Original Article

Bibenzyl compound **20c** protects against endoplasmic reticulum stress in tunicamycin-treated PC12 cells *in vitro*

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Aim: Accumulation of α -synuclein (α -syn) in the brain is a characteristic of Parkinson's disease (PD). In this study, we investigated whether treatment with tunicamycin, an endoplasmic reticulum (ER) stress inducer, led to the accumulation of α -syn in PC12 cells, and where α -syn protein was accumulated, and finally, whether bibenzyl compound **20c**, a novel compound isolated from *Gastrodia elata* (Tian ma), could alleviate the accumulation of α -syn and ER stress activation in tunicamycin-treated PC12 cells.

Methods: PC12 cells were treated with tunicamycin for different time (6 h, 12 h, 24 h, 48 h). Cell viability was determined by a MTT assay. Subcellular fractions of ER and mitochondria were extracted with the Tissue Endoplasmic reticulum Isolation Kit. The levels of α -syn protein and ER-stress-associated downstream chaperones were detected using Western blots and immunofluorescence.

Results: Treatment of PC12 cells with tunicamycin (0.5–10 μ g/mL) dose-dependently increased the accumulation of α -syn monomer (19 kDa) and oligomer (55 kDa), and decreased the cell viability. Accumulation of the two forms of α -syn was observed in both the ER and mitochondria with increasing treatment time. Co-treatment with **20c** (10^{-5} mol/L) significantly increased the viability of tunicamycin-treated cells, reduced the level of α -syn protein and suppressed ER stress activation in the cells, evidenced by the reductions in phosphorylation of eIF2 α and expression of spliced ATF6 and XBP1.

Conclusion: Tunicamycin treatment caused accumulation of α -syn monomer and oligomer in PC12 cells. Bibenzyl compound **20c** reduces the accumulation of α -syn and inhibits the activation of ER stress, which protected PC12 cells against the toxicity induced by tunicamycin.

Keywords: *Gastrodia elata*; bibenzyl compound **20c**; PC12 cells; tunicamycin; α -synuclein; ER stress; Parkinson's disease; neuroprotection

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Introduction

Parkinson's disease (PD) is an irreversible neurodegenerative disease. Lewy bodies (LBs) and Lewy neuritis (LNs) are the hallmarks of PD pathogenesis^[1, 2]. Fibrillar aggregates of ubiquitinated, phosphorylated, and/or *S*-nitrosylated forms of α -syn are major components of LBs and LNs^[3]. Numerous studies have suggested that the accumulation of pathological proteins in the brain is a common characteristic of most neurodegenerative diseases, including PD, Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS)^[4]. These neurodegenerative diseases are clas-

sified as protein-misfolding disorders (PMDs). Endoplasmic reticulum (ER) dysfunction is suggested to be one of the primary shared characteristics of these disorders^[5]. The accumulation of misfolded proteins is sensed by ER resident molecules, which initiate the expression of target genes, impact ER capacity^[6] and activate ER stress. Subsequently, a complex signaling pathway to regulate cell responses to ER stress called the unfolded protein response (UPR) is initiated. The initiation of the UPR directly activates three central transmembrane ER signaling chaperones, including PRKP-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6). These signaling pathways increase the expression of ER chaperones, inhibit protein entry into ER and accelerate the degradation of retrograde misfolded proteins, which can ameliorate the accumulation of misfolded

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proteins in the ER^[6, 7]. The activation of the PERK pathway promotes the phosphorylation of eIF2 α and the expression of transcription factor ATF4, which increases the expression of ER chaperones to reduce protein entry into the ER. During ER stress, ATF6 translocates to the Golgi and releases transcription factors to migrate into the nucleus and regulate gene expression. When IRE1 is activated, it catalyzes the splicing of the mRNA encoding the X-box-binding protein 1 (XBP1) to produce XBP1 and regulate target genes^[8].

Although the mechanisms of PD pathogenesis remain unclear, many studies have indicated that the accumulation of α -syn may be a neurotoxic factor^[9, 10]. However, the mechanisms underlying α -syn accumulation and how α -syn accumulation contributes to neurodegeneration remain poorly understood. Studies have indicated that sodium butyrate, which is an ER stress inducer, can induce an increase in the oligomeric forms of α -syn in 3D5 cells, and this effect was blocked by co-treatment with the ER stress inhibitor salubrinal, which suggested that ER stress could promote the aggregation of α -syn in 3D5 cells^[11]. However, whether ER stress is neurotoxic to PC12 cells is still unknown. Furthermore, the impact of ER stress on the accumulation of the monomeric and oligomeric forms of α -syn and the location of α -syn during ER stress remain unclear.

Gastrodia elata (Tian ma) is a traditional herb that is used to treat headaches, hypertension and neurodegenerative diseases. Recent studies have found that treatment with this herb can enhance cognitive function and help prevent oxidation^[11–13]. The compound **20c** (2-[4-hydroxy-3-(4-hydroxybenzyl)-4-(4-hydroxybenzyl) phenol] (Figure 3C) was isolated from *Gastrodia elata* and is a novel bibenzyl compound. Based on previous data from our laboratory, **20c** can protect PC12 cells against damage induced by rotenone, which suggests that **20c** is a compound with potential neuroprotective effects against PD (data not shown). However, the impact of **20c** on the accumulation of α -syn has yet to be determined, and no evidence has been reported that reveals the impact of **20c** on the activation of ER stress.

In this study, our data suggest that tunicamycin, which is an ER stress inducer, increased the expression of the monomeric and oligomeric forms of α -syn and that these impacts were associated with the tunicamycin concentration and treatment time. Furthermore, the accumulation of two forms of α -syn in the ER and mitochondria was induced by tunicamycin in a time-dependent manner. **20c** reduced the protein level of α -syn and inhibited ER stress by suppressing UPR activation. Together, ER stress increased the accumulation of the monomeric and oligomeric forms of α -syn, and **20c** attenuated the damage induced by tunicamycin and promoted PC12 cell survival.

Materials and methods

Reagents

The compound **20c** was obtained from the Department of Chemical synthesis, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing,

China). **20c** was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 mol/L as a stock solution, which was stored at -80 °C until it was used.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), tunicamycin, and DMSO were obtained from Sigma-Aldrich (St Louis, MO, USA). DMEM (Dulbecco's modified Eagle's medium), horse serum (ES) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). The following primary antibodies were used: anti- α -syn, anti-calnexin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Grp78, anti-CHOP, anti-p-eIF2 α , anti-eIF2 α (1:1000, Cell Signaling Technology, Danvers, MA, USA); anti-ATF6 (1:500, Enzo Life Sciences, New York, NY, USA); anti-XBP1, anti-COX4 (1:1000, Abcam, Cambridge, UK); and anti- β -actin (1:5000, Sigma, St Louis, MO, USA). The secondary antibodies were purchased from KPL (1:5000, Gaithersburg, MD, USA).

Cell culture and treatment

Rat pheochromocytoma PC12 cells were maintained in our laboratory. The cells were cultured in DMEM containing 5% FBS and 5% ES and placed in a water-saturated atmosphere of 5% CO₂ at 37 °C. The culture medium was changed every other day. PC12 cells were seeded at a density of 1×10⁵ cells·cm⁻².

Tunicamycin^[14] was dissolved in DMSO at a concentration of 10 mg/mL as a stock solution. The stock was stored at -80 °C until it was used. The PC12 cells were allowed to attach for 24 h before treatment. Then, the PC12 cells were treated with tunicamycin (0.5, 1, 2, 5, and 10 μ g/mL) or treated with tunicamycin (2 μ g/mL) and **20c** (10⁻⁵, 10⁻⁶, and 10⁻⁷ mol/mL) for 24 h. Following the treatment, cell viability assessment, Western blot analysis, and immunofluorescence analysis were performed.

Assessment of cell viability

The cells were treated with tunicamycin or with tunicamycin and **20c** for 24 h and cell viability was analyzed using an MTT assay^[15]. The cells were seeded in 96-well plates at a density of 5×10³ cells per well. After treatment for 24 h, 10 μ L MTT (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. Then, 100 μ L SDS-HCl was added to each well, and the plate was incubated overnight at 37 °C. The optical density (OD) was analyzed at 570 nm on a Microplate Reader (Thermo SkanIt Software 3.2, Germany)^[16].

Preparation of the subcellular fraction of the ER and mitochondria

PC12 cells were cultured in 10-cm plates at a density of 1×10⁵ cells·cm⁻² and were treated with tunicamycin (2 μ g/mL) for 6, 12, or 24 h. The PC12 cells were collected at 4 °C. The subcellular fractions of the ER and mitochondria were extracted using the Tissue Endoplasmic reticulum Isolation Kit (Genmed Scientific Inc, Netherlands). The collected ER and mitochondria were used for Western blot analysis.

Immunofluorescence

Cells were seeded on slides coated with PLL for 24 h. After treatment with tunicamycin (2 $\mu\text{g}/\text{mL}$) for 12, 24, and 48 h, the cells were washed with ice-cold PBS three times for 5 min each time. The cells were fixed with 1% paraformaldehyde and then permeabilized with 0.1% Triton-X 100 for 10 min at room temperature. After washing the cells with PBS three times, the cells were blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min at room temperature and incubated with primary antibodies, including anti-CHOP or anti- α -syn (1:100), overnight at 4°C. After washing the cells with PBS three times, the cells were incubated with an Alexa Fluor 488-conjugated secondary antibody, an Alexa Fluor 546-conjugated secondary antibody (1:500, Invitrogen, New York, USA) and Hoechst 33342 (1:1000, Life Technologies, NY, USA) for 1 h at room temperature. Images were obtained using a Leica inverted microscope equipped for fluorescence analysis (Leica Microsystems, Germany)^[17].

Western blot analysis

Following the treatments, the cultured medium was removed and cells were harvested in an ice bath. Total proteins were extracted as previously described^[17]. PC12 cells were lysed in NP-40 lysis buffer (150 mmol/L NaCl, 1% Nonidet P-40, 50 mmol/L Tris, pH 7.4, and 1 mmol/L ethylenediamine tetraacetic acid) with a proteinase inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). The cell lysates were centrifuged at 12000 $\times g$ for 20 min at 4°C. The supernatant (soluble part) and the precipitant (insoluble part) were collected. The concentration of total protein for each sample was quantified with the Bicinchoninic Acid kit (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded into the SDS-PAGE gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA). The membranes were blocked with 3% BSA and incubated with different primary antibodies at 4°C overnight. The membranes were washed with TBST 3 times for 10 min each and incubated with secondary antibodies for 1 h at room temperature. Then, the membranes were washed with TBST three times, and the protein bands were detected with an enhanced chemiluminescence (ECL) plus detection system. The protein bands were analyzed using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analyses

All data were analyzed using one-way analysis of Variance (ANOVA) with Dunnett's test. The data are shown as the mean $\pm$ SEM. Differences with $P < 0.05$ were considered statistically significant. All statistical analyses were performed using Prism 5 (GraphPad Software, Inc).

Results

Tunicamycin induced an increase in the monomeric and oligomeric forms of α -syn in PC12 cells

Studies have suggested that α -syn pathology can initiate ER stress in α -syn A53T mutant transgenic mice (α A53T tg mice)

and that an inhibitor of ER stress, salubrinal, can significantly reduce the accumulation of α -syn in α A53T tg mice^[18]. To determine whether an ER stress inducer could be directly associated with the accumulation of α -syn, we treated PC12 cells with tunicamycin at different concentrations (0.5, 1, 2, 5, and 10 $\mu\text{g}/\text{mL}$) for 24 h. The extracted protein was analyzed by Western blot analysis. The two bands, including one at approximately 19 kDa and another at 55 kDa, were the monomeric and oligomeric forms of α -syn. After tunicamycin administration, the protein levels of the monomeric and oligomeric forms of α -syn were increased in a dose-dependent manner in both the soluble and insoluble parts of the protein extracted (Figure 1A). Subsequently, we treated the PC12 cells with tunicamycin (2 $\mu\text{g}/\text{mL}$) for 12, 24, and 48 h. The protein level of CHOP increased with the treatment time. Meanwhile, the protein expression of α -syn increased in a time-dependent manner and was co-located with the increase in CHOP (Figure 1B). These data suggest that tunicamycin led to the accumulation of the monomeric and oligomeric forms of α -syn and that this accumulation was associated with the concentration and duration of tunicamycin treatment.

Accumulation of α -syn induced by tunicamycin occurred in the ER and mitochondria

Because the accumulation of α -syn was induced by tunicamycin, we identified the disturbance of α -syn in the ER after treating the cells with tunicamycin. First, we extracted the microsome (ER) and mitochondria fractions of PC12 cells (Figure 2A) and analyzed the alteration of α -syn using Western blot analysis. Surprisingly, the protein levels of the monomeric form of α -syn in the ER increased in a time-dependent manner after tunicamycin treatment. After 24 h, the oligomeric form of α -syn in the ER increased as well. Additionally, the accumulation of the α -syn monomer and oligomer in mitochondria increased after tunicamycin treatment. After 24 h, the oligomeric form of α -syn had a similar increase in the mitochondria as it did in the ER (Figure 2B). All of the data suggest that the monomeric and oligomeric forms of α -syn were enriched in a time-dependent manner in the ER and mitochondria fractions after tunicamycin administration.

20c significantly attenuated the accumulation of α -syn induced by tunicamycin

The major component of LBs is α -syn. Numerous data suggest that increased levels of α -syn lead to neurodegeneration. New compounds to ameliorate or prevent the α -synucleinopathies have attracted a great deal of attention^[19]. First, we treated PC12 cells with tunicamycin at different concentrations (0.5, 1, 2, 5, and 10 $\mu\text{g}/\text{mL}$) and analyzed cell viability using an MTT assay. Compared to the control group, cell viability decreased according to the concentration of tunicamycin. When the PC12 cells were treated with tunicamycin (2 $\mu\text{g}/\text{mL}$), the cell viability declined to 51.29 % (Figure 3A). Therefore, 2 $\mu\text{g}/\text{mL}$ was used in subsequent tests. Previous data indicated that 20c protected PC12 cells against the neurotoxicity of rotenone. To determine whether 20c attenuated the damage induced by

Figure 1. Tunicamycin induced accumulation of the α -syn monomer and oligomer. (A) PC12 cells were treated with tunicamycin at different concentrations (0.5, 1, 2, 5, and 10 $\mu\text{g/mL}$) for 24 h. The total protein was collected and analyzed using Western blot analysis. The soluble part of the protein fraction was the supernatant collected after centrifugation. The insoluble part of the protein fraction was the precipitant collected after centrifugation, which included the insoluble aggregates of α -syn. Respective immunoblots of α -syn are shown. β -Actin was the loading control. (B) PC12 cells treated with tunicamycin for 12, 24, and 48 h were immunostained with antibodies against CHOP (green) and α -syn (red). Nuclei were detected with Hoechst 33342 (blue). Scale bar, 100 μm .

tunicamycin, we examined the effects of **20c** on cell viability after treating the cells with tunicamycin. **20c** (10^{-5} mol/L) significantly enhanced the cell viability to 69.85% (% control group), which suggested that **20c** could protect PC12 cells against the damage induced by tunicamycin (Figure 3B). Furthermore, to determine whether **20c** attenuated the accumulation of α -syn induced by tunicamycin, we treated the PC12 cells with tunicamycin or a combination of tunicamycin and **20c**. Compared with the tunicamycin group, **20c** (10^{-5} mol/L) notably decreased the protein level of α -syn (Figure 4). These data suggest that **20c** protected the PC12 cells against insults and decreased the protein level of α -syn induced by tunicamycin.

20c inhibited ER stress by suppressing the UPR

The ER is the central intracellular organelle that is responsible for the synthesis, quality control, and degradation of proteins. Because **20c** attenuated the increase of α -syn, we speculated that **20c** inhibited the activation of ER stress. After treating the PC12 cells with tunicamycin or with tunicamycin and **20c**, we analyzed the protein level of Grp78 (a marker of ER stress). Compared with the control group, tunicamycin significantly increased the protein level of Grp78. We found that **20c** (10^{-5} mol/L) remarkably decreased the expression of Grp78 (Figure 5). To determine the effects of **20c** on the activation of UPR,

we analyzed the protein levels of ER-stress-associated downstream chaperones. Compared to the control group, the phosphorylation of eIF2 α and the expression of transcription factor ATF4 were significantly increased by tunicamycin, which meant that the PERK signaling pathway was activated. We also determined that **20c** markedly reduced the phosphorylation of eIF2 α and the expression of ATF4 and that **20c** inhibited the PERK pathway (Figure 6A). Similar to the effects of **20c** on the PERK pathway, the activation of the IRE1 and the ATF6 pathways was notably inhibited by **20c** and was accompanied by a decrease in the protein levels of XBP1 and ATF6 (50) (Figure 6B). These data suggest that **20c** suppressed the activation of the major branches of the UPR, which suggests that **20c** can inhibit ER stress.

Discussion

The presence of potentially pathogenic protein aggregates is a common characteristic shared by PD and other PMDs^[20]. However, whether ER stress directly contributes to the accumulation or aggregation of α -syn has not been clarified. Our data indicate that the accumulation of α -syn is directly induced by an ER stress inducer and that the increase of the monomeric or oligomeric forms of α -syn is associated with the tunicamycin concentration and treatment time. Additionally, the two forms of α -syn accumulated in the ER and mitochondria.

Figure 2. Tunicamycin led to the accumulation of α -syn in the ER and mitochondria, and the accumulation increased with treatment time. (A) Protocol for the extraction of ER and mitochondria. (B) The expression of α -syn accumulated in the ER and mitochondria. Calnexin and COX4 were organelle-specific markers: calnexin for the ER and COX4 for the mitochondria. Respective immunoblots of α -syn in the ER and mitochondria are shown.

dria after treatment with tunicamycin, which suggests that ER stress was an active participant in the process of α -syn accumulation. Moreover, our studies provide evidence that **20c** exerts protective effects on tunicamycin-treated PC12 cells, reduces the expression of α -syn and inhibits the activation of the UPR, which suggest that **20c** is a neuroprotective compound worthy of future study.

The aggregation of α -syn leads to lysosomal dysfunction^[21], membrane disruption^[22], and dopaminergic neuron disorders^[20]. Numerous studies have proposed that the aggregation of α -syn or the increased Ser129 phosphorylation of α -syn are co-localized with UPR activation^[23, 24]. ER stress initiates the UPR to modulate cellular response to restore ER homeostasis. The UPR leads to the simultaneous activation of adaptive and pro-apoptotic responses^[25]. Under chronic ER stress, when the ER homeostasis fails to be recovered, the UPR triggers cell death through apoptosis^[3]. The ER-stress-associated apoptosis pathway is the central pathway for eliminating damaged cells. The best characteristic of the pro-apoptotic pathway is the production of the CHOP transcription factor, which is modulated by ATF4, and possibly by ATF6 and XBP1^[26, 27]. In our studies, tunicamycin increased the expression of α -syn in a time-dependent manner. Accompanied by enhancement of the ER-stress intensity, the viability of PC12 cells was reduced. That decrease in viability is the reason why, after 48 h of tunicamycin

Figure 3. Compound **20c** protected PC12 cells against the toxicity of tunicamycin. (A) PC12 cells were treated with tunicamycin at different concentrations (0.5, 1, 2, 5, and 10 μ g/mL). Cell viability was analyzed with an MTT assay. The data are shown as the mean $\pm$ SEM. $n=6$. One-way ANOVA with Dunnett's test was used for statistical analysis. ** $P<0.01$ vs the control group. (B) After the PC12 cells were cultured for 24 h in 96-well plates, the cells were treated with tunicamycin 2 μ g/mL or with tunicamycin 2 μ g/mL and **20c** (10^{-5} , 10^{-6} , and 10^{-7} mol/L). The data are shown as the mean $\pm$ SEM. $n=6$. One-way ANOVA with Dunnett's test was used for statistical analysis. ** $P<0.01$ vs the control group. ## $P<0.01$ vs the tunicamycin group. (C) The chemical structure of **20c**.

cin treatment, the number of cells in the image was less than other groups. On the other hand, **20c** attenuated the decrease in cell viability induced by tunicamycin, which indicated that **20c** could protect against ER-stress-associated insults. Caspase-12-caspase-3 is another ER-stress-associated apoptosis pathway. Caspase-12 can be specifically cleaved and activated after the activation of the IRE1 pathway^[28]. The effects of **20c** on the activation of caspase-12 have yet to be clarified.

The accumulation of α -syn in the ER due to tunicamycin treatment is consistent with recent studies suggesting that α -syn accumulates in the ER as PD progresses^[1]. The level of α -syn in the ER of presymptomatic α A53T tg mice was proportional to the total α -syn. Subsequently, higher molecular

Figure 4. Compound **20c** attenuated the increase of α -syn expression induced by tunicamycin (2 μ g/mL). Respective immunoblots and statistical analysis of the protein level of α -syn are shown. The data are shown as the mean $\pm$ SEM. $n=4-5$. One-way ANOVA with Dunnett's test was used for statistical analysis. ** $P<0.01$ vs the control group. ## $P<0.01$ vs the tunicamycin group.

Figure 5. Compound **20c** inhibited the activation of ER stress. Respective immunoblots and statistical analysis of the protein level of Grp78 are shown. The data are shown as the mean $\pm$ SEM. $n=4-5$. One-way ANOVA with Dunnett's test was used for statistical analysis. ** $P<0.01$ vs the control group. # $P<0.05$ vs the tunicamycin group.

weight α -syn was enriched in the ER in symptomatic tg mice. The accumulation also occurred selectively in pathologically affected brain fields, such as SNpc, which suggests that the accumulation of α -syn in the ER is a primary characteristic

of PD. The accumulation of α -syn could trigger ER stress by interacting with the ER chaperone Grp78 or by disturbing Ca^{2+} metabolism^[29, 30]. Moreover, the overexpression of α -syn in yeast blocked ER-Golgi vesicular trafficking, which contributed to toxicity, cell loss and selective impacts on dopamine-producing neurons^[19]. Furthermore, α -syn disrupts UPR by interacting with ATF6 and blocking the incorporation of ATF6 into COPII vesicles^[31]. These studies indicate that the accumulation of α -syn may trigger the activation of ER stress. Furthermore, the accumulation of α -syn in the mitochondria was induced after treatment with tunicamycin. Bir *et al* observed that the intracellular accumulation of α -syn in SHSY5Y cells led to mitochondrial impairment and cell death through interaction with the permeability transition pore complex in isolated preparations^[32]. Moreover, monomers and oligomers of α -syn A53T localized to the mitochondrial membranes, and this localization was associated with selective age-related mitochondrial complex I inhibition, which suggested that mitochondrial α -syn affects cells and led to mitochondria dysfunction^[33]. The impact of **20c** on the expression of α -syn in the ER and mitochondria should be further studied.

Based on the current studies and our data supporting a relationship between ER-stress-associated toxicity and the accumulation of α -syn, we propose a model (Figure 7A). Normally, α -syn is located in the lumen of ER. Upon stimulation by tunicamycin or other stimuli, the monomeric form of α -syn increases and aggregates into the oligomeric form of α -syn. Finally, the oligomeric form matures into insoluble aggregates as the disease progresses. The accumulation or aggregation of α -syn triggers the activation of ER stress, and chronic ER stress leads to the initiation of ER-stress-associated apoptosis. As feedback, ER dysfunction exacerbates the increase in α -syn expression. Given the therapeutic effects of salubrinal on reducing α -syn oligomers in the ER^[1], the abnormal accumulation of α -syn contributes to chronic ER stress and ER-stress-associated insults.

Current pharmacological therapies for PD only attenuate the symptoms and fail to prevent the progression of PD^[15]. Therefore, new therapeutic compounds should be very attractive to researchers. Previous studies in our laboratory provide evidence that **20c** protected against neurotoxicity in H_2O_2 - and rotenone-treated models. Based on the model that we have proposed, **20c** attenuated the accumulation of α -syn, which alleviated the activation of UPR by suppressing all three central downstream pathways. As a result, **20c** improved cell viability, which was decreased by tunicamycin (Figure 7B). However, the mechanism through which **20c** decreased the expression of α -syn has yet to be determined. Numerous pathological conditions trigger ER stress and result in cell death, including disturbance of ER Ca^{2+} homeostasis, ER-Golgi vesicular trafficking, and local oxidative stress^[5, 34]. Compound **20c** was observed to be an anti-oxidative compound. The next step would be to identify the mechanism of protection that **20c** exerts against the toxicity induced by tunicamycin and to determine whether **20c** attenuates the accumulation and inhibits the initiation of UPR through anti-oxidative effects and

Figure 6. Compound **20c** suppressed the initiation of UPR. (A, B) Respective immunoblots and statistical analysis of the protein levels of p-eIF2 α , eIF2 α , ATF4, CHOP, XBP1s/XBP1, and ATF6(50)/ATF6. Data are shown as the mean $\pm$ SEM. $n=4-5$. One-way ANOVA with Dunnett's test was used for statistical analysis. * $P<0.05$, ** $P<0.01$ vs the control group. # $P<0.05$, ## $P<0.01$ vs the tunicamycin group.

modulation of ER Ca²⁺ homeostasis.

Together, the data obtained in our studies suggest that tunicamycin treatment led to neurotoxicity, decreased cell viability, and increased the accumulation of the monomeric and oligomeric forms of α -syn. Additionally, tunicamycin

promoted the accumulation of two forms of α -syn in the ER and mitochondria in a time-dependent manner. Furthermore, **20c** reduced the accumulation of α -syn and inhibited the initiation of UPR. The data indicate that modulation of ER stress is involved in the neuroprotective action of **20c**. We therefore

Figure 7. A model showing the protective effects of **20c** on tunicamycin-treated cells based on our data. (A) Accumulation of α -syn triggers the activation of ER stress. ER stress initiates the UPR to restore ER homeostasis. When UPR fails to recover ER homeostasis, ER-stress-specific apoptosis is promoted to eliminate the damaged cell. (B) Compound **20c** may inhibit the accumulation of α -syn, which suppresses the activation of ER stress. Finally, **20c** inhibits the activation of PERK, IRE1, and ATF6 signaling pathways, which promotes cell survival. However, the mechanism through which **20c** reduces the accumulation of α -syn has yet to be clarified.

hope that **20c** will be a compound that leads to new PD therapies and that regulating ER stress will provide a new theoretical basis for additional studies.

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Author contribution

Zheng MOU and Nai-hong CHEN designed the research study; Zheng MOU performed experiments and data analysis; Yu-he YUAN, Yu-xia LOU, Ju-yang HUANG, Cong-yuan XIA, Yan GAO, Shi-feng CHU, Piao LUO, and Yang HENG helped prepare the paper; Cheng-gen ZHU and Jian-gong SHI contributed to the preparation of **20c**.

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