Cell-based assays for Parkinson’s disease using differentiated human LUHMES cells

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Aim: Lund human mesencephalic (LUHMES) cells can be differentiated to post-mitotic cells with biochemical, morphological and functional features of dopaminergic (DAergic) neurons. Given the limited scale of primary DAergic neuron culture, we developed differentiated LUHMES cell-based cytotoxicity assays for identifying neuroprotective agents for Parkinson’s disease (PD).

Methods: LUHMES cells were incubated in a differentiation medium containing cAMP and GDNF for 6 d, and then differentiated cells were treated with MPP+ or infected with baculovirus containing α-synuclein. Cytotoxicity was determined by measuring intracellular ATP levels and caspase 3/7 activity in the cells. DAergic neuron-specific marker protein and mRNA levels in the cells were analyzed using Western blotting and RT-PCR, respectively.

Results: LUHMES cells grew extensive neurites and became post-mitotic neuron-like cells during differentiation period, and three DAergic neuron markers TH, DAT and Nurr1 exhibited different expression profiles. MPP+ dose-dependently reduced ATP levels in the cells with an IC50 value of 65 μmol/L. MPP+ (80 μmol/L) significantly increased caspase 3/7 activity in the cells. Both the CDK inhibitor GW8510 and the GSK3β inhibitor SB216763 effectively rescued MPP+-induced reduction of ATP levels with EC50 values of 12 and 205 nmol/L, respectively. Overexpression of α-synuclein also significantly decreased intracellular ATP levels and increased caspase 3/7 activity in the cells. GW8510 and SB216763 effectively rescued α-synuclein overexpression-induced reduction of ATP levels, whereas GW8510, but not SB216763, ameliorated α-synuclein overexpression-induced increase of caspase 3/7 activity.

Conclusion: MPP+- and α-synuclein overexpression-induced cytotoxicity of differentiated LUHMES cells may serve as good alternative systems for identifying neuroprotective compounds for PD.

Keywords: LUHMES cell; dopaminergic neuron; cytotoxicity; MPP+; α-synuclein; GW8510; SB216763; cell-based assay; Parkinson’s disease
to ensure an efficient and meaningful HTS for the treatment of PD.

The Lund human mesencephalic (LUHMES) cell line may offer such a cellular system. These cells are derived from embryonic human mesencephalon and immortalized by introducing a tetracycline-responsive v-myc gene (TET-off)\cite{13}. They can be easily cultured and maintained, similar to other cell lines. After several-day differentiation using tetracycline, cyclic AMP (cAMP) and glial derived neurotrophic factor (GDNF), these cells exit cell cycle and gradually develop into dopaminergic-like neurons, which express dopaminergic neuron specific markers biochemically and develop extensive neurites morphologically\cite{13, 14}. Functionally, these post-mitotic cells display spontaneous electrical activities and are able to release/uptake dopamine, which suggests a high relevance between differentiated LUHMES cells and human dopaminergic neurons\cite{14}.

To establish cellular assays for PD, dopaminergic neuron-specific neurotoxins have been commonly used to induce neuronal death manifested as reduced cell viability (eg, reduced intracellular ATP levels) and cell apoptosis (programmed cell death with multiple biochemical events, such as increased caspase 3/7 activity). Among the neurotoxins, MPP⁺ (1-methyl-4-phenylpyridinium) is one of the most dopaminergic neuron specific toxins. MPP⁺ is the active metabolite of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), the chemical that is known to cause human parkinsonism after injection\cite{15}. MPP⁺ is taken up by DAT to enter into dopaminergic neurons. There, it interferes with oxidative phosphorylation in the mitochondria, reduces dopamine levels, and gradually causes cell death\cite{16, 17}. In addition to the neurotoxins, PD-linked genetic factors, such as α-synuclein, have also been used to develop cellular assays with more translatability. α-Synuclein is a 140 amino acid protein. Mutation or multiplication of the α-synuclein gene SNCA causes inheritable familial forms of PD\cite{18}. Pathologically, α-synuclein aggregates have been found as the primary structural component of Lewy bodies (LBs) in both sporadic and familial PD\cite{19, 20}. The physiological function of α-synuclein is still obscure, though it has been suggested to play a role in neuronal plasticity\cite{21}. In this study, by setting up the culture system of LUHMES cell line, we confirmed the dopaminergic neuron-related features of the differentiated cells, established a MPP⁺-induced cytotoxicity model and α-synuclein overexpression-induced cytotoxicity model in differentiated LUHMES cells, and validated these models using neuroprotective reference compounds. Application of these cellular models to HTS or phenotypic screening may greatly facilitate the discovery of neuroprotective agents for PD.

Materials and methods
LUHMES cell culture and differentiation
The LUHMES cell line used in the studies was purchased from ATCC (CRL-2927). Culture dishes were pre-coated with 50 μg/mL Poly-L-ornithine (Sigma, USA) and 1 μg/mL human plasma fibronectin (Gibco, USA) in distilled water (Gibco, USA) at 37°C for 4 h. Cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12, Gibco, USA), supplemented with 1× N-2 supplement (Invitrogen, USA), 1× GlutaMax (Gibco, USA) and 40 ng/mL human recombinant basic FGF (bFGF, R&D System, USA), at 37 °C in a humidified atmosphere of 5 % CO₂ incubator.

Cell differentiation was generally performed following the published protocol\cite{14}. Briefly, 4×10⁶ LUHMES cells were seeded onto a pre-coated 100-mm dish. Twenty-four-hour later, the cells were treated with differentiation medium containing DMEM/F12, N-2 supplement, GlutaMax, 1 μg/mL Doxycycline (Sigma, USA), 40 ng/mL human recombinant GDNF (prepared in house) and 1 mmol/L cAMP (Sigma, USA). After 2 d differentiation, cells were trypsinized and re-plated to a pre-coated 6-well plate (2×10⁶ cells per well) or 96-well plate (6×10⁴ cells per well). The cells were maintained in differentiation medium and differentiated for an additional 4 d.

Western blotting
Cells were differentiated and re-plated in 6-well plates. At d 0, d 2, and d 6 of the differentiation, cells were scraped from the plate and lysed in 1× RIPA buffer (Cell signaling, USA) supplemented with 1× protease inhibitor (Calbiochem, USA) and 1× phosphatase inhibitor (Calbiochem, USA). The lysate was centrifuged at 14 800 rounds per minute for 10 min at 4°C, and the supernatant was collected. Protein concentration was measured using a BCA kit (Thermo Scientific, USA). Each sample (31.25 μg) was boiled for 5 min and electrophoresed in NuPAGE 4%-12% Bis-Tris Gel. The gel was transferred onto a nitrocellulose membrane. The membranes were incubated with primary antibodies at 4°C overnight followed by secondary antibody for 1 h at room temperature. Membranes were scanned and analyzed using an Odyssey IR Fluorescence Scanner.

Primary antibodies used in the study included anti-TH antibody (Millipore, USA, 1:1000 dilution), anti-Nurr-1 antibody (Santa Cruz, USA, 1:1000 dilution), anti-GAPDH antibody (Sigma, USA, 1:2000 dilution), and anti-Tuj-1 antibody (Millipore, USA, 1:1000 dilution). Secondary antibodies used in the study included: goat anti-rabbit IRDye 800CW (Odyssey, USA, 1:20000 dilution), and goat anti-mouse IRDye 680CW (Odyssey, USA, 1:20000 dilution).

Real-time PCR
Cells were differentiated and re-plated in 6-well plates. At d 0, d 2, and d 6, RNA was extracted using RNeasy Plus Mini Kit (Qiagen, USA). The concentration of RNA was measured by Biophotometer (Eppendorf, Germany). RNA (1 μg per sample) was reverse transcribed to cDNA using iScript CDNA Synthesis Kit (BIO-RAD, USA). Real-time PCR was run in a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA).

Primer sequences of the tested genes were obtained from the study by Scholz et al (Table 1)\cite{14}.

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Cells were differentiated and re-plated in 96-well plates. On MPP⁺-induced cytotoxicity USA, 1:1000 dilution). 1:1000 dilution), goat anti-rabbit IgG Alexa488 (Invitrogen, USA, 1:1000 dilution), anti-α-synuclein antibody (Sigma, USA, 1:1000 dilution), anti-Tuj1 antibody (Invitrogen, USA) was used for nuclear staining. Images were taken using a Nikon A1R Confocal Microscope. Immunostaining

Table 1. Primer sequences.

| Gene          | Sequences                                |
|---------------|------------------------------------------|
| AADC          | 5′-ACG GGC AGC ATC CTC AAC TT-3′ (Forward) |
|               | 5′-AAA ACT GAC TGG CAG CAG CC-3′ (Reverse) |
| DAT           | 5′-TGTC TGG TGT TAG CCG TT-3′ (Forward)   |
|               | 5′-CAC CAT CTT CCA GGA GGC AGA TC-3′ (Reverse) |
| DRD2          | 5′-GCG GGA GGC ATT GCT GAT CAT C-3′ (Forward) |
|               | 5′-TGG AGG ATT CCC CAT ATG AA-3′ (Reverse) |
| EN1           | 5′-TGTC TCT TTG GGA ATG TG-3′ (Forward)   |
| GAPDH         | 5′-CCA CTG GGC TTG CAG CTT CG-3′ (Forward) |
|               | 5′-CCG CTG CCG GCT ACT GAA AA-3′ (Reverse) |
| GFRA1         | 5′-CCA CTG GGC TTG CAG CTT CG-3′ (Forward) |
|               | 5′-ACG GGC AGC ATC CTG AAC TT-3′ (Reverse) |
| KCNJ6         | 5′-AAA ACT GAC TGG CAG CAG CC-3′ (Forward) |
|               | 5′-TGG TCC TGT AGT TAG CCG TT-3′ (Reverse) |
| Nestin        | 5′-CAC CAT CTT CCA GGA GGC AGA TG-3′ (Forward) |
| Nurr1         | 5′-TGTC TGG CGC TAT GGG CT-3′ (Forward)   |
| PITX3         | 5′-GAC CAG AGC ATC AGA AAA GGC-3′ (Forward) |
|               | 5′-GTC CAC AGC GGC ATC TCC TC-3′ (Reverse) |
| RET           | 5′-GGTC TTT GTG TCG TCC CAG-3′ (Forward)  |
|               | 5′-GAG GAG ATC TAG CCG ATG C-3′ (Reverse) |
| SYP           | 5′-GCA GGT CGA GTT CCA GTG TCT CC-3′ (Forward) |
|               | 5′-AAT TCG GCT GAC GAG GAG-3′ (Reverse)   |
| SYN1          | 5′-GAC CTT CTA CCC CCA CAA CTA-3′ (Forward) |
| TIF3          | 5′-GCT TCG GAA GAC GTC GTG-3′ (Reverse)   |
| TH            | 5′-GCC GAG GAA GCT GAT TGC TGG-3′ (Forward) |
|               | 5′-AGT CCG CCA CTT CGT GST TG-3′ (Reverse) |
| Tuj1          | 5′-GCC CCA GTA TGA GGG AGA T-3′ (Forward)  |
| VLM3          | 5′-TGG GGA GGC TGC TTG GTG CT-3′ (Forward) |
|               | 5′-CCC ATA GAC GGA CAC GTG CC-3′ (Reverse) |

Immunostaining

Cells were differentiated and re-plated in 96-well plates. On the day of the experiment, cells were fixed with 4% PFA for 10 min, permeated with 0.1% Triton X-100 for 10 min, and blocked with 5% BSA for 2 h at room temperature. Cells were stained with primary antibodies at 4 °C overnight followed by secondary antibody for 1 h at room temperature. Hoechst (Invitrogen, USA) was used for nuclear staining. Images were taken using a Nikon A1R Confocal Microscope.

Primary antibodies used in the study included anti-TH antibody (Millipore, USA, 1:1000 dilution), anti-Tuj1 antibody (Millipore, USA, 1:1000 dilution), anti-MAP2 antibody (Sigma, USA, 1:1000 dilution), anti-α-synuclein antibody (Sigma, USA, 1:1000 dilution). Secondary antibodies used in the study included goat anti-mouse IgG Alexa546 (Invitrogen, USA, 1:1000 dilution), goat anti-rabbit IgG Alexa488 (Invitrogen, USA, 1:1000 dilution).

MPP⁺-induced cytotoxicity

Cells were differentiated and re-plated in 96-well plates. On d 6 of the differentiation, cells were treated with various concentration of neurotoxin MPP⁺ (Sigma, USA) for 2 d. Cell viability was determined by measuring intracellular ATP levels. Intracellular ATP levels were measured using CellTiter-Glo Luminescent Cell Viability assay kit (Promega, USA). Cell apoptosis was determined by measuring the activity of caspase 3/7. Caspase 3/7 activity was measured using Caspase-Glo 3/7 assay kit (Promega, USA). For testing reference compounds, on d 6 of the differentiation, cells were co-treated with 80 μmol/L MPP⁺ together with various concentration of compound SB216763 (Sigma, USA) or compound GW8510 (Sigma, USA) or their vehicle for 2 d. ATP levels and caspase 3/7 activity were measured. Immunostaining of Tuj1 was also performed to study morphological changes after the treatment.

Overexpression of α-synuclein-induced cytotoxicity

Cells were differentiated in 100-mm dishes for 2 d, and infected by Baculovirus containing α-synuclein gene (Virus prepared in house) or the virus containing GFP gene (Virus prepared in house) for 4 h. The infected cells were trypsinized and re-plated onto pre-coated 96-well plates. The cells were maintained in the differentiation medium. Cell viability was determined by intracellular ATP levels and cell apoptosis was determined by caspase 3/7 activity at d 3 and d 6 of the differentiation (1 d and 4 d after the infection). Immunostaining of α-synuclein was also performed. For reference compound treatment, after the cells were re-plated onto 96-well plates, the cells were treated with 1 μmol/L of compound SB216763 (Sigma, USA) or compound GW8510 (Sigma, USA) for 1 d or 4 d.

Rat primary dopaminergic neuron culture and MPP⁺ treatment

Rat primary dopaminergic neurons were cultured from ventral mesencephalon of E13 embryos[22]. The cells were seeded onto 384-well plates (2×10⁴ cells per well). On the 7th day in vitro (DIV7), the cells were treated with 100 μmol/L MPP⁺ for 24 h. The culture medium containing MPP⁺ was then completely replaced with the medium containing various concentration of compound GW8510. Four days after the compound treatment, the cells were immunostained with TH antibody. The total intensity of TH immune-reactivity was quantified by Acumen.

Statistical analysis

Data were presented as mean±SD. The results were subjected to one-way ANOVA followed by Dunnett’s test or Student’s t-test to determine statistical significance using GraphPad Prism v5.00. P<0.05 was considered as being significant. Dose-response curves were fitted by Origin 9.0 using 5-parametric logistic algorism to determine IC₅₀ or EC₅₀ value.

Results

Differentiated LUHMES cells expressed dopaminergic specific markers and displayed neuronal morphology

Before setting up the screening assays, the dopaminergic
nature of LUHMES cells after differentiation was studied and confirmed. Following similar differentiation protocol as described in the study published by Scholz et al[10], LUHMES cells were differentiated in the culture medium containing tetracycline (to suppress v-myc gene expression) and cAMP/GDNF (to promote dopaminergic features). The cells were differentiated in 100-mm dishes for two days and then re-plated in 6-well plates for another 4 d of differentiations (Figure 1A).

At d 0, d 2, and d 6 during the differentiation, protein and RNA samples were collected for Western blot and real-time PCR (RT-PCR) analyses to understand the expression level of neuronal markers and, more importantly, dopaminergic neuron specific markers.

For RT-PCR study, 4 neuronal markers (Potassium inwardly-rectifying channel, subfamily J, member 6, KCNJ6; Synapsin I, SYN1; Synaptophysin, SYP; Tubulin beta-3, Tuj1) were studied. The mRNA level of these markers increased dramatically during the whole differentiation process (Figure 1A and Table 2), suggesting that these cells were steered toward post-mitotic neuron-like cells. A panel of dopaminergic-neuron specific genes was then further analyzed. Three dopaminergic neuron markers tyrosine hydroxylase (TH), dopamine transporter (DAT), and nuclear receptor related 1 (Nurr1) showed different expression profiles during differentiation (Figure 1B). The expression of TH only increased dramatically (about 56 fold compared to d 0) on d 6, while DAT mRNA levels peaked on d 2 (about 43 fold compared to d 0) and went down on d 6. Nurr1 mRNA levels were generally not changed during differentiation, with only a slight increase at d 2 (2 fold compared to d 0). In addition to the three genes, the mRNA levels of other dopaminergic neuron markers including GDNF receptor (rearranged during transfection, RET and GDNF family receptor alpha-1, GFRα1), aromatic L-amino acid decarboxylase (AADC), DAT, engrailed-1 (EN1), dopamine D2 receptor (DRD2), vesicular monoamine transporter 2 (VMAT2) were also markedly enhanced at either d 2, d 6, or both days during differentiation (Table 2). Interestingly, the expression of dopaminergic neuron-specific transcription factor pituitary homeobox 3 (PITX3) was not changed significantly during the whole differentiation process.

The change of mRNA levels was further corroborated by protein levels measured by Western blot. TH protein levels were significantly increased at d 6, while the level of Nurr1 was not obviously changed. Tuj1 protein gradually increased during the whole differentiation process, consistent with mRNA results (Figure 1C).

To morphologically characterize the differentiated LUHMES cells, immunostaining was performed on the cells after 6-d differentiation. The undifferentiated cells generally did not contain neurites (data not shown). After differentiation, extensive neurites were grown as assessed by microtubule-associated protein 2 (MAP2) or Tuj1 staining (Figure 1D). In contrast to homogenous expression of MAP2 and Tuj1, TH was expressed highly only in a portion of the differentiated LUHMES cells (Figure 1D). The reason for this heterogeneous expression pattern of TH is unclear.

Table 2. mRNA level of the genes during differentiation. Besides GAPDH mRNA levels, all data were normalized to GAPDH first and then normalized to d 0 mRNA levels and presented as mean fold change±SD (n=2/group). GAPDH mRNA levels were normalized to its d 0 mRNA levels and presented as mean fold change±SD (n=2/group). The cycle threshold value (CT) value of RT-PCR was also provided as mean Ct value±SD.

| Gene       | d 0            | d 2 Mean fold change versus d 0±SD (means Ct value±SD) | d 6            |
|------------|----------------|--------------------------------------------------------|----------------|
| GAPDH      | 1.00±0.02 (17.91±0.27) | 0.47±0.01 (18.90±0.27)                                 | 0.35±0.05 (19.52±0.32) |
| KCNJ6      | 1.00±0.14 (33.76±0.48) | 240.52±29.50 (26.83±0.30)                              | 571.81±98.00 (26.21±0.24) |
| RET        | 1.00±0.40 (34.02±0.36) | 1.12±0.22 (34.79±0.31)                                 | 7.04±2.54 (32.81±0.32) |
| AADC       | 1.00±0.17 (34.70±0.53) | 0.59±0.42 (36.74±1.11)                                 | 43.33±8.38 (30.88±0.48) |
| Nestin     | 1.00±0.24 (26.25±0.64) | 0.10±0.01 (30.53±0.32)                                 | 3.03±0.62 (26.26±0.61) |
| GFRA1      | 1.00±0.10 (26.05±0.16) | 1.98±0.43 (26.07±0.31)                                 | 32.27±2.89 (22.65±0.36) |
| SYN1       | 1.00±0.33 (34.80±0.75) | 540.60±90.69 (26.66±0.33)                              | 4083.39±384.86 (24.35±0.44) |
| EN1        | 1.00±0.12 (24.90±0.13) | 0.38±0.08 (26.34±0.35)                                 | 0.77±0.22 (25.29±0.26) |
| SYP        | 1.00±0.12 (27.37±0.22) | 13.87±0.47 (24.55±0.14)                                | 25.95±2.80 (24.28±0.16) |
| DRD2       | 1.00±0.13 (30.28±0.16) | 235.89±29.03 (23.48±0.34)                              | 1407.76±60.46 (21.36±0.23) |
| PITX3      | 1.00±0.50 (32.15±0.80) | 0.57±0.26 (33.99±0.67)                                 | 1.18±0.58 (33.45±0.47) |
| VMAT2      | 1.00±0.57 (31.05±0.87) | 1.45±0.14 (31.45±0.09)                                 | 4.81±0.51 (30.19±0.28) |
| TUJ1       | 1.00±0.09 (22.29±0.34) | 15.93±1.62 (19.38±0.26)                                | 21.90±2.20 (19.39±0.36) |
| NURR1      | 1.00±0.20 (26.36±0.26) | 1.99±0.17 (26.43±0.30)                                 | 0.92±0.08 (28.00±0.15) |
| DAT        | 1.00±0.07 (26.36±0.19) | 43.12±15.70 (24.36±0.63)                               | 3.04±0.29 (28.57±0.36) |
| TH         | 1.00±0.73 (33.63±0.91) | 0.48±0.09 (35.49±0.43)                                 | 56.33±8.15 (29.06±0.39) |

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; KCNJ6, Potassium inwardly-rectifying channel, subfamily J, member 6; RET, Rearranged during transfection; AADC, Aromatic L-amino acid decarboxylase; GFRα1, GDNF family receptor alpha-1; SYN1, Synapsin I; EN1, Engrailed-1; SYP, Synaptophysin; DRD2, Dopamine D2 receptor; PITX3, Pituitary homeobox 3; VMAT2, Vesicular monoamine transporter 2; TUJ1, Tubulin beta-3; NURR1, Nuclear receptor related 1; DAT, dopamine transporter; TH, Tyrosine hydroxylase.
MPP⁺ dose dependently induced cytotoxicity in differentiated LUHMES cells

To establish a MPP⁺-induced cytotoxicity assay, LUHMES cells were differentiated and re-plated in a 96-well plate. On d 6 of the differentiation, LUHMES cells were treated with increasing concentrations of MPP⁺ ranging from 0.69 μmol/L to 4.5 mmol/L (1:3 serial dilutions starting from 4.5 mmol/L). After 2 d treatment, intracellular ATP levels were measured to study cell viability. MPP⁺ induced a very sharp drop down of ATP levels between 55 μmol/L and 167 μmol/L, with no change of ATP levels at 55 μmol/L but almost 100% ATP loss at 167 μmol/L (Figure 2A, left panel). Based on the dose response curve, the half-maximal inhibitory concentration (IC₅₀) of MPP⁺ to induce ATP loss was calculated as 65 μmol/L. In order to create a sublethal cytotoxicity, we further diluted MPP⁺ solution with smaller concentration interval. Differentiated LUHMES cells were treated with 60, 70, 80, 90, or 100 μmol/L MPP⁺ for 2 d. MPP⁺ induced a dose dependent reduction of ATP levels compared to the vehicle treatment (Figure 2A, right panel). There was certain variation in this study with coefficient of variation value higher than 20% for most of MPP⁺-treated groups. With this variation, we finally decided to use 80 μmol/L MPP⁺ for further experiments, as it was the lowest concentration of MPP⁺ to consistently generate sublethal cytotoxicity with sufficient assay window (about 80% loss of intracellular ATP).

The cytotoxic effect of MPP⁺ was further confirmed by measuring intracellular caspase 3 and caspase 7 activities, an indication of cell apoptosis. MPP⁺ (80 μmol/L) caused a significant increase of caspase 3/7 activity compared to vehicle (Figure 2B). Cell morphology was also markedly changed after 80 μmol/L MPP⁺ treatment for 2 d. As demonstrated by immunostaining of neuronal marker Tuj1, cells in the vehicle group displayed extensive Tuj1 positive neurites while in MPP⁺-treated group, due to significant cell death, most of the cells were washed off during the staining process. For the limited remaining cells, neurites were significantly degenerated.

Thus, we have developed a MPP⁺-induced cytotoxicity model...
in differentiated LUHMES cells and confirmed cell degeneration both biochemically and morphologically.

**MPP⁺-induced cytotoxicity was rescued by a CDK2 inhibitor or a GSK3β inhibitor**

A good cytotoxicity screening assay for potential neuroprotective treatments should have a reproducible robust assay window. It also should be validated using reference pharmaceutical compounds to confirm that the induced cell death could be rescued. In order to further demonstrate the validity of MPP⁺-induced cytotoxicity assay in differentiated LUHMES cells, we cherry-picked 35 commercially available compounds, which may exert neuroprotective effect as suggested by the literature, as the candidate of reference compounds for the assay.

LUHMES cells were cultured and differentiated for 6 d to become dopaminergic-like cells. The differentiated cells were then co-treated with 80 µmol/L MPP⁺ and 1 µmol/L of the compounds or vehicle control for two days. ATP levels were subsequently measured. Among the 35 compounds tested, several compounds showed significant cytotoxicity at the 1 µmol/L, which made it impossible to conclude whether they had neuroprotective effect or not (Figure 3A and Table 3). Among the compounds which were not cytotoxic at the tested concentration, a cyclin-dependent kinase (CDK) inhibitor GW8510 and a glycogen synthase kinase-3 beta (GSK-3β) inhibitor SB216763 consistently showed robust protective effects against MPP⁺-induce cytotoxicity (Figure 3A and Table 3). Therefore, we chose to further confirm the protective effect of these two compounds in a dose response study. There were other compounds which showed positive effect at 1 µmol/L (Figure 3A and Table 3), although the effect was not as robust as GW8510 or SB216763. Their effect will be further repeated and confirmed in future studies.

Compounds SB216763 and GW8510 both dose dependently rescued MPP⁺-induced ATP loss at the concentrations lower than 1 µmol/L (Figure 3B). The half-maximal effective concentration (EC₅₀) of the two compounds was calculated as 205 nmol/L for SB216763 and 12.3 nmol/L for GW8510. At the concentrations higher than 1 µmol/L, both compounds generated significant cytotoxicity.

To ensure that the effect of the two compounds was not restricted to modulating intracellular ATP levels, caspase 3/7 activity was further measured as an indication of cell apoptosis in the same experimental setting as that for the ATP study. MPP⁺ (80 µmol/L ) significantly increased caspase 3/7 activity. Both compounds at the tested three concentrations (0.11, 0.33, and 1 µmol/L) significantly ameliorated MPP⁺-induced cell apoptosis (Figure 3C). Furthermore, immunostaining of Tuj1 also suggested that the cells treated with 1 µmol/L of
Figure 3. (A) The percentage of protection against 80 μmol/L MPP⁺-induced toxicity of 35 commercially available compounds at 1 μmol/L, as determined by ATP levels. MPP⁺ alone group was normalized as 0% and vehicle group was normalized as 100%. Data were presented as mean±SD (n=5/group). (B) Dose-dependent protection by SB216763 and GW8510 against 80 μmol/L MPP⁺-induced toxicity. MPP⁺ alone group was normalized as 0% and vehicle group was normalized as 100%. Data were presented as mean±SD (n=3/group). (C) Caspase 3/7 activity of the cells co-treated with 80 μmol/L MPP⁺ and SB216763 or GW8510. Data were all normalized to vehicle group and presented as mean±SD (n=3/group). ³P<0.01 vs MPP⁺ alone group, one-way ANOVA followed by Dunnett’s test. (D) Immunocytochemistry of the cells co-treated with 80 μmol/L MPP⁺ and SB216763 or GW8510. TuJ1 (red), Nucleus (blue), magnification at ×200. Scale bar represents 50 μm. (E) Dose-dependent protection by GW8510 against MPP⁺-induced toxicity in rat primary dopaminergic neurons. TH was measured to assess the toxicity. MPP⁺ alone group was normalized as 0% and vehicle group was normalized as 100%. Data were presented as mean±SD (n=2/group).
Table 3. The Percentage of protection of 1 μmol/L compound against 80 μmol/L MPP⁺-induced intracellular ATP loss in the differentiated LUHMES cells. MPP⁺ alone group was normalized as 0% and vehicle group was normalized as 100%. Data were presented as mean±SD (n=5/group).

| Compound | Vender                  | Protection % (at 1 μmol/L) (mean±SD, n=5/group) |
|----------|-------------------------|-----------------------------------------------|
| SCH442416| Sigma                   | -1.19±0.02                                    |
| PJ34     | Sigma                   | -0.08±0.89                                    |
| Benzoylcarbonyl-Val-Ala-Asp (0Me) fluoromethylketone | EMD4Biosciences | 0.68±2.78                                   |
| SB216763 | Sigma                   | 104.63±13.37                                  |
| AR-A014418 | Sigma                | -1.14±0.03                                    |
| Simvastatin | Sigma              | 2.03±9.37                                     |
| Selegiline/deprenyl | Sigma | -0.43±1.61                                    |
| Roscovitine | Sigma                | -5.17±0.55                                    |
| GW7647   | Sigma                   | -6.16±0.00                                    |
| Fenofibrate | sigma               | -2.79±2.83                                    |
| Pioglitazone | Sigma              | -31.50±0.14                                   |
| 5-Aminomidazole-4-carboxamide ribonucleotide | Sigma | -30.84±0.26                                   |
| Isradipine | Sigma                | -31.00±0.20                                   |
| Nimodipine | Sigma                | -29.88±1.73                                   |
| (R)(+)-WIN 55212-2 mesylate salt | Sigma | -28.86±4.68                                   |
| AS-252424 | Sigma                  | 22.15±2.09                                    |
| Ellagic acid | Sigma               | 38.13±3.14                                    |
| PF-3845  | Cayman chemical        | -13.74±8.08                                   |
| N-nonyl-deoxyojirimycin | Sigma | 34.12±4.81                                    |
| N-Dodecyl-1-deoxyojirimycin | Sigma | 58.35±0.61                                    |
| PD166793 | Sigma                   | 44.35±34.49                                   |
| CP-471474 | Sigma                  | 54.89±21.17                                   |
| RS 504393 | ToCRis bioscience    | -126.37±25.79                                 |
| Bay 11-7085 | Sigma                | -146.49±0.56                                  |
| Celastrol | Sigma                  | -133.68±1.20                                  |
| Desipramine | Sigma               | 7.73±3.89                                     |
| Flupirtine maleate salt | Sigma | -0.17±4.72                                    |
| Imipramine hydrochloride | Sigma | 17.51±8.40                                    |
| Eicosapentaenoic acid | Enzo     | -21.28±19.79                                  |
| Valproic acid sodium salt | Enzo   | 41.12±18.61                                   |
| SU4312   | Enzo                    | 7.36±3.70                                     |
| Coenzyme Q10 | Sigma              | 5.34±4.27                                     |
| alpha-Lipoic acid | Sigma | 10.34±7.03                                    |
| O-Acetyl-L-carnitine hydrochloride | Sigma | 19.62±5.16                                    |
| GW8510   | Sigma                   | 154.26±14.84                                  |

from differentiated LUHMES cells, GW8510 dose dependently rescued MPP⁺-induced TH loss with an EC₅₀ of 110 nmol/L (Figure 3E).

**Rescue from cell death in differentiated LUHMES cells induced by overexpression human α-synuclein with a CDK2 inhibitor**

Though MPP⁺-induced cytotoxicity assays have been commonly used in the research area of PD, they usually only reflect mechanisms specifically associated with mitochondrial dysfunction. To explore other mechanisms possibly involved in dopaminergic neuron death and to establish a potentially more translatable cellular assay, we overexpressed human α-synuclein in differentiated LUHMES cells to induce cytotoxicity.

In this study, LUHMES cells were differentiated in 100-mm dishes for two days and infected by baculovirus to overexpress human wild type α-synuclein at different multiplicity of infection (MOI) (Figure 4A). Virus carrying GFP was used as the control. Four hours after the infection, the cells were re-plated in 96-well plates and cultured for additional days. α-Synuclein was markedly over-expressed with increasing MOI (Figure 4D). Significant cytotoxicity was observed in the cells infected with α-synuclein baculovirus. Higher level of α-synuclein overexpression was associated with lower level of intracellular ATP, which appeared to be progressive between d 3 and d 6 after the start of differentiation (1 d and 4 d after infection) (Figure 4B). Overexpression of α-synuclein also increased caspase 3/7 activity at three MOI tested, suggesting cell apoptosis was triggered. The increase of caspase 3/7 activity by α-synuclein overexpression was less marked on d 6 (d 4 after infection), possibly due to a significant increase of baseline level of caspase 3/7 at d 6 compared to d 3 (Figure 4B).

After establishing α-synuclein overexpression-induced cytotoxicity, we tried to validate the assay using the two reference compounds tested in MPP⁺-induced cytotoxicity assay. Immediately after the infected cells were re-plated into 96-well plate, these cells were treated with 1 μmol/L of the CDK inhibitor GW8510 or 1 μmol/L of the GSK-3β inhibitor SB216763 and cell survival was measured at d 3 and d 6 after the start of differentiation (1 d and 4 d after infection). The expression of α-synuclein was not markedly affected by the treatment of compound either GW8510 or SB216763, as suggested by immunostaining (Figure 4E). The two compounds showed consistent effect against α-synuclein overexpression-induced cytotoxicity at three MOI tested. For simplicity, the results for MOI of 50 were shown. At MOI of 50, both compounds at 1 μmol/L significantly rescued α-synuclein overexpression-induced cytotoxicity as assessed by intracellular ATP levels (Figure 4C). With regard to cell apoptosis, GW8510 effectively normalized α-synuclein overexpression-induced increase of caspase 3/7 activity at both tested time points. However, compound SB216763 only reduced caspase 3/7 activity at d 3, while further enhancing it at d 6. The exact reason for this effect of compound SB216763 was unclear.

To understand the relevance of differentiated LUHMES cell to primary dopaminergic neurons, we further tested the effect of CDK inhibitor GW8510 against MPP⁺-induced toxicity in rat primary dopaminergic neurons. Consistent with the results either compound dramatically rescued MPP⁺-induced neurite degeneration and cell death (Figure 3D). In conclusion, we validated MPP⁺-induced cytotoxicity assay in differentiated LUHMES cells using a CDK inhibitor GW8510 and a GSK-3β inhibitor SB216763.

To understand the relevance of differentiated LUHMES cell to primary dopaminergic neurons, we further tested the effect of CDK inhibitor GW8510 against MPP⁺-induced toxicity in rat primary dopaminergic neurons. Consistent with the results...
Figure 4. (A) Schematic diagram of cell differentiation, BacMam virus infection and compound treatment. (B) Intracellular ATP level (Left) and caspase 3/7 activity (right) of differentiated LUHMES cells infected with GFP or α-synuclein BacMam virus at different MOI. Data were all normalized to the d 3 medium control group, and presented as mean±SD (n=6 for medium control group, n=3 for the rest of groups), *P<0.01, Student’s t-test. (C) Intracellular ATP levels (left) and caspase 3/7 activity (right) of differentiated LUHMES cells infected with GFP or α-synuclein BacMam virus (MOI of 50) and treated with the reference compounds. Data were all normalized to d 3 medium control group, and presented as mean±SD (n=6 for medium control group, n=3 for the rest of groups), *P<0.01, Student’s t-test. (D) Immunocytochemistry of differentiated LUHMES cells infected with α-synuclein BacMam virus at different MOI. α-Synuclein (red), Nucleus (blue), magnification at ×200. Scale bar represents 100 μm. (E) Immunocytochemistry of differentiated LUHMES cells infected with α-synuclein BacMam virus at MOI of 50 and treated with the reference compounds. α-Synuclein (red), Nucleus (blue), magnification at ×200. Scale bar represents 100 μm.
Discussion

PD is primarily caused by the progressive degeneration of dopaminergic neurons in the substantia nigra\(^3\). To identify a neuroprotective therapeutic agent, a cellular system that recapitulates most aspects of neurodegeneration is highly valuable. Though LUHMES human mesencephalic neuron-derived cells have not been widely used for PD research or drug discovery, they do have multiple advantages over other cellular systems. Firstly, they originated from human tissue, and therefore may be preferred to other non-human cell lines when studying human diseases. Secondly, they are derived from mesencephalic neurons, and display dopaminergic features after differentiation. Given the difficulty of obtaining and culturing primary dopaminergic neurons from the brains of post mortem adult/elderly people, LUHMES may offer an alternative system to study dopaminergic neuron degeneration in vitro. Thirdly, the feasibility of large-scale generation of post-mitotic differentiated LUHMES cells with good consistency\(^{24}\) makes it possible to establish screening assays to look for neuroprotective compounds or targets. Therefore, in this study, we confirmed the dopaminergic nature of LUHMES cells and established the relevant cytotoxicity assays, in the hope of facilitating drug discovery for PD.

Consistent with what has been reported previously\(^{14}\), after differentiation, LUHMES expressed multiple dopaminergic neuron specific markers including TH, DAT, Nurr-1, etc (Figure 1B, and Table 2). Morphologically, the cells grew extensive neurites, which highly resembled primary neurons. Functionally, though we did not characterize the dopamine uptake capability of these differentiated cells, the sensitivity of the cells to MPP\(^+\) indirectly demonstrated that functional DAT was expressed on the cell membrane after differentiation.

MPP\(^+\) induced a dose dependent cytotoxicity as measured by intracellular ATP levels and caspase 3/7 activity, which could be rescued by a CDK inhibitor and a GSK-3β inhibitor (Figure 3). CDKs and GSK3β have been reported to be involved in neurodegeneration in PD\(^{23, 24}\). For example, neurotoxins activate cyclin E, phosphorylate Rb, activate CDKs and ultimately cause neuronal death via a mitochondrial death pathway\(^{25}\). Inhibition of CDKs using a pan inhibitor exerts neuroprotective effect against MPP\(^+\)-induced neuronal toxicity\(^{26}\). Similarly, GSK-3β is also activated by treatment with rotenone or MPP\(^+\), and inhibition of its activity pharmacologically or genetically has been suggested to be neuroprotective\(^{23, 27}\). Thus, it is not surprising for us to see that a CDK inhibitor and a GSK-3β inhibitor protected the differentiated LUHMES cells against MPP\(^+\)-induced cytotoxicity. These results not only are in line with literature reports\(^{25, 26}\), but also demonstrate the validity and reversibility of the assay, paving the way for future drug screening.

It should be noted that besides the CDK inhibitor and the GSK-3β inhibitor, several other compounds also showed positive effect at 1 µmol/L against MPP\(^+\)-induced toxicity (Figure 3A and Table 3). Dose response confirmation has not been carried out for these compounds. Thus, it is too early to conclude that they are neuroprotective.

In addition to MPP\(^+\), overexpression of wild type human α-synuclein also induced significant cytotoxicity in the differentiated LUHMES cells, which was rescued by the CDK inhibitor GW8510 (Figure 4). α-Synuclein is the major component of Lewy bodies, the pathological hallmark of PD\(^{28, 29}\). Although it is still unclear whether/how the abnormality of α-synuclein causes cell death, our assay provides an in vitro tool for screening compounds that could ameliorate α-synuclein-induced toxicity and studying the molecular mechanism associated with high expression of α-synuclein. Additional characterization of the current assay is needed to further enhance its robustness, relevance and translatability. For example, it is worthwhile to study whether any intracellular α-synuclein aggregates are formed after the overexpression. Another interesting area to explore is to understand whether α-synuclein mutants (A53T, A30P, E46K\(^{18}\)), which are found in familial PD, have differential role on cell viability compared to WT α-synuclein. Furthermore, recent studies have revealed that α-synuclein aggregates could be transmitted among neurons, and there as seeds to induce intracellular α-synuclein aggregation\(^{30, 31}\). Though we have not characterized whether there is any α-synuclein transmission in our assay system, we did observe that the non-infected cells also died after the treatment with baculovirus (data not shown).

Schildknecht et al have recently reported a stable α-synuclein-overexpressing LUHMES subclone\(^{32}\). In their characterization, the increased amount of α-synuclein in LUHMES cells was well tolerated and no cytotoxicity was observed after overexpression of α-synuclein. This is clearly different from what we have observed, where significant cytotoxicity was induced after overexpression of wild type α-synuclein. The discrepancy could be explained by the different methodologies used to establish α-synuclein overexpression system. In Schildknecht et al study, the stable cell line was generated after lentiviral infection, while in our study, transient overexpression using baculovirus was performed. Though without direct comparison, the transient overexpression may generate much higher level of α-synuclein and induce cytotoxicity thereafter. In addition, the time point for virus infection was also different in the two studies. Schildknecht et al infected the undifferentiated cells (d 0), while we infected the cells after two days (d 2) of differentiation by GDNF and cAMP. At d 2 during differentiation, the expression level of certain neuronal markers (eg, Tuj-1) has already been increased and LUHMES cells have been gradually differentiated into post-mitotic cells. Thus, different differentiation stage may also possibly affect the susceptibility of LUHMES cells to α-synuclein overexpression.

As with previously described cellular systems used for PD research, this LUHMES cell assay also has limitations. After differentiation using cAMP and GDNF, the LUHMES cells displayed a heterogeneous expression pattern of TH, which is inconsistent with what has been reported previously\(^{14}\) (Figure 1C). One explanation could be the difference of cell culture medium used in our studies (DMEM/F12 in our study, and advanced DMEM/F12 in the published study\(^{14}\)). Another
limitation/gap of the current assay system is the lack of a thorough understanding of the relevance and, more importantly, the difference between differentiated LUHMES cells and neurons. Although we have demonstrated that the differentiated LUHMES cells expressed many dopaminergic markers and displayed certain dopaminergic functions, it is unknown whether the regulatory signaling pathways in dopaminergic neurons in vivo have all been captured in LUHMES cells after differentiation. This could be a potential risk for drug discovery, especially when screening compounds/targets against certain cellular phenotypes, such as cell death. To mitigate the risk, after the screen using differentiated LUHMES cells, it is necessary to follow up and confirm the hits by cellular assays using rodent primary neurons or human induced pluripotent stem cells (IPSc)-derived neurons and in vivo animal models for PD.

In summary, we have established the assays of MPP⁺-induced and α-synuclein overexpression-induced cytotoxicity in differentiated LUHMES cells. Multiple potential applications for these assays exist along the drug discovery cascade for PD. For instance, in target-based drug discovery, these assays could be used as the functional readouts to rapidly screen inhibitors or activators of certain drug targets and confirm their neuroprotective effect. Alternatively, these assays could also be used as the primary phenotypic assay to screen a library of compounds (with or without target annotation) for the ones that halt or delay neurodegeneration. The hits from the screen can then be followed up to deconvolute the drug target to facilitate a target-based drug discovery campaign for PD.

Author contribution
Xiao-min ZHANG and Min-hua ZHANG designed research; Xiao-min ZHANG and Min-hua ZHANG performed research; Xiao-min ZHANG analyzed data; Xiao-min ZHANG and Min-hua ZHANG wrote the paper; Xiao-min ZHANG, Ming YIN, and Min-hua ZHANG reviewed the paper.

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