Neuroprotective effects of Etidronate and Trisphosphonates on PC12 cells against toxicity induced by glutamate

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Abstract Etidronate is one of the best known derivatives of Bisphosphonates (BP), it is often used as a reference study in research related to hypercalcemia and other common bone diseases. 2,3,3-Trisphosphonates (TrisPP) belongs to a brand new analogue of BP, containing also a 'germinal bisphosphonate' unit with an additional phosphoryl group attached in a close proximity to the BP unit. It is known that BP bind to calcium by chemisorptions to form Ca-BP complexes through (O)P-C-P(O) moiety and hydrogen coordinations, and so they suppress calcium flow by interfering Ca^{2+} channel operations. The mechanistic actions of BP involving interactions and regulations of Ca^{2+} are somewhat similar to the pathogenesis of well known neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). To investigate the neuroprotective effect exhibited by the compounds of our interests, we used rat adrenal pheochromocytoma cell line (PC12) as our *in vitro* model to observe any occurrence of neuron inter-reflection. We pre-treated these PC12 cells using Etidronate and TrisPP before injuring the cells using high dosage of neurotoxin Glutamate. Our data showed that the pre-treatment of 100 μM Etidronate ameliorated some cell viability (47%), whereas pre-treating cells using only 10-100 μM TrisPP showed remarkable cell recovery (78-86%). Moreover, cell pre-treatments using Etidronate or TrisPP both showed to have attenuated cell apoptosis, reactive oxygen species (ROS) generation, Ca^{2+} overloading and caspase-3 protein expression, which were associated with a remarkably increased level of superoxide dismutase (SOD) activity in our glutamate-injured PC12 cells.

Keywords: Etidronate; Trisphosphonates; PC12 cells; Glutamate; Neuroprotection

Introduction

Bisphosphonates (BP) is a category of drugs that are used to treat various bone diseases, such as osteoporosis, Paget's disease, bone malignancy and other skeletal complications involving excessive bone losses. It is often used as modelling drug samples to study interactions involving calcium metabolism [1]. All BP drugs share a common P-C-P functional unit in relation to their chemical structures and so this is called the 'geminal bisphosphonate' unit [2, 3]. Etidronate is one of the very first generation of BP that was used to treat osteoporosis. Etidronate has the simplest chemical structure (**Fig. 1A**) of all BP and it is often used as a reference for potency study of other BP analogues [4]. *In vitro* model, BPs process similar functions as pyrophosphate, it has similar inhibitory effects on calcium phosphate formation and dissolution *via* the calcium homeostasis pathway. As *in vivo*, BPs

are potent inhibitors of soft tissue calcification where they inhibit accretion of mineral into bone and resorption of bone. Therefore, in clinical practice, BPs is used to inhibit bone destruction, which lead to an increase in bone mineral density by suppressing bone resorption and bone turn over process [5, 6]. Etidronate owns three active binding sites, which allow ionic coordination to occur between Ca^{2+} in the hydroxyapatite and any of the oxygen atoms attached to the bisphosphonate or the α-Carbon moiety [7]. The 2,3,3-Trisphosphonate (TrisPP) we present in this work belongs to a brand new analogue of phosphonates, which was synthesised *in situ* by Cheong *et al* [8]. This novel TrisPP is comprised by a 'geminal bisphosphonate' unit, with an additional phosphoryl group attached in a close proximity to the BP unit (**Fig. 1B**). An increased binding sites presence in the TrisPP is expected to display a better inter-cellular Ca^{2+} binding affinity in our neuronal cell. As a consequence, the intra-cellular influx will be reduced and the overall intracellular Ca^{2+} concentration will be suppressed or even inhibited. Here in, is the first report documented *in vitro* studies of the neuroprotective effect introduced by novel 2,3,3-TrisPP.

Neurodegenerative disorders and osteoporosis are two common chronically progressive degenerative diseases, they share some common risk factors such as advanced age [9]. It is worth noting that patients suffer osteoporosis are more commonly found to also diagnose with neurodegenerative complications. In contrast, patients with Parkinson's disease are found to have lower bone mineral densities when comparing to age-matched controls [10-12]. Recent studies showed patients with osteoporosis who underwent BP therapy have a lower risk of dementia [13-15]. Moreover, recent research also found that the Alzheimer's disease with risk genes such as Aβ42 and APP are also involved as risk factors in osteoporosis [16, 17]. There is strong evidence that suggests links may exist inherently in relations to neurodegenerative disorder and bone loss. There are obvious reasons to believe that neurodegenerative disorders are associated with osteoporosis (and other bone diseases) [14] and that they may share the common pathogenesis.

It is known that neuronal cell apoptosis is caused by excessive intracellular calcium influx due to an over excitation of glutamate receptors (i.e. NMDA) and unspecific ion-channel opening triggered by glutamate bindings. A molecular interaction study reported by Rebecca K. Lane *et. al*. found mitochondrial dysfunction caused aberrant reactive oxygen species (ROS) generation and Ca^{2+} deregulation, which explain a conserved mechanism involved in neurodegenerative disorders and osteoporosis pathological alterations [18]. Our previous studies concerning neurotoxicity effects [19] have provided adequate evidence

showing the increase of oxidative stress was directly related to the excessive intracellular accumulations of ROS and Ca^{2+} influx, which led to apoptosis of differentiated PC12 cells [20, 21]. Overloading oxidative stress is certainly a key factor for PC12 cells apoptosis, the underlying intracellular mechanisms involved in such a process can be studied *via* their ROS generation and SOD activities [22]. It is worthwhile considering these important factors and to extend the evaluations of BP and their derivatives, to explore their effect on reactive oxygen species (ROS) generation and Ca^{2+} concentration. Also to monitor any occurrence of reversible neurodegenerative effects on neurons that were damaged by glutamate [23] or whether these compounds may act as neuron injury preventer. These *in vitro* assays would allow better understanding of the possible pathogenesis shared among neurodegeneration and bone diseases, with an aim to expand the pharmacological potential of clinical and novel BP.

Material and methods

Chemicals and reagents

Glutamate (purity >98 %) was purchased from Sigma (St. Louis, MO, USA). Etidronate (Sigma-Aldrich UK Etidronic acid >95%) and 2,3,3-Trisphosphonate (TrisPP) (synthesised *in situ*) samples were acquired from the University of Hertfordshire, Hatfield, UK. RPMI 1640 cell culture medium, trypsin–EDTA and fetal bovine serum (FBS) were supplied by the Gibco (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Sigma Chemicals. Hoechst 33258, superoxide dismutase (SOD) and ROS test kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The Annexin V-FITC propidium iodide (PI) apoptosis detection kit was obtained from Tianjin Sungene Biotech Co. Ltd (Tianjin, China). Rat Caspase-3 Elisa Kit was supplied by the Shanghai Lengton Bioscience Co. Ltd (Shanghai, China).

Cell culture and sample preparations

PC12 rat pheochromocytoma cells, which were obtained from Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. They were cultured in RPMI 1640 medium (pH 7.4) supplemented with 10 % FBS and 1 % antibiotics (penicillin-streptomycin). Cells were grown in an incubator at 37°C in 95 % humidified air with 5 % CO_2. In general, all PC12 cells were cultured and differentiated with 50 ng/ml nerve growth factor for 24 hours (h) prior the pre-treatments of Etidronate and TrisPP for 4 or 8 h. The resulting PC12 cells were washed several times using glutamate-RPMI 1640 medium and finally incubated at

37 °C for 24 h.

Cell viability assay by MTT

PC12 cells were seeded in 96-well culture plates at an approximate density of 2×10^4 cells in 200 μL medium per well and cultured 24 h for stabilization. To obtain the optimal viability result induced by glutamate (dissolved in RPMI 1640 medium), initial study was performed by treating PC12 cells with glutamate under different concentrations (5, 10, 15, 20, 25, 30 mM), these samples were incubated for 24 h, 48 h and 72 h. The subsequent viability tests were carried out by pre-treating PC12 cells with Etidronate or TrisPP under different concentrations (0.1, 1, 10, 100μM) for 4 h and 8 h, the resulting cultures were washed using RPMI 1640 and then incubated in 25 mM glutamate for 24 h. Finally 10 μL MTT (0.5mg/ml) indicator was added to each well and incubated for a further 4 h at 37 °C. Excess MTT was removed and the insoluble dark blue formazan crystal was dissolved in 150 μL DMSO. The absorbance of formazan was measured using a microplate reader at wavelength 578 nm. The cell viability was calculated as a percentage against the control group [24].

Cell apoptosis detection by flow cytometry

The apoptotic index of PC12 cells were marked using FITC-Annexin V/propidium iodide (PI) apoptosis assay kit and analysed by flow cytometry [25]. The PC12 cells were plated in six-well plates and were exposed to 100μM Etidronate or TrisPP for 8 h, the resulting cells were washed and treated with 25 mM glutamate for 24 h. The cells were then harvested, washed twice with a cold phosphate-buffered saline (PBS, pH 7.4) and finally suspended in a binding buffer containing 5 μL V-FITC and 10 μL PI at room temperature in a dark environment for 15 min before being examined by a flow cytometer (Beckman-Coulter, USA). The percentage of cell apoptosis was obtained as follows:

% cell apoptosis = (Level of assigned group/Level of control group) x 100

Nuclear morphology – Hoechst 33258

Hoechst 33258 staining was used to evaluate the morphological change in all cell groups [26]. The PC12 cells were seeded in 96 well plates and were exposed to 100μM Etidronate or TrisPP for 8 h, the resulting cells were washed and treated with 25 mM glutamate for 24 h. 100 μL Hoechst 33258 was added to each well and the cells were

incubated for 30 min at 37 °C in a dark enviroment. Each sample was washed twice with PBS and the Hoechst stained nuclei were visualised using a fluorescence microscope.

Measurement of ROS generation

The level of ROS was measured using dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe [27]. PC12 cells were seeded in six-well plates, which were then treated with 100μM Etidronate or TrisPP for 8 h, all cells were then washed and exposed to 25 mM glutamate for 24 h. Cells were incubated with the final concentration of 10 mM DCFH-DA and kept in the dark for 30 min at 37 °C. The methodology and procedures were followed as described in the ROS assay kit. The samples then washed twice with PBS and the fluorescence DCFH intensities were detected by flow cytometry.

Measurement of SOD enzyme activity

The level of SOD activity was measured using SOD kit. The PC12 cells were plated in six-well plates and exposed to 100μM Etidronate or TrisPP for 8 h, followed by a treatment of 25mM glutamate for 24 h. PC12 cells were homogenized with PBS and lysed with lysis butter for 30 min at 4 °C. The lysates were centrifuged at 12,000 rpm/min for 15 min. Then, the supernatant obtained was used for the determination of SOD enzyme activity according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader.

Measurement of intracellular Ca^{2+}

Intracellular calcium concentration was determined using Fluo-3/AM fluorescent dye. The PC12 cells were plated in six-well plates and then exposed to 100μM Etidronate or the TrisPP for 8 h, followed by a treatment of 25mM glutamate for 24 h. Afterwards, the cells were washed twice with cold PBS and incubated with the final concentration of 5μM Fluo-3/AM for 30 min in the dark at 37°C. The fluorescence intensity of Ca^{2+} was examined by Confocal Laser Scanning Microscopy (Olympus, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Data analysis and image processing were acquired using image J software.

ELISA to detect the expression of caspase-3

The method for sample preparation was the same as SOD. An expression of caspase-3

was detected by Rat Caspase-3 Elisa kit. The specific experimental method was followed using instructions provided by the manufacturer. The absorbance was measured at 492nm using a microplate reader.

Statistical analysis

All of the results were expressed as mean ± standard deviation (SD) for three separate experiments, which were analyzed by SPSS 16.0 and Graph Pad Prism 5. The statistical significance of the changes between tested groups and the control group were analyzed by ANOVA followed Dunnett's multiple comparison and a value of $P < 0.05$ was considered significant.

Results

Cell viability was determined by MTT, selective concentrations of Etidronate, TrisPP and glutamate were used to perform these tests. Standardized test were carried out by exposing PC12 cells under different concentrations (5, 10, 15, 20, 25, 30 mM) of glutamate for 24 h, 48h and 72h. As shown in **Fig. 2A**, the cell viability was gradually decreased in time and it appeared to be dose-dependent. In particular, cell viability was down to 57% at the concentration of 25 mM in 24 h and the viability was almost the same when cells were treated with 30 mM of glutamate. Based on results showed in **Fig. 2A**, we chose to use 25 mM concentration of glutamate as the injury dosage of our assays.

All PC12 cells were pretreated with different concentrations of Etidronate or TrisPP for 4h and 8 h, then incubating sample cells with 25 mM glutamate for 24 h. **Fig. 2B** shows the percentage of cell viability of PC12 cells when they were pretreated with 0.1, 1, 10 and 100 μM concentration of Etidronate in 4 and 8 h. Our data indicates, 100 μM concentration Etidronate provided the optimal cell recovery over 4 and 8 h (42.98% and 47.44%). This study was repeated using different concentrations of TrisPP (0.1, 1, 10 and 100 μM), interestingly, data obtained from the pretreatment of TrisPP showed much more significant cell recovery (**Fig. 2C**) when comparing to data obtained from glutamate standard and Etidronate cell viability assay. In 4 h, cell viability reached to 41.34%, 41.42%, 65.60%, 66.05% at 0.1, 1, 10 and 100 μM concentrations of TrisPP respectively. The optimal percentage of cell viability was recorded when PC12 cells were pretreated using 100 μM of TrisPP for 8 h, cell recovery value reached to 86.40%. Moreover, in 8 h, 45.06%, 47.50% and 78% of cell recovery were recorded when cells were pretreated with 0.1, 1 and 10 μM of TrisPP respectively. In order to show that Etidronate and TrisPP do not exhibit harmful

cytotoxic effects to neuronal cells, we treated the PC12 cells with 100 μ M Etidronate and TrisPP for 8h, no significant change was observed in cell viability when the corresponding result was used to compare with one obtained from the control group (**Fig. 2D**). This treatment concentrations (100 μM) and condition (8 hrs) were therefore chosen to apply in our subsequent *in vitro* experiments for intra-cellular mechanistic examinations.

Cell apoptosis were performed using FITC-Annexin V/propidium iodide (PI) and the apoptotic rate was monitor by flow cytometry. As shown in **Fig. 3**, the apoptotic rate was remarkably increased when PC12 cells were treated with glutamate standard (178 %) when comparing to the control group (100%). Whereas, a slower apoptosis rate was observed when PC12 cells were pretreated using 100μM Etidronate (145%) and TrisPP (137%). The result indicated that both Etidronate and TrisPP can be used to prevent/ reduce apoptosis in glutamate-injured PC12 cells.

The morphology of cell nuclei was stained using Hochest 33258 and analyzed by fluorescent microscope. Fluorescent images in **Fig. 4A-D** showed all PC12 cells underwent nuclear condensation, nuclear segmentation and appeared in their apoptotic form when cells were treated with glutamate standard alone or in the present of Etidronate or TrisPP. Although all of which, the glutamate treated cells represented a typical process of cell apoptosis, a more alleviated cell nuclei damages were observed when cells were pretreated with 100 μM Etidronate (**Fig. 4C**) and TrisPP (**Fig. 4D**) for 8 h.

ROS levels were determined using DCFH-DA and measured using flow cytometry. While *SOD activities* were examined using commercial SOD kit and the resulting absorbance were measured using a Microplate reader. As shown **Fig. 5**, the ROS level had significantly increased to 88 % when cells were treated with 25 mM of glutamate standard comparing to the control group (56.8 %). Whereas, when glutamate-injured cells were pre-treated using 100 μM Etidronate and TrisPP for 8 h, their ROS generation had significantly reduced to 77.43 % and 66.60 % respectively.

In contrast, as indicated in **Fig. 6**, cells pre-treated with Etidronate and TrisPP were found to have their SOD activities recovered to 12 U/mgprot and 14 U/mgprot from the low SOD activity induced by the cytotoxic glutamate (16 U/mgprot).

Intracellular Ca^{2+} concentration were examined using Fluo-3/AM fluorescent dye and the resulting emission data was acquired using a Confocal Laser microscope (*Ex*. 488 nm; *Em*. 525 nm). The high fluorescent intensity observed in **Fig. 7E** shows the intracellular Ca^{2+}

concentration has significantly elevated by the treatment of glutamate when comparing with the control group that is shown in **Fig. 7A-B**. While pre-treating glutamate-injured cells using 100 μM Etidronate found to give good effect on reducing the intracellular Ca^{2+} level (**Fig. 7c**) even better result were obtained from the pre-treatment using TrisPP (**Fig. 7d**), this reading was very close to matching values obtained from the control group.

The expression of caspase-3 is a known technique used for examining the apoptosis index, therefore in our study, the level of caspase-3 was recorded and the results were used to illustrate neuroprotective effects induced by the present of Etidronate and TrisPP. In **Fig. 3B**, again, as glutamate-injured cells were showing a high level of caspase-3 expression (1.5 ng/ml), pre-treating these injured cells with 100 μM of Etidronate (1.1 ng/ml) and TrisPP (0.9 ng/ml), the level of caspase-3 were able to recover to near the values acquired from the control group (0.8 ng/ml).

Discussion

Neurodegenerative disorders are progressive multi-factorial autoimmune disorders, the category group of diseases is normally characterized by the injury and apoptosis of neurons. Glutamate excitotoxicity is one of the most known factors, which trigger cell death in many Central Nervous system disorders. Several clinical glutamate antagonists such as Simvastatin and Estrogen (17Beta-Rstradiol) are prescribed to patients to promote the neuroprotective effects [28]. Although BP work as anti-resorptive drugs and are the best known treatments for Paget's disease, osteoporosis, hypercalcemia and other bone diseases [1-4, 29]. A number of articles have been published in regard of using BP as alternative treatments for anti-parasitic [30], anti-infective [31] and anti-cancer therapies [32-35]. As it has been recently recognized that BP inhibits osteoclast activities by undergoing common mechanistic pathways to manipulate the isoprenoid and cholesterol biosynthesis [36]. As mentioned earlier, there are strong evidence showing links and associations between neurodegenerative and bone disorders [14]. To initiate our exploration in this area, we performed a series of *in vitro* assay to study the neuroprotective effect of 'Etidronate' and '2,3,3-TrisPP', as well as to understand their underlying bio-mechanisms. Etidronate is one of the conventional analogue of BP that we used as a reference in our study, while 2,3,3-TrisPP is a novel analogue of BP. With regard to the Structure Activity Relationship (SAR) of the 2,3,3-TrsiPP, it has rather small molecular size when comparing to other glutamate antagonists, which is essential for crossing the Blood Brain Barrier (BBB). It is believed that

removing the –OH group in general BP molecule reduces bone-binding ability and with the present of a more lipophilic substituent enhances cell or tissue penetration [36]. Therefore, the present of COOH group with an additional phosphoryl unit in the TrisPP imply a practical application as a neuroprotective agent as well as retaining the anti-resorptive function for bone loss complications.

Extracellular glutamate binds to N-methyl-D-aspartate (NMDA) receptors in a calcium channel membrane, where it mediates transportations of positive ions (i.e. Ca^{2+}). High calcium concentration in the cells is an indication of excessive accumulation of glutamate, hence glutamate acts as neurotoxin, which may lead to learning disability and memory loss [37, 38]. Mechanistic studies have proved that neuronal death induced by glutamate often caused by excessive ROS production and Ca^{2+} influx [39, 40]. PC12 cells, a rat pheochromocytoma cell line, possess neuron characteristics have been widely used as an *in vitro* model system for neuronal damage studies [41]. Thus, we expect a thorough examination of the ROS level and Ca^{2+} influx in glutamate-neurotoxin induced PC12 cells using Etidronate and TrisPP will provide crucial information for the exploration of potential neuroprotective medications.

To examine the neuroprotective effects of the compounds of our interests, we pre-treated our PC12 cells using different concentrations (0.1, 1, 10 and 100 μM) of Etidronate and TrisPP for 4 and 8 h prior injuring the cells using the optimal concentration (25 mM) of neurotoxin 'glutamate' selected from our initial cell viability assay (**Fig. 2A**). As shown in **Fig. 2B**, our cell viability data suggested that PC12 cells reached to optimum recovery of 47.4% when they were pre-treated with 100 μM of Etidronate for 8 h. Astonishing results were observed as cultures were pre-treated using lower concentrations of TrisPP (10 and 100 μM) for 4 and 8 h, cells recovery reached 66-86% (**Fig. 2C**). Although TrisPP showed to have excellent recovery rate and seemed to exert much higher protective effect on the glutamate-injured PC12 cells than Etidronate, the data obtained from cell apoptosis assay (**Fig.3**) of the two were indeed comparable. The apoptosis rates fell to 145-137 % when PC12 cells were pretreated 100 μM of Etidronate and TrisPP for 8 h, which still significantly lower than glutamate treated cells (178 %). Morphological images of nuclei obtained from Hochest 33258 staining tests show PC12 cells were severely damaged *via* nuclei condensation (**Fig.4B**). In contrast, only very mild nuclei damage was observed if PC12 cells were pre-treated using Etidronate or TrisPP. Although both Etidronate and TrisPP appeared to exhibit anti-apoptosis ability on the injured PC12 cells, a stronger protective effect was again observed from the pre-treatment of TrisPP.

In order to understand the bio-mechanisms of neuroprotective effects exhibited by Etidronate and TrisPP, we acquired further intracellular *in vitro* measurements to quantify the levels of ROS, SOD activities, Ca^{2+} influx and Caspase-3 expression in the glutamate-injured PC12 cells.

It is known that neurodegenerative disorders such as AD and PD are associated with neuron cell apoptosis, which is closely related to ROS and intracellular Ca^{2+} productions [42, 43]. Excessive accumulation of intracellular Ca^{2+} was an important signal of apoptosis and ROS generation [44, 45]. SOD is an important antioxidant, which denotes antioxidant capacity of cells and regulates the level of ROS [22]. Our experiments indicated that pretreatments with Etidronate and TrisPP prevented cell apoptosis induced by glutamate *via* an inhibition of ROS generation (**Fig. 5A-E**) and stimulation of SOD activity (**Fig. 6**). These results observed was clearly associated with the intracellular Ca^{2+} influx being suppressed by the pre-treatments of Etidronate and TrisPP (**Fig.7A-E**), which overall had reduced the apoptotic rate. Gather all the data and evidence collected from our *in vitro* experiments, we propose these analogues of BP exhibit neuroprotective effects by attenuating the apoptosis process through interaction with calcium to ameliorate the intra-cellular Ca^{2+} overload and mitochondrial oxidative stress, as illustrated in schematic **Fig. 8**. To further support our proposed mechanism of the neuroprotective effect occurred during cell apoptosis, we used ELISA to detect the Caspase-3 (an apoptosis factor) expression in our cell samples. The absorbance data acquired showed pretreatment of Etidronate and TrisPP had significantly down-regulated the caspase-3 expression against cell apoptosis, as expected, this observation was in-line with all other results collected from the ROS (**Fig. 5E**) and Ca^{2+} (**Fig. 7E**) measurements. Finally, it is important to note that our quantitative results obtained from pre-treatment using 100 μM Etidronate and TrisPP for 8 h induce ultimate neuroprotection against the glutamate-injured PC12 cells. As shown in **Fig. 5E**, **6**, **7E** and **8**, the experimental results acquired from TrisPP pre-treatments were very close to matching data generated by the control group, this has convinced us to further explore such novel compound and perhaps open up a fruitful area in drug discovery for the treatment of autoimmune diseases.

Conclusion

Our research has confirmed that both Etidronate and TrisPP provide excellent neuroprotective effects on PC12 cells against glutamate-induced apoptosis. All our *in vitro* results indicate TrisPP by far exerts much better protective effects than Etidronate under the applied concentrations of 0.1–100 μM. Preliminary mechanistic study suggested that both

Etidronate and TrisPP exhibit neuroprotective effect through attenuating the ROS generation, stimulating SOD activity, suppressing Ca^{2+} influx and down-regulating the caspase-3 expression (**Fig. 9**). With all the results collected, we are convinced that Etidronate, especially TrisPP and perhaps other BP analogues can be used as alternative medicines for patients suffer neurodegenerative disorders, such as Alzheimer's and Parkinson diseases. We also strongly believed that our study has introduced the concept of applying a single treatment to simultaneously target dual complications cause by neuron and bone degenerations. Further exploration will focus on *in vivo* assays using brain slices and animal models to carry out a more comprehensive study on the neuroprotective effected promoted by 2,3,3-TrisPP and other BP analogues.

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Figure legends

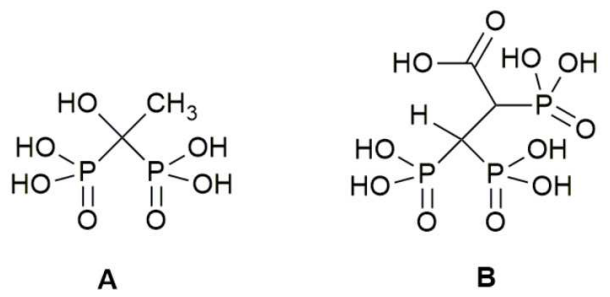

Fig. 1 Chemical structure of **(a)** Etidronate and **(b)** 2,3,3-Trisphosphonates.

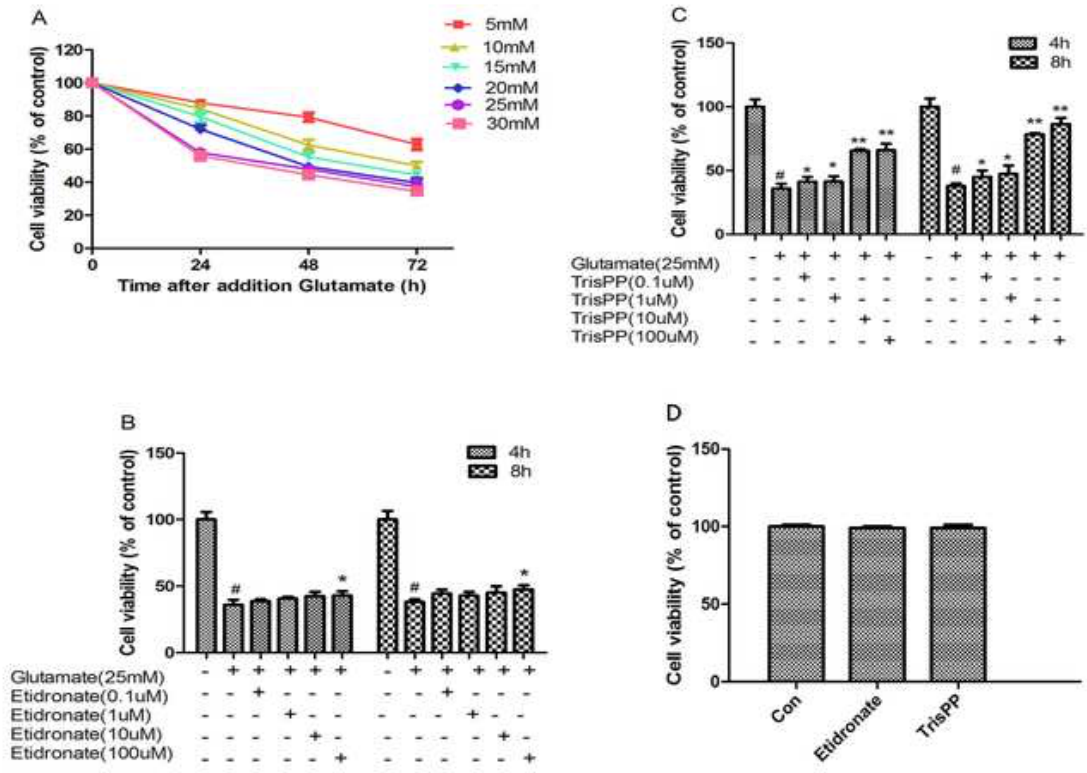

Fig. 2 Effects of Etidronate and TrisPP on the viability of damaged PC12 cells induced by glutamate. **a,** PC12 cells were exposed to 5-30 mM glutamate for 24 h, 48 h or 72 h. **b**, PC12 cells were pretreated with 0.1-100 μM Etidronate and then exposed to 25mM glutamate for 24 h. **c,** PC12 cells were pretreated with 0.1-100 μM TrisPP and then exposed to 25mM glutamate for 24 h. **d,** PC12 cells were treated with 100 μ M Etidronate and TrisPP for 8 h. Values are expressed as mean ± SD from three experiments. $^{\#}P < 0.05$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. group exposed to glutamate alone.

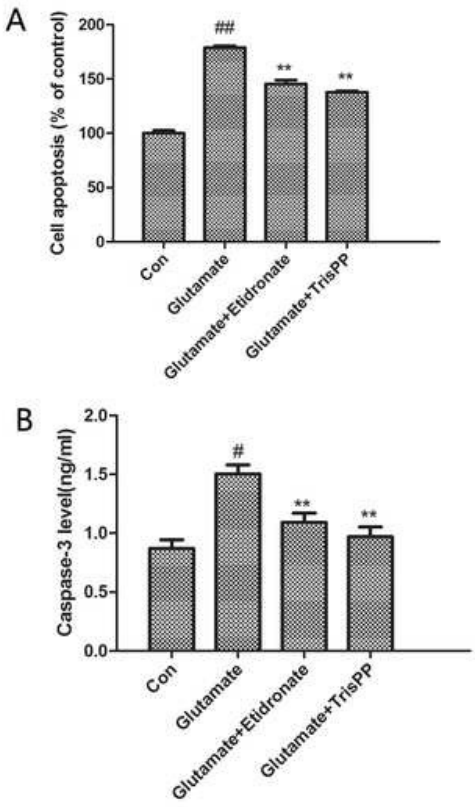

Fig. 3 Effects of Etidronate and TrisPP on apoptosis. The apoptosis of PC12 cell was analysis by flow cytometry. Values are expressed as mean ± SD from three experiments. $^{\#}P < 0.05$ vs. control group; $^{**}P < 0.01$ vs. group exposed to glutamate alone.

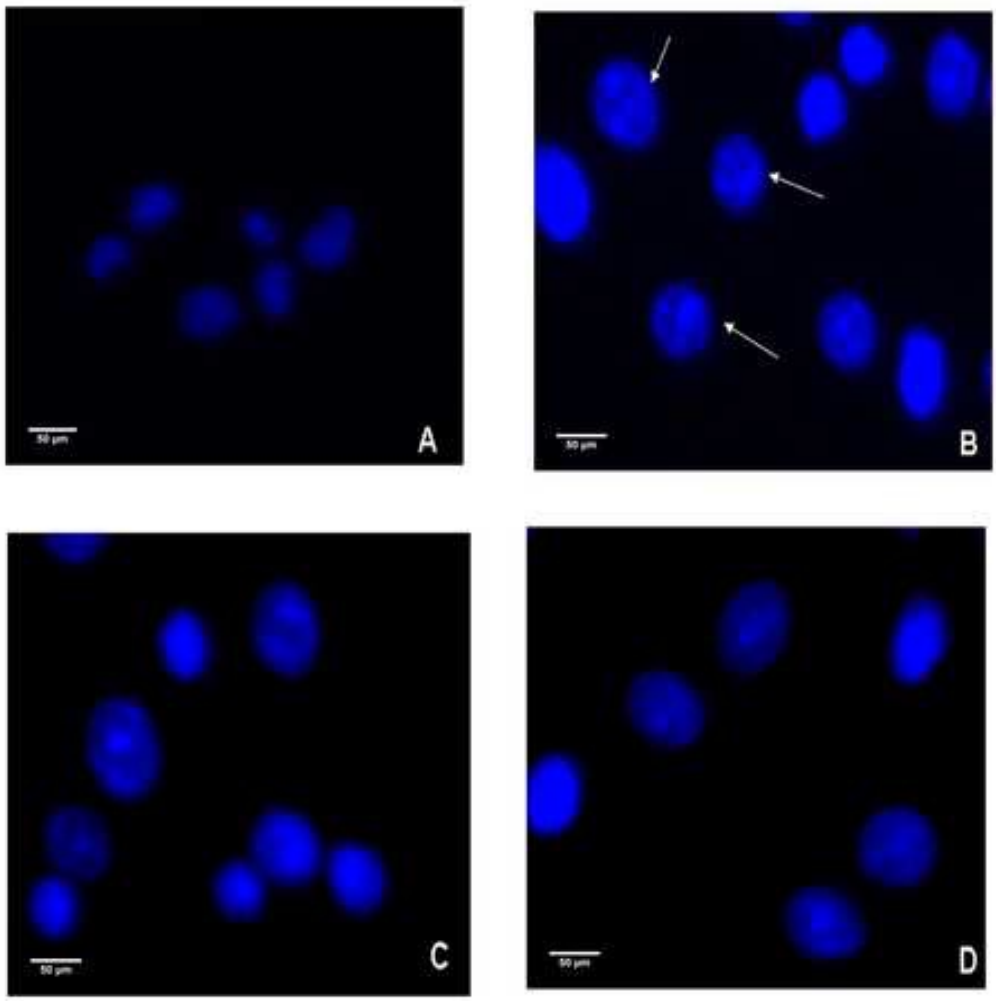

Fig. 4 The nuclear morphology of apoptotic cells imaged by fluorescence microscope (200×). **a**, Control group; **b**, 25mM glutamate alone; **c**, 100 μM Etidronate + 25mM glutamate; **d**, 100 μM TrisPP + 25mM glutamate. White arrows indicate nuclear condensation.

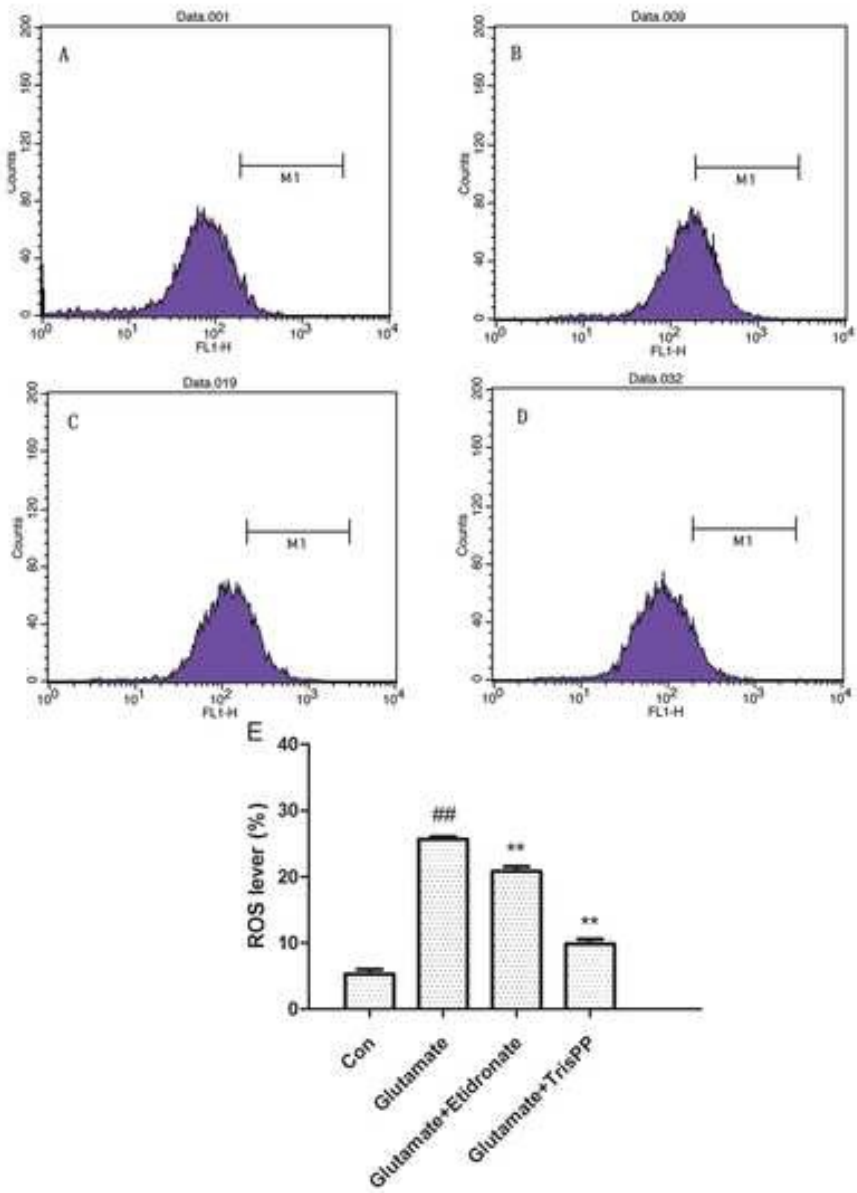

Fig. 5 Effects of Etidronate and TrisPP on the level of ROS in damaged PC12 cells induced by glutamate. **a**, Control group; **b**, 25mM glutamate alone; **c**, 100 μM Etidronate + 25mM glutamate; **d**, 100 μM TrisPP + 25mM glutamate; **e**, Data are expressed as the ROS level of each group. Values are expressed as mean ± SD from three experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group; $*P < 0.05$， $**P < 0.01$ vs. group exposed to glutamate alone.

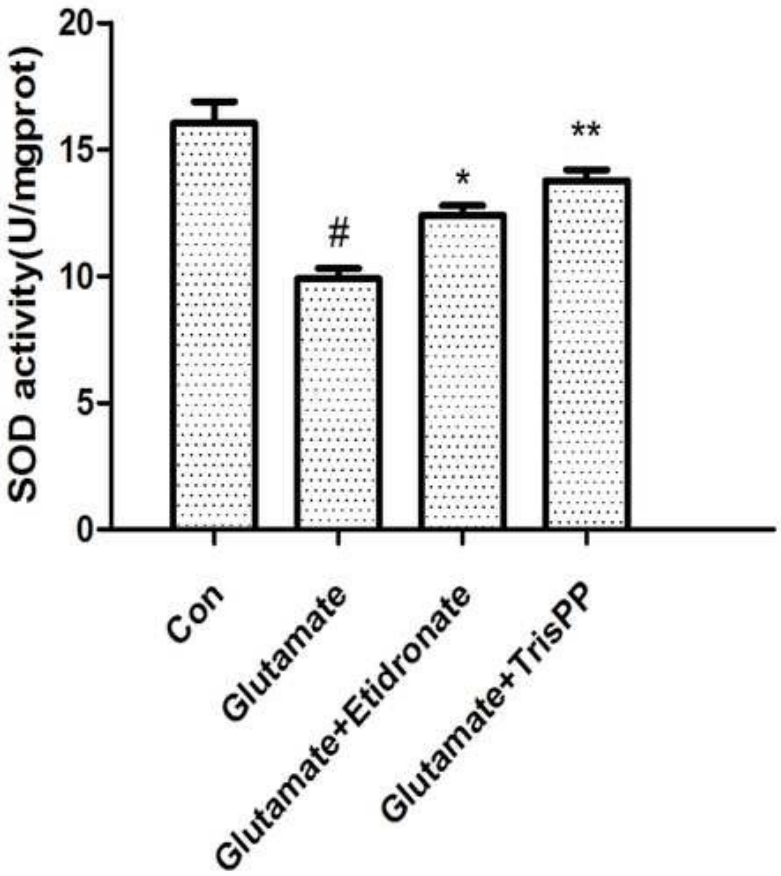

Fig. 6 Effects of Etidronate and TrisPP on SOD activities in damaged PC12 cells induced by glutamate. Values are expressed as mean ± SD from three experiments. $^{\#}P < 0.05$ vs. control group; $^{*}P < 0.05$，$^{**}P < 0.01$ vs. group exposed to glutamate alone.

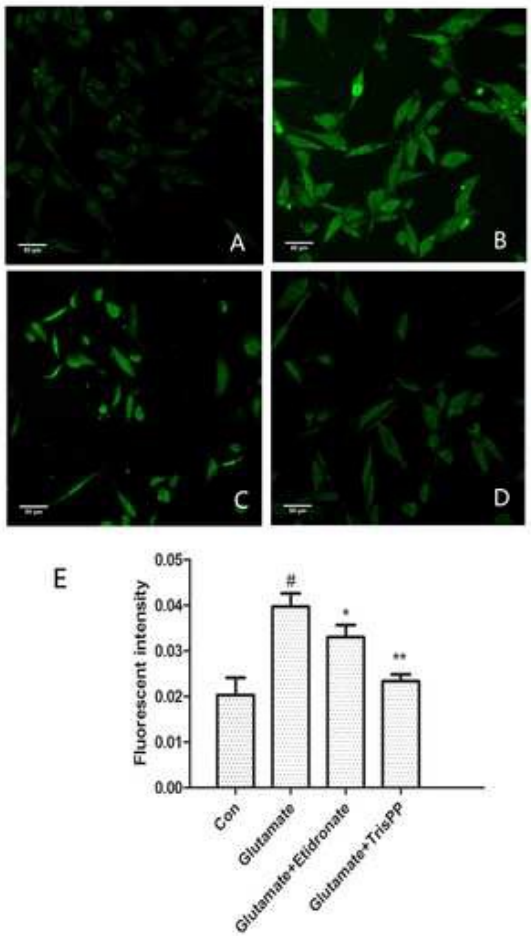

Fig. 7 Effects of Etidronate and TrisPP on the intracellular Ca^{2+} concentration in damaged PC12 cells induced by glutamate (x200). **a** Control group; **b** 25 mM glutamate alone; **c** 100 μM Etidronate + 25 μM glutamate; **d** 100 μM TrisPP + 25 μM glutamate; **e** data is expressed as the fluorescence intensity of each group; *P <0.05 versus control group; * P <0.05, ** P <0.01 versus group exposed to glutamate alone. Scale bar is 50 μm.

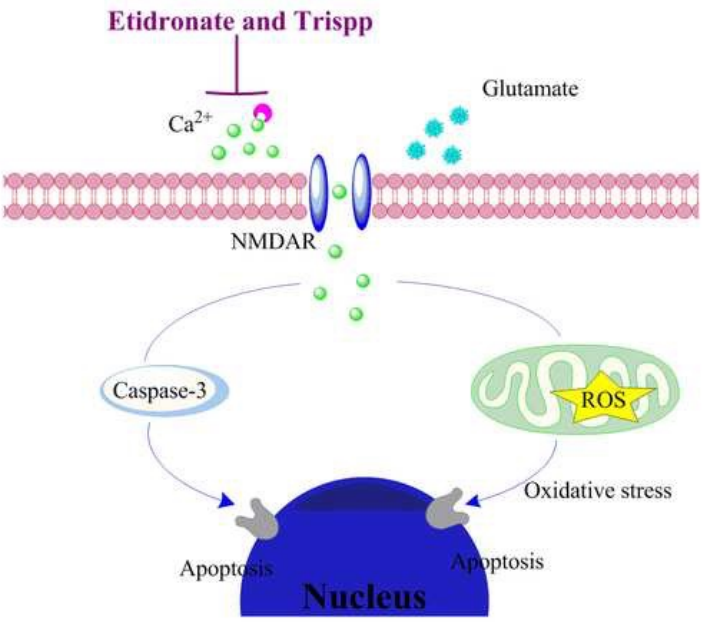

Fig. 8 Schematic figure illustrates the mechanism of Etidronate and TrisPP on protecting PC12 cells against glutamate-induced apoptosis. They mediated apoptosis through chelating calcium to ameliorate Ca^{2+} overload and mitochondrial oxidative stress which exerted neuroprotective effect.