

## Protective effects of pinostilbene, a resveratrol methylated derivative, against 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells

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### Abstract

Resveratrol (3,4',5-*trans*-trihydroxystilbene) is a phytoalexin with emerging lines of evidence supporting its beneficial effects on cardiovascular systems and inhibition of carcinogenesis. It has also been reported that certain methylated resveratrol derivatives are more effective than resveratrol in the prevention/treatment of cancer. However, little is known about the impact of resveratrol and its derivatives on the development of Parkinson's disease. In this study, we compared the neuroprotective effects of resveratrol with four methylated (fully or partially) resveratrol derivatives against parkinsonian mimetic 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in SH-SY5Y cells. Release of lactate dehydrogenase and activity of caspase-3 triggered by 6-OHDA were significantly reduced by resveratrol and one of the methylated derivatives, pinostilbene (3,4'-dihydroxy-5-methoxystilbene), in a dose-dependent manner. In addition, pinostilbene exerted a potent neuroprotective effect with a wider effective concentration range than resveratrol. By using high-performance liquid chromatography, we found that uptake of pinostilbene into SH-SY5Y cells was significantly higher than that of resveratrol. Enhanced bioavailability may thus be a major factor contributing to the neuroprotective activity of pinostilbene. Moreover, Western blot analysis demonstrated that pinostilbene markedly attenuated the phosphorylation of JNK and c-Jun triggered by 6-OHDA. Besides, mammalian target of rapamycin kinase may be an intracellular target accounting for the neuroprotective effects of pinostilbene. Our findings demonstrate the potential of methylated stilbenes in neuroprotection and provide important information for further research in this field.

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**Keywords:** Parkinson's disease; 6-Hydroxydopamine; Resveratrol; Pinostilbene; Neuroprotection

### 1. Introduction

Resveratrol (3,4',5-*trans*-trihydroxystilbene) is a stilbene-type phytoalexin in plants such as grapes, peanuts, berries and pines [1]. It is produced in these plants to counteract environmental stresses such as UV irradiation and fungal infection. Among the wide range of biological and pharmacological activities, resveratrol has been intensively investigated as a cancer chemopreventive agent. It is

reported to suppress the growth of several tumor cell lines such as leukemic, prostate, colonic, breast and esophageal cells through inhibiting tumor initiation, promotion and/or progression [1,2]. Resveratrol is also reported to be one of the active constituents of Itadori tea, which has been used as a traditional medicine mainly for curing heart disease and stroke in China and Japan [2]. There are considerable epidemiological reports pointing to an inverse association between moderate consumption of red wine and the incidence of coronary heart disease; resveratrol is widely regarded as one of the major phenolic compounds in red wine attributing to cardioprotection. Several mechanisms have been proposed to account for resveratrol's cardioprotective activities such as free-radical scavenging and the inhibition of cyclooxygenase and hydroperoxidase; the latter two result in attenuating platelet aggregation and reducing lipid peroxidation, respectively [1,3].

Studies on the neuroprotective activity of resveratrol have mainly focused on animal models with disorders or injury to the central nervous system such as Alzheimer's disease (AD),

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Parkinson's disease (PD), Huntington's disease, cerebral ischemia or traumatic brain injury [4,5]. Increasing lines of evidence suggest that acute or chronic treatment with resveratrol can confer protective effects on neurons against colchicine-, 3-nitropropionic-acid- or trauma-induced cognitive and motor impairment as well as hippocampal neuron loss [6–9]. The underlying mechanisms can be attributed to the ability of resveratrol to alleviate oxidative stress by reducing the elevated malondialdehyde, lipid peroxidation, nitric oxide and xanthine oxidase. On the other hand, it can restore the levels of glutathione and increasing succinate dehydrogenase activity in the brains from animal experiments. In addition, administration of resveratrol protects cerebral neurons from ischemia-induced damage [10] and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced motor coordination impairment, hydroxyl radical overloading and neuronal loss probably via scavenging of free radicals [11].

The usefulness of resveratrol, however, is limited by its instability upon exposure to light and oxygen or in environments with drastic pH conditions. These stimuli may cause *trans*–*cis* transformation or oxidation that leads to reduction in bioavailability and bioactivity [12]. An effective approach to stabilize resveratrol is accomplished by methylation of its hydroxyl groups to form trimethylated resveratrol. It has been demonstrated that the trimethylated resveratrol is more effective than resveratrol in preventing CCl<sub>4</sub>-induced liver damage, via inhibiting lipid peroxidation and serum enzyme activity of  $\gamma$ -glutamyl transpeptidase. Trimethylated resveratrol has been postulated to act as a prodrug of resveratrol, which improves the bioavailability of resveratrol in target tissues [13]. Similarly, trimethylated resveratrol or 3,5-dimethoxy-4'-hydroxystilbene has been reported to exert up to 100-fold stronger cytotoxicity than resveratrol in cancer cell lines by depleting the intracellular pool of polyamines and by altering microtubule polymerization [14]. However, little is known about the relative efficacy of methylated resveratrol and resveratrol in terms of neuroprotection. This motivated us to synthesize partially and fully methylated resveratrol derivatives and investigate their

protective effects on Parkinsonian mimetic 6-hydroxydopamine (6-OHDA)-induced neurotoxicity. The synthesized compounds include 3,4',5'-trimethoxystilbene (R1), 3,4'-dimethoxy-5'-hydroxystilbene (R2), 3,4'-dihydroxy-5'-methoxystilbene (R3, pinostilbene) and 3,5-dihydroxy-4'-methoxystilbene (R4, desoxyrhapontigenin) (Fig. 1). Their neuroprotective activities were evaluated in dopaminergic human SH-SY5Y cells by monitoring their effects on the level of lactate dehydrogenase (LDH) release and the activity of caspase-3 triggered by 6-OHDA. Bioavailability and stability of resveratrol and the methylated derivatives were analyzed by high-performance liquid chromatography (HPLC). Furthermore, their effects on the inhibition of 6-OHDA-activated JNK pathway as well as the modulation of mammalian target of rapamycin (mTOR) kinase activity were also evaluated with Western blot analysis.

## 2. Materials and methods

### 2.1. Materials

Materials used for SH-SY5Y cell cultures were purchased from Gibco-BRL (Burlington, Ontario, Canada). For organic synthesis of stilbenes, all starting materials with the highest quality were obtained from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were obtained from the following companies: resveratrol, 6-OHDA, 1,1-diphenyl-2-picrylhydrazyl (DPPH), protease inhibitor cocktail, phosphatase inhibitor cocktail and anti- $\beta$ -actin monoclonal antibody were obtained from Sigma-Aldrich, Inc. Caspase-3 substrate (Ac-DEVE-pNA) was purchased from Calbiochem, Inc. (La Jolla, CA, USA). LDH cytotoxicity assay kit was obtained from Roche Diagnostics (Mannheim, Germany). HPLC analysis was performed on a Shimadzu LC-20AT system equipped with a diode array detector and LC-Solution software. Rabbit polyclonal anti-phosphorylated JNK (Thy183/Tyr185) antibody, rabbit monoclonal anti-phosphorylated c-Jun-1 (Ser73) antibody, rabbit monoclonal anti-phosphorylated mTOR (Ser 2448) antibody and rabbit monoclonal anti-phosphorylated GSK-3 $\beta$  (Ser 9) antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish-peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were obtained from DAKO (Glostrup, Denmark). PVDF membrane was from Bio-Rad (Richmond, CA, USA). Biomax X-ray film was from Kodak (Tokyo, Japan). Visualizer Spray & Glow ECL Western Blotting Detection System was from Millipore (Billerica, MA, USA).

### 2.2. Synthesis of methylated RES derivatives

Dry potassium carbonate (1.38 g, 10 mmol) was added to a solution of resveratrol (1.163 g, 5 mmol) in dry acetone (16.25 ml). Methyl iodide (0.472 ml, 7.5 mmol) was added dropwise. The reaction mixture was stirred for 24 h at room temperature and then poured onto 20 g of ice, and acetone was removed under reduced pressure by a rotary evaporator. The obtained aqueous phase was extracted with ethyl acetate (3 $\times$ 20 ml). The organic phase was dried with MgSO<sub>4</sub>, and ethyl acetate was removed under reduced pressure. The crude residue from above was then subjected to flash chromatography on silica gel (chloroform/ethyl acetate, 7:1) to afford R1 (85.0 mg) and R2 (290.1 mg), a subfraction with two components and the starting resveratrol. The impure subfraction was further purified by gel-filtration chromatography on Sephadex LH-20 column (chloroform/methanol, 1:1) to offer R3 (154.5 mg) and R4 (363.9 mg). The structures of these stilbene compounds were confirmed by analysis and comparison of spectral data (NMR and MS) with literature [15].

### 2.3. Cell culture and treatment

Dopaminergic SH-SY5Y neuroblastoma cells (passage number  $\leq$ 22) were cultured with MEM supplemented with 10% heat-inactivated FBS, L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50  $\mu$ g/ml) at 37°C in a humidified 5% CO<sub>2</sub> incubator. Stock solutions of methylated derivatives and RES (10 mM) were prepared in dimethyl sulfoxide (DMSO). All treatments were performed when cells were at  $\sim$ 80% confluence. Just before treatment, the culture medium was replaced with treatment medium [MEM supplemented with 3% FBS, 1% L-glutamine (2 mM), 1% penicillin (50 U/ml) and streptomycin (50  $\mu$ g/ml)]. Different concentrations of stilbenes were diluted in the treatment medium. The vehicle DMSO alone (maximal concentration: 0.5  $\mu$ l/ml treatment medium) had no influence on the growth of the cells. Cells pretreated with or without compounds for 30 min were exposed to 25  $\mu$ M 6-OHDA.

### 2.4. LDH activity assay

When cells undergo necrosis, LDH can be released from inside cells with damaged membrane. To evaluate the general cytotoxicity, LDH activity assay was

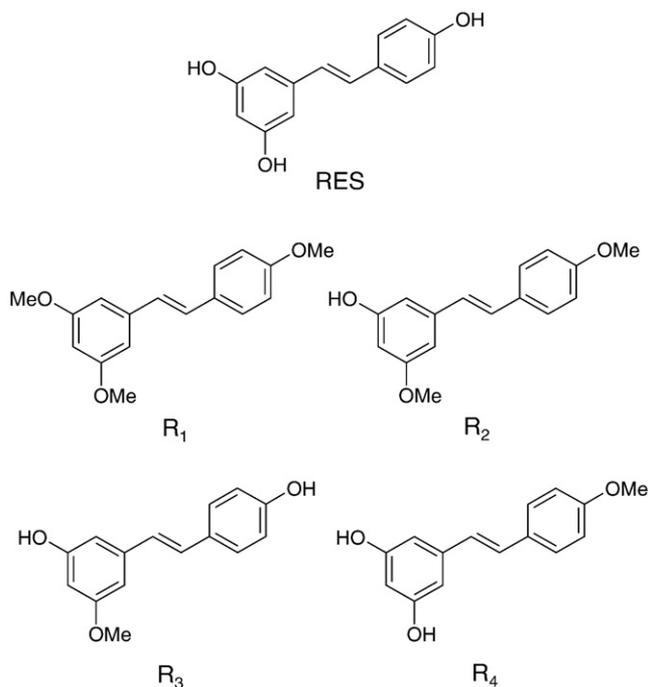


Fig. 1. Structures of resveratrol and synthesized methylated resveratrol derivatives.

carried out as described elsewhere [16–19]. Briefly, 46  $\mu$ l of culture medium was incubated with the same volume of LDH assay reaction mixture in the dark at room temperature (triplicate of each sample). After 30 min, the absorbance at 492 nm was measured to determine the level of LDH release. Results were expressed as fold of control.

### 2.5. Caspase-3-like activity assay

To determine the effects of resveratrol and its methylated derivatives on 6-OHDA-triggered apoptosis, we performed caspase-3-like activity assay. After different treatments, cells were harvested for the assay as described in our previous publications [17,20–25]. Briefly, 60  $\mu$ g of cellular protein was incubated with caspase-3 substrate (Ac-DEVD-pNA) for 2 h at 37°C. The active caspase-3 can interact with the colorimetric Ac-DEVD-pNA and produce a yellow product (pNA), determined by reading the absorbance at 405 nm. The specific activity (in pmol/min/ $\mu$ g) of caspase-3 was obtained by calculation. Results were expressed as fold of control.

### 2.6. Cellular uptake and stability analyzed by HPLC

To test the cellular permeability and metabolic situation of resveratrol or R3, we used a procedure similar to what we described in our previous publication [26]; cells were treated with resveratrol or R3 at 0 and 10  $\mu$ M, respectively. After 6 and 24 h incubation, cells from each treatment were washed with ice-cold Tris-buffered saline (pH 7.4) and then harvested with 120  $\mu$ l ice-cold lysis buffer containing DTT (5 mM), EDTA (0.1 mM), HEPES (50 mM, pH 7.4) and Triton X-100 (0.2%). The cell slurry was transferred into an Eppendorf tube, vortexed and sonicated for 3 s. By centrifuging at 20,000 $\times$ g for 15 min at 4°C, an aliquot (120  $\mu$ l) of the supernatant was immediately drawn out and filtered into an HPLC vial. Twenty microliters of each sample was auto-injected into the Shimadzu LC-20AT system equipped with a Phenomenex Luna C<sub>18</sub> column (250 $\times$ 4.6 mm, 5  $\mu$ m); flow rate, 1 ml/min; detection wavelength, UV 310 nm; the mobile phase was 40% aqueous acetonitrile containing 0.1% HCOOH.

### 2.7. Stable free-radical scavenging activity assay (DPPH assay)

The stable free-radical scavenging activity was determined by a modified procedure as described elsewhere [26–28]. Briefly, 0.2 ml of 80  $\mu$ M DPPH dissolved in ethanol was mixed with 0.6 ml of sample solution containing different concentrations of R3 or resveratrol for  $A_{\text{sample}}$  and 0.2 ml of DPPH solution was mixed with 0.6 ml of ethanol for  $A_{\text{control}}$ . The mixture was incubated in the dark at

room temperature for 30 min. Absorbance was measured at 517 nm by a Perkin-Elmer UV/VIS spectrophotometer (Lambda 2). The percentage of DPPH discoloration of each sample was calculated according to the following equation:

$$\% \text{ of discoloration} = \left[ 1 - \left( A_{\text{sample}} / A_{\text{control}} \right) \right] \times 100$$

The degree of discoloration was proportional to the scavenging activities of substances. The half-maximal inhibitory concentration ( $IC_{50}$ ) value was accordingly calculated by linear regression analysis.

### 2.8. Western blot analysis

To determine the phosphorylated level of JNK, c-Jun, mTOR and GSK-3 $\beta$  after different treatments, we collected cellular proteins from neurons using the methods in our previous publications [16–23,29,30]. Briefly, SDS-polyacrylamide gel (10%) electrophoresis was used to separate proteins, followed by transferring the proteins onto PVDF membrane (Bio-Rad). Thereafter, nonspecific binding was blocked with 5% nonfat dry milk in TBST [Tris-buffered saline (pH 7.4) containing 0.1% Tween-20] for 1 h. The membrane was then incubated with rabbit anti-phosphorylated c-Jun-1 (Ser 73) (1:1000), rabbit anti-phosphorylated mTOR (Ser 2448) (1:1000) or rabbit anti-GSK-3 $\beta$  (Ser 9) (1:2000) for 3 h at room temperature, with rabbit anti-phosphorylated JNK (Thr 183/Tyr 185) (1:1000) overnight in 4°C cold room. After washing, the membrane was then incubated with horseradish-peroxidase-conjugated secondary antibodies (1:2000) for 1 h at room temperature. Bands were developed on Biomax X-ray film using Visualizer Spray & Glow ECL Western-Blotting Detection System. By stripping with stripping buffer (50 mM glycine, 2% SDS, pH 2.0), the membranes were reprobed with monoclonal mouse anti- $\beta$ -actin (1:5000) as primary antibody and goat anti-mouse-HRP (1:10000) as secondary antibody.

### 2.9. Statistical analysis

Results were expressed as the mean $\pm$ S.E.M. from at least three independent tests. Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Student–Newman–Keuls test was used as post hoc test according to the statistical program SigmaStat (Jandel Scientific, Chicago, IL, USA).

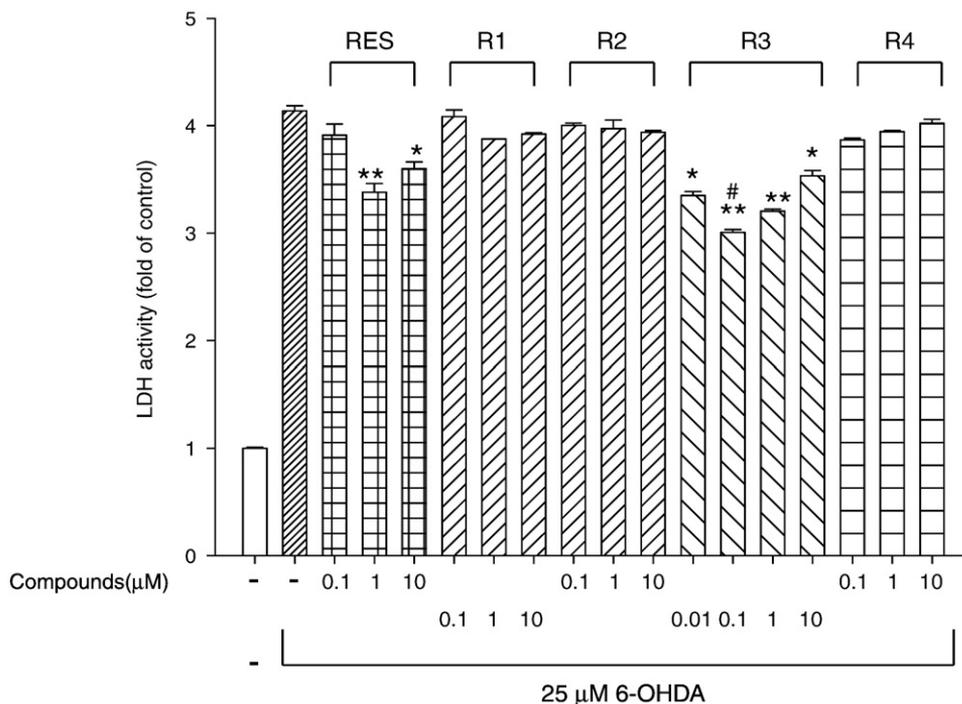


Fig. 2. Neuroprotective effects of resveratrol and its methylated derivatives. Neurons were pretreated with either resveratrol or methylated resveratrol derivatives (0.01–10  $\mu$ M) for 30 min, followed by incubation with or without 6-OHDA (25  $\mu$ M) for 24 h. After treatment, LDH activity assay was performed. Results are expressed as mean $\pm$ S.E.M. from at least three independent experiments. \* $P$ <0.01, \*\* $P$ <0.001 versus the group treated with 6-OHDA; # $P$ <0.05 versus the group treated with 1  $\mu$ M resveratrol and 6-OHDA only by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test. No statistically significant differences were analyzed between groups treated with R1, R2 and R4 and the group treated with 6-OHDA.

### 3. Results

#### 3.1. Neuroprotective effects of resveratrol and its methylated derivatives

It has been well established that neurons can undergo necrosis and apoptosis due to intracellular reactive oxygen species (ROS) triggered by 6-OHDA, where the LDH level and caspase-3 activity of neurons can serve as biomarkers for the induced neurotoxicity. In our experiments, SH-SY5Y cells were pretreated with the test compounds at selected concentrations for 30 min, followed by exposure to 25  $\mu\text{M}$  6-OHDA for 24 h. We then compared the protective efficacy of resveratrol with its methylated derivatives by evaluating the changes in LDH level and caspase-3 activity. It was found that exposure of neurons to 25  $\mu\text{M}$  6-OHDA resulted in a  $4.1 \pm 0.0$ -fold increase in LDH activity (Fig. 2) and  $2.4 \pm 0.1$ -fold increase in caspase-3 activity (Fig. 3) relative to the control. Pretreatment of cells with resveratrol from 1 to 10  $\mu\text{M}$  significantly reduced LDH release and caspase-3 activity. The most effective dosage was 1  $\mu\text{M}$ . Compared to resveratrol, R3 was found to be more effective in reducing 6-OHDA-triggered LDH release (Fig. 2) and elevation of caspase-3 activity (Fig. 3), and the inhibitory effects were observed over a wide concentration range (0.1–10  $\mu\text{M}$ ). However, as shown in Fig. 2, the other methylated derivatives, R1, R2 and R4, failed to protect neurons from necrosis triggered by 6-OHDA within the concentration range being tested.

#### 3.2. Comparison of stable free-radical (DPPH) scavenging ability between resveratrol and R3

We examined the DPPH free-radical scavenging activity of resveratrol and R3 in a cell-free system. As expected, resveratrol showed stronger radical scavenging activity than R3. The  $\text{IC}_{50}$  with respect to scavenging of DPPH radicals was 36.9 and 47.1  $\mu\text{M}$  for resveratrol and R3, respectively (data not shown).

#### 3.3. Cellular uptake of resveratrol and R3

To assess the capability of resveratrol and R3 to cross the cell membrane barrier and, thus, act as intracellular protective agents for neurons, extracts of lysed cells were subjected to HPLC analysis to

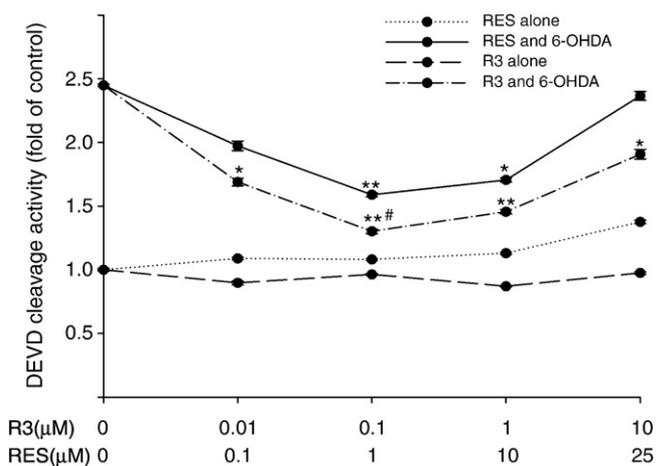


Fig. 3. Comparison of neuroprotective effects between resveratrol and R3 by measurement of caspase-3-like activity. Neurons were pretreated with either resveratrol or R3 (0.01–10  $\mu\text{M}$ ) for 30 min, followed by treating with or without 6-OHDA (25  $\mu\text{M}$ ) for 24 h. After incubation, colorimetric caspase-3-like activity assay was performed. Results are expressed as mean  $\pm$  S.E.M. from at least three independent experiments. \* $P < 0.01$ , \*\* $P < 0.001$  versus the group treated with 6-OHDA; # $P < 0.01$  versus the group treated with 1  $\mu\text{M}$  resveratrol and 6-OHDA only by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test.

reveal the changes in intracellular concentration of the test compounds with time. Results showed that both compounds were taken up by neurons from the culture medium that had been administered with 10  $\mu\text{M}$  resveratrol or R3. Intracellular concentrations of resveratrol and R3 were significantly higher after incubation for 24 h compared to that after 6 h. This observation indicated that resveratrol and R3 were gradually taken up by the cells within the time frame of the test (Fig. 4). By linear regression analysis, R3 was found to be more effectively (approximately two times) taken up into the cells than resveratrol.

#### 3.4. Western blot analysis of the JNK–c-Jun pathway and mTOR, GSK-3 $\beta$ signaling

Upon 6-OHDA stimulation, the phosphorylation level of JNK and c-Jun can be up-regulated [31,32]. In addition, Akt pathway is involved in protecting neurons against 6-OHDA-triggered neurotoxicity [33,34]. In a previous study we have demonstrated that dietary oxyresveratrol (2,3',4,5'-tetrahydroxystilbene) up-regulated phosphorylation levels of Akt. Downstream mediators of Akt, such as mTOR kinase and GSK-3 $\beta$  signaling, are also believed to play a role in protection against 6-OHDA toxicity. To investigate the responses of the JNK and Akt pathways in neurons challenged by 6-OHDA and attenuated by resveratrol and R3, Western blot analysis were conducted to evaluate changes in the phosphorylation states of the abovementioned kinases. As shown in Fig. 5, 6-OHDA led to an increase in the levels of phospho-JNK-1, phospho-JNK-2 and phospho-c-Jun-I in the neurons. Pretreatment of cells with R3 (0.1 and 1.0  $\mu\text{M}$ ) or resveratrol (1.0  $\mu\text{M}$ ) markedly attenuated their phosphorylation levels. On the contrary, exposure to 6-OHDA significantly suppressed the phosphorylation of mTOR and GSK-3 $\beta$  of the neurons. Pretreatment of the cells with resveratrol or R3 could counteract 6-OHDA-induced repression of mTOR and GSK-3 $\beta$  phosphorylation; the protective effect was especially prominent with respect to mTOR.

### 4. Discussion

In this study, we synthesized four methylated resveratrol derivatives and examined their neuroprotective effects on 6-OHDA-induced neurotoxicity. Among the four methylated resveratrol derivatives, only R3 demonstrated a potent neuroprotective effect, indicated by a marked attenuation of LDH release and caspase-3-like activity. Compared to resveratrol, the protective effect of R3 was observed over a wider concentration range. Subsequent HPLC analysis suggested that R3 could penetrate the cell membrane more readily than resveratrol, thus resulting in a higher effective intracellular dosage. Furthermore, inhibition of 6-OHDA-activated JNK pathway and modulation of mTOR kinase activity may also be involved in the neuroprotective mechanism of R3.

#### 4.1. 4'-Hydroxyl group is required for the free-radical scavenging activity and neuroprotective activity of stilbenes

6-OHDA is an analogue of catecholamine and is widely used as a neurotoxin to induce oxidative stress to neurons. It can be transported into dopaminergic neurons via dopamine transporter, wherein free-radical-mediated oxidation could lead to mitochondrial dysfunction [35]. 6-OHDA-induced ROS can also damage cellular lipids, proteins and nucleic acids, eventually inducing necrosis and apoptosis. In our previous study [26], we demonstrated that oxyresveratrol could protect neurons from 6-OHDA-induced toxicity by attenuating the release of LDH and caspase-3 activity. Scavenging intracellular ROS may be partially responsible for mediating the neuroprotective effects. Similarly, studies have shown that potent dietary antioxidants such as curcumin from tumeric and polyphenols in blueberry,

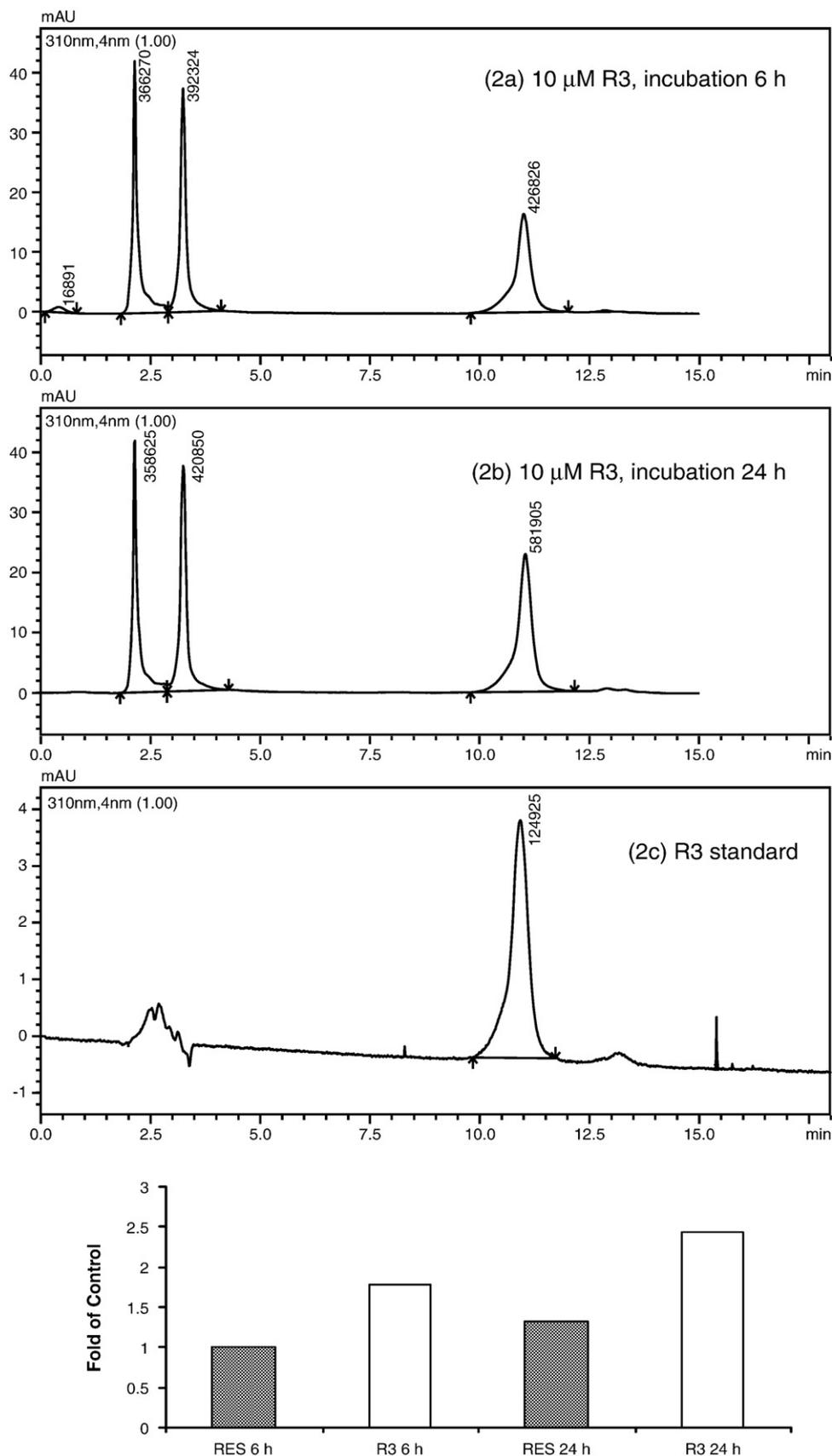


Fig. 4. HPLC chromatogram of intracellular metabolic situation of R3. Neurons were treated in the absence (control) or presence of 10  $\mu$ M resveratrol or R3. After incubating for 6 and 24 h, 120  $\mu$ l of intracellular extracts was aliquoted, and 20  $\mu$ l for each sample was subjected to HPLC analysis. We first examined the intracellular substances at different time points (6 and 24 h) that can be detected in the control group (chromatograms not shown). The retention time of intracellular resveratrol or R3 was confirmed by injecting related standards, respectively, under the same condition. The chromatogram demonstrated the metabolic situation of intracellular R3. The chromatograms for resveratrol were not shown.

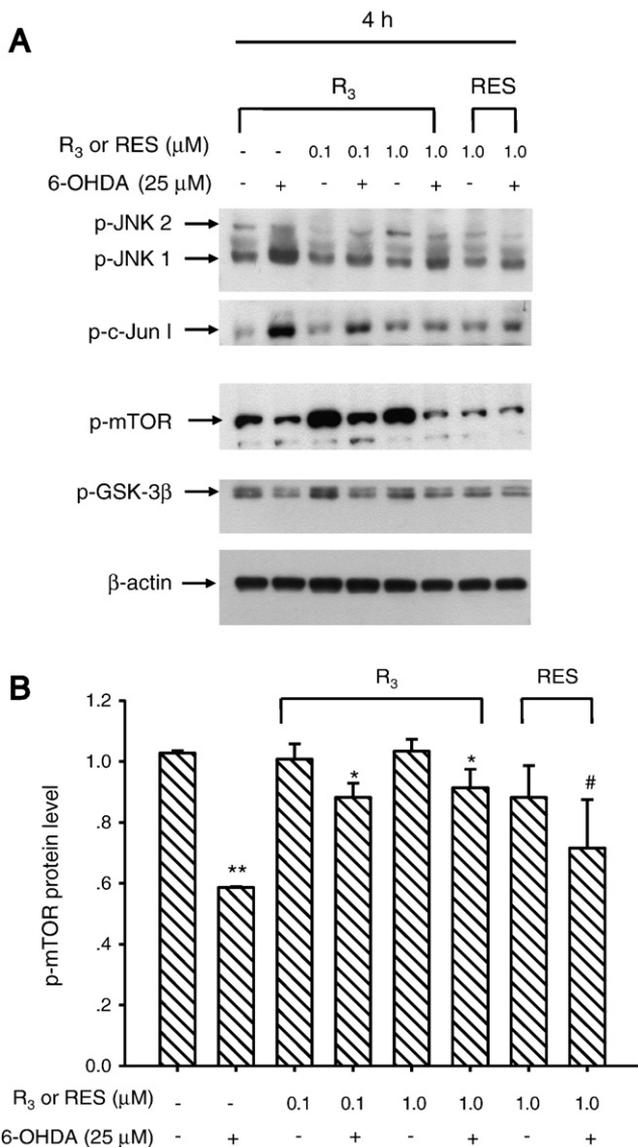


Fig. 5. Western blot analysis of the JNK–c-Jun pathway and mTOR, GSK-3 $\beta$  signaling in neurons treated with resveratrol and R<sub>3</sub> with or without 6-OHDA. Neurons were pretreated with R<sub>3</sub> (0.1 and 1.0  $\mu$ M) and resveratrol (1.0  $\mu$ M) for 30 min, followed by exposure to 25  $\mu$ M 6-OHDA for 4 h. Proteins extracted were subjected to Western blot analysis to detect the level of phospho-JNK, phospho-c-Jun, phospho-mTOR, phospho-GSK-3 $\beta$  and  $\beta$ -actin (loading control). Results are expressed from at least three independent experiments (a). Phospho-mTOR protein levels were analyzed by Image J software. \* $P$ <0.01 versus the group treated with 6-OHDA, \*\* $P$ <0.001 versus the control group, # $P$ =.46 versus the group treated with 6-OHDA (suggesting no significant difference) only by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test (b).

strawberry, green tea, black tea, grape and red wine possessed neuroprotective effects in both in vivo and in vitro models of AD and PD, which could be attributed to their free-radical scavenging and anti-apoptotic activity [36,37]. Ma et al. [38] also showed that caffeic acid phenethyl ester attenuated 6-OHDA-induced neurotoxicity by blocking free-radical generation.

Since both the number and position of hydroxyl groups are the critical structural determinants for the antioxidant activity of polyphenols [39,40], methylation of the hydroxyl groups of resveratrol is expected to lower its free-radical scavenging capacity. As expected, DPPH assay showed that the four methylated resveratrol derivatives had lower radical scavenging capacity than resveratrol and

that their relative activity was in the following order: R<sub>3</sub>>R<sub>4</sub>>R<sub>2</sub>>R<sub>1</sub> (data not shown). As a result, we initially suspected that resveratrol, the most potent free-radical scavenger among the compounds studied, could be the most potent neuroprotectant against 6-OHDA-triggered neurotoxicity. However, R<sub>3</sub>, whose 5-hydroxyl group is methylated, was found to demonstrate even better neuroprotective effects compared to resveratrol. Interestingly, methylation of the hydroxyl groups at other positions did not prove any enhancement of the neuroprotective activity of the resultant derivatives. Stivala et al. [41] have suggested that hydrogen abstraction from 4'-OH bond is favored as a consequence of greater resonance stabilization energy, while the reactions at the 3-OH or 5-OH groups are less exothermic and, therefore, less favorable. It has also been shown that the *para*-OH (4'-OH) in *trans*-resveratrol is more active in scavenging free radicals than the hydroxyl groups at the *meta* positions (3-OH and 5-OH) [40]. Careful comparison of the structures among four resveratrol derivatives revealed that R<sub>3</sub> differentiates from the other three derivatives by possessing a free 4'-hydroxyl group, which might thus play an important role in executing the antioxidant activity and the resulting neuroprotective effects [42].

#### 4.2. Methylation increased the hydrophobicity and the cellular uptake of R<sub>3</sub>

Although both resveratrol and R<sub>3</sub> possess a 4'-OH in common, R<sub>3</sub> exerted a stronger protective effect at 0.1  $\mu$ M that was 10-fold lower than the effective dose for resveratrol. HPLC analysis showed that resveratrol and R<sub>3</sub> were both accumulated in SH-SY5Y cells over time. It appeared that the more hydrophobic R<sub>3</sub> could penetrate the cell membrane more readily, thus leading to an increase (two times) in intracellular dosage compared to resveratrol. However, R<sub>1</sub>, which is the most hydrophobic among the compounds tested (hydrophobicity: R<sub>1</sub>>R<sub>2</sub>>R<sub>4</sub>>R<sub>3</sub>>resveratrol), failed to elicit any neuroprotective effects. These data imply that while methylation is desirable to improve cellular uptake of the purported neuroprotective agents, it is important to note that the 4'-hydroxyl group is retained as it is a critical structural requirement for the neuroprotective activity of stilbenes to effect. Our present findings are in agreement with a previous study [43], which reported that gallic acid, being much more hydrophilic than its esters (methyl gallate, *n*-propyl gallate, *n*-octyl gallate and *n*-dodecyl gallate), was much weaker in protecting neurons from 6-OHDA-induced oxidative stress than its esters.

#### 4.3. R<sub>3</sub> was not demethylated by *O*-demethylase in neuron cell culture

Trimethylated resveratrol has been shown to attenuate CCl<sub>4</sub>-induced liver damage more effectively than resveratrol. It was proposed to act as a prodrug of resveratrol and thereby increase the half-life of resveratrol after demethylation to the parent compound in vivo [13]. Deng et al. [44] speculated that cellular *O*-demethylase may be involved in the transformation of fully methylated curcumin into an active metabolic product that protects target tissues from free-radical-induced oxidative haemolysis in vitro. Assuming that *O*-demethylase is active in our neuron cell culture model, metabolites of all the methylated resveratrol derivatives should display protective effects at least to some extent, if not potently. Since only R<sub>3</sub>, but not the other methylated forms, was active, it appeared that *O*-demethylase was not involved in the neuroprotective effect of R<sub>3</sub> in the present model. Moreover, no peak corresponding to resveratrol was found in the HPLC chromatograms of the cellular extracts from cells treated with R<sub>3</sub> alone, suggesting that R<sub>3</sub> could not be demethylated into resveratrol inside the neurons. However, we could not preclude the possibility that methylated compounds can be demethylated in vivo, which has a much more sophisticated metabolic system than do the neuron cells.

#### 4.4. Direct modulation of cellular signaling cascades is also involved in R3's neuroprotective activity

As O-demethylase was not likely involved in the action mechanism of R3, we proposed that R3 could directly modulate the cell signaling to mediate its neuroprotective effects. Methylated resveratrol derivatives can readily enter cell plasma and reach the intracellular targets exerting either toxic or protective effects. A series of 75 synthesized resveratrol analogues without hydroxyl groups have been found to be more active than resveratrol in inhibiting the human tumor necrosis factor alpha-induced activation of NF- $\kappa$ B. The analogues were also shown to inhibit LPS-induced COX-2 expression in BV-2 microglial cells [45]. Similar findings have been reported in other investigations [46,47]. These data suggested that methylated stilbenes could directly modulate cellular signaling cascades.

A previous study [48] showed that the inhibition of mTOR kinase activity appears to be involved in the mechanism of 6-OHDA-induced neuron death in PC12 cells. We found that 6-OHDA treatment resulted in dephosphorylation and, subsequently, inactivation of mTOR kinase in SH-SY5Y cells. Pretreatment with R3 or resveratrol could prevent mTOR kinase inactivation induced by 6-OHDA. It has been demonstrated that 6-OHDA can inhibit activity of Akt and its downstream GSK3 by dephosphorylation in SH-SY5Y cells, PC12 cells and cultured rat cerebellar granule neurons. 6-OHDA-induced cleavage of caspase-3 and poly(ADP-ribose) polymerase, DNA fragmentation and cell death can be prevented by blocking GSK3 activity using selective inhibitors (lithium, TDZD-8 and L803-mts), while antioxidants had little effect on 6-OHDA-induced GSK3 activation [49]. This is in agreement with our observation that resveratrol and R3 could only slightly increase the activity of GSK-3 $\beta$  dephosphorylated by 6-OHDA.

Taken together, we discovered that methylated resveratrol derivative R3 is an effective neuroprotective agent against free-radical-mediated oxidative stress triggered by 6-OHDA in SH-SY5Y cells. H-atom abstraction from the 4'-OH group of R3 and its moderate hydrophobicity are proposed to be responsible for the neuroprotective effects. In addition, mTOR kinase is postulated to be a potential target for the pharmacological action of methylated stilbenes. These experimental findings are of great importance for design of stilbene-type neuroprotective agents.

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