

## Full-length article

# A cell-based model of $\alpha$ -synucleinopathy for screening compounds with therapeutic potential of Parkinson's disease<sup>1</sup>

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## Key words

 $\alpha$ -synuclein; A53T; Parkinson's disease; cell model; SH-SY5Y; apoptosis

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## Abstract

**Aim:** To develop a cell-based model by stable transfection of SH-SY5Y with mutant A53T human  $\alpha$ -synuclein, recapitulating neurotoxicity of  $\alpha$ -synuclein overexpression. **Methods:** The overexpression of mutant  $\alpha$ -synuclein was analyzed by Western blotting, immunocytochemistry, and RT-PCR. Cell viability was processed when treated with different concentrations of 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) and exogenous dopamine (DA) for 24, 48, and 72 h by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Early apoptosis and late apoptosis/necrosis were analyzed by flow cytometry using Annexin V and propidium iodide double staining, respectively. DNA was isolated and applied to agarose gel for electrophoresis; the typical DNA "ladder" represented severe apoptosis. We also used this model to screen 99 compounds with therapeutic potential by MTT assay. **Results:** One of the stably-transfected clones overexpressed exogenous genes on both the protein level and the transcriptive level. Significant differences in cytotoxicity were found between the pcDNA3.1(+) group and the pcDNA3.1(+)-hm $\alpha$ -synuclein group in the presence of the same concentration of MPP<sup>+</sup> and DA within the same incubation time. The level of either early apoptosis or late apoptosis/necrosis was remarkably increased in transfected cells compared with the control after treatment with 100  $\mu$ mol/L MPP<sup>+</sup> for 24 h. In addition, the presence of the typical DNA "ladder" was observed in the pcDNA3.1(+)-hm $\alpha$ -synuclein group when treated with 200  $\mu$ mol/L MPP<sup>+</sup> for 48 h. After the screening experiment, 12 of the 99 compounds were found to decrease DA-induced cytotoxicity on cell viability. **Conclusion:** We established a cell-based model which is useful for studying the function of  $\alpha$ -synuclein and screening compounds with therapeutic potential. In addition, it was identified that cells overexpressing A53T mutant  $\alpha$ -synuclein were significantly vulnerable against MPP<sup>+</sup> or dopamine exposures.

## Introduction

Parkinson's disease (PD), characterized pathologically by the selective loss of dopaminergic neurons and the presence of Lewy bodies (LB) in the substantia nigra, is the second most common neurodegenerative disorder after Alzheimer's disease, affecting people in mid and late life<sup>[1]</sup>. Mutations of the parkin and  $\alpha$ -synuclein genes have been associated with autosomal recessive and dominant parkin-

sonism pedigrees, respectively<sup>[2]</sup>.  $\alpha$ -Synuclein is a highly-conserved, 140 amino acid protein which is mainly expressed in neurons and concentrated in presynaptic nerve terminals<sup>[3,4]</sup>. It was first isolated as a constituent of protein aggregates from Alzheimer brains, distinct from the  $\beta$ -amyloid component<sup>[5]</sup>. Although the native function of  $\alpha$ -synuclein is still being investigated, evidence suggests that its potential roles are in neural plasticity and the regulation of synaptic vesicle pools<sup>[6]</sup>, modulation of dopamine release<sup>[7,8]</sup>, alterations in

dopamine synthesis<sup>[9]</sup>, and targeting the dopamine transporter to the plasma membrane<sup>[10]</sup>.  $\alpha$ -Synuclein pathology is involved in a large number of neurodegenerative diseases, including PD, dementia with LB, Lewy body variant of Alzheimer's disease, neurodegeneration with brain iron accumulation type-1, and multiple system atrophy, which is mostly due to the remarkable discovery in dominantly inherited  $\alpha$ -synuclein substitutions. Missense mutations (A53T, A30P, and E46K) in the  $\alpha$ -synuclein gene were found to be the cause of autosomal dominant PD in a small group of Mediterranean<sup>[11]</sup>, German<sup>[12]</sup>, and Spanish families<sup>[13]</sup>. Among them, A53T was found in at least 12 families with familial PD, although these families likely to share a common ancestor<sup>[14,15]</sup> and A53T seems to induce more severe harm either *in vivo* or *in vitro* than A30P. The pathological consequences of the A53T  $\alpha$ -synuclein mutation is closely associated with PD, whose symptoms include reduced spontaneous movement, static tremor, muscular rigidity, progressive inability to maintain erect posture, and shortening of the step length<sup>[16]</sup>, and neurons in the substantia nigra are predominantly, but not exclusively affected<sup>[17]</sup>.

1-Methyl-4-phenylpyridinium (MPP<sup>+</sup>), the active metabolite of 1-methyl-4-phenyl-2,3,6-tetrahydropyridine, can enter the mitochondria to induce oxidative stress and impair energy metabolism<sup>[18]</sup> by inhibiting mitochondrial complex I<sup>[19,20]</sup>, inducing a syndrome closely resembling PD. The neurotoxicity of exogenous dopamine (DA) has been described in *in vivo* primary cultures and several cell lines<sup>[21,22]</sup>. In dopaminergic neurons, DA is oxidized easily *in vitro* and *in vivo* to a variety of neurotoxic metabolites such as highly cytotoxic quinone molecules<sup>[23,24]</sup>, which participate in the generation of reactive oxygen species. Many studies indicate that an imbalance between cytoplasmic and vesicular DA may lead to oxidative stress and cell degeneration<sup>[25]</sup>. DA may cause apoptosis, but most studies on DA toxicity agree that non-apoptotic mechanisms are also involved in DA-induced cell death<sup>[26,27]</sup>.

Because a slowly progressive brain disorder is difficult to study in humans, a simple, reliable, and valid method is needed for furthering our understanding of the role of  $\alpha$ -synuclein in PD and for screening compounds with therapeutic potential. SH-SY5Y neuroblastoma is a widely and extensively used target cell line in the assessment of neurotoxicity and neuroprotection. So we sought to create a cell-based model by overexpressing mutant A53T human  $\alpha$ -synuclein in SH-SY5Y cells.

## Materials and methods

**Cell culture** Human dopaminergic neuroblastoma cells

SH-SY5Y (American Type Culture Collection, ATCC, Manassas, VA, USA) were maintained at 37 °C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 0.1 g/L streptomycin (Gibco, USA).

**Plasmid construction** Total RNA was extracted from cultured cells by the TRIzol (Invitrogen, Carlsbad, CA, USA) extraction method. From 2  $\mu$ g of total RNA, cDNA was synthesized using 200 units of reverse transcriptase (Superscript<sup>TM</sup> III RT, Invitrogen, USA) and oligo (dT) primers in a final volume of 20  $\mu$ L. The following primer pairs were used when processing PCR with pfuUltra (Stratagen, La Jolla, CA, USA): primers  $\alpha$ -synuclein-F (5'-GAA CTC GAG GGA CTC AGT GTG GTG-3' with the *Xho* I site) and  $\alpha$ -synuclein-R (5'-CT TCT AGA GGA TGG AAC ATC TGT CAG C-3' with the *Xba*I I site) for  $\alpha$ -synuclein (524 bp), and GAPDH-F (5'-CTC ATG ACC ACAG TC CAT GC-3') and GAPDH-R (5'-CACC ACCT GTT GCT GTA GC-3') for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 456 bp). PCR amplification was conducted under the following conditions: for  $\alpha$ -synuclein, initial denaturation was at 94 °C for 2 min, 30 s at 94 °C, 30 s at 57 °C, and 50 s at 72 °C for 30 cycles, followed by 10 min at 72 °C. For GAPDH, initial denaturation was at 94 °C for 2 min, 30 s at 94 °C, 30 s at 59 °C, and 30 s at 72 °C for 30 cycles, followed by 10 min at 72 °C. After amplification, 50  $\mu$ L aliquots were electrophoresed in 1% agarose gel (Biowest, Miami, FL, USA), followed by photographic recording of the gel stained with ethidium bromide. Then, the product of the PCR was obtained by agarose gel DNA purification (TaKaRa, Tokyo, Japan) and ligated into the pMD18-T simple vector (TaKaRa, Japan) after adding DeoxyAdenosine Triphosphate (dATP) to the blunt terminal of the fragment. The positive transformants were screened by bacteria PCR and DNA sequencing after transforming to competent *Escheria coli* TG-1. The A53T mutant, human  $\alpha$ -synuclein gene was procured by gene splicing and overlap extension (SOE) on site-directed mutagenesis of wild-type human  $\alpha$ -synuclein which was inserted into a pMD18-T simple vector purified by PureLink<sup>TM</sup> HiPure plasmid DNA purification kits (Invitrogen, USA). The following primers were used in the SOE: forward (5'-TGC ATG GTG TGA CAA CAG TGG CTG AGA-3'), reverse (5'-GCC ACTGTT GTCACACCATGCACCACTC-3'). The A53T, mutant human  $\alpha$ -synuclein cDNA was subcloned into the pcDNA3.1(+) vector (Invitrogen, USA) after double digestion of the *Xho* I and *Xba*I I restriction enzymes to either insert DNA or the vector.

**Transfection and selection** One day before transfection, 2 $\times$ 10<sup>5</sup> cells were seeded in 500  $\mu$ L of growth medium without

antibiotics in a 24-well format so that cells would be 90%–95% confluent at the time of transfection. DNA was diluted in 50  $\mu$ L Opti-MEM I Reduced Serum Medium (Invitrogen, USA) without serum, and the appropriate amount of Lipofectamine™ 2000 (Invitrogen, USA) was diluted in 50  $\mu$ L of Opti-MEM I Medium. After incubation for 5 min at room temperature, the diluted DNA was combined with the diluted Lipofectamine™ 2000, mixed gently, and incubated for 20 min at room temperature. We added 100  $\mu$ L complexes to each well containing the cells and medium. The cells were incubated at 37 °C in a CO<sub>2</sub> incubator for 18–48 h. During incubation, the medium was changed after 4–6 h, and then cells were passaged at 1:10 into fresh growth medium after incubation. The cells were incubated in the medium containing 0.6 g/L G418 (Gibco, USA) for 14 d to select the stably-transfected cells, and then the cells were collected for monoclonal screening. 100  $\mu$ L medium containing 0.3 g/L G418 was added to all of the wells in the 96-well plate except well A1 which was left empty. 200  $\mu$ L cell suspension was added to well A1, then 100  $\mu$ L cell suspension was quickly transferred from the first well to well B1 by gently pipetting and repeating the same procedure as H1. 100  $\mu$ L of cell suspension was discarded from H1 so that it ended up with the same volume as the wells above it. An additional 100  $\mu$ L medium was added to each well in column 1 (A1–H1), and then 100  $\mu$ L cell suspension was quickly transferred from the wells in column 1 to those in column 2 (A2–H2) with gently pipetting and repeating the same procedure as that of column 12. Cell suspension 100  $\mu$ L was discarded from each well in the last column (A12–H12) so that all of the wells ended up with 100  $\mu$ L of cell suspension. The final volume of all the wells was brought to 200  $\mu$ L by adding 100  $\mu$ L medium to each well. Each well that contained just a single cell was checked and marked so that these monoclonal cells could be subcultured from the wells into larger vessels. We did the subsequent work listed below after the cells were cultured in DMEM medium containing 0.3 g/L G418 for about 3 months.

**Western blotting** Protein concentration was determined by the Bradford assay (Applygen, Beijing, China). Equal amounts of protein were loaded onto each lane, and electrophoresed on SDS-PAGE with Tris-glycine running buffer. They were then transferred to nitrocellulose membranes by wet electrotransfer for 90 min at 100 mA. The blocked membranes were incubated overnight at 4 °C with the polyclonal antihuman  $\alpha$ -synuclein antibody (R&D, Minneapolis, USA 1:1000 dilution). Following 1 h of incubation at room temperature with a horseradish-peroxidase (HRP)-coupled secondary antibody (1: 5000 dilution), the blots were washed and immunodetection was carried out using enhanced

chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ, USA). The blots were then stripped and reprobed with the monoclonal antibody against  $\beta$ -actin (Sigma, St Louis, CA, USA, 1:1000 dilution) and detected as described earlier.

**Immunocytochemistry** After fixation with 4% paraformaldehyde, the cell slides were rinsed in phosphate buffer solution (PBS) and pre-incubated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Then, the cells were blocked with normal rabbit serum for 15 min at room temperature. The following primary antibody (R&D, USA, 1:100 dilution) was then added for overnight incubation at 4 °C in a humidified chamber. After rinsing for 3 $\times$ 3 min in PBS, the cells were incubated with secondary biotinylated antibody at 37 °C for 15 min, followed by rinsing for 3 $\times$ 3 min in PBS. Then, the cells were incubated with HRP-labeling streptavidin/avidin working solution at 37 °C for 15 min. The final reaction product was visualized with chromogen diaminobenzidine (DAB; Sigma, USA).

**RT-PCR** A total RNA of 2  $\mu$ g from the transfected and non-transfected cells as mixed with the RT reaction mixture (Invitrogen, USA). After reverse transcription, Platinum Taq DNA polymerase (Invitrogen, USA) and the samples (total volume 50  $\mu$ L) were placed in a thermal cycler. The following primer pairs were used when processing PCR: primers  $\alpha$ -synuclein-F (5'-GAAGCAGAGGACTCAGTGTGGTG-3') and  $\alpha$ -synuclein-R (5'-CT TGT ACA GGA TGG AAC ATC TGT CAG C-3') for  $\alpha$ -synuclein (524 bp),  $\beta$ -actin-F (5'-CCT CGCCTTTGCCGATCC-3'), and  $\beta$ -actin-R (5'-GGA TCTTCA TGA GGT AGT CAG TC-3') for  $\beta$ -actin (620 bp). PCR amplification of  $\alpha$ -synuclein was conducted under the same conditions listed above in plasmid construction, and the amplification of  $\beta$ -actin was conducted under the following conditions: initial denaturation at 94 °C for 2 min, 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C for 30 cycles, followed by 10 min at 72 °C. After PCR, 10  $\mu$ L aliquots of the reaction mixtures were resolved on 1% agarose gel containing ethidium bromide to identify the DNA amplicons generated.

**Cell viability** MTT assay is a standard method used to assess cell viability. Briefly, the cells ( $5 \times 10^3$  cells/well) were seeded in 96-well plates. After exposure to various concentrations (100, 200, and 500  $\mu$ mol/L) of MPP<sup>+</sup> and DA for 24, 48, and 72 h, 10  $\mu$ L MTT, (5 g/L in PBS, Sigma, USA) solution was added to each well and the plates were incubated for an additional 4 h at 37 °C. Then, the MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystals formed in the viable cells, 200  $\mu$ L DMSO was added to each well. The absorbance was read at 570 nm with DMSO as the blank.

**Flow cytometry (Fluorescence-activated cell sorter analysis)** To detect early apoptosis and late apoptosis/necrosis, the cells were stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (PI, BD Clontech San Jose, CA, USA) after being treated with 100  $\mu\text{mol/L}$  MPP<sup>+</sup> for 24 h. Approximately  $1 \times 10^6$  cells were washed with cold PBS before being resuspended in 200  $\mu\text{L}$  cold  $1 \times$  binding buffer. 10  $\mu\text{L}$  Annexin V-FITC and 5  $\mu\text{L}$  PI were added and incubated for 15 min at room temperature in the dark. A further 300  $\mu\text{L}$  binding buffer was added to terminate the reaction, and flow cytometric analysis was conducted immediately with 20000 cells. The experiment was repeated 3 times and the results were averaged.

**DNA fragmentation assay** The cells were grown to about 80% confluence and then treated with 200  $\mu\text{mol/L}$  MPP<sup>+</sup> for 48 h. After treatment, the cells were resuspended in 100  $\mu\text{L}$  DNA lysis buffer (TaKaRa, Japan), and then the supernatant was moved to another tube. The above step was repeated, and 200  $\mu\text{L}$  of the final supernatant was collected. The liquid was incubated at 56  $^\circ\text{C}$  for 1 h by adding 20  $\mu\text{L}$  10% SDS and 20  $\mu\text{L}$  proteinase K, and then reacted at 37  $^\circ\text{C}$  for 1 h after adding 20  $\mu\text{L}$  RNase to allow complete RNA digestion. The DNA was precipitated with 950  $\mu\text{L}$  100% ethanol and 130  $\mu\text{L}$  precipitant for 2 h at -70  $^\circ\text{C}$ . The DNA was pelleted at 12000 $\times$ g for 15 min and washed twice with 80% ethanol. The DNA was dissolved in Tris-EDTA buffer, and analyzed on 1.5% agarose gel for electrophoresis. DNA bands were visualized with ethidium bromide under ultraviolet light and photographed.

**Compound screening** The procedures were done as the part of cell viability, but the cytotoxin was 50  $\mu\text{mol/L}$  DA for 24 h of incubation. Ninety-nine compounds were added to the medium at the concentration of  $1 \times 10^{-5}$  mol/L, respectively. These compounds were extracted from *Fraxinus sielboldiana* blume, belonging to the Oleaceae family, which is widely distributed in the east of Asia, especially in the south of China.

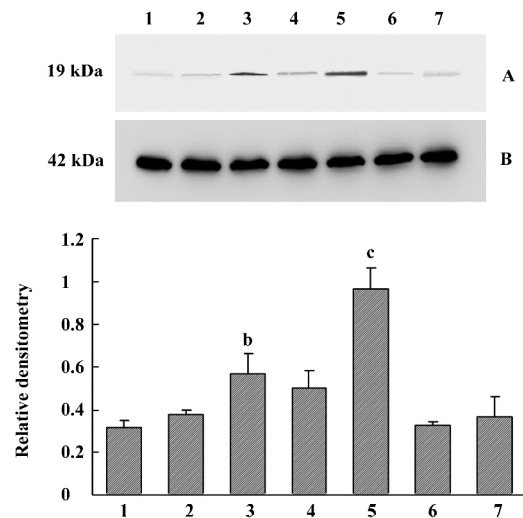
**Statistical analysis** All results were expressed as mean $\pm$ SD. Statistical analysis was performed between the 2 groups by Student's *t*-test.  $P < 0.05$  indicated significant difference.

## Results

**Construction of the eukaryotic expression system of mutant A53T human  $\alpha$ -synuclein** We obtained the mutation using the SOE method and then identified it by DNA sequencing blasted to GeneBank N<sub>0</sub> BC013293 and named it pcDNA3.1(+)-hm $\alpha$ -synuclein. After the double digestion with the *Xho* I and *Xba* I restriction enzymes, the fragments

of inserted DNA and pcDNA3.1(+) were 517 bp and 4911 bp as expected.

**Increased  $\alpha$ -synuclein protein expression in the cell culture model** It was identified that  $\alpha$ -synuclein expression increased in human SH-SY5Y cells after 90 d of stable transfection by Western blotting and immunocytochemistry. In the normal control or pcDNA3.1(+)-alone transfected group, the protein expression was maintained at a normal level. However, transfected monoclones showed different levels (Figure 1). Through analysis by Gel-Pro Analyzer software 3.1 (Media Cybernetics, LR, USA), we found that clone 1 ( $P < 0.05$ ) and clone 3 ( $P < 0.01$ ) were significantly different compared with the control, so clone 3 was used as a cell model for our study due to the high level expression of exogenous mutant  $\alpha$ -synuclein. The other 3 transfected groups seemed to only express the exogenous resistance gene designed in the pcDNA3.1(+) vector for their survival in the culture medium containing G418. Different clones showed different expression levels, which suggested that random integration of exogenous gene into genome was not the same copies. We also used immunocytochemistry in cell slides to

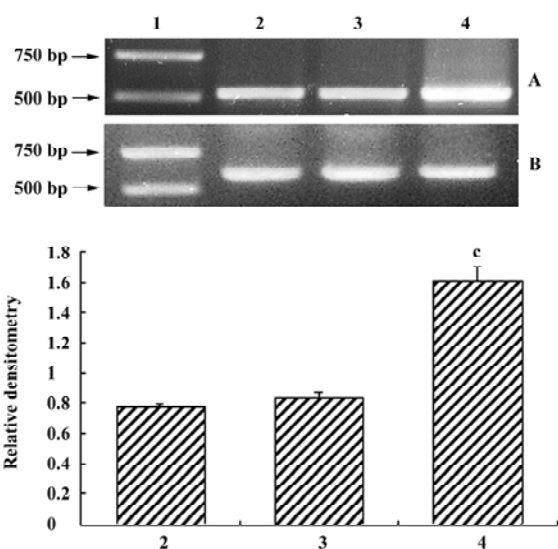
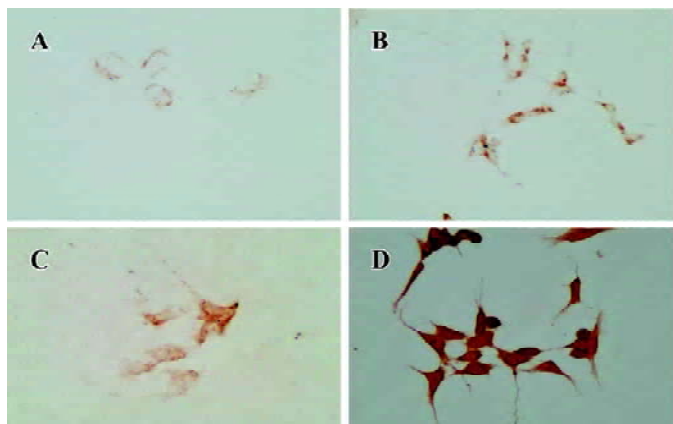


**Figure 1.** Western blotting analysis of stable expression of A53T mutant  $\alpha$ -synuclein in the transfected SH-SY5Y cells. The cells were lysed by incubating with 20 mmol/L lysis buffer containing detergents. Lysates were subjected to SDS-PAGE and blotted to a nitrocellulose (NC) membrane. The membrane was incubated with a polyclonal antibody against  $\alpha$ -synuclein (1:1000 dilution) and then with HRP-conjugated anti-goat IgG (1:5000 dilution). Detection was performed by incubation with a chemiluminescence substrate, and immunodetection was carried out using ECL detection reagents. (A)  $\alpha$ -synuclein; (B)  $\beta$ -actin. 1, normal control; 2, pcDNA3.1(+) group; 3-7, pcDNA3.1(+)-hm $\alpha$ -synuclein groups named clones 1-5.  $n=3$ . <sup>b</sup> $P < 0.05$ ; <sup>c</sup> $P < 0.01$  vs pcDNA3.1(+) group.

characterize the cellular localization and expression of  $\alpha$ -synuclein. The results showed that positive immunostaining of overexpression was widely localized in the cytoplasm and synapse compared with the control. Almost no staining was detected if the primary antibody was omitted (Figure 2).

**Increased  $\alpha$ -synuclein transcriptional level in the cell model** Given the change of protein expression, we sought to validate the transcriptional level obtained by semiquantitative RT-PCR. Clearly,  $\alpha$ -synuclein was upregulated in the transfected cells (Figure 3).

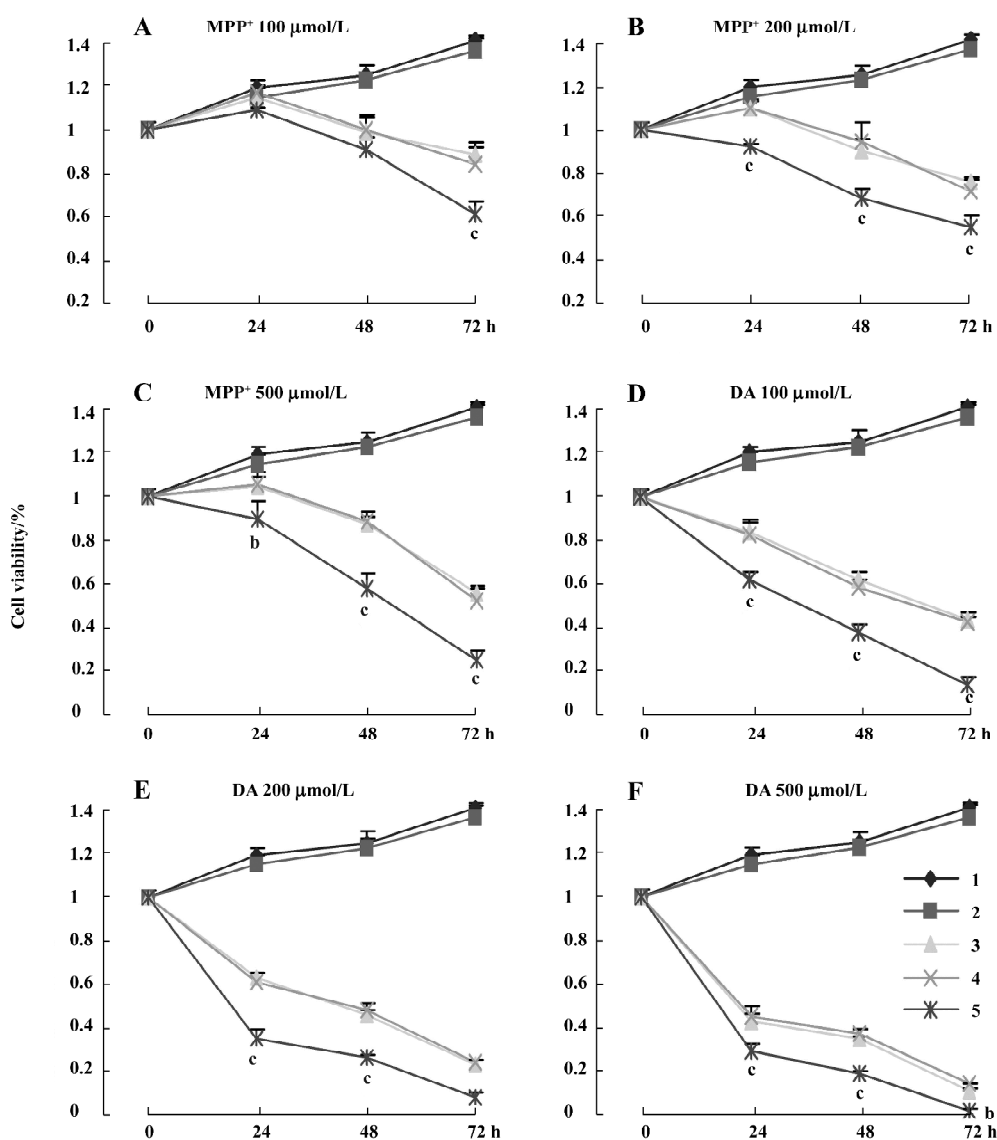
**Comparison of MPP<sup>+</sup> and DA-induced cytotoxicity between normal and stably transfected cells** All groups of cells were treated with a range of concentrations (100, 200, and 500  $\mu$ mol/L) of MPP<sup>+</sup> and DA for different times (24, 48, and 72 h), and cell viability was determined by MTT assay. MPP<sup>+</sup> and DA induced an evident dosage and time-dependent loss in cell viability. The treatment of cells with 100, 200, and 500  $\mu$ mol/L MPP<sup>+</sup>, resulted in a cell viability loss of about 2.6%, 8.1%, and 11.7% for 24 h; 20.2%, 24.6%, and 28.9% for 48 h; and 40.3%, 49.2%, and 62.8% for 72 h in the pcDNA3.1(+)-alone transfected group. However, cell viability loss was about 9.3%, 14.8%, and 25.1% for 24 h; 27.3%, 29.4%, and 45.3% for 48 h; and 49.5%, 53.8%, and 75.1% for 72 h in the clone 3 group, respectively (Figure 4A–4C). The incubation of cells with 100, 200, and 500  $\mu$ mol/L DA resulted in a cell viability loss of about 56.1%, 57.6%, and 61.8% for 24 h; 69.7%, 77.1%, and 78.5% for 48 h; and 82.8%, 89.9%, and 95.3% for 72 h in the pcDNA3.1(+)-alone transfected group, while there was a loss of about 65.2%, 70.3%, and 75.8% for 24 h; 77.6%, 78.9%, and 84.2% for 48 h; and 90.0%, 94.0%, and 98.0% for 72 h in the clone 3 group, respectively (Figure 4D–4F). It was found that more rapid loss of cell proliferation occurred with the incubation of DA than MPP<sup>+</sup>, and we concluded that there was significant difference between the pcDNA3.1(+) group and the clone 3 group in cell viability when treated with MPP<sup>+</sup> and DA. However, there was no



**Figure 3.** Analysis of cDNA synthesized in the transfected SH-SY5Y cells. Total RNA was extracted from transfected and non-transfected cells, and single strand cDNA was synthesized using primer oligo (dT). cDNA specific for  $\alpha$ -synuclein or  $\beta$ -actin was amplified by PCR, and the products were analyzed by electrophoresis. (A)  $\alpha$ -synuclein; (B)  $\beta$ -actin. 1, DL 2,000 DNA marker; 2, normal control; 3, pcDNA3.1(+) group; 4, clone 3.  $n=3$ . <sup>e</sup> $P<0.01$  vs pcDNA3.1(+) group.

statistical difference between normal cells and transfected cells without MPP<sup>+</sup> or DA, and it was indicated that the cell model would not show the more severe cytotoxicity unless it was exposed to an exogenous cytotoxin.

We identified early apoptotic cells, late apoptotic/necrotic cells, and viable cells by double staining the cells with PI and Annexin V. There was a significant difference between the pcDNA3.1(+)-alone transfected group and the clone 3 group after the cells were treated with 100  $\mu$ mol/L MPP<sup>+</sup> for 24 h. The percentage of both Annexin V<sup>+</sup>/PI<sup>-</sup> cells and Annexin V<sup>+</sup>/PI<sup>+</sup> cells increased from 0.98% to 1.56% and



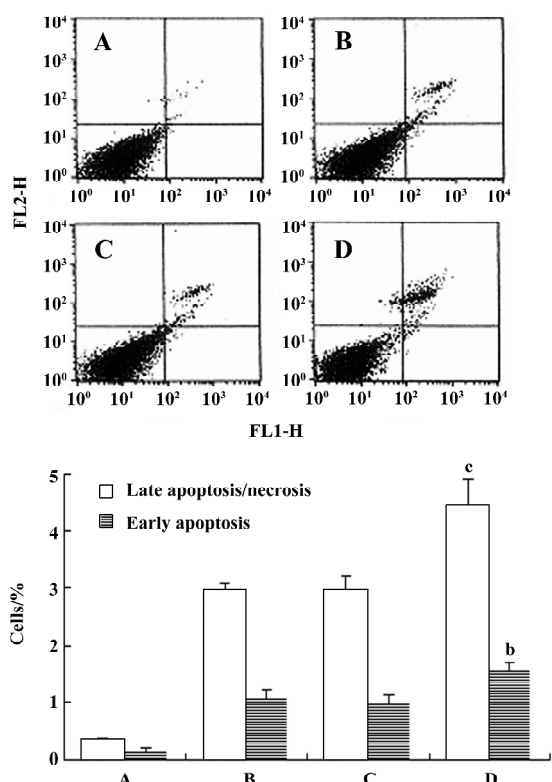
**Figure 4.** Effects of MPP<sup>+</sup> and DA on cell viability. Cells were exposed to different concentrations (100, 200, and 500 μmol/L) of MPP<sup>+</sup> and DA for 24, 48, and 72 h, and then 10 μL MTT solution was added to each well. After the MTT solution in medium was aspirated off, 200 μL DMSO was added to each well to achieve solubilization of the formazan crystals formed in the viable cells. Absorbance was read at 570 nm with DMSO as the blank. 1, normal control; 2, clone 3; 3, normal cells treated with MPP<sup>+</sup> or DA; 4, pcDNA3.1(+) group treated with MPP<sup>+</sup> or DA; 5, clone 3 treated with MPP<sup>+</sup> or DA. (A), 100 μmol/L MPP<sup>+</sup>; (B), 200 μmol/L MPP<sup>+</sup>; (C), 500 μmol/L MPP<sup>+</sup>; (D), 100 μmol/L DA; (E), 200 μmol/L DA; (F), 500 μmol/L DA. *n*=3. <sup>b</sup>*P*<0.05; <sup>c</sup>*P*<0.01 vs pcDNA(+) group treated with MPP<sup>+</sup> or DA.

from 2.98% to 4.45%, respectively (Figure 5).

MPP<sup>+</sup> has been described as an effective inducer for cell apoptosis, and a ladder pattern of DNA degradation is considered a molecular hallmark of apoptosis. Here, we proceeded to study the DNA from cells treated with 200 μmol/L MPP<sup>+</sup> for 48 h by means of electrophoresis in agarose gels (Figure 6). In the transfected cells, an apoptotic pattern of DNA degradation was easily and reproducibly detected. However, this apoptotic pattern could not be detected in

non-transfected cells.

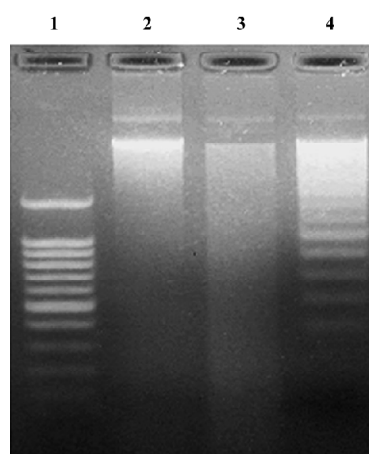
**Compounds screening** In the primary screening experiment, 12 of the 99 compounds showed anticytotoxic activity at the concentration of  $1 \times 10^{-5}$  mol/L (Table 1). After further screening, we found N<sub>0</sub>66 (F.mar-m-1), N<sub>0</sub>67 (F.mar-m-27), and N<sub>0</sub>80 (F.mar-m-16) displayed definite anticytotoxic activity in different concentration from  $1 \times 10^{-8}$  to  $1 \times 10^{-4}$  mol/L. The EC<sub>50</sub> were  $1.376 \times 10^{-6}$ ,  $8.409 \times 10^{-7}$ , and  $2.842 \times 10^{-6}$  mol/L, respectively (Figure 7).



**Figure 5.** Effect of MPP<sup>+</sup> (100 μmol/L for 24 h) on apoptosis by flow cytometry. It was possible to differentiate between early apoptotic cells (Annexin V positive, PI negative), late apoptotic/necrotic cells (Annexin V/PI double positive), and viable cells (Annexin V/PI double negative). (A) Normal control; (B) Normal cells treated with MPP<sup>+</sup>; (C) pcDNA3.1(+) group treated with MPP<sup>+</sup>; (D) Clone 3 treated with MPP<sup>+</sup>. *n*=3. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs pcDNA(+) group treated with MPP<sup>+</sup>.

## Discussion

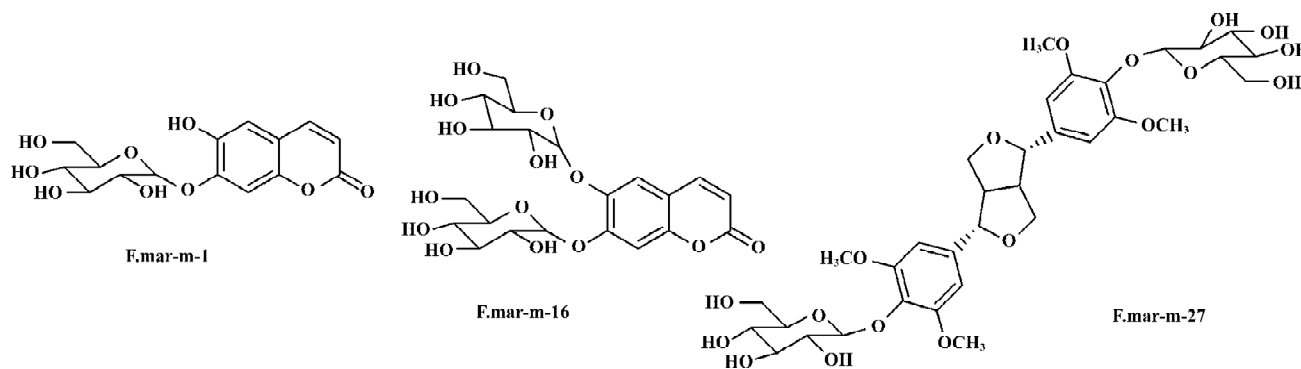
In the search for a better approach to compound screens



**Figure 6.** Effect of MPP<sup>+</sup> (200 μmol/L for 48 h) on DNA fragmentation. DNA was isolated and applied to agarose gel for electrophoresis; the bands were then visualized under a UV transilluminator. 1, 100 bp DNA markers; 2, normal control; 3, pcDNA3.1(+) group treated with MPP<sup>+</sup>; 4, clone 3 treated with MPP<sup>+</sup>.

for PD, which on the one hand targets a specifically related protein, and on the other, demonstrates anticytotoxic effects against the cytotoxin. In this study, we constructed a cell-based model of α-synucleinopathy to successfully develop a SH-SY5Y cell line expressing corresponding A53T mutant α-synuclein, which could maintain the expression level continually. In other studies<sup>[28–30]</sup>, several kinds of models were established for the research of α-synuclein, but not for compound screens and drug development. With the advantage of persistent expression, the cell model was suitable for long-time research work without repeated transfection.

Overexpression or aggregates of α-synuclein are found in a number of neurodegenerative disorders, now termed synucleinopathies<sup>[31]</sup>. Despite the physiological localization of α-synuclein in presynaptic terminals, the aggregates are



**Figure 7.** Chemical structure of the 3 compounds, F.mar-m-1, F.mar-m-16, and F.mar-m-27.

**Table 1.** Compound screening using the cell model. Ninety-nine compounds were added into the cell culture at a final concentration of  $1 \times 10^{-5}$  mol/L, and cell viability was analyzed by MTT assay (in the item, control means cell model, 50  $\mu$ mol/L, DA means cell model treated with 50  $\mu$ mol/L DA for 24 h).  $n=4$ . <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs 50  $\mu$ mol/L DA.

No	Groups	Cell viability (%)	No	Groups	Cell viability (%)
	Control	1	50	H-8a	0.1596±0.01257
	50 $\mu$ mol/L DA	0.4183±0.02584	51	B6-1	0.1557±0.01457
1	Le-25	0.3776±0.1574	52	CF-3	0.1865±0.01942
2	Le-25	0.416±0.02359	53	CF-7	0.2468±0.0094
3	Le-7	0.3873±0.01748	54	RP-5-12-13	0.2446±0.01439
4	Le-6	0.3857±0.03584	55	h-1	0.2055±0.01385
5	Le-16	0.4407±0.02358	56	h-2	0.1818±0.01235
6	Le-1	0.5642±0.01698 <sup>c</sup>	57	h-6	0.1706±0.01429
7	Le-12	0.4087±0.02513	58	h-7	0.1717±0.01852
8	Le-26	0.4008±0.02784	59	h-20	0.1612±0.01484
9	MSZS	0.3415±0.01684	60	h-25	0.2165±0.02451
10	YECS	0.4077±0.03158	61	ksh-3	0.1694±0.01352
11	DXS	0.4029±0.03624	62	k-5	0.2042±0.02158
12	LDS	0.4109±0.02413	63	df-1	0.1528±0.01385
13	HYC-13	0.3978±0.02135	64	F.mar-M-2	0.5071±0.01188 <sup>c</sup>
14	HYC-5	0.3913±0.01435	65	F.mar-M-4	0.4843±0.03195 <sup>b</sup>
15	HYC-2	0.4203±0.02351	66	F.mar-M-1	0.4736±0.04891 <sup>b</sup>
16	HYC-12	0.3792±0.03217	67	F.mar-M-27	0.5323±0.02171 <sup>c</sup>
17	HYC-11-1	0.3535±0.02416	68	F.mar-M-36	0.3953±0.0708
18	HYC-3	0.3381±0.02714	69	F.mar-23-2	0.393±0.0630
19	HXS-4-6	0.4074±0.03017	70	F.mar-M-12	0.327±0.03568
20	HXS-7	0.4105±0.02591	71	F.mar-M-28a	0.4933±0.02748 <sup>b</sup>
21	HXS-6	0.3327±0.01428	72	F.mar-18	0.4803±0.05874 <sup>b</sup>
22	EA-2-5	0.3728±0.02685	73	F.mar-21-1	0.3666±0.02658
23	EA-3-1	0.3769±0.03216	74	F.mar-27-9	0.4173±0.0015
24	EA-3-4	0.389±0.02484	75	F.mar-M-17	0.39866±0.01548
25	IBU-1	0.406±0.03152	76	F.mar-M-9	0.39233±0.02365
26	AC-9	0.3784±0.01498	77	F.mar-23-1	0.4273±0.04852
27	EA-3-2	0.3851±0.02845	78	F.mar-M-20	0.4223±0.03658
28	IP-2	0.3555±0.03541	79	F.mar-M-5	0.45633±0.03125
29	IS-4	0.4008±0.03154	80	F.mar-M-16	0.50366±0.01548 <sup>c</sup>
30	ICE-1	0.4127±0.02147	81	F.mar-M-38	0.3596±0.03325
31	IED-1	0.3781±0.01954	82	F.mar-M-39	0.4006±0.01548
32	EA-3-3	0.3676±0.03514	83	F.mar-M-32	0.3543±0.02584
33	Er-1	0.3502±0.02541	84	F.mar-M-40a	0.4366±0.02584
34	Er-2	0.3341±0.02484	85	F.mar-M-43	0.39733±0.03584
35	Er-3	0.3631±0.01487	86	F.mar-M-45	0.4693±0.02658 <sup>b</sup>
36	Er-4	0.3054±0.03241	87	F.mar-M-46	0.4046±0.03585
37	Er-5	0.4692±0.02841 <sup>c</sup>	88	F.mar-M-33	0.4603±0.02586 <sup>b</sup>
38	Er-6	0.3601±0.02795	89	F.mar-M-6	0.402±0.01584
39	LJV-2	0.3648±0.03147	90	F.mar-M-8	0.4096±0.03225
40	Hr16-1	0.3965±0.03841	91	F.mar-M-24	0.4681±0.02265 <sup>b</sup>
41	Hr19-5	0.3349±0.03217	92	F.mar-M-41	0.3633±0.03162
42	Hr-16-4	0.3237±0.02514	93	F.mar-M-15	0.375±0.02745
43	Hr4-1	0.2926±0.02954	94	LS-1	0.36033±0.01217
44	2#-1-2	0.3411±0.03274	95	LS-2	0.3596±0.01576
45	Hx-6	0.1838±0.01452	96	LS-3	0.4±0.01264
46	Hr19-4	0.1814±0.01217	97	LS-4	0.384±0.02686
47	Hr11-5	0.1725±0.02134	98	LS-5	0.37966±0.03541
48	Hr9-4	0.1753±0.02148	99	LS-6	0.4123±0.02163
49	Hr2-1	0.1555±0.01238			



present throughout the cell body. In our study, similarly altered localization of  $\alpha$ -synuclein was widespread in the cell model which expressed the A53T mutant version.

Effort in developing a therapy against neurodegeneration is therefore aimed at the inhibition of overexpression or aggregate formation<sup>[32,33]</sup>, but the role of proteins in the pathogenesis of human neurodegenerative disease has become an issue of debate<sup>[34]</sup> because a number of studies have not supported their toxicity<sup>[35,36]</sup>. It was demonstrated that overexpression of wild-type human  $\alpha$ -synuclein rescues mesencephalic dopaminergic cells from MPP<sup>+</sup>-induced apoptotic cell death by attenuating cytochrome c release, caspase-3 activation, and proteolytic activation of Protein Kinase C- $\delta$  (PKC- $\delta$ ). However, overexpression of the A53T human  $\alpha$ -synuclein mutant exacerbates MPP<sup>+</sup>-induced apoptosis by augmenting proteolytic cleavage of PKC $\delta$ <sup>[37]</sup>. So by using this cell model, we can perform the studies from elementary compound screens to mechanism research of the selected compounds for drug development as this model either imitates the reaction of human neurons *in vitro* or simultaneously represents the interactions between overexpression of mutant  $\alpha$ -synuclein and active compounds.

Although it has been suggested that  $\alpha$ -synuclein aggregation may stimulate apoptosis, it remains elusive whether programmed cell death itself is directly responsible for neuronal loss, especially considering the observations that  $\alpha$ -synuclein may also exert anti-apoptotic activity<sup>[38,39]</sup>. So its potential cytotoxic effects when encountering cytotoxin were tested in this study between normal and stably-transfected cells with MPP<sup>+</sup> and DA on cell viability. The addition of MPP<sup>+</sup> or DA led to more severe  $\alpha$ -synuclein-dependent growth inhibition in transfected cells than in non-transfected cells. It was suggested that the model overexpressing mutant  $\alpha$ -synuclein could show much more vulnerability to cytotoxin compared with normal cells. When compound screens were carried out, the candidates with anticytotoxic activity would be screened out by the cell model.

Apoptosis and necrosis are 2 modes of cell death in nucleated eukaryotic cells. Apoptosis is a programmed cell death characterized by changes in condensation of nuclear chromatin, cytoplasmic blebbing, and exposure of phosphatidylserine (PS) residues on the outside of the plasma membrane<sup>[40]</sup>. Necrosis, on the other hand, is accidental cell death and indicates the features of mitochondrial swelling, rupture of the plasma membrane, and the release of cytoplasmic constituents<sup>[41]</sup>. A key event of apoptosis in the early stage is that PS of the inner

Leaflet of the cell membrane appears in the outer leaflet, becoming a molecular marker for phagocytosis. Annexin V,

which preferentially binds to PS, is very useful in detecting the expression of PS on the surface of apoptotic cells. Although neuronal apoptosis is a normal event during development, many neurodegenerative diseases are thought to involve abnormal cell death that leads to the damage of the nervous system. In our study, when MPP<sup>+</sup> existed, there was a significant difference between the control and the transfected group, reflecting the increase of early apoptosis and late apoptosis/necrosis in addition to the appearance of the DNA "ladder". It was concluded that the cell model showed much more sensitivity to cytotoxin than non-transfected cells, which could demonstrated the validity of the cell model.

After high throughput screening of 99 compounds, we found that 12 could decrease DA-induced cytotoxicity, and 3 of the 12 had definite anticytotoxic activity. The preliminary result indicated that the 3 compounds deserved further research because of their therapeutic potential.

In summary, this study presented a cell-based model that recapitulated pathological properties of mutant  $\alpha$ -synuclein, which is particularly associated with the severer phenotype of familial PD<sup>[42]</sup>, and demonstrated the potential cytotoxicity of A53T mutant  $\alpha$ -synuclein. This cellular model can be used for further, fundamental studies associated with the underlying mechanisms of overexpression and genetic or environmental effects of  $\alpha$ -synuclein-mediated cytotoxicity. The model also can be applied to the screening of drugs with therapeutic potential for synucleinopathies.

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