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Isoquinoline-1,3,4-trione and its derivatives attenuate β-amyloid-induced apoptosis of neuronal cells

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Caspase-3 is a programmed cell death protease involved in neuronal apoptosis during physiological development and under pathological conditions. It is a promising therapeutic target for treatment of neurodegenerative diseases. We reported previously that isoquinoline-1,3,4-trione and its derivatives inhibit caspase-3. In this report, we validate isoquinoline-1,3,4-trione and its derivatives as potent, selective, irreversible, slow-binding and pan-caspase inhibitors. Furthermore, we show that these inhibitors attenuated apoptosis induced by β-amyloid(25–35) in PC12 cells and primary neuronal cells.

Caspases are involved in apoptosis and the inflammatory response. Of the 14 members of this protease family, caspase-3 is the key effector of caspase-dependent apoptosis, and is activated in nearly every model of apoptosis, including those with different signaling pathways. Caspase-3-deficient mice die prematurely with a vast excess of cells in their central nervous systems, apparently as a result of decreased apoptosis of neuronal cells, although apoptosis in other organs seems to occur normally [1,2]. Recent studies show that caspase-3 activation may be involved in other acute and chronic neurodegenerative processes, and treatment with caspase inhibitors may protect neurons from apoptotic cell death. Therefore, caspase-3 is a promising target for treatment of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke, amyotrophic lateral sclerosis [3–6].

Caspase-3 plays a prominent role in the pathology of Alzheimer's disease [7]. β-Amyloid (Aβ) is the major component of senile plaques and is regarded as playing a causal role in the development and progression of Alzheimer's disease. There is compelling evidence that Aβ-induced cytotoxicity is mediated through oxidative and/or nitrosative stress and induces neuronal apoptosis. Aβ is derived from cleavage of amyloid precursor protein (APP) by caspases [8]. Of the caspases, caspase-3 is predominantly responsible for APP cleavage, which is consistent with the marked elevation in the concentration of caspase-3 in dying neurons during Alzheimer's disease [9–11]. Caspase-3 also cleaves presenilin-1, presenilin-2, and tau, key proteins in the pathogenesis of Alzheimer's disease [12,13]. Aβ can induce neuronal stress and cell apoptosis via the cascade of caspase-3-mediated signal transduction pathways.

Abbreviations
Aβ, β-amyloid; APP, amyloid precursor protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

Several studies have suggested that inhibition of caspase-3 activity can block induction of apoptosis by Aβ in primary neuronal cells and PC12 cells [14]. The neurotoxicity of Aβ seems to depend on its ability to aggregate, and the active portion of the Aβ molecule appears to be the amino acids 25–35 fragment [15,16].

Most caspase-3 inhibitors are peptidyl inhibitors. Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-DEVD-fmk) and N-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) (peptides that compete for specific recognition sites on the substrate of caspase-3) block cell death in animal models of stroke, myocardial ischemia-reperfusion injury, liver disease, sepsis, and traumatic brain injury [3,17–20]. Although some peptidyl caspase inhibitors are effective, the pharmacokinetics of these inhibitors prevent their use in clinical environments. Small molecules that inhibit caspase-3 activity would be valuable for treatment of diseases involving excessive cell death.

Few small-molecule inhibitors against caspase-3 have been reported [21–24]. Isatin sulfonamide and its analogues are potent and selective inhibitors of apoptosis of chondrocytes and mouse bone marrow neutrophils in cell-based models of osteoarthritis [25]. M-791 reduces mortality by 80% in murine and rat sepsis models by preventing apoptosis of B and T cells [23]. Recently, two caspase inhibitors were subjected to clinical trials. VX-740 (pralnacasan, Vertex Pharmaceuticals), a potent inhibitor specific for caspase 1, is undergoing phase II clinical trials for osteoarthritis. However, it was recently withdrawn for treatment of rheumatoid arthritis because of evidence of abnormal liver toxicity in a long term phase II animal study [26]. The irreversible pan-caspase inhibitor, IDN-6556 (IDUN Pharmaceuticals) has successfully completed phase I studies. IDN-6556 prevents cold- and ischemia-induced apoptosis in donor livers and reduces sinusoidal endothelial cell apoptosis and caspase-3 activity by 94%. It has recently been granted orphan drug status for liver and solid organtransplantation (diseases that affect $< 200\,000$ patients in the USA) [27–29].

We previously identified isoquinoline-1,3,4-trione and its derivatives as caspase-3 inhibitors [30] and showed that they protect human Jurkat T cells against apoptosis induced by camptothecin. In this study, we validated isoquinoline-1,3,4-trione and its derivatives as selective, irreversible, slow-binding, pan-caspase inhibitors. This compound and its derivatives protected PC12 cells and primary cortical neuronal cells against apoptosis induced by Aβ(25–35).

Results

Preparation of active caspases

His6-labeled caspase 2, 3, 6, 7, 8, and 9 were purified from supernatants of cell lysates using HiTrap affinity chromatography. The caspase solutions were 90% pure, and 15% SDS/PAGE revealed they contained 20 kDa and 10 kDa subunits, which is consistent with previous reports on their autocleavage and activation.

Selectivity of isoquinoline-1,3,4-trione derivatives for proteases

The selectivity of seven inhibitory compounds (Fig. 1) against five other cysteine or serine proteases and five other caspases were determined. Although keto-amide compounds are thought to inhibit the activities of cysteine or serine proteases, the results of our selectivity experiments indicated that the compounds we tested had better selectivity for caspases than the other five cysteine or serine proteases, suggesting that these compounds are not general protease inhibitors (Table 1).

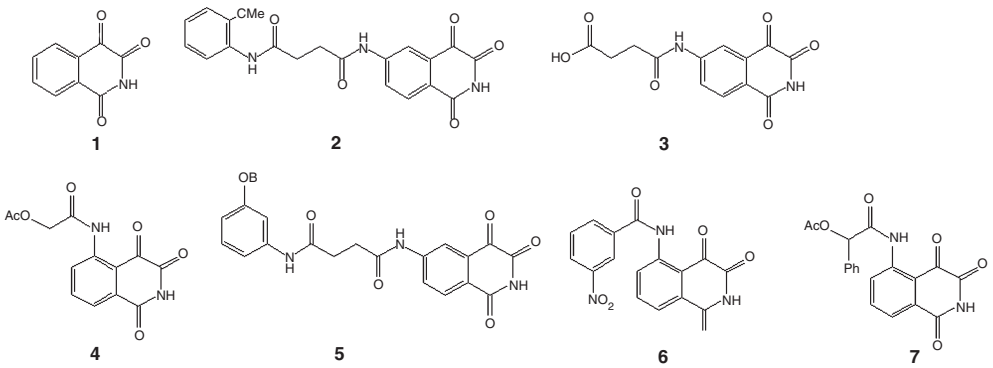

Fig. 1. Structures of isoquinoline-1,3,4-trione and its derivatives.

Table 1. Selectivity of isoquinoline-1,3,4-trione and derivatives on cysteine or serine proteases [IC_{50} (μM)]. Data from compounds **1**, **2**, **3** and **7** is from [30].

	Caspase-3	Calpain 1	Proteasome	Papain	Trypsin	Thrombin
Compound **1**	0.149 ± 0.015	> 5	> 5	> 5	> 5	> 5
Compound **2**	0.113 ± 0.011	> 5	> 5	> 5	> 5	> 5
Compound **3**	0.068 ± 0.006	> 5	> 5	> 5	> 5	> 5
Compound **4**	0.064 ± 0.004	> 5	> 5	> 5	> 5	> 5
Compound **5**	0.055 ± 0.004	> 5	> 5	> 5	> 5	> 5
Compound **6**	0.053 ± 0.002	> 5	> 5	> 5	> 5	> 5
Compound **7**	0.040 ± 0.003	> 5	> 5	> 5	> 5	> 5

Table 2. Selectivity of isoquinoline-1,3,4-trione and derivatives on caspases [IC_{50} (μM)]. Data from compounds **1**, **2**, **3** and **7** is from [30].

	Caspase 2	Caspase-3	Caspase 6	Caspase 7	Caspase 8	Caspase 9
Compound **1**	1.529 ± 0.241	0.149 ± 0.015	0.474 ± 0.083	0.386 ± 0.034	1.913 ± 0.152	1.574 ± 0.284
Compound **2**	0.537 ± 0.035	0.113 ± 0.011	0.137 ± 0.006	0.218 ± 0.024	0.835 ± 0.016	1.300 ± 0.127
Compound **3**	0.859 ± 0.073	0.068 ± 0.006	0.201 ± 0.006	0.136 ± 0.014	1.122 ± 0.043	1.640 ± 0.089
Compound **4**	0.657 ± 0.086	0.064 ± 0.004	0.148 ± 0.031	0.113 ± 0.016	2.360 ± 0.155	1.811 ± 0.315
Compound **5**	0.303 ± 0.051	0.055 ± 0.004	0.079 ± 0.027	0.151 ± 0.009	0.684 ± 0.023	0.933 ± 0.152
Compound **6**	0.268 ± 0.032	0.053 ± 0.002	0.079 ± 0.017	0.057 ± 0.007	0.987 ± 0.025	2.104 ± 0.708
Compound **7**	0.233 ± 0.027	0.040 ± 0.003	0.216 ± 0.014	0.063 ± 0.007	0.425 ± 0.055	0.860 ± 0.155

However, with respect to selectivity among caspase family members, isoquinoline-1,3,4-trione and its derivatives were most potent against caspase-3 and 7, but were still active against caspase 6, 8, and 9 (five-fold increase in IC_{50}). Therefore, they should be considered broad-spectrum caspase inhibitors (Table 2).

Isoquinoline-1,3,4-trione and derivatives inhibit caspase-3 irreversibly

The reversibility of inhibition is easily determined by measuring the recovery of enzymatic activity after a rapid and large dilution of the enzyme–inhibitor complex. If the inhibition is reversible, enzymatic activity will recover to 90% of the initial value; if the inhibition is irreversible, enzymatic activity will not recover.

After dilution, the caspase-3 concentration was equal to that used in typical applications, but for compounds **1** and **7**, the concentration decreased from 10 times the IC_{50} to 0.1 times the IC_{50}. Caspase-3 activity recovered to 90% of initial activity at 0 min when incubated with the reversible inhibitor, Ac-DEVD-CHO, but caspase-3 activity did not recover between 0 min and 30 min when incubated with compound **1** or compound **7** (Fig. 2A). These results indicate that isoquinoline-1,3,4-trione and derivatives inhibited caspase-3 activity irreversibly.

Reversible inhibitors can be removed from the reaction solution by dialysis, whereas irreversible inhibitors cannot be removed. Figure 2B shows that the caspase-3 activity inhibited with compound **7** was even lower after the dialysis, than that before the dialysis. The result showed the inhibition of compound **7** to caspase-3 was not recovered, indicating that compound **7** is an irreversible caspase-3 inhibitor.

Isoquinoline-1,3,4-trione and its derivatives are slow-binding inhibitors. The hallmark of slow-binding inhibition is that the degree of inhibition at a fixed concentration of compound varies over time because equilibrium between the free and enzyme-bound forms of the compound is established slowly. The true affinity of such compounds can only be assessed after the system has reached equilibrium. The IC_{50} of compound **7** for caspase-3 is 0.128 μM without preincubation. However, the IC_{50} decreased significantly after 15 min, and equilibrium was reached between 20 min and 40 min. The IC_{50} of compound **7** for caspase-3 was 38 nM at 30 min (Fig. 2C).

Protective effects of isoquinoline-1,3,4-trione on PC12 cell injury induced by Aβ(25–35)

The biological activities of compound **1** were initially evaluated using PC12 cells. Using a phase-contrast microscope, we observed significant morphological changes of PC12 cells treated with Aβ(25–35) and caspase-3 inhibitors after 48 h (Fig. 3A). In cells treated with 20 μM Aβ(25–35), membrane blebbing and

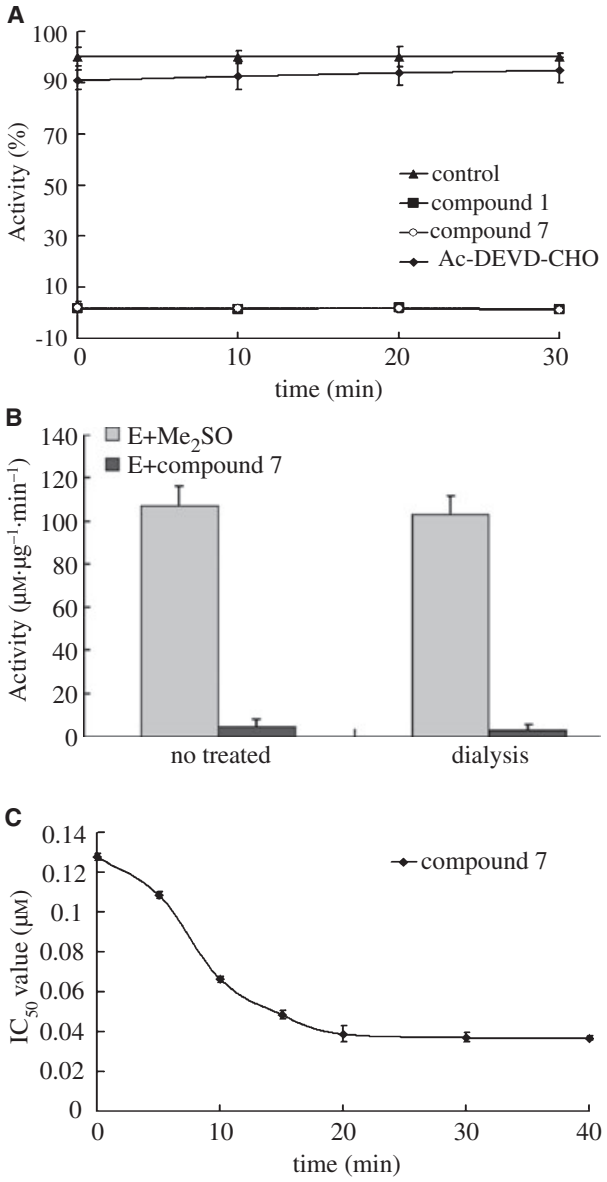

Fig. 2. Characteristic studies on caspase-3 inhibitors. (A) The first methods for the irreversibility of compound **1**, **7**, and the reversibility of Ac-DEVD-CHO. After diluted caspase-3 preincubation with compound **1**, **7**, its activity was not recovered with reversible inhibitor Ac-DEVD-CHO. (B) The dialysis methods for the irreversibility of compound **7**. After dialyzed 12 h caspase-3 preincubation with compound **7**, its activity was not recovered (C). Slow-binding inhibition of isoquinoline-1,3,4-trione derivative (compound **7**). 20 nM caspase-3 preincubated with a range of concentrations of compound **7**, and IC_{50} was determined at different time.

cell shrinkage were prominent, normal morphological characteristics disappeared, and an apoptotic body was evident. However, cells treated with caspase-3 inhibitors had normal morphological characteristics. These results indicate that caspase-3 inhibitors can block PC12 apoptosis induced by Aβ(25–35) and that they are not toxic for PC12 cells.

The effect of compound **1** on cytotoxicity induced by Aβ was assessed using the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and PC12 cells after incubation with Aβ(25–35) in the presence or absence of caspase-3 inhibitors for 48 h. Aβ(25–35) decreased cell viability, and this effect was blocked completely by the selective peptide inhibitor Ac-DEVD-CHO at 10 μM (Fig. 3B). Compound **1** also blocked cell death dose-dependently and was blocked completely at 20 μM. Moreover, compound **1** was nontoxic and caspase-3 inhibitors did not affect PC12 cell viability, even at 40 μM.

Apoptotic cells with degraded DNA appear as cells with hypodiploid DNA content and are represented by the so-called sub-G1 peaks on DNA histograms. After PC12 cells had been treated with Aβ(25–35) for 24 h, flow cytometry revealed the presence of a typical sub-G1 peak indicative of an apoptosis ratio of 4.73%. As the duration of treatment increased to 48 and 72 h, the ratio increased to 19.91% and 31.71%. In contrast, apoptosis ratios were 4.01% and 4.09% when PC12 cells were treated with 2 μM Ac-DEVD-CHO and 28 μM compound **1**, respectively. Apoptosis ratios were 2.27% and 2.15%, respectively, when cells were treated with 2 μM Ac-DEVD-CHO and 28 μM compound **1** but not with Aβ(25–35). The apoptosis ratio without Aβ(25–35) or caspase-3 inhibitors was 1.89%. Therefore, caspase-3 inhibitors protected PC12 cells from the apoptosis induced by Aβ(25–35) (Fig. 3C).

We evaluated the effects of caspase-3 inhibitors on apoptosis by measuring hydrolysis of the caspase-3 specific substrate, Ac-DEVD-pNA. When PC12 cells were exposed to 20 μM Aβ(25–35) for 10 h, caspase-3 activity was equivalent to 4.92 ± 0.72 $mOD \cdot min^{-1} \cdot \mu g^{-1}$ (absorbance increment, mOD). After the treatment with 2 μM Ac-DEVD-CHO, the caspase-3 activity was reduced to 1.56 ± 0.36 $mOD \cdot min^{-1} \cdot \mu g^{-1}$. After the treatment with compound **1**, the caspase-3 activity was reduced in a dose-dependent manner (Fig. 3D).

Isoquinoline-1,3,4-trione derivatives protect neurons from Aβ(25–35)-induced neurotoxicity

In a fashion similar to that of PC12 cells, morphological changes of cortical neurons were significant 48 h after addition of Aβ(25–35) and treatment with caspase-3 inhibitors (Fig. 4A). Control primary neurons grew with axons and dendrites. The axons and dendrites of neurons gradually diminished as the con-

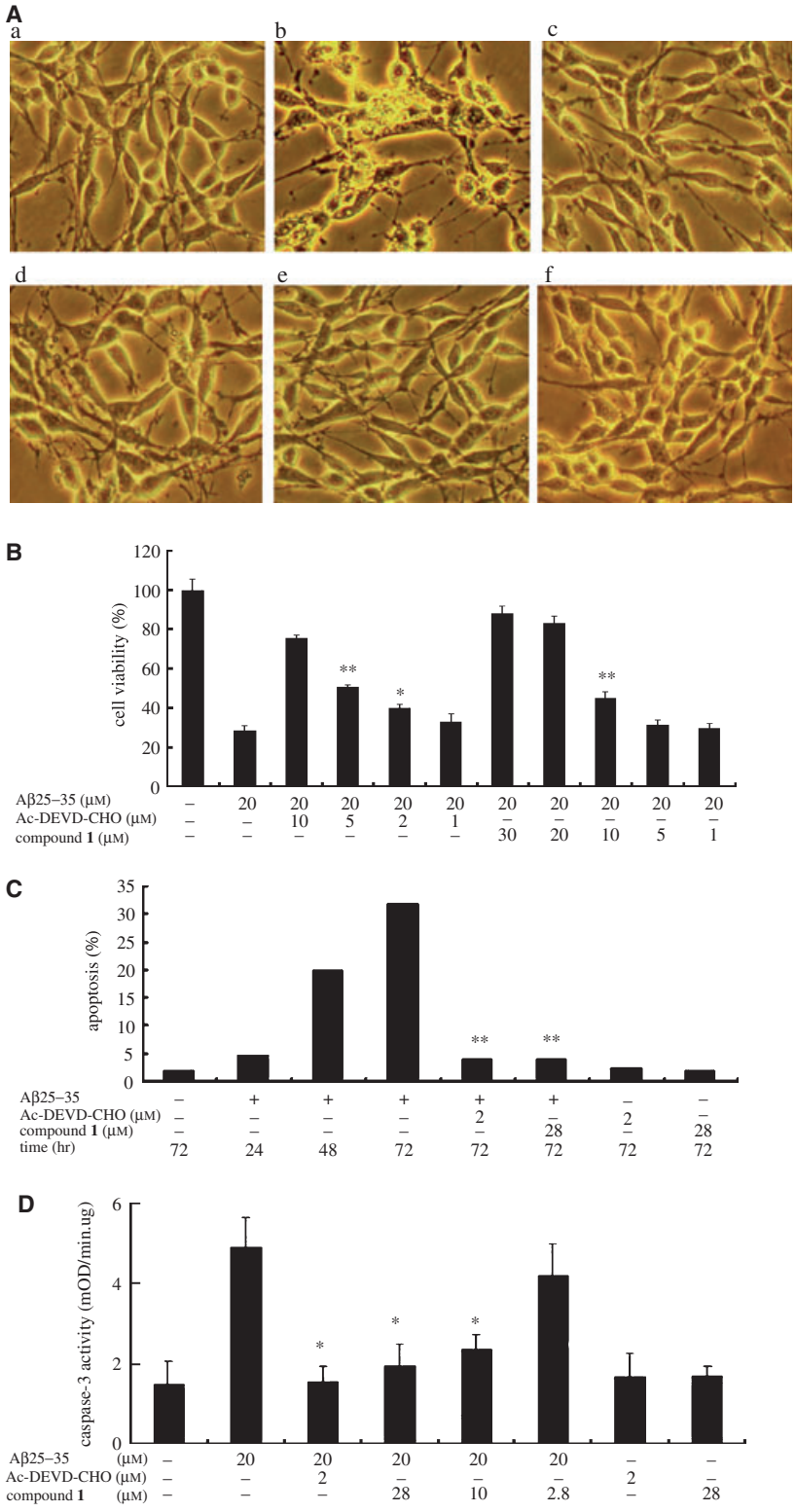

Fig. 3. Effect of isoquinoline-1,3,4-trione on PC12 cell apoptosis induced by Aβ(25–35). (A) Morphology of cells exposed to Aβ(25–35) for 48 h observed with phase-contrast microscope (×200). (a) No treatment. (b) Aβ(25–35) (20 μM), a majority of cells show obvious cytotoxicity. (c) Aβ(25–35) (20 μM) and Ac-DEVD-CHO (10 μM). (d) Aβ(25–35) (20 μM) and compound **1** (25 μM). (e) Ac-DEVD-CHO (10 μM). (f) Compound **1** (25 μM). The data indicated Aβ(25–35) induced a majority of cells show cytotoxicity, isoquinoline-1,3,4-trione reduced this cytotoxicity induced by Aβ(25–35), protected cell natural morphology. (B) Caspase-3 inhibitors increased cell viability of PC12 cells after incubation with 20 μM Aβ(25–35) for 48 h. Compound **1** protects cells from Aβ(25–35) with dose-dependence the same as positive inhibitor, Ac-DEVD-CHO, and was blocked completely at 30 μM. (C) The result of flow cytometry of PC12 cell treated with 20 μM Aβ(25–35) and caspase-3 inhibitors. Compound **1** apparently blocked cell apoptosis rate at 28 μM induced by the neurotoxicity of Aβ(25–35) without toxicity to PC12 cells, and Ac-DEVD-CHO protected apoptosis at 2 μM. (D) Caspase-3 activity of PC12 cell on 20 μM Aβ(25–35) and caspase-3 inhibitors after 10 h. A dose-dependent decrease in caspase-3 activity following treatment with compound **1** was observed. Significant differences between cells treated with Aβ(25–35) are indicated by *, $P < 0.05$ and **, $P < 0.01$.

centration of Aβ(25–35) increased, and cells lost their normal morphological characteristics and developed apoptotic bodies at an Aβ(25–35) concentration of 20 μM. However, treatment with three caspase-3 inhibitors prevented the morphological changes that were induced by Aβ(25–35). The protection conferred

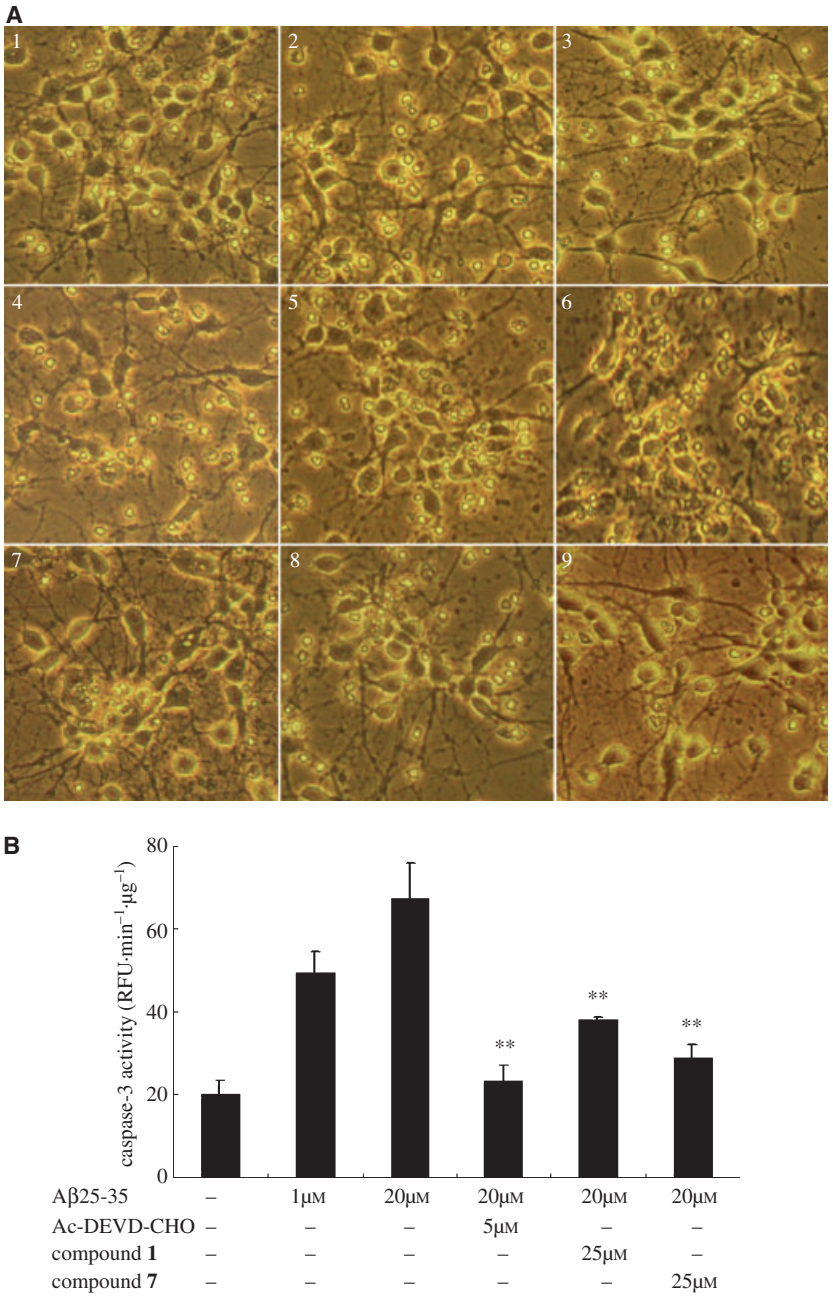

Fig. 4. Isoquinoline-1,3,4-trione and derivatives block neurons from Aβ(25–35)-induced neurotoxicity. (A) Morphology of neurons exposed to Aβ(25–35) for 48 h observed with phase-contrast microscope (×200). (1) No treatment. (2) Compound **1** (25 μM) (3) Compound **7** (25 μM) (4) Aβ(25–35) (1 μM). (5) Aβ(25–35) (5 μM). (6) Aβ(25–35) (20 μM), a majority of cells show obvious cytotoxicity. (7) Aβ(25–35) (20 μM) and Ac-DEVD-CHO (2 μM). (8) Aβ(25–35) (20 μM) and compound **1** (25 μM). (9) Aβ(25–35) (20 μM) and compound **7** (25 μM). The data indicated isoquinoline-1,3,4-trione reduced this cytotoxicity induced by Aβ(25–35), protected cell natural morphology. (B) Caspase-3 activity of neuronal on 20 μM Aβ(25–35) and caspase-3 inhibitors after 48 h. A dose-dependent decrease in caspase-3 activity following treatment with compound **1** was observed. Significant differences between cells treated with Aβ(25–35) are indicated by *, $P < 0.05$ and **, $P < 0.01$.

by compounds **1** and **7** on Aβ-mediated neurotoxicity was similar to that conferred by Ac-DEVD-CHO.

The effects of caspase-3 inhibitors on cellular caspase-3-like enzyme activity were determined by measuring the hydrolysis of the fluorogenic substrate Ac-DEVD-AMC (Fig. 4B). In contrast to the caspase-3 activity of the control [20.11 ± 3.40 RFU·min^{-1}·μg^{-1} (relative fluorescence units)], caspase-3 activity in neuronal cells was increased to 49.44 ± 5.04 RFU·min^{-1}·μg^{-1} and 67.29 ± 8.47 RFU·min^{-1}·μg^{-1} after induction of 1 μM or 20 μM Aβ(25–35), respectively, for 48 h. After treatment with 25 μM compound **1**, compound **4**, or 5 μM Ac-DEVD-CHO, caspase-3 activity in neuronal cells was reduced to 37.99 ± 0.81 RFU·min^{-1}·μg^{-1}, 27.84 ± 3.27 RFU·min^{-1}·μg^{-1}, or 23.10 ± 3.90 RFU·min^{-1}·μg^{-1}, respectively. The inhibitory effect of compound **7** was stronger than that of compound **1**, the original hit from random screening. Our results showed that compounds **1** and **7** attenuated the apoptosis and cell death of primary neurons induced by Aβ(25–35).

Discussion

Isoquinoline-1,3,4-trione is a novel small-molecule inhibitor of caspase-3 that was identified by high-throughput screening of a library of 22 800 organic compounds with diverse chemical structures [30]. Based on the relationship between the structure and activity of isoquinoline-1,3,4-trione, a series of its derivatives were designed and synthesized. Most of the derivatives inhibited caspase-3 activity (with IC_{50} values in the nanomolar range). Compound **7** had an IC_{50} value of 40 nM, which means that its inhibition potency was almost four times that of compound **1**. Isoquinoline-1,3,4-trione derivatives are structurally distinct from the other known classes of nonpeptide caspase-3 inhibitors. The results of dilution and dialysis experiments indicated that our compounds are irreversible and slow-binding inhibitors. Research on the inhibitory mechanism of isoquinoline-1,3,4-trione and its derivatives is under way.

The results of selectivity experiments indicated that isoquinoline-1,3,4-trione and its derivatives have excellent selectivity for five cysteine or serine proteases, which suggests that these compounds are not general protease inhibitors. However, these compounds inhibited all five caspases (IC_{50} values in the nanomolar to micromolar range) to various extents. Therefore, they had low selectivity and could be considered broad spectrum caspase inhibitors. Given that apoptosis signal transduction involves activation of multiple caspases and that the most promising caspase inhibitors tested in clinical trials are pan-caspase inhibitors, the ability to inhibit most of the caspases is a desirable feature of isoquinoline-1,3,4-trione and its derivatives. Moreover, caspases play an important role in mediating the effects of inflammatory cytokines (interleukin-1, Fas-l) and pathological processes in inflammatory diseases such as Crohn's disease, rheumatoid arthritis, ankylosing spondylitis, juvenile rheumatoid arthritis, psoriatic arthritis, and psoriasis. Therefore, some caspases, especially caspase 1 and caspase-3, are also good therapeutic targets for many inflammatory diseases [17,29]. The effects of our compounds on caspase 1 and the immune system will be the subject of further study.

Caspase-3 inhibitors prevented cell death in other assays based on adherent and nonadherent cells. Previously, we reported that isoquinoline-1,3,4-trione and its derivatives protect human Jurkat T cells against the induction of apoptosis by camptothecin [30]. In this study, we found that isoquinoline-1,3,4-trione and its derivatives protected PC12 cells and rat cortical primary neurons against the induction of apoptosis by Aβ(25–35). The PC12 cell line was derived from a pheochromocytoma of the rat adrenal medulla. PC12 cells stop dividing and undergo terminal differentiation when treated with nerve growth factor, making the line a useful model system for nerve cell differentiation. It has been suggested that Aβ, the major protein component of senile plaque, plays an important role in the pathogenesis of Alzheimer's disease. Studies have shown that Aβ-induced apoptosis is mediated by caspase activation in many cell types. Not only are caspase 2, 3, 8 and 9 activated, but cytochrome *c* is released from mitochondria, a process in which caspase-3 plays a significant role. A recent study showed that caspase-3 is involved in apoptosis that directly results in the death of neurons, but it also acts as an initiator by cleaving the amyloid protein precursor to produce Aβ. The results of measurements of morphology, cell viability, and cellular caspase-3 activity, and flow cytometry analysis indicated that isoquinoline-1,3,4-trione and its derivatives attenuated the apoptosis of PC12 cells induced by Aβ(25–35), but had no obvious toxicity for PC12 cells.

We also demonstrated that isoquinoline-1,3,4-trione and its derivatives protected the growth of axons and dendrites of neurons treated with Aβ(25–35) and attenuated neuronal apoptosis. Moreover, the protection afforded by the derivatives of isoquinoline-1,3,4-trione was stronger than that of isoquinoline-1,3,4-trione. Further study is under way to determine the effects of these compounds on APP cleavage and Aβ production.

Conclusions

In summary, we have developed a series of nonpeptide, small-molecule, irreversible, broad spectrum caspase inhibitors, which protect neuronal cells against Aβ(25–35)-induced apoptosis by attenuating the activation of caspases and associated caspase cascades. Further study is in progress to verify their therapeutic effects in animal models of Alzheimer's disease and to optimize their structures to increase their potency and efficiency *in vivo*. It is promising because some derivatives selected for primary animal brain ischemia studies in the widely accepted transient middle cerebral artery occlusion stroke model showed obvious protection efficiency [30]. Our findings may initiate a new approach to drug discovery for clinical therapies of neurodegenerative diseases.

Experimental procedures

The plasmid pET32b and *Escherichia coli* strain BL21(DE3) plysS were purchased from Novagen (Madison, WI, USA). The plasmid pGEMEX-1 and *E. coli* strain JM109 were

purchased from Promega (San Luis Obispo, CA, USA). The restriction enzymes and Ex Taq™ polymerase were from Takara (Dalian, China). The human proteasome was a gift from J. Wu (Centre hospitalier de l'Université de Montréal, QC, Canada). Human trypsin, thrombin, papain, calpain 1, MTT and amyloid-β(25–35) were purchased from Sigma Aldrich (St Louis, MO, USA). Caspase peptide substrates Ac-DEVD-pNA, Ac-DEVD-AMC, N-acetyl-Val-Asp-Val-Ala-Asp-p-nitroanilide (Ac-VDVAD-pNA), N-acetyl-Val-Glu-Ala-Asp-p-nitroanilide (Ac-VEAD-pNA), and N-acetyl-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-pNA) were synthesized in this laboratory. Peptide inhibitor Ac-DEVD-CHO and peptide substrates Suc-LY-AMC, Ac-LLVY-pNA, and N-b-FVR-pNA were purchased from Bachem Bioscience (King of Prussia, PA, USA). Rat PC12 cells were generously provided by X.-C. Tang (Shanghai Institute of Materia Medica, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, newborn calf serum, neurobasal medium, and B27 supplement were obtained from Gibco BRL (Grand Island, NY, USA). Analytical grade reagents and solvents were used.

PCR was performed using a GeneAmp PCR System2400 from PerkinElmer (Boston, MA, USA). HiTrap Chelating HP and HiPrep 26/10 Desalting columns were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Continuous kinetic monitoring of enzyme activity was performed on a SPECTRAmax 340 or a Flexstation^{2-}384 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and controlled by SOFTMAX software (Molecular Devices). Liquid handling for random screening was carried out with a Biomek FX liquid handling workstation integrated with an ORCA system from Beckman Coulter (Fullerton, CA, USA) and HYTRA-96 semiautomated 96-channel pipettors from Robbins (Sunnyvale, CA, USA).

Expression and purification of human caspase 2, 3, 6, 7, 8 and 9

The nucleotide fragments encoding human caspase 2, 3, 6, 7, 8 and 9 catalytic domains (no prodomains) were amplified by RT-PCR using RNA from Jurkat and HeLa cells or from EST clones and the human fetal brain cDNA library [31,32]. After separate digestion with *Nde*I/*Xho*I and *Nhe*I/*Xho*I, caspase 2, 3, 7 and 9 cDNA with the nucleotide fragments encoding the His6 tag at the C-terminus of the recombinant proteins were cloned into pET32b expression vectors, and caspase 6 and 8 cDNA with the nucleotide fragments encoding the His6 tag at the C-terminus were cloned into pGEMEX-1 expression vectors. The nucleotide sequences cloned into the recombinant plasmids were confirmed by DNA sequencing. The recombinant plasmids were then transformed into *E. coli* BL21(DE3)plysS for expression. BL21(DE3)plysS cells containing the recombinant plasmid were grown in a litre of Luria–Bertani medium in the presence of ampicillin (100 mg·L^{-1}) with shaking at 37 °C. Isopropyl thio-β-D-galactoside was added to a concentration of 500 μM when the cell density reached a D_{600} of 0.8–1.0. Cells were cultured for 8 h at 30 °C and harvested by centrifugation for 2 min at 7000 ***g*** (rotor R12A3, Hitachi, Tokyo, Japan). After washing twice with lysis buffer (50 mM Hepes pH 7.4, 100 mM NaCl, 2 mM EDTA), the cells were lysed by sonication for 3 min on ice. After centrifugation at 12 000 ***g*** for 15 min (rotor R20A2, Hitachi), the supernatant was loaded onto a 5 mL HiTrap Chelating HP column previously equilibrated with 50 mM Hepes pH 7.4 and the His6-tagged caspases were eluted with 100–250 mM imidazole in 50 mM Hepes pH 7.4. The eluted fractions were then loaded onto a 50 mL HiPrep desalting column preequilibrated with 50 mM Hepes pH 7.4, 10 mM dithiothreitol, and 5 mM EDTA to remove imidazole. Protein samples from the purification procedure were analyzed by 15% reducing SDS/PAGE and their protein concentrations were determined by the Bradford method with BSA as the standard.

Caspase-3 enzymatic assay and inhibition of catalytic activity

The enzymatic activity of caspase-3 at 35 °C was determined by measuring the change in absorbance at 405 nm caused by the accumulation of pNA from hydrolysis of Ac-DEVD-pNA. A typical 100 μL assay mixture contained 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 100 μM Ac-DEVD-pNA, and recombinant caspase-3. Enzymatic activity was monitored continuously and the initial rate of hydrolysis was determined from the early linear region of the enzymatic reaction curve.

Ac-DEVD-CHO, a selective peptide inhibitor of caspase-3, competitively inhibits caspase-3 by covalently and reversibly binding to the catalytic active site [20]. Ac-DEVD-CHO solution was prepared as a positive control and inhibition assays were performed with 20 nM recombinant enzyme, 100 μM Ac-DEVD-pNA in 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. Dilutions of inhibitors were based on estimated IC_{50} values. The IC_{50} was calculated from a nonlinear curve of percent inhibition vs. inhibitor concentration [I] using the equation, percentage inhibition $= 100/[1 + (IC_{50}/[I])^k]$, where k is the Hill coefficient.

Characterization of caspase-3 inhibitors

To characterize the hit from high-throughput screening and its derivatives, two different assays were carried out to test the reversibility [33]. In the first assay, a solution containing 2 μM recombinant caspase-3 (100-fold higher concentration than required for typical activity assays) was preincubated for 30 min with Ac-DEVD-CHO and compounds **1** or **7**, the concentrations of which were 10 times that of the IC_{50}. The mixture was then diluted 100-fold into a standard assay solution containing Ac-DEVD-pNA to initiate the enzymatic

reaction. The activities of caspase-3 were determined at various intervals and compared with those obtained when 20 nM caspase-3 was incubated and diluted in the absence of inhibitor. In the second assay, a solution of recombinant caspase-3 and inhibitor was preincubated for 30 min and then dialyzed before determination of enzymatic activity. Briefly, compound **7** (1 μM, about 40 times the IC_{50}) was preincubated at 4 °C in typical assay buffer (2 mL) containing 100 $\mu g \cdot mL^{-1}$ caspase-3 for 2 h, following which the mixture (1 mL) was dialyzed twice in 250 mL buffer. Me_2SO was used as a negative control. Caspase-3 activity and protein concentration were determined after dialysis for 10 h.

To determine whether the isoquinoline-1,3,4-trione derivative, compound **7**, is a slow-binding inhibitor, 20 nM caspase-3 was preincubated with a range of concentrations of compound **7** and the IC_{50} was determined at various intervals.

Caspase-3 inhibitor selectivity

Caspase 2, 6, 7, 8, and 9, human proteasome, human trypsin, thrombin, papain, and calpain 1 were used to study the selectivity of caspase-3 inhibition. Assays of the activities of caspase 2, 6, and 7 were performed using 100 μM Ac-VDVAD-pNA, Ac-VEAD-pNA, and Ac-DEVD-pNA, respectively, as substrates. Assays of the activities of caspase 8 and 9 were performed using 100 μM Ac-LEHD-pNA as substrate. The reactions were carried out in 50 mM Hepes, 150 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA at their optimum pH. Assays of proteasome activity were performed using 25 μM N-acetyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Ac-LLVY-AMC) as substrate in 100 mM Tris HCl, pH 8.2. Assays of the activities of papain, trypsin, and thrombin were performed using 100 μM N-benzoyl-Phe-Val-Arg-p-nitroanilide (*N*-β-FVR-pNA) as substrate under optimal conditions as described previously [34–36]. Assays of the activity of calpain 1 were performed using 100 μM succinyl-Leu-Tyr-7-amido-4-methylcoumarin (Suc-LY-AMC) as substrate in 50 mM Tris HCl, pH 7.5, 50 mM NaCl, 5 mM β-mercaptoethanol, and 100 mM CaCl2. The enzymes and inhibitors were preincubated for 30 min and the assays were initiated by adding the substrates. All assays were performed at 35 °C in a 96-well clear polystyrene microplate. The rate of production of pNA by hydrolysis was monitored continuously for 1–3 min by measuring absorbance at 405 nm using a SPECTRA max 340 PC. The rate of production of the hydrolysis product, 7-amino-4-methylcoumarin (AMC), was monitored continuously for 10 min by measuring fluorescence (λ_{ex}355, λ_{em}460) using a FlexStationII384. All the inhibitors were dissolved and diluted in Me_2SO before addition to the assay mixture; the final Me_2SO concentration was 2%. Compounds were tested at a series of final concentrations (0.005–10 μg), and IC_{50} was determined for all compounds expressing measurable inhibitory activity.

Cell culture and treatment with Aβ(25–35)

Rat PC12 cells were maintained under 5% CO_2 air at 37 °C in DMEM supplemented with 10% newborn calf serum. Before the experiment, cells were seeded overnight at a concentration of 3×10^4 cells$\cdot mL^{-1}$ and cultured in the required plates. Primary cortical neurons were prepared from embryonic day 16–18 Sprague Dawley rats. Briefly, each pup was decapitated and the cortex was digested in 0.25% trypsin at 37 °C for 30 min. The tissue was dissociated in DMEM containing 10% fetal bovine serum by aspirating trituration. Cell were plated (1×10^6 cells$\cdot mL^{-1}$) onto poly-D-lysine-coated dishes and maintained in neurobasal medium containing 2% B27 supplement, 10 $U \cdot mL^{-1}$ penicillin, 10 $\mu g \cdot mL^{-1}$ streptomycin, 25 μM glutamate, and 0.5 mM glutamine for four days. The growth of non-neuronal cells was inhibited by this medium. The cells were used for the experiment on the fifth day of culture. Methods used ensured minimal pain and discomfort to experimental animals according to NIH guidelines.

Aβ(25–35) was prepared as a 1 mM stock solution in sterile water, incubated at 37 °C for 48 h, and diluted to the required concentration with cell culture medium. Cells were preincubated with caspase-3 inhibitors for 1 h before Aβ(25–35) treatment. Cells treated only with Me_2SO and cells treated with Aβ(25–35) and Me_2SO were used as positive and negative controls, respectively.

Cell viability measurement using MTT

Cell survival after treatment with Aβ(25–35) and caspase-3 inhibitors for 44 h was evaluated from the ability of cell cultures to reduce MTT, an indication of metabolic activity. The assay is based on the ability of the mitochondrial dehydrogenase enzyme of viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystals, which accumulate in healthy cells because cell membranes are largely impermeable to them. MTT (5 $mg \cdot mL^{-1}$) was added to the cultures at the indicated times. After four hours incubation, the media was removed and 100 μL Me_2SO was added to each well. The absorbance of each well at 550 nm (reference wave length = 690 nm) was determined using a SpectraMAX 340 microplate reader (Molecular Devices). Measurements were performed in triplicate.

Detection of caspase-3 activity

Aβ(25–35)-treated cells were washed once using $NaCl/P_i$ and resuspended in 200 μL lysis buffer composed of 50 mM Hepes (pH 7.5), 10 mM dithiothreitol, 5 mM EDTA, 10 $\mu g \cdot mL^{-1}$ proteinase K, 100 $\mu g \cdot mL^{-1}$ phenylmethysulfonyl fluoride, 10 $\mu g \cdot mL^{-1}$ pepstatin, and 10 $\mu g \cdot mL^{-1}$ leupeptin. Cells in lysis buffer were cooled to −80 °C and then

warmed to 4 °C four times to lyse them completely. The samples were centrifuged at 12 000 ***g*** for 20 min at 4 °C. The protein concentrations of supernatants were measured using the Bradford method. Caspase-3 activity was measured in a volume of 100 μL containing 50 mM Hepes pH 7.0, 150 mM NaCl, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 200 μM Ac-DEVD-pNA (PC12 cells) or 100 μM Ac-DEVD-AMC (primary neuronal cells), and 20 μL cell lysate. A sample composed of substrate and lysis buffer was used as a blank. Caspase-3 activity was normalized to equal protein concentrations.

Flow cytometry analysis of apoptosis

After treatment with caspase-3 inhibitors and Aβ(25–35), cells were digested using 0.05% trypsin, centrifuged at 200 ***g*** at 4 °C for 5 min, washed once in $NaCl/P_i$ and then resuspended in 70% ice-cold ethanol for fixing. The fixed cells were centrifuged and the pellet was resuspended in 1 mL $NaCl/P_i$. After addition of 100 μL of 200 $\mu g \cdot mL^{-1}$ DNase-free RNase A (Sigma), samples were incubated at 37 °C for 30 min. Then 50 $\mu g \cdot mL^{-1}$ propidium iodide (light sensitive) was added and the samples were incubated at room temperature for 15 min before they were transferred to 12 mm × 75 mm Falcon tubes. The number of apoptotic cells was measured using a linear amplification in the FL-2 channel of a FACScan flow cytometer (Becton Dickinson, Rockville, MD, USA) equipped with CELLQUEST software (Becton Dickinson).

Statistical analyses

Data are presented as the mean ± SE. Statistical analysis of multiple comparisons was performed using analysis of variance. For single comparisons, the significance of differences between means was determined using the *t*-test. $P < 0.05$ was considered significant and $P < 0.001$ was considered highly significant.

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