Evaluation of Antimelanogenic Activity and Mechanism of Galangin in Silico and in Vivo

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Abnormal pigmentation owing to excessive melanin synthesis can result in serious problems such as freckles, age-spots, and melanoma. Tyrosinase inhibitors have been an interesting target for the treatment of hyperpigmentation because tyrosinase is the rate-limiting enzyme in melanin synthesis. The screening for strong tyrosinase inhibitors led to the finding of the flavonoid galangin, which showed notable inhibitory effects on mushroom tyrosinase. The IC50 value of galangin (3.55±0.39 µM) was lower than that of kojic acid (48.55±1.79 µM), which was used as a positive control. In silico docking simulation and mechanistic studies demonstrated that galangin interacted with the catalytic sites of tyrosinase and competed with tyrosine. In B16F10 melanoma cells stimulated with α-melanocyte stimulating hormone, galangin inhibited tyrosinase activity as well as melanin production. Although high doses of galangin were cytotoxic, no cytotoxic effects were observed at low doses. In addition, the in vivo efficacy of galangin was evaluated in HRM2 melanin-possessing hairless mice. As measured by the skin-whitening index and melanin staining, repeated UVB exposure increased skin melanin synthesis. Galangin application significantly reduced melanogenesis induced by UVB exposure. Collectively, our data indicates that galangin shows strong tyrosinase inhibition activity, which suggests that it may be an effective skin-whitening agent.

Key words galangin; melanogenesis; pigmentation; tyrosinase

MATERIALS AND METHODS

Materials Galangin, kojic acid, mushroom tyrosinase, L-tyrosine, α-melanocyte stimulating hormone (α-MSH), and other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A).

Measurement of Tyrosinase Inhibitory Effect and Inhibitory Mechanism in a Cell Free System To evaluate the inhibitory efficacy and mechanisms of galangin on tyrosinase, mushroom tyrosinase was utilized as previously described. Briefly, 1000 units of mushroom tyrosinase was dissolved in 20 µL phosphate-buffered saline (PBS) and added to 170 µL of the assay mixture, which contained 1 mM L-tyrosine, 50 mM...
phosphate buffer (pH 6.5), and 10 µL of the test material. The mixture was incubated at room temperature (25°C) for 30 min. The amount of dopachrome produced was measured spectrophotometrically at 492 nm (OD492) in a microplate reader. The IC50 was calculated from repetitions of the experiment at different galangin doses. To determine the inhibitory mechanism of galangin, a tyrosinase kinetic assay was performed. Various concentrations of l-tyrosine (1, 2, 4, and 8 mM) were used for the inhibition assay. After examination, each value was converted into its reciprocal according to Lineweaver–Burk plots. The results showed the plot of 1/V versus 1/[S]. The intersection of each plot was used to determine the inhibitory mechanism.

**Docking Simulation** For the *in silico* protein-ligand docking simulation, we utilized Autodock4.2. Successful binding was obtained between the protein and the ligand. The three dimensional (3D) structure of tyrosinase used in the crystal structure was from *Agaricus bisporus* (PDB ID: 2Y9X) and a predefined binding site of tyrosine was used as the docking pocket. The docking simulations were performed between tyrosinase and galangin/kojic acid. The compounds were prepared for docking simulation by the following steps: (1) 2D structures were converted into 3D structures; (2) charges were calculated; and (3) hydrogen atoms were added using the ChemOffice program (http://www.cambridgesoft.com). The prediction of possible hydrogen bonding residues between the compounds and tyrosinase and the generation of pharmacophores were computed by LigandScout 3.0.

**Cell Culture System** Murine melanoma B16F10 cells obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) were first used to evaluate the effects of galangin on tyrosinase inhibition. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; WELGENE Inc., Korea, LM001-05) supplemented with 10% fetal bovine serum (FBS; WELGENE Inc., SI01-01) and penicillin/streptomycin (100 UI/50 µg/mL; WELGENE Inc., LS202-02) at 37°C in a humidified atmosphere containing 5% CO2 in air. To evaluate the effects of galangin on cell viability, a cell viability assay was conducted by using commercially available kit (EZ-1000, Korea’s modified Eagle’s medium (DMEM; WELGENE Inc., Korea, LM001-05) supplemented with 10% fetal bovine serum (FBS; WELGENE Inc., SI01-01) and penicillin/streptomycin (100 UI/50 µg/mL; WELGENE Inc., LS202-02) at 37°C in a humidified atmosphere containing 5% CO2 in air. To evaluate the effects of galangin on cell viability, a cell viability assay was conducted by using commercially available kit (EZ-1000, Dogen Bio, Korea). Briefly, the cells were cultured in 96-well plates and treated with different concentrations of galangin. After the indicated time, the absorbance was read at 450 nm by using a microplate reader. All cell experiments were performed at least three times to ensure reproducibility.

**Tyrosinase Activity** Tyrosinase activity in B16F10 cells was examined through the measurement of the rate of oxidation of L-DOPA. The cells were plated in 60-well dishes, incubated in the presence or absence of 1 µM α-MSH, and then treated for 24 h with or without galangin at 5 and 10 µM. The treatment with kojic acid was used as a positive control. The cells were lysed in 500 µL of 50 mM sodium phosphate buffer (pH 6.8) containing 25 µL 1% Triton X-100 and 25 µL 0.1 mM phenylmethyl sulfon- nyl fluoride and then frozen at −80°C for 30 min. After thawing and mixing, the cellular lysates were clarified by centrifugation at 12000 × g for 30 min at 4°C. The supernatants (80 µL) were placed in a 96-well plate, 20 µL L-DOPA (2 mg/mL) was added, and the absorbance was read at 492 nm every 10 min for 1 h at 37°C using a plate reader.

**Melanin Content** In the current study, the melanin content was used as an index of melanogenesis. Briefly, B16 cells were plated in a 60-well plate and incubated in the presence or absence of 1 µM α-MSH. The cells were then incubated for 24 h with or without galangin at 5 and 10 µM. The treatment with kojic acid was used as a positive control. After two washes with PBS, the samples were dissolved in 500 µL 1 N NaOH, incubated at 60°C for 1 h, and mixed to solubilize the melanin. The absorbance at 405 nm was measured by using a microplate reader.

**In Vivo Experiments** The *in vivo* depigmenting efficacy of galangin was assessed in animal experiments. The animal studies were designed by the Aging Tissue Bank, approved by the Institutional Animal Care Committee of Pusan National University, and performed in accordance with the guidelines for animal experimentation issued by Pusan National University. Six-week-old male HRM2 melanin-possessing hairless mice were obtained from Hoshino Laboratory Animals (Yashino, Saitama, Japan) and housed in controlled conditions (23±1°C, 55%±5% humidity, 12-h light/dark cycle) with free access to water and a standard laboratory diet. After an acclimation period of 1 weeks, the mice were randomly divided into four groups of six animals. Galangin (10 µM) and kojic acid (50 µM) were prepared in solution made up of propylene glycol and ethanol (3:7). Dissolved solution (200 µL) or vehicle was topically applied to the dorsal skin of the animal once per day. The animals were irradiated by UVB from a CROSSLINKER (BEX-800, Ultra-Lum Inc., CA, U.S.A.) at 50 mJ/cm2 in accordance with the animal experimental schedule. The colors of the skin sites were measured by using a spectrophotometer CR-10 (Konica Minolta Sensing, Inc., Sakai, Osaka, Japan) in which the colors were described by their L*, a*, and b* values in accordance with the Commission International de L’Eclairage color system. After the animals were sacrificed, the skins were collected, fixed in 4% paraformaldehyde overnight at room temperature, and stained for melanin by using a Fontana–Masson staining kit from American Master*Tech Scientific, Inc. (Lodi, CA, U.S.A.) in accordance with the manufacturer’s instructions. Briefly, sliced skins were stained with ammoniacal silver solution for 60 min at 60°C, incubated in 0.1% gold chloride, and then in 5% sodium thiosulfate.

**Statistical Analyses** The Student’s t-test was used to analyze differences between two groups and ANOVA was used to analyze intergroup differences. Values of p<0.05 were considered statistically significant. The analysis was performed using GraphPad Prism 5 (GraphPad software, La Jolla, CA, U.S.A.).

**RESULTS**

**In Silico Docking Simulation of Galangin on Tyrosinase**

Galangin is a naturally occurring phytochemical and contains a flavonoid moiety in its structure (Fig. 1). Initially, a docking simulation was performed between mushroom tyrosinase (tertiary structure) and compounds (galangin, kojic acid, and tyrosine). The docking site to which galangin and kojic acid were bound was previously determined as the active site of tyrosinase. This simulation was successful and a significant score was achieved. The binding energy between galangin and tyrosinase was −7.67 kcal/mol, as determined by analysis using Autodock4.2. The binding energy between tyrosinase and kojic acid (the positive control) was −4.09 kcal/mol, and −5.13 kcal/mol between tyrosinase and the original substrate.
tyrosine (Table 1). These results showed that galangin bound tyrosinase with a higher affinity than the other interactions. Based on the docking simulation results, we searched the interactions between tyrosinase and compounds using the LigandScout program. We found that galangin interacted with ASN-260, VAL-283, ALA-286, and PHE-292, whereas kojic acid only interacted with ASN-260 and MET280 (Fig. 2, Table 1). These interactions may therefore be the key determinants of inhibitor activity and have an important effect on docking score.

**Determinaton of Anti-melanogenic Effects and Mechanisms in Mushroom Tyrosinase** Based on the *in silico* results and other previously reported results, we evaluated the antimelanogenic effects and mechanisms using mushroom tyrosinase. When tested with the mushroom tyrosinase in cell free system, the inhibitory efficacy of galangin was stronger than that of kojic acid (Fig. 3A). In addition, galangin dose-dependently reduced tyrosinase activity (Fig. 3B). The IC₅₀ values of galangin and kojic acid were calculated and are presented in Table 2. The low IC₅₀ value of galangin (3.55±0.39 µM) indicated that the potency was significantly higher than that of kojic acid (48.55±1.79 µM). The inhibitory mechanism of galangin was further evaluated using Lineweaver–Burk double-reciprocal plots. Various concentrations of L-tyrosine (1, 2, 4, and 8 mM) and galangin (0, 7.5, and 15 µM) were used for inhibition assay. The time-dependent changes in absorbance were measured and the double-reciprocal results were plotted (Fig. 3C). The results indicated that the plot of 1/V versus 1/[S] produced three different lines with different slopes, which intersected on the same vertical axis. As the concentration of compound increased, the value of Kₘ increased, but the values of Vₘₐₓ remained the same, which suggested that galangin was a competitive inhibitor of binding to tyrosinase. These data agreed with the *in silico* results that galangin bound to, and inhibited, the active site of tyrosinase.

**Evaluating Depigmenting Activity of Galangin in B16F10 Murine Melanoma Cells** Next, the depigmenting
activity of galangin was evaluated using B16F10 murine melanoma cells. First, the cytotoxic effects of galangin on B16F10 murine melanoma cells were tested. As shown in Fig. 4A, galangin significantly decreased cell viability at doses above 25 µM. Therefore, to avoid cytotoxicity, we used 5 µM and 10 µM galangin in the experiments to evaluate the antimelanogenic effects. B16F10 cells were treated with galangin in the presence of 1 µM α-MSH. The total melanin synthesis caused by α-MSH treatment was visible to the naked eye, and showed that galangin potently inhibited α-MSH-triggered melanin synthesis (Fig. 4B). α-MSH treatment significantly increased tyrosinase activity and the melanin content of B16F10 cells (Figs. 4C, D). Galangin treatment inhibited cellular melanogenesis, which was augmented by α-MSH in a dose-dependent manner (Figs. 4C, D). Collectively, these results indicated that galangin could be a potent agent for the suppression of melanogenesis through the inhibition of tyrosinase.

Effects of Galangin on the in Vivo Skin Pigmentation
The inhibitory effects of galangin were subsequently examined in melanin-possessing hairless mice that were treated as described by the schedule in Fig. 5A. The colors of the skin sites were measured accurately by using a spectrophotometer. The UVB exposure of mice led to a decrease in the L* value, which was representative of pigmentation (Fig. 5B). The UVB-induced decreases in L* value were significantly blocked in the galangin-treated animals in comparison with that in the control animals treated with vehicle, which demonstrated the potent depigmenting efficacy of galangin (Fig. 5B). The ΔL* value also indicated the potency of galangin during the experimental periods (Fig. 5C). In addition, the skin colors detected by the naked eye at day 14 also indicated the antimelanogenic potency of galangin (Fig. 5D). Fontana–Masson staining, which highlights melanin, verified the effects of galangin. The skin samples of UVB-irradiated animals demonstrated increased melanin spots (Fig. 5E). In agreement with the numerical data from spectrophotometry, galangin-treated animals showed a decrease in the stained melanin spots in comparison with the UVB-irradiated control animals (Fig. 5E). Collectively, these data suggested that galangin was an effective antimelanogenic agent in the UVB-induced in vivo melanogenesis model.

DISCUSSION
It has been previously reported that galangin exerts various biological effects, including cytoprotection. Although the anti-tyrosinase activity of galangin has been previously studied, the mechanisms and accurate efficacy in cell culture systems were not completely described. Therefore, we aimed to explore the potency and inhibitory mechanisms of galangin. The inhibitory efficacy of galangin was initially evaluated by using

### Table 2. The Inhibition Percentage and IC₅₀ Values of Galangin and Kojic Acid

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Concentration</th>
<th>Inhibition percentage (%)</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galangin</td>
<td>0.5 µM</td>
<td>15.4 (±0.9)</td>
<td>3.55 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>19.2 (±1.1)</td>
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<tr>
<td></td>
<td>2.5 µM</td>
<td>35.6 (±1.3)</td>
<td></td>
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<tr>
<td></td>
<td>5 µM</td>
<td>70.5 (±1.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>82.7 (±2.4)</td>
<td></td>
</tr>
<tr>
<td>Kojic acid</td>
<td>5 µM</td>
<td>2.6 (±1.1)</td>
<td>48.55 ± 1.79</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>10.5 (±0.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 µM</td>
<td>28.7 (±2.5)</td>
<td></td>
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<tr>
<td></td>
<td>50 µM</td>
<td>52.5 (±2.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>73.5 (±3.2)</td>
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in silico docking simulation. The docking simulations revealed that the binding of galangin to the active site of tyrosinase was more stable than those of original substrate (tyrosine) or kojic acid (positive control). The inhibitory efficacy and mechanisms were also confirmed using mushroom tyrosinase. Finally, the inhibitory efficacy of galangin against melanogenesis was evaluated in α-MSH-stimulated B16F10 murine melanoma cells. In summary, the present study identified galangin as a potent antimelanogenic agent.

Computational docking simulation has been established as a powerful tool to screen and evaluate new pharmacological agents. Docking simulation results provide not only the binding sites, but also the binding affinity between the compound and the enzyme, which provides initial information without the need for physical experiments. Although the predictions are not always accurate and require further experiments, the docking simulation reduces the materials and resources required and also can support the biological experimental data. Our computational docking simulation indicated that galangin could bind more strongly to tyrosinase than tyrosine or kojic acid. Furthermore, the number of interacting residues between galangin and tyrosinase was larger in comparison with tyrosine or kojic acid. The initial docking data were further verified by using a tyrosinase enzyme assay, which showed that the IC₅₀ of galangin was lower than kojic acid; thus, the inhibitory effects of galangin on tyrosinase were more potent than kojic acid.

The inhibitory mechanism of galangin on tyrosinase was further examined by using mushroom tyrosinase. Because the docking simulation data suggested that galangin binds to the same active site that tyrosinase binds to, it was anticipated that galangin may be a competitive inhibitor of tyrosinase. The experimental results revealed that the galangin competitively inhibited tyrosinase from binding its original substrate, tyrosine, which confirmed the docking simulation results. Collectively, the results demonstrated the reliability of the computational docking simulation and its potential for screening inhibitors.

The efficacy of galangin was also evaluated in B16F10 murine melanoma cells. Galangin showed significant cytotoxic effects in B16F10 cells when administered at a high dose. This was consistent with previous reports of the anticancer activity of galangin in several cancer cell lines. Because of the cytotoxic effects, the experiments to verify the antimelanogenic effects of cells required careful selection of the conditions. However, the IC₅₀ value of galangin was very low when tested in a cell-free system that showed no cytotoxic effects. Indeed, the efficacy of galangin for the inhibition of melanogenesis was acceptable at low doses that did not exert cytotoxicity. Low doses of galangin effectively reduced the
melanin content, as well as the tyrosinase activity, in the cell culture system. Along with the cell-free system, galangin was shown to have a strong inhibitory effect on tyrosinase in the cell culture system.

Finally, the antimelanogenic effect of galangin was evaluated by using a HRM2 hairless mouse model. The HRM2 hairless mouse model provided a useful basis for in vivo melanogenesis experiments, as the mouse can produce melanin especially under environmental stresses. In this mouse model, galangin showed a significant antimelanogenic effect on UVB-induced melanin synthesis. To our knowledge, this is the first study to show the antimelanogenic efficacy of galangin using an in vivo melanogenesis model. It is important to establish in vivo efficacy, because many other melanogenesis inhibitors have failed to act in vivo owing to their inability to cross the stratum corneum of the skin. In summary, galangin was identified as a potent antimelanogenic agent in vivo. We concluded that the tyrosinase inhibitor galangin offered a novel drug candidate for the treatment of hyperpigmentation disorders.

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Conflict of Interest The authors declare no conflict of interest.
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