4-(6-Hydroxy-2-naphthyl)-1,3-benzendiols: A Potent, New Tyrosinase Inhibitor

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Tyrosinase is a key enzyme for melanin biosynthesis and known to be sensitive to ultraviolet light in the presence of oxygen. Therefore, finding effective tyrosinase inhibitors, either from synthetic or natural sources, can be beneficial in the treatment of melanin-related disorders. We synthesized 4-(6-hydroxy-2-naphthyl)-1,3-benzediols (HNB), a new family of hydroxyl substituted phenyl naphthalenes, as the isosteres of oxyresveratrol. This study investigated inhibitory effects of HNB on tyrosinase activity. HNB inhibited mushroom tyrosinase with an IC₅₀ value of 0.07 μ M, which is more potent than the anti-tyrosinase activity of kojic acid (IC₅₀=38.24), a well-known tyrosinase inhibitor. The kinetic analysis of tyrosinase inhibition revealed that HNB is a competitive inhibitor (K_i 4.78×10^{-9} M at 0.125 μ M and K_i 6.21×10^{-9} M at 0.25 μ M). We further found that HNB also inhibited melanin production in B16F10 melanoma cells (B16 cells). In addition to tyrosinase inhibiting activity, melanin biosynthesis was inhibited by HNB in the B16F10 cells. These data strongly suggest that HNB can suppress the production of melanin *via* the modulation of tyrosinase activity.

Key words 4-(6-hydroxy-2-naphthyl)-1,3-benzediols (HNB); tyrosinase inhibitor; melanin biosynthesis

Melanin is secreted by melanocytes localized in the basal layer of the dermis¹⁾ and is the major cellular component responsible for skin color. Although normal melanin pigmentation in the skin is the major defense mechanism against ultraviolet light, the production of abnormal pigmentation such as melasma, freckles, senile lentigines, and other forms of melanin hyperpigmentation can be a serious problem aesthetically.²⁾

The *de novo* synthesis of melanin involves the conversion of the amino acid, L-tyrosine to L-DOPA (3,4-dihydroxyphenylalanine), and then the oxidation of L-DOPA by tyrosinase, a rate-limiting enzyme, catalyzing step in melanin biosynthesis³⁾ that yields dopaquinone. This altered tyrosinase process often causes abnormal accumulation of melanin pigments.³⁾ Therefore, it has been common practice to include tyrosinase inhibitors such as kojic acid⁴⁾ in the depigmentation ingredients of cosmetic products.⁵⁾

Various tyrosinase inhibitors that have been reported include hydroquinone,⁶⁾ ascorbic acid derivatives,⁷⁾ azelaic

acid,⁸⁾ corticosteroids,⁹⁾ retinoids¹⁰⁾ and arbutin.¹¹⁾ Recently, phenolic compounds were shown to have a tyrosinase inhibitory effect, in addition to their known anti-oxidative and free radical-scavenging efficacies.¹²⁾ These compounds also are shown to exhibit anti-inflammatory,¹³⁾ antiviral,¹⁴⁾ and anti-allergic activities in various biological systems.¹⁵⁾ Some stilbenes have polyphenolic compounds that are reported to possess various biological activities that are anti-carcinogenic,¹⁶⁾ anti-inflammatory,¹⁷⁾ and inhibit tyrosinase.^{18–22)}

Resveratrol, a hydroxystilbene present at high concentrations in grape seeds and red wine, was recently shown to have a wide range of biological and pharmacological activities including as a potent antioxidant. Oxyresveratrol, one of the ingredients of the Mori cortex,²³⁾ is reported to exert a potent inhibitory effect on tyrosinase's DOPA oxidase activity. At present, the production of skin-whitening cosmetics with oxyresveratrol as its basis depends on Mori cortex extract, because the quantity of the ingredient is limited and an effective method of synthesis has yet to be established.^{24,25)}

Therefore, a search for alternative materials continues, as with polyphenolic compounds, which have high bioactivity and easy accessibility. Through our continuous effort, we have found several potent tyrosinase inhibitors from natural materials^{26,27)} that have an even greater potency than the well-known kojic acid.²⁸⁾ Recently, we succeeded in the synthesis of a new compound (patent application number: 10-2006-0071433), "HNB", as a polyphenolic compound (Fig. 1).

The aim of this current study was to characterize the inhibition mode of HNB on mushroom-derived tyrosinase. We also investigated the inhibitory effects of HNB on melanin biosynthesis, cell viability, and its depigmenting effect on the murine B16F10 melanoma cell line (B16 cells).

Fig. 1. Chemical Structure of 4-(6-Hydroxy-2-naphthyl)-1,3-benzediols (HNB)

Hydroquinone, kojic acid and resveratrol are included as comparisons.

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MATERIALS AND METHODS

Materials Mushroom tyrosinase, L-tyrosine [3-(4-hy-

droxyphenyl)]-L-alanine (*S*)-2-amino-3-(4-hydroxyphenyl) propionic acid), kojic acid [5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one] and α -MSH (alpha-melanocyte stimulating hormone) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.) Solvents used for organic syntheses were redistilled. All other chemicals and solvents were of analytical grade and used without further purification.

Cell Culture B16 cells (obtained from the Korean Cell Line Bank) were cultured in DMEM with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (100 IU/50 μ g/ml) in a humidified atmosphere containing 5% CO₂ in air at 37 °C. B16 cells were cultured in 24-well plates for the melanin quantification and enzyme activity assays.

Cell Viability Cell survival was quantified by a colorimetric MTT assay that measures mitochondrial activity in viable cells. This method is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to MTT-formazan crystal by mitochondrial enzyme as previously described.²⁹⁾ Briefly, cells seeded at a density of 3×10^4 /cell in a Corning 48-well plate (Corning, NY, U.S.A.), were allowed to adhere overnight; the culture medium was then replaced with fresh serum free DMEM. MTT was freshly prepared at 5 mg/ml in phosphate-buffered saline (PBS). Aliquots of 500 μ l of MTT stock solution were added to each well, and the plate was incubated at 37 °C for 4 h in an humidified 5% CO₂ incubator. After 4 h, the medium was removed. To each well, 500 μ l of EtOH-DMSO (1:1 mixture solution) was added to dissolve formazan. After 10 min, the optical density of each well was measured spectrophotometrically with a 560 nm filter. Results are shown from three experiments.

Assay to Measure Inhibitory Effects on Mushroom Tyrosinase Mushroom-derived tyrosinase was used as the source of the enzyme for the entire study. Tyrosinase activity was determined as described previously with minor modification.¹²⁾ Briefly, 20 μ l of aqueous solution of mushroom tyrosinase (1000 units) was added to a 96-well microplate (Nunc, Denmark), in a total volume of 200 μ l assay mixture containing 1 mM L-tyrosine solution and 50 mM phosphate buffer (pH 6.5). The assay mixture was incubated at 25 °C for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm (OD₄₉₂) using a microplate reader (Hewlett Packard). IC₅₀, inhibitory concentration-50, is the concentration of a drug that inhibits a standard 50% response. IC₅₀ is a value derived from the *X*-axis and is determined from the alignment of the dose-response curve on the dependent *Y*-axis. In the present study, dose-dependent inhibition experiments were performed in triplicate to determine the IC₅₀ of a drug. According to the inhibition percentage of three doses in each experiment, the log-linear curves and their equations were determined. Then, the individual IC₅₀ was calculated as the concentration when the *Y*-axis equalled 50% inhibition. Results are shown from three experiments.

Kinetic Analysis of Tyrosinase Inhibition Various concentrations of L-tyrosine (2 to 32 mM) as substrate, 20 μ l of aqueous solution of mushroom tyrosinase (1000 units), and 50 mM potassium phosphate buffer (pH 6.5) with or without test samples [0.125 and 0.25 μ M of 4-(6-hydroxy-2-naphthyl)-1,3-benzendiol] were added to a 96-well plate in a total volume assay mixture of 200 μ l. Using a microplate reader,

the initial rate of dopachrome formation from the reaction mixture was determined by the increase of absorbance at wavelength 492 nm per min (Δ OD₄₉₂/min). Michaelis constant (K_m) and maximal velocity (V_{max}) of the tyrosinase activity were determined by the Lineweaver-Burk's plot at various concentrations of L-tyrosine as a substrate.³⁰⁾ The reaction kinetics required a modification of the Michaelis-Menten equation due to competitive inhibition by HNB together with substrate inhibition by L-tyrosine. The result is shown from three experiments.

Assay of Murine Tyrosinase Activity Tyrosinase activity was estimated by measuring the rate of oxidation of L-DOPA.³¹⁾ Cells were plated in 24-well dishes at a density of 5×10^4 cells/ml. B16 cells were incubated in the presence or absence of 100 nM MSH and then treated for 24 h at various concentrations of 4-(6-hydroxy-2-naphthyl)-1,3-benzendiol (0–50 μ M). Cells were washed and lysed in 100 μ l of 50 mM sodium phosphate buffer (pH 6.5) containing 1% Triton X-100 (Sigma) and 0.1 mM PMSF (phenylmethylsulfonyl fluoride) and then frozen at –80 °C for 30 min. After thawing and mixing, cellular extracts were clarified by centrifugation at 12000 rpm for 30 min at 4 °C. Eighty microliters of the supernatant and 20 μ l of L-DOPA (2 mg/ml) were put in a 96-well plate, and the absorbance at 492 nm was read every 10 min for 1 h at 37 °C using an ELISA plate reader. The final activity was expressed as Δ O.D./min for each condition. Results are shown from three experiments.

Determination of Melanogenesis in B16 Cells Determination of melanin content was performed using a modified method of Bilodeau *et al.*³²⁾ The amount of melanin was used as an index of melanogenesis in the current study. The B16 cells (5×10^4) were plated on 24-well dishes and incubated in the presence or absence of 100 nM MSH. The cells were then incubated for 24 h at various concentrations of 4-(6-hydroxy-2-naphthyl)-1,3-benzendiol (0–50 μ M). After washing twice with PBS, samples were dissolved in 100 μ l of 1 N NaOH. The samples were incubated at 60 °C for 1 h and mixed to solubilize the melanin. Absorbance at 405 nm was compared with a standard curve of synthetic melanin. Results are shown from three experiments.

Statistical Analysis The inhibition of tyrosinase activity is expressed as a percentage of inhibition based on: $100 - [(A \cdot 100)/B]$, where $A = OD_{492}$ with a test sample and $B = OD_{492}$ without a test sample. Data collected have a mean standard error ($n=3$). The biological significance $p < 0.05$ was determined by the Student's *t*-test.

RESULTS

The Ability of HNB to Inhibit Tyrosinase In the present study, we investigated the effect of HNB on tyrosinase inhibition in the hope of finding a new and effective substance for skin whitening and for the prevention of hyper-pigmentation. We found that HNB clearly reduced tyrosinase activity in a dose-dependent manner. The tyrosinase inhibitory effects of hydroquinone, kojic acid, resveratrol and HNB are presented in Table 1. HNB showed a very strong inhibitory effect against tyrosinase. HNB at the low concentration of 1.56 μ M exhibited 80.97% inhibition of DOPA oxidase activity in mushroom tyrosinase, whereas 50% inhibition was shown at the concentration of 0.07 μ M. As shown in Fig. 2,

Table 1. Inhibitory Effects of HNB, Hydroquinone, Kojic Acid and Resveratrol on Mushroom Tyrosinase Activity

Compound	Inhibition (%)						IC ₅₀ ^{b)} AVG (μM)
	1.56 μM ^{a)}	3.13 μM ^{a)}	12.5 μM ^{a)}	25 μM ^{a)}	50 μM ^{a)}	100 μM ^{a)}	
HNB	80.97±5.41***	87.25±7.83***	72.86±2.98***	94.40±1.75**	95.46±1.00	95.72±1.41	0.07
Hydroquinone	3.60±2.94	11.05±1.03	22.17±1.30	62.27±5.41	95.27±0.78	96.69±0.43	33.48
Kojic acid	14.49±3.61*	26.03±4.37**	32.04±6.03	60.45±10.99	69.85±10.77***	87.56±6.56*	38.24
Resveratrol	17.44±6.21*	27.14±6.21*	35.22±12.22**	37.79±9.29*	52.22±4.79***	69±6.40***	55.61

a) Values represent means±S.E. of three experiments. b) 50% inhibitory concentration (IC₅₀). **p*<0.05, ***p*<0.01, ****p*<0.001, compared to the hydroquinone.

Fig. 2. Dose-Dependent Inhibitory Effects of HNB on Tyrosinase Activity

Tyrosinase activity was measured using L-tyrosine as the substrate. Values are mean±S.E. of *n*=3 determinations. The optical density value of control at 492 nm was 0.486±0.007.

Fig. 3. Dose-Dependent Inhibitory Effects on Mushroom Tyrosinase by HNB, Hydroquinone, Kojic Acid, and Resveratrol

Samples shown are HNB (circle), hydroquinone (diamond), kojic acid (square) and resveratrol (triangle). Effects on tyrosinase activity by the samples as a function of concentration are represented as inhibition %, mean±S.E. of three independent tests. **p*<0.05, ***p*<0.01, ****p*<0.001, compared to the hydroquinone.

Fig. 4. Lineweaver-Burk Plot of Mushroom Tyrosinase in the Presence of HNB

Data were obtained as mean values of $1/V$, inverse of the increase of absorbance at a wavelength 492 nm per min ($\Delta A_{492}/\text{min}$), of three independent tests with different concentrations of L-tyrosine as a substrate. Inhibitors of the enzyme were with a new compound 0.125 μM (rectangle), 0.25 μM (triangle) and no HNB (circle). The modified Michaelis-Menten equation is: $1/V_{\text{max}} = 1/K_m (1/[I]/K_i)$. V denotes velocity of the reaction, S for L-tyrosine concentration, K_i for inhibitor constant.

HNB showed dose-dependent inhibitory effects on the mushroom tyrosinase activity and exhibited 65.77% inhibition at a very low concentration of 0.049 μM on DOPA oxidase activity of mushroom tyrosinase. Hydroquinone, kojic acid, resveratrol and HNB showed dose-dependent inhibitory effects on the mushroom tyrosinase activity (Fig. 3).

Inhibition Pattern of Mushroom Tyrosinase by HNB

The mode of inhibition of the enzyme was determined by Lineweaver-Burk plot analysis as shown in Fig. 4, in which the inhibitory kinetics exhibited K_i 4.78×10^{-9} M at 0.125 μM and K_i 6.21×10^{-9} M at 0.25 μM. HNB at 0.125 and 0.25 μM showed the same V_{max} value of 1.79×10^{-2} and K_m values of 7.70×10^{-1} M and 7.71×10^{-1} M, respectively. As L-tyrosine substrate concentration varies, the K_m value of tyrosinase increased in a dose-dependent manner, without changing V_{max} , thereby indicating HNB as a competitive tyrosinase inhibitor.

Melanin Production and Cell Viability of B16 Cells

The results from the cell viability assay using MTT for B16 cells are given in Fig. 5. At doses of 20, 50, and 100 μM/ml of HNB, cell viability was 96.60%, 93.86%, and 87.36% respectively, during 24-h treatments, indicating the non-cytotoxic nature of HNB in B16 cells.

To gain further evidence of HNB's involvement in tyrosinase and melanogenesis, the inhibition of melanin production in B16 cells was examined. As shown in Fig. 5, the melanin content of the HNB-treated cells decreased in a dose-dependent manner, showing 96.09% at 10 μM, 95.94% at 25 μM, 92.71% at 50 μM, 35.94% at 100 μM compared to control (the MSH-treated group as 100%). In addition, we investigated the inhibitory effect of HNB on tyrosinase activity of B16 cells treated with MSH (Fig. 6). After 24 h incubation with

	0μM ^{a)}	20μM ^{a)}	50μM ^{a)}	100μM ^{a)}
Cell Viability(%)	100	96.60±0.48**	93.86±1.49*	87.36±4.97
Melanin Production(%)	100	95.94±5.51	92.71±0.41***	35.94±3.39***

a) Values represent means ± S.E. of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the untreated control.

Fig. 5. The Effect of HNB on Cell Viability and Melanin Production in B16 Cells

Cells were treated with various doses of HNB (20–100 μM) and were examined by MTT assay. Melanin contents were measured at 405 nm. Values represent the mean ± S.E. of three experiments. Data are expressed as % of Control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the untreated control.

	0μM ^{a)}	10μM ^{a)}	25μM ^{a)}	50μM ^{a)}	100μM ^{a)}
Tyrosinase activity(%)	100	64.77±18.13	54.40±0	37.31±3.24***	27.98±2.37***

a) Values represent means ± S.E. of three experiments. *** $p < 0.001$, compared to the untreated control.

Fig. 6. Effect of HNB on Tyrosinase Activity

In the presence of 100 nM MSH, B16 cells were treated with various doses of HNB (10–100 μM) for 24 h. Results are expressed as % of control and each column represents the mean ± S.E. of three determinations. *** $p < 0.001$, compared to the untreated control.

HNB, tyrosinase activities were suppressed to 64.77% at 10 μM, 54.40% at 25 μM, 37.31% at 50 μM, 27.98% at 100 μM, and 23.32% at 200 μM compared to the control MSH-treated group (100%), which correlates well with the extent of reduced melanin content.

DISCUSSION

As stated, we measured the effect of HNB on tyrosinase inhibition in the hope of finding a new and effective substance for skin whitening and the prevention of hyperpigmentation; we found that HNB clearly reduced tyrosinase activity in a dose dependent manner. In a kinetics study, HNB was identified as a competitive inhibitor of the mushroom tyrosinase with the same V_{max} value. Therefore, HNB might indicate the enzyme inhibitory activity by binding at the same site as the copper active site of mushroom tyrosinase.

In the B16 cell culture system, we found tyrosinase activ-

ity and melanin levels were significantly reduced by the addition of HNB to the incubation medium containing cells pretreated with MSH. It is interesting to note that HNB exhibited a stronger effect on mushroom tyrosinase activity compared to murine tyrosinase activity.

Treatment of B16 cells with HNB significantly inhibited melanin formation, as less amounts of melanin were formed when cells were treated with HNB at various doses (0–100 μM) for 24 h, and when induced by 100 nM MSH. The melanin content decreased in a dose-dependent manner by the HNB treatments.

As a potential skin-lightening agent, HNB should be safe, without adverse cytotoxic side effects. Results from the cell viability test presented in Fig. 5 show that HNB suppressed tyrosinase activity and total melanin content without adversely affecting cell viability. These data suggest that HNB has strong depigmenting activity without any discernable cytotoxicity.

Based on the current data, we believe that HNB could be used as a skin whitening agent preferable to kojic acid because it showed more potent anti-tyrosinase activity: $IC_{50} = 0.07 \mu M$ vs. $IC_{50} = 35.05 \mu M$, respectively.

It may be of interest to mention other polyphenolic compounds. Oxyresveratrol, for example, is shown to have a potent inhibitory effect on tyrosinase's DOPA oxidase activity. One drawback of oxyresveratrol is its instability due to its stilbene structure, which is sensitive to oxygen and moisture. HNB, which may prevent the instability of oxyresveratrol, was designed and synthesized to show excellent inhibition of mushroom tyrosinase.

In conclusion, our newly synthesized HNB is a safe and effective tyrosinase inhibitor that can be produced in large quantities. We propose HNB as a potent new tyrosinase inhibitor that could be considered for skin lightening purposes.

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