Inhibition of Melanogenesis by Selina-4(14),7(11)-dien-8-one Isolated from Atractylodis Rhizoma Alba

Yun-Hee Chang, CheongTaek Kim, Minhwan Jung, Young-Hee Lim, Sanghwa Lee, and Sangjin Kang

To develop effective skin-lightening agents, we tested medicinal herbal extracts for their melanogenic-inhibitory activities. We isolated a sesquiterpenoid compound from the extract of Atractylodis Rhizoma Alba using the bioactivity-guided fractionation and identified it as selina-4(14),7(11)-dien-8-one (compound 1) with spectroscopic methods. Compound 1 dramatically reduced melanin synthesis of melan-a cells without any apparent cytotoxicity. Compound 1 did not inhibit cell-free tyrosinase activity but decreased tyrosinase activity in melanocytes. These effects were attributed to reduced expression of melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2). These results suggest that compound 1 may be an effective skin-lightening agent that regulates expression of melanogenic enzymes.

Key words: Atractylodis Rhizoma Alba; melanin; tyrosinase; pigmentation

The level of UV radiation emitted by the sun is increasing due to the destruction of the ozone layer. Our skin is more exposed to UV radiation and often suffers from various harmful effects of UV. Melanin production in human skin is an important defense mechanism against UV and a major determinant of skin color. Melanocytes residing within the basal layer of the epidermis synthesize melanin in specialized organelles, designated as melanosomes, by coordinated action of various melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2). Then, melanin-containing melanosomes are transferred to keratinocytes, providing protection against UV radiation and eventually disappear with desquamation of the skin. Despite the protective role of melanins, abnormal hyperpigmentation such as freckles, chloasma, and other forms of melanin hyperpigmentation sometimes can be serious aesthetic problems. Therefore potent active agents for the improvement of hyperpigmentation have been sought for cosmetic use. Many chemicals such as hydroquinone, arbutin, kojic acid, and ascorbic acid are well known for their skin-lightening effects to develop a skin-lightening agent from traditional medicinal plants and found that Atractylodis Rhizoma Alba, which has been used in the treatment and prevention of diabetes and ulceration, has strong melanogenic-inhibitory activity. Its active compound was purified using bioactivity-guided fractionation and identified as selina-4(14),7(11)-dien-8-one (compound 1) by spectroscopic methods.

The effects of compound 1 were evaluated on melan-a cells. Compound 1 markedly inhibited melanogenesis of melan-a cells by regulating the expression of melanogenic enzymes.

MATERIALS AND METHODS

Materials Atractylodis Rhizoma Alba was purchased at Kyeongdong market in Seoul. Silica gel 60 (230—400 mesh, Merck) for column chromatography, silica gel 60F254 (Merck art. 5715) for TLC, and YMC pack-sil column for HPLC were used. For structural elucidation, a Micromass AutoSpec mass spectrometer and Bruker DMX-400 FT-NMR spectrometer were used. All tissue culture medium and components were purchased from Gibco BRL (Long Island, NY, U.S.A.). Arbutin, L-tyrosine, l-Dopa, phenylmethylsulfonyl fluoride (PMSF), sodium phosphate, β-mercaptoethanol, HCl, and charcoal were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protease inhibitor cocktail was from Boehringer-Mannheim (Indianapolis, IN, U.S.A.), and l-[U-14C] tyrosine were from Amersham (Piscataway, NJ, U.S.A.).

Isolation of Compound 1 (Selina-4(14),7(11)-dien-8-one) Atractylodis Rhizoma Alba (2 kg) was extracted with methanol (three times, each 5 l) at 60 °C. The concentrated methanol extract (350 g) was added to water and then partitioned successively with n-hexane, ethyl acetate (EtOAc), and 1-butanol. The n-hexane layer (35 g), which showed significant melanogenic-inhibitory activity (IC50 = 100 μg/ml), was fractionated using silica gel column chromatography with n-hexane–ethyl acetate stepwise elution (n-hexane : EtOAc = 80 : 1—2 : 1). Five fractions were obtained and fraction III showed the strongest activity (IC50 = 20 μg/ml). This fraction was subjected to silica gel column chromatography with n-hexane–ethyl acetate (40 : 1—1 : 1). Compound I was further purified by HPLC with n-hexane–ethyl acetate (90 : 1) as eluting solvents (Chart 1). The molecular formula of compound I was determined on the basis of the spectral data (1H and 13C-NMR, MS).

Cells and Culture Melan-a melanocytes were a kind gift from Dr. Bennette D.C. (St. George’s Hospital, London, U.K.). Melan-a cells are a nontumorigenic melanocyte cell line established from a patient with malignant melanoma. Melan-a cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids, 1% penicillin, and 1% streptomycin at 37°C in a humidified incubator with 5% CO2.
line originally derived from C57BL mice and more similar to the primary mouse melanocytes than tumorigenic B16 melanoma cell. Melan-a cells were grown in a humidified incubator at 37 °C in a humidified atmosphere of 5% CO₂. Cells were routinely passaged in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, β-mercaptoethanol 100 μM, penicillin 50 U/ml, streptomycin 50 μg/ml, L-glutamine 2 mM, and PMA 200 nM.21)

**Cell Treatment** Melan-a cells were seeded at a density of 2.5 × 10⁶ cells/well of 6-well plates and allowed to attach for 24 h. Then, triplicate cultures were fed with fresh medium containing various concentrations of compound 1. After 48 h, the medium was replaced with the same, fresh test medium. After a further 48 h, cells were harvested with 0.5 ml of 0.25% trypsin/EDTA. After dislodging the cells with occasional agitation, 2 ml of medium was immediately added to inactivate the trypsin, and 100 μl aliquots were seeded into 96-well plates for MTT assay, as described below. The remaining cell suspensions were centrifuged for 5 min at 1500 x g, washed with PBS, and then solubilized in 200 μl of extraction buffer (1% Nonidet P-40, 0.01% SDS, Tris–HCl 0.1 M, pH 7.2, and protease inhibitor cocktail). Extracts were solubilized at 4 °C for at least 1 h and then assays were conducted for each sample in triplicate.22)

**Melanin Content Assay** After treating and extracting as described above,23) cell extracts were centrifuged for 5 min at 15000 rpm. The resulting pellets were lysed with 200 μl of 1 N NaOH and transferred to 96-well plates in triplicate. The relative melanin content was determined by absorbance at 405 nm in a PowerWave X340 ELISA reader (Bio-tech Instruments, Inc.). Arbutin, a known tyrosinase inhibitor, was used as a positive control.15)

**Measurement of Cell Proliferation** The MTT assay was used to determine cell proliferation. After treating as described above,22) 100 μl aliquots of harvested cells were plated. Cells were allowed to attach and grow overnight at 37 °C. The media were discarded, and 100 μl of 0.5 mg/ml MTT was added to each well and incubated at 37 °C for 4 h. After incubation, the plate was centrifuged for 10 min at 3000 rpm. Supernatants were discarded, 200 μl of isopropanol was added, and the plate was incubated at room temperature for 4 h. The formazan precipitates were quantitated based on absorbance at 562 nm in an ELISA reader (PowerWave X340, Bio-tech Instruments, Inc.).

**Measurement of Tyrosinase Activity in Compound 1-Pretreated Cell Extract** Tyrosinase activity was measured according to the method of Virador et al. with some modifications.24) Briefly, the tyrosinase assay was performed in 96-well plates by adding 30 μl of cell extracts prepared as described above, 10 μl of L-[U-14C]tyrosine, and 10 μl of L-Dopa cofactor 0.25 mM in sodium phosphate buffer 1 M, pH 7.2, containing 0.01% albumin. Reactions were incubated for 4 h at 37 °C and the reaction was stopped by cooling. The contents of each well were transferred to 2.5-cm diameter prelabeled 3MM filter disks (Whatman, U.K.) and air-dried. The disks were washed three times with 0.1 N HCl containing excess cold L-tyrosine, twice with 95% ethanol, and then with acetone. Each disk was air-dried and put into a liquid scintillation vial, mixed with scintillation cocktail, and its radioactivity determined using the LS 6500 scintillation system (Beckman, U.S.A.).

**Measurement of Cell-Free Tyrosinase Activity** To prepare solubilized tyrosinase, 5 × 10⁶ cells were removed from stock culture flasks and centrifuged at 1000 x g for 10 min, and the cell pellet was sonicated in 0.5 ml of sodium phosphate buffer 0.1 M (pH 6.8) containing 1% Triton X-100 and PMSF 0.1 mM. After 1 h at 4 °C, the resulting extract was centrifuged at 40000 x g for 20 min at 4 °C. Fifty microliters of extract was added to 1 ml of a reaction mixture containing L-tyrosine 0.1 mM, [3H]-tyrosine 2 μCi per ml, L-DOPA 0.1 mM, PMSF 0.1 mM, and various concentrations of compound in sodium phosphate buffer 0.1 M (pH 6.8). After 3 h at 37 °C, the reaction was terminated by the addition of 1 ml of charcoal (10% wt/vol in 0.1 N HCl). Samples were centrifuged at 2000 g for 10 min, the supernatant was removed and mixed with scintillation cocktail, and the radioactivity was determined using the LS 6500 scintillation system (Beckman, U.S.A.).

**Northern Blotting** Total cellular RNA was extracted from melan-a cells treated with compound 1 using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Total RNA (20 μg) was size-fractionated on 1% agarose/formaldehyde gels, then transferred to a nylon membrane (Amersham), and fixed by UV cross-linking. The blots were prehybridized in Rapid Hyb Buffer (Amersham) according to the manufacturer’s instructions. They were then hybridized in Rapid Hyb Buffer with murine tyrosinase, TRP-1 or TRP-2 cDNA that had been random prime-labeled with [α-32P]dCTP (Amersham) at 60 °C for 1 h. The membranes were washed twice in 2x SSC/0.1% SDS for 10 min at room temperature, and twice in 0.1x SSC/0.1% SDS for 30 min at 65 °C. Specific bands indicating hybridization to RNA samples were visualized autoradiographically. As an internal loading control, the membranes were rehybridized with a human β-actin cDNA probe (Clontech) that had been random prime-labeled with [α-32P]dCTP.

**Western Blotting** Cells were seeded at a density of 2.5 × 10⁶ cells/well of 6-well plates and allowed to attach for 24 h. Then, triplicate cultures were fed with fresh medium containing various concentrations of compound 1. After 48 h, the medium was replaced with the same, fresh test medium. After a further 48 h, the cells were harvested with 0.5 ml of 0.25% trypsin/EDTA. Cell pellets were lyzed in extraction buffer containing 1% Nonidet P-40, 0.01% SDS, and protease inhibitor cocktail. Protein contents were determined with a BCA assay kit (Pierce, Rockford, IL, U.S.A.), equal amounts of each protein extract (10 μg per lane) were resolved using 8% SDS polyacrylamide gel (Koma Biotech, Korea) and transferred onto nitrocellulose membranes (Amersham), and the membranes were blocked with 5% nonfat milk in TBS buffer. After blocking, the membranes were incubated with specific antibodies (1:1000 dilution), αPEP7, αPEP1, and αPEP8, which recognize the COOH-terminal of tyrosinase, TRP-1, and TRP-2, respectively. αPEP7, αPEP1, and αPEP8 were a kind gift from Dr. Vincent J. Hearing (National Cancer Institute, National Institutes of Health, U.S.A.). The membranes were then incubated with HRP-conjugated anti-rabbit IgG at a dilution of 1:2000. Immunoreactive bands were detected with an ECL kit (Amersham).

**Statistical Analysis** The results of each group are expressed as mean ± standard deviation (S.D.). Data were ana-
analyzed using one-way ANOVA with Dunnett’s *post hoc* test between control and sample-treated groups. A *p* value of ≤ 0.05 was considered to represent a statistically significant difference.

RESULTS

**Isolation and Structural Identification of Compound 1**

The concentrated methanol extract of crushed Atractylodis Rhizoma Alba showed melanogenic-inhibitory effect in melan-a cells. Then, bioassay-guided fractionation of the extract was carried out. The extract was partitioned successively with *n*-hexane, ethyl acetate, and 1-butanol. The active fraction, the *n*-hexane layer was further separated with silica gel column chromatography to afford 5 fractions (fr. I—fr. V). Fraction III showed the strongest activity (IC₅₀ 20 mg/ml). This fraction was subjected to silica gel column chromatography to afford compound 1 (Chart 1). The structure of compound 1 was determined by comparison of its MS, UV, and NMR spectral data (Table 1) with those reported in the literature. Compound 1 was a colorless oil. The EI-MS spectrum showed a molecular ion peak at *m/z* 218.34. The molecular formula was determined to be C₁₅H₂₂O on the basis of the elemental analysis; C 82.52%, H 10.16%, O 7.33%. The structure was determined based on the NMR spectra. The ¹H-¹H COSY and HMQC spectra revealed the presence of two structural elements: CH—CH₂—CH₂— and –CH₂–CH—. We determined the structure by combining the information of two partial structures as mentioned and ³JCH or ⁴JCH couplings observed in the HMBC spectrum. Accordingly, compound 1 was confirmed to be selina-4(14),7(11)-dien-8-one by comparison between the above data and the references (Fig. 1).

**Effects of Compound 1 on Melanin Production**

To investigate the effects of compound 1 on melanin production, melan-a cells were treated with various concentrations of compound 1 for 4 d. Arbutin was used as a positive control. As shown in Fig. 2, compound 1 showed a remarkable depigmenting effect in a dose-dependent manner. At concentrations of 5 mg/ml and 10 mg/ml, compound 1 decreased melanin content by almost 40% and 50%, respectively.

**Effects of Compound 1 on Cell Proliferation**

When selecting skin-lightening compounds for cosmetic formulations, one of the important points is that they should have minimal effects on melanocyte cell proliferation. Thus proliferation of cells treated with compound 1 for 4 d was evaluated with the MTT assay. Compound 1 showed little inhibitory effects on cell proliferation at the tested concentrations (2, 5, 10 µg/ml), suggesting that the inhibitory effect of compound 1 on melanin production was not attributable to its cytotoxicity (Fig. 3).

**Effects of Compound 1 on Tyrosinase Activity**

To determine the effects of compound 1 on tyrosinase, a rate-limiting enzyme of melanogenesis, we measured the activity of tyrosinase purified from compound 1-treated melan-a cells by counting ¹⁴C-tyrosine incorporation into nascent insoluble melanin. Compared with the untreated control, com-

---

**Table 1. DEPT, ¹H- and ¹³C-NMR, ¹H-¹H COSY, and HMBC Data for Compound 1 (CDCl₃)**

<table>
<thead>
<tr>
<th>Atom</th>
<th>DEPT</th>
<th>¹H (ppm)</th>
<th>¹³C (ppm)</th>
<th>COSY</th>
<th>HMBC (H—C, 10 Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CH₂</td>
<td>1.51, 1.40</td>
<td>41.24</td>
<td>2</td>
<td>2, 3, 5, 9, 10, 15</td>
<td></td>
</tr>
<tr>
<td>2 CH₂</td>
<td>1.64, 1.53</td>
<td>23.12</td>
<td>1, 3</td>
<td>1, 3, 4, 10</td>
<td></td>
</tr>
<tr>
<td>3 CH₂</td>
<td>2.37, 2.04</td>
<td>36.76</td>
<td>2,14 (w)</td>
<td>1, 2, 4, 5, 14</td>
<td></td>
</tr>
<tr>
<td>4 Q</td>
<td>—</td>
<td>149.06</td>
<td>5</td>
<td>1, 3, 4, 6, 10, 12, 13, 14, 15</td>
<td></td>
</tr>
<tr>
<td>5 CH</td>
<td>2.21</td>
<td>47.06</td>
<td>6</td>
<td>4, 5, 7, 8, 10, 11, 14, 15</td>
<td></td>
</tr>
<tr>
<td>6 CH₂</td>
<td>2.23, 2.72</td>
<td>29.17</td>
<td>15 (w)</td>
<td>6, 9, 12, 13</td>
<td></td>
</tr>
<tr>
<td>7 Q</td>
<td>—</td>
<td>131.57</td>
<td>15 (w)</td>
<td>6, 9, 12, 13</td>
<td></td>
</tr>
<tr>
<td>8 Q</td>
<td>—</td>
<td>201.25</td>
<td>15 (w)</td>
<td>1, 5, 7, 8, 10, 15</td>
<td></td>
</tr>
<tr>
<td>9 CH₂</td>
<td>2.27</td>
<td>57.61</td>
<td>15 (w)</td>
<td>1, 5, 6, 9, 15</td>
<td></td>
</tr>
<tr>
<td>10 Q</td>
<td>—</td>
<td>38.17</td>
<td>15 (w)</td>
<td>1, 5, 6, 9, 15</td>
<td></td>
</tr>
<tr>
<td>11 CH₃</td>
<td>1.81</td>
<td>22.26</td>
<td>15 (w)</td>
<td>5, 6, 7, 8, 11, 12</td>
<td></td>
</tr>
<tr>
<td>12 CH₃</td>
<td>1.98</td>
<td>23.19</td>
<td>15 (w)</td>
<td>5, 6, 7, 8, 11, 12</td>
<td></td>
</tr>
<tr>
<td>13 CH₃</td>
<td>4.85, 4.61</td>
<td>106.91</td>
<td>15 (w)</td>
<td>2, 3, 4, 5, 6</td>
<td></td>
</tr>
<tr>
<td>15 CH₃</td>
<td>0.77</td>
<td>17.30</td>
<td>15 (w)</td>
<td>1, 5, 9, 10</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1. Structure of Compound 1**

**Chart 1. Extraction and Isolation of Compound 1 from Atractylodis Rhizoma Alba**

---

**April 2007**

**721**
Compound 1 reduced tyrosinase activity by 40% and 60% at 5 μg/ml and 10 μg/ml, respectively (Fig. 4). To examine whether this reduced tyrosinase activity is caused by direct inhibition of tyrosinase action, we performed a cell-free tyrosinase assay using solubilized tyrosinase from untreated cells. Unlike arbutin, a known direct tyrosinase inhibitor, compound 1 did not show any inhibition of cell-free, solubilized tyrosinase, suggesting that compound 1 is not a direct inhibitor of tyrosinase catalytic activity (Fig. 5).

Effects of Compound 1 on Expression of Tyrosinase, TRP-1, and TRP-2 To explore the mechanism responsible for the decreased pigmentation, we examined changes in the mRNA levels and protein levels of three important melanogenic enzymes (tyrosinase, TRP-1, and TRP-2) using Northern blotting (Fig. 6) and Western blotting (Fig. 7), respectively. Melan-a cells were treated with 5 μg/ml and 10 μg/ml of compound 1 for 4 d and then each mRNA level and protein level were examined. When compared with the untreated control, mRNA and protein levels of the three melanogenic enzymes were dose dependently reduced.

DISCUSSION

Although melanins protect our skin from UV-induced damage, abnormal hyperpigmentation caused by increased production of melanins can cause psychosocial and cosmetic
Fig. 7. Compound 1 Inhibited Protein Expression of Melanogenic Enzymes

Melan-a cells were treated with compound 1 (5, 10 μg/ml) for 4 d and then harvested. Total protein was extracted and subjected to Western blotting as described in Materials and Methods. Specific detection of tyrosinase, TRP-1, and TRP-2 was performed using αPEP7 (anti-tyrosinase), αPEP7 (anti-TRP-1), and αPEP1 (anti-TRP-2), respectively.

Problems. Many studies have been performed to screen and develop depigmenting agents. Depigmenting agents can be classified on the basis of interfering mechanisms such as regulating: 1) the expression or activity of tyrosinase, TRP-1, TRP-2, and/or peroxidase; 2) the uptake and distribution of melanosomes in keratinocytes; and 3) melanin and melanosome degradation and turnover of pigmented keratinocytes. An ideal depigmenting agent should have a potent and selective skin-lightening effect on the undesired pigmented region such as freckles and chloasma without any side effects. For example, although hydroquinone, introduced in 1961, has been demonstrated to be therapeutically effective, its use has been limited because of its cytotoxicity to melanocytes.

In this study, in an effort to develop a safe and effective skin-whitening agent, we screened medicinal herbal extracts that have long been used for therapeutic purposes. We found that Atractylodis Rhizoma Alba has strong melanogenic-inhibitory activity. The active compound was further purified and identified as selina-4(14),7(11)-dien-8-one (compound 1). At a concentration of 10 μg/ml of compound 1, melanin contents of melan-a cells were reduced by almost 50% compared with the untreated control. The effective concentration of compound 1 was not cytotoxic to melan-a cells. This result suggests that the inhibitory effects of compound 1 on melanin production was not attributable to its cytotoxicity and compound 1 can be a safe skin-lightening agent candidate without influencing melanocyte growth. Some well-known skin-lightening agents such as arbutin and kojic acid have been known to inhibit tyrosinase, a rate-limiting enzyme of melanogenesis, directly. However, compound 1 did not inhibit cell-free tyrosinase but decreased tyrosinase activity in cells. Therefore we concluded that compound 1 is not a direct inhibitor of tyrosinase. Northern blotting and Western blotting indicated that the decreased melanin production and tyrosinase activity resulted from the reduced mRNA and protein expression of tyrosinase, TRP-1, and TRP-2. Because microphthalmia-associated transcription factor (MITF) is known to regulate the mRNA level of tyrosinase, TRP-1, and TRP-2 by binding to the M-box promoter of those proteins, we should examine whether compound 1 decreases expression of the melanogenic proteins by regulating MITF in further study. In conclusion, we demonstrated that selina-4(14),7(11)-dien-8-one isolated from Atractylodis Rhizoma Alba decreases melanin production by regulating tyrosinase, TRP-1, and TRP-2.

REFERENCES