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Structure–activity relationship of human GLO I inhibitory natural flavonoids and their growth inhibitory effects

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Abstract—Glyoxalase I (GLO I) is the rate-limiting enzyme for detoxification of methylglyoxal (MG), a side product of glycolysis, which is able to induce apoptosis. Since GLO I is known to be highly expressed in the most tumor cells and little in normal cells, specific inhibitors of this enzyme have been expected as effective anticancer drugs. The purpose of this study is a good construction of the human GLO I/inhibitor pharmacophore to obtain unique human GLO I inhibitory seed compounds for the development of useful anticancer drugs. Here, we selected natural flavonoid compounds that possess a plane configuration of *cis* C-4 ketone and C-5 hydroxy groups as the substrate (MG) transition-state mimetic structure. These compounds were examined the inhibitory abilities to human GLO I activity and analyzed their structure–activity relationships to determine an important pharmacophore of flavonoids for the human GLO I binding. Our results point to the contribution of hydroxy groups at the B ring of flavonoids to the effective inhibition of the human GLO I. Based on the binding mode of flavonoids, we constructed the human GLO I/inhibitor pharmacophore. This work delivers the first three-dimensional (3D) structural data and explains certain flavonoids interact specifically with the human GLO I.

1. Introduction

Glyoxalase I (GLO I) is a key enzyme in pathways leading to the detoxification of methylglyoxal (MG), one of the side products of glycolysis, which is highly reactive with DNA and proteins and thereby induces apoptosis.[1] GLO I catalyzes the conversion of cytotoxic MG (as the glutathione (GSH) thiohemiacetal) to nontoxic *S*-D-lactoylglutathione[2,3] via a proton-transfer mechanism involving a Zn^{2+}-bound enediolate intermediate [GS] C(OH) = C(O^-)CH3, where [GS] equals glutathionyl.[4] This enzyme system is ubiquitously distributed in all mammalian cells and is involved in tissue maturation and cell death.[5]

Abnormal expression or higher activity of GLO I has been demonstrated in many human tumors including colon, prostate, and lung.[6–8] Moreover, GLO I has been shown to be highly expressed in anti-tumor agent-resistant human leukemia cells.[9] The overexpression of GLO I confers resistance to anti-tumor agents, such as Etoposide and Adriamycin.[9] These observations indicate that the increase of GLO1 expression is closely associated with carcinogenesis[6–8] and drug resistance.[9] Thus, inhibitors of GLO I are expected to offer possibility for inhibiting carcinogenesis and overcoming drug resistance, because the inhibition of GLO I activity has potential ability to induce elevated concentrations of apoptosis-inducible MG in tumor cells.[10]

Inhibitors of GLO I have long been sought as possible anticancer agents, which selectively kill drug-resistant tumors especially overexpressed GLO I. In support of this hypothesis, a GSH analog, *S*-*p*-bromobenzylglutathione (BBG)[8] inhibits the growth of murine and human tumors in laboratory mice at dosing levels of 80 and 100 mg/kg, respectively. However, the ubiquity of GSH in nature means that it is likely to fall prey to a number

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of destructive enzyme systems (e.g., cleavage by γ-glutamyl transpeptidase), limiting the usefulness of the native GSH structure as a model for drug design in in vivo using.

Human GLO I is a homodimeric Zn^{2+} metalloenzyme with one active site per monomer, whereas yeast GLO I is a monomeric enzyme with two active sites.[11,12] Since human GLO I is not commercially available, we produced recombinant His-tagged human GLO I protein in the Baculovirus expression system. In the aspect of the protein structural differences, recombinant human GLO I (rhGLO I) is suitable to discover seed and lead compounds for development of GLO I inhibitory anticancer drugs against human cancers.

So far, several natural compounds, such as flavones and coumarins, have been reported to inhibit GLO I.[13,14] In order to obtain the information of GLO I/inhibitor pharmacophore for in silico design of GLO I inhibitors, we selected natural flavonoid compounds which possess a plane configuration of *cis* C-4 ketone and C-5 hydroxy groups as the substrate (MG) transition-state mimetic structure. We evaluated the inhibitory effects of these natural flavonoid compounds on human GLO I activity and analyzed the structure–activity relationship of these compounds to obtain the pharmacophore information of the human GLO I/inhibitor interaction. Our data provide new information of the human GLO I/inhibitor pharmacophore from the specific interaction of certain flavonoids with the human GLO I and the role that flavonoids may play in cancer treatment and prevention.

2. Results

2.1. Evaluation of in vitro human GLO I inhibitory effect of the natural flavonoid compounds which possess C-4 ketone and C-5 hydroxy groups

In order to obtain a good pharmacophore information of the human GLO I/inhibitor binding, we selected here several natural flavonoid compounds that possess a plane configuration of *cis* C-4 ketone and C-5 hydroxy groups (the distance between these groups is 2.8 Å) as the substrate (MG) transition-state mimetic structure (Fig. 1A). These compounds are predicted to mimic the enediolate intermediate (the distance between ketone and hydroxy groups is approximately 2.5 ± 0.5 Å) that forms along the reaction pathway of GLO I (Fig. 1B).

Since the pure human GLO I protein was needed for evaluating inhibitory activities in in vitro GLO I assay, we produced recombinant His-tagged human GLO I protein (rhGLO I) in the Baculovirus expression system. The rhGLO I was effectively inhibited by *S-p*-bromobenzylglutathione (BBG), a well-known inhibitor of GLO I (Fig. 2). The IC_{50} of this control inhibitory compound was calculated to be 23 μM in our in vitro assay system.

To evaluate the inhibitory effects on the human GLO I of the selected natural flavonoid compounds shown in Figure 3 (Baicalein, Baicalin, Kaempferol, Hyperin, Luteolin, Myricetin, Naringenin, Oroxylin A glucuronide, and Quercetin), our in vitro GLO I assay was performed with rhGLO I. Among these compounds, Baicalein, Kaempferol, Luteolin, Myricetin, and Quercetin showed very effective inhibition on rhGLO I at 100 μM (Fig. 4). Naringenin showed 50% inhibition of rhGLO I at 100 μM. In contrast, Baicalin, Hyperin, and Oroxylin A glucuronide were found to be very poor inhibitors.

We next evaluated the dose-dependency and determined the IC_{50} values of the potential inhibitors, Baicalein, Kaempferol, Luteolin, Myricetin, and Quercetin. As shown in Figure 5, the IC_{50} values of these compounds are 11.0, 20.6, 7.7, 0.56, and 3.2 μM, respectively. These results suggested that the hydroxy groups at the B ring of flavonoids greatly contribute to the human GLO I inhibitory activity of the flavonoid compounds.

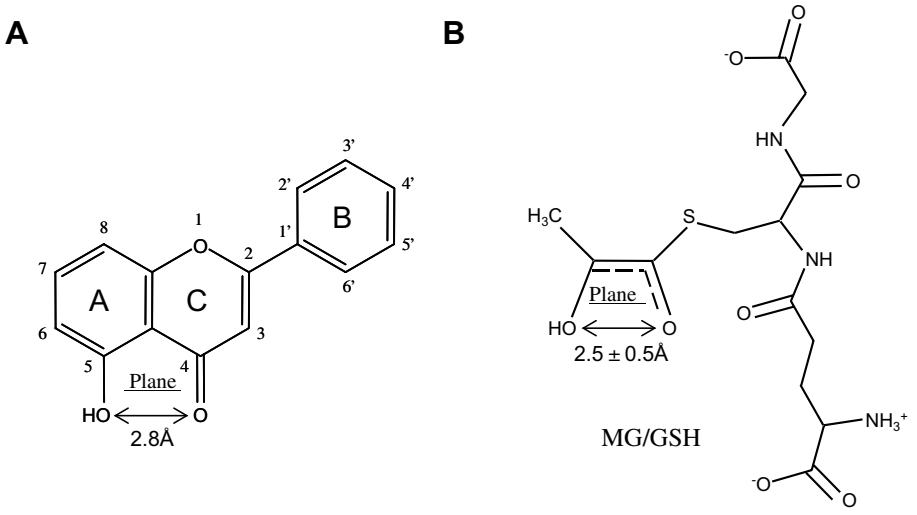


Figure 1. A basic structure of natural flavonoids and the enediolate intermediate that forms along the reaction pathway of GLO I. (A) A basic structure of natural flavonoid compounds. (B) A transition-state of the reaction between methylglyoxal (MG) and glutathione (GSH) on GLO I.

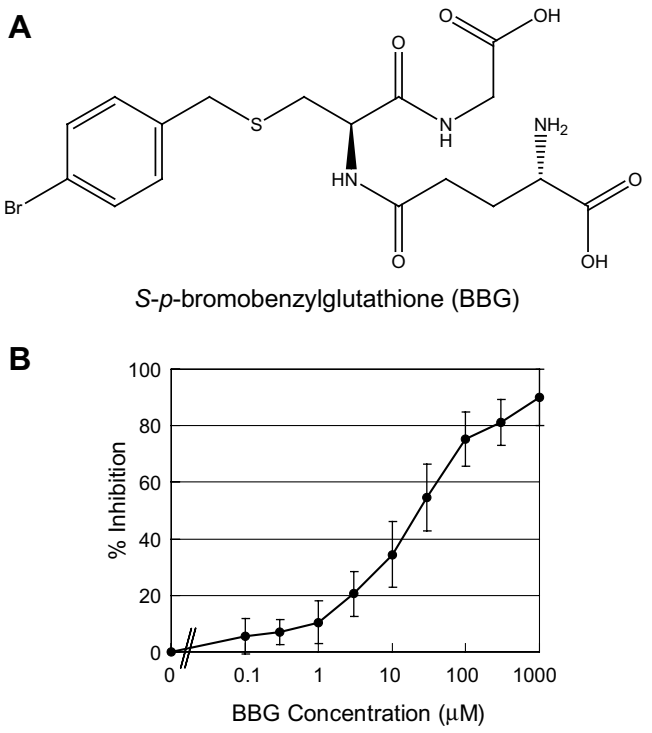


Figure 2. The inhibitory effect of BBG on rhGLO I activity. (A) *S-p*-bromobenzylglutathione (BBG). (B) The inhibitory effect of BBG on rhGLO I was investigated as described under Section 4. Data are the averages of three independent experiments and *bars* show the SD values.

2.2. Antiproliferative effects of the human GLO I inhibitory flavonoid compounds on HL-60 cells (MTS assay)

BBGC is a potent cell-permeable inhibitor of GLO I.[1,20] Previous report demonstrated that BBGC sensitized drug-resistant leukemia cells to Etoposide.[9] Also, BBGC itself was reported to have antiproliferative activity in human leukemia cells.[1] These results indicate that the human GLO I inhibitors have potential as therapeutic agents against human leukemia cells.

To test whether the human GLO I inhibitory flavonoid compounds are also effective in suppressing growth of human promyelotic leukemia HL-60 cells, we examined the antiproliferative effect of those compounds on HL-60 cells. BBGC, as a positive control, effectively suppressed the growth of HL-60 cells (Fig. 6). Importantly, the flavonoid compounds that showed the human GLO I inhibitory effects by in vitro assay suppressed the growth of HL-60 cells (Fig. 6). In contrast, the compounds which have little inhibitory effect on the human GLO I activity in vitro did not suppress cell growth. Good correlations were observed between the antiproliferative activities and the in vitro IC_{50} values of Myricetin, Quercetin, and Kaempferol. These results indicate that the human GLO I inhibitory flavonoid compounds suppress the growth of HL-60 cells, and that their antiproliferative effects were related to the in vitro human GLO I inhibitory effects.

3. Discussion

In this report, we evaluated the inhibitory effects on the human GLO I of several natural flavonoid compounds which possess a plane configuration of *cis* C-4 ketone and C-5 hydroxy groups as the substrate (MG) transition-state mimetic structure. From our results of in vitro rhGLO I assay, the order of inhibitory potencies of Myricetin, Quercetin, and Kaempferol was Myricetin > Quercetin > Kaempferol. These results suggest that the hydroxy groups at the B ring (Fig. 3, red circles) contribute to the inhibitory effects on rhGLO I. Luteolin showed stronger inhibition than Kaempferol. This result suggests that the C-5′ hydroxy group is seemed to contribute greatly to the inhibitory effects (Fig. 3, red circles at the B ring). However, the possibility that C-3 hydroxy group may reduce the inhibitory effects is not excluded. Hyperin showed weaker inhibition than Quercetin. This indicates that the bulky functional groups at C-3 sites reduce the inhibitory effects on rhGLO I (Fig. 3, blue circles). Baicalin showed weaker inhibition than Baicalein, suggesting that the bulky functional groups at C-7 sites reduce the inhibitory effects on rhGLO I (Fig. 3, blue circles). Oroxylin A glucuronide showed weaker inhibition than Baicalin. This result indicates that the replacement of C-6 hydroxy group with a methoxy group reduces the inhibitory effects (Fig. 3, blue circles). Naringenin showed 50% inhibition at 100 μM, whereas Kaempferol showed 60% inhibition. This implies that the double bond between C-2 and C-3 is not necessary to the inhibitory effects (Fig. 3, a green circle).

We next evaluated the effects of the human GLO I inhibitory flavonoid compounds on the proliferation of HL-60 cells. Expectedly, the flavonoid compounds that possess the in vitro rhGLO I inhibitory activities suppressed the growth of HL-60 cells. The good correlations were observed between antiproliferative activities and in vitro IC_{50} values of Myricetin, Quercetin, and Kaempferol. The human GLO I inhibitory flavonoid compounds showed stronger inhibition than BBG in vitro, however, antiproliferative activity of these compounds was weaker than that of BBGC. BBGC is a cyclopentyl diester form of BBG, which can cross the cell plasma membranes, and is de-esterified within the cytoplasm to form BBG, which in turn inhibits the human GLO I. Therefore, the weak antiproliferative effects of these flavonoid compounds may be attributed to their poor cell permeabilities. These observations indicate that the human GLO I inhibitory flavonoid compounds may be able to suppress growth of tumor cells, and that the anti-tumor effects may be due to the induction of apoptosis with accumulated MG by GLO I inhibition.

The purpose of our study is to obtain ultimately the human GLO I specific inhibitors for anticancer drugs. Flavonoid structure is probably useful for designing human GLO I specific inhibitors. Based on the experimental data in this report, we constructed the pharmacophore for in silico designing of the human GLO I inhibitors. From our results of in vitro GLO I assay (Fig. 5), the hydroxy groups at the B ring in the basic structure shown in Figure 1A were considered to greatly contrib-

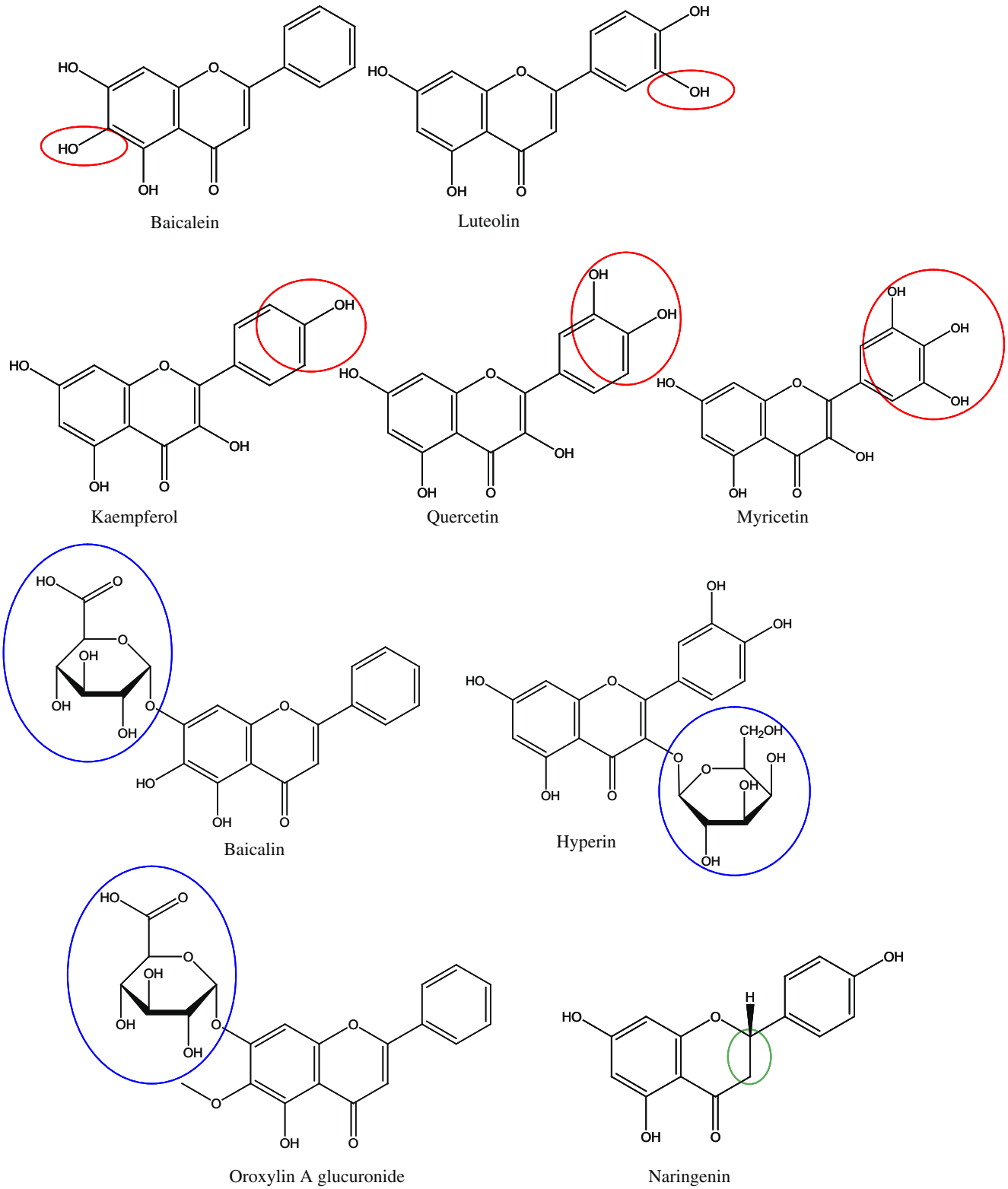


Figure 3. The natural flavonoid compounds that possess a plane configuration of *cis* C-4 ketone and C-5 hydroxy groups. Red circles show the hydroxy groups which contribute to the inhibitory effects on the human GLO I activity. Blue circles show the groups that reduce the inhibitory effects on the human GLO I activity.

ute to the human GLO I inhibitory activity of the flavonoid compounds. Therefore, these hydroxy groups are suggested to interact with the polar amino acid existing at S2 hydrophilic pocket (Fig. 7). In this binding mode, the three polar atoms (O and N–H atoms on Asn103B, N–H atom on Arg37B) on the human GLO I (PDB code 1FRO) are capable of forming hydrogen bonds to the compounds. Based on the above information, we con-

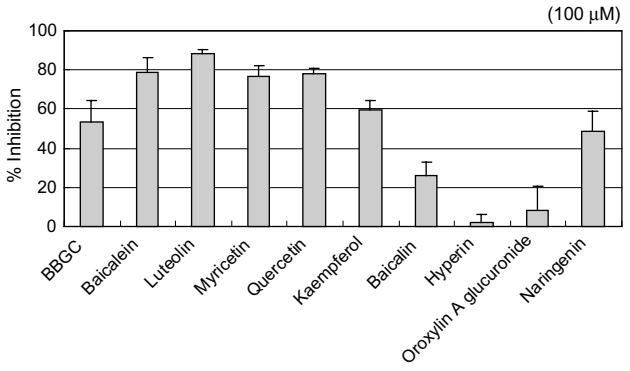


Figure 4. The human GLO I inhibitory effects of the flavonoid compounds. The inhibitory effects of the flavonoid compounds at 100 μM to rhGLO I were investigated as described under Section 4. Data are the averages of three independent experiments and *bars* show the SD values.

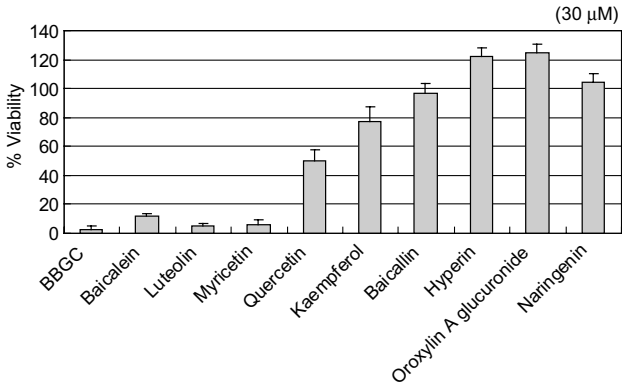


Figure 6. The antiproliferative effects of the human GLO I inhibitory flavonoid compounds. HL-60 cells were treated with 30 μM BBGC or the human GLO I inhibitory flavonoid compounds for 48 h. % Viabilities were measured by using MTS assay. Data are the averages of three independent experiments and *bars* show the SD values.

structed the GLO I/inhibitor pharmacophore presented in Figure 8. In order to refine this pharmacophore, we are trying to evaluate the structure–activity relationship of several unnatural synthetic flavonoid(-like) compounds that possess a plane configuration of *cis* C-4 ketone and C-5 hydroxy groups. Now, structure-based virtual screening (SBVS) and structure-based drug design (SBDD) of the human GLO I inhibitory lead compounds that have novel structures are under investigation.

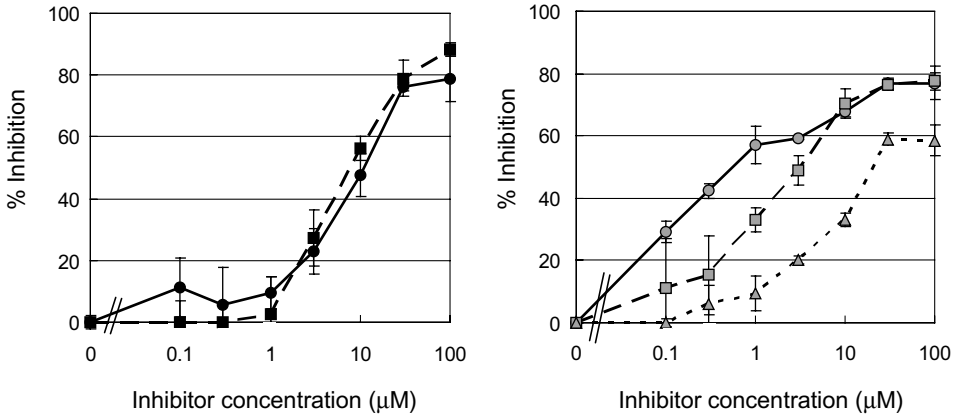
4. Experimental

4.1. Materials

S-p-Bromobenzylglutathione (BBG) and *S-p*-bromobenzylglutathione cyclopentyl diester (BBGC) were generous gifts from Taiho Pharmaceutical Co., Ltd (Tokyo, Japan). Baicalein, Baicalin, Kaempferol, Hyperin, Luteolin, Myricetin, Naringenin, Oroxylin A glucuronide, and Quercetin were purchased from Sigma and Fluka. All other chemicals were of reagent grade.

4.2. Expression and purification of recombinant His-tagged GLO I protein in the Baculovirus expression system

Human GLO I was cloned into the insect donor vector pAcHLT-A (BD Biosciences) giving rise to an N-terminal hexahistidine tag to aid in purification. The Sf21 insect cell line *Spodoptera frugiperda* was cultured in Grace's insect medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) at 27 °C. Following the BaculoGold system protocol, the Sf21 cells were cotransfected with BaculoGold™ DNA (BD Biosciences) and recombinant transfer vector pAcHLT-A/hGLOI. The recombinant virus stock was amplified by several rounds of culture, and a high titer virus stock solution was harvested and used to infect Sf21 cells. Protein purification was performed following the manufacturer's protocol (BD Biosciences). Briefly, 2×10^7 cells



	Baicalein	Luteolin	Myricetin	Quercetin	Kaempferol
IC_{50}	11.0 μM	7.7 μM	0.56 μM	3.2 μM	20.6 μM

Figure 5. The dose-dependencies and IC_{50} values of the potential flavonoid inhibitors of rhGLO I, The dose-dependencies and IC_{50} values of rhGLO I inhibitory flavonoid compounds, Baicalein (—●—), Luteolin (—■—), Myricetin (—○—), Quercetin (– □ –), and Kaempferol (···△···), were measured by in vitro GLO I assay as described under Section 4. Data are the averages of three independent experiments and *bars* show the SD values.

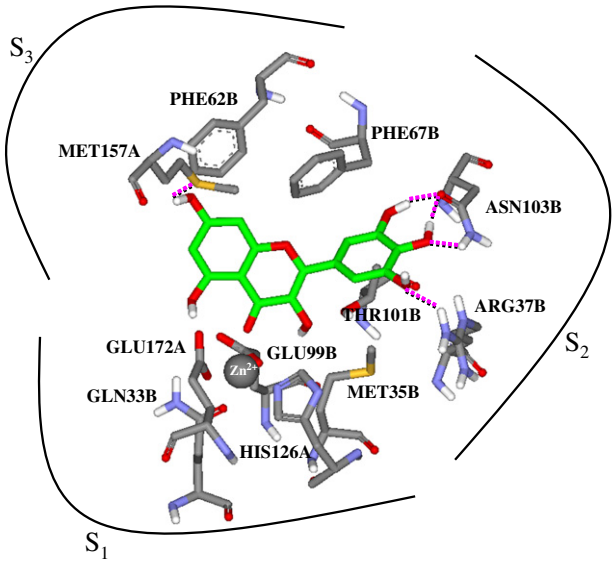


Figure 7. The predicted binding mode of Myricetin on the human GLO I (PDB code 1FRO). The putative binding mode was obtained from computational molecular docking study as described in Section 4. Carbon, nitrogen, oxygen, sulfur, and hydrogen are shown in green (Myricetin)/gray (human GLO I), blue, red, yellow, and white, respectively. Zn^{2+} ion is shown in gray (CPK type).

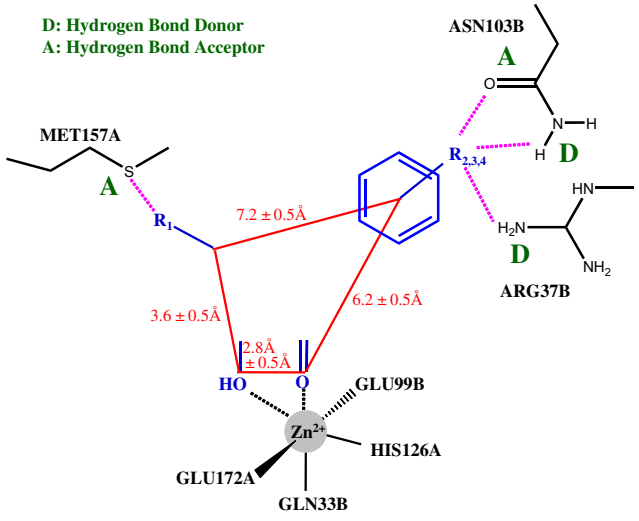


Figure 8. A deduced pharmacophore for the human GLO I inhibitors.

were pelleted by centrifugation at 2500 × g for 5 min. The cells were lysed in ice-cold Insect Cell Lysis Buffer containing reconstituted Protease Inhibitor Cocktail on ice for 45 min. Lysate was cleared by centrifugation at 20,000 × g for 45 min, and the supernatant loaded directly onto Ni-NTA Agarose preequilibrated in 6× His Wash Buffer. Then the Ni-NTA Agarose bead slurry was washed in 6× His Wash Buffer. Elution was carried out with 6× His Elution Buffer. The resultant solution was stored for use in in vitro GLO I assay.

4.3. In vitro GLO I assay

The GLO1 assay was performed according to a spectrophotometric method monitoring the increase in absorbance at 240 nm due to the formation of *S*-D-lactoylglutathione for 5 min at 25 °C.[15] The standard assay mixture contained 7.9 mM MG, 1 mM glutathione, 14.6 mM magnesium sulfate, and 182 mM imidazole–HCl, pH 7.0. Before initiating the reaction by adding recombinant human GLO I to the assay mixture, the mixture was allowed to stand for 15 min to ensure the equilibration of hemithioacetal formation.

4.4. Cell culture

Human myeloid leukemia HL-60 cells were maintained in RPMI1640 (Sigma) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS. The cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

4.5. Measurements of cell growth inhibition

The sensitivities of HL-60 cells to BBGC and GLO I inhibitor candidate flavonoid compounds were evaluated by the inhibition of cell growth after 48 hr incubation. The number of viable cells was estimated by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) method[16] using CellTiter 96 AQ_{ueous} One Solution cell proliferation assay (Promega, Tokyo, Japan).

4.6. Computational molecular docking

The complex structure of human glyoxalase I (hGLO I) and *S*-benzylglutathione was obtained from the Protein Data Bank (PDB) (code 1FRO). Water was removed from the PDB file. Energy minimization of the complex structure was performed using Amber8 package with Amber94 force field.

Molecular docking was carried out using AutoDock 3.0.[17] The binding free energy scoring function in the AutoDock is based on an empirical function derived by linear regression analysis of a large set of diverse protein–ligand complexes with known inhibition constants. There are many successful examples of structures of protein–ligand system structures studied by the AutoDock program.[18,19] The Docking energy grid (grid maps with 60 × 60 × 60 points, grid spacing 0.375 Å) was produced with AutoGrid Program.[17] The grid box was centered on the center of the ligand from the corresponding crystal structure complex (PDB code 1FRO). The Lamarckian Genetic Algorithm (LGA) was utilized and energy evaluations were set at 5×10^6. Simulation was performed for a total of 100 runs. Zn^{2+} parameters were set as radius 0.79 Å, well depth 0.35 kcal/mol, and charge +2.00e. Ligands using docking were prepared in CDX format using ChemDraw package (Cambridge Soft) and then converted to three-dimensional structure and energy minimized by molecular mechanics (MM2) and by MOPAC (PM3) calculation using Chem3D package (Cambridge Soft). Molecular visualizations were carried out in DS Viewer Pro (Accelrys, Inc., San Diego, CA).

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