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ORIGINAL ARTICLE

The protective effects of oxyresveratrol imine derivative against hydrogen peroxide-induced cell death in PC12 cells

J. Hur[1], S. Kim[2], P. Lee[3], Y. M. Lee[1] & S. Y. Choi[1]

[1]*Korea Food Research Institute, Seongnam, South Korea,* [2]*College of Pharmacy, Seoul National University, Seoul, South Korea, and* [3]*Department of Natural Medicine Resources, Semyung University, Jecheon, South Korea*

Abstract

Oxyresveratrol (2′,3,4′,5-tetrahydroxystilbene) is a naturally occurring ingredient found in mulberries that shows potential as an antioxidant, anti-inflammatory, and neuroprotective agent. This study was performed to identify materials similar to oxyresveratrol that may have more effective antioxidant properties. We synthesized a stilbene analog referred to as Compound 1 (2′,3,4′,5-tetramethoxystilbene); a benzamide analog referred to as Compound 2 ((2,4-dimethoxyphenyl)-3,5-dimethoxybenzamide); and three imine analogs referred to as Compound 3 (3,5-dimethoxybenzylidene)-(2,4-dimethoxyphenylamine), Compound 4 ((4-methoxybenzylidene)-(3-methoxyphenyl) amine), and Compound 5 ((4-methoxybenzylidene)phenylamine). The cytoprotective effects of these compounds were subsequently evaluated using hydrogen peroxide-treated PC12 cells. The cytoprotective effects of the imine analogs were greater than the effects of oxyresveratrol and the other analogs at concentrations of 200 μM. The Compound 3, which is the most effective imine analog of oxyresveratrol, exhibited these cytoprotective effects against hydrogen peroxide-induced oxidative stress through the regulation of heme oxygenase-1 (HO-1) expression and the translocation of nuclear factor E2-related factor 2 (Nrf2). Our results suggest that imine analogs of oxyresveratrol may be useful agents in reducing neuronal oxidative damage.

Keywords: oxyresveratrol, derivative, anti-oxidant, cytoprotection, heme oxygenase

Introduction

Increases in oxidative stress are linked to cardiovascular disease, aging, and many neurodegenerative diseases [1]. Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a component of red wine that reduces the risk of cardiovascular disease and most likely underlies the phenomenon known as the "French paradox" [2–4]. The physiological benefits of resveratrol are most likely due to its antioxidative and free radical scavenging activities [5,6]. In addition, resveratrol has exhibited direct neuroprotective effects *in vitro* against neuronal cell death induced by hydrogen peroxide (H_2O_2)-induced oxidative stress or other oxidative agents [7–10].

Oxyresveratrol is a naturally occurring analog of hydroxystilbene, which has one more hydroxy group than resveratrol. Oxyresveratrol has recently been reported to be a more effective tyrosinase inhibitor and antioxidative agent than resveratrol [11,12]. Moreover, oxyresveratrol strongly prevents neuronal apoptotic cell death in an animal model of cerebral ischemia [13–15]. Therefore, we proposed that a search for new antioxidative materials among oxyresveratrol derivatives might reveal other neuroprotective agents that are more potent than oxyresveratrol.

Heme oxygenase-1 (HO-1) is one of the phase II antioxidant enzymes regulated by transcriptional factor nuclear factor E2-related factor 2 (Nrf2). Under unstressed conditions, Nrf2 binds to kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. However, under stress conditions, Nrf2 is released and translocates to the nucleus [16,17]. In addition, Yu et al. have reported that mitogen-activated protein kinase (MAPK) involves the modulation of ARE-mediated gene expression such as Nrf2 [18]. Natural plant derivatives, such as curcumin and sesamine, have been reported to upregulate Nrf2 expression, subsequently increasing HO-1 enzyme expression [17,19].

For the identification of a new class of antioxidative agents, we examined the cytoprotective effects of resveratrol, oxyresveratrol, and five synthetic oxyresveratrol derivatives (Compounds 1–5) on PC12 cells exposed to hydrogen peroxide. Compounds 1–3 (stilbene, benzamide and imine analogs, see Figure 1) all possess four methoxy groups at the same position, as well as a transformed connection chain between the two benzene rings; Compounds 4 and 5 are also imine analogs. In addition, we also examined the inhibitory activity on intracellular reactive oxygen species (ROS) generation via HO-1 and Nrf2 signaling in MAPK dependent manners.

Materials

Generals

UV spectra were obtained using a Molecular Devices E09090 microplate reader (Sunnyvale, CA) TLC and

Correspondence: Sang Yoon Choi, PhD, Korea Food Research Institute, 516 Baekhyeon-dong, Bundang-gu, Seongnam 463-746, South Korea. Tel: + 82-31-780-9307. Fax: + 82-31-709-9876. E-mail: sychoi@kfri.re.kr

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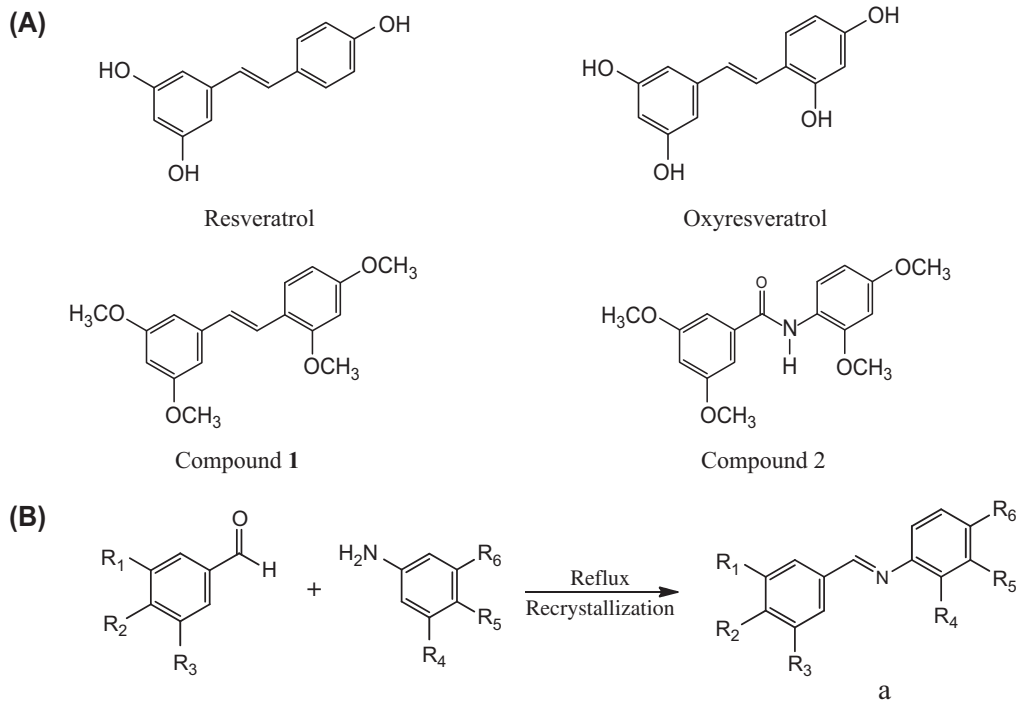


a	R_1	R_2	R_3	R_4	R_5	R_6
Compound **3**	OCH_3	H	OCH_3	OCH_3	H	OCH_3
Compound **4**	H	OCH_3	H	H	OCH_3	H
Compound **5**	H	OCH_3	H	H	H	H

Figure 1. The chemical structure of resveratrol, oxyresveratrol, Compounds 1 and 2 (A) and synthesis of compounds 3–5 (B).

column chromatography were carried out on precoated silica gel F254 plates (Merck, Whitehouse Station, NJ) and Silica gel 60 (Merck, 70–230 mesh). All other solvents were of analytical grade. Solvents for chromatography and organic synthesis were used after further distillation.

Chemicals and synthesis

Resveratrol was purchased from Sigma (St. Louis, MO). Oxyresveratrol and compounds 1–4 were synthesized as previously reported [20,21]. Compound 5 ((4-methoxybenzylidene)phenylamine) was synthesized using the following method: *p*-anisaldehyde (1.2 ml, 10.0 mmol) and aniline (910 µl, 10.0 mmol) were dissolved in methanol (50 ml), and the reaction mixture was refluxed for 12 h. The product (27 mg) was recrystallized at –20°C.

(4-methoxybenzylidene)phenylamine (Compound 5)

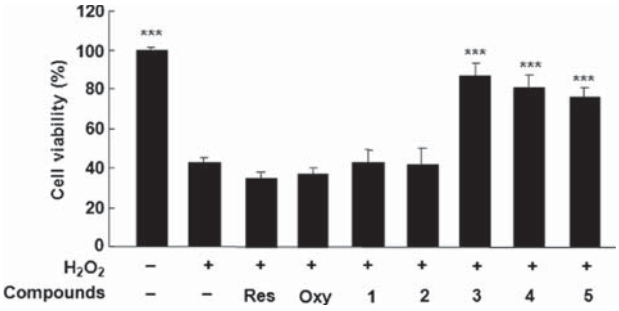
Yellowish solid; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 8.39 (1H, s), 7.87 (1H, t, J = 2.7 Hz), 7.84 (1H, t, J = 2.7 Hz), 7.38 (2H, m), 7.21 (3H, m), 7.00 (1H, t, J = 2.7), 6.97 (1H, t, J = 2.7 Hz), 3.88 (3H, s). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 162.24, 159.68, 152.35, 131.98, 130.50, 129.26, 129.08, 125.54, 120.85, 114.17, 55.41. MS (EI^+) *m/z* 211(M^+, 100), 195(6), 167(24), 77(47).

Cell culture

PC12 rat pheochromocytoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modification of Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 5% horse serum, 2 mM glutamine, and 1% of penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD). Cells were cultivated in 5% CO_2 at 37°C incubator. The cells were grown on culture dishes precoated with poly-D-lysine (50 µg/ml).

Exposure to H_2O_2 and cytoprotective effect

PC12 cells were pretreated with the test samples for 90 min before exposure to H_2O_2 (300 µM). After a 24-h incubation, the lactate dehydrogenase (LDH) levels in the culture supernatants were measured with a modification of the method reported by Koh and Choi [22]. Briefly, 30 µl of substrate (1.4 mM β-nicotinamide adenine dinucleotide in 0.75 mM pyruvate) and 30 µl of medium were mixed in a 96-well plate. After a 30-min incubation at 37°C, 30 µl of 2,4-dinitrophenylhydrazine (1 mM in 1 N HCl) was added to the mixture and incubated for 20 min at room temperature. Subsequently, 100 µl of 0.4 N NaOH was added to each well, and the UV absorption at 405 nm was measured.



	Cytoprotective effects (%)		
	2 μM	20 μM	200 μM
Resveratrol	4.9 ± 5.1	29.8 ± 4.5	–16.7 ± 4.4
Oxyresveratrol	4.8 ± 6.2	33.1 ± 5.8	–9.6 ± 6.3
Compound **1**	0.5 ± 4.0	3.1 ± 4.6	0.6 ± 2.7
Compound **2**	0.9 ± 3.4	5.5 ± 4.9	0.2 ± 1.9
Compound **3**	9.3 ± 5.6	29.9 ± 5.6	81.2 ± 6.1
Compound **4**	9.6 ± 4.8	25.3 ± 6.8	70.5 ± 7.0
Compound **5**	6.1 ± 4.9	14.6 ± 5.4	63.7 ± 5.5

Figure 2. The cytoprotective effects of resveratrol, oxyresveratrol, and Compounds 1–5 against hydrogen peroxide-induced injury in PC12 cells. The graph shows the effect of the 200 μM compounds under control conditions and when 300 μM H_2O_2 was added. The viability of the untreated cells was set to 100%. Data are presented as the mean ± S.E. of three independent experiments. ***$p < 0.001$ compared with the control.

Extracellular hydrogen peroxide-reducing activity

Hydrogen peroxide-reducing activity was determined by the method of catalase assay kit (Sigma). A 25 μl of 200 mM H_2O_2 solution was added to 75 μl test samples in 50 mM potassium phosphate buffer (pH 7.0), and the mixtures were shaken vigorously. After an incubation for 5 min at room temperature, 900 μl of 15 mM sodium azide in water was added to the mixture. To 10 μl of the reaction, 1 ml of the 150 mM potassium phosphate buffer (pH 7.0) containing 0.25 mM 4-aminoantipyrine and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid was added, mixed, and incubated for 15 minutes at room temperature for color development. The absorbance was measured at 520 nm.

Intracellular ROS generation

Intracellular ROS generation was measured by fluorescent microscopy using cells stained with the ROS-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCF-DA, Sigma). PC12 cells were incubated with H_2O_2

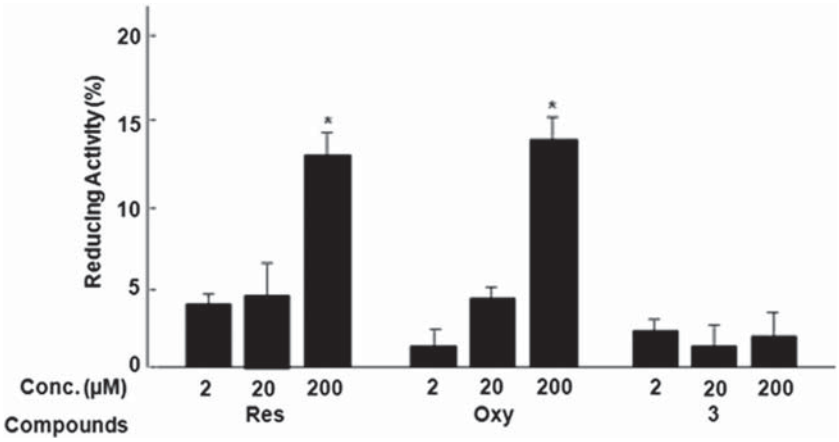


Figure 3. Changes in extracellular hydrogen peroxide levels by resveratrol, oxyresveratrol and Compound 3. H_2O_2-reducing activity was measured using a catalase assay kit (Sigma CAT100). Data are presented as the mean ± S.E. of three independent experiments. *$p < 0.05$ compared with the control.

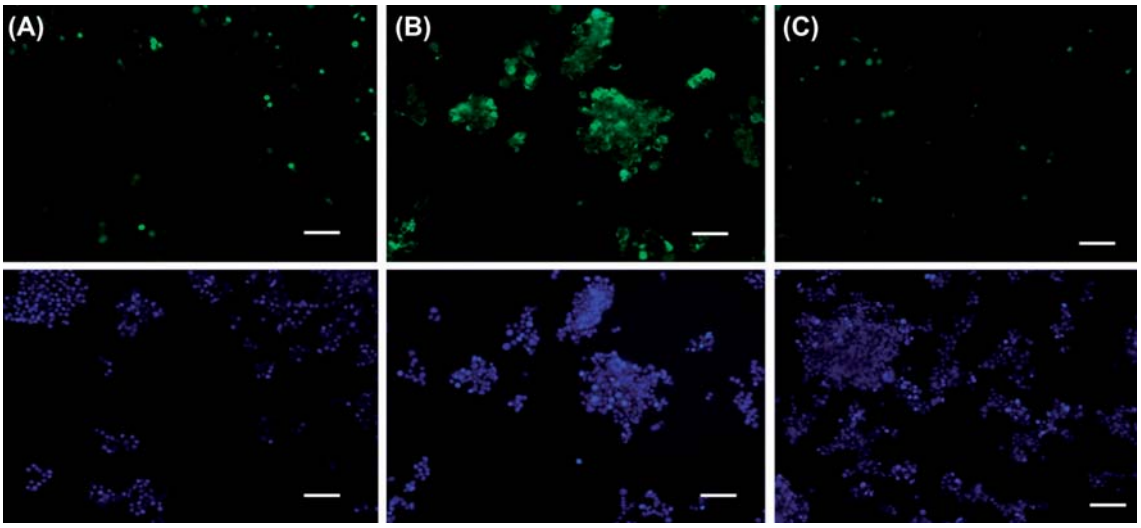


Figure 4. The inhibitory effect of Compound 3 on intracellular ROS production in PC12 cells. The ROS levels were then analyzed by fluorescent microscopy (Nickon Eclips Ti) using cells stained with the ROS-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCF-DA). A, Control; B, Hydrogen peroxide; C, Hydrogen peroxide and 200 μM Compound 3. Scale bar = 100 micrometers. DAPI was used for nuclei marker.

for 1 h with or without Compound 3 (200 μM). PC12 cells were washed with PBS and incubated with 3 μM DCF-DA for 30 min at 37°C. DCF-DA positive cells were counted from ten randomly chosen microscopic fields (Nickon). These ten measurements averaged and regarded as a data point. Fluorescence was observed using a fluorescent microscope (Nickon, Eclips Ti microscopy) at 485–535 nm and DAPI dye was used for nucleus marker.

Western blot analysis

The mitochondrial and cytosolic components were isolated using a nucleic/cytosolic fractionation kit (Bio-Vision, Mountain View, CA) according to the manufacturer's instruction. An equal amount of protein from each sample was separated by SDS-poly-acrylamide gel electrophoresis (10% gel) and transferred to Hybond ECL nitrocellulose membrane. The membranes were blocked with 5% skim milk and incubated with rabbit anti-Nrf2 (1:1000 dilution; Abcam, Cambridge, UK), rabbit anti-HO-1(1:2000 dilution; Enzo life science, Farmingdale, NY), anti-Erk, phosphor-Erk, JNK, phosphor JNK antibody (1: 1000 dilution; Cell signaling Technology, Danvers, MA), mouse beta-actin (1:4000 dilution; Santa-Cruz Scientific, Santa Cruz, CA), and HRP-conjugated secondary antibodies (1:1000 dilution; Cell signaling Technology), this step was followed by enhanced chemiluminescence detection (Bio-Rad, Hercules, CA).

Statistical analysis

All data were presented as the mean ± SE from three independent experiments. Statistical comparisons between the different treatments were conducted with a Student's *t*-test.

Results

Cytoprotection against H_2O_2-induced Injury

To measure the cytoprotective effect of Compounds 1–5 against oxidative stress, we used PC12 rat adrenal pheochromocytoma cells. It is generally accepted that these cells are similar to dopaminergic neuronal cells and are quite sensitive to heavy metal ions and free radical attack [9]. For the experiments, we pretreated PC12 cells with Compounds 1–5 (Figure 1), resveratrol or oxyresveratrol for 90 min and then exposed them to H_2O_2 for 24 h. The parent compound oxyresveratrol exhibited 33.1% of its protective effect at 20 μM. However, at a concentration of 200 μM, oxyresveratrol did not reduce the H_2O_2-induced oxidative cell injury (Figure 2). In contrast, the same dose of imine analog Compounds 3, 4, and 5 mediated 81.2, 70.5, and 63.0% reductions, respectively. The stilbene (Compound 1) and benzamide (Compound 2) analogs did not exhibit significant cytoprotective effects on 300 μM H_2O_2-induced injury at any concentration we tested (Figure 2). These results demonstrate that compared to oxyresveratrol, imine analog Compounds 3, 4, and 5 more effectively protect PC12 cells from H_2O_2-induced cytotoxicity at higher concentrations.

Extracellular H_2O_2-reducing activity

We used a catalase assay to ascertain the extracellular H_2O_2 decomposing and scavenging ability of Compound 3. Although a difference in mechanical properties between decomposing and scavenging activity existed, both the activities commonly could have led to a reduction of the amount of H_2O_2. Our results demonstrated that resveratrol and oxyresveratrol reduced the H_2O_2 level in a dose-dependent manner (Figure 3). However, Compound 3 did not exhibit H_2O_2-reducing activity at the same dose.

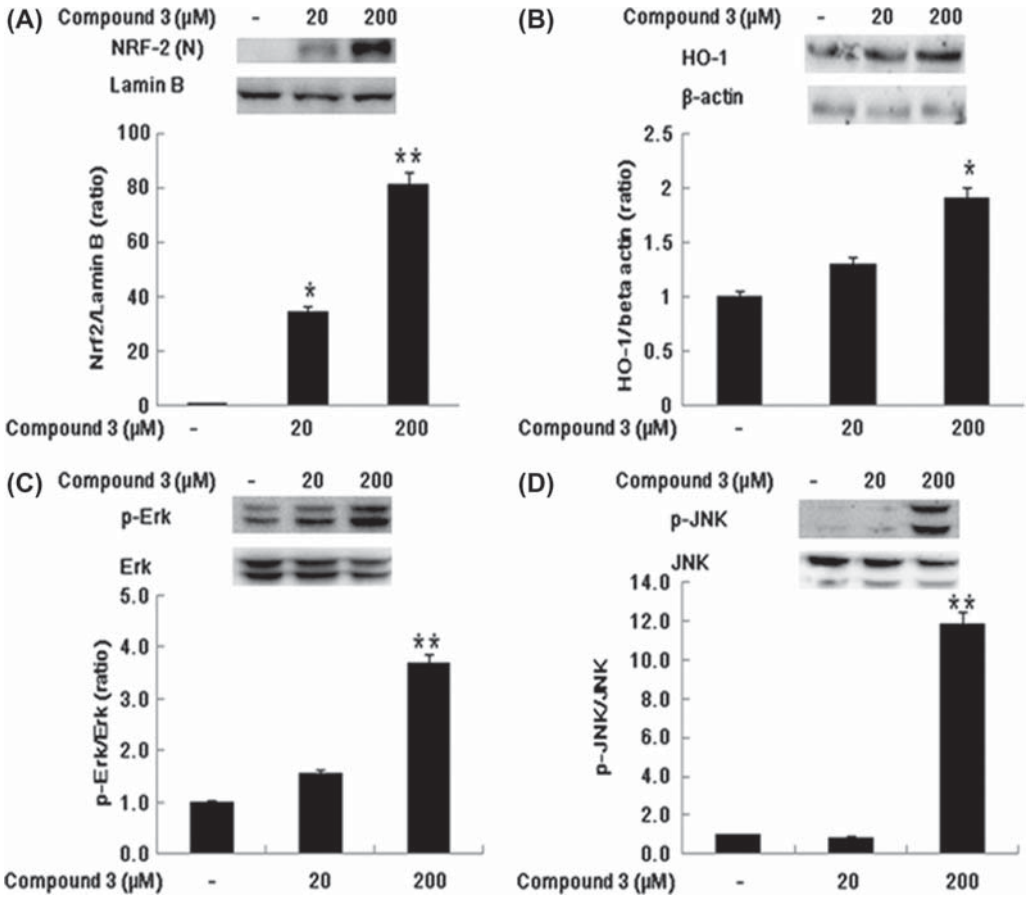


Figure 5. Compound 3 induced Nrf2 and HO-1 expression in MAPK dependent manner. PC12 cells were treated with Compound 3 (20, 200 μM) for 16 h. The effects of Compound 3 on the expression of HO-1 in the whole fraction and of Nrf2 (NRF2(N)) in the nuclear fraction (A, B); The effects of Compound 3 on Erk and JNK phosphorylation (C, D). The results shown are representative of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control.

Inhibition of ROS generation in PC12 cells

Because Compound 3 did not cause extracellular H_2O_2-reducing activity, we examined the effect of Compound 3 on ROS generation in PC12 cells. The excessive production of ROS may lead to oxidative stress, loss of cell function, and ultimately apoptosis [23]. To investigate whether Compound 3 inhibits intracellular ROS in PC12 cells, we measured the level of ROS in PC12 cells treated with H_2O_2 by using the fluorescent dye DCF-DA. Fewer DCF-DA positive cells showed in the non-treated control cells (Figure 4A) However, H_2O_2-treated PC12 cells showed a 2-fold increase in ROS production (Figure 4B). In contrast, PC12 cells treated with 200 μM of Compound 3 exhibited significantly reduced DCF fluorescence (Figure 4C).

Upregulation of HO-1 expression and Nrf2 translocation in a MAPK dependent manner

To investigate the mechanism of ROS inhibition in PC12 cells, we examined the expression of HO-1 and Nrf2. HO-1, which is regulated by Nrf2, is an important protein that provides a cellular defense against oxidative stress. The protein levels of HO-1 and Nrf2 are shown in Figure 5A and B). PC12 cells treated with Compound 3 showed significant increases in HO-1 and Nrf2 protein levels by over 2.0 and 80 fold, respectively, when treated with 200 μM of Compound 3. These results suggest that the antioxidative effects of Compound 3 may be mediated through the upregulation of HO-1 and Nrf2. To investigate the upstream pathway, we examined the activation of Erk and JNK, which have protective role in oxidative stress. As shown Figure 5C and D, Compound 3 increases the phosphorylation of Erk and JNK, however, p38 is not phosphorylated by Compound 3 at16 h incubation (data not shown).

Discussion

Oxidative stress increases oxidative damage that plays a role in neurodegenerative statements [24]. Recently, it has been reported that many antioxidants may attenuate neurodegenerative diseases [25]. In this study, we demonstrated for the first time that oxyresveratrol derivatives, such as Compound 3, have antioxidant properties on H_2O_2-induced PC12 cell damage. Because Compounds 1–5 are modifications of the oxyresveratrol structure, we compared the antioxidant effects of Compounds 1–5

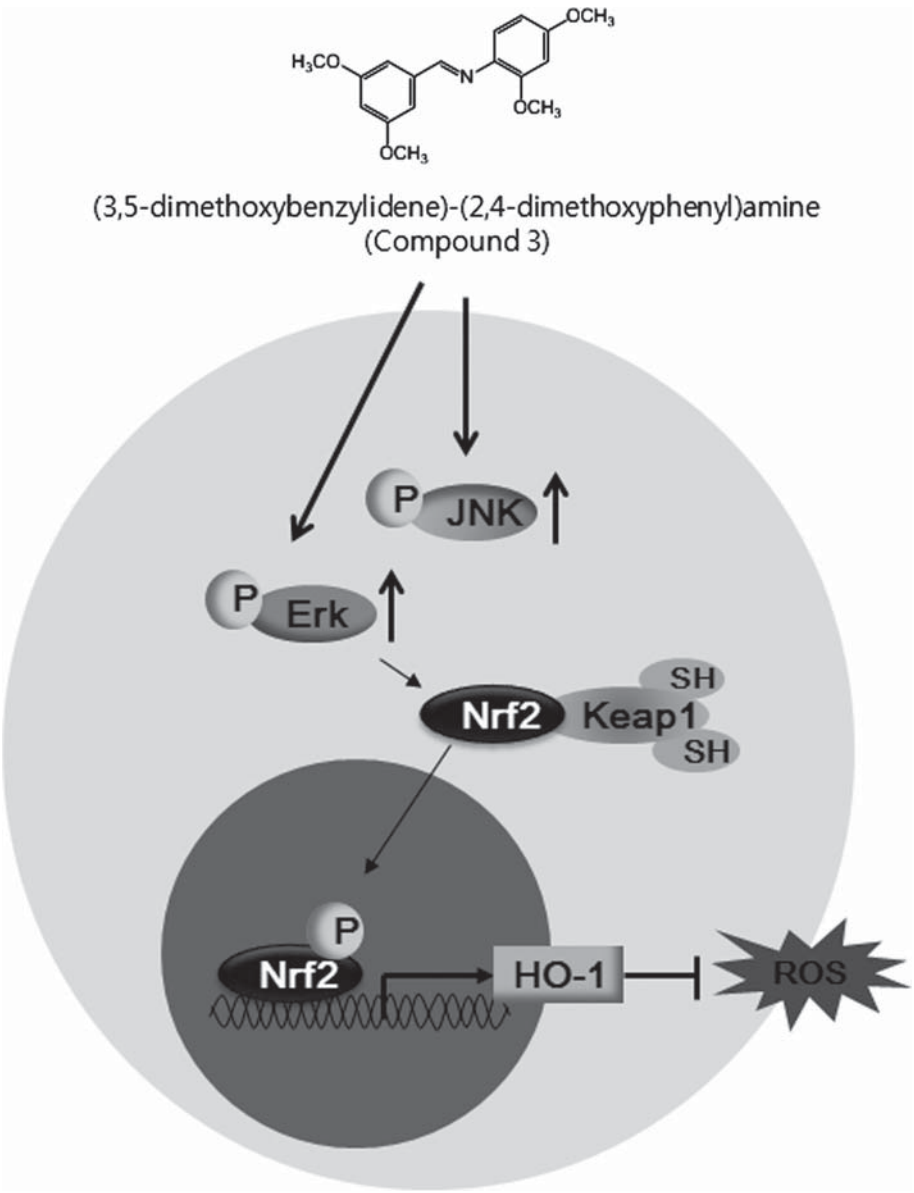


Figure 6. Scheme of the intracellular ROS inhibition mechanism by Compound 3. Compound 3 inhibited intracellular ROS generation via HO-1 and Nrf2 signaling in MAPK dependent manners. The Erk and JNK pathways are involved in Compound 3-induced HO-1 expression.

to those of oxyresveratrol using cell-based and H_2O_2 reducing assays. Our results demonstrate the superior cytoprotective effects of imine analog Compounds 3–5 compared to oxyresveratrol. As shown in Figure 2, Compounds 3, 4, and 5 more effectively prevented the H_2O_2-mediated cell death of PC12 cells compared to oxyresveratrol. Compound 3 was most effective among them. Moreover, PC12 cells treated with Compound 3 showed a reduction of intracellular ROS production. To confirm the intracellular ROS inhibition effect of Compound 3, we measured the ROS using DCF-DA. Our data indicate that Compound 3 effectively blocked H_2O_2 released from the cells (Figure 4). However, Compound 3 did not affect extracellular H_2O_2-reducing activity. These results suggest that the cytoprotective effects of Compound 3 were not due to direct H_2O_2-reducing abilities but result from inhibition of intracellular ROS production.

HO-1 is regarded as an important enzyme against oxidative stress and a therapeutic enzyme in neurodegenerative disease [26]. In addition, many researches have reported that MAPK and other protein kinase is an important pathway for Nrf2 activation which related to HO-1 [27,28]. To investigate the intracellular signaling mechanism of Compound 3, we measured the expression of HO-1 and Nrf2 expression in PC12 cells treated with Compound 3. As we expected, Compound 3 increased the expression levels of these two proteins in the cytoplasm and nucleus, respectively. Moreover, our results showed that the Erk and JNK pathways are involved in Compound 3 induced HO-1 (Figure 6). However, other kinase study which is related the nrf2 translocation remains to be elucidated.

In the present study, we are the first to report that oxyresveratrol imine derivatives can effectively protect cells

from oxidative damage, opening up the possibility of their use as potent antioxidants.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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