1 Introduction

There is increasing interest in searching for cancer-preventive agents from edible plant resources. Carcinogenesis is usually a long process that requires many years before developing into intractable metastasis stage, and therefore can be subjected to dietary intervention before becoming full-blown cancer [1–3].

Induction of phase 2 detoxifying/antioxidant enzymes is assumed to be one of the most effective ways to prevent carcinogenesis by both endogenous and exogenous carcinogens. For instance, sulforaphane, which is present abundantly in broccoli sprouts, has been reported to prevent carcinogenesis by inducing phase 2 detoxifying/antioxidant enzymes such as NAD(P)H:quinone oxidoreductase (NQO1), glutathione S-transferase (GST), glutathione reductase (GR) and heme oxygenase-1 (HO-1) [4, 5]. In fact, hundreds of compounds have been reported to increase phase 2 detoxifying enzymes, and these may act as chemopreventive agents. Interestingly, there is no apparent common structural feature among phase 2 enzyme inducers, except that many of them are Michael reaction ac-
ceptors and possess antioxidant activity [6, 7]. Most of them are known to be induced by promoting nuclear translocation of NF-E2-related factor-2 (Nrf2) and its subsequent binding to the antioxidant response element (ARE) sequence in those enzymes, leading to transcriptional activation. In unstressed cells, Nrf2 is known to remain in the form of a Nrf2-Keap1 complex, mostly in the cytosolic compartment. Once activated by exogenous stimuli, such as the entry of some phytochemicals or perturbation in intracellular redox potential, Nrf2 is released from Keap1 and migrate into nucleus, acting as transcriptional activator [8, 9].

Soybean isoflavones are known to be estrogenic, with potential beneficial health effects in humans [10]. Glyceollins, prenylated isoflavones derived from daidzein in soybean exposed to some kinds of fungi, were also found to have various biological activities including anti-estrogenic and anticancer activities [11–13]. Our previous study confirmed that a mixture of glyceollin I, II and III are efficiently produced during sprouting of a high-isoflavone variety of soybean (Aga 3) in the presence of Aspergillus sojae, and exhibited antioxidant, anti-fungal, and anti-diabetic activities [13–15].

Since some compounds with antioxidant activity share some common structural or physiological features with known phase 2 enzyme inducers, we investigated the potential of glyceollins to cause Nrf2-mediated induction of phase 2 detoxifying enzymes. Electrophilic compounds have been reported to cause cysteine modification of Keap1, facilitating its release from Nrf2, which migrates into nucleus to act as transcription factor for several antioxidant enzymes genes [16–18].

In addition, molecular modeling and computational methods have also been applied to gain further insight into the Nrf2-release mechanism by glyceollins from the Keap1-Nrf2 complex.

2 Materials and methods

2.1 Preparation of glyceollin mixture

Induction of de novo biosynthesis of glyceollins and isolation of glyceollins from elicited soybean has been described previously [11, 14]. Briefly, soybeans (Aga no. 3), which have an exceedingly high level of isoflavones (~10 mg/g), were obtained from Kyungpook National University Soyventure Co., Ltd. (Daegu, South Korea) and were subjected to elicitation by A. sojae, an edible fungus, for de novo biosynthesis of glyceollins [11–13, 19]. An A. sojae spore suspension (10 µL) was applied to the cut surface of soybean seed, followed by storing in a chamber at 26°C in the dark for 3 days and extraction with 15 mL 80% ethanol at 50°C for 1 h. Extracts were filtered through a sterile filter with a 0.45-µm pore size (Sartorius Biotech GmbH, Göttingen, Germany). Most crude extracts were freeze-dried and dissolved in dimethylsulfoxide at a concentration of 100 mg/mL prior to use.

For analysis, the freeze-dried crude extract was suspended in deionized water and partitioned with hexane and ethyl acetate (EtOAc). Glyceollins were purified by silica gel column chromatography using EtOAc and MeOH mixtures (1:1) of increasing polarity (50:1 to 5:1). The concentration of purified glyceollin isomers (hereafter called ‘glyceollins’) was ~90% as assayed by HPLC. The relative ratio of glyceollin I, II, and III in the mixture was 12:1.3:1. The chemical structures of glyceollin isomers are shown in Fig. 1.

2.2 Cell culture

Hepa1c1c7 and its mutant (BPRc1) cells, obtained from American Type Culture Collection (Rockville, MD, USA), were plated at density of $3 \times 10^5$ and $5 \times 10^5$ cells per 100-mm plate (Nunc, Rochester, NY) in 10 mL α-MEM (Gibco-BRL; Gaithersburg, MD, USA) supplemented with 10% FBS (Hyclone), respectively. The HepG2-C8 cell line established in Dr. Kong’s lab at Rutgers, the State University of New Jersey, by transfecting human hepatoma HepG2 cells with pARE-TI-luciferase construct was used for the reporter assay [20]. HepG2-C8 cells were maintained in modified DMEM supplemented with 10% FBS, GlutaMax (Gibco No. 35050-061), 100 U/mL penicillin, and 0.5 mg/mL G418. Cells were normally starved overnight in 0.5% FBS-containing medium before treatment. The cells used in this study were cultured in a humidified in-
cubator (Sanyo MCO-15AC, Gunma, Japan) in 5% CO₂ at 37°C, and were routinely passaged every 3–4 days. For experiments, cells were pre-cultured for 48 h, exposed to various concentrations of the sample for another 24 h, followed by biochemical assays.

2.3 Biochemical assays

NQO1 enzyme activity was measured by a spectro-photometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm [21]. α-Butylhydroquinone (20 µM or 3.3 µg/mL), a known NQO1 inducer, was used as a positive control in all biochemical assays. The specific activity of enzymes was normalized to the protein concentration, which was determined in triplicate using a Protein Assay Kit (Bio-Rad). All values are reported as the mean ± SD whenever possible.

2.4 Reporter assay

HepG2-C8 cells were plated in six-well plates at a density of 5 × 10⁵ cells/well. After 16-h incubation, cells were cultured in fresh modified DMEM with high glucose containing 0.5% FBS for 12 h prior to sample treatment. After culturing cells for another 16 h in the presence of varying concentrations of sample, cells were collected and the luciferase activity was assayed according to the protocol provided by the manufacturer (Promega Corp., Madison, WI, USA).

2.5 Preparation of nuclear protein extract

Nuclear and cytosolic protein extracts were prepared using a kit (NE-PER Nuclear and Cytosolic Extraction Reagents; Thermo Scientific, Rockford, IL) according to the supplier’s protocol. Briefly, cells were cultured on 100-mm dishes to 90% confluence and treated with various doses of glyceollins for 24 h. Cells were then washed with PBS twice and harvested by scraping in ice-cold PBS, followed by centrifugation at 500 × g for 5 min. Cells were lysed with buffer A (10 mM HEPES-KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) on ice for 20 min, and then centrifuged at 14 000 × g for 15 min at 4°C. The supernatants were saved as the cytoplasmic fractions. The nuclear pellets were washed three times with buffer A and resuspended in buffer B (20 mM HEPES, 0.5 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.9) for 30 min at 4°C on a rotating wheel and then centrifuged at 14 000 × g for 15 min at 4°C. The nuclear fraction was subjected to immunoblot analysis using anti-Nrf2 and anti-lamin B antibodies.

2.6 Western blot

The levels of proteins investigated, including phase 2 detoxifying/antioxidant enzymes, were measured by western blot for whole cell extracts or nuclear fraction [22]. The primary antibodies including anti-NQO1, anti-HO-1, anti-gamma glutamylcysteine synthase (γGCS), anti-GR, anti-Nrf2, anti-β-actin, anti-lamin B, and horseradish peroxidase-conjugated secondary antibody anti-goat or anti-rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The bands were detected using a chemiluminescence kit (Pierce, Cheshire, UK). Densitometry analysis was performed with Lab Image software (GE Fuji LAS-4000 mini, Piscataway, NJ, USA).

2.7 Molecular modeling of Keap1 (residues 77–287) and electrostatic calculations

The Keap1 domain from residue 77 to 287 was built with homology modeling techniques. Both a fold-recognition search and a BLAST search against the Protein Data Bank (PDB) identified two suitable highly related templates: the crystal structure of the crystal structure of the Btb-Back domains of Human Klhl11 (PDB id 3I3N) with 45% sequence identity, and the crystal structure of the Spomath-xBTB3-Box-Pucsbc1 (PDB id 3HQI) with 47% sequence identity [23]. After a careful multiple-sequence alignment with ClustalW V2.0.9, 100 models were generated with Modeler V9.5 [24, 25]. The best model, according to the intrinsic modeler function, was subjected to side-chains positioning using the SCWRL4 program [26]. The model with side chains accurately positioned was then further energy minimized with spatial restraints (CNS V1.2). Stereochemistry was checked with PROCHECK [27]. The model was manually inspected with Coot [28, 29]. Structural overlay was done with the SSM algorithm in Coot 0.6.1. Electrostatics calculations were performed with APBS V1.2.1, and the molecular surfaces with the electrostatics properties were rendered with both VMD V1.8.7 and PyMOL V1.2 [29].

2.8 Docking and binding energy

Three series of docking experiments were performed with AutoDock Vina 1.1.1 with a grid size of 14 × 12 × 14 Å, 15 × 13 × 15 Å, or 16 × 14 × 16 Å centered on Cys151, which encompassed the whole binding area around Cys151 and was large.
enough to explore the range of binding [30]. AutoDock Vina parameters were: maximum number of binding modes to generate set to 50; exhaustiveness of the global search set to 10; maximum energy difference between the best binding mode and the worst one displayed set to 6 kcal/mol. Atomic structures of glyceollin I, glyceollin II, glyceollin III, dehydroglyasperin C, t-butylhydroxyquinone and sulforaphane were downloaded from Pubchem (http://pubchem.ncbi.nlm.nih.gov/).

AutoDockTools 1.5.5 were used to add the partial charges and non-polar hydrogen atoms on the receptor (Keap1 residues 77–287), and determine the appropriate grid size. AutoDock 4.2 C-shell scripts and python code (prepare_ligand4.py) were used to add the partial charges and non-polar hydrogen atoms to prepare the ligand files for docking. Docking calculations were run on Intel dual core Core2duo 3.06 Ghz and Intel i5 quad core 2.66 GHz MacOS X workstations. Relative docking energies from the three series of runs were averaged and ranked.

2.9 Statistical analysis

The statistical significance of data was tested by t-tests using SPSS software (SPSS Inc., Chicago, IL). $p < 0.05$ was considered to be statistically significant.

3 Results

3.1 Effect of glyceollins on NQO1 activity

To investigate the effect of glyceollins on NQO1 enzyme activity, we used mouse hepatoma Hepa1c1c7 and its mutant BPRc1, which lacks the arylhydrocarbon receptor nuclear translocator (ARNT). Phase 2 detoxifying enzyme inducers are classified into bifunctional and monofunctional inducers. Monofunctional inducers are usually considered to be better cancer-preventive agents because they promote the phase 2 enzymes selectively without affecting phase 1 enzymes that are involved in bioactivation of procarcinogens. Use of the BPRc1 cell line facilitates the identification of monofunctional inducers, which only induce phase 2 enzymes in BPRc1, while bifunctional inducers are...
not able to induce phase 2 enzymes in this cell line [31].

As shown in Fig. 2, glyceollins did not show any significant toxicity at 3 µg/mL or lower, and induced NQO1 activity in a dose-dependent manner both in Hepa1c1c7 and BPRc1 cells in the range of 0.187–3 µg/mL, suggesting that glyceollins are categorized into monofunctional inducers (Fig. 3).

### 3.2 Induction of phase 2 enzymes by glyceollins

Expression of phase 2 detoxifying enzymes and antioxidant enzymes, including NQO1, HO-1, and GR, were induced by glyceollins in a dose-dependent fashion in both Hepa1c1c7 and BPRc1 cells, except gamma glutamylcysteine synthase (γGCS), which showed relatively weak response to glyceollins. The phase 2 detoxifying enzyme induction was more prominent in BPRc1 cells than Hepa1c1c7 cells (Fig. 4).

### 3.3 Nuclear translocation of Nrf2 by glyceollins

Nrf2 modulates the expression of phase 2 enzyme genes, i.e., upon phosphorylation of Nrf2 and/or structural modification of Keap1, Nrf2 is released from Nrf2-Keap1 complex and migrates into the nucleus. There it binds to ARE, a specific DNA sequence in the promoter region of genes of some antioxidant and detoxifying enzymes, increasing the transcription of the related genes. Nuclear translocation of Nrf2 was increased by treatment with glyceollins in a dose-dependent manner from 0 to 3 µg/mL (Figs. 5 and 6). Accumulation of Nrf2 in the nucleus was observed both in Hepa1c1c7 and BPRc1 cells, suggesting that translocation of Nrf2 to the nucleus is independent of ARNT.

Furthermore, glyceollins were found to not only promote nuclear translocation of Nrf2 but also increase the total intracellular level of Nrf2 (Fig. 6), probably through transcriptional activation and increased translational efficiency [31].

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**Figure 4.** Effect of glyceollins on the expression of phase 2 detoxifying enzymes including NQO1, γ-GCS, GR, HO-1 in Hepa1c1c7 and BPRc1 cells. (A) Hepa1c1c7 and (B) BPRc1 cells were treated with various doses of glyceollins for 24 h, followed by immunoblot analyses as described in the Materials and methods section. NQO1: NAD(P)H:quinone acceptor oxidoreductase 1; GST: glutathione S-transferase; HO-1: heme oxygenase; γ-GCS: γ-glutamylcysteine synthase; GR: glutathione reductase.
3.4 ARE-mediated transcriptional activation by glyceollins

To investigate whether glyceollins induces phase 2 enzymes through interacting with the ARE, cis-element in the promoter region of phase 2 enzymes, HepG2-C8 cells containing the reporter ARE-luciferase gene were treated with glyceollins for various periods. Luciferase activity in the cells was increased proportionately with exposure time at doses of 1.5 and 3.0 µg/mL (Fig. 7). In particular, the luciferase activity of HepG2-C8 cells was more prominently enhanced in the cells treated with 3 µg/mL glyceollins compared to those treated at the dose of 1.5 µg/mL.

3.5 Involvement of Akt phosphorylation in nuclear translocation of Nrf2 by glyceollins

To examine whether nuclear translocation of Nrf2 by glyceollins is mediated by the phosphoinositide 3-kinase (PI3K) signaling pathway, nuclear Nrf2 levels were measured in Hepa1c1c7 and BPrC1 cells treated with glyceollins plus various concentrations of LY294002, a PI3K inhibitor. Glyceollins resulted in significantly increased phosphorylation of Akt, a kinase downstream from PI3K, in both Hepa1c1c7 and BPRc1 cells (Fig. 8A), suggesting regulation of PI3K signaling pathway by glyceollins. This result suggests the possibility that nuclear migration of Nrf2 could be mediated by activation of the PI3K signaling pathway by glyceollins. Furthermore, both nuclear Nrf2 accumulation and ARE-luciferase activity induced by glyceollins was abrogated by LY294002, again suggesting that the PI3K signaling pathway is involved in nuclear translocation of Nrf2 (Fig. 8B).

3.6 Molecular modeling of hKeap1 (77–286), binding area around Cys151 and docking

The best template to model the N-terminal region of the human Keap1 (residues 77–286 out of 624) was identified as the X-ray crystal structure of the Btb-Back domains of human Klhl11 (PDB 3I3N) with 45% sequence identity and the crystal structure of the MATH-BTB protein spop (PDB 3HQI) with 47% sequence identity. In homology modeling, the selection of template(s) is undoubtedly a critical step. However, it is generally assumed that two proteins whose sequences share at least 40% identity have similar structures. Homology modeling under such conditions is then expected to generate models whose accuracy is close to that of an experimental structure. The accuracy of the sequence alignment is another critical step in the homology
modeling workflow, as a shift of one single residue in the alignment introduces locally a 3.8-Å distortion of the model Cα-backbone [30]. In that matter, the sequence alignment used has been carefully checked for any errors. Another critical step in building high-resolution models, is the accurate prediction of the side-chain conformations for a given backbone architecture. We used the program SCWRL4 that usually has a success rate of prediction ranging from 78 to 89% for the chi1 and chi2 angles of residues in the core of the protein [32]. In addition, we performed an energy minimization of the final model to avoid any steric clashes. By carefully validating every step, we are therefore confident that we have built a sound model for Keap1 77–287 with the best available template to date.

The electrostatic surface analysis around Cys151 of Keap1 revealed a large 8-Å-radius positively charged binding pocket (Fig. 9). It contrasts with the overall negatively charged area outside the Cys151 binding area.

AutoDock Vina was used to dock glyceollin I, glyceollin II, glyceollin III, dehydroglyasperin C, t-butylhydroxyquinone and sulforaphane around Cys151 of Keap1 (Table 1). All the compounds bind with a negative relative docking energy that supports a stabilization step in the binding area. Among the six compounds tested, the glyceollin isomers bound significantly better in the current study.

The compound properties (LogP partition coefficient, polar surface area and molecular surface area) were calculated with both Spartan’08 from Wavefunction, Inc. and FieldView V2.01 from Cresset Biomolecular Discovery Ltd.
A

(i) p-AKT

(ii) p-AKT

(iii) p-AKT

(iv) p-AKT

β-actin

β-actin

Glycolellins (µg/mL)

Glycolellins (µg/mL)

Protein level (% of control)

Protein level (% of control)

Glycolellins (3 µg/mL)

LY294002 (µM)

- - + + + +

- - 2.5 5 10

pAKT/AKT

pAKT/AKT

AKT/β-actin

AKT/β-actin

p-AKT relative to AKT (% of control)

p-AKT level relative to AKT (% of control)

Glycolellins (3 µg/mL)

LY294002 (µM)

- - + + + +

- - 2.5 5 10

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4 Discussion

Glyceollins are de novo synthesized from daidzein in soybean exposed to abiotic or biotic stress [10, 33, 34]. Three isomers (I, II, III) have been reported in glyceollin mixtures and their relative ratio was analyzed as 12:1.3:1 by HPLC-MS [14]. The compounds were reported to have various bioactive functions, such as anticancer, anti-diabetic and anti-fungal activities as well as anti-estrogenic property [11–14]. They also share common features with isoflavones and some phase 2 detoxifying enzyme inducers in that they are electrophiles and possess antioxidant property. As expected from their structures containing a phenolic hydroxyl group, glyceollins were shown to have potential to induce phase 2 detoxifying enzymes, which was mediated by Nrf2. The expression of most phase 2 detoxifying or anticarcinogenic enzymes is regulated at the transcriptional level by Nrf2, which exists normally in an Nrf2-Keap1 complex in the cytosolic compartment. Current data suggested that glyceollins promote separation of Nrf2 from the complex and translocation into nucleus, leading to transcriptional activation of several phase 2 detoxifying or anticarcinogenic enzymes.

However, it is not clear how glyceollins facilitate the release of Nrf2 from Keap1. Two main mechanisms have been proposed with regard to translocation of Nrf2 into nucleus. First, Keap1 loses affinity to Nrf2 when its sulphydryl groups are modified by exogenous factors, and is separated from Nrf2, which can freely travel to nucleus [5, 16, 35–37]. Our study did not examine whether glyceollins could modify Keap1 and thereby facilitate its entry into nucleus. Nonetheless, from the virtual docking study it is quite plausible for the compounds to interact with Keap1, releasing Nrf2 from Keap1-Keap2 complex. For instance, electrophilic compounds such as tBHQ, ebselen and 1,2-naphthoquinone have been reported to react with cysteine residues in Keap1 and cause Nrf2-mediated induction of phase 2 enzymes [18].

Second, exogenous and endogenous factors that activate the PI3K signaling pathway phosphorylate Nrf2, which, in turn, reduces its affinity to Keap1, facilitating migration into nucleus [7, 38, 39]. As shown in Fig. 8A, glyceollins enhanced the phosphorylation of Akt without change in the level of Akt protein. Furthermore, transcriptional activation of reporter ARE gene in HepG2 cells caused by
glyceollins was abolished by LY294002, a PI3K inhibitor, suggesting that glyceollins induce phase 2 enzymes by regulating the PI3K signaling pathway followed by phosphorylation of Nrf2.

Taken together, it appears that both phosphorylation of Nrf2 and keap1 modification are prerequisite to nuclear translocation of Nrf2. In fact, inhibition of PI3K suppressed nuclear accumulation of Nrf2 induced by glyceollins (Fig. 8).

In the recent study by Kobayashi et al. [35], the authors hypothesized the role of Keap1-Cys151 as a key residue in the release mechanism of Nrf2 from Keap1. Among the cysteine residues in Keap1, Cys151 appear to be the only crucial residue for the release of Nrf2. To shed some light on the Keap1-Nrf2 release mechanism by glyceollin isomers, we used molecular modeling and computational methods to get further insight into the binding of glyceollin on Keap1. Sulforaphane is an isothiocyanate found in cruciferous vegetables such as broccoli, and it is a known Nrf2/Keap1 pathway modulator. Sulforaphane binds Keap1-Cys151 covalently, triggering the release of Nrf2. Therefore, it appeared extremely interesting to investigate the binding of glyceollins around Cys151 of Keap1. A shallow binding pocket, mostly positively charged all around, surrounds Cys151, favoring polar compounds. It contrasts with the negatively charged area outside the Cys151-binding domain. The docking simulations revealed a good structural and electrochemical match with the glyceollin isomers (Fig. 9, Table 1). Although the docking energy values do not represent the real experimental values, it gives a valid estimate of the affinity between a ligand and a receptor. The glyceollin isomers appear to tightly bind around Cys151 with an average docking energy of -4.9 kcal/mol. Surprisingly, sulforaphane, a known Cys151 binder, appear to be the weakest binder among the six compounds assessed, with a value of -2.3 kcal/mol. This is explained by sulforaphane having a smaller polar area with a higher molecular surface/polar area ratio compared to the glyceollins, thus reducing its affinity with the large polar area around Cys151 of Keap1 (Table 1). In addition, sulforaphane is firstly stabilized around Cys151 by electrostatic interactions before being covalently attached to it. It also contrasts with glyceollins, which are most likely not covalently bound to Cys151. Dehydroglyasperin C has a binding energy and molecular properties close to the glyceollin isomers (Table 1, Fig. 1). However, unlike dehydroglyasperin C, the glyceollin isomers bind by being wrapped around Cys151 and by maximizing the polar contacts with the binding pocket. Noteworthy, AutoDock does not take into account the disulfide bond formation, as it simulates only non-covalent binding events.

As suggested by Kobayashi et al. [35], the surface area around Cys151 might be one of the key-binding domains with Nrf2 due to its electrochemical nature. Our molecular docking data strongly suggest that the glyceollin isomers tightly bind into the binding pocket around Cys151. It blocked the area, preventing Nrf2 from docking to Keap1. However, it is difficult to differentiate between the glyceollins isomers as all three bind virtually identically around Cys151. Furthermore, we speculate that phenyl hydroxyl group of glyceollins may play major role in the interaction with Keap1 domain around Cys 151 because it was involved in antioxidant activity of glyceollins.

In conclusion, glyceollins appear to cause a significant induction of phase 2 detoxifying and antioxidant enzymes, which was mediated by nuclear translocation of Nrf2 through interaction with Keap1 domain around Cys 151 residue.

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5 References

