Tenuigenin Attenuates α -Synuclein-Induced Cytotoxicity by Down-Regulating Polo-Like Kinase 3

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SUMMARY

Background and aims: Tenuigenin (Ten) is a Chinese herbal extract with antioxidative and antiinflammatory effects on toxin-induced cell models of Parkinson's disease (PD); however, its effects on α -synuclein toxicity-based PD models remain unknown. α -synuclein hyperphosphorylation is a key event in PD pathogenesis and potential target of therapeutic interventions. We tested whether Ten alleviates α -synuclein-induced cytotoxicity via reducing kinases that phosphorylate α -synuclein. **Methods:** SH-SY5Y cells transiently transfected with wild-type or A53T mutant α -synuclein were used to evaluate the effect of Ten on the levels of α -synuclein phosphorylation-related kinases. Cells treated with 10 μ M Ten for 24 h were measured for viability (proliferation and apoptosis assays) and cellular proteins harvested and fractioned. The levels of total and phosphorylated α -synuclein and five associated kinases (polo-like kinase [PLK] 1–3, casein kinase [CK] 1–2) were evaluated by Western blotting. **Results:** Overexpression of either wild-type or A53T mutant α -synuclein decreased cell viability and increased α -synuclein phosphorylation. Ten treatment-protected cells from this α -synuclein-induced toxicity and dramatically reduced α -synuclein phosphorylation and PLK3 (but not other kinase) levels. **Conclusion:** In α -synuclein cell model of PD, Ten is effective in attenuating α -synuclein-induced toxicity and α -synuclein phosphorylation probably via targeting PLK3, suggesting it could be an efficient therapeutic drug to treat α -synuclein-related neurodegeneration.

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The first two authors contributed equally to this work.

Introduction

Tenuigenin (Ten) is a natural extract from the *Polygala tenuifolia* root, a traditional Chinese herb that has been widely prescribed in traditional Chinese medicine for amnesia, neurasthenia, palpitation, insomnia, and cognitive dysfunction for thousands of years. Two random, double-blind clinical studies show that Ten improves cognitive function in the elderly [1] and enhances memory in healthy humans [1,2] demonstrating a neurotrophic function under normal conditions. Moreover, laboratory studies show that Ten attenuates stress-induced memory impairments [3] and amyloid- β -induced toxicity both *in vivo* and *in vitro* [4,5], indicating a neuroprotective effect in models of Alzheimer's disease and related cognitive disorders. The recent finding that Ten alleviates

toxin-induced (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP], 1-methyl-4-phenylpyridinium [MPP⁺], 6-hydroxydopamine [6-OHDA], and lipopolysaccharides [LPS]) models of dopaminergic neuronal injury [5–9], raises the possibility of its therapeutic benefit for patients with Parkinson's disease (PD). However, such data cannot distinguish whether Ten has a generic effect on cell loss pathways [10,11] or also acting on any disease specific mechanism.

Cellular changes in α -synuclein characterize PD, with genetic studies demonstrating that familial PD can be caused by multiplications (duplication and triplication) of or mutations (A53T, A30P, and E46K) in the α -synuclein gene [12–14]. Hence, cells or animals overexpressing α -synuclein (wild-type or mutant) are the most commonly used models to study PD pathogenesis and therapeutic

interventions [10,11,15]. α -Synuclein is a small (140 amino acid), presynaptic, cytosolic protein [16] important in the regulation of dopamine release, vesicle recycling, and synaptic plasticity [17]. In PD, α -synuclein pathologically accumulates and aggregates into cytoplasmic inclusions called Lewy bodies (LB) [18]. While there is strong evidence from genetic multiplications that cellular toxicity is caused by simply increasing the monomeric form of α -synuclein [13,19], an increasing number of studies suggest α -synuclein may need to be post-translationally modified to acquire its pathological properties [20,21] especially phosphorylation at Ser129 which is associated most with LB formation [22,23]. Several kinase families have been identified as capable of phosphorylating α -synuclein at Ser129, including polo-like kinases (PLK1-3) [24,25], casein kinases (CK1-2) [26], and G-protein coupled receptor kinases (GRK1, 2, 5, and 6) [27]. Whether Ten affects these α -synuclein-related pathways remains unknown, although such data would appear essential prior to suggesting any potential therapeutic benefit for patients with PD. The current study aims to test whether Ten can alleviate α -synuclein-induced cellular toxicity and the levels of any of the kinases involved in its phosphorylation.

Materials and Methods

Cell Culture Model

Human neuroblastoma SH-SY5Y cells (Peking Union Medical College, Beijing, China) were maintained in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12; GIBCO, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; GIBCO) at 37°C in 5% CO₂. pcDNA3.1 plasmid expressing human wild-type α -synuclein has been previously described [28], and pcDNA3.1 plasmid expressing A53T mutant was constructed by introducing the G209A via site-directed mutagenesis kit (Promega, Madison, WI, USA). All constructs were confirmed by sequencing. SH-SY5Y cells were plated and when 70–80% confluent transiently transfected with pcDNA3.1 plasmids expressing human wild-type or A53T mutant α -synuclein using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Plasmids encoding the pcDNA3.1 vector (Invitrogen) alone were transfected into cells as controls using the same protocol. Opti-medium (GIBCO) was changed 6 h after transfection. To determine transfection efficiency, SH-SY5Y cells were also transfected with plasmids expressing GFP-tagged α -synuclein (kind gifts from P.J. McLean, Massachusetts General Hospital East, Charlestown, MA, USA) using the same procedures, revealing that around 40–50% transfection efficiency was achieved.

Drug Treatment Regimen

Ten powder (molecular formula C₃₀H₄₅ClO₆, average molecular weight 537.14 kDa) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (NICBP, 111572-200702) with a purity of 98.7%. Twenty milligram of Ten powder was dissolved in 500 μ L of dimethyl sulfoxide (DMSO) and filtered through a 0.22 μ m filter (Millipore, Billerica, MA, USA) to prepare a 0.74 M Ten stock solution. Ten working

solutions were freshly prepared by diluting the stock solution in DMEM/F12 with 5% FBS. Control solutions were prepared by adding the same volume of DMSO to DMEM/F12 with 5% FBS. To determine the optimal dose of Ten, SH-SY5Y cells were directly incubated with 0.1 μ M to 1 mM Ten working solutions or related control solutions. For the drug treatment of the cell culture model, Opti-medium was changed into optimized Ten working solutions or related control solution 6 h after transfection and the cells grown for another 24 h prior to harvesting.

Outcome Measures and Analyses

Cell Viability (Proliferation and Apoptosis) Assays

Cell proliferation was assessed using a cell proliferation assay (CellTiter 96 AQ; Promega) according to the manufacture's protocol. Briefly, SH-SY5Y cells were seeded at a density of 1.5×10^4 cells/well in 96-well plates. After transfection and drug treatment, previous medium was removed from cells and replaced by 100 μ L mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) with phenazine methosulfate (20:1) diluted in culture medium (1:10). Cells were then incubated for 1–3 h at 37°C with protection from light prior to measuring the 490 nm light absorbance using a microplate reader (PerkinElmer Inc, Waltham, MA, USA).

Cell apoptosis was determined using an FITC Annexin V Apoptosis Detection Kit (BD Bioscience, San Jose, CA, USA) according to the manufacture's protocol. Briefly, 1×10^5 cells were resuspended in $1 \times$ Binding buffer and incubated with the mixture of 5 μ L of FITC Annexin V and 5 μ L of propidium iodide for 15 min at room temperature in the dark. Detection and measurement of stained cells were performed by flow cytometry (GE Healthcare, Pittsburgh, PA, USA).

Western Blotting of Cellular Proteins

Cellular proteins from the SH-SY5Y cells were assessed whole or fractionated with Triton X-100 into Triton-soluble and Triton-insoluble protein extracts using different lysis buffers and centrifuge speeds. For whole cell lysates preparation, cells were harvested in RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA; Beyotime Institute of Biotechnology, Shanghai, China) with $1 \times$ protease inhibitor (Roche, Shanghai, China) and $1 \times$ phosphatase inhibitor (Roche) by sonicating for 10 s on ice, then centrifuging at 700 g for 15 min, and the supernatant was labeled as whole cell lysates.

For subcellular fractionation, detergent-soluble proteins were separated from detergent insoluble fractions by lysing cells in TBST buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) with $1 \times$ protease inhibitor (Roche) and $1 \times$ phosphatase inhibitor (Roche) by pipetting up and down for 10 times and incubating on ice for 30 min. The lysate was centrifuged at 16,000 g for 15 min at 4°C with the supernatant being the Triton-soluble fraction (also known as TBST fraction). The remaining pellet was washed in TBS buffer (50 mM Tris pH 7.4, 150 mM NaCl) and resuspended in SDS-Urea buffer (1% SDS, 6 mM Urea, 5 mM EDTA) with $1 \times$ protease inhibitor

(Roche) and $1\times$ phosphatase inhibitor (Roche), centrifuged at 16,000 g at room temperature with the supernatant being the Triton-insoluble fractions (also known as SDS fraction). Protein concentrations were determined using a BCA kit (Thermo Fisher Scientific Inc, Waltham, MA, USA).

Semiquantitative Western blotting was performed as previously described [29]. Briefly, equal amounts of protein sample were dissolved in an equal volume aliquot of $5\times$ Laemmli buffer (Beyotime Institute of Biotechnology), boiled at 95°C for 5 min and loaded onto SDS-PAGE gels. After separation, proteins were transferred onto $0.22\text{-}\mu\text{m}$ nitrocellulose filter membranes (Bio-Rad, Hercules, CA, USA). Blots were blocked with 5% milk in washing buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) and probed using the primary antibodies (anti- α -synuclein [syn-1, 1:2000; BD Transduction Laboratories, San Diego, CA, USA]; anti-Ser129-phospho- α -synuclein [S129P, 1:5000; Elan Pharmaceuticals, South San Francisco, CA, USA]; anti-PLK1 [1:300; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA]; anti-PLK2 [1:500; Santa Cruz Biotechnology Inc]; anti-PLK3 [1:1000; BD Transduction Laboratories]; anti-CK1 [1:500; Santa Cruz Biotechnology Inc]; anti-CK2 [1:500; Santa Cruz Biotechnology Inc]; and anti- β -actin [1:15,000; Sigma, St. Louis, MO, USA]) suspended in 5% milk in washing buffer at 4°C overnight. The antibody to β -actin was used as an internal control to ensure that the same amount of protein was loaded for each sample. Membranes were washed three times with washing buffer and probed with the appropriate fluorescently labeled secondary antibody (1:10,000; LI-COR Biosciences, Lincoln, NE, USA) suspended in 5% milk in washing buffer, applied for 1 h at room temperature. After washing, membranes were scanned using an Odyssey infrared imaging system (LI-COR Biosciences). Scanned images were exported in TIFF format, and the intensity of each band quantified with Quantity One software (Bio-Rad Laboratories) and related to the intensity of the β -actin band for that sample.

Statistical Analyses

SPSS 18 (IBM, Chicago, IL, USA) was used for all analyses, and significance established when P was <0.05 . Analysis of variance with Bonferroni post hoc protection was used to determine any differences between cell groups.

Results

α -Synuclein Overexpressing SH-SY5Y Cells Have Increased Phosphorylation of α -Synuclein at Ser129 and Reduced Cell Viability

As expected, cells overexpressing α -synuclein (either wild-type and A53T) had dramatic increases in the levels of total α -synuclein compared with vector controls (4.2–4.5-fold increase, Figure 1A–F). Intriguingly, while the levels of endogenous S129P α -synuclein in the vector controls were too low to detect, cells overexpressing α -synuclein (both wild type and A53T) had significant levels of S129P α -synuclein in all protein fractions extracted (Figure 1A–F). These increases in the levels and phosphorylation of α -synuclein in SH-SY5Y cells were associated

Figure 1 Characterization of the α -synuclein overexpressing SH-SY5Y cell models. SH-SY5Y cells were transiently transfected with wild-type α -synuclein, A53T mutant α -synuclein or pCDNA3.1 vector control for 24 h and harvested for semiquantitative Western blotting (A–F) or cell viability measurements (G, H). Representative Western blots of proteins prepared from whole cell lysates (A) or from serially extracted protein fractions (C, E) showing the increased expression of total α -synuclein (syn-1) and S129P α -synuclein relative to the loading control (β -actin) in the differently transfected cells (A, C, E). The relative quantitation of the data showed that both wild-type and A53T mutant α -synuclein transfections produced over a 4-fold increase in total cellular α -synuclein levels compared with vector controls (B, D, F). Note that endogenous α -synuclein is low and primarily in the more soluble TBST fraction (vector in c compared with e and d compared with f) and has little Ser129 phosphorylation. The relative increase in S129P α -synuclein is disproportionately higher than the increased level of total cellular α -synuclein, particularly in the more soluble fraction (D). Overexpression of wild-type and A53T mutant α -synuclein in SH-SY5Y cells significantly decreased proliferation (G) and increased apoptosis (H) compared with vector groups. Data are the mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$.

with decreased proliferation (by $18\text{--}23 \pm 4\%$ compared with the vector controls, $P < 0.001$; Figure 1G) and increased apoptosis (by $21\text{--}33 \pm 5\%$ compared with vector controls, $P \leq 0.003$; Figure 1H). No significant differences were observed between cells transfected with wild-type versus A53T mutant α -synuclein ($P > 0.57$).

Identification of Nontoxic Ten Concentrations for the Treatment of the Cell Model

SH-SY5Y cells were incubated with different concentrations of Ten working solutions from 0.1 μ M to 1 mM for 24 h. Cell viability assays showed that 1 mM Ten was toxic to SH-SY5Y cells, reducing cell viability by $53 \pm 2\%$ ($P < 0.001$, Figure 2A), 100 μ M Ten slightly affects cell viability but not significant, and 0.1–10 μ M of Ten was not toxic to SH-SY5Y cells (Figure 2A).

Figure 2 Effects of Ten treatment on SH-SY5Y cell proliferation (A, B) and apoptosis (C). SH-SY5Y cells were incubated with different concentrations of Ten working solutions from 0.1 to 1 mM for 24 h and cell viability assessed. 1 mM Ten significantly decreased cell proliferation, while 100–0.1 μ M Ten did not alter the proliferation of SH-SY5Y cells. (B and C) SH-SY5Y cells overexpressing α -synuclein (wild-type or A53T mutant) were treated with 10 μ M Ten for 24 h and cell viability assessed. 10 μ M Ten treatment increased proliferation (B) and decreased apoptosis (C) only in cells overexpressing α -synuclein (wild-type or A53T mutant) and not in the vector controls (B, C). Data are the mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$.

Ten Treatment Alleviates α -Synuclein-Induced Cytotoxicity

To investigate the effects of Ten on α -synuclein-related cytotoxicity, SH-SY5Y cells overexpressing α -synuclein (wild-type or A53T mutant) were treated with 10 μ M, 1 μ M, and 0.1 μ M Ten for 24 h, respectively, and cell proliferation and apoptosis assessed. Ten micromolar Ten had the most significant protective effects on α -synuclein overexpressing cell models. In the α -synuclein overexpressing groups, Ten treatment significantly increased cell proliferation by $12\text{--}20 \pm 3\%$ ($P \leq 0.001$, Figure 2B) and decreased apoptosis by $22\text{--}29 \pm 4.5\%$ ($P \leq 0.031$, Figure 2C). No significant changes in either cell proliferation or apoptosis were observed with Ten treatment compared with vector controls (Figure 2B,C). These results indicate that Ten treatment attenuates cytotoxicity induced by the overexpression of α -synuclein but, at the same potency, does not alter normal SH-SY5Y cell viability.

Ten Treatment Down-Regulates Ser129 Phosphorylation of α -Synuclein

As Ten attenuated α -synuclein-induced cytotoxicity, we tested whether Ten can also reduce α -synuclein Ser129 phosphorylation. SH-SY5Y cells overexpressing α -synuclein (wild-type or A53T mutant) were treated with 10 μ M Ten for 24 h, the levels of total and phosphorylated α -synuclein (syn-1 total and S129P) and that of related kinases were assessed using Western blotting in the different protein extracts. It should be noted that no specific oligomeric forms of α -synuclein were observed in our Western blotting condition, so quantification of the monomeric α -synuclein bands was performed.

In vector control lysates, S129P α -synuclein was undetectable, and no changes were observed with Ten treatment (Figure 3A,B). However, the increased S129P α -synuclein observed in the α -synuclein overexpressing cells (both wild type and A53T) was reduced by 30–35% with Ten treatment (Figure 3A,B), representing a 25–29% decrease in the relative level of S129P to total α -synuclein in the whole lysates (Figure 3B) rather than a reduction in the amount of α -synuclein overexpressed.

To determine whether the Ten effect occurred preferentially in the more soluble α -synuclein pool which is thought to pathologically aggregate and become insoluble to finally form intracellular LB [18], we compared the ratio of S129P to total α -synuclein proteins in the more soluble TBST fraction and less soluble SDS fractions after Ten treatment. In the TBST protein fraction, Ten treatment reduced S129P α -synuclein levels by 18–30%, reducing the ratio of S129P to total α -synuclein by 15–24% (Figure 3C,D). In the SDS protein fraction, Ten treatments reduced S129P α -synuclein levels by 38–43%, reducing the ratio of S129P to total α -synuclein by 25–30% (Figure 3E,F). These data show that Ten reduces the Ser129 phosphorylation of α -synuclein and that this effect is not confined to the more soluble forms of the protein.

Ten Treatment Down-Regulates Polo-Like Kinase-3 Levels in SH-SY5Y Cells

To determine whether the decrease in S129P α -synuclein levels observed following Ten treatment is associated with alternations

Figure 3 Effects of 10 μ M Ten treatment on the phosphorylation of α -synuclein at Ser129. SH-SY5Y cells overexpressing α -synuclein (wild-type or A53T mutant) were treated with 10 μ M Ten for 24 h, and the levels of total (syn-1) and S129P α -synuclein assessed using Western blotting in the differently prepared protein fractions. Representative Western blots (**A**, **C**, **E**) and graphs of the relative quantitation of the change in levels of S129P α -synuclein and the ratio of S129P to total α -synuclein in whole lysates (**A**, **B**) as well as in the separate protein fractions (**C**, **D**, **E**, **F**). The data show that compared with their vector controls, 10 μ M Ten treatment of cells overexpressing wild-type or A53T α -synuclein reduced their levels of S129P α -synuclein and the ratio of S129P to total α -synuclein in whole lysates (**A**, **B**) as well as in the separate protein fractions (**C**, **D**, **E**, **F**). Data are the mean $\pm$ SD.

Figure 4 Effects of 10 μ M Ten treatment on PLK3 expression in SH-SY5Y cells. SH-SY5Y cells overexpressing α -synuclein (wild-type or A53T mutant) were treated with 10 μ M Ten for 24 h and kinase levels assessed using semiquantitative Western blotting in the differently prepared protein fractions. Representative Western blots (**A**, **C**, **E**) and graphs of the relative quantitation of the change in levels of PLK3 (**B**, **D**, **F**) showing that Ten treatment reduced endogenous PLK3 levels in all groups in all protein fractions. The reduction in PLK3 levels did not differ between the vector controls or cells overexpressing α -synuclein (wild or A53T) (**A**–**F**). Data are the mean $\pm$ SD.

in α -synuclein kinase candidates, we measured the protein levels of the two major α -synuclein phosphorylation-associated kinase families (PLK1-3 [25,30] and CK1-2 [26,30]) by semiquantitative Western blotting. The protein level of the five individual kinases

was not affected by α -synuclein transfection, as no significant differences were found between α -synuclein-expressing cells and their vector controls (Figure 4A,C,E). Remarkably, Ten treatment selectively decreased PLK3 levels in all cell groups regardless their

α -synuclein-expressing status (Figure 4A–F). In contrast, Ten treatment had no effect on the levels of CK1, CK2, PLK1, or PLK2 protein levels (Figure S1).

Discussion

To the best of our knowledge, this is the first study to assess the effects of Ten on PD pathogenesis using α -synuclein-based cell models. The data show that Ten attenuates α -synuclein-induced cytotoxicity, down-regulating excessive phosphorylation of α -synuclein at Ser129 probably by reducing the amount of PLK3 in this neuronal cell model. Such data hold considerable promise that Ten may be a potential neuroprotective agent for the hyperphosphorylation of α -synuclein in PD and related synucleinopathies.

The overexpression of wild-type and A53T mutant α -synuclein in SH-SY5Y cells was successfully utilized in our PD model of cytotoxicity, with cells overexpressing 4 $\times$ their normal levels of the protein and having decreased proliferation and increased apoptosis. A53T mutant α -synuclein-induced toxicity is widely accepted and considered to associate with a gain-of-function [10,11,31]; however, the modes of toxicity of overexpressing wild-type α -synuclein in cells are hotly debated [32–34]. Part of the discrepancy could be explained by the utilization of different plasmids and transfection methods. In this study, we used full-length α -synuclein DNA cloned to pc-DNA3.1 vector, and utilized a transient transfection system rather than stable transfections, which may affect the biological characteristics of the cell colonies selected by their antibiotic resistance. We confirm that the overexpression of either wild-type or A53T mutant α -synuclein is toxic to SH-SY5Y neurons in this system. Such a finding is consistent with genetic observations in patients with PD [12–14]. Such overexpressing cell models have mostly been used to study the accumulation and aggregation of α -synuclein, and so the relatively high level of S129P α -synuclein in this cell model was a significant finding and suggests that, in addition to aggregation and toxicity, significant hyperphosphorylation occurs as a consequence of such increased α -synuclein expression in neurons. This greater increase in Ser129 phosphorylation is relevant to the finding of increased Ser129 phosphorylation in sporadic patients with PD without increase in total α -synuclein protein level [29,35,36] and reveals a new characteristic of this cell model. The role of hyperphosphorylated α -synuclein in neurodegeneration is still not clear, with some studies showing Ser129 phosphorylation is harmful [37,38] while others suggest the opposite [39,40]. Some of these discrepancies can be attributed to the use of the phosphorylation-mimicking mutant S129D which may not truly reflect the biophysical and biochemical properties of authentic S129P α -synuclein [38]. In the present study, high levels of S129P α -synuclein resulted from the overexpression of wild-type or A53T mutant α -synuclein showing that in neurons, an increase in α -synuclein levels increases Ser129 phosphorylation above the ~10% expected level [29], providing a simple model system to assess the effects of α -synuclein phosphorylation and pathways.

Our finding that Ten treatment-protected SH-SY5Y cells from α -synuclein-induced cytotoxicity suggests that Ten should be considered as a potential drug for the treatment of α -synuclein-related neurodegeneration. Previous studies have shown that

Ten prevented MPTP/MPP⁺ and 6-OHDA-induced dopaminergic neuronal apoptosis and LPS-induced inflammation in cell and animal models of PD [6–8]. In these toxin-induced PD models, the therapeutic effect of Ten was considered to be due to its antioxidant and antiinflammatory properties [6–8]. Such properties could indicate that Ten is effective in any condition where there is increased neuronal oxidation and inflammation. It is unknown whether Ten affects protein kinase signaling pathways or alters functions of cellular organelle (mitochondria, lysosome, proteasome, Golgi, etc.). Here, our data suggest that the effect of Ten is more specific to the hyperphosphorylation of α -synuclein known to occur in sporadic PD [29,35,36], and so Ten may be especially beneficial early in the disease process, as such phosphorylation appears to precede aggregation in patients with PD [29,35,36]. In the present study, Ten treatment decreased S129P α -synuclein levels and increased cell viability, suggesting that these processes are linked. In addition, we show that Ten treatment specifically reduced the levels of PLK3 in the SH-SY5Y cells, while the overexpression of α -synuclein did not alter the levels of PLK3 or the other kinases analyzed. This indicates that PLK3 may be a specific target of Ten. PLK3 is a member of PLKs, a serine/threonine kinase family containing two polo box regions, which bind to phosphoserine/threonine motifs. While five mammalian PLK family members have been identified so far (PLK1–5), the neuronal PLK2 and PLK3 have been shown to completely phosphorylate α -synuclein *in vitro* and specifically at Ser129 [24,25,30]. Intriguingly, we found that Ten treatment only reduced the levels of PLK3 to attenuate the α -synuclein-induced cytotoxic increase in Ser129 phosphorylation, consistent with studies showing that PLK3 is efficient in phosphorylating α -synuclein at Ser129 [25]. Overall, the data supports the concept that the phosphorylation of α -synuclein at Ser129 by PLK3 is toxic in this cell model of PD and that Ten treatment can reduce the levels of PLK3 and mitigate such toxicity. Further studies are required to test this theory and determine the potential of Ten to change the early detrimental increases in the molecular levels of phosphorylated α -synuclein that are observed in PD and related synucleinopathies.

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Conflicts of Interest

The authors have no conflicts of interest.

References

- Shin KY, Lee JY, Won BY, et al. BT-11 is effective for enhancing cognitive functions in the elderly humans. *Neurosci Lett* 2009;**465**:157–159.
- Lee JY, Kim KY, Shin KY, Won BY, Jung HY, Suh YH. Effects of BT-11 on memory in healthy humans. *Neurosci Lett* 2009;**454**:111–114.
- Shin KY, Won BY, Heo C, et al. BT-11 improves stress-induced memory impairments through increment of glucose utilization and total neural cell adhesion molecule levels in rat brains. *J Neurosci Res* 2009;**87**:260–268.
- Jia H, Jiang Y, Ruan Y, et al. Tenuigenin treatment decreases secretion of the Alzheimer's disease amyloid beta-protein in cultured cells. *Neurosci Lett* 2004;**367**:123–128.
- Lv J, Jia H, Jiang Y, et al. Tenuifolin, an extract derived from tenuigenin, inhibits amyloid-beta secretion in vitro. *Acta Physiol (Oxf)* 2009;**196**:419–425.
- Choi JG, Kim HG, Kim MC, et al. Polygalae radix inhibits toxin-induced neuronal death in the Parkinson's disease models. *J Ethnopharmacol* 2011;**134**:414–421.
- Yuan HL, Li B, Xu J, et al. Tenuigenin protects dopaminergic neurons from inflammation-mediated damage induced by the lipopolysaccharide. *CNS Neurosci Ther* 2012;**18**:584–590.
- Liang Z, Shi F, Wang Y, Lu L, Zhang Z, Wang X. Neuroprotective effects of tenuigenin in a SH-SY5Y cell model with 6-OHDA-induced injury. *Neurosci Lett* 2011;**497**:104–109.
- Naito R, Tohda C. Characterization of anti-neurodegenerative effects of Polygala tenuifolia in Abeta (25–35)-treated cortical neurons. *Biol Pharm Bull* 2006;**29**:1892–1896.
- Chesselet MF. In vivo alpha-synuclein overexpression in rodents: a useful model of Parkinson's disease? *Exp Neurol* 2008;**209**:22–27.
- Ulusoy A, Decressac M, Kirik D, Bjorklund A. Viral vector-mediated overexpression of alpha-synuclein as a progressive model of Parkinson's disease. *Prog Brain Res* 2010;**184**:89–111.
- Chartier-Harlin MC, Kachergus J, Roumier C, et al. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* 2004;**364**:1167–1169.
- Singleton AB, Farrer M, Johnson J, et al. Alpha-synuclein locus triplication causes Parkinson's disease. *Science* 2003;**302**:841.
- Zarranz JJ, Alegre J, Gomez-Esteban JC, et al. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann Neurol* 2004;**55**:164–173.
- Lashuel HA, Hirling H. Rescuing defective vesicular trafficking protects against alpha-synuclein toxicity in cellular and animal models of Parkinson's disease. *ACS Chem Biol* 2006;**1**:420–424.
- Lee HJ, Choi C, Lee SJ. Membrane-bound alpha-synuclein has a high aggregation propensity and the ability to seed the aggregation of the cytosolic form. *J Biol Chem* 2002;**277**:671–678.
- Trexler AJ, Rhoades E. Function and dysfunction of alpha-synuclein: probing conformational changes and aggregation by single molecule fluorescence. *Mol Neurobiol* 2013;**47**:622–631.
- Tong J, Wong H, Guttman M, et al. Brain alpha-synuclein accumulation in multiple system atrophy, Parkinson's disease and progressive supranuclear palsy: a comparative investigation. *Brain* 2010;**133**:172–188.
- Fuchs J, Nilsson C, Kachergus J, et al. Phenotypic variation in a large Swedish pedigree due to SNCA duplication and triplication. *Neurology* 2007;**68**:916–922.
- Beyer K, Ariza A. alpha-synuclein posttranslational modification and alternative splicing as a trigger for neurodegeneration. *Mol Neurobiol* 2013;**47**:509–524.
- Oueslati A, Fournier M, Lashuel HA. Role of post-translational modifications in modulating the structure, function and toxicity of alpha-synuclein: implications for Parkinson's disease pathogenesis and therapies. *Prog Brain Res* 2010;**183**:115–145.
- Anderson JP, Walker DE, Goldstein JM, et al. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J Biol Chem* 2006;**281**:29739–29752.
- Braithwaite SP, Stock JB, Mouradian MM. Alpha-synuclein phosphorylation as a therapeutic target in Parkinson's disease. *Rev Neurosci* 2012;**23**:191–198.
- Inglis KJ, Chereau D, Brigham EF, et al. Polo-like kinase 2 (PLK2) phosphorylates alpha-synuclein at serine 129 in central nervous system. *J Biol Chem* 2009;**284**:2598–2602.
- Mbeo MK, Paleologou KE, Boucharaba A, et al. Phosphorylation of synucleins by members of the Polo-like kinase family. *J Biol Chem* 2010;**285**:2807–2822.
- Waxman EA, Giasson BI. Specificity and regulation of casein kinase-mediated phosphorylation of alpha-synuclein. *J Neuropathol Exp Neurol* 2008;**67**:402–416.
- Pronin AN, Morris AJ, Surguchov A, Benovic JL. Synucleins are a novel class of substrates for G protein-coupled receptor kinases. *J Biol Chem* 2000;**275**:26515–26522.
- Kragh CL, Lund LB, Febbraro F, et al. alpha-synuclein aggregation and Ser-129 phosphorylation-dependent cell death in oligodendroglial cells. *J Biol Chem* 2009;**284**:10211–10222.
- Zhou J, Broe M, Huang Y, et al. Changes in the solubility and phosphorylation of alpha-synuclein over the course of Parkinson's disease. *Acta Neuropathol* 2011;**121**:695–704.
- Waxman EA, Giasson BI. Characterization of kinases involved in the phosphorylation of aggregated alpha-synuclein. *J Neurosci Res* 2011;**89**:231–247.
- Bertoncini CW, Fernandez CO, Griesinger C, Jovin TM, Zweckstetter M. Familial mutants of alpha-synuclein with increased neurotoxicity have a destabilized conformation. *J Biol Chem* 2005;**280**:30649–30652.
- Ko LW, Ko HH, Lin WL, Kulathinal JG, Yen SH. Aggregates assembled from overexpression of wild-type alpha-synuclein are not toxic to human neuronal cells. *J Neuropathol Exp Neurol* 2008;**67**:1084–1096.
- Xu J, Kao SY, Lee FJ, Song W, Jin LW, Yankner BA. Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat Med* 2002;**8**:600–606.
- Sidhu A, Wersinger C, Moussa CE, Vernier P. The role of alpha-synuclein in both neuroprotection and neurodegeneration. *Ann N Y Acad Sci* 2004;**1035**:250–270.
- Lue LF, Walker DG, Adler CH, et al. Biochemical increase in phosphorylated alpha-synuclein precedes histopathology of Lewy-type synucleinopathies. *Brain Pathol* 2012;**22**:745–756.
- Walker DG, Lue LF, Adler CH, et al. Changes in properties of serine 129 phosphorylated alpha-synuclein with progression of Lewy-type histopathology in human brains. *Exp Neurol* 2012;**240C**:190–204.
- Chen L, Feany MB. alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease. *Nat Neurosci* 2005;**8**:657–663.
- Sato H, Arawaka S, Hara S, et al. Authentically phosphorylated alpha-synuclein at Ser129 accelerates neurodegeneration in a rat model of familial Parkinson's disease. *J Neurosci* 2011;**31**:16884–16894.
- Azeredo da Silveira S, Schneider BL, Cifuentes-Diaz C, et al. Phosphorylation does not prompt, nor prevent, the formation of alpha-synuclein toxic species in a rat model of Parkinson's disease. *Hum Mol Genet* 2009;**18**:872–887.
- McFarland NR, Fan ZY, Xu K, et al. Alpha-synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease. *J Neuropathol Exp Neurol* 2009;**68**:515–524.

Supporting Information

The following supplementary material is available for this article:

Figure S1. Representative Western blots of PLK1-2 and CK1-2.