Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 586 (2008) 35–43
www.elsevier.com/locate/ejphar

# Tournefolic acid B attenuates amyloid β protein-mediated toxicity by abrogating the calcium overload in mitochondria and retarding the caspase 8-truncated Bid-cytochrome *c* pathway in rat cortical neurons

Chih-Wen Chi [a], Yun-Lian Lin [b], Ying-Hsiu Wang [b], Chieh-Fu Chen [a], Chuen-Neu Wang [b,*], Young-Ji Shiao [b,c,*]

[a] *Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan, ROC*
[b] *National Research Institute of Chinese Medicine, Taipei, Taiwan, ROC*
[c] *Institute of Biopharmaceutical Science, National Yang-Ming University, Taipei, Taiwan, ROC*

Received 2 November 2007; received in revised form 29 January 2008; accepted 13 February 2008
Available online 29 February 2008

**Abstract**

The effect of tournefolic acid B (TAB) on amyloid β protein-mediated neurotoxicity and the underlying mechanisms were investigated. Amyloid β protein 25–35 elicited neuronal death as determined by calcein/ethidium homodimer-1 staining. 10 μM amyloid β protein 25–35 caused cell death at a level of 41.5±3.8% by MTT reduction. 50 μM TAB attenuated the amyloid β protein 25–35-induced cell death by 49.7±11.1%. TAB also abrogated amyloid β protein-induced activation of caspases 8 and 9 by about 50–60%. Furthermore, TAB significantly diminished the amyloid β protein 25–35-induced elevation of calcium level in mitochondria, whereas it did not affect the calcium level in cytosol or endoplasmic reticulum. TAB markedly retarded the amyloid β protein-mediated release of cytochrome *c* from mitochondria. Amyloid β protein 25–35 elevated mitochondrial truncated BH3 interacting domain death agonist (tBid) and decreased the level of B-cell leukemia/lymphoma-2α (Bcl-2α) in mitochondria. Moreover, amyloid β protein induced a slight up-regulation of Bcl-2 agonist killer 1 (Bak) in cytosol. 50 μM TAB decreased the amyloid β protein-induced elevation of mitochondrial tBid and the level of Bak, whereas it did not affect the amyloid β protein-mediated decrease in mitochondrial Bcl-2α. Caspase 8 inhibitor significantly inhibited the amyloid β protein-mediated increase in mitochondrial tBid and the release of cytochrome *c*. Therefore, TAB blocked the overload of calcium in mitochondria and impaired the amyloid β protein-mediated activation of the caspase 8–tBid–cytochrome *c* pathway, thereby conferring its neuroprotective effects on amyloid β protein-mediated neurotoxicity.


*Keywords:* Tournefolic acid B; Amyloid β protein; Calcium; Caspase 8; Bid; Cytochrome *c*

## 1. Introduction

Amyloid β protein is the major protein component of senile plaque, which is a hallmark of Alzheimer's disease and has been suggested to play an important role in the pathogenesis of Alzheimer's disease (Selkoe, 1990; Smith, 1998). Amyloid β protein is derived proteolytically from amyloid precursor protein (Buxbaum and Greengard, 1996; Selkoe, 1996). Abundant evidence has demonstrated that amyloid β protein is a cause of neuronal death and neuritic changes (Loo et al., 1993; Maurice et al., 1996; Estus et al., 1997; Mattson et al., 1998). Several different mechanisms have been suggested to be involved in amyloid β protein-induced neurotoxicity, which may be attributable to disturbing calcium homeostasis and accumulating reactive oxygen species (Maurice et al., 1996; MacManus et al., 2000). Reactive oxygen species can compromise membrane integrity by provoking membrane damage, which increases the permeability of ions, including calcium.

* Corresponding authors. Shiao is to be contacted at National Research Institute of Chinese Medicine, NO. 155-1. Sec. 2, LiNung ST., Peitou, Taipei, Taiwan, ROC. Tel.: +886 2 2820 1999x4171; fax: +886 2 28264266. Wang, National Research Institute of Chinese Medicine, NO. 155-1. Sec. 2, LiNung ST., Peitou, Taipei, Taiwan, ROC. Tel.: +886 2 2820 1999x4163; fax: +886 2 28264266.
*E-mail addresses:* cnwang@nricm.edu.tw (C.-N. Wang), yshiao@nricm.edu.tw (Y.-J. Shiao).

0014-2999/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2008.02.058

Apoptosis plays a pivotal role in amyloid β protein-induced cell death (Nakagawa et al., 2000; Wang et al., 2001; Morais Cardoso et al., 2002). There are two main pathways leading to apoptosis in mature neurons, the extrinsic and intrinsic pathways. The death receptor, Fas-associated death domain (FADD), and the caspase-cascade are implicated in the extrinsic apoptotic pathway. The interaction of anti-apoptotic proteins (such as B-cell leukemia/lymphoma-2 (Bcl-2)) and pro-apoptotic proteins (such as B-cell lymphoma 2-associated protein X (Bax) and Bcl-2 agonist killer 1 (Bak)) plays a crucial role in the intrinsic apoptotic pathway (Adams and Cory, 1998; Antonsson and Martinou, 2000). In the intrinsic apoptotic pathway, mitochondrial activation triggers the release of cytochrome *c* via Bax and subsequently activates caspase-9 and caspase-3 (Kluck et al., 1997; Wolter et al., 1997). BH3 interacting domain death agonist (Bid) is a molecular linker bridging intrinsic and extrinsic apoptotic pathways. Bid was originally discovered as a binding partner of both Bcl-2 and Bax (Wang et al., 1996), and later identified as a substrate of caspase-8 (Li et al., 1998; Luo et al., 1998; Gross et al., 1999). The truncated Bid (tBid), a 15 kDa C-terminal fragment, is derived from caspase cleavage and consequently redistributes from cytosol to mitochondria and promotes the release of cytochrome *c* (Yin, 2006). Evidence has shown that amyloid β protein-induced cell death is concomitant with the down-regulation of Bcl-2 (Wei et al., 2000; Tamagno et al., 2003), and/or up-regulation of Bax (Paradis et al., 1996; Tamagno et al., 2003). However, whether tBid participates in the apoptotic signaling pathways induced by amyloid β protein remains to be determined.

Several agents have been shown to be neuroprotective in the *in vitro* system by targeting specific pathways in amyloid β protein-induced toxicity. These agents include anti-oxidants (Pappolla et al., 1997; Ueda et al., 1997), blockers of calcium channel (Ueda et al., 1997; Ekinci et al., 1999), growth factors (Prehn et al., 1996), caspase inhibitors (Troy et al., 2000), and flavonoids (Wang et al., 2001). A variety of polyphenolic compounds have been isolated from *Tournefortia sarmentosa* Lam. (Boraginaceae), widely used in Taiwan as detoxicants and anti-inflammatory agents (Lin et al., 2002). Most of these polyphenolic compounds possess anti-oxidative activity including tournefolic acid B (TAB) (Fig. 1). In the present study, we attempted to investigate the neuroprotective effects of TAB on amyloid β protein-mediated toxicity in primary cultures of rat cortical neurons. The underlying mechanisms by which TAB conferred its effect were also elucidated. The results showed that the caspase 8–tBid–cytochrome *c* pathway was implicated in the neurotoxicity of amyloid β. The neuroprotective effect of TAB on amyloid β protein-mediated neurotoxicity was mediated by abrogating amyloid β protein-induced $Ca^{2+}$-loading in mitochondria and attenuating the caspase 8–tBid–cytochrome *c* pathway.

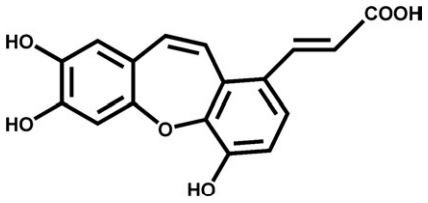


Fig. 1. Structure of tournefolic acid B (TAB).

## 2. Materials and methods

### 2.1. Materials

Enhanced chemiluminescence detection reagents, anti-rabbit IgG antibody conjugated with horseradish peroxidase, and anti-mouse IgG antibody conjugated with horseradish peroxidase were obtained from Amersham Biosciences (Buckinghamshire, UK). Fura-2 AM, mag-fura-2 AM, rhod-2 AM, calcein AM/ethidium homodimer-1, MitoTracker Green FM, and ER-tracker Red were purchased from Molecular Probes (Eugene, OR, USA). Monoclonal anti-cytochrome *c* antibody was obtained from BD Biosciences (San Jose, CA, USA). Polyclonal anti-Bak antibody, monoclonal anti-Bax antibody, monoclonal anti-Bcl-2α antibody, and colorimetric substrates for caspases were obtained from BioSource (Nivelles, Belgium). Polyclonal anti-adenine nucleotide translocator (ANT) and anti-Bid/tBid antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Monoclonal anti-actin antibody was from Chemicon International Inc (Temecula, CA, USA). IETD-CHO (cell permeable caspase 8 inhibitor I) was purchased from Merck Biosciences (Darmstadt, Germany). All other reagents were purchased from Sigma (St Louis, MO, USA) or Merck (Darmstadt, Germany).

### 2.2. Extraction and isolation

Stems of *T. sarmentosa* Lam. were collected from Nei-Men, Kaohsiung County, Taiwan, in August 1998. The plant was identified by comparison with voucher specimens deposited earlier at the Herbarium of the Department of Botany, National Taiwan University, Taipei, Taiwan (no. TAI175693, collected April 1, 1979). The extraction of *T. sarmentosa* and the isolation and purification of TAB were described previously (Lin et al., 2002). The purity of TAB was greater than 95% using HPLC analysis.

### 2.3. Cell culture

Primary cultures of neonatal cortical neurons were prepared as described previously (Wang et al., 2001). The Institutional Animal Care and Use Committee at the National Research Institute of Chinese Medicine had approved the animal protocol. Briefly, the cortex isolated from Sprague–Dawley rat pups by decapitation was digested in 0.5 mg/ml papain at 37 °C for 15 min. The tissue was dissociated in Hibernate A medium (containing B27 supplement) by aspirating trituration. Cells were plated and maintained in Neurobasal medium containing B27 supplement, 10 U/ml penicillin, 10 μg/ml streptomycin, and 0.5 μg/ml glutamine. The cells were used for experiments on the fifth day.

### 2.4. Measurement of cell viability

The calcein/ethidium homodimer-1 staining and the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were used to evaluate cell viability. Cells were loaded with 1 μM of both calcein AM and ethidium homodimer-1 at room temperature for 30 min. The cells were observed by Leica CS SP confocal fluorescence microscope (Wetzlar, Germany). Cells were incubated with 0.5 mg/ml MTT for 1 h. The formazan particles were dissolved with DMSO. $OD_{600\ nm}$ was measured using an ELISA reader.

### 2.5. Measurement of intracellular, mitochondrial, and endoplasmic reticulum calcium

The levels of calcium in cytoplasm, mitochondria, and endoplasmic reticulum were measured as described previously (Wang et al., 2006). Cells were loaded with fura-2 AM (1 μM, 60 min), mag-fura-2 AM (5 μM, 60 min), and rhod-2 AM (1 μM, 15 min) to detect the calcium levels in cytoplasm, mitochondria, and endoplasmic reticulum, respectively. The loaded cells were treated with vehicle or 50 μM TAB for 30 min then transferred to a microscope-equipped humidity chamber at 37 °C and 5% $CO_2$. Cells were exposed to 10 μM amyloid β protein 25–35 and detected with a Leica DMIRB fluorescence microscope with dual excitation wavelengths of 340 and 380 nm for fura-2 and mag-fura-2, and with excitation wavelength of 555 nm for rhod-2. Time-lapse images were captured every 15 s over a 60-min period using MetaFluor software (Universal Imaging Co., West Chester, PA, USA). The validity of using mag-fura-2 and rhod-2 to measure $Ca^{2+}$ in endoplasmic reticulum and mitochondria, respectively was confirmed by the co-localization with endoplasmic reticulum and mitochondria tracker, respectively. The treatment with carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, a strong uncoupler) declined the fluorescence intensity of rhod-2 further supporting that the fluorescence intensity measured represents mitochondrial rhod-2 (data not shown).

### 2.6. Measurement of cellular activity of caspases

Treated cells were harvested in cell lysis buffer (50 mM Hepes, pH 7.4, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Chaps, and 0.1% Triton X-100). The cellular lysates were prepared and subjected to assay for caspase activity. Intracellular activity of caspases 2, 3, 6, 8, and 9 was determined by the ability to cleave Ac-VDVAD-pNA, Ac-DEVD-pNA, Ac-VEID-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA, respectively. The detailed experiments were performed according to the manufacturer's protocol.

### 2.7. Subcellular fractionation

Treated cells were collected in harvest buffer (50 mM Hepes, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 10 μg/ml leupeptin) and passed through a 1 ml syringe with 25 gauge needle 20 times to disrupt cells. The cellular lysates were centrifuged at 150 ×*g* for 5 min and 600 ×*g* for 10 min to remove the unbroken cells and nuclei, respectively. The

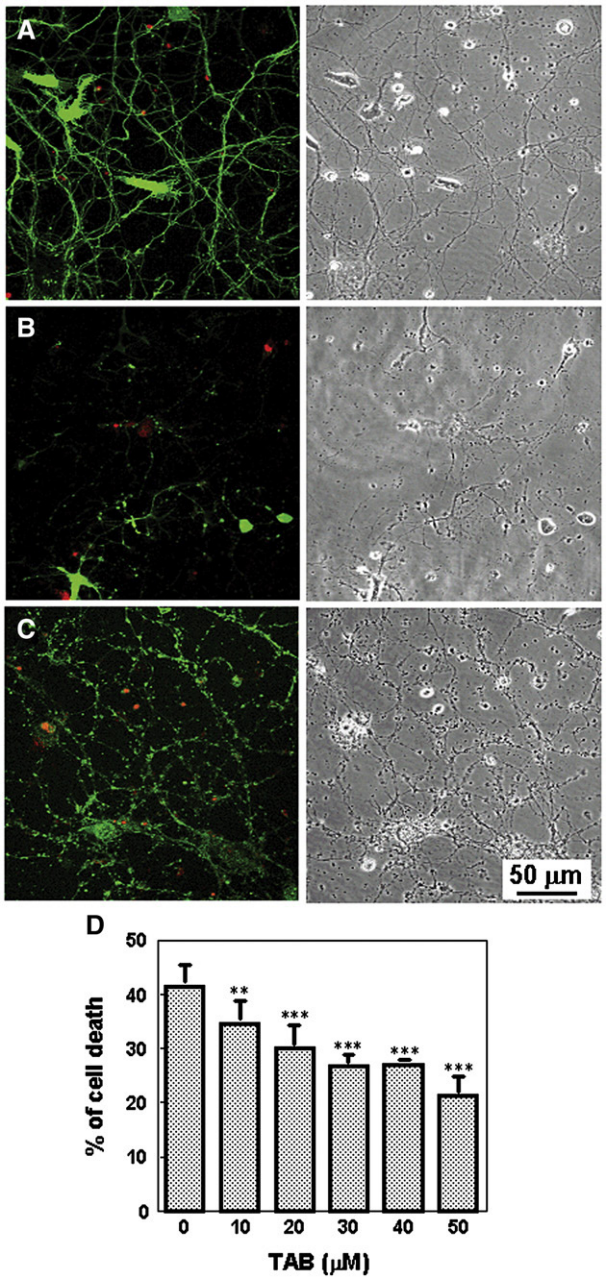


Fig. 2. TAB decreased amyloid β protein 25–35-mediated cell death. Cortical neurons were treated with vehicle (0.1% DMSO, v/v) (A, B) or 50 μM TAB (C) for 2 h then exposed to 10 μM amyloid β protein 25–35 for 24 h (B. C). Cells were subjected to calcein/ethidium homodimer-1 staining. The left parts of panels A, B, and C are the representative photographs of calcein (green) and ethidium homodimer-1 (red) fluorescent staining. The right parts are photographs of phase contrast. Similar results were repeated for three times. Cortical neurons were treated with vehicle or 0–50 μM TAB (D) for 2 h. Afterwards, cells were challenged with 10 μM amyloid β protein 25–35 for 40 h. Cell viability was measured by MTT reduction. Results are means±S.D. from five independent experiments, and are expressed relative to control cells. Significant differences between cells treated with amyloid β protein and amyloid β protein plus TAB are indicated by **, $P<0.01$; and ***, $P<0.001$.

supernatants were centrifuged at 10,000 ×*g* for 30 min to obtain the mitochondrial fraction (pellet). The supernatants were further centrifuged at 85,000 ×*g* for 90 min to obtain the cytosol fraction (supernatant).

### 2.8. Immunoblotting

Equal protein amounts of cellular fractions were prepared and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting. Specific antibodies for cytochrome *c*, Bid/tBid, Bak, Bax, and Bcl-2 were used to detect the mitochondrial and cytosolic levels of cytochrome *c*, Bid/tBid, Bak, Bax, and Bcl-2, respectively. Fujifilm LAS-3000 (Tokyo, Japan) was used to detect and quantify the immunoreactive protein.

### 2.9. Statistical analysis

Results are expressed as mean±S.D. and were analyzed by ANOVA with post-hoc multiple comparisons with a Bonferroni test.

## 3. Results

### 3.1. TAB attenuated amyloid β protein-mediated neurotoxicity and the activation of caspases

10 μM amyloid β protein 25–35 fibril provoked significant decrease of calcein staining concomitant with the appearance of neurites breakage (Fig. 2B). 50 μM TAB attenuated the amyloid β protein 25–35-induced decrease of calcein staining and neurites breakage (Fig. 2C). 10 μM amyloid β protein 25–35 elicited cell death at a level of 41.5±3.8% as measured by MTT reduction (Fig. 2D). Treatment with TAB abrogated the amyloid β protein 25–35-induced cell death in a concentration-dependent manner. TAB at 20, 40, and 50 μM diminished the cell death by 25.1±10.7, 37.3±8.3 and 49.7±11.1%, respectively. TAB at 10 to 100 μM did not exert cytotoxicity as measured by MTT reduction (data not shown).

The *in vitro* assay was conducted to evaluate the effects of amyloid β protein 25–35 and TAB on the activity of caspases. Treatment with 10 μM amyloid β protein 25–35 for 24 h increased the activity of caspases 2, 3, 6, 8 and 9 to 5.0±1.4, 4.2±0.7, 3.9±0.4, 2.5±0.3, and 4.0±0.4-fold of control, respectively (Fig. 3). TAB abrogated amyloid β protein-induced activation of caspases 6, 8 and 9 to a higher extent than that of caspase 2 and 3. 50 μM TAB attenuated the amyloid β protein 25–35-mediated increased activity of caspases 2, 3, 6, 8, and 9 by about 43.2, 20.8, 73.2, 63.8, and 50.1%, respectively.

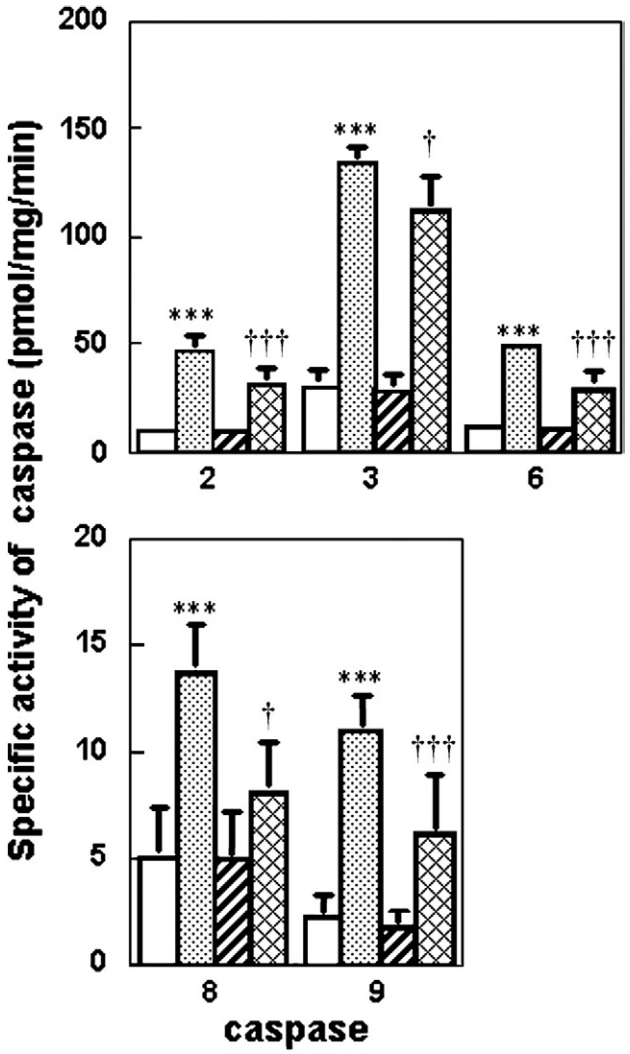


Fig. 3. TAB attenuated the amyloid β protein 25–35-induced increase in caspase activities. Cells were treated with vehicle (unfilled column) or 50 μM TAB (hatched column) only for 24 h. Otherwise, cells were pre-treated with vehicle (dotted column) or 50 μM TAB (crosshatched column) for 2 h, then exposed to 10 μM amyloid β protein 25–35 for 24 h. Cells were harvested to assay the activity of caspases 2, 3, 6, 8, and 9. Results are means±S.D. (where large enough to be shown) from four independent experiments. Significant differences between control cells and cells treated with amyloid β protein are indicated by ***, $P<0.001$. Significant differences between cells treated with amyloid β protein and cells treated with amyloid β protein plus TAB are indicated by †, $P<0.05$; and †††, $P<0.001$.

### 3.2. TAB attenuated the amyloid β protein 25–35-mediated increase in $Ca^{2+}$ in mitochondria

Fura-2, mag-fura-2, and rhod-2 were employed to determine the levels of $Ca^{2+}$ in cytosol, endoplasmic reticulum, and mitochondria, respectively. 10 μM amyloid β protein 25–35 immediately elevated the free $Ca^{2+}$ in cytosol. The ratiometric value of fura-2 was increased from 0.59±0.03 to 0.77±0.06 for 10 min, and the ratiometric value was maintained to 60 min (Fig. 4A). Treatment with 10 μM amyloid β protein 25–35 gradually increased the level of $Ca^{2+}$ in endoplasmic reticulum. The ratiometric value of mag-fura-2 was increased from 0.31±0.02 to 0.51±0.05 and 0.57±0.06 for 30 and 60 min, respectively (Fig. 4B). Treatment with TAB did not alter the amyloid β-mediated increase in $Ca^{2+}$ levels in cytosol and endoplasmic reticulum.

10 μM amyloid β protein 25–35 elevated the fluorescence intensity of rhod-2 to 1.29±0.11, 1.37±0.08, and 1.35±0.03-

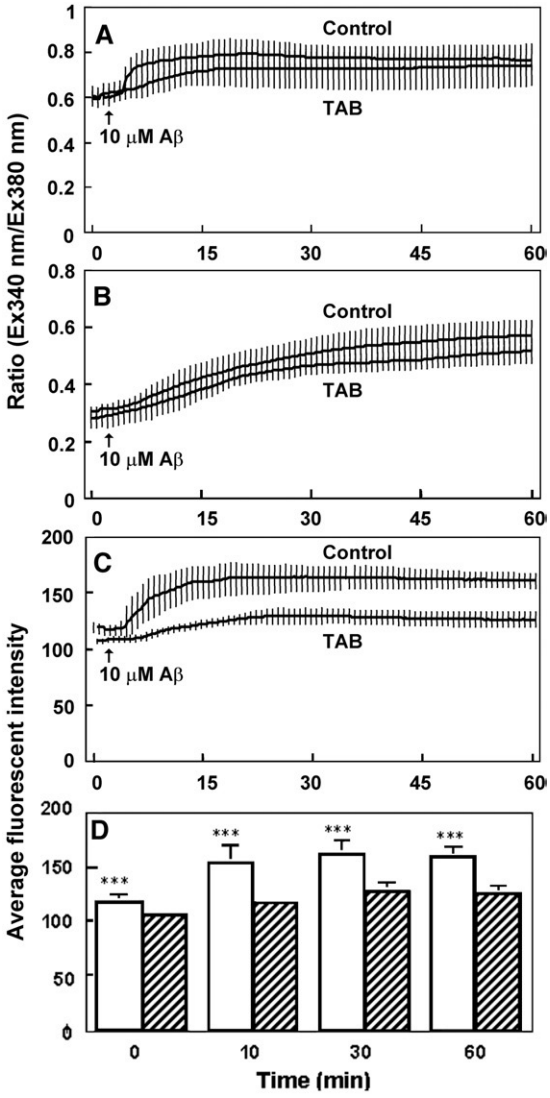


Fig. 4. The effect of TAB on the amyloid β protein 25–35 (Aβ)-induced elevation of $Ca^{2+}$ in cytosol, endoplasmic reticulum, and mitochondria. Cells were loaded with fura-2 AM, mag-fura-2 AM, and rhod-2 AM to monitor the level of $Ca^{2+}$ in cytosol (A), endoplasmic reticulum (B), and mitochondria (C, D), respectively. The loaded cells were treated with vehicle (unfilled column) or 50 μM TAB (hatched column) for 30 min then transferred to a microscope-equipped humidity chamber at 37 °C and 5% $CO_2$. Cells were exposed to 10 μM amyloid β protein 25–35 at the 1-min time point and detected by fluorescence microscope with dual excitation wavelengths of 340 and 380 nm for fura-2 and mag-fura-2, and with excitation wavelength of 555 nm for rhod-2. The time-lapse images over a 60-min period were captured using MetaFluor software. Results are means±S.D. from six independent experiments (where large enough to be shown). Panels A, B, and C are the average of time-course effects of TAB on the amyloid β protein-induced elevation of fluorescence in cytosol, endoplasmic reticulum, and mitochondria, respectively. Panel D is the fluorescence intensity of rhod-2 in mitochondria at 0, 10, 30, and 60 min. Significant differences between cells treated with amyloid β protein and cells treated with amyloid β protein plus TAB are indicated by ***, $P<0.001$.

fold of initial fluorescence intensity for 10, 30, and 60 min, respectively (Fig. 4B, D). Treatment with 50 μM TAB decreased the basal fluorescence intensity by 10.5±4.3%. TAB impeded the amyloid β protein 25–35-mediated elevation of fluorescence intensity by 62.5±18.6, 47.4±16.5, and 53.1±12.6% for 10, 30, and 60 min, respectively.

### 3.3. TAB blocked the amyloid β protein 25–35-induced release of cytochrome c from mitochondria and the generation of mitochondrial tBid

Several mitochondria-related apoptotic proteins were measured to assess the effects of amyloid β protein 25–35 and TAB on apoptosis. The time-course study was performed to select the specific time point at which amyloid β protein 25–35 exerted the most pronounced effect (data not shown). Treatment with 10 μM amyloid β protein 25–35 elicited the release of cytochrome *c* from mitochondria to cytosol by 1.6±0.2-fold of control coinciding with the decrease in mitochondrial cytochrome *c* (Fig. 5). 50 μM TAB completely reversed the amyloid β protein-mediated attenuation of mitochondrial cytochrome *c* from 67.5±

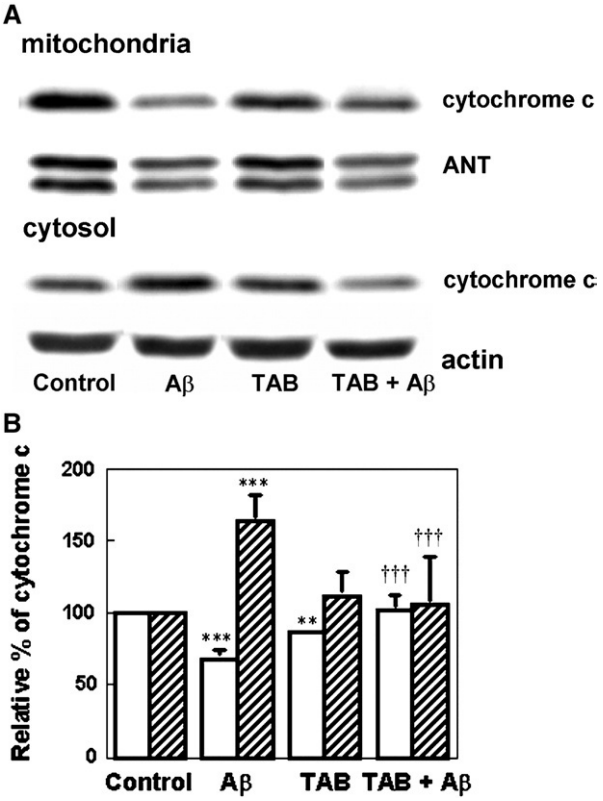


Fig. 5. TAB attenuated the amyloid β protein 25–35 (Aβ)-induced release of cytochrome *c* from mitochondria to cytosol. Cells were treated with vehicle or 50 μM TAB for 2 h. Cells were then incubated with or without 10 μM amyloid β protein 25–35 for 16 h. After incubation, cells were collected and subjected to subcellular fractionation. The same amount of proteins were subjected to immunoblotting to detect the level of cytochrome *c* in mitochondria (unfilled column) or in cytosol (hatched column). Panel A shows the representative immunoblots of cytochrome *c*, ANT, and actin. Panel B shows the relative level of cytochrome *c* in mitochondria and cytosol. The level of cytochrome *c* in mitochondria and cytosol has been normalized with ANT and actin, respectively. Results are means±S.D. (where large enough to be shown) from three independent experiments, and are expressed relative to control cells. Significant differences between control cells and cells treated with amyloid β protein, TAB, or amyloid β protein plus TAB are indicated by **, $P<0.01$; and ***, $P<0.001$. Significant differences between cells treated with amyloid β protein and cells treated with amyloid β protein plus TAB are indicated by †††, $P<0.001$.

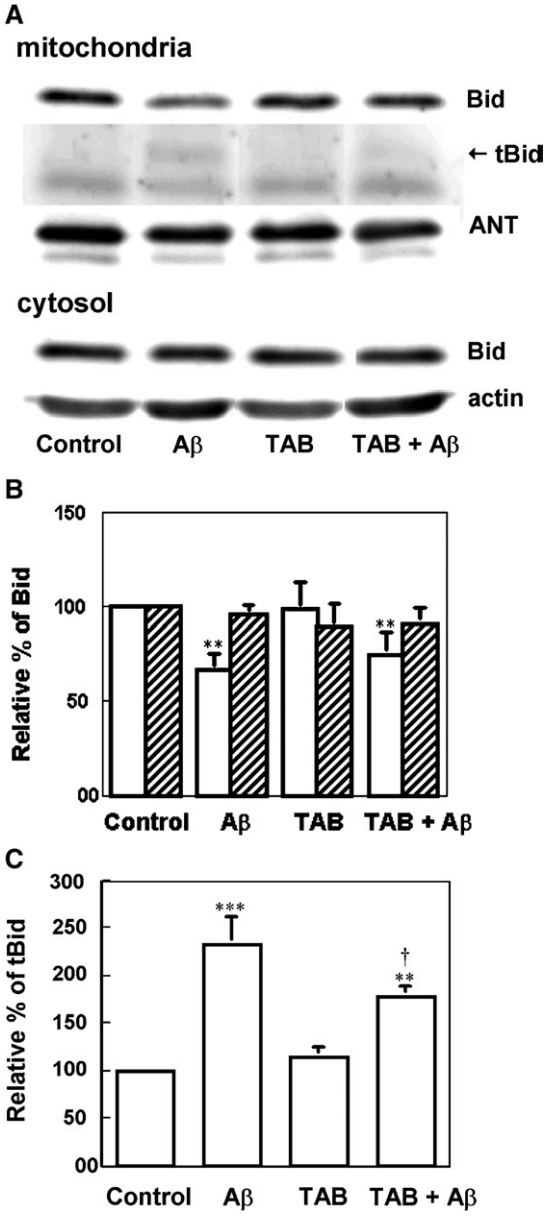


Fig. 6. TAB counteracted the amyloid β protein 25–35 (Aβ)-induced decrease in Bid and elevation of tBid in mitochondria. Cells were treated with vehicle or 50 μM TAB for 2 h. Cells were then incubated with or without 10 μM amyloid β protein 25–35 for 6 h. After incubation, cells were collected and subjected to subcellular fractionation. The same amount of proteins were subjected to immunoblotting to detect the level of Bid and tBid in mitochondria (unfilled column) or in cytosol (hatched column). Panel A shows the representative immunoblots of Bid, tBid, ANT, and actin. Panel B shows the relative level of Bid in mitochondria and cytosol. Panel C shows the relative level of tBid in mitochondria. The level of Bid/tBid in mitochondria and cytosol has been normalized with ANT and actin, respectively. Results are means ± S.D. from three independent experiments, and are expressed relative to control cells. Significant differences between control cells and cells treated with amyloid β protein, TAB, or amyloid β protein plus TAB are indicated by **, $P<0.01$; and ***, $P<0.001$. Significant difference between cells treated with amyloid β protein and cells treated with amyloid β protein plus TAB is indicated by †, $P<0.05$.

7.0 to 101.1 ± 11.8% of control. The amyloid β protein-mediated elevation of cytosolic cytochrome *c* was also inhibited from 163.6 ± 17.8 to 105.5 ± 33.3% of control.

10 μM amyloid β protein 25–35 provoked a decrease in the mitochondrial Bid by 34.0 ± 9.1%, whereas it did not affect the cytosolic Bid (Fig. 6A, B). The tBid in mitochondria was elevated to 232 ± 29.9% of control by treatment with 10 μM amyloid β protein 25–35 (Fig. 6C). TAB (50 μM) blocked the amyloid β protein 25–35-mediated increase in mitochondrial tBid by about 52.6%.

10 μM caspase 8 inhibitor (IETD-CHO) reduced cell death by 29.2 ± 6.2% as determined by MTT reduction (Table 1). Caspase 8 inhibitor increased the mitochondrial cytochrome *c* from 64.1 ± 8.6 to 84.5 ± 6.7% of control, coinciding with the decrease in cytochrome *c* release. Caspase 8 inhibitor also blocked the amyloid β protein 25–35-mediated decrease in mitochondrial Bid concomitant with abrogating the generation of mitochondrial tBid.

### *3.4. Effects of amyloid β protein 25–35 and TAB on the distribution of Bak, Bax, and Bcl-2α between mitochondria and cytosol*

10 μM amyloid β protein 25–35 increased the level of cytosolic Bak to 127.1 ± 14.4% of control (Fig. 7A, B). However, amyloid β protein 25–35 failed to show an effect on the content of mitochondrial Bak. 50 μM TAB diminished the amyloid β protein 25–35-mediated elevation of cytosolic Bak from 127.1 ± 14.4% to 83.8 ± 14.6% of control. TAB also exerted a similar effect on the level of mitochondrial Bak in amyloid β protein 25–35-treated cells.

For Bax, amyloid β protein 25–35 showed no effects on the level of Bax either in mitochondria or in cytosol (Fig. 7C, D). However, in amyloid β protein 25–35-treated cells, pre-treatment

Table 1
Caspase 8 inhibitor attenuated amyloid β protein 25–35-mediated cell death, release of cytochrome *c* from mitochondria to cytosol, and increase of mitochondrial tBid

| | Amyloid β protein 25–35 (10 μM) | Caspase 8 inhibitor + amyloid β protein 25–35 |
|---|---|---|
| | Relative % of control | |
| Cell death (48 h) | 40.2 ± 3.7 | 28.3 ± 2.5[b] |
| Cytochrome *c* (16 h) | | |
| Mitochondria | 64.1 ± 8.6 | 84.5 ± 6.7[a] |
| Cytosol | 143.7 ± 15.6 | 102.9 ± 26.5[a] |
| Bid (6 h) | | |
| Mitochondria | 76.7 ± 9.3 | 98.5 ± 15.7 |
| Cytosol | 97.1 ± 10.2 | 98.5 ± 15.7 |
| tBid (6 h) | | |
| Mitochondria | 196.4 ± 6.3 | 121.1 ± 18.0[b] |

Cortical neurons were treated with vehicle (0.1% DMSO, v/v) or 10 μM caspase 8 inhibitor (IETD-CHO) for 2 h. Cells were then exposed to 10 μM amyloid β protein 25–35 for time indicated. The cell death was determined by MTT reduction. Otherwise, cells were collected and subjected to subcellular fractionation. The levels of cytochrome *c*, Bid, and tBid in mitochondria or cytosol were measured by immunoblotting using equal amount of protein. The levels of cytochrome *c*, Bid, and tBid in mitochondria and cytosol have been normalized with ANT and actin, respectively. Results are means ± S.D. from four independent experiments, and are expressed relative to control cells. Significant differences between cells treated with amyloid β protein 25–35 and amyloid β protein 25–35 plus caspase 8 inhibitor are indicated by [a], $P<0.05$; and [b], $P<0.01$.

with 50 μM TAB decreased the level of mitochondrial and cytosolic Bax by 32.5±2.1% and 19.6±1.8%, respectively. The effects of amyloid β protein 25–35 and TAB on Bcl-2α were also elucidated. Amyloid β protein 25–35 (10 μM) decreased mitochondrial Bcl-2α to 74.7±12.6% of control (Fig. 8), whereas

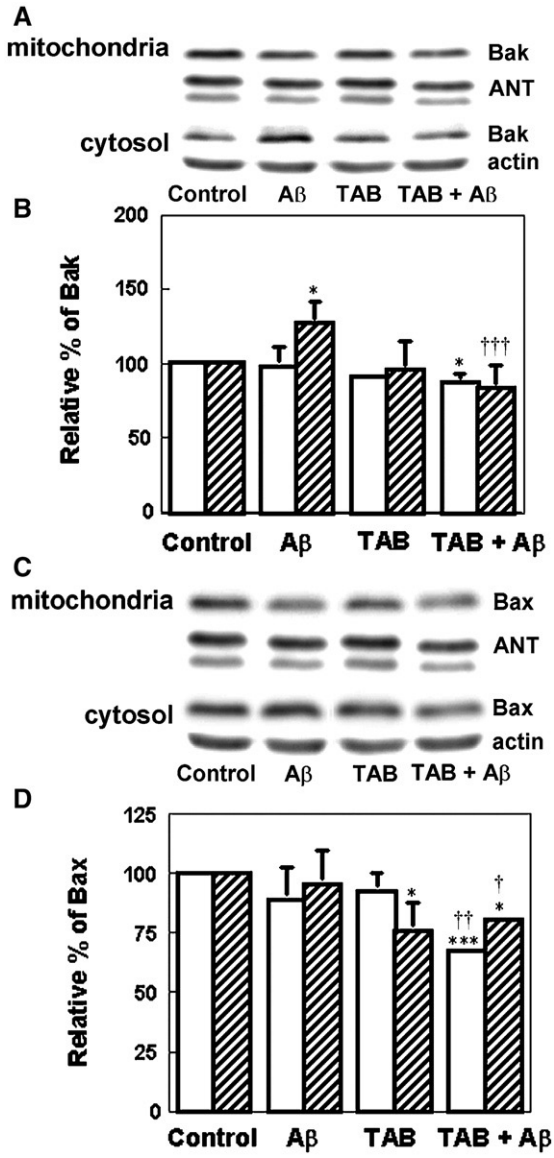


Fig. 7. Effects of TAB and amyloid β protein 25–35 (Aβ) on the level of Bak and Bax in mitochondria and cytosol. Cells were treated with vehicle or 50 μM TAB for 2 h. Cells were then incubated with or without 10 μM amyloid β protein 25–35 for 6 h. After incubation, cells were collected and subjected to subcellular fractionation. The same amount of proteins were subjected to immunoblotting to detect the level of Bak and Bax in mitochondria (unfilled column) or in cytosol (hatched column). Panel A and panel C show the representative immunoblots of Bak, Bax, ANT, and actin. Panel B and panel D show the relative level of Bak and Bax in mitochondria and cytosol, respectively. The levels of Bak/Bax in mitochondria and cytosol have been normalized with ANT and actin, respectively. Results are means±S.D. (where large enough to be shown) from three independent experiments, and are expressed relative to control cells. Significant differences between control cells and cells treated with amyloid β protein, TAB, or amyloid β protein plus TAB are indicated by *, $P<0.05$; and ***, $P<0.001$. Significant differences between cells treated with amyloid β protein and cells treated with amyloid β protein plus TAB are indicated by †, $P<0.05$; ††, $P<0.01$; and †††, $P<0.001$.

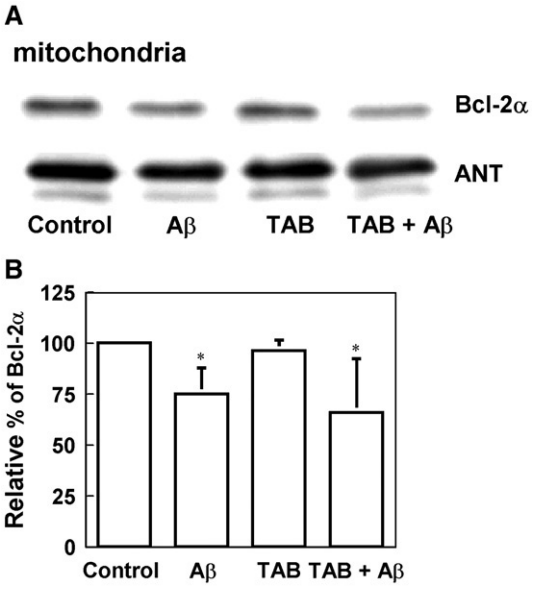


Fig. 8. Effects of TAB and amyloid β protein 25–35 (Aβ) on the level of Bcl-2α in mitochondria. Cells were treated with vehicle or 50 μM TAB for 2 h and were then incubated with or without 10 μM amyloid β protein 25–35 for 1 h. After incubation, cells were collected and subjected to subcellular fractionation. The same amount of proteins were subjected to immunoblotting to detect the level of Bcl-2α in mitochondria. Panel A shows representative immunoblots of Bcl-2α and ANT. Panel B shows the relative level of Bcl-2α in mitochondria. The level of Bcl-2α in mitochondria has been normalized with ANT. Results are means± S.D. from three independent experiments, and are expressed relative to control cells. Significant differences between control cells and cells treated with amyloid β protein, TAB, or amyloid β protein plus TAB are indicated by *, $P<0.05$.

TAB did not show any effect on the level of Bcl-2α in control or amyloid β protein 25–35-treated cells.

## 4. Discussion

Amyloid β protein triggers a massive influx of $Ca^{2+}$ via L-type voltage-sensitive $Ca^{2+}$ channels into the intracellular compartment (Davidson et al., 1994; Ueda et al., 1997). Endoplasmic reticulum and mitochondria function as the intracellular stores to sequester the excessive amount of cytosolic $Ca^{2+}$. It is well established that disturbance of $Ca^{2+}$ homeostasis in mitochondria has detrimental effects. The overload of calcium in mitochondria causes the deregulation of reactive oxygen species production and the impairment of bioenergetic metabolism ultimately culminates in neuron death (Murphy et al., 1999; Nicholls and Budd, 2000; Pivovarova et al., 2004). TAB abolished the amyloid β protein-induced sustained elevation of calcium levels in mitochondria. This event prevents the disturbance of $Ca^{2+}$ homeostasis in mitochondria and consequently abrogates reactive oxygen species production, maintains mitochondrial integrity, and attenuates the trigger of intrinsic apoptosis. The attenuated elevation of mitochondrial $Ca^{2+}$ therefore may account, in part, for the neuroprotection of TAB.

The mitochondrion is the main target compartment for multiple anti-apoptotic and pro-apoptotic molecules such as Bcl-2, Bax, Bad, Bid, etc. (Polster and Fiskum, 2004). Bcl-2 family proteins are central mediators of the mitochondria-

dependent cell death pathway, functioning as either anti- or pro-apoptotic factors that regulate the release of apoptosis-inducing proteins from these organelles (Reed, 1997; Adams and Cory, 1998; Chao and Korsmeyer, 1998; Danial and Korsmeyer, 2004; Green and Kroemer, 2004). The BH3-only member Bid serves the unique function of interconnecting the extrinsic death receptors to the mitochondrial amplification loop of the intrinsic pathway. tBid either directly or indirectly activates Bax and Bak. The activation of Bax and Bak is associated with their oligomerization in mitochondrial membranes. The pore-forming capability of oligomerized Bax and Bak results in the destabilization of the mitochondrial outer membrane and the subsequent release of death molecules from the confines of these organelles (Li et al., 1998; Luo et al., 1998; Korsmeyer et al., 2000; Zha et al., 2000).

Amyloid β protein ubiquitously elicited the activation of caspases 2, 3, 6, 8, and 9 in previous studies and the present report, indicating that both extrinsic and intrinsic apoptotic signaling pathways were implicated in amyloid β protein-mediated apoptosis (Wang et al., 2001). In the present study, we further demonstrated that the Bid-mediated crosstalk between extrinsic and intrinsic apoptosis was also implicated in the neurotoxicity of amyloid β protein. Our results showed that a small portion of Bid was cleaved by caspase 8 to produce tBid which subsequently translocated to the mitochondrial outer membrane. The data on tBid production may be underestimated as the half-life of tBid is much less than our treatment time period (1.5 h *versus* 6 h). The decline of mitochondrial Bid introduces several speculative reasons such as that tBid is derived from the cleavage of mitochondrial Bid, or that the balance of mitochondrial and cytosolic Bid is altered by amyloid β protein treatment. Based on the fact that cleavage of Bid by caspase 8 occurs in the cytosol, we therefore propose that amyloid β protein treatment may disturb the balance of mitochondrial and cytosolic Bid or amyloid β protein may directly modulate the total level of Bid. We used ANT, a component of the permeability transition pore, to eliminate the variability of mitochondrial isolation. However, the results indicated that amyloid β protein alone or in combination with TAB decreased the level of ANT. Therefore, the content of mitochondrial cytochrome *c* and Bid/tBid in the present study may be underestimated (the content of these two proteins was normalized with ANT in the present study). The amyloid β protein-mediated decline of ANT may also be implicated in the amyloid β protein-induced mitochondrial energy failure (Reddy and Beal, 2005; Reddy, 2006).

The results with caspase 8 inhibitor indicate that blocking the caspase 8–tBid–cytochrome *c* pathway is not sufficient to account for the inhibitory extent of TAB on amyloid β protein-mediated cell death. Furthermore, TAB only exhibited a slight inhibition of the activity of executive caspases such as caspase 3. There is evidence that oxidative stress and endoplasmic reticulum stress also contribute to the amyloid β protein-mediated neurotoxicity (Ekinci et al., 1999; Nakagawa et al., 2000; Wang et al., 2001; Hitomi et al., 2004). TAB is classified as a polyphenolic compound possessing anti-oxidative activity. It is therefore conceivable that TAB may counteract the amyloid β protein-induced oxidative stress or interfere with other pathways. TAB retarded the caspase 8–tBid–cytochrome *c* pathway in combination with other effects to confer its neuroprotective effect on amyloid β protein toxicity.

## Acknowledgements

This study was supported by Grant NSC-94-2323-B-077-002 from the National Science Council, and Grants 95DBCMR-2 and 95DBCMR-3 from the National Research Institute of Chinese Medicine, Taiwan, ROC.

## References

Adams, J.M., Cory, S., 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281, 1322–1326.

Antonsson, B., Martinou, J.C., 2000. The Bcl-2 protein family. Exp. Cell Res. 256, 50–57.

Buxbaum, J.D., Greengard, P., 1996. Regulation of APP processing by intra- and intercellular signals. Ann. N.Y. Acad. Sci. 777, 327–331.

Chao, D.T., Korsmeyer, S.J., 1998. BCL-2 family: regulators of cell death. Annu. Rev. Immunol. 16, 395–419.

Danial, N.N., Korsmeyer, S., 2004. Cell death: critical control points. Cell 116, 205–219.

Davidson, R.M., Shajenko, L., Donta, T.S., 1994. Amyloid beta-peptide (A beta P) potentiates a nimodipine-sensitive L-type barium conductance in N1E-115 neuroblastoma cells. Brain Res. 643, 324–327.

Ekinci, F.J., Malik, K.U., Shea, T.B., 1999. Activation of the L voltage-sensitive calcium channel by mitogen-activated protein (MAP) kinase following exposure of neuronal cells to β-amyloid. MAP kinase mediates β-amyloid-induced neurodegeneration. J. Biol. Chem. 274, 30322–30327.

Estus, S., Tucker, H.M., van Rooyen, C., Wright, S., Brigham, E.F., Wogulis, M., Rydel, R.E., 1997. Aggregated amyloid-β protein induces cortical neuronal apoptosis and concomitant "apoptotic" pattern of gene induction. J. Neurosci. 17, 7736–7745.

Green, D.R., Kroemer, G., 2004. The pathophysiology of mitochondrial cell death. Science 305, 626–629.

Gross, A., Yin, X.M., Wang, K., Wei, M.C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., Korsmeyer, S.J., 1999. Caspase cleaved BID targets mitochondria and is required for cytochrome *c* release while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J. Biol. Chem. 274, 1156–1163.

Hitomi, J., Katayama, T., Eguchi, Y., Kudo, T., Taniguchi, M., Koyama, Y., Manabe, T., Yamagishi, S., Bando, Y., Imaizumi, K., Tsujimoto, Y., Tohyama, M., 2004. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. J. Cell Biol. 165, 347–356.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., Newmeyer, D.D., 1997. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275, 1132–1136.

Korsmeyer, S.J., Wei, M.C., Saito, M., Weiler, S., Oh, K.J., Schlesinger, P.H., 2000. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. Cell Death Differ. 7, 1166–1173.

Li, H., Zhu, H., Xu, C.J., Yuan, J., 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94, 491–501.

Lin, Y.L., Chang, Y.Y., Kuo, Y.H., Shiao, M.S., 2002. Anti-lipid-peroxidative principles from *Tournefortia Sarmentosa*. J. Nat. Prod. 65, 745–747.

Loo, D.T., Copani, A., Pike, C.J., Whittemore, E.R., Walencewicz, A.J., Cotman, C.W., 1993. Apoptosis is induced by β-amyloid in cultured central nervous system neurons. Proc. Natl. Acad. Sci. U. S. A. 90, 7951–7955.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., Wang, X., 1998. Bid, a Bcl2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. Cell 94, 481–490.

MacManus, A., Ramsden, M., Murray, M., Henderson, Z., Pearson, H.A., Campbell, V.A., 2000. Enhancement of $^{45}Ca^{2+}$ influx and voltage-dependent $Ca^{2+}$ channel activity by β-amyloid-(1-40) in rat cortical synaptosomes and cultured cortical neurons. Modulation by the proinflammatory cytokine interleukin-1β. J. Biol. Chem. 275, 4713–4718.

Mattson, M.P., Partin, J., Begley, J.G., 1998. Amyloid β-peptide induces apoptosis-related events in synapses and dendrites. Brain Res. 807, 167–176.

Maurice, T., Lockhart, B.P., Privat, A., 1996. Amnesia induced in mice by centrally administered β-amyloid peptides involves cholinergic dysfunction. Brain Res. 706, 181–193.

Morais Cardoso, S., Swerdlow, R.H., Oliveira, C.R., 2002. Induction of cytochrome *c*-mediated apoptosis by amyloid beta 25–35 requires functional mitochondria. Brain Res. 931, 117–125.

Murphy, A.N., Fiskum, G., Beal, M.F., 1999. Mitochondria in neurodegeneration: bioenergetic function in cell life and death. J. Cereb. Blood Flow Metab. 19, 231–245.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B.A., Yuan, J., 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. Nature 403, 98–103.

Nicholls, D.G., Budd, S.L., 2000. Mitochondria and neuronal survival. Physiol. Rev. 80, 315–360.

Pappolla, M.A., Sos, M., Omar, R.A., Bick, R.J., Hickson-Bick, D.L.M., Reiter, R.J., Efthimiopoulos, S., Robakis, N.K., 1997. Melatonin prevents death of neuroblastoma cells exposed to the Alzheimer amyloid peptide. J. Neurosci. 17, 1683–1690.

Paradis, E., Douillard, H., Koutroumanis, M., Goodyer, C., LeBlanc, A., 1996. Amyloid β peptide of Alzheimer's disease downregulates Bcl-2 and upregulates bax expression in human neurons. J. Neurosci. 16, 7533–7539.

Pivovarova, N.B., Nguyen, H.V., Winters, C.A., Brantner, C.A., Smith, C.L., Andrews, S.B., 2004. Excitotoxic calcium overload in a subpopulation of mitochondria triggers delayed death in hippocampal neurons. J. Neurosci. 24, 5611–5622.

Polster, B.M., Fiskum, G., 2004. Mitochondrial mechanisms of neural cell apoptosis. J. Neurochem. 90, 1281–1289.

Prehn, J.H., Bindokas, V.P., Jordán, J., Galindo, M.F., Ghadge, G.D., Roos, R.P., Boise, L.H., Thompson, C.B., Krajewski, S., Reed, J.C., Miller, R.J., 1996. Protective effect of transforming growth factor-β 1 on β-amyloid neurotoxicity in rat hippocampal neurons. Mol. Pharmacol. 49, 319–328.

Reed, J.C., 1997. Double identity for proteins of the Bcl-2 family. Nature 387, 773–776.

Reddy, P.H., 2006. Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. J. Neurochem. 96, 1–13.

Reddy, P.H., Beal, M.F., 2005. Are mitochondria critical in the pathogenesis of Alzheimer's disease? Brain Res. Brain Res. Rev. 49, 618–632.

Selkoe, D.J., 1990. Deciphering Alzheimer's disease: the amyloid precursor protein yields new clues. Science 248, 1058–1060.

Selkoe, D.J., 1996. Amyloid β-protein and the genetics of Alzheimer's disease. J. Biol. Chem. 271, 18295–18298.

Smith, M.A., 1998. Alzheimer disease. Int. Rev. Neurobiol. 42, 1–54.

Tamagno, E., Parola, M., Guglielmotto, M., Santoro, G., Bardini, P., Marra, L., Tabaton, M., Danni, O., 2003. Multiple signaling events in amyloid-β induced, oxidative stress-dependent neuronal apoptosis. Free Radic. Biol. Med. 35, 45–58.

Troy, C.M., Rabacchi, S.A., Friedman, W.J., Frappier, T.F., Brown, K., Shelanski, M.L., 2000. Caspase-2 mediates neuronal cell death induced by β-amyloid. J. Neurosci. 20, 1386–1392.

Ueda, K., Shinohara, S., Yagami, T., Asakura, K., Kawasaki, K., 1997. Amyloid β protein potentiates $Ca^{2+}$ influx through L-type voltage-sensitive $Ca^{2+}$ channels: a possible involvement of free radicals. J. Neurochem. 68, 265–271.

Wang, C.N., Chi, C.W., Lin, Y.L., Chen, C.F., Shiao, Y.J., 2001. The neuroprotective effects of phytoestrogens on amyloid β protein-induced toxicity are mediated by abrogating the activation of caspase cascade in rat cortical neurons. J. Biol. Chem. 276, 5287–5295.

Wang, C.N., Pan, H.C., Lin, Y.L., Chi, C.W., Shiao, Y.J., 2006. Ester derivatives of tournefolic acid B attenuate *N*-methyl-D-aspartate-mediated excitotoxicity in rat cortical neurons. Mol. Pharmacol. 69, 950–959.

Wang, K., Yin, X.M., Chao, D.T., Milliman, C.L., Korsmeyer, S.J., 1996. BID: A novel BH3 domain-only death agonist. Genes Dev. 10, 2859–2869.

Wei, H., Leeds, P.R., Qian, Y., Wei, W., Chen, R., Chuang, D., 2000. β-amyloid peptide-induced death of PC 12 cells and cerebellar granule cell neurons is inhibited by long-term lithium treatment. Eur. J. Pharmacol. 392, 117–123.

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G., Youle, R.J., 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol. 139, 1281–1292.

Yin, X.M., 2006. Bid, a BH3-only multi-functional molecule, is at the cross road of life and death. Gene 369, 7–19.

Zha, J., Weiler, S., Oh, K.J., Wei, M.C., Korsmeyer, S.J., 2000. Posttranslational *N*-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science 290, 1761–1765.