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In vitro melanogenesis inhibitory effects of *N*-feruloyldopamine

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Synopsis

Tyrosinase is the rate-limiting enzyme in the melanogenesis process. It remains the most efficient way to downregulate melanin production and improve unsightly pigmentary disorders. The aim of our investigations was to find a structurally characterized molecule with better efficacy than existing molecules without cell toxicity. We focused our investigations on compounds that could act as substrate-mimicking inhibitors of tyrosinase and identified *N*-feruloyldopamine as the best candidate. *In vitro*, *N*-feruloyldopamine inhibited human tyrosinase with higher efficacy than the reference inhibitor arbutin without cell toxicity at least up to 100 µM as measured in cultured normal human epidermal melanocytes (NHEMs). Moreover, the inhibition appeared to be specific to mammalian tyrosinases as shown by a very poor inhibition of mushroom tyrosinase, but a significant decrease of total melanin in B16-F10 cells. The antioxidant capacity assessed using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was comparable to that of vitamin C and finally, *N*-feruloyldopamine exerted a significant inhibition of Pmel17 gene expression when used at 100 µM on cultured NHEM. Taken together, these results suggest that *N*-feruloyldopamine is a serious candidate for *in vivo* application as complexion-brightening ingredient.

INTRODUCTION

Besides increasing concerns about deleterious effects of excessive sun exposure, skin complexion color has become a crucial parameter in the perception of health and beauty (1). Pigmentation disorders that can occur at different moments of a lifetime (pregnancy, old age …), e.g., melasma, freckles, or age spots, can dramatically affect one's self-confidence. Medicinal or cosmetic complexion-lightening agents have proved growing popularity.

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In mammalian melanocytes, melanin polymers are produced within specific lysosome-related organelles called melanosomes. Melanosomes contain three major pigmentary enzymes: tyrosinase, tyrosinase-related protein-1, and tyrosinase-related protein-2 (dopachrome tautomerase). By catalyzing the two rate-limiting steps in melanogenesis, i.e., the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and its subsequent oxidation to dopaquinone (2,3), tyrosinase plays a critical role in the melanogenesis process (4).

Melanosomes undergo a four-step maturation process characterized by different morphological stages (I to IV). As observed using transmission electron microscopy, melanosomes in stage I appear as round, clear, unpigmented organelles with intralumenal vesicles. Late stage I melanosomes exhibit proteinaceous fibrils that are completely formed in not yet pigmented melanosomes in stage II. The production of internal matrix fibers as well as the maturation of melanosomes from stages I to II depend on the presence of the structural protein Pmel17, also known as gp100 or SILV. Shortly after its delivery from the trans-Golgi network to stage I melanosomes, Pmel17 is cleaved into several fragments, of which some will form the fibrillar matrix of the organelle (5,6). Pmel17 expression, stability, trafficking, and processing are principally affected by another melanosomal protein called MART-1 (7). The two proteins form a complex, which suggests that MART-1 acts as a chaperone-like structural component for Pmel17 (8).Once the fibrous striations are fully formed in ellipsoidal stage II melanosomes, tyrosinase is transported to stage III melanosomes, which triggers melanin synthesis. Melanin polymers deposit on the fibrils, resulting in their progressive thickening and darkening. Stage IV of so-called mature melanosomes is finally described as the stage where internal structures are no longer distinguishable (9).

Development of safe yet effective melanogenesis inhibitors is one of the challenges for the dermatological research and cosmetics industry. Despite numerous chemical steps involving several enzymes, transport, and structural proteins, tyrosinase is considered the rate-limiting enzyme of melanogenesis. As a consequence, the majority of commercially available skin-lightening ingredients used over the past decades act—at least *in vitro*—as tyrosinase inhibitors. Kojic acid (10), arbutin (11), licorice extract (12), *n*-butylresorcinol (13) are among the best known members. However, there are some exceptions such as ascorbic acid and niacinamide (14) that were shown to exert their depigmenting effect through significant antioxidant activity and by inhibiting the transfer of melanosome to keratinocytes, respectively.

The aim of our research was to develop a structurally characterized tyrosinase inhibitor devoid of cell toxicity. For this, we chose to focus our investigations on compounds having high structural homology with the natural substrates of human tyrosinase, namely L-tyrosine and L-DOPA and that would thus act as substrate-mimicking inhibitor. This way, we selected *N*-feruloyldopamine (also referred to hereafter as *N*-feruloyldopamine), a naturally occurring ferulic acid derivative as the best candidate.

To demonstrate the efficacy of this molecule at inhibiting melanogenesis, its ability to inhibit mushroom and human tyrosinases *in vitro* as well as melanin production *in vitro* was assessed. Furthermore, knowing that antioxidant capacities can downregulate melanin production, its radical-scavenging property was also evaluated. Finally, its capacity to act on melanosome maturation was also assessed by measuring gene expression of three proteins involved in melanosome formation, namely Pmel17, MART-1, and Protein P.

MATERIALS AND METHODS

CHEMICALS

Ferulic acid, 3-hydroxytyramine, EDCI (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride), L-DOPA, synthetic melanin, 3-methyl-2-benzothiazolinone hydrazone (MBTH), mushroom tyrosinase, dimethyl sulfoxide (DMSO), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

SYNTHESIS OF *N*-FERULOYLDOPAMINE

N-feruloyldopamine was obtained in a one-step synthesis by means of a peptide coupling reaction between ferulic acid and 3-hydroxytyramine in basic medium using EDCI as water soluble coupling agent. Subsequent crystallization affords obtaining *N*-feruloyldopamine with around 50% yield. The structure of the synthesized compound was confirmed by nuclear magnetic resonance and mass spectrometry analyses and further characterized by infrared and high-performance liquid chromatographic (HPLC) analyses (data not shown). The final purity of *N*-feruloyldopamine was ≥93% as measured using appropriate HPLC method.

CELL VIABILITY

Cells [normal human epidermal melanocytes (NHEMs) or B16-F10 cells] were seeded at a density of 8000 cells/well in 96-well plates.

The B16-F10 cells were cultured in RPMI 1640 medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (FBS; Invitrogen).

NHEMs were cultured in keratinocyte serum-free medium (KSFM; Invitrogen) supplemented with 2% FBS, basic fibroblast growth factor, endothelin-1, α-melanocyte–stimulating hormone (α-MSH), and isoproterenol for 24 h, and then switched to the same medium containing the compounds to test at increasing concentrations and incubated for additional 72 h. After incubation, the medium was removed and 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was then added. After 2 h of incubation, the MTT solution was discarded and finally DMSO was added. Absorbance [optical density (OD)] was read at 550 nm using Wallac Victor 2 Spectrophotometer (Perkin Elmer, Turku, Finland). Each condition was tested in sextuplicate ($n = 6$).

HUMAN TYROSINASE INHIBITION ASSAY

NHEM were seeded in 24-well plates at a density of 80000 cells per well and grown to confluence. Inhibitors (*N*-feruloyldopamine or positive references) were diluted in DMSO and next applied in the culture medium for 24 h at 37°C under 5% CO_2. After incubation, the culture medium was removed and human tyrosinase was extracted by lysis of melanocytes by thermal shock. After centrifugation, the supernatants containing tyrosinase were incubated with MBTH and L-DOPA solutions for 30 min before the OD was read at 490 nm. Results are expressed as percentage of tyrosinase activity compared

to untreated control (cells grown in the culture medium alone) after normalization to the total protein content beforehand quantified using BCA Assay Kit (Interchim)(15). Kojic acid at 700 µM and arbutin 1 mM were used as positive controls. Each condition was tested in triplicate.

MUSHROOM TYROSINASE INHIBITION ASSAY

Twenty microliters of inhibitor molecule (*N*-feruloyldopamine or positive references) diluted in DMSO were incubated for 5 min with 20 µl of a 200 U/ml mushroom tyrosinase solution adjusted with 140 µl of phosphate-buffered saline (PBS; Sigma-Aldrich). Then, 20 µL of a 10 mM solution of L-DOPA were added, and the OD was read at 490 nm after 10 min of incubation at room temperature. Kojic acid at 70 µM was used as positive control.

INHIBITION OF MELANIN SYNTHESIS

B16-F10 murine melanoma cells at the 12th passage were seeded in 96-well plates. Cells were grown for 24 h in DMEM medium (Invitrogen) supplemented with 10% FBS (Invitrogen). The compounds to test were then added together with NDP-MSH 10^{-7}M, and cells were incubated for additional 72 h. The media were subsequently removed and cells were rinsed in PBS. After cell lysis, total melanin content was assessed by measuring the absorbance at 405 nm and by referring to a standard curve obtained beforehand using synthetic melanin.

Results are expressed as percentage of melanin content compared to untreated control (culture medium + DMSO) after normalization to the total protein content.

EVALUATION OF THE ANTIOXIDANT CAPACITY

N-feruloyldopamine was dissolved in ethanol at 2*X*, *X* representing the final concentration in wells. *N*-feruloyldopamine (100 µl) were dissolved in an ethanolic solution of DPPH at 60 µM. Cysteine at 20 µM was used as positive control. After 30 min of incubation at room temperature, the OD was read at 530 nm. Results are expressed as percentage of radical-scavenging capacity relative to untreated control.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE PCR

NHEM were first cultured at 37°C under 5% of CO_2 in KSFM (Invitrogen) supplemented with 100 µg/ml geneticin (Sigma) and 0.3% normycin (Invitrogen).

Cells were then seeded into 24-well plates and cultured for 48 h in KSFM added with the products to test.

After washing in PBS, total RNA was extracted using SV 96 Total RNA Isolation System kit (Promega, Charbonnières-lès-Bains, France) according to the manufacturer's instructions. After quantification at 260 nm, 100 µL of purified total RNA were kept for each sample. Primers used were the following:

	Forward primer	Reverse primer
Actin	GTGGGGCGCCCCAGGCACCA	CTCCTTAATGTCACGCACGATTTC
Pmel17 gene	GGTGGAGACCACAGCTAGAGA	GCGGAACCTGCCCAAGGCCTGCT

One-step quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the "iScript One-Step RT-PCR Kit with SYBR Green" (Biorad, Marnes-la-Coquette, France). The reaction mix contained the SYBR Green buffer 1×; the two specific primers at 0.6 µM; 1 µl of enzyme mix; 50 ng of total sample RNA; and qsp 50 µl with RNase-free water. The reaction was run in 96-well plates using CHROMO4® thermocycler (Biorad). Results were normalized to the actin transcript levels.

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett's procedure for multiple comparisons versus untreated control group.

RESULTS AND STATISTICAL ANALYSIS

Results are presented as means ± SD for experiments conducted at least in triplicate ($n = 3$). The statistical significance between groups was assessed using Student's *t-test* for positive controls and one-way ANOVA followed by either Tukey' test or Dunnett's method for *N*-feruloyldopamine.

RESULTS

SCREENING RESULTS OF COMPOUNDS HAVING STRUCTURAL HOMOLOGY WITH TYROSINASE SUBSTRATE

The conserved catalytic center of tyrosinase is composed of two copper atoms bound to six histidine residues. Tyrosinase substrates, i.e., the amino acids L-Tyrosine and L-DOPA dock to this dinuclear copper center by their phenol function and catechol group, respectively (16,17).

Using docking approach, Khatib *et al.* (18) have shown that addition of a short two–carbon lipophilic alkyl chain enhanced the tyrosinase-inhibiting properties of resorcinol. In addition, we have previously shown that amides derived from p-coumaric acid with a two–carbon alkyl chain separating the amide function from a phenol ring was a potent structure for tyrosinase inhibition (19).

In our approach of searching for a substrate-mimicking tyrosinase inhibitor, *N*-feruloyldopamine was selected as the best candidate (Figure 1). Indeed, this molecule exhibits all the structural features that are reported to support a tyrosinase–substrate interaction or a tyrosinase-inhibiting effect, i.e., a catechol moiety and a phenol substituent in *para* position of a two-carbon alkyl chain (20).

EVALUATION OF *N*-FERULOYLDOPAMINE CYTOTOXICITY

Before an evaluation of its inhibiting properties, the cytotoxicity of *N*-feruloyldopamine at increasing concentrations was measured both in human melanocytes and in murine

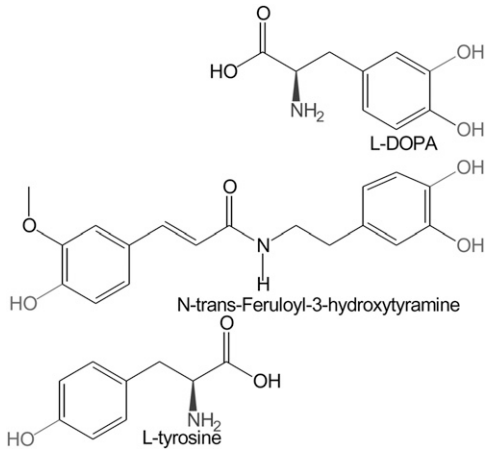

Figure 1. Chemical structures of *N*-feruloyldopamine, L-tyrosine, and L-DOPA.

B16-F10 cells. After 72 h of incubation, *N*-feruloyldopamine did not significantly alter cell growth for concentrations up to 50 µM for B16-F10 cells and for concentrations at least up to 100 µM for NHEMs (Figure 2).

HUMAN AND MUSHROOM TYROSINASE INHIBITIONS

The effect of *N*-feruloyldopamine on tyrosinase activity was first assessed in NHEM using MBTH assay (21). Results show that *N*-feruloyldopamine dose dependently inhibited tyrosinase activity for concentrations ranging from 5 to 100 µM (Figure 3A). From 50 µM, the inhibitory effect of *N*-feruloyldopamine was significantly higher than that of the positive control arbutin ($p < 0.01$).

Contrary to human tyrosinase, no significant inhibition of mushroom tyrosinase could be observed (Figure 3B) although the positive control kojic acid significantly inhibited mushroom tyrosinase activity, thus validating the experiment.

INHIBITION OF MELANIN SYNTHESIS IN B16-F10 CELLS

After 72 h of incubation with *N*-feruloyldopamine at 10 µM, melanin synthesis was significantly decreased by 31.6% ($p < 0.01$) compared to untreated control. *N*-feruloyldopamine used at 30 µM provided a significant 65.5% inhibition ($p < 0.01$) and a 75.6% inhibition when used at 50 µM ($p < 0.01$) (Figure 4).

N-FERULOYLDOPAMINE ANTIOXIDANT CAPACITY

To investigate further melanogenesis inhibitory action of *N*-feruloyldopamine *in vitro*, its antioxidant capacity was evaluated using DPPH *in tubo* assay. *N*-feruloyldopamine showed a dose-dependent antioxidant capacity that reached a plateau with around 70% of radical-scavenging activity for concentrations equal or above 30 µM. From 10 µM, the radical-scavenging activity of *N*-feruloyldopamine was comparable to that of cysteine 40 µM (Figure 5).

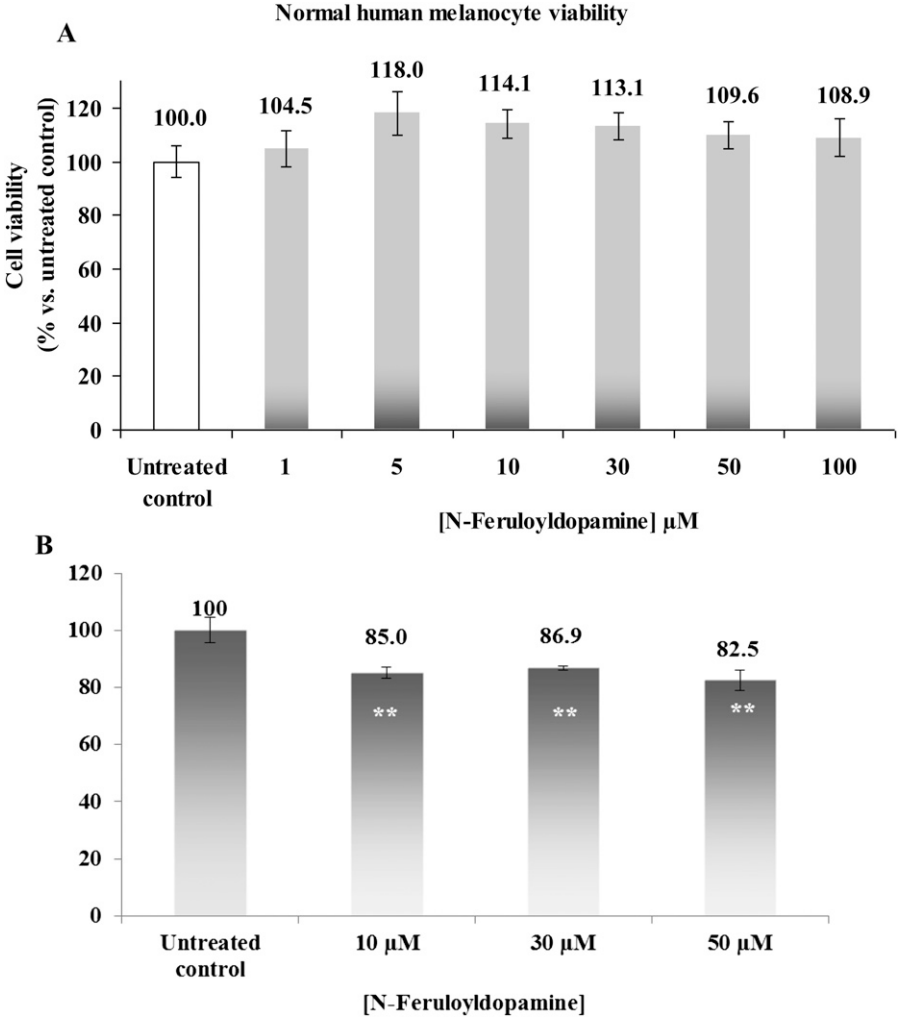

Figure 2. Cell viability assay. MTT assay of (A) NHEMs. (B) B16-F10 cells. Mean ± SD, $n = 6$. *, **Statistically significant vs. untreated control, $p < 0.05$ and $p < 0.01$, respectively.

ACTIVITY OF *N*-FERULOYLDOPAMINE ON MELANOSOME MATURATION GENES

After 48 h of incubation with *N*-feruloyldopamine, Protein P mRNA expression remained unchanged as measured using qRT-PCR methods. On the other hand, MART-1, described as a chaperone protein for Pmel17, was moderately decreased (data not shown). Finally, incubation with *N*-feruloyldopamine significantly decreased Pmel17 mRNA expression in a dose-related manner with a statistically significant 53% decrease when used at 100 µM (Figure 6) ($p < 0.05$ Dunn's procedure).

DISCUSSION

Tyrosinase catalyzes the first two limiting steps of melanogenesis. It is thus the most often targeted protein for melanogenesis inhibition purposes. In our research program

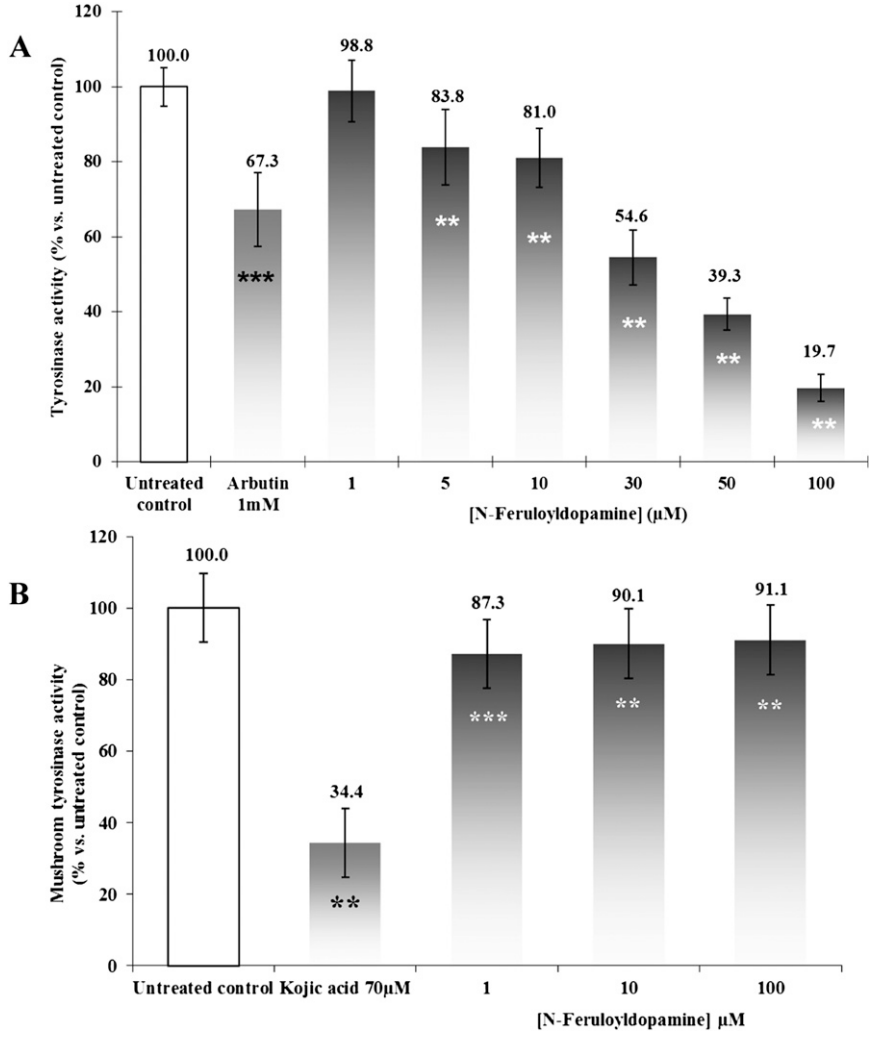

Figure 3. Measurement of (A) human and (B) mushroom tyrosinase activities. Mean ± SD, $n = 6$. **, ***Statistically significant vs. untreated control, $p < 0.01$ and $p < 0.001$, respectively.

to identify a novel tyrosinase inhibitor, we decided to screen compounds combining the structural features known to participate in the interaction between tyrosinase and its substrates or inhibitors. This way, we have selected *N*-feruloyldopamine as the most potent substrate-mimicking inhibitor of tyrosinase.

N-feruloyldopamine has been previously isolated from *Atraphaxis spinosa* (22) with poor yield. We used organic synthesis to obtain this molecule in sufficient amounts to evaluate its melanogenesis inhibition abilities *in vitro*.

Results show that *N*-feruloyldopamine exerted 45% inhibition when used at 30 µM in cultured NHEMs. Using linear regression, the IC50 of *N*-feruloyldopamine in this model was evaluated at 48.3µM (% inhibition = $-0.8042 \times$ [*N*-feruloyldopamine] + 90.698; $R^2 = 0.9068$). Contrarily, *N*-feruloyldopamine did not exert any inhibitory effect toward

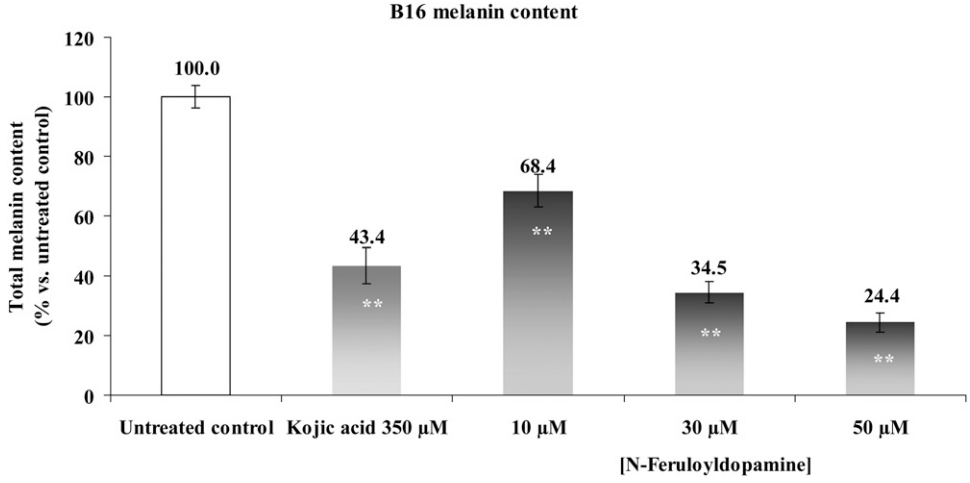

Figure 4. Total melanin content of murine melanoma B16-F10 cells. Mean ± SD for $n = 6$. **Statistically significant vs. untreated control, $p < 0.01$.

mushroom tyrosinase. Comparable results have previously been reported, notably with p-coumaric acid (23). Such a difference between the two models might be explained by the different structural features of human and mushroom tyrosinases. Although both tyrosinases exhibit high homology in their active sites, human tyrosinase is a monomeric protein while mushroom tyrosinase is mostly tetrameric. Moreover, regulation of mushroom tyrosinase differs significantly in several respects from mammalian tyrosinase, notably with regard to post-translational modifications (4). It is also worth noting that qRT-PCR results showed no effect of *N*-feruloyldopamine on tyrosinase gene expression in normal human melanocytes (data not shown).

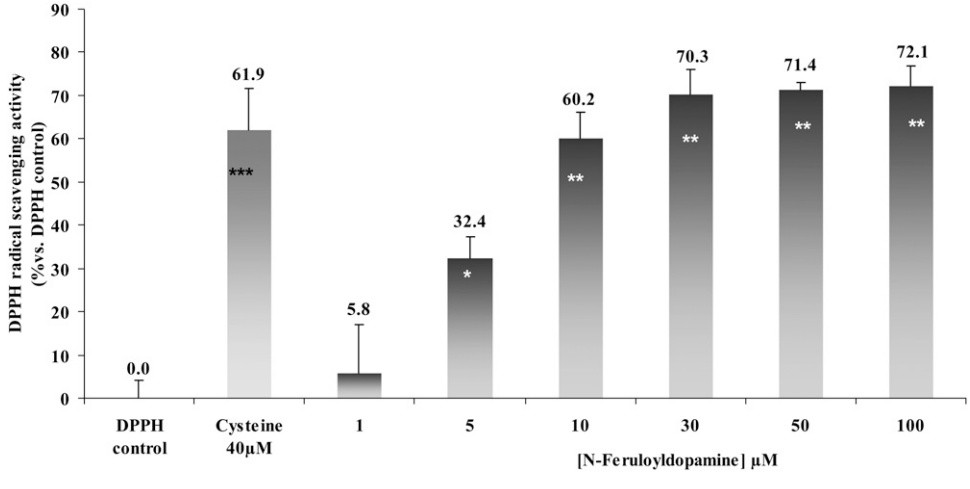

Figure 5. Radical-scavenging activity of *N*-feruloyldopamine. DPPH assay. Mean ± SD, $n = 6$. *,**Statistically significant vs. untreated control, $p < 0.05$ and $p < 0.01$, respectively.

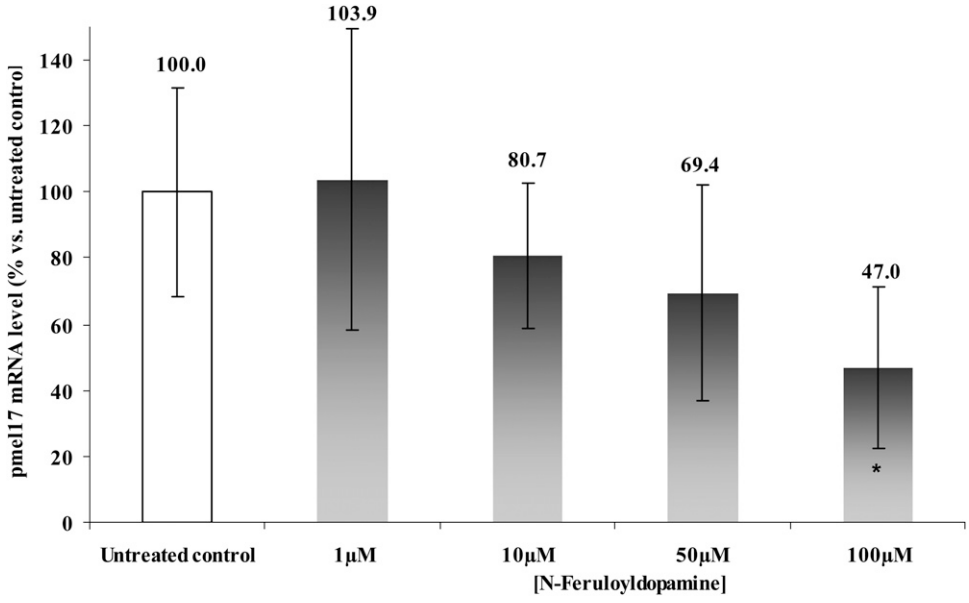

Figure 6. Effect of *N*-feruloyldopamine on Pmel17 mRNA level. qRT-PCR. Mean ± SD, $n = 7$. *Statistically significant vs. untreated control, $p < 0.05$.

N-feruloyldopamine was next shown to significantly decrease total melanin content in B-16-F10 murine cells. Over 30% inhibition was observed when *N*-feruloyldopamine was used at 10 μM. Using linear regression, IC 50 in that model system was evaluated at 27.1 μM (% inhibition $= -1.4758 \times$ [*N*-feruloyldopamine] $+ 90.03$; $R^2 = 0.9045$). Taken together, the results of human tyrosinase, mushroom tyrosinase, and melanin synthesis in B16 cells support the idea that tyrosinase inhibition by *N*-feruloyldopamine may be specific to mammalian-type tyrosinases.

To investigate other mechanisms that may contribute to a melanogenesis inhibitory effect of *N*-feruloyldopamine, radical-scavenging properties as well as melanosome maturation gene regulation were evaluated. Indeed, one of the reactions catalyzed by tyrosinase is the oxidation of L-DOPA to dopaquinone using copper and molecular oxygen. Some antioxidants can thus interfere with the oxidation reaction. Moreover, other antioxidants such as vitamin C act as hypopigmenting agents by reducing intermediates of melanin polymers (24,25). By stimulating the synthesis of α-MSH in keratinocytes, ultraviolet-induced reactive oxygen species (ROS) also stimulate melanin production (26). Reducing ROS generation can thus help minimize melanin production. *N*-feruloyldopamine showed significant antioxidant efficiency with around 70% of radical-scavenging activity for concentrations equal to or higher than 30 μM.

Using qRT-PCR methods, the effect of *N*-feruloyldopamine on Pmel17, MART-1, and Prot P—the best-described melanosome maturation genes—was evaluated. Despite poor inhibition of MART-1 and Protein P gene expressions, *N*-feruloyldopamine significantly decreased Pmel17 gene expression when used at 100 μM on cultured NHEM. Under physiological conditions, only mature melanosomes are transferred from melanocytes to the surrounding keratinocytes. Hence, factors that regulate melanosome maturation are expected to reduce skin pigmentation (27). In that respect, Pmel17

plays a crucial role as structural proteins on which melanin deposits after production in the presence of MART-1 (9).

In summary, our *in vitro* results show that *N*-feruloyldopamine, a molecule chosen for its structural analogies with the natural substrates and inhibitors of human tyrosinase, significantly decreased tyrosinase activity in NHEM as well as melanin synthesis in B16 cells. In addition, *N*-feruloyldopamine has demonstrated interesting antioxidant activity and a significant ability to downregulate Pmel17 gene expression in NHEM, both of which may enhance the melanogenesis inhibition effect. In the absence of cytotoxicity at efficient concentrations, *N*-feruloyldopamine represents an interesting alternative to the often-decried kojic acid, hydroquinone, and arbutin.

ACKNOWLEDGMENT

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