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Low levels of Bax inhibitor-1 gene expression increase tunicamycin-induced apoptosis in human neuroblastoma SY5Y cells****

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Abstract

A human SH-SY5Y neuroblastoma cell line with a low level of Bax inhibitor-1 expression was established by lentivirus-mediated RNA interference and fluorescence-activated cell sorting. In control SH-SY5Y cells, tunicamycin treatment induced endoplasmic reticulum stress-mediated apoptosis; however, after Bax inhibitor-1 gene knockdown, cell survival rates were significantly decreased and the degree of apoptosis was significantly increased following tunicamycin treatment. In addition, chromatin condensation and apparent apoptotic phenomena, such as marginalization and cytoplasmic vesicles, were observed. Our findings indicate that Bax inhibitor-1 can delay apoptosis induced by endoplasmic reticulum stress.

Key Words

Bax inhibitor-1; RNA interference; SH-SY5Y; endoplasmic reticulum stress; tunicamycin; apoptosis; neural regeneration

Abbreviations

PCD, programmed cell death; BI-1, Bax inhibitor-1; ER, endoplasmic reticulum; TUN, tunicamycin

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INTRODUCTION

In eukaryotes, programmed cell death (PCD), or apoptosis, is a genetically controlled series of events, and is an essential process during development, aging, and in the removal of damaged or infected cells after environmental and pathogenic injury^[1-2]. Dysregulation of PCD is an underlying cause or contributing factor in many diseases. Bcl-2 family proteins are centrally involved in the control of PCD, including both inhibitory (Bcl-2 and Bcl-XL) and pro-apoptotic (Bax and Bak) members^[3-4]. Bax inhibitor-1 (BI-1) was first identified as a suppressor of cell death that is activated by Bax in yeast and mammalian cells^[5]. BI-1 contains several transmembrane domains ER stress is associated with many diseases, including

and localizes to endoplasmic reticulum (ER) membranes^[6-7]. Overexpression of BI-1 provides protection against apoptosis induced by some stimuli, such as Bax, pathogens or abiotic stress, in a variety of cells from yeast, plant, and mammalian origins^[8-11]. Many processes, including those of cellular redox regulation, cause accumulation of unfolded proteins in the ER, which then trigger the unfolded protein response^[12]. If cells cannot relieve the ER stress, apoptosis is activated, representing a last resort of multicellular organisms to dispose dysfunctional cells^[13-14]. Several mechanisms have been proposed for the unfolded protein response, including direct activation of proteases, kinases, transcription factors, and Bcl-2 family proteins and their modulators^[12].

ischemia/reperfusion injury,

neurodegeneration and diabetes^[15]. Neurodegenerative diseases associated with inclusion body formation and protein aggregation, such as Parkinson's disease, Alzheimer's disease and Huntington's disease have also been linked to ER stress^[12]. These neurodegenerative diseases are often accompanied by neuronal loss and apoptosis. Neurons are highly susceptible to cell death induced by ER stress^[16]. Recently, fibroblasts, hepatocytes and neuronal cells isolated from BI-1 knockout mice were also shown to exhibit heightened apoptosis induced by ER stress^[17]. Watanabe *et al*^[18] found evidence for the role of *Arabidopsis* BAX inhibitor-1 as a modulator of ER stress-mediated PCD. Studies of hepatic or renal artery occlusion in BI-1^{-/-} mice revealed that BI-1 provides endogenous protection from ER stress and ischemia-reperfusion injury in the liver and kidney^[19]. Tunicamycin (TUN) can produce ER stress and induce apoptosis. The role of BI-1 in ER stress-mediated PCD in human neuronal cells remains unclear. In the present study, we aimed to clarify these issues by knocking down the expression of BI-1 in a human SH-SY5Y neuroblastoma cell line by lentivirus-mediated RNA interference.

RESULTS

Silencing effect of RNA interference on BI-1

Real-time PCR analysis demonstrated that the level of BI-1 mRNA in cells infected with small hairpin RNA (shRNA)-expressing lentivirus decreased to 54% of that in control cells infected with pLL3.7-expressing virus (Figure 1A). To confirm these results, BI-1 protein levels were determined by western blot analysis. The shRNA could effectively inhibit BI-1 protein expression. Absorbance analysis of western blots indicated the level of BI-1 protein extracted from the cells infected with shRNA-expressing lentivirus decreased to 62% (equal loading was confirmed by assessing the β -actin bands) (Figure 1B). Using green fluorescent protein expression from the pLL3.7 vector, infected cells were isolated by fluorescence-activated cell sorting and a cell line, SY5Y/B, with low BI-1 expression and a control cell line, SY5Y/P, were established. Enhanced green fluorescent protein (EGFP)-positive cells accounted for over 99% of cells following fluorescence-activated cell sorting (Figure 2).

Low level of BI-1 expression reduced the survival rate of SH-SY5Y cells under TUN-induced ER stress

SH-SY5Y cells in medium containing TUN at a concentration of 2 μ g/mL displayed abnormal morphology characterized by cell elongation and gradual loss of cell-cell adhesion. TUN inhibited the growth of wild-type SH-SY5Y cells in a dose-dependent manner (data not shown). The sensitivity to TUN was different between the

two cell lines studied. Indeed, TUN-induced cell death occurred earlier and was more severe in SY5Y/B cells than in SY5Y/P cells. Cell viability of SY5Y/B cells at 36 and 41 hours after TUN treatment was significantly lower than that of SY5Y/P cells ($P < 0.01$; Figure 3).

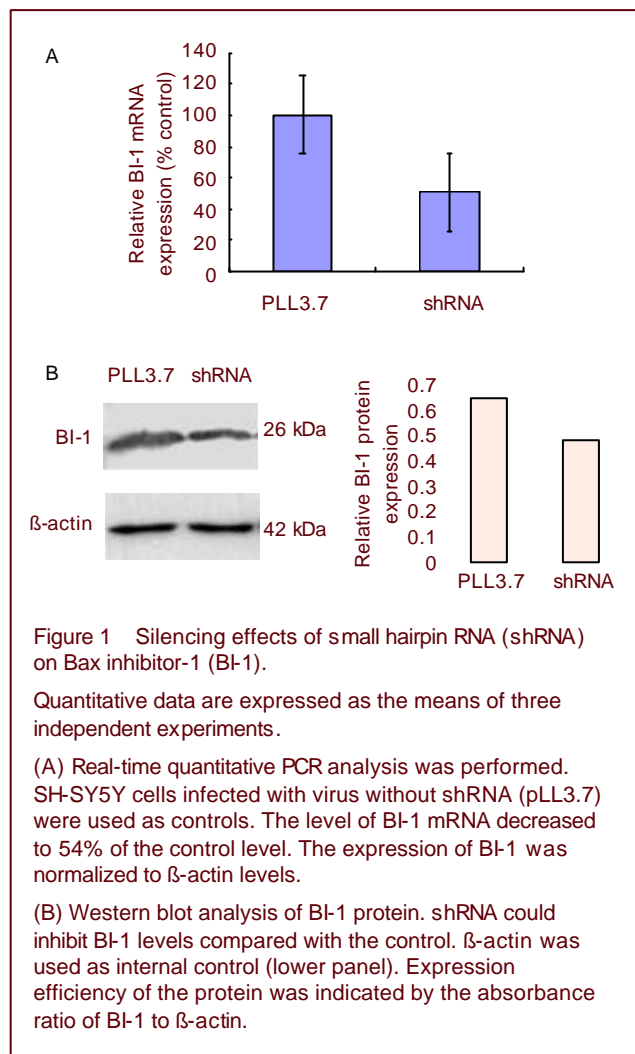


Figure 1 Silencing effects of small hairpin RNA (shRNA) on Bax inhibitor-1 (BI-1).

Quantitative data are expressed as the means of three independent experiments.

(A) Real-time quantitative PCR analysis was performed. SH-SY5Y cells infected with virus without shRNA (pLL3.7) were used as controls. The level of BI-1 mRNA decreased to 54% of the control level. The expression of BI-1 was normalized to β -actin levels.

(B) Western blot analysis of BI-1 protein. shRNA could inhibit BI-1 levels compared with the control. β -actin was used as internal control (lower panel). Expression efficiency of the protein was indicated by the absorbance ratio of BI-1 to β -actin.

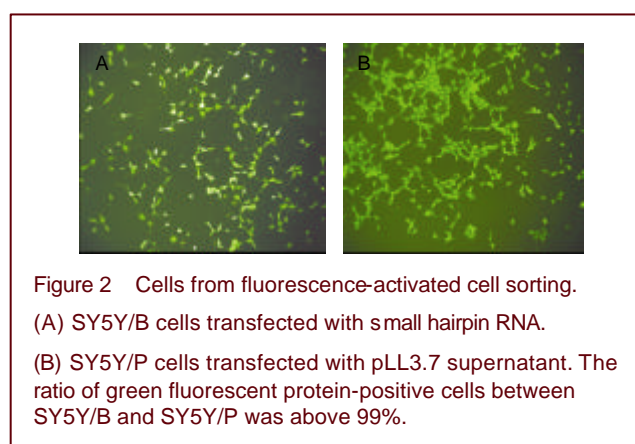


Figure 2 Cells from fluorescence-activated cell sorting.

(A) SY5Y/B cells transfected with small hairpin RNA.

(B) SY5Y/P cells transfected with pLL3.7 supernatant. The ratio of green fluorescent protein-positive cells between SY5Y/B and SY5Y/P was above 99%.

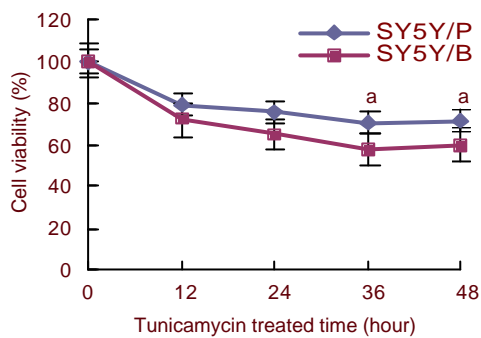


Figure 3 Different survival rates of the two cell lines treated with tunicamycin.

Cell viability of the two groups initially declined, but rose at 41 hours. Data are expressed as mean \pm SD from three independent experiments. ^a $P < 0.01$, vs. SY5Y/B cells (one-way analysis of variance).

SY5Y/B: SH-SY5Y cell line with low levels of Bax inhibitor-1 expression; SY5Y/P: SH-SY5Y cell line infected with empty control virus.

Low level of BI-1 expression increased apoptosis of SY5Y/B cells under TUN-induced ER stress

TUN-induced cell apoptosis was detected in both SY5Y/B and SY5Y/P cells, but the rate of apoptosis was increased in SY5Y/B cells compared with SY5Y/P cells. After treatment with 10 μ g/mL TUN for 24 hours, cell apoptosis increased 24% in SY5Y/B cells compared with 14% in SY5Y/P cells ($P < 0.05$; Figure 4). These results suggested that inhibition of BI-1 expression in SH-SY5Y cells significantly increased sensitivity to TUN-mediated PCD upon ER stress signaling.

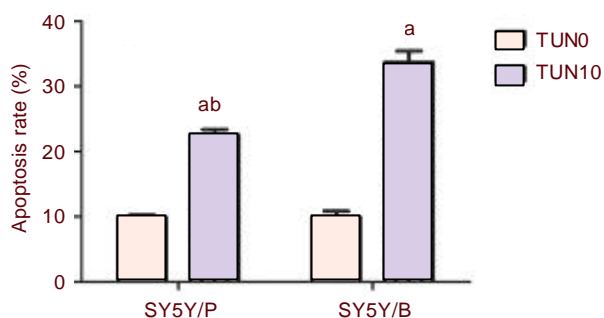


Figure 4 Effect of Bax inhibitor-1 on tunicamycin (TUN)-induced cell apoptosis.

Cell apoptosis was detected using the Annexin V-FITC kit after treatment with 10 μ g/mL TUN (TUN10) for 24 hours. Data (%) are expressed as mean \pm SD from three independent experiments.

^a $P < 0.05$, vs. 0 μ g/mL TUN-treated cells (TUN0); ^b $P < 0.05$, vs. SY5Y/B cells (one-way analysis of variance).

Ultrastructure of SY5Y/B cells under TUN-induced ER stress

Cell ultrastructure was observed by transmission electron microscopy (Figure 5). In the absence of TUN,

SY5Y/B cells did not exhibit obvious morphological changes associated with apoptosis compared with SY5Y/P cells, although the SY5Y/B cells had shrunken but intact cell membranes. After TUN treatment, SY5Y/B cells underwent severe apoptosis with condensed, marginalized chromatin and vacuolated cytoplasm. The degree of apoptosis was significantly higher in SY5Y/B cells than in SY5Y/P cells.

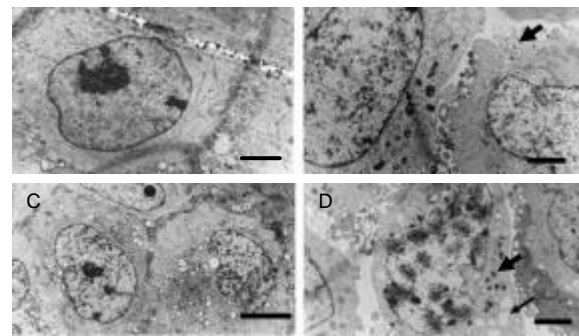


Figure 5 Ultrastructure of SY5Y/P and SY5Y/B cells (electron microscopy).

(A) Control cell line SY5Y/P without tunicamycin. The cells exhibit a lightly electron dense cytoplasm rich in organelles and are enclosed by an intact cell membrane, with clear nucleoli. Scale bar: 0.2 μ m.

(B) Control cell line SY5Y/P treated with 10 μ g/mL tunicamycin. The cells exhibited membrane blebbing (arrowhead) and slightly condensed nuclear chromatin, with a normal nuclear membrane margin. Scale bar: 0.2 μ m.

(C) Most SY5Y/B cells showed invaginated nuclear membranes and increased cytoplasmic electron density even without tunicamycin treatment. Scale bar: 0.5 μ m.

(D) SY5Y/B cells exposed to 10 μ g/mL tunicamycin show changes in features consistent with a later stage of apoptosis, including cytoplasmic vacuoles (small arrowhead), nuclear chromatin condensation, crystalline granular inclusion bodies (large arrowhead), absent nucleoli, and membrane blebbing. Scale bar: 0.2 μ m.

DISCUSSION

BI-1 protein is a member of the Bcl-2/Bax family and associates with Bcl-2 and Bcl-XL in mammalian cells. The BI-1 protective mechanism is characterized by suppression of Bax activation and translocation to mitochondria, preservation of mitochondria membrane potential and mitochondrial morphology. Low expression or loss of BI-1 is not lethal, and tissues from BI-1-deficient mice are histologically normal^[17]. Here we report that SH-SY5Y neuroblastoma cells with low expression levels of BI-1 could proliferate normally, but were more sensitive to TUN injury.

The SH-SY5Y cell line with low levels of BI-1 expression (SY5Y/B) was established using shRNA targeting the

region containing the start codon (-2 bp to 17 bp). This shRNA could effectively inhibit the expression of the BI-1 gene, while the inhibition efficiencies of three other shRNAs (two targeting the coding region and one targeting the 3' UTR) were all lower than 23% (data not shown). The shRNAs designed against different target sites resulted in different degrees of suppression, indicating that inhibition efficiency is mainly based on the shRNA binding site. Thus, the selected shRNA inhibits the translation initiation complex and synthesis of the mature mRNA.

TUN is a typical ER stress inducer. It can induce apoptosis mainly through inhibiting the glycosylation of glycoproteins. Bcl-2/Bax family proteins can modulate ER Ca²⁺ homeostasis and control cell death induced by ER stress agents^[18]. Recently, BI-1 has been linked to protection from apoptosis induced by ER stress^[17, 19]. The viability of BI-1-knockdown human neuroblastoma cells was not different from that of control cells 24 hours after treatment with TUN. However, a significant difference in cell viability between BI-1-knockdown and control cells was seen 36 hours after treatment with TUN. Thereafter, the cell viability rose again. Three reasons may account for these results. First, after treatment with a high concentration of TUN for a short time, apoptosis was initiated in the two cell lines and cell viability was not significantly different between the two. However, owing to the apoptosis, more and more cells died. The role of BI-1 in apoptosis was shown on the basis of cell viability. Second, ER stress is accompanied by the expression of molecular chaperones in the ER. Expression of stress proteins and molecular chaperones can reduce the accumulation of unfolded proteins in the ER lumen and ameliorate the physiological state of cells^[20]. It has been reported that overexpression of immunoglobulin binding protein was able to alleviate the poisoning effect of TUN on cells and in intact plants^[20-21]. Cells that did not undergo apoptosis after TUN treatment were resistant to ER stress and maintained a relatively balanced physiological state. Third, the ER stress inducer TUN partially degrades after long-term exposure, reducing its working concentration, resulting in a decrease in ER stress and an increase in proliferation in some cells.

It has been previously reported that ER stress is a common mediator of apoptosis in both neurodegenerative and non-neurodegenerative lysosomal storage disorders, such as Batten disease (neuronal ceroid lipofuscinosis 3)^[22]. Results of this study suggest that BI-1 is neuroprotective in human neuroblastoma cells (SH-SY5Y) via the ER pathway. After treatment with 10 μg/mL TUN for 24 hours, the rate of cell apoptosis was higher in SY5Y/B cells than in SY5Y/P cells. SY5Y/B cells displayed a statistically significant increase in sensitivity to TUN, which was consistent with the studies of

Dohm *et al*^[17, 23]. Electron microscopy observations demonstrated that most cells contained intact organelles and did not exhibit late apoptotic morphological changes, although there was a difference in the degree of apoptosis between the two cell lines. This could be due to two factors. One is that TUN mainly induces early apoptosis. The second is that the efficiency of BI-1 functional inhibition by RNA interference was not as complete as knockout of the BI-1 gene. Since the pathological mechanisms underlying degenerative diseases and the protective effects of BI-1 are associated with ER stress, and because degenerative diseases are accompanied by neuronal apoptosis, we hypothesize that an unidentified pathogenic component is involved in the development of neurodegenerative diseases, which may be linked to the ER.

ER stress is relevant to degenerative diseases. Further studies will determine whether BI-1 regulates ER stress pathways relevant to these diverse pathologies, and whether changes in ER Ca²⁺ are mediated by Bcl-2/Bax family proteins, including BI-1. The BI-1-knockdown cell line may help to further elucidate the pathological mechanisms associated with degenerative diseases and help to develop therapeutic strategies for the diseases.

MATERIALS AND METHODS

Design

An *in vitro* observational study involving genetic engineering.

Time and setting

Experiments were performed from January 2009 to March 2010 in the Laboratory of Gene Therapy, Department of Medical Genetics, Peking University Health Science Center, China.

Materials

293T virus packaging cells were purchased from ATCC, CRL-11268 (University Boulevard Manassas, VA, USA). SH-SY5Y cells were taken from the preserved stocks of the Laboratory of Gene Therapy, Department of Medical Genetics, Peking University Health Science Center, China.

Methods

Culture of 293T and SH-SY5Y cells

293T cells were cultured in Dulbecco's modified Eagle's medium (PAA Laboratories, Linz, Austria) containing 10% fetal bovine serum (PAA Laboratories) and penicillin (100 U/mL), streptomycin (100 μg/mL). SH-SY5Y cells were cultured in RPMI 1640 medium (PAA Laboratories) containing 15% fetal bovine serum and 1% penicillin/

streptomycin solution (PAN Biotech GmbH, Aidenbach, Germany). Cells were cultured at 37°C in a humidified incubator with 5% CO₂.

siRNA design targeting BI-1 gene

Four shRNAs were designed according to the nucleotide sequence of the human BI-1 gene (GenBank Accession No. NM_003217) using the online tool provided by Ambion (www.ambion.com). An *HpaI* site was introduced into the 5' end and an *XhoI* site was introduced into the 3' end of the shRNA sequence. The most effective shRNA sequence targeting ORF-2-17 bp of the human BI-1 gene was: 5'-TCC ATG AAC ATA TTT GAT CGT TCA AGA GAC GAT CAA ATA TGT TCA TGG TTT TTT C-3'. Other shRNA sequences tested were (5'-3') TTG ATC TTT ACC TGC TTC ACT TCA AGA GAG TGA AGC AGG TAA AGA TCA TTT TTT C, TGC TCA TGA TGA TCC TGG CCA TTC AAG AGA TGG CCA GGA TCA TCA TGA GCT TTT TTC and TGT ATA GTT TGG AGT TTG GCT TTC AAG AGA AGC CAA ACT CCA AAC TAT ACT TTT TTC.

pLL3.7/shRNA vector construction and virus packaging

120 pmol of oligonucleotides were annealed in 50 µL buffer (0.1 M NaCl, 10 mM Tris, pH 7.4). The mixture was incubated at 95 °C for 5 minutes, 70°C for 2 minutes, then gradually cooled to 4°C. One microliter of annealed oligonucleotides, 2 pmol of *HpaI/XhoI*-digested (TaKaRa, Dalian, China) pLL3.7 vector (a gift from the Department of Neurology, Peking University First Hospital, Beijing, China), 1 µL of 10 × ligation buffer, 1 µL of T4 DNA ligase (TaKaRa) and ddH₂O were combined. Ten microliters of ligation mixture was incubated at 4°C overnight. Five microliters of ligation products were transformed into competent *E. coli*/DH5a cells (TaKaRa). Primers used for identifying recombinant pLL3.7/shRNA vectors (primer2815: 5'-CTA TAA GAG ATA CAA ATA CTA AAT-3' and primer3090: 5'-CTA TTA ATA ACT AAT GCA TGG C-3') were synthesized by AOKE Company (Beijing, China).

293T cells were plated on 35-mm dishes at 2 × 10⁵ cells per dish in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Two micrograms of pLL3.7 or pLL3.7/shRNA, 1 µg of VSVG, RRE and REV were combined (all plasmids were provided by the Department of Neurology, Peking University First Hospital, China). Adherent cells were incubated with a mixture of plasmid and transfection reagent (Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) in serum-free medium for 6 hours. Then the cells were supplemented with additional medium (supplemented with 10% fetal bovine serum) and incubated for an additional 24 hours. After changing medium, cells were incubated for another 24 hours and culture supernatants were collected and filtered through

0.2 µm syringe filters.

Virus infection and real-time PCR detection of BI-1 RNA silencing in SH-SY5Y cells

SH-SY5Y cells were seeded in six-well plates (density 2 × 10⁵ cells/well) and allowed to adhere for 24 hours. The medium was replaced with a mixture of fresh medium and the collected viral supernatant at a ratio of 1:20 with 8 µg/mL polybrene (Sigma, St. Louis, MO, USA). The viral supernatant could induce 15% of the 293T cells to express EGFP. The infection efficiency was determined using a Calibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Total RNA was collected with Trizol Reagent (Invitrogen) 48 hours after infection and the inhibition level of BI-1 gene expression was determined by real-time PCR according to the protocol of the SYBR Premix Ex Taq kit (TaKaRa). The amplification primers were: 5'-ACG GAC TCT GGA ACC ATG AA-3' and 5'-AGC CGC CAC AAA CAT ACA A-3' and the product length was 170 bp. Beta-actin mRNA was used as an internal control, with the amplification primers 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3' and 5'-GGT AAC CGT TAC TCG CCA AGG CGA C-3' and a product length of 251 bp^[24].

Western blot analysis of BI-1 RNA interference silencing in SH-SY5Y cells

Infected SH-SY5Y cells were harvested and 5 × 10⁵ cells were lysed in 0.5 mM lysis buffer (mM; 137 NaCl, 1.5 MgCl₂, 2 EDTA, 10 sodium pyrophosphate, 25 β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 sodium orthovanadate, 1 phenylmethylsulfonyl fluoride and 20 Tris, pH 7.5) supplemented with 1× protease inhibitor cocktail. After incubation on ice for 10 minutes, cell lysates were precleared by centrifugation at 13 000 × g for 25 minutes. Twenty micrograms of total protein were loaded into each well and then separated by SDS-PAGE on 12% gels. Proteins were electro-transferred to nitrocellulose membranes. After blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20, the nitrocellulose membranes were incubated with a goat polyclonal antibody against human BI-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and donkey anti-goat IgG conjugated with horseradish peroxidase (1:5 000; Santa Cruz Biotechnology). Scanned bands were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA), and data were collected from three independent experiments. β-actin was used as control. A mouse monoclonal antibody against β-actin (1:5 000; Abcam, Cambridge, MA, USA) and goat anti-mouse IgG conjugated with horseradish peroxidase (1:2 000; Zhongshan Goldbridge, Beijing, China) were used.

Fluorescence-activated cell sorting

SH-SY5Y cells were infected with shRNA or empty control virus and the infected cell lines termed SY5Y/B or SY5Y/P, respectively. Cells were digested with trypsin at 48 hours after infection and adjusted to a concentration of 1×10^7 cells/mL. One milliliter of cell suspension was used in fluorescence-activated cell sorting. Infected cells expressing EGFP were isolated by fluorescence-activated cell sorting. The isolated cells were cultured and passaged.

Cell viability assay

Following fluorescence-activated cell sorting, passage 2 SY5Y/B and SY5Y/P cells were seeded in 96-well plates at a density of 1×10^4 cells/well. Cells were allowed to adhere for 24 hours and were then treated with $2 \mu\text{g/mL}$ TUN (Sigma, St. Louis, MO, USA). Cell viability was evaluated using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) every 12 hours until 41 hours, following the manufacturer's protocol. Briefly, after treatment, $10 \mu\text{L}$ of Cell Counting Kit-8 solution was added to each well. After incubation at 37°C for 2 hours in a humidified CO_2 incubator, absorbance at 540 nm was monitored using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The values were used to calculate cell viability. Each sample was tested in triplicate.

Flow cytometry detection of cell apoptosis

Passage 3 SY5Y/B and SY5Y/P cells were seeded at a passage ratio of 50%. After 24 hours, cells were treated with 0 or $10 \mu\text{g/mL}$ TUN for an additional 24 hours. Apoptotic cells were detected using the Annexin V-FITC kit (Jingmei Biotech, Shenzhen, Guangdong Province, China). In brief, cells were harvested by centrifugation at 1500 r/min for 10 minutes, washed twice with ice-cold PBS and then resuspended in $100 \mu\text{L}$ of binding buffer. Five microliters of AV-FITC stock solution and $5 \mu\text{L}$ of propidium iodide were mixed together and added to cell suspensions and incubated for 15 minutes at room temperature in the dark. After adding $400 \mu\text{L}$ of binding buffer, the cells were immediately analyzed on a FACStar Plus flow cytometer (Becton-Dickinson, Mountain View, CA, USA).

Electron microscopy observation of cell apoptosis

The pretreatment method was the same as that in Detection of cell apoptosis. Cell pellets of the harvested SY5Y/B and SY5Y/P cells were fixed overnight in 2% glutaraldehyde in 0.1 M PBS. The cell pellets were post-fixed in 1% osmium tetroxide in 0.1 M PBS for 1 hour, dehydrated, and embedded in epoxy resin Eponate. Sections of 60-90 nm thickness were cut with a Leica Ultracut UCT microtome (Solms, Germany) and

examined for ultra-structural features on a Hitachi H-7500 (Tokyo, Japan). SY5Y/B and SY5Y/P cells treated with $10 \mu\text{g/mL}$ TUN for 24 hours were also prepared as described above.

Statistical analysis

Data were expressed as mean \pm SD. One-way analysis of variance was used to compare differences using SPSS 13.0 software (SPSS, Chicago, IL, USA). A *P* value of less than 0.05 was considered to be statistically significant.

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Conflicts of interest: None declared.

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