounds [^{125}I]2–4 using NH_2-γ-Glu[-Dab(*N*-(*p*-bromobenzoyl)-*N*-hydroxyl)-Gly-OH]-OH (1) as a lead compound *in vitro*. [^{125}I]2, a conventional diester, features two carboxy groups esterified with cyclopentyl alcohol. A new design strategy was followed for [^{125}I]3 and [^{125}I]4, wherein the ester group at the Gly residue in [^{125}I]2 was replaced by a CH_2OH or CF_3 group. These compounds showed log $D_{7.4}$ values suitable for membrane permeability. Their cellular uptake increased in a time-dependent manner; however, that of [^{125}I]3 remained low. While the majority of metabolites of [^{125}I]2 comprised multiple unknown compounds, the main metabolite of [^{125}I]4 was monocarboxylic acid generated by the hydrolysis of an ester of γ-Glu residue. These results provide useful insights for developing glutathione-derived therapeutic agents.

Key words glutathione analog, intracellular delivery, cell membrane permeability, metabolism, glyoxalase I

INTRODUCTION

Glutathione (GSH: γ-Glu-Cys-Gly) is a ubiquitous tripeptide with multifunctional properties. GSH and GSH-related enzymes participate in the detoxification of products of oxidative stress and xenobiotics, and various regulatory processes, including cell signaling and apoptosis.[1,2] Therefore, not only derivatives of glutathione itself but also GSH-derived inhibitors of enzymes involved in GSH-related metabolism can be used as therapeutic agents against various diseases, such as neurodegenerative disorders,[3-5] cancer,[6,7] and infections.[8,9]

Glyoxalase I (GLO-I) is a key enzyme in the glyoxalase system involving GSH and plays a pivotal role in inhibiting apoptosis by metabolizing methylglyoxal. It is also implicated in carcinogenesis, malignancy, and anticancer drug resistance.[10] Consequently, ongoing studies have explored GLO-I inhibitors as potential new anticancer agents.[11,12] However, previously reported GLO-I inhibitors—GSH analogs—exhibit poor membrane permeability owing to high hydrophilicity, partly attributable to the two carboxy groups of the γ-Glu and Gly residues.[11,12] Therefore, to render these compounds suitable as therapeutic agents targeting intracellular proteins, they must be modified to decrease hydrophilicity and enhance membrane permeability.

Esterification is a conventional design strategy used to increase the lipophilicity of compounds with carboxy groups. Diester derivatives of GSH and GLO-I inhibitors and monoester derivatives of GSH, where only the carboxy group of the Gly residue is esterified, have been suggested as molecules that can efficiently translocate through cell membranes.[13-15] However, esterification of the carboxy group of the Gly residue might be unsuitable because, in GSH derivatives, the γ-Glu ester is rapidly cleaved; the cleavage of the Gly ester

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is slow.[16] Thus, GSH derivatives are likely to be excreted extracellularly as Gly monoesters before their deesterification to dicarboxylic acids which can interact with target proteins. In particular, the extracellular excretion of Gly monoester can occur easily under *in vivo* conditions because the extracellular concentration of GSH derivatives is rapidly decreased following clearance from the blood, unlike in *in vitro* conditions, where the extracellular concentration is maintained. Therefore, the development of a new design to increase the lipophilicity of GSH analogs may be useful.

One strategy to address this problem is the replacement of the carboxy groups of γ-Glu and Gly residues with an ester and a lipophilic group possessing similar biological properties to a carboxy group, respectively. Therefore, in this study, as a preliminary validation of the new design strategy for passive intracellular delivery of a GLO-I inhibitor, we aimed to evaluate the effect of substitution of one ester to another lipophilic group in a conventional diester derivative. To this end, we investigated the cellular uptake and intracellular metabolism of three model compounds based on GLO-I inhibitor **1**, which shows high stability against γ-glutamyltransferase participating in extracellular GSH breakdown.[17] One of the model compounds is a conventional diester, designated as [^{125}I]**2**, whereby the bromine in **1** was replaced by radioiodine to facilitate tracing the metabolic fate of the compound in cells. Additionally, cyclopentyl alcohol was used to esterify the two carboxy groups because the cyclopentyl diester of GLO-I inhibitors can diffuse readily across cell membranes.[15] The other two model compounds, [^{125}I]**3** and [^{125}I]**4**, were developed following our new design strategy. In these compounds, the ester group at the Gly residue in [^{125}I]**2** is replaced with CH_2OH and CF_3 groups, respectively. The CH_2OH group was selected to evaluate the effect of lipophilicity because it is more lipophilic than the COOH group.[18,19] The CF_3 group was selected because fluorine is a more lipophilic and classical bioisostere of oxygen.[18,20] These membrane-permeable forms [^{125}I]**2**, [^{125}I]**3**, and [^{125}I]**4** were designed to undergo intracellular hydrolysis for conversion into the metabolite forms [^{125}I]**5**, [^{125}I]**6**, and [^{125}I]**7**, respectively (Fig. 1). In this study, we synthesized these model compounds and evaluated their lipophilicity, cellular uptake, and intracellular metabolism *in vitro*.

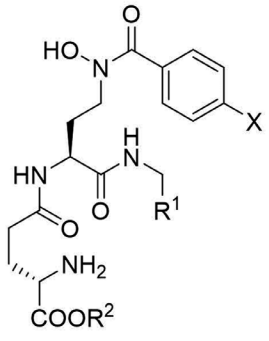
MATERIALS AND METHODS

Synthesis Compound **1** was synthesized following the method described by More *et al.*[17] [^{125}I]**2**–**7** and their non-radioactive counterparts **2**–**7** were synthesized as described in Supplementary Materials.

Partition Coefficient Measurement Each [^{125}I]**2**–**7** solution in PBS (185 Bq, pH 7.4, 500 μL) was mixed with 500 μL of 1-octanol. The mixture was vortexed for 20 s and allowed to stand for 40 s. After this procedure was repeated thrice, the mixture was centrifuged (2,000 × *g*, 1 min). A 350 μL aliquot of each phase was measured with a γ-counter (2470 WIZARD^2, PerkinElmer Inc., Shelton, CT, USA). The partition coefficient was subsequently calculated based on the ratio of radioactivity in 1-octanol and PBS layers.

Cellular Uptake Analysis U251MG cells (1 × 10^5 cells) were seeded in 24-well collagen I-coated plates. After 48 h, the medium was removed, and the cells were washed with 500 μL of cell culture medium. Subsequently, the cells were incubated with [^{125}I]**2**–**7** dissolved in cell culture medium (1.85 kBq, 500 μL) at 37°C for 5, 10, and 30 min. Following incubation, the medium was removed, and the cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (D-PBS) (-) (FUJIFILM Wako Pure Chemical Co., Tokyo, Japan) with 0.1% Tween 20 (500 μL). The cells were lysed with 0.3 M NaOH (500 μL). Radioactivity of each cell lysate was measured using a γ-counter. The protein concentration of each cell lysate was determined using the Micro-BCA assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) using bovine serum albumin as a protein standard. Results are presented as percentage dose per milligram protein (%dose/mg protein). Values are expressed as mean ± SD (n = 3). In the Student's unpaired *t*-test, *P* values less than 0.05 were considered to represent statistical significance.

Analysis of Intracellular Metabolites U251MG cells were seeded in a 150 mm cell culture dish at 5 × 10^6 cells and cultured at 37°C for 48 h. After the EMEM medium was removed, EMEM medium (10 mL) containing [^{125}I]**2** or [^{125}I]**4** (18.5 kBq) was added to the dish, and cells were incubated at 37°C for 30 min. Subsequently, the medium was removed, and the cells were washed twice with 5 mL of ice-cold D-PBS containing 0.1% Tween 20. Furthermore, 1 mL of ice-cold D-PBS



Compound		X	R^1	R^2
1		Br	COOH	H
Membrane-permeable form	[^{125}I]2	^{125}I	COO–	–O
	[^{125}I]3	^{125}I	CH_2OH	–O
	[^{125}I]4	^{125}I	CF_3	–O
Metabolite form	[^{125}I]5	^{125}I	COOH	H
	[^{125}I]6	^{125}I	CH_2OH	H
	[^{125}I]7	^{125}I	CF_3	H

Fig. 1. Chemical Structures of GLO-I Inhibitor **1** as a Lead Compound, Membrane-Permeable Forms [^{125}I]**2**, [^{125}I]**3**, and [^{125}I]**4**, and Their Metabolite Forms [^{125}I]**5**, [^{125}I]**6**, and [^{125}I]**7**

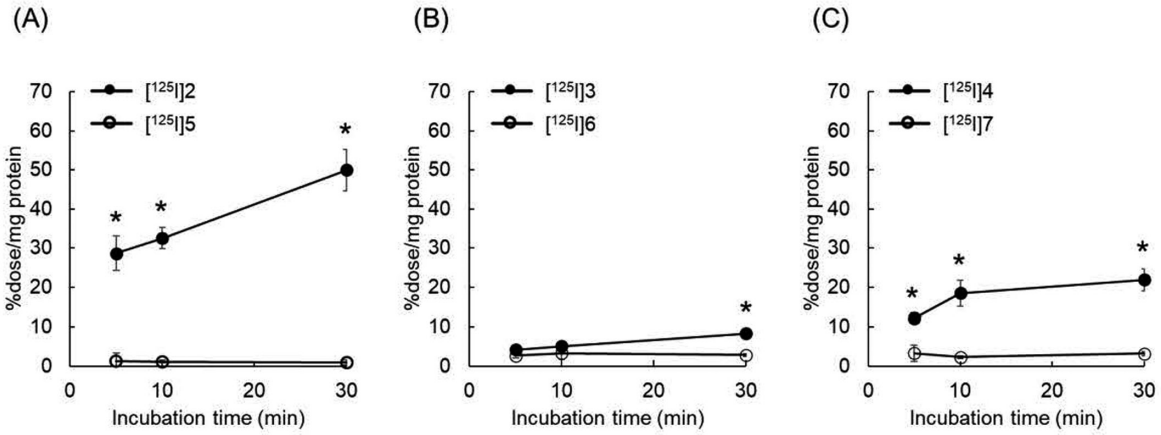


Fig. 2. Uptake of Membrane-Permeable Forms [^{125}I]**2**–**4** and Their Metabolite Forms [^{125}I]**5**–**7** in U251MG Cells

(A) [^{125}I]**2** and its metabolite form [^{125}I]**5**. (B) [^{125}I]**3** and its metabolite form [^{125}I]**6**. (C) [^{125}I]**4** and its metabolite form [^{125}I]**7**. Data represent means ± SD. Significance was determined by Student's *t*-test (*P<.01)

containing 0.1% Tween 20 was added, and cells were collected using a cell scraper. The cells were centrifuged at 2,000 × *g* for 1 min, and the supernatant was removed. To the cell pellet, 1 mL of ice-cold radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) with 5 μL of a protease inhibitor cocktail (Merck KGaA, Darmstadt, Germany) was added. The mixture was sonicated five times for 30 s at 30 s intervals at 4°C using a Q125 Sonicator (QSONICA L.L.C., Newton, MA, USA) as an ultrasonic homogenizer, followed by incubation for 20 min on ice. To 300 μL of cell lysate, 200 μL of methanol was added, vortexed for 1 min, and centrifuged at 2,000 × *g* for 1 min. The supernatants were analyzed by reverse-phase HPLC (RP-HPLC). The mobile phase of the RP-HPLC comprised 0.1% TFA/H_2O-methanol, with the percentage of methanol increasing linearly from 40% to 100% over 30 min at a flow rate of 2.0 mL/min. Eluates were collected in one-minute intervals, followed by measuring the radioactivity of fractions using a γ-counter.

RESULTS AND DISCUSSION

Synthesis Non-radioactive compounds **2**–**7** were obtained with overall yields of 0.15%, 0.68%, 0.35%, 13%, 2.1%, and 0.54%, respectively. The choice of ^{125}I was based on its relatively long half-life and low energy γ-rays, providing experimental convenience. [^{125}I]**2**–**7** were obtained with radiochemical yields of 33%, 11%, 22%, 38%, 8%, and 18%, respectively, and radiochemical purities of 95%, >99%, 97%, >99%, 99%, and 97%, respectively.

Lipophilicity and Cellular Uptake To assess lipophilicity, a key factor related to cell membrane permeability, we measured partition coefficients in 1-octanol-PBS (Table 1). The log $D_{7.4}$ values of metabolite forms [^{125}I]**5**–**7** were below 1, while those of membrane-permeable forms [^{125}I]**2**–**4**, where two carboxy groups were modified, were within the range 1–3. These values are considered suitable for membrane permeation.[21,22]

Therefore, to assess whether [^{125}I]**2**–**4** could translocate through cell membranes, cellular uptake experiments were performed using *in vitro* cultured cells. While the uptake levels of metabolite forms [^{125}I]**5**–**7** remained low, those of membrane-permeable forms [^{125}I]**2**–**4** increased in a time-dependent manner (Fig. 2). However, uptake of [^{125}I]**3** was notably low, attributed to its low log $D_{7.4}$ value. These results indicate that the monoester of the GSH analog would require appropriate modification of the other carboxy group to efficiently permeate the cell membrane.

Table 1. Partition Coefficients of [^{125}I]**2**–**7** in 1-octanol-PBS

Compounds	Membrane-permeable form		
	[^{125}I]**2**	[^{125}I]**3**	[^{125}I]**4**
log $D_{7.4}$	1.79 ± 0.03	1.30 ± 0.04	1.47 ± 0.05
Compounds	Metabolite form		
	[^{125}I]**5**	[^{125}I]**6**	[^{125}I]**7**
log $D_{7.4}$	-2.03 ± 0.06	-1.06 ± 0.06	0.05 ± 0.01

Intracellular Metabolites We analyzed the chemical forms of the radioactivity in cells after incubation with [^{125}I]**2** and [^{125}I]**4**, excluding [^{125}I]**3** owing to its low cellular uptake.

The cleavage of the Gly ester in GSH diethyl ester is slow,[16] so we speculated that [^{125}I]**2** would yield three main compounds: intact [^{125}I]**2** (Fig. 3 C), its metabolite form [^{125}I]**5** (Fig. 3 B), and a monocarboxylic acid in which the Gly ester is maintained. Contrary to our expectations, we observed multiple peaks, and most radioactivity was derived from various unknown chemical forms (Fig. 3 A). It has been reported that carboxypeptidases exist in cytosole.[23] Such known cytosolic enzymes and/or unknown ones could be responsible for the unexpected degradation of [^{125}I]**2** and [^{125}I]**5**. These results demonstrate that [^{125}I]**2** and [^{125}I]**5** are unstable in cells, suggesting that the conventional diester is unsuitable as a therapeutic agent. However, following incubation with [^{125}I]**4**, radio-HPLC analysis (Fig. 3D) showed that most radioactivity corresponded to retention times of the metabolite form [^{125}I]**7** and unchanged [^{125}I]**4**, eluted at 18.5 min and 22.5 min, respectively (Fig. 3E and F). These results demonstrate that the main intracellular metabolite of [^{125}I]**4** was [^{125}I]**7**, as expected. These findings suggest that replacing the carboxy group in GLO-I inhibitors with a structurally similar group at the Gly residue can result in compounds with high intracellular stability.

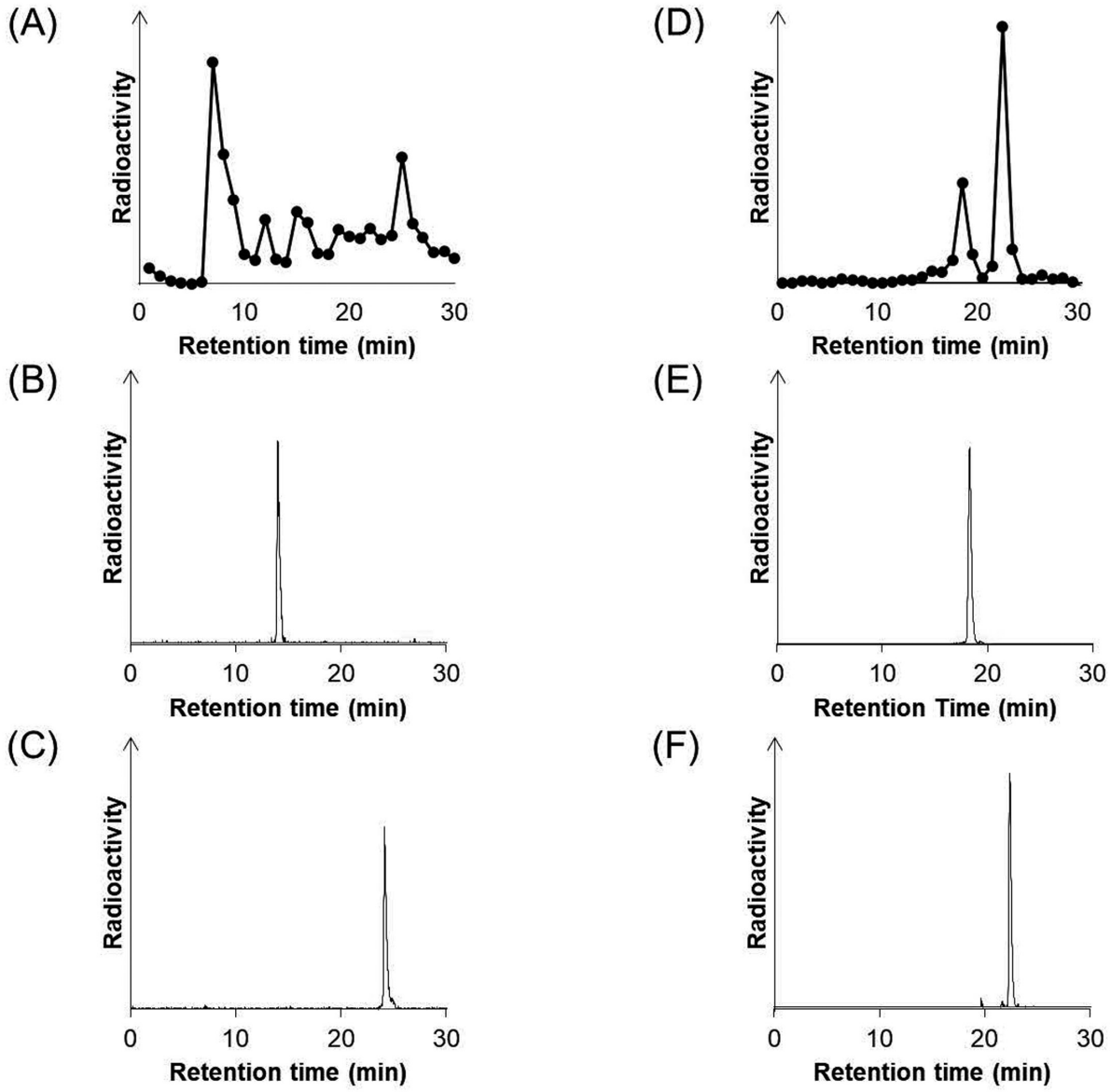


Fig. 3. HPLC Analysis of Radioactivity in Cells after Incubation with Membrane-Permeable Forms [^{125}I]**2** and [^{125}I]**4**

Cell lysate after incubation with [^{125}I]**2** (A) and [^{125}I]**4** (D), and intact [^{125}I]**5** (B), [^{125}I]**2** (C), [^{125}I]**7** (E), and [^{125}I]**4** (F) as references.

CONCLUSION

This study focused on evaluating the effect of replacing the conventional diester with the newly designed compound on cellular uptake and metabolism using model compounds [^{125}I]**2**–**4**. Although molecular size increased when bromine was substituted with iodine, the comparison remained valid because [^{125}I]**2**–**4** exhibited distinct uptake and metabolic behaviors. The results indicate that, to develop an intracellular delivery strategy for GLO-I inhibitors, replacing the carboxy groups of the γ-Glu and Gly residues with an ester and a lipophilic moiety that mimics a carboxy group, respectively, is advantageous. Further investigations, including the optimization of metabolite forms for intracellular target interaction, are necessary to advance GLO-I inhibitors as therapeutic agents based on our design strategy. When selecting bioisosteres, the substitutions of carboxy groups with nonclassical bioisosteres, such as tetrazole and sulfonamide,[24] and classical bioisosteric substitutions should be evaluated; additionally, careful consideration of lipophilicity is essential, as [^{125}I]**2**–**4** demonstrated markedly different cellular uptake levels corresponding to its log $D_{7.4}$ values ranging from 1.30 to 1.79. Successful development of GLO-I inhibitors through these studies would encourage applying our design strategy to a wider range of GSH-related compounds, including GSH itself.

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Conflict of interest The authors declare no conflict of interest.

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