Hydroxamic Acid Derivatives as Anti-melanogenic Agents: The Importance of a Basic Skeleton and Hydroxamic Acid Moiety

Ho Sik Rho, Heung Soo Baek, Soo Mi Ahn, Jae Won Yoo, Duck Hee Kim, and Han Gon Kim

R & D Center, AmorePacific Corporation, Yongin, Gyeonggi 446-729, Korea. *E-mail: thiocarbon@freechal.com
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The production of melanin is mainly regulated by melanogenic enzyme tyrosinase. Tyrosinase is bifunctional enzyme, which catalyzes the hydroxylation of L-tyrosine to L-dopa and enhances the oxidation of L-dopa to dopaquinone. Therefore, inhibitors of tyrosinase should be useful as therapeutic agents for the treatment of melanin hyperpigmentation. Many metal ion chelators, for example, kojic acid, flavonol, benzaldehyde, N-nitrosohydroxylamine, have been developed as tyrosinase inhibitors. Their inhibiting activities come from binding with copper in active site of tyrosinase. Hydroxamic acids were well known as metal ion chelators. However, studies on hydroxamic acids as tyrosinase inhibitors and depigmenting agents were rare. Recently we reported hydroxamic acid derivative 1 containing adamantane moiety strongly inhibits melanin synthesis (Fig. 1). However, compound 2 which is precursor of compound 1 showed no inhibitory activity. Moreover, salicylhydroxamic acid exhibited no inhibitory activity in tested concentration. These result revealed that hydroxamic acid moiety as well as the rest part play an important role for the anti-melanogenic activity.

To gain more insight into an anti-melanogenic activity of hydroxamic acid, several hydroxamic acid derivatives were synthesized and evaluated their inhibitory effects on tyrosinase and depigmenting effects in a murine melanocyte cell line were evaluated.

![Figure 1. Structure of hydroxamic acid derivatives](image_url)

Results and Discussion

The synthetic pathways of hydroxamic acid derivatives are shown in scheme 1 and 2.

The acids 5a-5d were reacted with ethylchloroformate and N-methylmorpholine in THF as solvent to convert the carboxylic acids to an anhydrides. The anhydrides were immediately reacted NH₂OH·HCl or NH₂OMe·HCl to produce the corresponding hydroxamic acid derivatives 3a-3d or methyl protected hydroxamic acids 6c and 6f (Scheme 1). 4-Substituted benzoic acids (5c, 5e, 5f, 5g and 5h) were refluxed in thionyl chloride to afford acid chlorides. These acid chlorides were reacted immediately with methyl 4-aminobenzoate in pyridine to produce corresponding amide derivatives (6a-6e). The ester groups were hydrolyzed under standard condition (NaOH, ethanol) to produce corresponding acids (7a-7e). The acids were reacted with ethylchloroformate and N-methylmorpholine in THF to convert the carboxylic acids to anhydrides. The anhydrides were reacted immediately with NH₂OH·HCl or NH₂OMe·HCl to produce the corresponding hydroxamic acid derivatives (6a-6e) or methyl protected hydroxamic acids 4f and 4g (Scheme 2).

The inhibitory activities of our synthetic hydroxamic acid derivatives on mushroom tyrosinase was initially investigated and compared with those of kojic acid and hydroquinone which are well known tyrosinase inhibitors. Acetoxyhydroxamic acid showed no inhibitory activity against tyrosinase. When methyl group was replaced by normal hexyl and cyclohexyl groups, mild activities were shown (IC₅₀ = 39.99 µM and IC₅₀ = 17.89 µM). Surprisingly, when methyl group was replaced by phenyl group, the resulting compound 3e showed potent inhibition activity (IC₅₀ = 0.28 µM). Naphthyl derivative 3d showed more potent inhibitory activity (IC₅₀ = 0.10 µM). However, when the NHOH group of 3e and 3d were methylated...
ted on the hydroxyl group, their inhibitory activities were completely lost. These results indicated that planar hydrophobic group and hydroxamic acid moiety are necessary for the inhibition of tyrosinase. In a next set of experiments, we changed planar naphthyl group of 3d into more bulky and a little flexible structure (Fig. 2).

Interestingly, 4-(phenylcarboxamido)-N-hydroxybenzamide 4a containing two phenyl groups connected with amide linkage showed decreasing activity (IC₅₀ = 2.29 µM). When methyl group was attached to the para position of phenylcarboxamido group, the resulting compound 4b showed decreased activity (IC₅₀ = 4.38 µM). Decreases in the activities were detected along with an increasing chain length such as ethyl, n-propyl and n-butyl (Table 1, 4c-4e). In tyrosinase assay, planar structure is important factor for the inhibitory activity of hydroxamic acid. Without planar structures such as phenyl and naphthyl, hydroxamic acid moiety may not bind tightly to the active site or other essential part of tyrosinase. However, more bulky groups than naphthyl also interrupted binding of hydroxamic acid to tyrosinase.

After testing tyrosinase inhibitory activity, we evaluated the inhibitory potency against the melanin formation. The synthesized derivatives were assayed for their cytotoxicities and inhibitory effects in a murine melanocytes cell line (Melan-a). The results obtained from these experiments revealed that acetohydroxamic acid showed no anti-melanogenic activity (IC₅₀ > 50 µM). In agreement with the results on tyrosinase activity, compounds 3e, 3f, 4f and 4g containing NHMe group showed no inhibitory activities. All other compounds presented inhibitory activities. However, anti-melanogenic activities in a murine melanocytes cell line are a little different from inhibitory potentials of tyrosinase. Compound 3d containing naphthyl group, which exhibited the highest activity in tyrosinase inhibition, showed moderate activity (IC₅₀ = 26.16 µM). More bulky and a little flexible hydroxamic acid, 4-(phenylcarboxamido)-N-hydroxybenzamide 4a, showed more

Table 1. Anti-melanogenic activities of hydroxamic acid derivatives

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Tyrosinase IC₅₀*</th>
<th>Melanin Inhibition IC₅₀</th>
<th>% Survival of melan-a cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetohydroxamic acid</td>
<td>&gt; 200 µM</td>
<td>&gt; 50 µM</td>
<td>93.77 (50 µM)</td>
</tr>
<tr>
<td>3a</td>
<td>39.99 µM</td>
<td>15.72 µM</td>
<td>105.27 (30 µM)</td>
</tr>
<tr>
<td>3b</td>
<td>17.89 µM</td>
<td>42.31 µM</td>
<td>98.22 (50 µM)</td>
</tr>
<tr>
<td>3c</td>
<td>0.28 µM</td>
<td>45.51 µM</td>
<td>96.63 (50 µM)</td>
</tr>
<tr>
<td>3d</td>
<td>0.10 µM</td>
<td>26.16 µM</td>
<td>98.34 (30 µM)</td>
</tr>
<tr>
<td>3e</td>
<td>&gt; 200 µM</td>
<td>&gt; 50 µM</td>
<td>97.23 (50 µM)</td>
</tr>
<tr>
<td>3f</td>
<td>&gt; 200 µM</td>
<td>&gt; 50 µM</td>
<td>94.25 (50 µM)</td>
</tr>
<tr>
<td>4a</td>
<td>2.29 µM</td>
<td>4.53 µM</td>
<td>96.30 (30 µM)</td>
</tr>
<tr>
<td>4b</td>
<td>4.38 µM</td>
<td>4.01 µM</td>
<td>100.21 (30 µM)</td>
</tr>
<tr>
<td>4c</td>
<td>7.31 µM</td>
<td>2.93 µM</td>
<td>92.10 (30 µM)</td>
</tr>
<tr>
<td>4d</td>
<td>12.51 µM</td>
<td>1.24 µM</td>
<td>97.38 (30 µM)</td>
</tr>
<tr>
<td>4e</td>
<td>27.41 µM</td>
<td>1.69 µM</td>
<td>95.31 (30 µM)</td>
</tr>
<tr>
<td>4f</td>
<td>&gt; 200 µM</td>
<td>&gt; 50 µM</td>
<td>103.64 (50 µM)</td>
</tr>
<tr>
<td>4g</td>
<td>&gt; 200 µM</td>
<td>&gt; 50 µM</td>
<td>99.23 (50 µM)</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>25.85 µM</td>
<td>1.10 mM</td>
<td>97.12 (3.0 mM)</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>9.81 µM</td>
<td>3.97 µM</td>
<td>87.27 (10 µM)</td>
</tr>
</tbody>
</table>

*Values were determined from logarithmic concentration-inhibition curves and are given as means of three experiments.
potent activity (IC_{50} = 4.54 µM). In contrast to the result of tyrosinase, increasing inhibitory activities were detected along with increasing chain length such as methyl, ethyl and n-propyl. Interestingly n-butyl group did not show any significant increase in the inhibitory activity. Among all derivatives, compound 4d showed stronger inhibitory activity (IC_{50} = 1.24 µM). These differences can be explained by two hypotheses. One is the difference of amino acid sequence between mushroom tyrosinase and murine tyrosinase. The other is different physical properties of hydroxamic acid derivatives for cell penetration. In case of cell based assay, synthetic derivatives must penetrate into the cell membrane to show inhibitory activity.

In conclusion, new hydroxamic acid derivatives 3a-3f and 4a-4g, containing various hydrophobic characters, were synthesized and evaluated as potent inhibitors on tyrosinase activity and melanin formation in melan-a cells. The hydroxamic acid moiety and basic skeleton having proper hydrophobic character are very important for anti-melanogenic activity. Among all compounds tested, compound 4d showed stronger inhibitory activity (IC_{50} = 1.24 µM) in cell based assay. Its activity was more potent than that of hydroquinone (IC_{50} = 3.97 µM) which is known as a most powerful anti-melanogenic agent.

Experimental Section

N-Hydroxyhexanamide (3a). To a solution of hexanoic acid 5a (1.0 g, 8.6 mmol) and N-methylmorpholine (960 mg, 9.5 mmol) in THF (15 ml) at 0°C were added ethylchloroformate (1.0 g, 9.5 mmol) dropwise and the mixture was stirred for 30 min. The solid was filtered off and the filtrate was added to the solution of hydroxyamine hydrochloride (894 mg, 12.9 mmol) and Et3N (1.3 g, 12.9 mmol) in DMF (20 ml) for 10 min. The reaction mixture was stirred for 30 min at 25°C. DMF was evaporated in vacuo. The residue was extracted with ethyl acetate (80 mL), washed with water. The solvent was dried over MgSO4 and evaporated to dryness. The crude product was purified by silica gel column chromatography to N-hydroxyhexanamide 3a (936 mg) in 83 % yields.

1H NMR (300 MHz, DMSO-d6) δ 10.34 (s, 1H), 8.68 (s, 1H), 1.95 (t, 2H, J = 7.2 Hz), 1.47 (m, 2H), 1.25 (m, 4H), 0.87 (t, 3H, 8.7 Hz). IR ν_{max} (KBr) 3253, 2954, 1622 cm^{-1}. FABMS, m/e 132 [M+1]+.

N-Hydroxycyclohexanecarboxamide (3b). 1H NMR (300 MHz, DMSO-d6) δ 10.33 (s, 1H), 8.64 (s, 1H), 1.94 (m, 1H), 1.02-1.80 (m, 10H). IR ν_{max} (KBr) 3190, 2921, 1625 cm^{-1}. FABMS, m/e 144 [M+1]+.

N-Hydroxybenzamide (3c). 1H NMR (300 MHz, DMSO-d6) δ 11.23 (s, 1H), 9.05 (s, 1H), 7.74 (m, 2H), 7.45 (m, 3H). IR ν_{max} (KBr) 3187, 3034, 1625 cm^{-1}. FABMS, m/e 138 [M+1]+.

N-Hydroxy-2-naphthamide (3d). 1H NMR (300 MHz, DMSO-d6) δ 11.37 (s, 1H), 9.14 (s, 1H), 8.36 (s, 1H), 8.00 (m, 3H), 7.82 (d, 1H, J = 8.4 Hz), 7.60 (m, 2H). IR ν_{max} (KBr) 3188, 3034, 1625 cm^{-1}. FABMS, m/e 188 [M+1]+.

N-Methoxybenzamide (3e). 1H NMR (300 MHz, DMSO-d6) δ 11.80 (s, 1H), 7.76 (m, 2H), 7.49 (m, 3H), 3.71 (s, 3H). IR ν_{max} (KBr) 3184, 3033, 1625 cm^{-1}. FABMS, m/e 152 [M+1]+.

N-Methoxy-2-naphthamide (3f). 1H NMR (300 MHz, DMSO-d6) δ 11.89 (s, 1H), 8.34 (s, 1H), 8.01 (m, 3H), 7.78 (d, 1H, J = 8.4 Hz), 7.62 (m, 2H), 3.73 (s, 3H). IR ν_{max} (KBr) 3142, 3006, 1623 cm^{-1}. FABMS, m/e 202 [M+1]+.

Preparation of methyl 4-(phenylcarbamoyl)benzoate (6a). Benzoic acid 5e (61.0 g, 0.50 mol) was dissolved in 100 mL of SOC1 at 0°C and refluxed for 1 h. The SOC1 was removed in vacuo, and crude acid chloride was dissolved in CH2Cl2 (50 mL). To a solution of methyl 4-aminobenzoate (7.5 g, 0.05 mol) in pyridine (100 mL) was added prepared acid chloride solution. The reaction mixture was stirred for 4 h at room temperature. The reaction mixture was concentrated in vacuo and the residue was extracted with ethyl acetate (250 mL), washed with water. The organic layer was dried with anhydrous MgSO4 and concentrated to give a crude product. The resultant was purified by crystallization from dichloromethane-hexane to give a methyl 4-(phenylcarbamoyl)benzoate 6a (10.3 g) in 81% yields.

1H NMR (300 MHz, DMSO-d6) δ 9.01 (s, 1H), 7.92 (d, 2H, J = 8.7 Hz), 7.83 (m, 2H), 7.71 (d, 2H, J = 8.7 Hz), 7.54 (m, 3H), 3.46 (s, 3H). FABMS, m/e 266 [M+1]+.

Preparation of 4-(phenylcarbamoyl)benzoic acid (7a). Methyl 4-(phenylcarbamoyl)benzoate 6a (7.6 g, 0.03 mol) was dissolved in KOH (0.5 M, 300 ml) solution and heated to 50°C. After obtaining clear solution, the clear solution was acidified with HCl (1 M) solution and resulting precipitate was gathered by filtration to give 4-(phenylcarbamoyl)benzoic acid 7a (8.3 g) in 87% yields.

1H NMR (300 MHz, DMSO-d6) δ 9.01 (s, 1H), 7.92 (d, 2H, J = 8.7 Hz), 7.83 (m, 2H), 7.71 (d, 2H, J = 8.7 Hz), 7.52 (m, 3H). FABMS, m/e 224 [M+1]+.

Preparation of 4-(phenylcarbamido)-N-hydroxybenzamide (4a). To a solution of 4-(phenylcarbamido)benzoic acid 7a (1000 mg, 4.1 mmol) and N-methylmorpholine (456 mg, 4.5 mmol) in THF (10 ml) at 0°C were added ethylchloroformate (488 mg, 4.5 mmol) dropwise and the mixture was stirred for 30 min. The solid was filtered off and the filtrate was added to the solution of hydroxyamine hydrochloride (427 mg, 6.1 mmol) and Et3N (617 mg, 6.1 mmol) in DMF (10 ml) for 10 min. The reaction mixture was stirred for 30 min at 25°C. DMF was evaporated in vacuo. The residue was extracted with ethyl acetate (50 mL), washed with water. The solvent was dried over MgSO4 and evaporated to dryness. The crude product was purified by silica gel column chromatography to afford 4-(phenylcarbamido)-N-hydroxybenzamide 4a (840 mg) in 81 % yields. 1H NMR (300 MHz, DMSO-d6) δ 11.02 (s, 1H), 10.44 (s, 1H), 9.01 (s, 1H), 7.79 (d, 2H, J = 8.4 Hz), 7.86 (d, 2H, J = 8.7 Hz), 7.74 (d, 2H, J = 8.7 Hz), 7.54 (m, 3H). IR ν_{max} (KBr) 3187, 3034, 1650, 1623 cm^{-1}. FABMS, m/e 257 [M+1]+.

4-((4-Methylphenyl)carbamido)-N-hydroxybenzamide (4b). 1H NMR (300 MHz, DMSO-d6) δ 11.15 (s, 1H), 10.37 (s, 1H), 8.99 (s, 1H), 7.87 (m, 4H), 7.73 (d, 2H, J = 8.7 Hz), 7.34 (d, 2H, J = 8.7 Hz), 2.39 (s, 3H). IR ν_{max} (KBr) 3255, 3033, 1654, 1620 cm^{-1}. FABMS, m/e 271 [M+1]+.

4-((4-Ethylphenyl)carbamido)-N-hydroxybenzamide (4c). 1H NMR (300 MHz, DMSO-d6) δ 11.15 (s, 1H), 10.37 (s, 1H), 8.99 (s, 1H), 7.88 (m, 4H), 7.74 (d, 2H, J = 8.7 Hz), 7.36 (d, 2H, J = 8.7 Hz), 2.70 (q, 2H, J = 7.5 Hz), 1.21 (t, 3H, J = 7.5 Hz).
Notes


7.5 Hz). IR \(\nu_{\text{max}}\) (KBr) 3188, 3050, 1654, 1621 cm\(^{-1}\). FABMS, \(m/e\) 285 [M+1]\(^{+}\).

4-(4-n-Propylphenyl)carboxamido)-N-hydroxybenzamide (4d). \(^{1}\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 11.15 (s, 1H), 10.37 (s, 1H), 8.98 (s, 1H), 7.83 (m, 4H), 7.73 (d, 2H, \(J = 8.7\) Hz), 7.37 (d, 2H, \(J = 8.7\) Hz), 2.66 (t, 2H, \(J = 7.5\) Hz), 1.66 (m, 2H), 0.93 (t, 3H, \(J = 7.5\) Hz). IR \(\nu_{\text{max}}\) (KBr) 3200, 3039, 2911, 1655, 1621 cm\(^{-1}\). FABMS, \(m/e\) 299 [M+1]\(^{+}\).

4-(4-n-Butylphenyl)carboxamido)-N-hydroxybenzamide (4e). \(^{1}\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 11.15 (s, 1H), 10.38 (s, 1H), 9.00 (s, 1H), 7.89 (m, 4H), 7.76 (d, 2H, \(J = 8.7\) Hz), 7.37 (d, 2H, \(J = 8.7\) Hz), 2.66 (t, 2H, \(J = 7.5\) Hz), 1.58 (m, 2H), 1.32 (m, 2H), 0.90 (t, 3H, \(J = 7.5\) Hz). IR \(\nu_{\text{max}}\) (KBr) 3187, 3034, 2910, 1650, 1623 cm\(^{-1}\). FABMS, \(m/e\) 313 [M+1]\(^{+}\).

4-(4-n-Butylphenyl)carboxamido)-N-methoxybenzamide (4f). \(^{1}\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 11.60 (s, 1H), 10.28 (s, 1H), 7.84 (m, 4H), 7.72 (d, 2H, \(J = 8.7\) Hz), 7.34 (d, 2H, \(J = 8.7\) Hz), 3.63 (s, 3H), 2.65 (t, 2H, \(J = 7.5\) Hz), 1.64 (m, 2H), 0.93 (t, 3H, \(J = 7.2\) Hz). IR \(\nu_{\text{max}}\) (KBr) 3201, 3035, 2910, 1652, 1624 cm\(^{-1}\). FABMS, \(m/e\) 313 [M+1]\(^{+}\).

Mushroom tyrosinase assay. Mushroom tyrosinase, L-tyrosine were purchased from Sigma Chemical. The reaction mixture for mushroom tyrosinase activity consisted of 150 µl of 0.1 M phosphate buffer (pH 6.5), 3 µl of sample solution, 8 µl of mushroom tyrosinase (2,100 unit/ml, 0.05 M phosphate buffer at pH 6.5), and 36 µl of 1.5 mM L-tyrosine. Tyrosinase activity was determined by reading the optical density at 490 nm on a microplate reader (Bio-Rad 3550, Richmond, CA, U.S.A.) after incubation for 20 min at 37 °C. The inhibitory activity of the sample was expressed as the concentration that inhibits 50% of the enzyme activity (IC\(_{50}\)).

Cell culture. Murine melan-a melanocytes were originally derived from C57BL/6 J (black, a/a) mice, a kind gift from Prof. Dorothy C. Bennett (St. George’s Hospital, London, U.K.). Melan-a cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 200 nM of phenol bol 12-myristate 13-acetate (TPA) at 37 °C in 10% CO\(_2\). The culture medium was changed twice every week, and the cells were subcultured once a week.

Measurements of cell viability. To evaluate the effects of hydroxamic acid derivatives on cell viability, the percentages of viable melan-a cells were determined using a modified crystal violet assay. \(^{17}\) After removing the medium from each well, the cells were washed with PBS and stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature. The cells were then rinsed four times with distilled water, and crystal violet retained by adherent cells was extracted with 95% ethanol at room temperature for 10 min. Crystal violet absorption was measured at 590 nm (Molecular Devices Co., Sunnyvale, CA, U.S.A.).

Measurements of melanin content. The melanin content was measured using the method reported by Hosoi et al. with a slight modification. \(^{13}\) The cells (2 × 10\(^5\) cells/ml) were seeded into 24-well plates and the test compounds were added in triplicate. The medium was changed daily and after 4 d of culture, the cells were lysed with 1 ml of 1 N NaOH. Then 200 µL of each crude cell extract was transferred into 96-well plates. The relative melanin content was measured at 400 nm with a microplate reader (Molecular Devices).

References

9. Acetohydroxamic acid was purchased from Aldrich.