Introduction

Tissue transglutaminase (tTG) or transglutaminase-2 (TG2) belongs to the transglutaminase family (EC 2.3.2.13) and has a molecular mass of 77–85 kDa. The transglutaminase family consists of nine members: factor XIII-A subunit, TG1, tTG (TG2), TG3, TG4 through TG7 and Band 4.2[1]. The first five members have been found in active forms in humans, but only TG1, tTG, TG3, TG6, and TG7 were detected in the brain, especially in the frontal cortex[2, 3], and tTG showed the highest expression. tTG has been reported to be expressed in both neurons and glial cells[4] and was localized in the cytosol, membranes, extracellular fractions, and even in the nuclei of neuroblastoma cells[4].

Tissue transglutaminase is a calcium-dependent enzyme that catalyzes an acyl transfer reaction between the γ-carboxamide group of a polypeptide-bound glutamine residue and the ε-amino group of a polypeptide-bound lysine residue to form an γ-glutamyl-ε-lysine covalent isopeptide bond (called isopeptide below)[5, 6]. This covalent bond was termed a cross-link. The catalyzed cross-links can be either intramolecular or intermolecular. Intramolecular cross-links change protein conformation, while intermolecular ones lead to the formation of rigid, stable and highly insoluble protein complexes[7, 8].

Alzheimer’s disease (AD) is a devastating neurodegenerative disease characterized by progressive cognitive impairment culminating in dementia. The main pathological mechanism is extracellular aggregations of β-amyloid (Aβ) as senile plaques (SPs)[9, 10] and intracellular accumulation of hyperphosphorylated Tau protein as neurofibrillary tangles (NFTs)[11]. Given that tTG-catalyzed cross-linking can cause Aβ or Tau accumulation and aggregation in AD-related brain regions, it is pos-
sible that tTG is involved in forming isopeptide bonds. This suggests that tTG-catalyzed cross-linking of Aβ and Tau may contribute to the pathogenesis of AD.

Neuronal loss from cell death is a typical feature of AD progression. Apoptosis is a pattern of regulated cell death, which could be identified by a variety of morphological and biochemical changes to the cell architecture that alters the cells for removal by phagocytic cells. The major biochemical and morphological changes that typify apoptosis are the activation of a subset of caspase family proteases, including caspase-3, 6, 7, 8, and 9. Protein expression changes, such as alterations in B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax), have been reported in apoptosis as critical components of the neuronal response to injury. The balance between the pro-apoptotic factor Bax and the anti-apoptotic factor Bcl-2 is important in apoptosis. Once activated, Bax can relocate from the cytoplasm to the mitochondria and cause the mitochondrial permeability transition and trigger an apoptotic cascade. Bax forms a heterodimer with Bcl-2 to block anti-apoptotic processes.

The SH-SY5Y neuroblastoma cell line is a well-characterized neuro-like cell line, which has been extensively used to study neurodegenerative diseases. When treated with all-trans-retinoic acid (RA), SH-SY5Y cells stop dividing and undergo neuronal differentiation. In particular, these cells overexpress tTG protein. These properties make the cell line suitable to assess the effects of enhanced tTG expression in neurodegenerative diseases.

NTU283 (1,3-dimethyl-2-[(2-oxopropyl) sulfanyl]-1H-imidazol-3-ium) is a 2-[(2-oxopropyl)thioimidazolium derivative. It has been used to irreversibly inhibit tTG activity as reported previously. NTU283 can block the enzyme activity by covalently modifying the catalytic site of the enzyme and then preventing substrate binding.

Although tTG inhibitors have been assessed in several diseases, few studies focused on their applications in AD and other neurodegenerative diseases. Our previous study reported that tTG expression and activity were increased in patients with AD and a transgenic mouse model of AD with age. In addition, the isopeptide immunoreactivity colocalized with the neurodegeneration-related proteins neurofilament, Tau, and Aβ in both AD patients and AD transgenic mice. These results suggest that tTG may be a key factor in the pathogenesis of abnormal protein aggregation in the AD process. Because they are highly resistant to proteolysis, the formation of isopeptide bonds can induce protein aggregation and deposition. Thus, we hypothesized that tTG may be used as a target in AD studies. To address this hypothesis, in our present study, the tTG inhibitor NTU283 was applied to RA-stimulated SH-SY5Y cells to determine whether tTG could alleviate the pathological features of AD.

Materials and methods
Reagents
NTU283 was provided by the Mayo Clinic, Jacksonville. All-trans-retinoic acid (RA) was purchased from Sigma-Aldrich (Louis, MO, USA). Synthetic human Aβ[1-40, 42] was purchased from Bachem (Torrance, CA, USA). Anti-tTG (transglutaminase-2) and anti-isopeptide (153-81D4) antibodies were obtained from Abcam (Cambridge, MA, USA). Anti-Bax, anti-Bcl-2, anti-β-actin and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from ProteinTech (Chicago, IL, USA). Near-infrared fluorescent secondary antibodies (IRDye 680RD, IRDye 800CW) were obtained from LI-COR (Lincoln, NE, USA).

Cell culture and treatments
SH-SY5Y neuroblastoma cells, obtained from the American Type Culture Collection (ATCC), were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 (1:1), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin and maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Cells were seeded in 6-well, 24-well or 96-well plates at a density of 1×10⁵ cells/mL and cultured for 24 h. The medium was removed and replaced by cell culture medium with 3% (v/v) FBS and 20 μmol/L RA every 3 d. Six days after RA administration, the medium was removed and replaced by serum-free medium containing NTU283 at different concentrations (0, 20, 100, and 500 μmol/L). After a 2 h pretreatment, 1 μmol/L Aβ[1-40,42] (called Aβ below) was added to the medium. An equivalent volume of medium was added to the culture as a control. Cells were harvested for further measurement and analysis after 24 h.

Western blot analysis
SH-SY5Y cell samples were harvested from 6-well plates and lysed in RIPA buffer containing protease inhibitor cocktail and phenylmethanesulfonyl fluoride (PMSF). Lysates were incubated for 30 min on ice and then centrifuged at 10000×g for 10 min at 4°C. The protein concentration in the supernatant was determined using BCA kits with bovine serum albumin (BSA) as a standard. Samples were denatured in protein sample buffer (100 mmol/L Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate, 0.2% Bromophenol Blue, 20% glycerol, 20% H₂O₂, and 200 mmol/L dithiothreitol) at 100°C for 5 min. Equal amounts of protein (30 μg per lane) were separated with 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membrane was blocked in TBST (Tris-buffered saline with 0.05% Tween) containing 5% non-fat milk for 1 h at room temperature (RT) and then incubated with primary antibodies (tTG, 1:2000; isopeptide, 1:500; Bax, 1:1000; Bcl-2, 1:500; β-actin, 1:10000) overnight at 4°C. After 3×10 min washes in TBST, immunodetection was performed using HRP-conjugated secondary antibodies. The membrane was visualized with an electrochemiluminescence (ECL) system and then detected on a Bio-Imaging System (DNR Lumi BIS, Jerusalem, Israel). The densities of the target bands were measured by Image-Pro Plus 6.0 and expressed as relative levels with respect to the β-actin loading control.
In-cell Western

In-cell Western (ICW) is an assay that can detect protein expression in situ\(^{[29]}\). It was performed using the Odyssey Application Protocol Manual (LI-COR). The ICW assay is a quantitative immunofluorescence-based technique, which directly detects the antigen in the cells. It can reduce sample loss compared to regular Western blots with a more complicated procedure. In addition, this assay has high sensitivity and can decrease background with near-infrared fluorescence detection. Additionally, it can be used for high throughput detection with a double-labeled method in 96-well plates.

After treatment with NTU283 or Aβ, the medium of SH-SY5Y cells, which were seeded in 96-well black plates with a clear bottom (REF655090, Greiner Bio-One, Germany), was removed. Cells were fixed by 4% polyformaldehyde in PBS for 20 min at RT, and then permeabilized by PBST-X (0.1% Triton X-100 in PBS) for 4×5 min. After 2 h blocking with 10% goat serum, primary antibodies (tTG+β-actin or isopeptide+β-actin) were incubated overnight at 4 °C. The primary antibodies were chosen in different hosts [tTG+β-actin: rabbit (IgG)+mouse (IgG); isopeptide+β-actin: mouse (IgM)+rabbit (IgG)]. The plates were washed with PBST (PBS with 0.1% Tween-20), and then, near-infrared fluorescent secondary antibodies (LI-COR) were added and incubated for 1 h at RT. Double-labeled secondary antibodies (IRDye 680RD, IRDye 800CW) for target proteins and β-actin were chosen depending on the hosts of the primary antibodies. Quantitative fluorescence signals were detected with an Odyssey SA Imaging System (LI-COR) and analyzed with Image Studio Ver 3.1. tTG and isopeptide protein levels were expressed as relative values to the β-actin levels.

Cell viability assay

To evaluate cell viability, we detected total mitochondrial activity with a Cell Counting Kit-8 (CCK-8) (Beyotime, Haimen, China). After the cells were treated with various concentrations of NTU283 (1, 4, 20, 100, and 500 μmol/L) for 24 h, the cells were lysed with one-tenth volume of CCK-8 reagent and incubated for 4 h at 37 °C. The absorbance at 450 nm and 630 nm of all samples was measured using a microplate reader (PerkinElmer, USA). The OD value was calculated to determine the viability of SH-SY5Y cells.

Hoechst 33342 and PI co-staining

Apoptotic or necrotic cell death was characterized using Hoechst 33342 and propidium iodide (PI) double staining\(^{[29,30]}\). SH-SY5Y cells with different treatments were stained with Hoechst 33342 and PI for 30 min on ice. After washing with D-Hanks’ solution, the cells were observed and imaged with an imaging system attached to a fluorescence microscope (Nikon, ELWD, 0.3, JP) at 350 nm and 550 nm excitation wavelength. Five random photographs were captured in each sample in different sections, and Image-Pro Plus 6.0 was used to analyze the apoptotic cell count.

Caspase-3 and caspase-7 activity assay

The caspase-3 and caspase-7 activity kits were purchased from Promega (Madison, WI, Apo-ONE\(^{[3]}\)). The substrate was diluted 1:100 with the buffer to obtain the working reagent at the desired volume. SH-SY5Y cells were treated in 96-well black plates (REF655090, Greiner Bio-One) and divided into following groups: blank (reagent+cell culture medium), negative control (reagent+vehicle-treated SH-SY5Y cells), and assay (reagent+treated SH-SY5Y cells). Reagent was added to each well at a volume of 100 μL (total volume 200 μL) and mixed thoroughly, followed by incubation for 4 h in the dark at RT. The fluorescence signal was detected on a microplate reader (PerkinElmer, USA) with a 499 nm excitation wavelength and a 521 nm emission wavelength.

Statistical analysis

All data are expressed as the mean±standard error of the mean (SEM). Statistical analyses were performed with one-way ANOVA followed by post hoc comparison and linear regression analysis with a threshold of \(P<0.05\) using SPSS 17.0 software. The results from individual experiments were averaged within each experimental group.

Results

NTU283 did not affect SH-SY5Y cell viability

We first examined the toxicity of NTU283 on SH-SY5Y cells by comparing the cells treated with 5 concentrations of NTU283 to the untreated control. No significant decrease in cell viability was observed following incubation of the cells with up to 500 μmol/L of NTU283 (data not shown).

Isopeptide level increased after co-treatment with NTU283 and Aβ

To test the inhibitory effects of NTU283 on tTG, we examined tTG and isopeptide (representing the enzyme activity of tTG) protein levels by Western blot analyses. Although NTU283 did not decrease the tTG protein level (Figure 1A), NTU283 reduced the isopeptide level (Figure 1B), suggesting this chemical may inhibit tTG enzyme activity to decrease isopeptide cross-linking.

Neither NTU283 nor Aβ alone or combined altered the tTG protein levels, while the levels of isopeptide were significantly reduced by NTU283 alone \((P<0.05, \text{ Lane 4})\) in a concentration-dependent manner (Figure 1B), indicating the inhibition of tTG activity. As expected, Aβ alone increased the isopeptide level (Lane 5 compared to Lane 1). However, when cells were pretreated with NTU283, addition of Aβ substantially increased the level of isopeptide at the same concentrations (Figure 1B). The highest level was observed at 100 μmol/L NTU283 \((P<0.01, \text{ Lane 7})\).

To confirm the results of the Western blot analyses, we further examined tTG and isopeptide protein levels using in-cell Western assays. Consistent with the results above, no significant changes in tTG level were observed following NTU283 or Aβ treatment or a combination (Figure 2A). However, isopeptide levels were significantly enhanced in the cells co-treated...
with NTU283 and Aβ (P<0.01), although NTU283 alone inhibited the formation of isopeptide cross-linking (Figure 2B).

**NTU283 promotes Aβ-induced apoptosis**

To explain the unexpected findings above, we determined whether apoptosis had occurred. Double staining of Hoechst and PI was used to detect apoptosis. There was almost no neuronal apoptosis detected following treatment with NTU283 alone at different concentrations for 24 h. Aβ at 1 μmol/L could induce low levels of apoptosis in SH-SY5Y cells, as shown by the Hoechst-positive cells with condensed, fragmented or degraded nuclei, as well as the sparse numbers of PI-labeled cells compared to vehicle-control cells (Aβ-free) (P<0.01) (Figure 3A). However, when the cells were pretreated with NTU283 and then co-incubated with Aβ, a dramatic increase in apoptotic cells was observed in a concentration-dependent manner. Only a few necrotic cells were observed. The highest apoptotic rate (approximately 50%) was observed following co-treatment with 100 μmol/L NTU283 and Aβ (P<0.01) (Figure 3B).

Caspase-3 and caspase-7 enzyme activities were further detected to confirm the above findings. The results demonstrated (Figure 3C) that caspase activities were higher in Aβ-treated cells compared to those of the vehicle control (Aβ-free) (P<0.01). Consistent with the apoptotic rate, after pretreating the cells with different concentrations of NTU283, the activities of caspase-3 and caspase-7 were significantly enhanced by Aβ stimulation (P<0.01).

Bax and Bcl-2 were detected by Western blot analysis as well. Bax showed higher expression in Aβ-treated cells compared to that of the vehicle control (Aβ-free) (Figure 4A). Significant differences were observed at a concentration of 100 μmol/L of NTU283 (P<0.05, Lane 3 vs Lane 7). Treatment with NTU283 alone did not significantly increase Bax protein levels. However, co-treatment of cells with NTU283 and Aβ substantially increased the Bax level, with a peak at 100 μmol/L (P<0.01, Lane 7). Meanwhile, lower levels of Bcl-2 were observed in the groups treated with Aβ alone and pretreated with NTU283 (Figure 4B). A high concentration of NTU283 (500 μmol/L) also decreased Bcl-2 expression (P<0.05, Lane 4). Regardless of Aβ co-treatment, the Bax/Bcl-2 ratio increased with NTU283 concentration (Figure 4C). Consistent with the above results, co-treatment of cells with NTU283 and Aβ enhanced the ratio of Bax/Bcl-2 significantly, although NTU283 alone also increased the ratio to some extent. The peak was observed at 100 μmol/L of NTU283 with Aβ (P<0.01).

In summary, NTU283 can promote Aβ-induced apoptosis, although NTU283 itself cannot induce apoptosis in SH-SY5Y cells alone.

**Discussion**

tTG expression and activity have been reported to be increased in Alzheimer’s disease. tTG can catalyze Aβ cross-linking to form isopeptide polymers[3, 12]. Being highly resistant to proteolysis, these bonds can induce protein aggregation and deposi-
It has been ever hypothesized that multiple factors, such as trauma[31, 32], inflammation[33, 34], or ischemic damage[35, 36], in sporadic AD or overproduction of Aβ in familial AD may lead to cross-linking of AD-related proteins. The overexpression of tTG in turn catalyzed protein cross-linking, and aggravated the pathogenesis of AD[16]. Thus, tTG may be an AD therapeutic target.

We tested the effect of a tTG inhibitor on SH-SY5Y cells, which were stimulated to overexpress tTG by RA. Moreover, Aβ was added to mimic an AD pathological condition because it is involved in protein aggregation and neural toxicity in AD pathogenesis[42, 43]. The Aβ oligomers were toxic to neurons and may trigger oxidative stress and apoptosis[44]. tTG-mediated cross-linking may also play a role in Aβ aggregation and accumulation in cells. In our study, apoptosis was induced by Aβ stimulation in SH-SY5Y cells as verified by morphological staining, caspase activities and Bax/Bcl-2 protein immunoblotting. However, instead of apoptosis inhibition, increased apoptosis was observed after NTU283 and Aβ co-treatment, which was contrary to our expectations. These results suggested that tTG enzyme activity is involved in the regulation of apoptosis.

Previous studies have indicated that transglutaminases are involved in the regulation of apoptosis[45, 46]. tTG has crucial roles in many cell biological processes, including differentiation, adhesion, extracellular matrix formation, and cell apoptosis, by modifying its substrate proteins[47]. It was suggested that tTG not only regulated apoptosis but also interacted with apoptosis-related factors[48]. Recent studies have indicated that
Figure 3. Apoptotic detection of SH-SY5Y by Hoechst 33342/PI co-staining and caspase assays. (A) SH-SY5Y cells were pretreated as indicated in 24-well plates. Hoechst 33342 and PI co-staining were applied and then detected with fluorescence microscopy at 350 nm excitation and 550 nm excitation wavelength. Then, 150–250 cells were observed per vision field. Representative images of Hoechst (blue), PI (red), and Merged (purple) are shown on the left. Aβ (-) groups included different NTU283 concentrations: 0 μmol/L (a–c), 20 μmol/L (d–f), 100 μmol/L (g–i), and 500 μmol/L (j–l), as well as Aβ (+) groups containing 0 μmol/L (m–o), 20 μmol/L (p–r), 100 μmol/L (s–u), and 500 μmol/L (v–x) NTU283. (B) Cell apoptosis determined by Hoechst 33342/PI co-staining was analyzed by Image-Pro Plus 6.0. Bright blue nuclear staining without red staining indicated apoptotic cells. The rate was calculated by the number of apoptotic cells/the number of total cells. Five independent captured images from each sample of three experimental groups (n=3) were analyzed. (C) Caspase-3 and caspase-7 enzyme activities were detected in SH-SY5Y cells that were pretreated as indicated and plated in 96-well black plates. Caspase kit reagent was added to each well at a volume of 100 μL (total volume 200 μL) and mixed thoroughly, and the samples were incubated for 4 h in the dark at RT. Fluorescence intensity was detected by a microplate reader. Statistical data were calculated from three replicate samples in each independent group from three experimental groups and are expressed as the mean±SEM. *P<0.05, **P<0.01 vs each control (0 μmol/L NTU283) with or without Aβ. ***P<0.01 Aβ(-) group vs Aβ(+) group.
the complexes formed by tTG and other proteins could inhibit apoptotic cascades. Additionally, tTG can interact with the pro-apoptotic protein Bax to inhibit apoptosis. Moreover, because of the calcium-binding activity of tTG, increased free calcium may be released to the cytoplasm and extracellular matrix after irreversible inhibition of the enzyme. Under these conditions, high concentrations of free calcium would cause cell stress and finally lead to cell death.

From our data and previous reports on the roles of tTG in apoptosis, we hypothesized that the effects of the tTG inhibitor NTU283 on Aβ-induced apoptosis may be related to inhibition of tTG interacting with the Bax protein, resulting in the release of free Bax and activation of apoptotic cascades. With increased tTG inhibition, more active Bax would aggravate cell apoptosis. Meanwhile, a high concentration of calcium was released to the cellular environment. An increased risk of apoptosis and calcium-induced cell stress may directly lead to neural cell death. To alleviate cell death and lysis, tTG may catalyze more proteins, including neurodegenerative-related proteins, fibronectin, and fibrinogen to form isopeptide cross-linking bonds on the cell surface for packing cell as a cocoon, which is involved in wound healing and adhesion functions of tTG. Highly resistant to proteolysis, these proteins would protect cells from apoptotic death or other external risks. This may explain why the isopeptide level was increased with NTU283 and Aβ co-treatment. This hypothesis should be assessed in further studies.

In conclusion, tissue transglutaminase can catalyze Aβ cross-linking as γ-glutamyl-ε-lysine isopeptide bonds in AD, resulting in protein aggregation and deposition. Thus, tTG may be a target for alleviating AD. Inhibition of tTG activity with NTU283 alone can reduce isopeptide protein aggregation in SH-SYSY cells. However, in contrast to our expectations, the isopeptide levels were increased following Aβ and NTU283 co-treatment. Moreover, it should be emphasized that irreversible inhibitors such as NTU283 could promote Aβ-induced neuron apoptosis, although NTU283 itself cannot induce apoptosis. This type of irreversible inhibitor treatment must be evaluated in future studies of AD because its irreversible inhibitory effects may affect the normal functions of tTG.

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Author contribution
Rui WANG designed and guided the research, analyzed the data and polished the paper; Ji ZHANG designed and performed the research, analyzed the data and wrote the paper; Yi-rong DING assisted in performing the research.

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