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A novel synthetic compound PHID (8-Phenyl-6a, 7, 8, 9, 9a, 10-hexahydro-6H-isoindolo [5, 6-g] quinoxaline-7, 9-dione) protects SH-SY5Y cells against MPP^+-induced cytotoxicity through inhibition of reactive oxygen species generation and JNK signaling

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ABSTRACT

1-Methyl-4-phenylpyridinium ion (MPP^+), a neurotoxin selective to dopaminergic neurons and an inhibitor of mitochondrial complex I, has been widely used as an etiologic model of Parkinson's disease. In this study, we investigated the protective effects of a novel synthetic compound, 8-Phenyl-6a,7,8,9,9a,10-hexahydro-6H-isoindolo [5,6-g]quinoxaline-7,9-dione (PHID), on MPP^+-induced cytotoxicity in SH-SY5Y cells. MPP^+ induced apoptosis characterized by generation of reactive oxygen species, caspase-3 activation, poly ADP ribose polymerase proteolysis and increase in Bax/Bcl-2 ratio were blocked by PHID in a dose-dependent fashion. Furthermore, MPP^+-mediated activation of stress-activated protein kinase/c-Jun N-terminal kinase (JNK) was also inhibited by PHID in a dose-dependent manner. The results indicate that PHID protects against MPP^+-induced apoptosis by blocking reactive oxygen species stimulation and JNK signaling pathways in SH-SY5Y cells, implicating the novel compound in the prevention of progressive neurodegenerative diseases such as Parkinson's disease.

1. Introduction

Neuronal cell death is an important feature of both acute and chronic neurodegenerative diseases (Yuan and Yankner, 2000; Mattson, 2000). The hallmark of Parkinson's disease is a loss of dopaminergic neurons in the substantia nigra. Although the etiology of Parkinson's disease remains unclear, accumulating evidence strongly suggests the involvement of oxidative stress and mitochondrial dysfunction (Mattson, 2000; Pieczenik and Neustadt, 2007; Szeto, 2006). The major mitochondrial defect in Parkinson's disease appears to be associated with inhibition of respiratory chain complex I. 1-Methyl-4-phenylpyridinium ion (MPP^+), the active metabolite of 1-methyl-4-phenyl-2, 3, 6-tetrahydropyridine (MPTP), is a neurotoxin that selectively and potently inhibits complex I of the mitochondrial electron transport chain (Singer and Ramsay, 1990) and induces a condition closely resembling Parkinson's disease in cellular and animal models (Eberhardt and Schulz, 2003; Przedborski et al., 2004; Przedborski and Jackson-Lewis, 1998). It is now believed that the dramatic loss of dopaminergic neurons involving oxidative stress and/or mitochondrial impairment results from the activation of an apoptotic cascade (Mattson, 1990; Fiskum et al., 2003). Therefore, the suppression of dopaminergic neuronal cell death by regulation of intracellular reactive oxygen species and modification of the apoptotic cascade could be an effective therapeutic approach in the alleviation of the progression of neurodegeneration.

For several years, our group has synthesized compounds from isoindoloquinoxaline, an antitumor agent (Diana et al., 2008; Jung et al., 2006), and screened for anti-apoptotic activities by neuron cell-based assays. One of these compounds, 8-Phenyl-6a,7,8,9,9a,10-hexahydro-6*H*-isoindolo[5,6-*g*]quinoxaline-7,9-dione (PHID), was presently investigated. Here, we report the inhibitory effects of PHID upon cell viability loss, increase in reactive oxygen species generation, caspase-3 activation, poly ADP ribose polymerase (PARP) proteolysis and Bax/Bcl-2 ratio induced by MPP^+ in SH-SY5Y cells. Further, we have examined MPP^+-mediated activation of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) in SH-SY5Y cells.

2. Materials and methods

2.1. Reagents

MPP^+, 3-(3,4-dimehylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), poly-D-lysine and caspase-3 assay kit were obtained from Sigma-Aldrich (St Louis, MO, USA). Six-well and 96-well tissue culture plates and 100 mm-diameter culture dishes were purchased from Nunc

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(North Aurora Road, IL, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL Technologies (Carlsbad, CA, USA). Lactate dehydrogenase (LDH) assay kits were purchased from Dojindo (Rockville, MD, USA). 2, 7-Dichlorofluorescein diacetate (DCFH-DA) and propidium iodide (PI) were supplied by BD Clontech (Mountain View, CA, USA). Antibodies against apoptosis signal-regulating kinase 1 (ASK1), phospho-ASK1, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), phospho-SAPK/JNK, c-Jun, phospho-c-Jun, p38 MAPK kinase, phospho-p38 and poly ADP ribose polymerase (PARP) were obtained from Cell Signaling Technology (Danvers, MA. USA). Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used in this study were of analytical grade and were obtained, unless otherwise noted, from Sigma-Aldrich (St Louis, MO, USA).

2.2. Cell culture and treatments

Human neuroblastoma SH-SY5Y and mouse neuroblastoma Neuro-2a cells were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% (v/v) inactivated fetal bovine serum, and 100 U/ml penicillin/streptomycin. Cells were maintained at 37 °C in 5% CO_2 and 95% humidified air incubator for the indicated time. Mouse dopaminergic MN9D cells were kindly provided by Dr. Young J Oh (Yonsei University, Seoul, f Korea). MN9D cells were plated on 96-well plates or 6-well plates coated with 25 μg/ml poly-D-lysine. Cultures were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, San Diego, CA) in an incubator with an atmosphere of 10% CO_2 at 37 °C for 3 days. Cells were subsequently switched to serum-free N2 medium (Bottenstein and Sato, 1979). All experiments were carried out 24–48 h after cells were seeded. SH-SY5Y, Neuro-2a and MN9D cells were pretreated for 4 h with 0.1, 1, or 10 μM PHID before incubation in medium containing 1 mM, 0.5 mM and 25 μM MPP^+, respectively. For morphological studies, SH-SY5Y cells maintained in DMEM were kept in a humidified atmosphere at 37 °C and 5% CO_2. Cells were allowed to attach for 1 h before the addition of 10 μM (final concentration) all trans-retinoic acid (RA, made in ethanol as a 10 mM stock solution; Sigma-Aldrich, St Louis, MO, USA) that was used to induce neuronal differentiation.

2.3. Assessment of cell viability

Cell viability was measured by using a previously described quantitative colorimetric MTT assay of mitochondrial activity in living cells (Datki et al., 2003). MTT dissolved in phosphate-buffered saline (PBS) was added at the end of incubation to a final concentration of 0.5 mg/ml. After incubation for 4 h at 37 °C and 5% CO_2, each supernatant was removed and the formazan crystals that had formed in the viable cells were measured at 550 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The release of the intracellular enzyme LDH into the medium was also used as a quantitative measurement of cell viability. Cells were incubated in 96-well plates with the indicated concentration of PHID and 1 mM of MPP^+ for 48 h and each assay included control cells that were not treated with PHID. Each cell suspension was centrifuged (4000×*g*, 5 min, 4 °C) and then the supernatant was collected. LDH activity in the supernatant was performed by using a cytotoxicity assay kit according to the manufacturer's instructions (Takara Bio, Shiga, Japan). Absorbance was read at 440 nm and cytotoxicity (%) was calculated as:

$$\{(\text{supernatant value} - \text{blank value}) \div [(\text{supernatant value} - \text{blank value}) + (\text{upper control value} - \text{blank value})]\} \times 100.$$

Each experiment was performed in triplicate.

2.4. Isolation of total RNA and expression analysis

SH-SY5Y cells (1×10^6 cells/well) were cultured in 6-well plates, and the total RNA was isolated by extraction with TRIzol (Invitrogen, Carlsbad, CA, USA). For the reverse transcription-polymerase chain reaction (RT-PCR), 2.5 μg of total RNA were reverse transcribed using a First Strand cDNA synthesis kit (Invitrogen) and the resulting complimentary DNA was used as the template for PCR. The following primers were used for PCR: Bcl-2 sense, 5′- ACTTTGCAGAGATGTCCAGT-3′; Bcl-2 anti-sense, 5′-CGGTTCAGGTACTCAGTCAT-3′; Bax sense, 5′-CTGGACAGTAACATGGAGC-3′; Bax anti-sense, 5′-TCTTCTTCCAGATGGTGAGT-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH; which was used as an internal control to evaluate the relative expressions of Bcl-2 and Bax) sense, 5′-GCAGTGGCAAAGTGGAGATTG-3′; and GAPDH anti-sense 5′-TG CAGGATGCATTGCTGACA-3′. RT-PCR products were electrophoresed on a 1% (w/v) agarose gel, stained with ethidium bromide, and bands were visualized by ultraviolet light.

2.5. Immunoblot analysis

To obtain the total cell lysate, 0.1 or 0.05 ml of RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), with freshly added protease inhibitor cocktail; (Calbiochem, San Diego, CA, USA) was added to SH-SY5Y and Neuro-2a cells cultured in 6-well plates. The cells were scraped from the well surface, incubated for 10 min on ice, and centrifuged at 14,000× rpm for 10 min at 4 °C. The protein concentration was determined by the DC protein assay (Bio-Rad, Hercules, CA, USA), and 15 μg of whole cell lysate was loaded for 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed and the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) using an electroblotting apparatus (Bio-Rad, Hercules, CA, USA). Each membrane was blocked for 1 h in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% dry milk prior to incubation overnight with a 1:1000 dilution of anti-PARP or anti-phosphor-c-Jun primary antibody followed by incubation for 1 h with a 1:10000 dilution of HRP-conjugated secondary antibody. The optical densities of the antibody-specific bands were analyzed by a LAS-3000 luminescent image analyzer (Fuji, Tokyo, Japan).

2.6. Flow cytometry detection of apoptotic cells

SH-SY5Y cells (1×10^6 cells/well) were collected by centrifugation following MPP^+ exposure for 60 h and washed two times with ice-cold PBS. The pellets were resuspended in ice-cold 70% ethanol and fixed at 4 °C for 24–48 h. Cells were washed and resuspended in 1 ml of DNA staining reagent containing 50 μg/ml RNase, 0.1% Triton X-100, 0.1 mM EDTA (pH 7.4), and 50 μg/ml PI. The staining was stable at 4 °C for 30 min (Telford et al., 1991). Red fluorescence (DNA) was detected through a 563–607 nm band pass filter by using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Ten thousand cells in each sample were analyzed and the percentage of apoptotic cells accumulating in the sub-G1 peak was calculated by Cell Quest software (Becton Dickinson San Jose, CA, USA).

2.7. Caspase-3 activity assay

Caspase-3 activity was detected by use of the colorimetric caspase-3 assay kit according to the manufacturer's protocol. In brief, the reaction mixture (total volume 100 μl) contained 5 μl of cell lysate and 10 μl of caspase-3 substrate (Ac-DEVD-pNA; final concentration, 200 μM) in assay buffer, and the assay was carried out in a 96-well plate. To account for non-specific hydrolysis of substrate, a control reaction mixture contained 5 μl of cell lysate and 10 μl of the specific caspase-3 inhibitor (Ac-DEVD-CHO; final concentration, 20 μM) in

assay buffer. Both mixtures were incubated for 90 min at 37 °C and the absorbance was read at 405 nm.

2.8. Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species production based on the formation of hydrogen peroxide generated by an oxidative metabolic burst was measured using a non-fluorescent compound (DCFH-DA) as previously described (Bass et al., 1983). Viable cells can deacetylate DCFH-DA to 2′,7′-dichlorofluorescin (DCFH), which is not fluorescent. This compound reacts quantitatively with oxygen species within the cell to produce a fluorescent dye 2′, 7′-dichlorofluorescein (DCF), which remains trapped within the cell and can be measured to provide an index of reactive oxygen species level. Cells (1×10^6 cells/well) were collected and loaded with 20 μM DCFH-DA dissolved in dimethyl sulfoxide for 30 min at 37 °C. After washing out the excess probe, the cells were measured by a FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.9. Statistical analysis

The results were expressed as means ± S.E.M of at least three independent experiments run in duplicate or triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by *post hoc* multiple comparisons using the Bonferroni method with the Sigmaplot 11.1 software (Systat Software, San Jose, CA, USA). $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Synthesis of PHID

A solution of quinoxalinosultine (100 mg, 0.45 mM) and *N*-phenylmaleimide (3 equiv) in toluene (4 ml) was sealed in a 40 ml Pyrex tube and heated at 200 °C for 24 h. The solution was then cooled to room temperature. The solvent was evaporated under vacuum, and the residue was subjected to flash silica gel column chromatography using dichloromethane/ethyl acetate (from 9:1 to 1:4) as the eluent to yield PHID (purity > 98%; Fig. 1). The product was obtained as a white solid in 93% yield; mp 250–251 °C; IR (KBr) 1706, 1502, 1376 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 8.82 (s, 2H, hetAr-H), 7.96 (s, 2H, Ar-H), 6.82–7.06 (m, 5H, Ar-H), 3.51–3.58 (m, 4H, CH_2, CH), and 3.25–3.28 (m, 2H, CH_2).

3.2. PHID ameliorated MPP^+-induced reduction in cell viability

Treatment of SH-SY5Y cells with 1 mM MPP^+ alone for up to 48 h resulted in neuronal cell loss in almost half of the cells as evaluated by MTT assay. When the cells were pretreated with non-toxic doses of PHID (0.1, 1, and 10 μM) for 4 h prior to the addition of 1 mM MPP^+, cell viability was significantly improved in a dose-dependent fashion. PHID alone did not affect the overall cell viability. When cell viability under serum-free conditions was defined as 100% survival, viability of cells treated with 1 mM MPP^+ decreased to 50.2 ± 0.4%. The viability of cells incubated with 0.1, 1 and 10 μM of PHID was 53.3 ± 3.4%, 61.4 ± 1.3%, and 71 ± 0.7% of the control, respectively (Fig. 2A).

The level of cell death was also estimated by LDH assay which detected the release of LDH in culture medium. Exposure of SH-SY5Y cells to MPP^+ resulted in 1.6 ± 0.1-fold increase in the release of LDH when compared to the control cultures ($P<0.001$ vs. control group). Pretreatment of PHID at 0.1, 1 and 10 μM for 4 h significantly reduced MPP^+-induced LDH release dose responsibly. The viability of cells treated with MPP^+, incubated with indicated concentrations of PHID was 1.52 ± 0.03 at 0.1 μM PHID ($P<0.05$), 1.45 ± 0.03 at 1 μM PHID ($P<0.005$) and 1.38 ± 0.01 at 10 μM PHID ($P<0.001$) respectively, compared with 1 mM MPP^+ only group (Fig. 2B).

Further, to prove that PHID has similar effect in dopaminergic cells and independent of the cell line used we performed MPP^+-induced reduction in cell viability loss using MTT assay on mouse dopaminergic MN9D and mouse neuroblastoma Neuro-2a cells. Our data revealed that exposure of MN9D cells to 25 μM MPP^+ induced a reduction in cell viability. When pretreated with PHID at 0.1, 1, and 10 μM for 4 h prior to the addition of 25 μM MPP^+, cell viability was significantly improved which occurred in a concentration dependent manner (Fig. 2C). Similar effects were shown with Neuro-2a cells treated with 0.5 mM MPP^+ with or without various concentrations of PHID (Fig. 2D). PHID treatment alone did not affect the overall cell viability tested in both these cell lines. These results strongly indicate that MPP^+-induced loss of cell viability can be fully attenuated by PHID in a dose-dependent manner in all the cell types used.

To investigate the morphology, we observed the effect of PHID in MPP^+-induced cellular morphology changes in differentiated SH-SY5Y cells. As shown in Fig. 2E (lower panel), concentration dependent protection to neurite outgrowth was observed in differentiated SH-SY5Y cells on pretreatment with PHID upon MPP^+ insult. However, PHID alone does not show any morphological changes.

To verify the inhibitory effect of PHID on the increase in apoptotic cells, SH-SY5Y cells were labeled with PI and histogram analysis-related nuclear DNA contents were ascertained by flow cytometry. DNA content

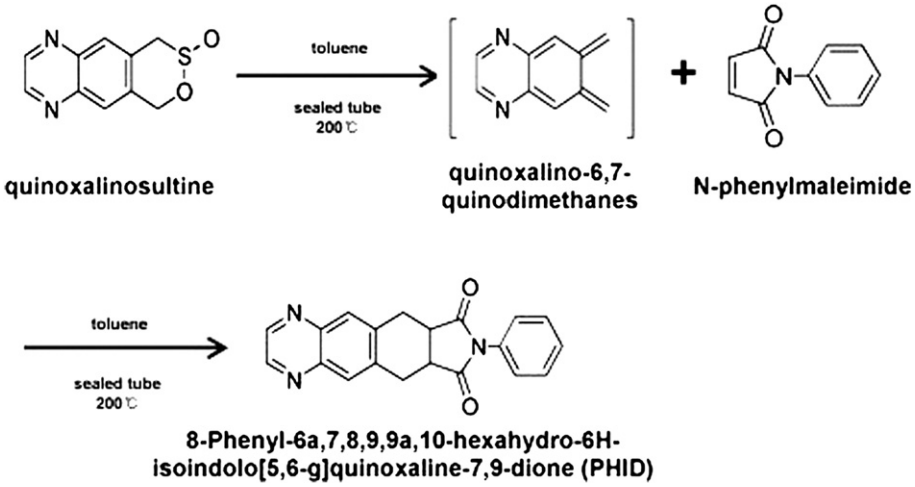


Fig. 1. The structure and synthesis of PHID.

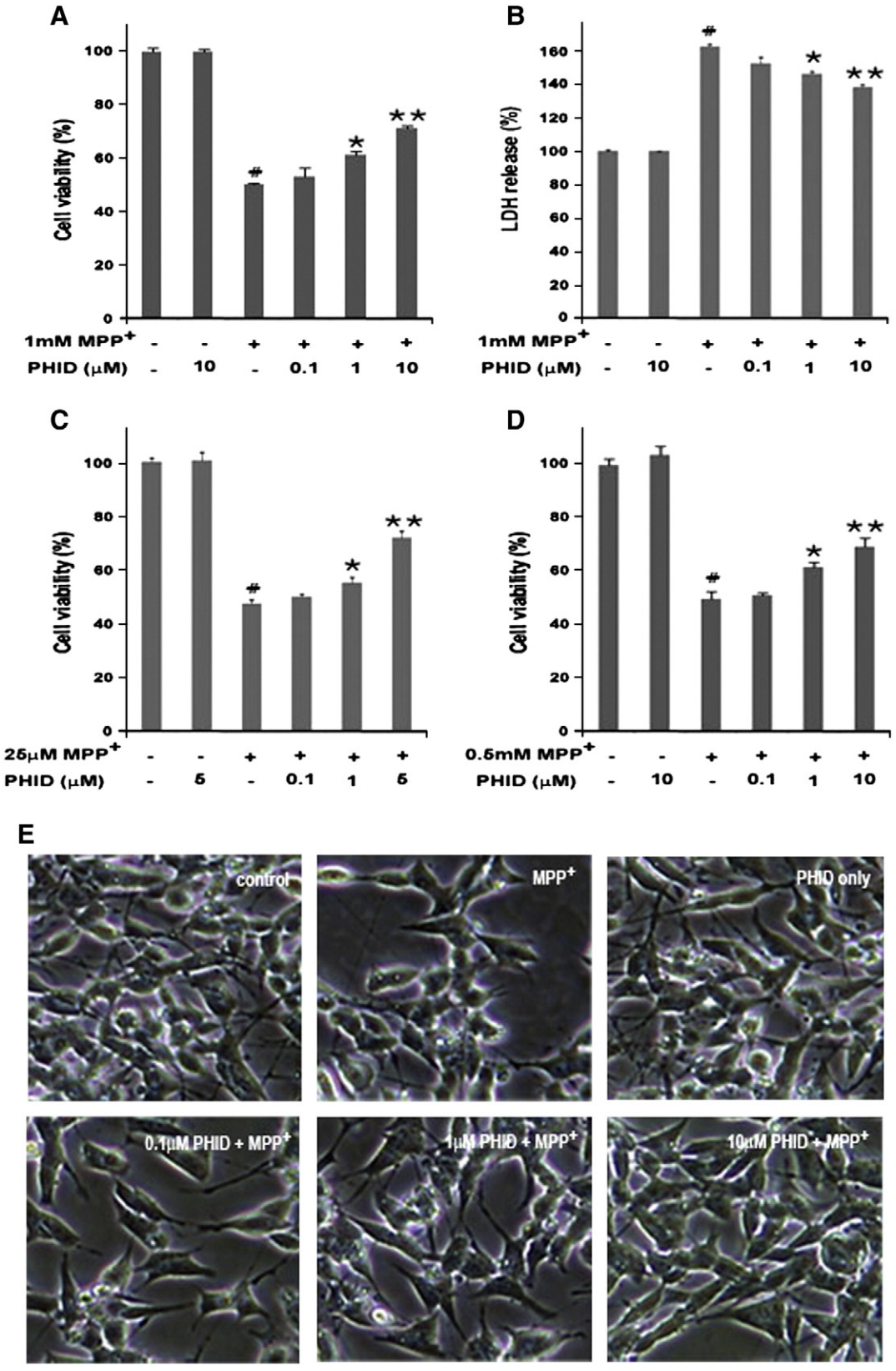


Fig. 2. Effect of PHID on MPP^+-induced cell death in SH-SY5Y, MN9D and Neuro-2a cells. Cells were exposed to MPP^+ for 48 h and cell viability was assessed by MTT assay or LDH assay. Cells were treated with indicated doses of MPP^+ in the absence or presence of PHID (0.1, 1, and 10 μM). (A) Cell viability assessed by MTT assay in SH-SY5Y cells. (B) Cell viability assessed by LDH assay in SH-SY5Y cells. (C) Cell viability assessed by MTT assay in MN9D cells. (D) Cell viability assessed by MTT assay in Neuro-2a cells. (E) Microscopic examination of morphological changes treated with MPP^+ with or without PHID in SH-SY5Y cells differentiated with retinoic acid. Data are expressed as the percentage of values in untreated control cultures. Each value represents mean ± S.E.M. ($n = 3$). $^{\#}P<0.001$, compared with control group; and $^{*}P<0.05$ and $^{**}P<0.001$ compared with MPP^+-treated group by one-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparison.

histograms were obtained after cells were exposed to 1 mM MPP^+ and with various concentrations of PHID. Cells with DNA content below G1 phase (defined as hypodiploid sub-G1 peak) were regarded as apoptotic cells (Liu et al., 2000, 2001; Zhang and Zhao, 2003; Ormerod, 2002). When cells were incubated in medium alone, a typical single peak of nuclei with diploid DNA content was observed (Fig. 3A) and there was only about 2%–3% cell death. In the presence of 1 mM MPP^+, apoptotic cells (apparent as a characteristic hypodiploid DNA content peak which shows sub-G0-G1 apoptotic populations) were distinguishable in the population of 34.7 ± 0.96% (Fig. 3B). PHID alone did not show any effect on the overall population of cells (Fig. 3C). Following treatment with PHID (0.1, 1 and 10 μM), the apoptotic population was reduced to 34.5 ± 0.48% (Fig. 3D), 26.8 ± 0.56% (Fig. 3E), and 18.25 ± 0.38% (Fig. 3F), respectively.

3.3. PHID suppresses MPP^+-induced caspase-3 activation and PARP proteolysis

Caspases are the molecular machinery that drives apoptosis (Grutter, 2000). Presently caspase-3, a crucial biomarker of the neuronal apoptosis that also act as an apoptotic executor (Hartmann et al., 2000) was investigated in both SH-SY5Y and MN9D cells. Treatment with 1 mM MPP^+ markedly increased caspase-3 activity and addition of PHID attenuated MPP^+-induced caspase-3 expression to 349.33 ± 15.14%, 266 ± 18.68%, and 191.33 ± 20.25%, respectively, in SH-SY5Y cells in a dose-dependent manner (Fig. 4A).

Caspase-3 also plays a major role in PARP cleavage early during apoptosis in many different cell lines (Lazebnik et al., 1994; Le et al., 2002). We further examined the cleavage of PARP. A previous study reported that MPP^+ induces obvious increase in PARP proteolysis at 48 h when compared to the control cultures (Kitamura et al., 1998). Cleavage of PARP was detected using polyclonal antibody against cleaved PARP fragments (85 kDa). Following the treatment with 1 mM MPP^+, PARP proteolysis markedly increased to 2.34 ± 0.43 fold, while PHID at concentrations of 0.1, 1, and 10 μM, attenuated MPP^+-induced PARP proteolysis to 2.36 ± 0.47, 1.89 ± 0.48, and 1.67 ± 0.39 fold, respectively in SH-SY5Y cells dose-dependently. The western blot data and the band density ratio to β-actin were represented in Fig. 4B. Similar effects were observed in attenuation of MPP^+-induced caspase-3 activation and PARP proteolysis in MN9D cells and the data was represented in Fig. 4C and D, respectively.

3.4. PHID suppressed MPP^+-induced increase of reactive oxygen species in SH-SY5Y cells

Mounting evidence suggests that oxidative damage and overproduction of reactive oxygen species can cause severe impairment of cellular functions, and may contribute to the apoptotic process found in Parkinson's disease (Zhou et al., 2008; Choi and Suk, 2007; Kehrer and Smith, 1994). MPP^+ stimulates the production of the reactive oxygen species and induces cell death in SH-SY5Y cells (Adams et al., 1993; Cassarino et al., 1999; Domingues et al., 2008). Therefore, we investigated whether PHID was capable of modulating intracellular reactive oxygen species generation by MPP^+. The fluorescent probe DCFH-DA was used to assess the generation of reactive oxygen species. The relative fluorescent intensity graph and the typical flow cytometric histogram of DCF were shown (Fig. 5). Cells exposed to MPP^+ showed the obvious increase in DCF fluorescent intensity at 24 h when compared to the control cultures (Fig. 5B). PHID treatment at 10 μM alone did not show any significant changes in the reactive oxygen species production (Fig. 5C). Whereas the reactive oxygen species production in cells exposed to 1 mM MPP^+ with 0.1, 1, and 10 μM of PHID were suppressed to 89.45 ± 5.06%, 79.93 ± 2.06%, and 64.70 ± 3.25% respectively in a dose-dependent fashion as compared to that in MPP^+-treated group (Fig. 5D–F). Flow cytometric analysis also indicated that PHID effectively blocked ROS production mediated by MPP^+, as evident by a shift to the left of the fluorescence intensity thus reflecting a reduction in reactive oxygen species generation, and suggesting that PHID possesses reactive oxygen species scavenging properties.

3.5. PHID suppresses MPP^+-induced increase in JNK and p38 activation, but not extracellular signal-regulated kinase

Stress-activated protein kinases are considered as one of the important upstream signals which lead to neuronal degeneration. Neuronal apoptosis in neurodegenerative diseases was observed in response to various stimuli via the c-Jun N-terminal kinases (JNK) and p38 kinases (Mielke and Herdegen, 2000). Phosphorylated forms of JNK and p38 are recognized to be highly expressed in postmortem brains of PD (Ferrer et al., 2001). Activation of SAPK/JNK and its transcription factor targets are prominent mediators of dopaminergic toxicity in a MPP^+-induced model of Parkinson's disease (Luo et al., 1998; Oo et al., 1999; Choi et al., 1999; Saporito et al., 1999, 2000; Gearan et al., 2001; Chun et al., 2001; Xia et al.,

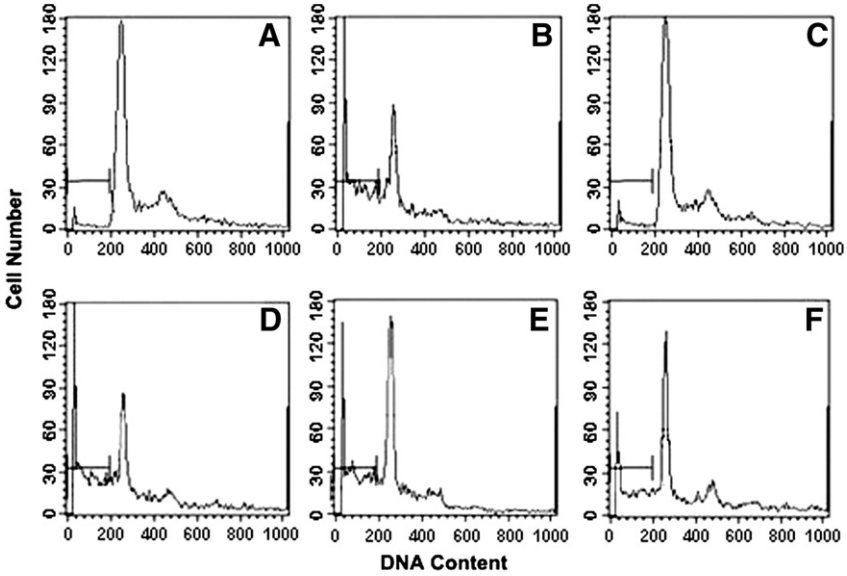


Fig. 3. Effect of PHID against MPP^+-induced neurotoxicity in cultured SH-SY5Y cells as detected by flow cytometric DNA analysis. (A) Control cells; (B) cells exposed to 1 mM MPP^+ alone; (C) cells exposed to 10 μM PHID alone; and cells pretreated with PHID at 0.1 (D), 1 (E), and 10 μM (F), in the presence of 1 mM MPP^+. Bar (├┤) represents a sub-G0/G1 peak or hypodiploid DNA fraction. The results are representative of one of three independent experiments.

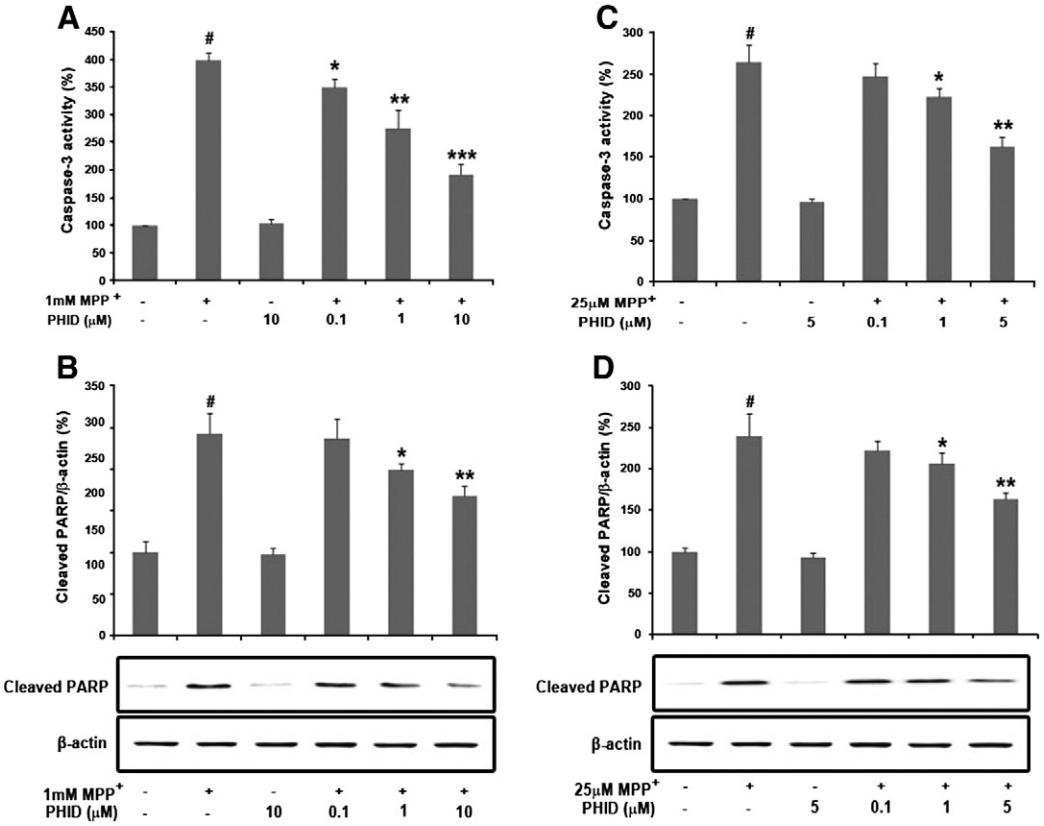


Fig. 4. PHID inhibits MPP^+-induced activation of caspase-3 and PARP proteolysis SH-SY5Y.and MN9D cells. Cells were treated with MPP^+ in the absence or presence of PHID. Caspase-3 activity in SH-SY5Y cells was measured by a colorimetric assay kit (A) and PARP proteolysis was measured by immunoblot analysis in SH-SY5Y cells. The band intensity was quantified and is presented as relative to the level of β-actin (B). Caspase-3 activity in MN9D cells was measured by a colorimetric assay kit (C) and PARP proteolysis was measured by immunoblot analysis. The band intensity was quantified and is presented as relative to the level of β-actin (D). Cell lysate samples (20 μg protein/lane) were subjected to immunoblot with specific antibodies against the cleaved site of human PARP. Data represent mean ± S.E.M. ($n = 3$). $^{\#}P<0.001$ compared with control group; and $^{*}P<0.05$, $^{**}P<0.005$ and $^{***}P<0.001$ compared with MPP^+-treated group by one-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparison.

2001). Also, overexpression of ASK1 activates JNK and induces apoptosis in nerve growth factor-differentiated PC12 cells and primary rat sympathetic neurons. While expression of dominant-negative form of ASK1 inhibits such induced apoptosis in these cells (Kanamoto et al., 2000). To investigate a possible effect of PHID on MPP^+-stimulated JNK signaling cascade, we measured the changes in phosphorylation levels of ASK1, SAPK/JNK, c-Jun and p38 in SH-SY5Y cells (Fig. 6). Western blot analysis of the total protein levels and the expression of phospho-ASK1 (Thr845), phospho-SAPK/JNK (Thr183/Tyr185), phospho-c-Jun (Ser73) and p38 were performed to provide an estimate of the relative level of expression of the proteins. After 24 h of incubation, 1 mM MPP^+ significantly increased phosphorylation of ASK1 to 1.95 ± 0.25 fold (Fig. 6A), p54-SAPK/JNK to 3.23 ± 0.25 fold (Fig. 6B), c-Jun to 3.15 ± 0.33-fold (Fig. 6C) and p38 to 4.24 ± 0.24 (Fig. 6D) as compared to controls. Following PHID treatment (0.1, 1, and 10 μM), p-ASK1 was reduced to 1.91 ± 0.09, 1.51 ± 0.12 and 1.20 ± 0.05 fold, phosphor-p54-SAPK/JNK to 3.08 ± 0.15, 2.89 ± 0.09, 1.92 ± 0.14 fold, phospho-c-Jun to 2.81 ± 0.22, 2.53 ± 0.15, 1.88 ± 0.38 fold and phosphor-p38 to 3.97 ± 0.33, 3.30 ± 0.15, 2.61 ± 0.26 fold (Fig. 6A–D) respectively, in a concentration dependent fashion. PHID pretreatment alone did not affect the total expression of these proteins but only inhibited the activated forms indicating that PHID suppresses the MPP^+-induced increase in JNK activation.

Experiments in cell culture and animal model systems showed that ERK1/2 play a key role in a variety of cellular signaling including dopaminergic cell death (Hunot et al., 2004; Peng et al., 2004). Activation of ERK1/2 by the neurotoxin MPP^+ has been well documented (Gómez-Santos et al., 2002; Zhang et al., 2007). Given that ERK1/2 confers survival/neuroprotective effects (Kyriakis and Avruch, 2001), we investigated activation of ERK1/2 in MPP^+-induced cell death by Western blot analysis using phospho-ERK1/2 antibody. PHID treatment alone and in the presence of 1 mM MPP^+ did not significantly affect phosphorylation of ERK 1/2, indicating that the protective effect of PHID was not regulated via the mitogen-activated protein kinase (MAPK)/ERK signaling pathway (Fig. 6E). Our result correlated with earlier study that treatment with 1 mM MPP^+ for 24 h did not induce any activation in the expression of phosphorylated ERK in SH-SY5Y cells (Gómez-Santos et al., 2002) and was only activated when high doses of MPP^+ (>2.5 mM) was administered (Zhu et al., 2007; Halvorsen et al., 2002).

3.6. PHID affected the expression of Bcl-2 and Bax in MPP^+-treated cells

One of the main mechanisms involved in the induction of the mitochondrial apoptotic pathway is a decrease in the levels of Bcl-2 or, alternatively, an increase in the levels of Bax (Cory and Adams, 2002). In this study, we investigated whether PHID has any effect on the expression of Bcl-2 and Bax in MPP^+-treated cells using expression analysis. As shown in Fig. 7A, Bax expression was increased significantly in the MPP^+-treated group compared with control cells, a finding consistent with previous studies (Gao et al., 2008; Cheng et al., 2009). However, PHID treatment suppressed Bax mRNA expression in a dose-dependent manner. In contrast, the level of Bcl-2 in the MPP^+-treated group was significantly decreased compared with that of control cells, while expression of Bcl-2 was recovered following PHID treatments. The Bax/Bcl-2 ratio in cells exposed to 1 mM MPP^+ was increased 3.2 ± 0.4 fold than the control group, while in cells pretreated with 0.1, 1, and 10 μM PHID, the ratio decreased in a dose-dependent manner, suggesting that PHID treatment shifted the balance between pro- and anti-apoptotic members towards cell survival (Fig. 7B). PHID treatment alone did not significantly alter the Bax/Bcl-2 ratio (Fig. 7B).

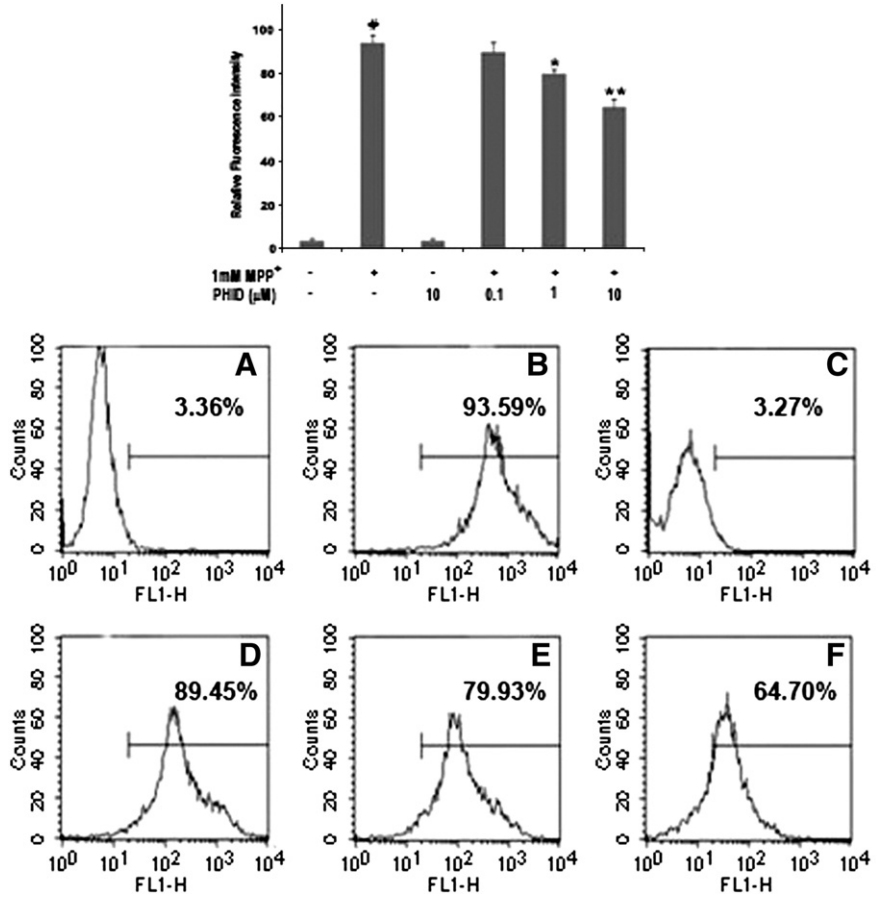


Fig. 5. Effects of PHID on reactive oxygen species generation in SH-SY5Y cells exposed to MPP^+. Cells were exposed to 1 mM MPP^+ in the absence or presence (0.1, 1, or 10 μM) of PHID. Reactive oxygen species generation was detected by fluorometric analysis of the fluorescent intensity of DCF following conversion of DCFH-DA. Upper panel: The relative fluorescent intensity graph. Lower panels: (A) control cells; (B) cells exposed to 1 mM MPP^+ only; (C) cells exposed to 10 μM PHID only; and cells pretreated with 0.1 (D), 1 (E), and 10 μM (F) PHID in the presence of 1 mM MPP^+. Data represent mean ± S.E.M. ($n = 3$). $^{\#}P<0.001$ compared with control group; and $^{*}P<0.05$ and $^{**}P<0.005$ compared with MPP^+-treated group. FL1-H: relative DCF fluorescence intensity, and counts: cell number.

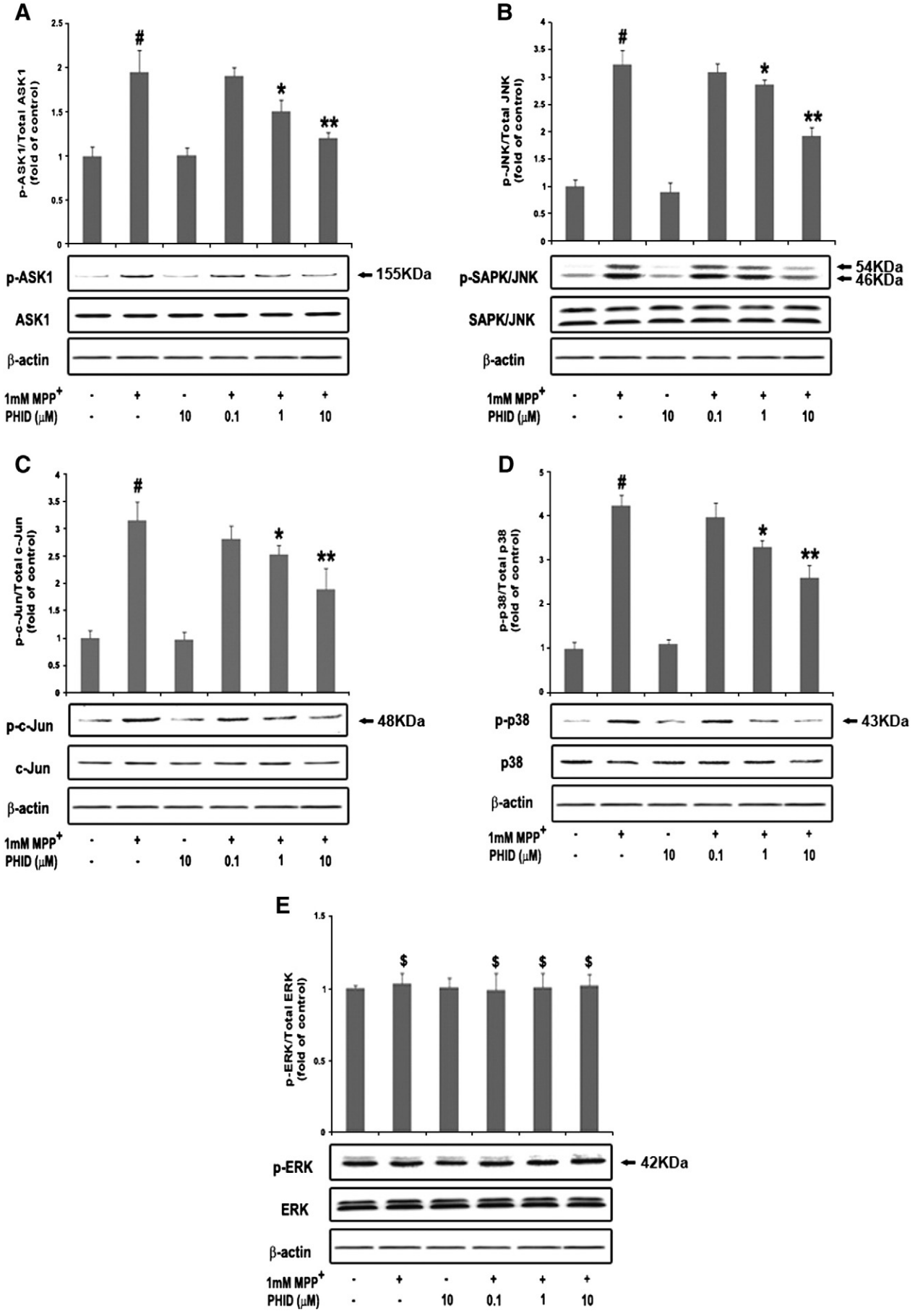
4. Discussion

SH-SY5Y cells treated with MPP^+ have been widely used as an *in vitro* cellular model for studying neurodegenerative events that occur in Parkinson's disease (Sheehan et al., 1997). It is interesting to note that several compounds like flavopiridol, 17-(Allylamino)-17-demethoxy-geldanamycin, and protocatechuic acid having profound antitumor activities also possess marked neuroprotective function (Pallas et al., 2005; Waza et al., 2006; Tseng et al., 1996). In search for developing novel therapeutic agents for treating neurodegenerative diseases including Parkinson's disease, we systematically screened isoindoloquinoxaline. Isoindoloquinoxaline and its derivatives have been earlier reported for their potent antitumor activities (Diana et al., 2008), but their use in neuroprotection was untouched. Our search yielded a synthetic compound PHID, derived from isoindoloquinoxaline showing significant neuroprotective effects in MPP^+-induced cytotoxicity in SH-SY5Y cells.

Here, we present evidence that non-toxic concentrations of PHID protect SH-SY5Y cells against MPP^+-induced cytotoxicity in several aspects. At sub-toxic doses, PHID attenuated MPP^+-induced cell loss in human neuroblastoma SH-SY5Y, mouse neuroblastoma neuro-2a and mouse dopaminergic MN9D cells. Further, PHID prevented caspase-3 activation and PARP proteolysis in SH-SY5Y and MN9D cells. PHID also inhibited the MPP^+-induced morphological changes and cell loss in SH-SY5Y cells differentiated with retinoic acid. Further, PHID significantly suppressed MPP^+-induced reactive oxygen species production, decreased the Bax/Bcl-2 ratio, and inhibited SAPK/JNK and c-Jun phosphorylation in SH-SY5Y cells. When compared with a known drug apomorphine at 5 μM concentration in SH-SY5Y cells, the inhibition of MPP^+-induced neuronal cell death, Bax/Bcl-2 ratio, caspase-3 activation and PARP proteolysis were similar with that of PHID treated at 10 μM, proving the compound as a promising candidate and can be further evaluated for development as a potent therapeutic agent in treating neurodegenerative diseases (supplementary data).

It is well-known that many types of chemical and physiological neurotoxins including MPP^+ induce oxidative stress are able to cause an initial reactive oxygen species increase and initiate oxidative-dependent cascades in apoptotic dopaminergic cell death (Wong et al., 1999). Previous reports indicated that oxidative damage occurs in the Parkinsonian brain (Mattson, 2000) and overproduction of reactive oxygen species can cause severe impairment of cellular functions and may contribute to the apoptotic process found in Parkinson's disease (Kehrer and Smith, 1994). Presently, treatment with MPP^+ resulted in significant cell viability loss and reactive oxygen species overproduction. Pretreatment with PHID decreased the MPP^+-induced cytotoxicity and accumulation of reactive oxygen species in a dose-dependent

Fig. 6. Effects of PHID on JNK signaling cascade in MPP^+-induced apoptosis in SH-SY5Y cells. Cells were treated with 1 mM MPP^+ in the absence or presence of PHID. The immunoblot analysis using specific antibodies and the densitometric analysis of total and phosphorylated forms of each protein (20 μg protein/lane) in cell lysate samples was represented. (A) Total ASK1 and phosphorylated form of ASK1. (B) Total SAPK/JNK and phospho-SAPK/JNK. (C) Total c-Jun and phospho-c-Jun. (D) Total p38 and phospho-p38 levels and (E) total ERK and phospho-ERK levels. Data represent mean ± S.E.M. ($n = 3$). $^{\#}P<0.001$ compared with control group; $^{*}P<0.05$ and $^{**}P<0.005$ compared with MPP^+-treated group; and $^{\$}$no significant difference compared with MPP^+ treated group and control group by one-way analysis of variance (ANOVA) and post hoc Bonfferoni multiple comparison.


A
p-ASK1/Total ASK1 (fold of control)
p-ASK1
ASK1
β-actin
155KDa
B
p-JNK/Total JNK (fold of control)
p-SAPK/JNK
SAPK/JNK
β-actin
54KDa
46KDa
C
p-c-Jun/Total c-Jun (fold of control)
p-c-Jun
c-Jun
β-actin
48KDa
D
p-p38/Total p38 (fold of control)
p-p38
p38
β-actin
43KDa
E
p-ERK/Total ERK (fold of control)
p-ERK
ERK
β-actin
42KDa
1mM MPP+ - + - + + +
PHID (μM) - - 10 0.1 1 10

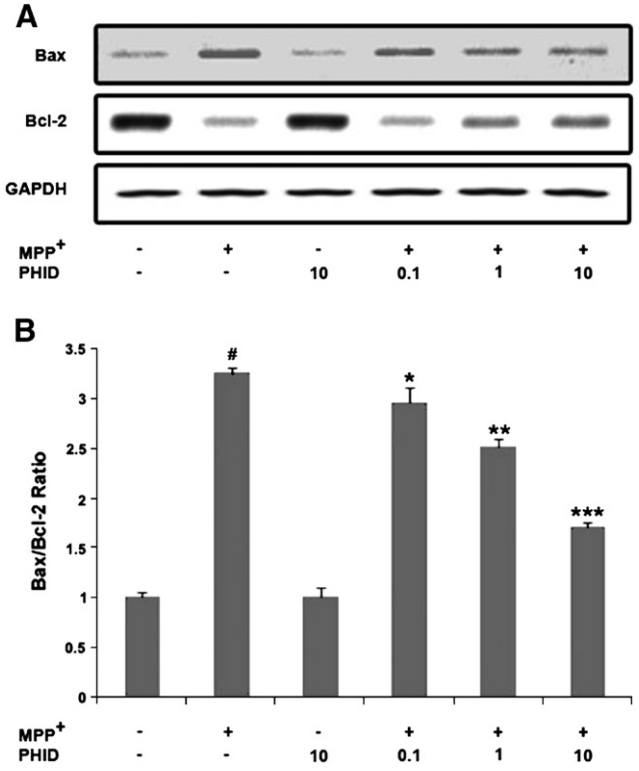


Fig. 7. Effects of PHID on the expressions of Bcl-2 and Bax in SH-SY5Y. Cells were treated with 1 mM MPP^+ in the absence or presence of PHID, and total RNA was collected for semi-quantitative RT-PCR. The levels of Bax and Bcl-2 were quantified by densitometric analysis (A) and the Bax/Bcl-2 ratio was determined (B). Data represent mean ± S.E.M. ($n=3$). $^{\#}P<0.001$ compared with control group; and $^{*}P<0.05$, $^{**}P<0.005$ and $P<0.001$ compared with MPP^+-treated group by one-way analysis of variance (ANOVA) and post hoc Bonferroni multiple comparison.

manner. Treatment with up to 10 μM PHID did not produce any cytotoxic effects on overall cell viability as evaluated by MTT and LDH assays.

Caspase-3, an important biomarker which plays an important role in apoptotic processes is well documented. Caspase-3 activation can act directly on its substrate, PARP, causing hydrolysis (Hartmann et al., 2000; Nicholson et al., 1995). PARP is an abundant nuclear enzyme that normally functions in DNA repair, but extensive PARP activation can promote cell death (Li et al., 2008). In this study, the observation that treatment with MPP^+ lead to an increase in caspase-3 activity is in agreement with previous findings (Blum et al., 2001). However, co-treatment with PHID effectively suppressed MPP^+-induced activation of capase-3, and prevented activation of PARP proteolysis in both the cell types.

Cell survival in the apoptotic cascade depends on the balance between the pro- and anti-apoptotic members of the Bcl-2 family. Bax and Bcl-2, the two main members of this family, influence the permeability of the mitochondrial membrane, and play a crucial role in the mitochondrial apoptotic pathway (Cory and Adams, 2002). Thus, the Bax/Bcl-2 ratio may better predict the decision between cell survival and death, and any shift in the balance of pro- and anti-apoptotic members may affect cell death (Cory and Adams, 2002). Bcl-2 family members are intimately involved in cell death processes caused by MPP^+ (Blum et al., 2001). Presently, the ratio of the pro-apoptotic Bax to the anti-apoptotic Bcl-2 increased significantly in a dose-dependent manner, thereby ameliorating the MPP^+-induced Bax/Bcl-2 ratio elevation in SH-SY5Y cells. Therefore, the effect of PHID on MPP^+-induced apoptosis may be, at least in part, mediated by regulation of Bax and Bcl-2 expressions.

Among the many signaling pathways that respond to stress, MAPK family members are crucial for the maintenance of cells. The ERK signaling pathway regulates cell proliferation, differentiation, and survival, while the SAPK/JNK and p-38 MAPK pathways are activated in response to cytokines, growth factors, and a variety of environmental stresses (Ryan et al., 2007). Reactive oxygen species can activate both SAPK/JNK and apoptosis in a variety of cell systems (Chen et al., 2003; Verheij et al., 1996). Recent studies have also linked activation of the JNK signaling pathway converging on the phosphorylation of c-Jun with the induction of apoptosis in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice and blocking of the JNK signaling pathway may be effective in the control of the disorder (Saporito et al., 2000; Xia et al., 2001).

Thus, we examined whether the protective effects of PHID could be involved via the SAPK/JNK, p38 and ERK signaling pathways. Although MPP^+ treatment to SH-SY5Y cells induced the activation of these signaling cascades, treatment with PHID suppressed the phosphorylation of SAPK/JNK, c-Jun and p38, but did not affect the phosphorylation of ERK 1/2. These observations support with the earlier published reports that treatment at 1 mM concentration of MPP^+ did not alter the expression of p-ERK but increased activation was observed only at high doses (Zhu et al., 2007; Halvorsen et al., 2002). Another study also reported that CEP-1347 (KT7515), a neuroprotective agent inhibits JNK but not ERK 1/2 activation in motor neurons (Maroney et al., 1998).

The discovery of new drug molecules that act by interrupting the signaling pathways that mediate cell death or activate cell survival pathways are an effective way of protecting dopaminergic cells from apoptotic insult in Parkinson's disease. Our present observations that pretreatment with PHID inhibited phosphorylation of ASK1, SAPK/JNK, c-Jun and p38 indicates that PHID may act at an early stage of apoptosis. PHID pretreatment also enhanced the expression of Bcl-2 and inhibited caspase-3 activation in a dose-dependent manner.

In conclusion, our results show that PHID protects SH-SY5Y cells against MPP^+-induced cytotoxicity. The data obtained was highly convincing with our supporting experiments conducted in MN9D, Neuro-2a and differentiated SH-SY5Y cells. The compound's antioxidative and anti-apoptotic properties render this molecule potentially protective against MPP^+-induced cytotoxicity. Furthermore, PHID exerts its potent anti-apoptotic effects by blocking intracellular reactive oxygen species production up-regulated by the neurotoxin MPP^+, and inhibited phosphorylation of JNK signaling in SH-SY5Y cells. Further studies of the neuroprotective mechanism of PHID in MPTP-intoxicated animal models, its dosage regimen, and better understanding of the delivery to the brain through the blood brain barrier will be required. The present data should spur research on PHID as a potential therapeutic strategy for the prevention of progressive neurodegenerative diseases such as Parkinson's disease.

Supplementary data to this article can be found online at doi:10.1016/j.ejphar.2010.09.063.

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