Bioorganic & Medicinal Chemistry 18 (2010) 7029–7033

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Delphinidin, a dietary anthocyanidin in berry fruits, inhibits human glyoxalase I

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ARTICLE INFO

Article history:
Received 30 June 2010
Revised 3 August 2010
Accepted 4 August 2010
Available online 9 August 2010

Keywords:
Glyoxalase I
Inhibitor
Anthocyanidins
Delphinidin
Apoptosis

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, inhibitors of this enzyme has been expected to be new anticancer drugs. Here, we examined the inhibitory abilities to the human GLO I of anthocyanidins, such as delphinidin, cyanidin and pelargonidin. Among them, delphinidin was found to have the most potent inhibitory effect on human GLO I. Also, only delphinidin-induced apoptosis in HL-60 cells in a dose- and time-dependent manner. Furthermore, we determined a pharmacophore for delphinidin binding to the human GLO I by computational simulation analyses of the binding modes of delphinidin, cyanidin and pelargonidin to the enzyme hot spot. These results suggest that delphinidin could be a useful lead compound for the development of novel GLO I inhibitory anticancer drugs.

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 non-toxic *S*-D-lactoylglutathione.[2,3] This enzyme system is ubiquitously distributed in all mammalian cells and is involved in tissue maturation and cell death.[4]

Importantly, abnormal expression or higher activity of GLO I has been demonstrated in many human tumors including colon, prostate and lung.[5–7] Moreover, GLO I has been shown to be highly expressed in antitumor agent-resistant human leukemia cells.[8] These observations indicate that the increase of GLO I expression is closely associated with carcinogenesis[5–7] and drug resistance.[8] Thus, the inhibitors of GLO I are expected to offer possibilities for inhibiting carcinogenesis and overcoming drug resistance by the mechanism of accumulations of apoptosis-inducible MG in tumor cells.[9]

Berry fruits, such as bilberry, blackberry, black raspberry and cranberry, are widely consumed in our diet and have attracted much attention by their potential human health benefits. They have been shown to be rich sources of phytochemicals with the potential to prevent human cancers.[10–12] Delphinidin, the major anthocyanidin present in berry fruits, has been shown to possess strong antioxidant, anti-inflammatory and anticancer effects.[13,14] However, the mechanism of the anticancer effect has not yet been fully elucidated.

Previously, we have shown that the natural flavonoid compounds that possess C-4 ketone group and C-5 hydroxy group, such as baicalein, luteolin, myricetin and quercetin, effectively inhibit human GLO I.[15] Considering our interest in inhibitors of GLO I and based on the rationale mentioned above, structurally related compounds, anthocyanidins, were considered to have inhibitory effects on the human GLO I. Our working hypothesis was that similar to flavonoid compounds, the polyphenolic compounds may active to the human GLO I.

In this study, we investigated the human GLO I inhibitory activities of the major anthocyanidins, delphinidin, cyanidin and pelargonidin, and their apoptosis inducibilities. Among the anthocyanidins, delphinidin was found to have both strong abilities of the human GLO I inhibition and apoptosis induction. Furthermore, we analyzed their structure–activity relationships and provided an important pharmacophore of delphinidin for the human GLO I binding. These data are useful to develop novel GLO I inhibitory agents for cancer chemotherapy.

2. Results

2.1. Effects of anthocyanidins on the human GLO I

In order to investigate whether the major anthocyanidins, delphinidin, cyanidin and pelargonidin (Fig. 1), inhibit the human GLO I, in vitro GLO I assay was performed with our prepared

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doi:10.1016/j.bmc.2010.08.012

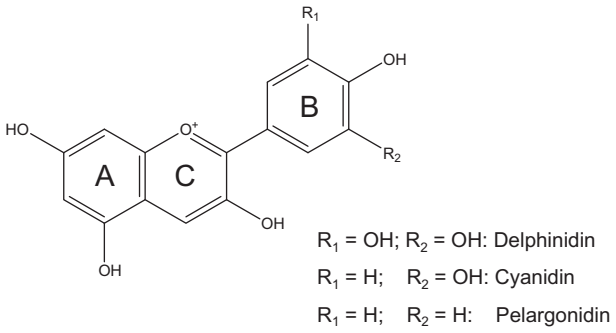
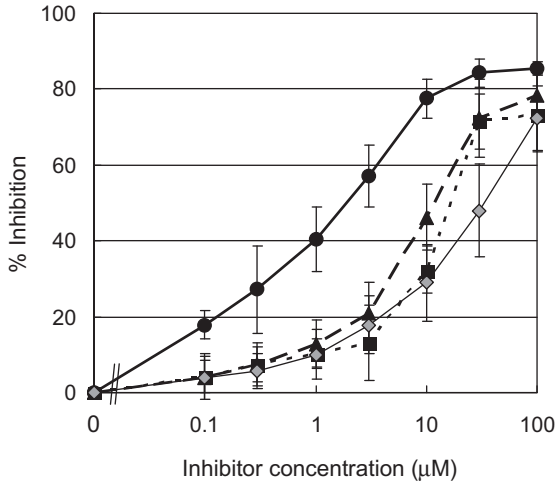


Figure 1. Structures of anthocyanidins (delphinidin, cyanidin and pelargonidin).

recombinant human GLO I (rhGLO I). We evaluated the dose-dependencies and determined the IC_{50} values of these three compounds (Fig. 2). Among them, delphinidin showed the most potent inhibition on rhGLO I activity. The IC_{50} values of delphinidin, cyanidin and pelargonidin are calculated to be 1.9, 11.7 and 16.4 μM, respectively. These results suggest the importance of the specific interactions of the different numbers of hydroxy groups on the B ring of the three anthocyanidins with the human GLO I molecule for the inhibitory potencies.

2.2. Binding modes of anthocyanidins on the human GLO I

To examine the structure–activity relationships of the inhibitory activities of the three anthocyanidins, we analyzed their binding modes by computational molecular docking study. As shown in Figure 3A–C, three hydroxy groups on the B ring of delphinidin forms four hydrogen bonds to three amino acids (Asn103B, Arg122A and Arg37B) of the human GLO I (Fig. 3A). On the other hand, cyanidin which has two hydroxy groups on the B ring forms three hydrogen bonds to two amino acids (Arg122A and Arg37B) and does not bind to Asn103B of the human GLO I (Fig. 3B). Pelargonidin has only one hydroxy group and forms two hydrogen bonds to Arg122A and Arg37B of the human GLO I (Fig. 3C). The docked energies of delphinidin, cyanidin and pelargonidin to the human GLO I are calculated to be −9.46, −9.00 and −8.93 kcal/mol, respectively. These results suggest that the hydroxy groups on the B ring, especially at the R_1 position, greatly contribute to the inhibition potency and specificity of anthocyanidins on the human GLO I activity.



	Delphinidin	Cyanidin	Pelargonidin	BBG
IC_{50}	1.9 μM	11.7 μM	16.4 μM	33.2 μM

Figure 2. The effects of anthocyanidins on the human GLO I. The effects of anthocyanidins (delphinidin, cyanidin and pelargonidin) and a GSH analog GLO I inhibitor *S*-*p*-bromobenzylglutathione (BBG) on rhGLO I were investigated. The dose-dependencies and IC_{50} values of delphinidin (—●—), cyanidin (—▲—), pelargonidin (···■···) and BBG (—◇—) 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.

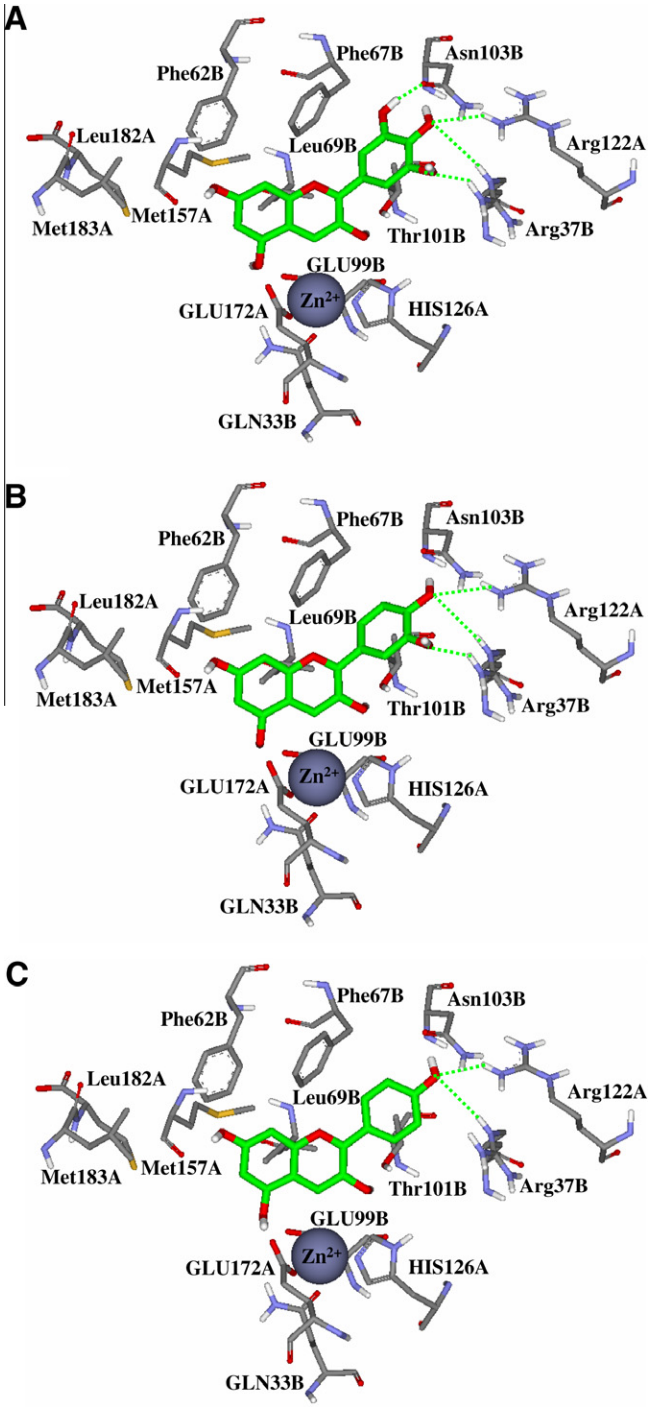


Figure 3. The predicted binding modes of the anthocyanidins on the human GLO I (PDB code 1FRO). The putative binding modes were obtained from computational molecular docking study as described in 'Section 4'. Carbon, nitrogen, oxygen, sulfur, and hydrogen are shown in green ((A) delphinidin; (B) cyanidin; (C) pelargonidin)/gray (human GLO I), blue, red, yellow and white, respectively. Zn^{2+} ion is shown in blue-gray (CPK type).

2.3. Inhibition of cell proliferation and induction of apoptosis by delphinidin

To test whether the human GLO I inhibitory anthocyanidins are effective in suppressing growth of human promyelotic leukemia HL-60 cells, we examined the antiproliferative effects of delphinidin, cyanidin and pelargonidin on HL-60 cells. Expectedly, delphinidin that has stronger inhibitory effect on the human GLO I activity in vitro suppressed the growth of HL-60 cells in a dose-dependent manner (Fig. 4). Importantly, this suppressive effect was found to be a time-dependent manner, suggesting the accumulation of cytotoxic MG (Fig. 4). The IC_{50} values of delphinidin for HL-60 cells at 24 h (Fig. 4A) and 48 h (Fig. 4B) treatments were about 80 μM and 40 μM, respectively.

In contrast, cyanidin and pelargonidin, which have less inhibitory effects on the human GLO I activity in vitro, had little inhibitory effect on the cell proliferation. These results indicate that the inhibition of GLO I is an important contribution to the antiproliferative effect on the tumor cells, and that its suppressive effect is due to the accumulation of MG in the cells.

To prove the antiproliferative effect of delphinidin is due to induction of apoptosis, we examined an important hallmark of apoptosis, nucleosomal DNA fragmentation. Analysis of the DNA from delphinidin-treated HL-60 cells by agarose gel electrophoresis showed a pattern of internucleosomal cleavage characteristic of apoptosis (DNA ladder) (Fig. 5). The ladder formation was in a dose- and time-dependent manner. These observations suggest the apoptosis inducibility of delphinidin.

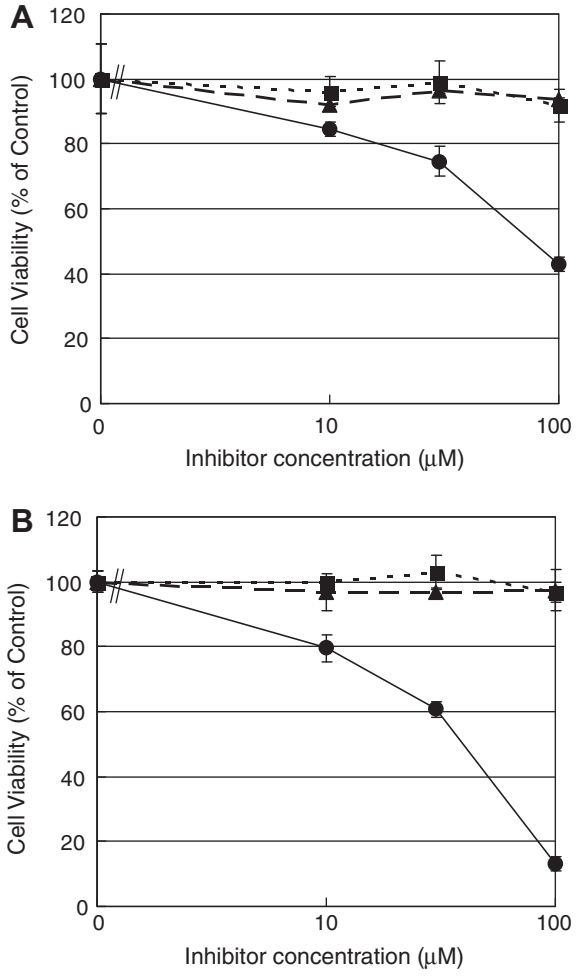


Figure 4. The antiproliferative effects of anthocyanidins. HL-60 cells were treated with indicated concentrations of delphinidin (—●—), cyanidin (—▲—) and pelargonidin (···■···) for (A) 24 and (B) 48 h. Cell viability (% of control) was measured by MTS assay. Data are the averages of three independent experiments and *bars* show the SD values.

3. Discussion

In this study, we first demonstrated the inhibitory effects of anthocyanidins, delphinidin, cyanidin and pelargonidin, on the human GLO I activity. Among them, delphinidin was found to be the most effective inhibitor of the human GLO I. Previously, we have shown that several natural flavonoid compounds which possess C-4 ketone and C-5 hydroxy groups effectively inhibit the human GLO I.[15] The analysis of structure–activity relationships of the flavonoid compounds showed that the hydroxy groups on the B ring contribute to the inhibitory effects on the human GLO I.[15] Consistent with the previous observations, the present results of in vitro GLO I assay (Fig. 2) revealed that the hydroxy groups on the B ring of anthocyanidins, especially at the R_1 position (Fig. 1), greatly contribute to the inhibitory activities on the human GLO I. The comparison of the computationally predicted binding modes of these three anthocyanidins suggests that delphinidin can form more hydrogen bonds to bind one more amino acid (Asn103B) of the human GLO I than cyanidin and pelargonidin via its hydroxy group at the R_1 position (Fig. 3). Thus, the three hydroxy groups on the B ring of delphinidin are considered to be involved in the specific interaction with the hot spot constructed especially by Asn103B, Arg122A and Arg37B on the human GLO I molecule.

Since GLO I is an attractive target for development of new anticancer drugs, we next evaluated the effects of the anthocyanidins on the proliferation of HL-60 cells. As expected, delphinidin that possessed strong inhibitory activity to the human GLO I activity in vitro suppressed the growth of HL-60 cells in a dose- and time-dependent manner (Fig. 4). In contrast, cyanidin and pelargonidin which have less inhibitory effects on the human GLO I had little suppressive effects on the cell growth. Furthermore, delphinidin was appeared to induce cell death by apoptosis (Fig. 5). Since the kinetics of delphinidin-induced apoptosis is slow, it is likely that the onset of apoptosis by delphinidin treatment is the consequence of the accumulation of MG by GLO I inhibition. These observations indicate that the GLO I inhibition is an important contribution to the antiproliferative and apoptosis inducing activities. It should be emphasized that more experiments are necessary to further elucidate whether delphinidin actually enters the cells,

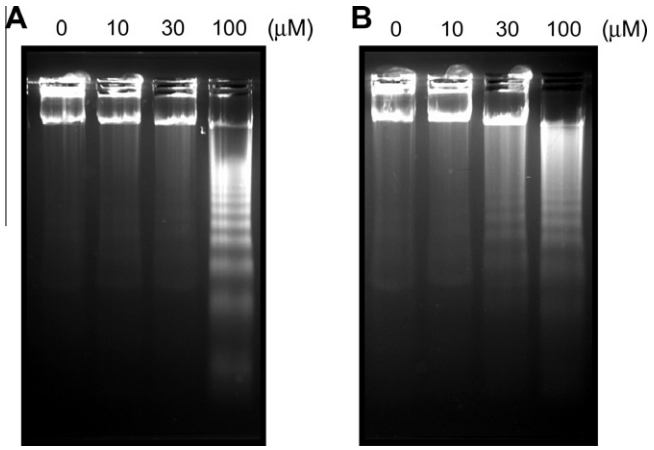


Figure 5. Delphinidin induced apoptosis of HL-60 cells in a dose and time-dependent manner. HL-60 cells were treated with indicated concentrations of delphinidin for (A) 24 and (B) 48 h. DNA extracted from delphinidin-treated HL-60 cells was analyzed by agarose gel electrophoresis as described under 'Section 4'.

inhibits GLO in cells and induced the accumulation of MG. These issues should be addressed in future studies.

It is now widely appreciated that agents capable of inducing apoptosis in cancer cells can potently lead to the development of mechanism-based prevention and treatment approaches for cancer. Our results suggest that delphinidin could be an important natural lead compound for the development of GLO I inhibitory anticancer drugs. That is, the structure of delphinidin could provide a valuable scaffold to design the human GLO I specific inhibitors. In the predicted binding mode shown in Figure 3, the four polar atoms (O atom on Asn103B, N–H atom on Arg122A, two N–H atoms on Arg37B) on the human GLO I (PDB code 1FRO) are capable of forming hydrogen bonds to delphinidin. Taken together with these in silico and in vitro data, we tried to construct a delphinidin/GLO I pharmacophore illustrated in Figure 6. Probably, the specific interaction of delphinidin/GLO I is due to the flavylium ion in delphinidin and Phe67B in the human GLO I and the A aromatic ring and Leu69B. This pharmacophore may be useful for computational screening and design of novel GLO I specific inhibitors.

The purpose of our study is to obtain ultimately the human GLO I specific inhibitors leading to anticancer drugs. Now, by using the unique pharmacophore of GLO I/delphinidin, structure-based virtual screening (SBVS) and structure-based drug design (SBDD) of lead small molecular compounds that have specific inhibitory effects on the human GLO I are under investigation.

4. Experimental

4.1. Materials

S-*p*-Bromobenzylglutathione (BBG) was generous gifts from Taiho Pharmaceutical Co., Ltd. Delphinidin chloride, cyanidin chloride and pelargonidin chloride were purchased from TOKIWA Phytochemical Co., Ltd and EXTRASYNTHESE. All other chemicals were of reagent grade.

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

Human GLO I (hGLO I) cDNA fragment was subcloned into pET-28a (Novagen), and the resulting construct was transformed into BL21 to produce recombinant hGLO I (rhGLO I) as hexahistidine fusion proteins. rhGLO I protein was purified with the TALON Metal Affinity Resins (Clontech) in native condition following the manufacturer's protocol.

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.[15,16] 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 rhGLO 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 promyelocytic leukemia HL-60 cells were maintained in RPMI1640 (Sigma) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS.[15] 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 anthocyanidins were evaluated by the inhibition of cell proliferation after 24 and 48 h treatment with various concentrations of delphinidin, cyanidin and pelargonidin. The number of viable cells was estimated by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

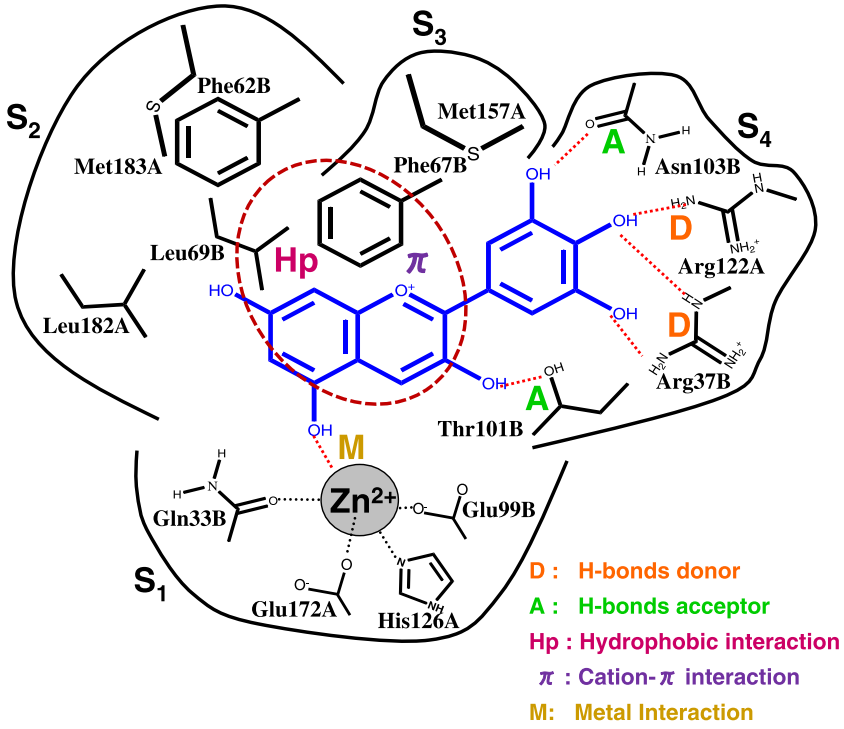


Figure 6. A deduced pharmacophore of delphinidin to the human GLO I.

sulfophenyl)-2*H*-tetrazolium] (MTS) method using CellTiter 96 AQ_{ueous} One Solution cell proliferation assay (Promega).[15]

4.6. Detection of apoptotic DNA fragmentation

HL-60 cells were lysed in lysis buffer [50 mM Tris–HCl (pH 7.8), 10 mM EDTA and 0.5% (w/v) sodium *N*-lauroylsarcosinate] and incubated with 0.5 mg/ml RNase A at 50 °C for 1 h. Then 0.5 mg/ml Proteinase K was added, and the lysates were incubated for 1 h.[17] DNA thus prepared was subjected to 1.8% agarose gel electrophoresis. The DNA was visualized by UV illumination after ethidium bromide staining.

4.7. 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 AUTODOCK3.0.[18] 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 complexes studied by the AUTODOCK program.[19,20] The Docking energy grid (grid maps with $60 \times 60 \times 60$ points, grid spacing 0.375 Å) was produced with AUTOGRID Program.[18] 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 1.5×10.[6] Simulation was performed a total of 10 runs. Zn^{2+} parameters were set as radius 0.79 Å and charge +2.00e.[15] Other parameters were set to default values. Ligands using docking were prepared in CDX format using ChemDraw package (Cambridge Soft) and then converted to three-dimensional structure by MOPAC (PM3) calculation using Chem3D package (Cambridge Soft). Molecular visualizations were carried out in DS Viewer Pro (Accelrys, Inc., San Diego, CA).

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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