# *N-trans*-Feruloyltyramine as a Melanin Biosynthesis Inhibitor

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**In this study, we examined the effect of *N-trans*-feruloyltyramine (FA) on melanogenesis in mouse B16 melanoma cells. Melanogenesis was inhibited by FA in a dose-dependent manner. FA exhibited a greater potency than kojic acid as a standard inhibitor of melanogenesis. Moreover, treatment of B16 melanoma cells with FA was found to cause marked decreases in the expression levels of tyrosinase. FA-induced downregulation of tyrosinase resulted in suppression of melanin biosynthesis in murine B16 melanoma cells.**

**Key words** melanin; melanogenesis; tyrosinase

Melanin production is principally responsible for skin color and plays an important role in the prevention of sun-induced skin injury. Melanin biosynthesis proceeds through a complex series of enzymatic and chemical reactions in melanocytes.[1—4] Synthesis of melanin starts from the conversion of the amino acid L-tyrosine to dopaquinone by tyrosinase, the enzyme catalyzing the rate-limiting step for melanin biosynthesis.[2] This tyrosinase process is involved in abnormal accumulation of melanin pigments (hyperpigmentation).[5,6] Therefore, tyrosinase inhibitors such as kojic acid and albutin have been established as important constituents of cosmetic products and depigmenting agents for hyperpigmentation. Several new melanin biosynthesis inhibitors from plants have been reported in cultured B-16 mouse melanoma cells, such as *N*-feruloylserotonin, *N*-(*p*-coumaroyl)serotonin, acacetin, esculetin, and nobiletin.[7—9]

As part of efforts to discover phytochemicals with melanin biosynthesis inhibitory activity, we screened compounds isolated from *Enicosanthum membranifolium* Sinclair (Annonaceae) as melanin biosynthesis inhibitors. *N-trans*-feruloyltyramine (FA) showed potent inhibitory activity against melanogenesis in cultured B-16 mouse melanoma cells. This compound was also isolated from *Enicosanthum cupulare* (King) Airy-Shaw. To the best of our knowledge, this is the first report of the melanin biosynthesis inhibitory effects of FA.

## MATERIALS AND METHODS

**Materials** 3,4-Dihydroxyphenylalanine (L-DOPA) and synthetic melanin were purchased from Sigma. Protease inhibitor mixture (Complete) was from Roche. The antibody to tyrosinase was from Santa Cruz Biotechnology. Anti-rabbit antibody conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit were obtained from Amersham Pharmacia. Other reagents were of the highest quality available.

**Extraction and Purification of FA from *E. membranifolium* Sinclair** The methanol extract (369 g) of branches of *E. membranifolium* Sinclair was poured into water and fractionated successively with *n*-hexane, ethyl acetate, and *n*-butanol. Using various chromatographic methods, FA was isolated together with *R*-(−)-mellein, clerodermic acid, and salicifoline chloride. For the details, see our recent paper.[10]

**Extraction and Purification of FA from *E. cupulare*** Air-dried bark of *E. cupulare* (King) Airy-Shaw (5 kg) was finely ground and macerated at room temperature in methanol. The mixture was subsequently filtered and concentrated *in vacuo* to give the methanol extract (185 g). The methanol extract was partitioned successively with *n*-hexane, ethyl acetate, and *n*-butyl alcohol. The ethyl acetate extract (20 g) was fractionated on a silica-gel 60 (Kanto Chemical Co., Ltd., 40—50 $\mu$m) column ($\phi$ 60 mm×500 mm, 300 g), eluted with $CHCl_3$–acetone and methanol in turn. This fractionation process gave 10 fractions (fr. A to J). Fr. D (1.18 g) was separated on high-performance liquid chromatography (Wakosil-II 5C18 HG Prep, $\phi$ 20 mm×250 mm, Wako Pure Chemical Industries, Ltd., Tokyo) to give FA (40.9 mg).

**Spectral Data of FA** mp 92—93 °C. UV (EtOH) $\nu_{max}$ 207, 321 nm. IR (KBr) $\nu_{max}$: 3410, 1651 $cm^{-1}$. $^1$H-NMR (500 MHz, $CD_3OD$): $\delta$ 2.75 (2H, t, $J$=7.2 Hz, H-$\beta$), 3.46 (2H, t, $J$=7.2 Hz, H-$\alpha$), 3.86 (3H, s, $OCH_3$), 6.40 (1H, d, $J$=15.5 Hz, H-2), 6.72 (2H, d, $J$=8.6 Hz, H-3′, H-5′), 6.78 (1H, d, $J$=8.6 Hz, H-8), 7.00 (1H, dd, $J$=2.3, 8.6 Hz, H-9), 7.04 (2H, d, $J$=8.6 Hz, H-2′, H-6′), 7.09 (1H, d, $J$=2.3 Hz, H-5), 7.43 (1H, d, $J$=15.5 Hz, H-3). $^{13}$C-NMR (125 MHz, $CD_3OD$): $\delta$ 35.8 (C-$\beta$), 42.5 (C-$\alpha$), 56.3 ($OCH_3$), 111.5 (C-5), 116.3 (C-3′, C-5′), 116.5 (C-8), 118.6 (C-2), 123.3 (C-9), 128.0 (C-4), 130.7 (C-2′, C-6′), 131.3 (C-1′), 142.1 (C-3), 149.4 (C-6), 150.2 (C-7), 156.9 (C-4′), 169.2 (C-1). EI-MS $m/z$: $[313]^+$.

**Cell Culture** Mouse B16 melanoma cells were purchased from Riken Cell Bank (Tsukuba, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml of penicillin, and 100 $\mu$g/ml of streptomycin at 37 °C in a humidified, $CO_2$-controlled (5%) incubator.

**Determination of Melanin Content** The B16 cells were washed with PBS and dissolved in 2 N NaOH for 1 h at 60 °C. The absorbance at 470 nm was measured and melanin content was measured using the authentic standard of synthetic melanin. The cell viability was determined using the trypan blue exclusion test.

**Measurement of Tyrosinase Activity** The B16 cells were lysed by incubating at 4 °C for 30 min in radioimmunoprecipitation (RIPA) buffer (Tris–HCl 10 mM, pH 7.5, 1%

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NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), NaCl 150 mM, EDTA 1 mM) containing protease inhibitors (Complete protease inhibitor mixture). The lysates were centrifuged at 15000×***g*** for 30 min to obtain the supernatant as source of tyrosinase. The reaction mixture contained phosphate buffer 50 mM, pH 6.8, 0.05% L-DOPA and the supernatant. After incubation at 37 °C for 20 min, dopachrome formation was monitored by measuring absorbance at the wavelength of 470 nm.

**Protein Assay** Protein concentrations were assayed using the DC protein assay reagent (Bio-Rad) with bovine serum albumin (BSA) as a standard.

**Western Blot Analysis** Cells were lysed by incubating at 4 °C for 30 min in RIPA buffer containing protease inhibitors and centrifuged at 15000×***g*** for 30 min. The resultant supernatant (solubilized proteins) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide gel. Proteins were transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membrane. Blocking was performed in Tris-buffered saline containing 5% skim milk powder and 0.05% Tween-20. Blots were incubated with the appropriate primary antibodies and then further incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using an enhanced chemiluminescence (ECL) detection system.

## RESULTS AND DISCUSSION

The methanol extract of branches of *E. membranifolium* was poured into water fractionated with successively *n*-hexane, ethyl acetate, and *n*-butanol. The ethyl acetate fraction was repeatedly subjected to column chromatography on silica gel and further purified by HPLC. The structure of the isolated compound was analyzed using IR, MS, $^{1}$H- and $^{13}$C-NMR, DEPT, and 2D NMR spectra techniques (HMBC, HMQC, and COSY). Finally, the structure was confirmed by comparison with the reference data, and it was identified as FA. This compound was also found in the ethyl acetate fraction of *E. cupulare* (KING) AIR-SHAW (Chart 1).

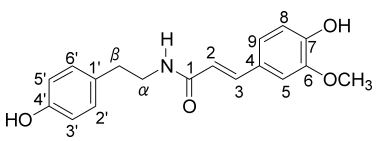


Chart 1. *N-trans*-Ferulolytyramine (FA)

FA, one of the phenylpropanoids,[11] was previously isolated from *Annona glabra*,[12] *Annona cherimola*,[13] *Cananga odorata*,[14] *Polyalthia suberosa*,[15] *Porcelia macrocarpa* (Annonaceae),[16] *Salsola tetrandra*,[17] *Spinacia oleracea* (Chenopodiaceae),[18] *Piper caninum* (Piperaceae),[19] and *Hypecoum* sp. (Papaveraceae).[20] The compound has been reported to show bioactivities such as antimicrobial and anti-HIV activities.[21—24]

From the preliminary tests of FA, R-(−)-mellein, clerodermic acid, and salicifoline chloride, isolated from *E. membranifolium*, FA showed the most potent inhibitory effect against melanogenesis. Further studies of FA were carried out to define the mechanism of its inhibitory activity on melanogenesis in mouse B16 melanoma cells. Mouse B16 melanoma cells were cultured in the presence of FA for 3 d. The melanin content in FA-treated cells was found to be decreased compared with that in the control cells (Fig. 1).

As shown in Fig. 2A, the melanin content as reduced in a concentration-dependent manner by FA, with the maximal reduction at 20 μM. To exclude the possibility that the inhibitory effects of FA on melanogenesis might be caused by the inhibition of cell growth, we examined the number of viable cells grown in the presence of FA. Various concentrations of FA did not suppress cell growth significantly (Fig. 2B), and FA showed more potent inhibitory activity than the positive control of Kojic acid (Fig. 2C).

Tyrosinase, the enzyme catalyzing the rate-limiting step in melanin biosynthesis, is a well-characterized marker of dif-

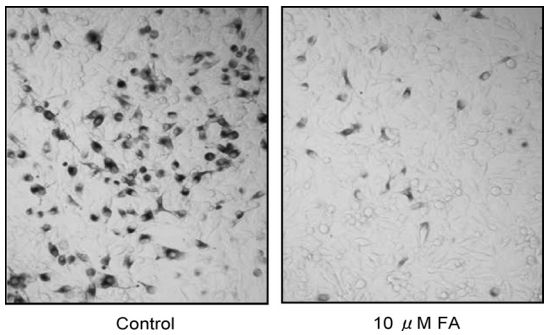


Fig. 1. Microscopic Observations of B16 Melanoma Cells after Treatment without or with FA

The cells were treated with 10 μM FA for 72 h. The cells were viewed under a phase-contrast microscope.

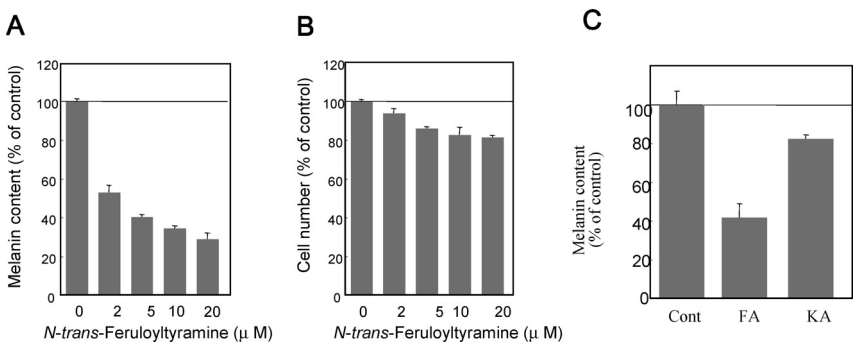


Fig. 2. Effect of FA on Melanin Content and Cell Number in B16 Melanoma Cells

(A) The cells were treated with various concentrations of FA for 72 h. The melanin content was determined as described in Materials and Methods. (B) Cell viability was determined using the trypan blue dye-exclusion test. (C) Cells were treated with FA 10 μM or kojic acid (KA) 10 μM for 72 h. The melanin content was determined as described in Materials and Methods. All data represent the mean±S.D. of two different experiments each carried out in duplicate.

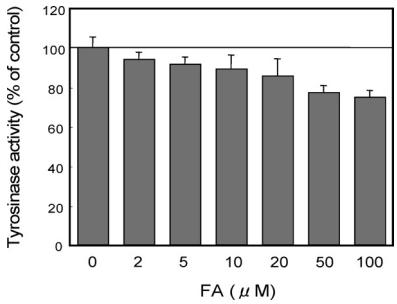


Fig. 3. Effect of FA on Murine Tyrosinase Activity

Tyrosinase activity was determined by measuring the formation of dopachrome as described under Materials and Methods. Data represent the mean±S.D. of two different experiments each carried out in duplicate.

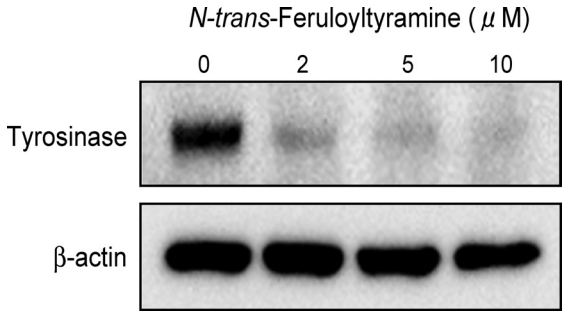


Fig. 4. Effect of FA on the Expression of Tyrosinase Protein in B16 Melanoma Cells

The cells were treated with various concentrations of FA for 72 h. The expression level of tyrosinase protein was examined using Western blot analysis with specific antibody as described in Materials and Methods.

ferentiation in melanocytes and melanoma cells. We examined the inhibitory effects of FA on murine tyrosinase activity using L-DOPA as substrate. As shown in Fig. 3, FA showed marginal inhibition of tyrosinase activity, indicating that the inhibitory effects of FA on melanogenesis are not due to direct inhibition of tyrosinase activity.

To investigate the mechanism underlying the inhibition of melanogenesis, we examined the effects of FA on the expression of tyrosinase protein using Western blot analysis with the specific antibody against tyrosinase. As shown in Fig. 4, the levels of tyrosinase protein expression were decreased by treatment with FA.

Several intracellular signaling pathways have been reported to involve expression of tyrosinase. The activation of microphthalmia-associated transcription factor (MITF), a transcription factor that regulates tyrosinase gene expression, is known to be a critical step during melanogenesis. Extracellular signal-regulated kinase (ERK) phosphorylates MITF and promotes its degradation, thereby resulting in inhibition of tyrosinase expression and melanogenesis. In addition, previous reports demonstrated that the phosphatidylinositol 3-kinase (PI3K) pathway is involved in the regulation of tyrosinase gene expression. Although it is likely that FA is implicated in these proposed signaling pathways, further investigations are required to determine the precise role of FA.

In summary, the present study demonstrated that FA suppresses melanogenesis by preventing expression of tyrosinase protein in mouse B16 melanoma cells.

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