Inhibitory Effect of Gnetin C, a Resveratrol Dimer from Melinjo (*Gnetum gnemon*), on Tyrosinase Activity and Melanin Biosynthesis

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Tyrosinase is the key enzyme involved in melanogenesis. The aim of this study was to investigate the *in vitro* inhibitory effects of gnetin C, a resveratrol dimer isolated from melinjo (*Gnetum gnemon*) seeds, on tyrosinase activity and melanin biosynthesis in murine B16 cells. The inhibitory activities of gnetin C and resveratrol were shown to be almost equal against tyrosinase and melanin biosynthesis in the cells. The IC₅₀ values of gnetin C activity against tyrosinase and melanin biosynthesis were 7.0 and 7.6 μM, respectively, whereas resveratrol demonstrated IC₅₀ values of 7.2 and 7.3 μM, respectively. These results indicated that gnetin C inhibited melanogenesis, in a manner similar to that of resveratrol, by inhibiting tyrosinase and may therefore function as a new skin-whitening agent. However, the direct effects of gnetin C and resveratrol on murine tyrosinase activities were not equal. The IC₅₀ value of resveratrol was 10.1 μM, while gnetin C only exhibited a 25.2% enzyme inhibition at 16 μM. The IC₂₅ values for gnetin C and resveratrol were 15.5 and 4.0 μM, respectively. Therefore, it is suggested that the effects of gnetin C may be due to mechanisms other than the direct inhibition of tyrosinase activity.

Key words gnetin C; tyrosinase; melanin; melinjo; resveratrol dimer

Melanogenesis is the process primarily responsible for the production of melanin, the pigment responsible for skin color and prevention of sun-induced skin injury.¹⁾ In the sequential pathway of melanin formation, tyrosinase (Tyr, EC 1.14.18.1) is the rate-limiting enzyme that catalyzes tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and oxidizes DOPA to dopaquinone.²⁾ This tyrosinase activity is also involved in the abnormal accumulation of melanin (hyperpigmentation). Therefore, tyrosinase inhibitors may be of importance in treating hyperpigmentation and as skin-whitening agents.

Gnetin C, a resveratrol dimer (Fig. 1), is found abundantly in melinjo (*Gnetum gnemon*) seeds. Melinjo is a plant widely cultivated in Southeast Asia; its fruits and seeds are common ingredients in Indonesian foods. The seeds contain phytochemicals, such as gnetin C, resveratrol and other resveratrol dimers. Both resveratrol and its dimers have been reported to possess antioxidant and antimicrobial activities.³⁾ Resveratrol is a natural polyphenolic compound commonly found in the skin of grapes, and is well known for its various pharmacological properties, including anti-inflammatory, anti-carcinogenic, anti-oxidant, anti-platelet, anti-microbial, estrogenic, anti-obesity, and anti-aging properties,^{4–7)} as well as for its inhibitory effect on tyrosinase activity.^{8,9)}

Recently, many compounds from natural sources have been reported to be tyrosinase inhibitors.^{9–13)} For example, oxyresveratrol and hydroxystilbene also inhibit tyrosinase.⁹⁾ In addition, gnetol, isolated from the roots of melinjo, has been reported to strongly inhibit tyrosinase activity in murine B16 melanoma cells.¹⁰⁾ A previous study reported that resveratrol oligomers, ranging from monomers to tetramers, demonstrate significant reductions in inhibitory potency that are associated with increasing polymerization.¹¹⁾ In this study, we evaluated the inhibitory effects of gnetin C, a resveratrol dimer, on

tyrosinase activity and melanin biosynthesis.

MATERIALS AND METHODS

Chemicals Resveratrol and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), supplemented with 25 mM dextrose and GlutaMAXTM, was obtained from Life Technologies (Carlsbad, CA, U.S.A.). The Cell Proliferation Kit I (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay), Protease Inhibitor Cocktail Set I, bicinchoninic acid (BCA) Protein Assay Kit, and fetal bovine serum (FBS) were purchased from Roche Diagnostics (Basel, Switzerland), Merck Chemicals (Darmstadt, Germany), Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.), and Equitech-Bio Inc. (Kerrville, TX, U.S.A.), respectively.

Isolation of Gnetin C An ethanol extract of melinjo seeds (EEMS) was purchased from Hosoda SHC Co. (Fukui, Japan) and the isolation of gnetin C was performed by a previously described method.³⁾ In brief, the EEMS (23 g), containing 63.2% melinjo extract, as a solid, was subjected to octadecyl silica (ODS) column chromatography (Cosmosil 75C₁₈-PREP, 50×475 mm, Nacalai Tesque, Kyoto, Japan) with

Fig. 1. Chemical Structures of Gnetin C and Resveratrol (A) gnetin C, (B) resveratrol.

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10%, 25%, 40%, 60%, and 100% methanol (each 1 L/fraction) as the eluent. The fraction eluted with 60% methanol was subjected to medium-pressure liquid chromatography (MPLC) with methanol (3-min hold at 28%, 3–12-min linear gradient to 28–36%, and 12–18-min hold at 36%) in CHCl_3 as the eluent. MPLC was carried out on a Yamazen MPLC system using a Yamazen Hi-Flash silica gel column (W004, 46×130mm, Yamazen, Osaka, Japan). Fractions (60 mL each) were collected at a flow rate of 60 mL/min. Fractions 6–8 were further purified by HPLC with CH_3CN (10-min linear gradient at 20–22%, 10–20-min linear gradient at 22–30%, and 20–35-min hold at 37%) in 0.1% trifluoroacetic acid to yield gnetin C (69.6 mg; t_R , 35 min). The structure was confirmed by comparing the data with published NMR and MS data.³⁾ Gnetin C was dissolved in dimethyl sulfoxide (DMSO) for the *in vitro* experiments.

Measurement of Tyrosinase Activity Murine melanoma B16 cells were cultured in supplemented DMEM, containing 10% FBS in a 5% CO_2 atmosphere at 37°C. Tyrosinase activity in B16 cells was assessed by measuring the rate of oxidation of L-DOPA, as previously described,¹⁴⁾ with modification. In brief, cells were cultured in 96-well plates to a density of 5×10^3 cells/well. B16 cells were incubated overnight and then treated for 3 d with various concentrations of gnetin C or resveratrol. Following incubation, the cells were washed with ice-cold phosphate buffered saline (PBS) and lysed with 90 μL of 1% Triton X-100 in PBS. Ten microliters of 10 mM L-DOPA was added to the wells containing the cell lysates and incubated at 37°C for 2 h. After incubation, the absorbance was measured at 492 nm and used to calculate the tyrosinase activity per 10^4 cells.

A cell-free assay system was used to test for the direct effects on tyrosinase activity. B16 cells were stirred in 1% Triton X-100 in PBS at 4°C for 2 h and then centrifuged ($20000 \times g$) at 4°C for 30 min. The protein concentration of the supernatant was measured with a BCA Protein Assay Kit. Aliquots (10 μL) of the supernatant, adjusted to a protein concentration of 0.5 mg/mL, were mixed with 10 μL of 10 mM L-DOPA, 70 μL of PBS, and 10 μL of various concentrations of gnetin C or resveratrol in wells of a 96-well plate, and incubated at 37°C for 1 h. After incubation, the absorbance at 492 nm was measured. The inhibitory effect on tyrosinase activity was calculated from the absorbance ratio *versus* a control well that lacked the test compounds.¹²⁾

Cell Viability Cell viability was determined using an MTT assay. The assay provided a quantitative measure of the number of viable cells by determining the amount of formazan produced by metabolic activity in treated or control cells. In brief, 5×10^3 cells/well were incubated in a 96-well plate overnight. After the cells were exposed to various concentrations of gnetin C or resveratrol for 3 d, MTT labeling reagent was added to each well and incubated for 4 h at 37°C. Thereafter, the solubilization solution was added to each well and incubated overnight at 37°C. After incubation, the absorbance of each well was read at 550 nm.

Inhibition of Melanin Biosynthesis B16 cells were cultured with various concentrations of resveratrol or gnetin C for 3 d. Subsequently, the cells were washed with PBS and collected by centrifugation ($100 \times g$), at 4°C, for 3 min. The cell pellet was dissolved in 1 N NaOH after being adjusted to a density of 2.5×10^5 cells per 100 μL . The suspensions were

incubated at 60°C for 30 min to solubilize the melanin, and the absorbance was measured at 492 nm. The inhibition rate (%) was calculated with respect to the control without addition of the compounds.^{10,12)}

Statistical Analyses Statistical analyses were performed using the Dunnett test, and the data are expressed as mean $\pm$ S.D. A *p* value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Inhibition of Tyrosinase Activity and Melanin Biosynthesis in B16 Cells The inhibitory properties of gnetin C and resveratrol on B16 cell tyrosinase activity are shown in Fig. 2A. The inhibitory effects of these agents on tyrosinase activity were very similar. The IC_{50} values for gnetin C and resveratrol against tyrosinase activity were 7.0 and 7.2 μM , respectively. Figure 2B shows that gnetin C and resveratrol were not cytotoxic to B16 cells over the concentration range

Fig. 2. Effects of Gnetin C and Resveratrol on Tyrosinase in B16 Cells

After cells were cultured with various concentrations of gnetin C or resveratrol for 3 d, tyrosinase activity (A) and cell viability (B) were measured. Data are the mean $\pm$ S.D. of 4 independent experiments. Statistically significant differences, as compared to controls, are indicated (* $p < 0.05$, ** $p < 0.01$).

Fig. 3. Effects of Gnetin C and Resveratrol on Melanin Biosynthesis in B16 Cells

The inhibitory effect on melanin biosynthesis was measured as the cellular melanin content after culturing the cells for 3 d in the presence of gnetin C or resveratrol. Cellular melanin, solubilized with 1N NaOH, was measured by absorbance at 492nm. Data are mean±S.D. of at least 3 independent experiments. Statistically significant differences, as compared to controls, are indicated (** $p < 0.01$).

Fig. 4. Direct Effects of Gnetin C and Resveratrol on Tyrosinase Activity

To test the direct effects of gnetin C and resveratrol on tyrosinase, the effect of gnetin C and resveratrol on murine tyrosinase activity was measured in a cell-free system. Data are mean±S.D. of at least 3 independent experiments. Statistically significant differences, as compared to controls, are indicated (* $p < 0.05$, ** $p < 0.01$).

of 2–8 μM. As shown in Fig. 3, the test compounds were almost equally effective in inhibiting melanogenesis, based on the cellular melanin content of the cells following treatment with gnetin C or resveratrol. The IC_{50} values of gnetin C and resveratrol on melanin biosynthesis were 7.6 and 7.3 μM, respectively. Thus, the inhibitory activities of gnetin C and resveratrol on tyrosinase and melanin biosynthesis in B16 cells were virtually equivalent. However, the inhibitory effect of other resveratrol oligomers has previously been shown to be modest, as compared to resveratrol activity.¹¹⁾ Therefore, the present data suggested that gnetin C may be an effective substance for use as a skin-whitening agent and for the prevention of hyperpigmentation.

Previous data have suggested that anti-melanogenic activity may be related to a compound's anti-oxidative properties.¹⁴⁾ For example, the inhibitory activities of the resveratrol analog piceatannol (PICE) on tyrosinase and melanogenesis were

shown to be more potent than those of resveratrol. PICE activity against reactive species generation was also shown to be more effective than that of resveratrol; the anti-melanogenic action of PICE is likely mediated by its anti-oxidative properties.¹⁴⁾ The resveratrol dimer, gnetin C, has been reported to also possess anti-oxidative activity.³⁾ This effect might be due to the mechanism by which gnetin C inhibits tyrosinase; however, further research is needed to confirm this possibility. Considering the importance of counteracting oxidative stress, caused by ultra violet radiation, as a means of preventing skin damage, it is important to find a multifunctional skin-whitening agent that inhibits melanin biosynthesis and possesses anti-oxidant properties.

Direct Effects of Gnetin C and Resveratrol on Tyrosinase Activity To estimate the direct effects of gnetin C and resveratrol on tyrosinase activity, B16 cell lysates were used as a source of murine tyrosinase. These compounds showed inhibitory effects on DOPA oxidation by murine tyrosinase (Fig. 4). Resveratrol showed a 64.1% inhibition of DOPA oxidation at 16 μM, while gnetin C only exhibited a 25.2% inhibition at same concentration. The IC_{25} values for gnetin C and resveratrol were 15.5 and 4.0 μM, respectively. The effect of gnetin C on tyrosinase activity was less potent than that of resveratrol. These data are consistent with other similar studies.^{11,15)} The di-, tri-, and tetramers of resveratrol were found to be weak inhibitors of murine tyrosinase. The direct effects of some oligomeric stilbenes on tyrosinase inhibition have also been reported; all of them showed much less inhibitory effect than did the monomer. Such results indicate that the olefinic double bond in the parent stilbene skeleton and an appropriate molecular size are necessary for tyrosinase inhibition.¹¹⁾

Resveratrol has been reported to inhibit tyrosinase activity and melanogenesis in cultured cells.⁸⁾ It was shown that at least 2 mechanisms underlie the inhibition of tyrosinase activity by resveratrol. The first mechanism causes a reduction in tyrosinase protein levels, and the second involves the direct inhibition of tyrosinase when resveratrol was added to cell extracts.^{8,9)} Although the inhibitory mechanism of gnetin C has not been clarified, it is suggested that the effects may be due to mechanisms other than, or in addition to, direct inhibition of tyrosinase activity. For instance, the intracellular concentration of gnetin C may be higher than that of resveratrol. The underlying mechanism remains to be elucidated.

In summary, the depigmenting action of gnetin C, a resveratrol dimer, was demonstrated. The results showed that gnetin C has an inhibitory effect on tyrosinase activity, leading to the suppression of melanin biosynthesis. Gnetin C is a promising compound that may be useful for treating hyperpigmentation and as a skin-whitening agent.

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