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Neurocytoprotective Effects of Aliphatic Hydroxamates from Lovastatin, a Secondary Metabolite from *Monascus*-Fermented Red Mold Rice, in 6-Hydroxydopamine (6-OHDA)-Treated Nerve Growth Factor (NGF)-Differentiated PC12 Cells

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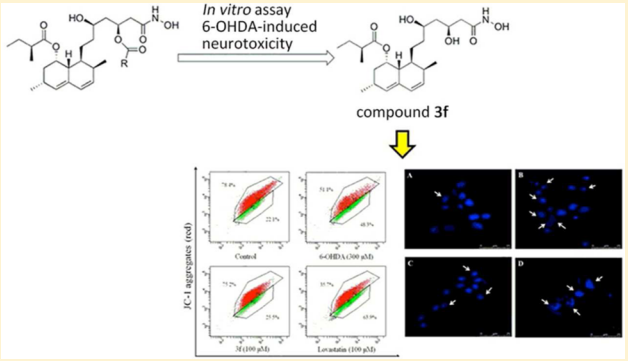
Supporting Information

ABSTRACT: Lovastatin, a secondary metabolite isolated from *Monascus*-fermented red rice mold, has neuroprotective activity and permeates the blood−brain barrier. The aim of this study was to enhance the activity of lovastatin for potential use as a treatment for neuronal degeneration in Parkinson's disease. Six lovastatin-derived compounds were semisynthesized and screened for neurocytoprotective activity against 6-hydroxydopamine (6-OHDA)-induced toxicity in human neuroblastoma PC12 cells. Four compounds, designated as **3a**, **3d**, **3e**, and **3f**, significantly enhanced cell viability. In particular, compound **3f** showed excellent neurocytoprotective activity (97.0 ± 2.7%). Annexin V-FITC and propidium iodide double staining and 4′,6-diamidino-2-phenylindole staining indicated that compound **3f** reduced 6-OHDA-induced apoptosis in PC12 cells. Compound **3f** also reduced caspase-3, -8, and -9 activities, and intracellular calcium concentrations elevated by 6-OHDA in a concentration-dependent manner, without inhibiting reactive oxygen species generation. JC-1 staining indicated that compound **3f** also stabilized mitochondrial membrane potential. Thus, compound **3f** may be used as a neurocytoprotective agent. Future studies should investigate its potential application as a treatment for Parkinson's disease.



KEYWORDS: *Lovastatin-derived hydroxamate, 6-hydroxydopamine, neurocytoprotective, mitochondrial membrane potential, Parkinson's disease*

As the population of older adults increases, age-related illnesses and their associated medical costs pose a great challenge to families. Parkinson's disease (PD) is one of the most common neurodegenerative disorders in humans,[1] and its prevalence increases with age.[2] This disease is characterized by motor dysfunction, including rigidity of movement, hand tremors, and postural instability[3] caused by the irreversible degeneration of dopaminergic neurons in the substantia nigra pars compacta.[4] Agents preventing such neuronal damage are required for treating and preventing PD progression.

Oxidative damage has been reported to play a key role in neurodegeneration and in the peripheral tissues of PD patients.[4] Oxidative stress affects several intracellular signaling pathways, including mitogen-activated protein kinases (MAPKs), c-jun N-terminal kinase (JNK), p38,[5] the re-entry of postmitotic neurons into the cell cycle,[6] and the mitochondrial caspase cascade[7] that induces apoptosis. 6-Hydroxydopamine (6-OHDA) is a neurotoxin reported to damage cathecolaminergic neurons through reactive oxygen species (ROS) generation.[7] This drug is thus widely used to generate PD-like models in vitro and in vivo.[8]

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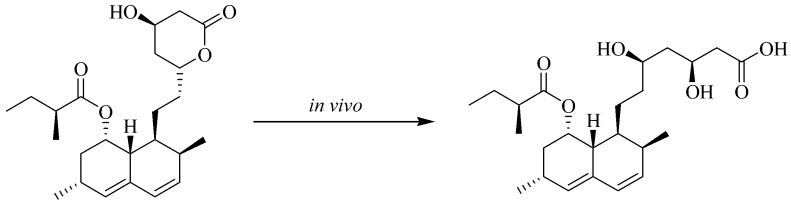
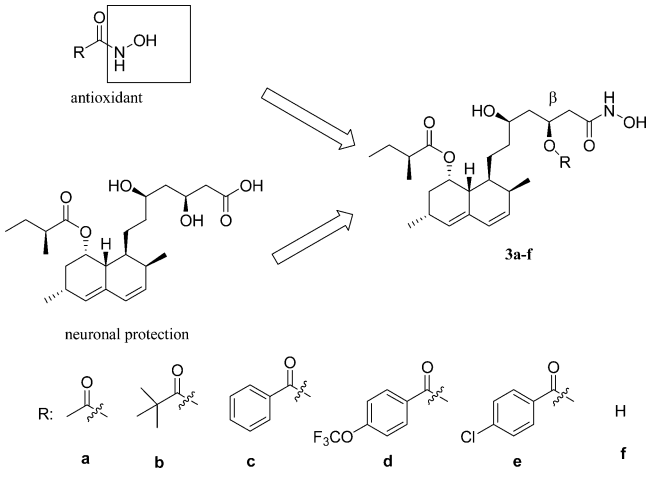


Figure 1. In vivo conversion of lovastatin into the active form.

We discovered potent neuroprotective substances from naturally occurring compounds. Lovastatin (monacolin K) is a secondary metabolite isolated from *Monascus*-fermented red rice mold.[9] Lovastatin contains a lactone moiety that is enzymatically hydrolyzed in vivo into β-hydroxy acid to generate a form that is active against HMG-CoA reductase (Figure 1).[10] Lovastatin has various pharmacological activities, including lowering cholesterol and reducing inflammation,[11] as well as anticancer,[12] antioxidant,[13] and, in particular, neuroprotective effects.[14] The mechanism underlying these effects remains to be clarified but may involve reducing oxidative damage.[15] Animal and clinical studies in vivo have indicated that lovastatin can pass through the blood–brain barrier because of its lipophilicity.[16] On the basis of these reports, we hypothesized that lovastatin serves as a lead compound in executing structural modifications to enhance its neuroprotective activity.

Dimerumic acid,[17] desferioxamine,[18] and ciclopiroxamine[19] contain a hydroxamate group and demonstrate antioxidant activity, suggesting that this moiety plays a crucial role in the mechanism underlying protection against oxidative stress. By combining the antioxidant core structure hydroxamate with a neuroprotective lovastatin scaffold, we hope to generate a therapeutically superior compound for treating neurodegenerative diseases (Scheme 1). In this study, we converted lovastatin into a series of aliphatic chain hydroxamates (**3a–f**), each with a different substitution at the β-position of the hydroxamate group (Scheme 1). We subsequently screened these compounds for neuroprotection against 6-OHDA-induced cytotoxicity in nerve growth factor (NGF)-differentiated PC12 cells and elucidated the signaling pathways involved in their activity.

Scheme 1. Design of lovastatin-Based Hydroxamates 3a–f with Neuronal Protection Activity



RESULTS AND DISCUSSION

Determination of the Cytotoxicity of Lovastatin-Derived Compounds in Nerve-Growth-Factor-Differentiated PC12 Cells. NGF treatment induces PC12 cells to develop into neurites.[20] These cells thus serve as a model for evaluating potential neuroprotective compounds. Lovastatin-derived compounds (**3a–f**) were initially screened for their cytotoxicity to NGF-differentiated PC12 cells at a high concentration (100 μM). According to the WST-8 cell proliferation assay, compounds **3a**, **3d**, **3e**, and **3f** conferred a cell viability of more than 85%, 24 h after treatment (Figure 2A). Thus, compounds **3a**, **3d**, **3e**, and **3f** were further examined for neurocytoprotective effects.

Evaluation of the Neurocytoprotective Effects of Lovastatin-Derived Compounds in 6-Hydroxydopamine-Induced Nerve-Growth-Factor-Differentiated PC12 Cells. 6-OHDA is a selective catecholaminergic neurotoxin that causes the degeneration of dopaminergic neurons, including cultured cells.[21] NGF-differentiated PC12 cells were preincubated with each compound (**3a, 3d, 3e, 3f,** and lovastatin) at 100 μM and then treated with 6-OHDA (300 μM, which reduced the cell viability to 50%). The viability of cells treated with compounds **3a**, **3d**, **3e**, and **3f** was 73.0 $\pm$ 1.8%, 88.3 $\pm$ 4.7%, 87.6 $\pm$ 2.4%, and 97.0 $\pm$ 2.7%, respectively (Figure 2B).

Compound **3f** was the most potent of the four compounds having neurocytoprotective activity. This compound lacks a β-substituent relative to the hydroxamate moiety, suggesting that the substituent at the β-position limits the neuroprotective activity. Furthermore, the effects of compound **3f** were concentration dependent within the concentration range of 12.5–100 μM, prompting further study of its activity (Figure 2C).

Effect of compound 3f on 6-Hydroxydopamine-Induced Apoptosis in Nerve-Growth-Factor-Differentiated PC12 Cells. 6-OHDA induces cell apoptosis.[21] The population of apoptotic NGF-differentiated PC12 cells preincubated with or without compound **3f** was determined using flow cytometry. The apoptotic cells were analyzed after staining with propidium iodide (PI) and FITC-labeled Annexin V (AV-FITC) (Figure 3A). Most cells in the control group were viable, with 78.9% normal, 5.6% early apoptotic, 8.7% late apoptotic, and 6.8% necrotic cells. Exposure of NGF-differentiated PC12 cells to 6-OHDA significantly increased the number of early (AV-FITC) and late (PI) apoptotic cells (66.2% and 24.2%, respectively). When cells were pretreated with various concentrations of compound **3f**, the percentage of apoptotic cells decreased significantly (50 μM, 9.2% necrotic,

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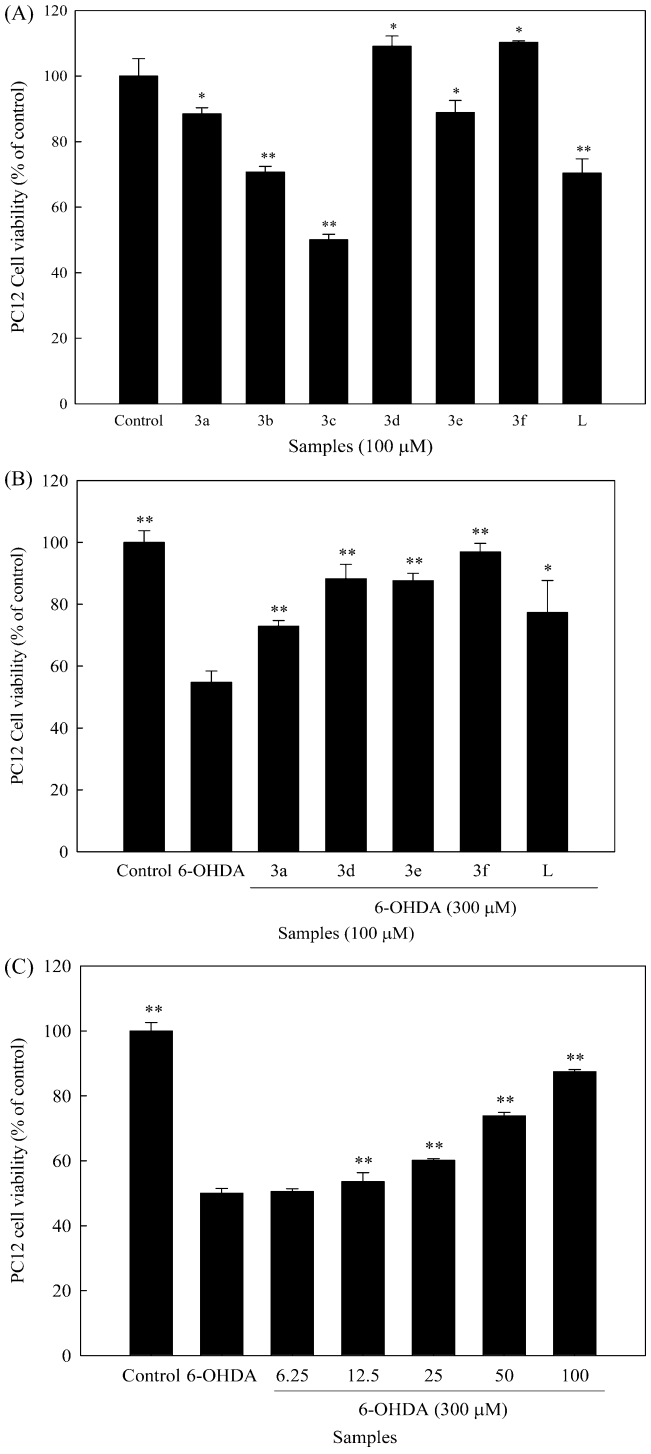

Figure 2. Cytotoxicity and neurocytoprotective effects of novel statin derivatives in PC12 cells. Nerve-growth-factor-differentiated PC12 cells were incubated with (A) statin derivatives **3a**–**f** (100 μM) for 6 h and (B) statin derivatives **3a** and **3d**–**f** (100 μM) for 6 h, and subsequently incubated with 6-hydroxydopamine (6-OHDA) (300 μM) for another 24 h. (C) Cells were incubated with various concentrations of compound **3f** (6.25, 12.5, 25, 50, and 100 μM) for 6 h and subsequently incubated with 6-OHDA (300 μM) for another 24 h. Cell viability was evaluated using the WST-8 assay. L, lovastatin. Data are expressed as the percentage of untreated control cells (n = 3). *P < 0.05; **P < 0.01 compared with 6-OHDA-treated cells.

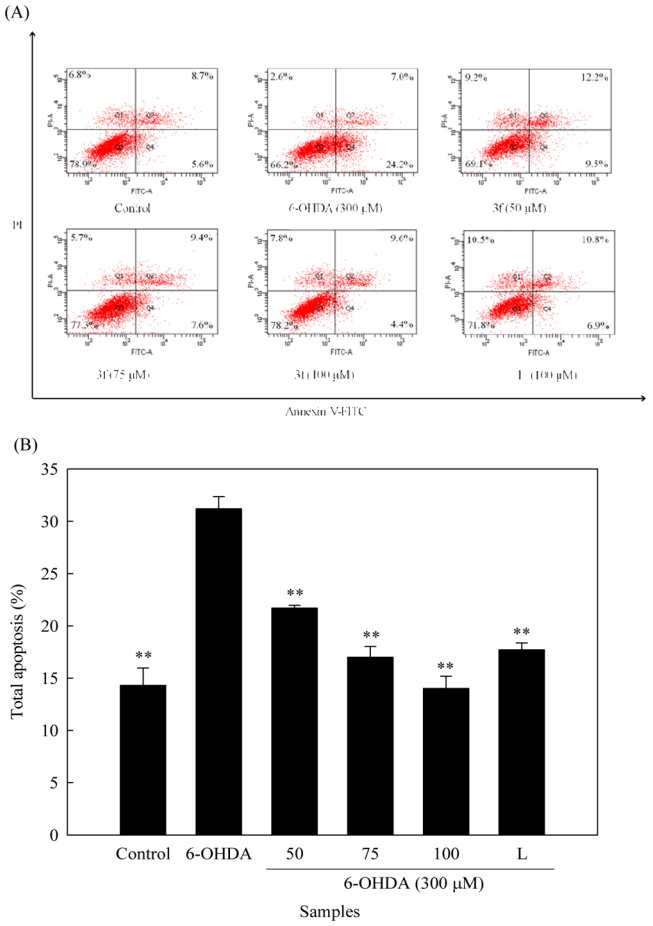

Figure 3. Protective effect of compound **3f** against 6-hydroxydopamine (6-OHDA)-induced apoptosis in PC12 cells. Nerve-growth-factor-differentiated PC12 cells were incubated with compound **3f** (50, 75, and 100 μM) and lovastatin (100 μM) for 6 h; 6-OHDA (300 μM) was added, and incubation continued for 24 h. (A) The apoptotic cell fraction was determined through flow cytometry by using Annexin V-FITC and propidium iodide double staining. Q1, Q2, Q3, and Q4 represent necrotic, late apoptotic, normal, and early apoptotic cells, respectively. (B) Histogram showing the proportion of apoptotic cell relative to total cells (n = 3). L, lovastatin. **P < 0.01 compared with cells treated with 6-OHDA.

12.2% late apoptotic, 69.1% normal, and 9.5% early apoptotic; 75 μM, 5.7% necrotic, 9.4% late apoptotic, 77.3% normal, and 7.6% early apoptotic; 100 μM, 7.8% necrotic, 9.6% late apoptotic, 78.2% normal, and 4.4% early apoptotic; 100 μM lovastatin alone as the positive control, 10.5% necrotic, 10.8% late apoptotic, 71.8% normal, and 6.9% early apoptotic). In this study, the proportion of cells undergoing total apoptosis was calculated as the sum of the proportions of early and late apoptotic cells (Figure 3B). The population of apoptotic cells increased to 31.2% when they were treated with 6-OHDA (300 μM), compared with 14.3% in the control. Pretreatment with compound **3f** at 0, 75, and 100 μM reduced the apoptotic cell fractions to 21.7%, 17.0%, and 14.0%, respectively. These results suggest that the neurocytoprotective activity of compound **3f** is concentration dependent.

Evaluation of the Effect of Compound 3f on 6-Hydroxydopamine-Induced Caspase-3, -8, and -9 Activities in Nerve-Growth-Factor-Differentiated PC12 Cells. Caspase activation is a characteristic of apoptosis. Caspase-8 is localized upstream in the caspase cascade and activates downstream caspases-3, -6, and -7, which cleave key cellular substrates, resulting in cell apoptosis. In addition,

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caspase-9 can promote caspase-3 activation, resulting in cell death.[22] 6-OHDA induces caspase-3 activation via caspase-8 in a mitochondria-independent manner.[23] We further evaluated whether the inhibition of caspase proteases plays a crucial role in apoptotic inhibition by compound **3f**. NGF-differentiated PC12 cells exposed to 300 μM 6-OHDA for 6 h exhibited 2.7-, 2.4-, and 1.8-fold increases in caspase-3, -8, and -9 activities (Figure 4), respectively, relative to the control. Treating cells

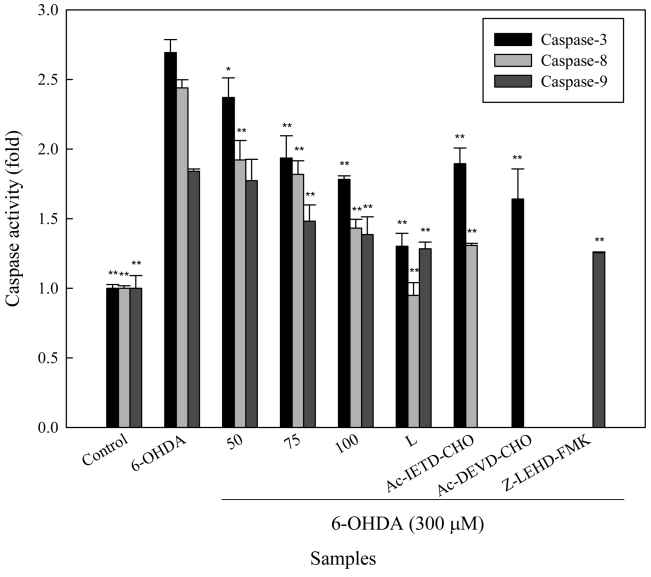

Figure 4. Effect of compound **3f** on caspase-3, -8, and -9 activities in PC12 cells. Nerve-growth-factor-differentiated PC12 cells were incubated with compound **3f** (50, 75, and 100 μM) and lovastatin (100 μM) for 6 h; 6-hydroxydopamine (6-OHDA) (300 μM) was then added, and incubation continued for 4 or 6 h. Caspase-3, -8, and -9 activities were determined by measuring fluorescence with excitation and emission at 360 and 440 nm (caspase-3 and -8), 400 and 505 nm (casepase-9), respectively. Ac-DEVD-CHO, caspase-3 inhibitor (10 μM); Ac-IETD-CHO, caspase-8 inhibitor (10 μM); Z-LEHD-FMK, caspase-9 inhibitor (10 μM). L, lovastatin. Data are expressed as fold increases in the fluorescence intensity (n = 3) over that of controls. *P < 0.05 and **P < 0.01 compared with cells treated with 6-OHDA.

with differing concentrations of compound **3f** resulted in the significant inhibition of caspase-3 and -8 activities in a concentration-dependent manner. The IC_{50} values for caspases-3, -8, and -9 were 90.4, 75.4, and 91.3 μM, respectively. Caspase-8 inhibitor (Ac-IETD-CHO) also inhibited caspase-3 activity. Compound **3f** partially inhibited downstream events in the pathway leading to mitochondria-mediated apoptotic cell death.

Evaluation of the Effect of Compound 3f on 6-Hydroxydopamine-Induced Intracellular Reactive Oxygen Species Production in Nerve-Growth-Factor-Differentiated PC12 Cells. The oxidation of 6-OHDA, yielding ROS, is the main cause of 6-OHDA-induced cytotoxicity.[23] To further examine whether the protective effects of compound **3f** on 6-OHDA-induced neurotoxicity involved antioxidant mechanisms, we determined the level of intracellular ROS accumulation by measuring the intensity of fluorescence emitted from the oxidative product of CM-H_2DCFH. Treatment with 6-OHDA increased the fluorescence intensity in a time-dependent manner over 180 min (Figure 5). The ROS level increased 2.33-fold compared with that of the control, and

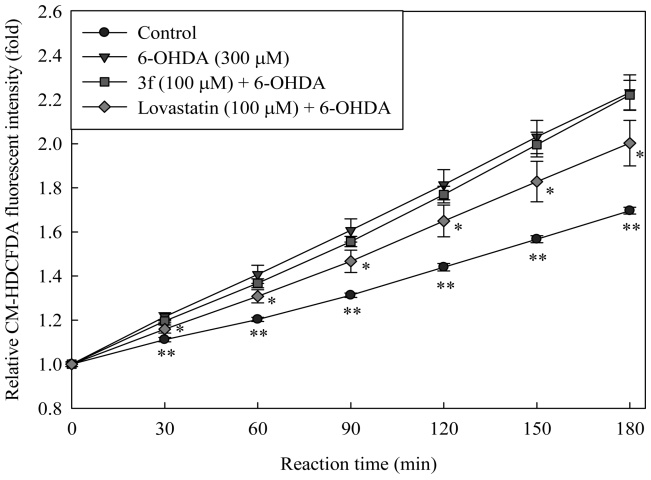

Figure 5. Effect of compound **3f** on 6-hydroxydopamine (6-OHDA)-induced intracellular reactive oxygen species (ROS) accumulation. Nerve-growth-factor-differentiated PC12 cells were incubated with compound **3f** (100 μM) and lovastatin (100 μM) for 6 h; 6-OHDA (300 μM) was added, and incubation continued for 24 h. Intracellular ROS levels were determined by measuring fluorescence with excitation and emission at 484 and 535 nm, respectively. Data are expressed as fold increases in the fluorescence intensity (n = 3). *P < 0.05 and **P < 0.01 compared with cells treated with 6-OHDA.

pretreatment with compound **3f** did not prevent this increase. These results suggested that the protective activity of compound **3f** against 6-OHDA-induced cytotoxicity in PC12 cells is not associated with ROS.

Effect of Compound 3f on 6-Hydroxydopamine-Induced Intracellular Calcium in Nerve-Growth-Factor-Differentiated PC12 Cells. 6-OHDA-induced Ca^{2+} elevation leads to the apoptosis of PC12 cells. To investigate the effect of compound **3f** on intracellular Ca^{2+}, we monitored the Ca^{2+} levels by measuring the fluorescence intensity emitted by Fluo-4 AM/Ca^{2+}conjugates. Treating PC12 cells with 6-OHDA clearly increased the fluorescence intensity 1.6-fold relative to that of the control. Preatment with compound **3f** at 50, 75, and 100 μM reduced the fluorescence intensity to 1.47-, 1.39-, and 1.22-fold, respectively, in a concentration-dependent manner. These results demonstrated that compound **3f** slightly affected the intracellular calcium levels induced by 6-OHDA treatment in NGF-differentiated PC12 cells (Figure 6).

Effect of Compound 3f on the Nuclear Morphology of Nerve-Growth-Factor-Differentiated PC12 Cells. Changes in the nuclear morphology reportedly associated with cell apoptosis include cell shrinkage, membrane blebbing, nuclear fragmentation, and apoptotic body formation.[24] The nuclear morphology of NGF-differentiated PC12 cells was analyzed using 4′,6-diamidino-2-phenylindole (DAPI) staining. Exposure to 6-OHDA alone resulted in marked cell shrinkage and membrane blebbing relative to the morphology of controls (Figure 7). Pretreatment with compound **3f** at 100 μM significantly reduced the number of apoptotic cells compared with that of untreated cells; however, pretreatment with lovastatin at concentrations similar to that of compound **3f** did not attenuate the 6-OHDA-induced apoptotic effect. These results suggested that compound **3f** effectively protects NGF-differentiated PC12 cells from 6-OHDA-induced apoptosis.

Effect of Compound 3f on 6-Hydroxydopamine-Induced Mitochondrial Membrane Depolarization Change ($\Delta\Psi_m$) in Nerve-Growth-Factor-Differentiated

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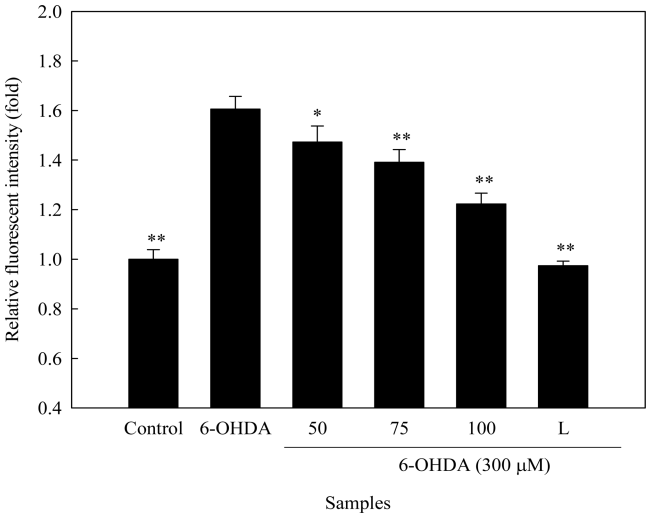

Figure 6. Effect of compound **3f** on 6-hydroxydopamine (6-OHDA)-induced intracellular calcium accumulation. Nerve-growth-factor-differentiated PC12 cells were incubated with compound **3f** (100 μM) and lovastatin (L) (50, 75, and 100 μM) for 6 h; 6-OHDA (300 μM) was added, and incubation continued for 2 h. Intracellular calcium concentrations were determined by measuring fluorescence with excitation and emission at 484 and 535 nm, respectively. Data are expressed as fold increases in the fluorescence intensity (n = 3). *P < 0.05 and **P < 0.01 compared with cells treated with 6-OHDA.

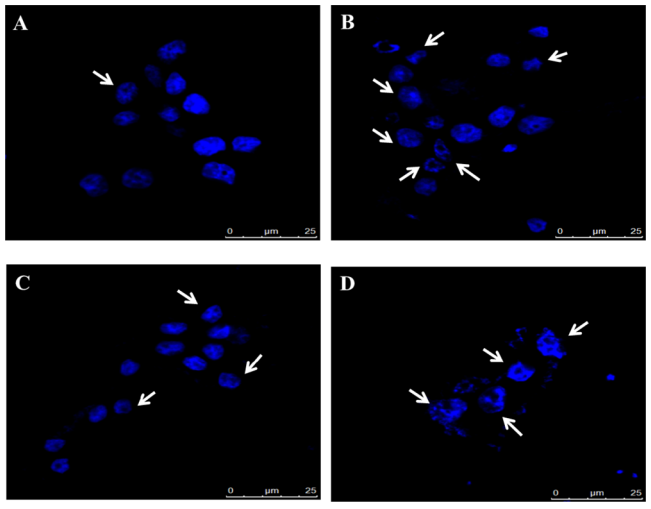

Figure 7. Effect of compound **3f** on 6-hydroxydopamine (6-OHDA)-induced changes in the nuclear morphology of PC12 cells. Nerve-growth-factor-differentiated PC12 cells were incubated with compound **3f** (100 μM) and lovastatin (L) (100 μM) for 6 h; 6-OHDA (300 μM) was added, and the cells were incubated for 24 h. Nuclear morphology was analyzed using a confocal microscope. (A) control, (B) 6-OHDA (300 μM), (C) compound **3f** (100 μM), and (D) lovastatin. White arrows indicate apoptotic cells. Scale bars, 25 μm.

PC12 Cells. Mitochondrial membrane depolarization (MMP) results in apoptosis through the release of cytochrome c, which triggers the intrinsic apoptotic pathway cascade. MMP changes were detected using JC-1, which aggregates to emit red fluorescence in mitochondria with normal MMP but remains monomeric and emits green fluorescence in depolarized mitochondria. Treating cells with 6-OHDA reduced the fraction of cells with normal MMP from 78.8% to 51.1% in untreated controls. Preatment with compound **3f** at 100 μM increased this fraction to 75.2%, whereas lovastatin treatment at concentrations similar to that of compound **3f** reduced this fraction to 35.7%. The ratio of red to green flouoresence (R/G) was 3.55, 1.06, 2.95, and 0.56 for controls, 6-OHDA, compound **3f**, and lovastatin treatment, respectively. Moreover, the R/G ratio verified that cell apoptosis accompanied the MMP change. Preatment with compound **3f** significantly increased the ratio to 2.95, which is comparable to that of normal cells. These results suggest that compound **3f** has antiapoptotic effects on 6-OHDA-treated NGF-differentiated PC12 cells that could occur through the mitochondrial intrinsic pathway (Figure 8).

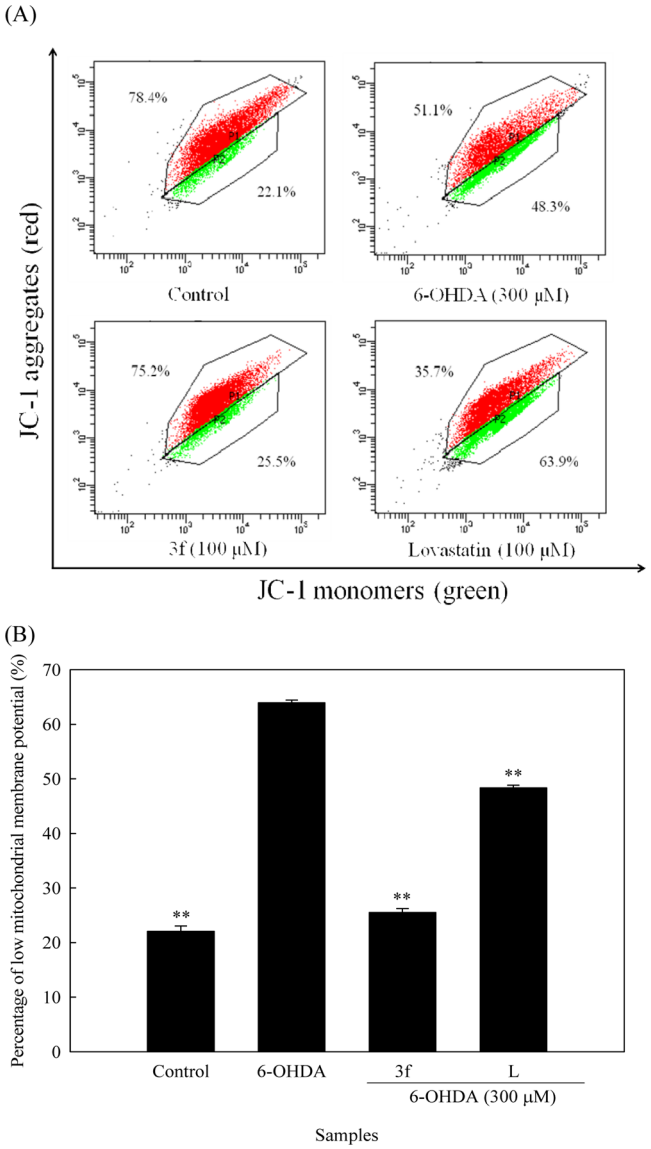

Figure 8. Effect of compound **3f** on 6-hydroxydopamine (6-OHDA)-induced changes in mitochondrial membrane potential (MMP). Nerve-growth-factor-differentiated PC12 cells were incubated with compound **3f** (100 μM) and lovastatin (100 μM) for 6 h and 6-OHDA (300 μM) for 24 h. (A) PC12 cells were stained with JC-1 fluorescent dye, and changes in MMP were determined through flow cytometry by using green (FITC) and red (propidium iodide) channels. (B) Histogram indicates the proportion of low-MMP cells relative to total cells (n = 3). L, Lovastatin. **P < 0.01 compared with cells treated with 6-OHDA.

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In this study, we developed an aliphatic hydroxamate series by incorporating the antioxidant core hydroxamate into the naturally occurring compound lovastatin. We observed that this compound has neurocytoprotective activity, inhibiting caspases-3, -8, and -9 and intracellular calcium accumulation and signaling pathways in 6-OHDA-treated NGF-differentiated PC12 cells. In NGF-differentiated PC12 cells, four of the compounds generated (**3a**, **3d**, **3e**, and **3f**) showed no toxicity, and in particular, compound **3f** had the most potent protective activity against 6-OHDA-induced cytotoxicity. Although its effect in suppressing caspases-3, -8, and -9, inhibiting ROS generation, and repressing intracellular calcium accumulation was lower than that of lovastatin, compound **3f** had a higher neurocytoprotective activity than lovastatin, showing a concentration-dependent manner. Notably, the apoptotic inhibitory activity of compound **3f** was higher than that of lovastatin; this effect accompanied a decrease in MMP changes. Two major pathways are involved in apoptosis. The extrinsic (death receptor) pathway, involved in caspase-8 activation, is mediated by death receptors, and the intrinsic (mitochondrial) pathway is activated by DNA damage, ROS, or loss of trophic support.[25] Caspase-8 was shown to activate caspase-3 in the extrinsic apoptotic pathway.[22] Caspase-9 could cleave and activate the downstream executioner caspases, including caspase-3, and then cause the apoptosis-associated morphological changes, DNA fragmentation, and cytoskeletal disruption.[26] Compared with lovastatin, compound **3f** showed a strong effect on neurocytoprotection and MMP change reduction in contrast to its weak anticaspase-3, -8, and -9 activities. These results indicated that the compound-**3f**-induced antiapoptotic effect on 6-OHDA-mediated cytotoxicity in NGF-differentiated PC12 cells may mainly result from the intrinsic/mitochondrial pathway. Furthermore, a lovastatin derivative, simvastatin, was shown to increase neuronal protein and gene levels of the antiapoptotic Bcl-2 in vitro and in vivo.[27] Bcl-2 was recognized as a major molecule in the intrinsic apoptotic pathway. Compound **3f** may also exhibit the antiapoptotic effect through stimulated Bcl-2.[28] Fonseca and co-workers reported that lovastatin can attenuate the increase in cytosolic calcium levels, inhibit ROS accumulation, and exhibit a neuroprotective effect against β-amyloid-mediated cytotoxicity in PC12 cells.[29] These results may correlate with our findings that the activities of lovastatin against ROS production and calcium accumulation were much stronger than those of compound **3f**. Regarding antiapoptotic signal transduction, caspase-8 is the upstream regulator of caspase-3 in the extrinsic pathway, and inhibiting p53 activity can block apoptosis by downregulating the expression of apoptotic proteins, including caspases-9 and -3, and their activity in the intrinsic pathway.[30] We showed that compound **3f** not only partially inhibited the extrinsic pathway but also effectually blocked the intrinsic pathway. The cell viability assay showed that the cytotoxicity of compound **3f** against NGF-differentiated PC12 cells was much lower than that of lovastatin, suggesting that compound **3f** may provide a therapeutic index superior to that of lovastatin. In addition, Johnson-Anuna and co-workers reported that lovastatin, pravastatin, and simvastatin can cross the blood−brain barrier in mice.[22,27c] However, LC/MS/MS profiling indicated that after the administration of these three statins, in the cerebral cortex, the levels of all of the statins decreased remarkably within 6 h. This reduction was likely induced by active efflux transporters or metabolic enzymes, which may specifically interact with these statins or their metabolic acids. Compound **3f**, differing from lovastatin and its acid in chemical structure, may resist these elimination mechanisms in the brain when administered in vivo, exhibiting an improved pharmacokinetic profile compared with that of statins. These results suggested that compound **3f** is a potential neurocytoprotective compound for use against 6-OHDA-induced apoptosis. Further research for identifying the underlying molecular mechanism of this compound is being undertaken.

METHODS

Materials. Phosphate-buffered saline (PBS), 6-OHDA, luteolin, *m*-aminophenylboronic acid-agarose, 5′,5′-dithiobis(2-nitrobenzoic acid), 3-carboxy-4 nitrophenyl disulfide, caspase-3, -8, and -9 kits, RPMI 1640 medium, and Hanks' balanced salt solution (HBSS) were purchased from Sigma-Aldrich (St. Louis, MO). WST-8 was purchased from Nacalai Tesque, Japan. Horse and fetal bovine serum (FBS) were obtained from Gibco. NGF was obtained from Alomone Laboratories (Jerusalem, Israel). H_2DCFH and CM-H_2DCFDA were purchased from Invitrogen (Carlsbad, CA).

Synthesis of Compounds 3a−f. The synthesis of lovastatin-based hydroxamates **3a−f** is described in Scheme 1S of the Supporting Information. Details on the synthetic procedure and spectral data of the intermediates and final products are also provided in the Supporting Information. The compounds tested using the in vitro assay were estimated to be at least 95% pure through HPLC analysis.

Cell Cultures. PC12 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% FBS[31] at 37 °C in a humidified 5% CO_2 atmosphere. Neuronal differentiation was induced in the medium by treating the cells with NGF and 1% serum (1:100) for 5 days. PC12 cells were derived from a transplantable rat adrenal pheochromocytoma and are widely used in neurobiological and neurotoxicological studies.[32] When treated with NGF, these cells differentiate into neuronal-type cells.[33]

Cell Viability Analysis. After differentiation, the cells were incubated with the test compounds for 24 h, and the cell viability was evaluated using the WST-8 assay[34] as follows. In brief, the cells were treated with the WST-8 reagent (200 μL) and incubated for 4 h. The optical density was measured at 450 nm on a model μQuant microplate reader (Bio-Tek Instruments, Winooski, VT). The viability was calculated as follows: (test sample absorbance/media-only absorbance) × 100%.

Neurocytoprotective Activity Analysis. The NGF-differentiated PC12 cells were incubated with the test compounds for 5 h before exposure to 6-OHDA. After incubation for 24 h, cell viability was evaluated as mentioned previously.[34]

Apoptosis Analysis by Using Annexin V-FITC/Propidium Iodide Double-Labeled Flow Cytometry. The Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson) was used to analyze cell apoptosis.[35] NGF-differentiated PC12 cells on 6-well plates were incubated with the test compounds for 6 h before exposure to 6-OHDA. After incubation for 24 h, the cells were trypsinized, centrifuged, washed twice with ice-cold PBS, and resuspended in a 1× binding buffer. The cell density was adjusted to 1×10^6 cells/mL. The cell suspension (100 μL), Annexin V-FITC (10 μL), and PI (10 μL) were added to a 5 mL centrifuge tube. The mixtures were incubated in the dark at room temperature for 15 min, added to a 1× binding buffer (400 μL), and analyzed using an EPICS ALTRAII flow cytometer (Beckman Coulter, Brea, CA) at an excitation wavelength of 488 nm. Twenty thousand cells were collected from the analyzed sample. Cells positive for Annexin V and negative for PI were defined as early apoptotic cells, and cells positive for Annexin V and PI were defined as late apoptotic cells.[36]

Measurement of Caspase-3, -8, and -9 Activities. Caspase-3 and -8 activities were measured using fluorometric assay kits, according to the manufacturer instructions (Sigma, St. Louis, MO), to detect fluorescence emitted from the hydrolysis of a caspase-3 or -8 substrate.[37] The caspase-9 activity was measured using fluorometric assay kits (Biovision, San Diego, CA). NGF-differentiated PC12 cells

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seeded in 6 cm dishes were incubated with the test compounds for 6 h before exposure to 6-OHDA. After incubating for 4 h (caspases-8 and -9) or 6 h (caspase-3), the cells were harvested, centrifuged, and resuspended in 200 μL of a cold cell lysis buffer in an ice bath for 10 min. The cells were then centrifuged at 10 000 rpm for 10 min, and the supernatant was transferred to a tube. Equal amounts of protein, an assay buffer (1×), and Ac-IETD-AMC (150 μM, caspase-8 substrate), Ac-DEVD-AMC (150 μM, caspase-3 substrate), or LEHD-AFC (50 μM, caspase-9 substrate) were added to each of the 96 wells. The intensity of caspase-3 and -8 fluorescence, emitted by AMC (excitation, 360 nm; emission 440 nm), and that of caspase-9 fluorescence, emitted by AFC (excitation, 400 nm; emission 505 nm), were measured using a microplate reader (Bio-Tek Instruments) at 15 min intervals for 120 min. Caspase-3, -8, or -9 activity in the experimental samples was compared with that in controls.

Intracellular ROS Measurement. NGF-differentiated PC12 cells on 96-well plates were treated with the test compounds for 6 h before exposure to 6-OHDA. The plates were washed with PBS and incubated with CMH_2DCFDA in the dark at 37 °C for 40 min.[35] After the plates were washed with HBSS twice, 6-OHDA was added to the plates, and the mixture was incubated for an additional 2 h. ROS generation was measured as the intensity of fluorescence emitted by an oxidized product, CM-DCFH (excitation, 485 nm; emission, 535 nm) (Bio-Tek Instruments), and is reported as fold values.

Intracellular Calcium Ion Measurement. NGF-differentiated PC12 cells on 96-well plates were treated with the test compounds for 6 h before exposure to 6-OHDA. After incubation for 2 h, Fluo-4 AM was added to each well, and the mixture was incubated at 37 °C for 30 min. Calcium ions were measured as the intensity of fluorescence emitted by Ca-conjugated Fluo-4 A (excitation, 485 nm; emission, 520 nm) by using a microplate reader (Bio-Tek Instruments), and the concentrations are reported as fold values.[38]

Analysis of Apoptotic Cell Nuclear Morphology by Using 4′,6-Diamidino-2-phenylindole Staining. NGF-differentiated PC12 cells were incubated in 6-well plates with the test compounds for 6 h before exposure to 6-OHDA. After being treated with 6-OHDA for 24 h, the cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS, treated with 0.1% Triton-X100 in PBS for 10 min, and soaked in 0.1% BSA for 20 min. The mixture was incubated with DAPI (2 μg/mL) in the dark for 10 min and washed with PBS. Nuclear morphology was visualized using a fluorescence microscope (Leica, TCS SP5, Germany).

Mitochondrial Membrane Potential Measurement. NGF-differentiated PC12 cells on 96-well plates were incubated with the test compounds for 6 h before exposure to 6-OHDA. After incubating for 24 h, the cells were collected, washed twice with ice-cold PBS, and incubated with JC-1 (4 μg/mL) for 30 min in the dark. The MMP change ($\Delta\Psi_m$) was measured using an EPICS ALTRAII flow cytometer (Beckman Coulter).[39]

Statistical Analysis. Data are expressed as the mean ± standard deviation and were analyzed using one-way analysis of variance with the Student−Newman−Keuls method. A P value of <0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic scheme, general methods, synthetic procedures, and physical and spectral data for the intermediates and final products. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine; NGF, nerve growth factor; PI, propidium iodide; MMP, mitochondrial membrane depolarization; ROS, reactive oxygen species; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; DAPI, 4′,6-diamidino-2-phenylindole

■ REFERENCES

(1) Roy, A., and Pahan, K. (2011) Prospects of statins in Parkinson disease. *Neuroscientist 17*, 244−255.

(2) Earhart, G. M., and Falvo, M. J. (2013) Parkinson disease and exercise. *Compr. Physiol. 3*, 833−848.

(3) Gazewood, J. D., Richards, D. R., and Clebak, K. (2013) Parkinson disease: an update. *Am. Fam. Physician 87*, 267−273.

(4) Sanders, L. H., and Greenamyre, J. T. (2013) Oxidative damage to macromolecules in human Parkinson disease and the rotenone model. *Free Radicals Biol. Med. 62*, 111−120.

(5) Tian, X., Sui, S., Huang, J., Bai, J. P., Ren, T. S., and Zhao, Q. C. (2014) Neuroprotective effects of *Arctium lappa* L. roots against glutamate-induced oxidative stress by inhibiting phosphorylation of p38, JNK and ERK 1/2 MAPKs in PC12 cells. *Environ. Toxicol. Pharmacol. 38*, 189−198.

(6) Sykora, P., Wilson, D. M., 3rd, and Bohr, V. A. (2013) Base excision repair in the mammalian brain: implication for age related neurodegeneration. *Mech. Ageing Dev. 134*, 440−448.

(7) Hanrott, K., Gudmunsen, L., O'Neill, M. J., and Wonnacott, S. (2006) 6-Hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase Cdelta. *J. Biol. Chem. 281*, 5373−5382.

(8) Soria, G., Aguilar, E., Tudela, R., Mullol, J., Planas, A. M., and Marin, C. (2011) *In vivo* magnetic resonance imaging characterization of bilateral structural changes in experimental Parkinson's disease: A T2 relaxometry study combined with longitudinal diffusion tensor imaging and manganese-enhanced magnetic resonance imaging in the 6-hydroxydopamine rat model. *Eur. J. Neurosci. 33*, 1551−1560.

(9) (a) Endo, A., and Hasumi, K. (1993) HMG-CoA reductase inhibitors. *Nat. Prod. Rep. 10*, 541−550. (b) Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., and Springer, J. (1980) Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc. Natl. Acad. Sci. U. S. A. 77*, 3957−3961. (c) Endo, A. (1979) Monacolin K, a new hypocholesterolemic agent produced by a *Monascus* species. *J. Antibiot. 32*, 852−854. (d) Lee, C. L., Kung, Y. H., Wu, C. L., Hsu, Y. W., and Pan, T. M. (2010) Monascin and ankaflavin act as novel hypolipidemic and high-density lipoprotein cholesterol-raising agents in red mold dioscorea. *J. Agric. Food Chem. 58*, 9013−9019.

DOI: 10.1021/cn500275k
ACS Chem. Neurosci. 2015, 6, 716−724

(10) Corsini, A., Maggi, F. M., and Catapano, A. L. (1995) Pharmacology of competitive inhibitors of HMG-CoA reductase. *Pharmacol. Res. 31*, 9−27.
(11) Davignon, J., and Laaksonen, R. (1999) Low-density lipoprotein-independent effects of statins. *Curr. Opin. Lipidol. 10*, 543−559.
(12) (a) Lee, S. J., Ha, M. J., Lee, J., Nguyen, P., Choi, Y. H., Pirnia, F., Kang, W. K., Wang, X. F., Kim, S. J., and Trepel, J. B. (1998) Inhibition of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase pathway induces p53-independent transcriptional regulation of p21(WAF1/CIP1) in human prostate carcinoma cells. *J. Biol. Chem. 273*, 10618−10623. (b) Lee, J., Lee, I., Park, C., and Kang, W. K. (2006) Lovastatin-induced RhoA modulation and its effect on senescence in prostate cancer cells. *Biochem. Biophys. Res. Commun. 339*, 748−754.
(13) (a) Weitberg, A. B. (2007) The antioxidant effect of lovastatin on phagocyte-induced DNA damage: implications for cancer prevention. *J. Exp. Clin. Cancer Res. 26*, 583−586. (b) Chen, L., Haught, W. H., Yang, B., Saldeen, T. G., Parathasarathy, S., and Mehta, J. L. (1997) Preservation of endogenous antioxidant activity and inhibition of lipid peroxidation as common mechanisms of antiatherosclerotic effects of vitamin E, lovastatin and amlodipine. *J. Am. Coll. Cardiol. 30*, 569−575.
(14) (a) Elkind, M. S., Sacco, R. L., Macarthur, R. B., Peerschke, E., Neils, G., Andrews, H., Stillman, J., Corporan, T., Leifer, D., Liu, R., and Cheung, K. (2009) High-dose lovastatin for acute ischemic stroke: results of the phase I dose escalation neuroprotection with statin therapy for acute recovery trial (NeuSTART). *Cerebrovasc. Dis. 28*, 266−275. (b) Dolga, A. M., Nijholt, I. M., Ostroveanu, A., ten Bosch, Q., Luiten, P. G. M., and Eisel, U. L. M. (2008) Lovastatin induces neuroprotection through tumor necrosis factor receptor 2 signaling pathways. *J. Alzheimer's Dis. 13*, 111−122. (c) Elkind, M. S. V., Sacco, R. L., MacArthur, R. B., Fink, D. J., Peerschke, E., Andrews, H., Neils, G., Stillman, J., Corporan, T., Leifer, D., and Cheung, K. (2008) The neuroprotection with statin therapy for acute recovery trial (NeuSTART): an adaptive design phase I dose-escalation study of high-dose lovastatin in acute ischemic stroke. *Int. J. Stroke 3*, 210−218.
(15) van der Most, P. J., Dolga, A. M., Nijholt, I. M., Luiten, P. G. M., and Eisel, U. L. M. (2009) Statins: Mechanisms of neuroprotection. *Prog. Neurobiol. 88*, 64−75.
(16) (a) Botti, R. E., Triscari, J., Pan, H. Y., and Zayat, J. (1991) Concentrations of pravastatin and lovastatin in cerebrospinal fluid in healthy subjects. *Clin. Neuropharmacol. 14*, 256−261. (b) Saheki, A., Terasaki, T., Tamai, I., and Tsuji, A. (1994) *In vivo* and *in vitro* blood-brain barrier transport of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. *Pharm. Res. 11*, 305−311. (c) Tsuji, A., Saheki, A., Tamai, I., and Terasaki, T. (1993) Transport mechanism of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors at the blood-brain barrier. *J. Pharmacol. Exp. Ther. 267*, 1085−1090.
(17) Aniya, Y., Ohtani, I. I., Higa, T., Miyagi, C., Gibo, H., Shimabukuro, M., Nakanishi, H., and Taira, J. (2000) Dimerumic acid as an antioxidant of the mold, Monascus anka. *Free Radical Biol. Med. 28*, 999−1004.
(18) Adgent, M. A., Squadrito, G. L., Ballinger, C. A., Krzywanski, D. M., Lancaster, J. R., and Postlethwait, E. M. (2012) Desferrioxamine inhibits protein tyrosine nitration: mechanisms and implications. *Free Radical Biol. Med. 53*, 951−961.
(19) Sato, E., Kohno, M., Nakashima, T., and Niwano, Y. (2008) Ciclopirox olamine directly scavenges hydroxyl radical. *Int. J. Dermatol. 47*, 15−18.
(20) (a) Meng, L., Jiang, A., Chen, R., Li, C. Z., Wang, L., Qu, Y., Wang, P., Zhao, Y., and Chen, C. (2013) Inhibitory effects of multiwall carbon nanotubes with high iron impurity on viability and neuronal differentiation in cultured PC12 cells. *Toxicology 313*, 49−58. (b) Lin, Y. P., Chen, T. Y., Tseng, H. W., Lee, M. H., and Chen, S. T. (2009) Neural cell protective compounds isolated from Phoenix hanceana var. formosana. *Phytochemistry 70*, 1173−1181.
(21) Bove, J., and Perier, C. (2012) Neurotoxin-based models of Parkinson's disease. *Neuroscience 211*, 51−76.
(22) Johnson-Anuna, L. N., Eckert, G. P., Keller, J. H., Igbavboa, U., Franke, C., Fechner, T., Schubert-Zsilavecz, M., Karas, M., Muller, W. E., and Wood, W. G. (2005) Chronic administration of statins alters multiple gene expression patterns in mouse cerebral cortex. *J. Pharmacol. Exp. Ther. 312*, 786−793.
(23) Saito, Y., Nishio, K., Ogawa, Y., Kinumi, T., Yoshida, Y., Masuo, Y., and Niki, E. (2007) Molecular mechanisms of 6-hydroxydopamine-induced cytotoxicity in PC12 cells: involvement of hydrogen peroxide-dependent and -independent action. *Free Radical Biol. Med. 42*, 675−685.
(24) Tao, L., Li, X., Zhang, L., Tian, J., Li, X., Sun, X., Li, X., Jiang, L., Zhang, X., and Chen, J. (2011) Protective effect of tetrahydroxystilbene glucoside on 6-OHDA-induced apoptosis in PC12 cells through the ROS-NO pathway. *PLoS One 6*, e26055.
(25) Bulat, N., and Widmann, C. (2009) Caspase substrates and neurodegenerative diseases. *Brain Res. Bull. 80*, 251−267.
(26) Viswanath, V., Wu, Y., Boonplueang, R., Chen, S., Stevenson, F. F., Yantiri, F., Yang, L., Beal, M. F., and Andersen, J. K. (2001) Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease. *J. Neurosci. 21*, 9519−9528.
(27) (a) Butterick, T. A., Igbavboa, U., Eckert, G. P., Sun, G. Y., Weisman, G. A., Muller, W. E., and Wood, W. G. (2010) Simvastatin stimulates production of the antiapoptotic protein Bcl-2 via endothelin-1 and NFATc3 in SH-SY5Y cells. *Mol. Neurobiol. 41*, 384−391. (b) Franke, C., Noldner, M., Abdel-Kader, R., Johnson-Anuna, L. N., Gibson Wood, W., Muller, W. E., and Eckert, G. P. (2007) Bcl-2 upregulation and neuroprotection in guinea pig brain following chronic simvastatin treatment. *Neurobiol. Dis. 25*, 438−445. (c) Johnson-Anuna, L. N., Eckert, G. P., Franke, C., Igbavboa, U., Muller, W. E., and Wood, W. G. (2007) Simvastatin protects neurons from cytotoxicity by up-regulating Bcl-2 mRNA and protein. *J. Neurochem. 101*, 77−86.
(28) Yan, J. Q., Ma, Y. J., Sun, J. C., Bai, S. F., and Huang, L. N. (2014) Neuroprotective effect of lovastatin by inhibiting NMDA receptor1 in 6-hydroxydopamine treated PC12 cells. *Int. J. Clin. Exp. Med. 7*, 3313−3319.
(29) Fonseca, A. C., Proenca, T., Resende, R., Oliveira, C. R., and Pereira, C. M. (2009) Neuroprotective effects of statins in an *in vitro* model of Alzheimer's disease. *J. Alzheimer's Dis. 17*, 503−517.
(30) Liu, M., Xu, Y., Han, X., Liang, C., Yin, L., Xu, L., Qi, Y., Zhao, Y., Peng, J., and Sun, C. (2014) Potent effects of flavonoid-rich extract from Rosa laevigata Michx fruit against hydrogen peroxide-induced damage in PC12 cells *via* attenuation of oxidative stress, inflammation and apoptosis. *Molecules 19*, 11816−11832.
(31) Liu, C. S., Chen, N. H., and Zhang, J. T. (2007) Protection of PC12 cells from hydrogen peroxide-induced cytotoxicity by salvianolic acid B, a new compound isolated from Radix Salviae miltiorrhizae. *Phytomedicine 14*, 492−497.
(32) Das, K. P., Freudenrich, T. M., and Mundy, W. R. (2004) Assessment of PC12 cell differentiation and neurite growth: a comparison of morphological and neurochemical measures. *Neurotoxicol. Teratol. 26*, 397−406.
(33) Greene, L. A., and Tischler, A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A. 73*, 2424−2428.
(34) Taniguchi, H., Yamamoto, H., Akutsu, N., Nosho, K., Adachi, Y., Imai, K., and Shinomura, Y. (2007) Transcriptional silencing of hedgehog-interacting protein by CpG hypermethylation and chromatic structure in human gastrointestinal cancer. *J. Pathol. 213*, 131−139.
(35) Lin, C. M., Lin, R. D., Chen, S. T., Lin, Y. P., Chiu, W. T., Lin, J. W., Hsu, F. L., and Lee, M. H. (2010) Neurocytoprotective effects of the bioactive constituents of Pueraria thomsonii in 6-hydroxydopamine (6-OHDA)-treated nerve growth factor (NGF)-differentiated PC12 cells. *Phytochemistry 71*, 2147−2156.
(36) Xiong, X. I., Jia, R. H., Yang, D. P., and Ding, G. H. (2006) Irbesartan attenuates contrast media-induced NRK-52E cells apoptosis. *Pharmacol. Res. 54*, 253−260.

DOI: 10.1021/cn500275k
ACS Chem. Neurosci. 2015, 6, 716−724

(37) Villa, P., Kaufmann, S. H., and Earnshaw, W. C. (1997) Caspases and caspase inhibitors. *Trends Biochem. Sci. 22*, 388−393.
(38) Lin, Y. J., Lai, C. C., Lai, C. H., Sue, S. C., Lin, C. W., Hung, C. H., Lin, T. H., Hsu, W. Y., Huang, S. M., Hung, Y. L., Tien, N., Liu, X., Chen, C. L., and Tsai, F. J. (2013) Inhibition of enterovirus 71 infections and viral IRES activity by Fructus gardeniae and geniposide. *Eur. J. Med. Chem. 62*, 206−213.
(39) Yue, G. G., Chan, B. C., Hon, P. M., Lee, M. Y., Fung, K. P., Leung, P. C., and Lau, C. B. (2010) Evaluation of *in vitro* anti-proliferative and immunomodulatory activities of compounds isolated from *Curcuma longa*. *Food Chem. Toxicol. 48*, 2011−2020.

DOI: 10.1021/cn500275k
ACS Chem. Neurosci. 2015, 6, 716−724