



Neuroprotective effect of naturally occurring RXR agonists isolated from *Sophora tonkinensis* Gagnep. on amyloid- β -induced cytotoxicity in PC12 cells

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Abstract

Neuronal cell death induced by amyloid- β (A β) oligomers is implicated in neuronal degeneration and is a leading cause of Alzheimer's disease (AD). Therefore, to identify effective therapeutic agents for AD, we investigated the neuroprotective effects of two naturally occurring retinoid X receptor (RXR) agonists (SPF1 and SPF2), isolated from the root of *Sophora tonkinensis* Gagnep., on the A β_{25-35} -induced cytotoxicity against nerve growth factor-differentiated rat pheochromocytoma (PC12) cells. Pretreatment with SPFs significantly prevented A β_{25-35} -induced apoptosis in PC12 cells, similarly to the synthetic RXR agonist bexarotene. These effects were blocked by the RXR antagonist PA452. When the effects of SPFs were studied in the presence of the liver X receptor (LXR) agonist T0901317, the protective effects of SPFs were enhanced, suggesting that RXR/LXR heterodimers may play a key role in the neuroprotective effects of SPFs. SPFs and T0901317 induced ATP-binding cassette transporter 1 (ABCA1) protein expression in PC12 cells when administered alone or in combination. Intriguingly, a functional inhibitor of ABCA1 cyclosporine A negated the neuroprotective effects of SPFs or T0901317. Taken together, these results demonstrate that the RXR agonists SPF1 and SPF2 protect PC12 cells from A β_{25-35} -induced neurotoxicity in an RXR-dependent manner and that their effects are markedly enhanced by the LXR agonist T0901317, in part related to ABCA1 function. These results suggest a novel approach to the treatment or prevention of AD.

Keywords Retinoid X receptor · Liver X receptor · Naturally occurring agonist · Alzheimer's disease · Amyloid β · Neurotoxicity

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and a major cause of dementia, characterized by the deposition of amyloid- β (A β) peptide, a principal component of senile plaques and intracellular neurofibrillary

tangles composed of hyperphosphorylated tau protein [1, 2]. Currently, the precise mechanism underlying the pathogenesis of AD has not been fully elucidated, but the predominant theory for AD, the "amyloid cascade hypothesis," postulates that the deposition of A β peptides in the brain is intimately linked with neuronal degeneration leading to cognitive deficit. A β s sequentially cleaved from amyloid precursor protein have multiple forms, some of which are highly neurotoxic and exert diverse effects on neuronal functions, finally leading to neuronal cell death. Neurotoxic oligomers such as A β_{42} oligomer interact with various receptors, including the receptor for advanced glycation end products (RAGE), NMDA-type glutamate receptor (NMDAR), calcium-sensing receptors (CaSRs), and cellular prion protein (PrP^c) expressed on the surface of neurons, which trigger signals to induce inflammation, endoplasmic reticulum (ER) stress, and oxidative stress, and elevate intracellular Ca²⁺ levels [3, 4]. The failure of several recent clinical trials using anti-A β antibody to reduce A β deposits has provoked controversy as

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to whether A β plaque deposits are functionally associated with the pathogenesis of AD [5, 6]. However, at present, it seems likely that several interacting pathophysiological processes induced by A β oligomers might be intricately involved in neuronal degeneration. Given the urgent need for the development of novel and effective therapeutic strategies for the prevention and treatment of AD, it is worth exploring therapeutic agents that protect neuronal cells from A β oligomer-evoked neurotoxicity.

Nuclear receptors (NRs) belong to a superfamily of ligand-activated transcription factors that regulate the expression of diverse genes involved in a wide variety of biological processes, such as cellular metabolism and neuronal function, by binding to sequence-specific DNA elements. Over the past decade, numerous studies have revealed that agonists against NRs, including peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXR), retinoic acid receptor, and retinoid X receptor (RXR), have a broad range of salutary effects in murine models of AD [7–11]. The beneficial effects of NR agonists are broadly categorized into stimulation of A β clearance, suppression of A β generation, regulation of neuronal function, and anti-inflammatory action [12]. Animal studies have reported promising results, although there have been some inconsistencies between reports [1]. Additionally, the clinical efficacy for AD treatment has not yet been proven because of low blood–brain barrier (BBB) permeability and serious adverse effects inherent in some NR agonists [13]. Overall, even though some problems remain to be addressed, the activation of NRs seems to be a potential therapeutic strategy for AD treatment.

The RXR selective agonist bexarotene (BEX) was reported to enhance soluble A β clearance, reduce A β plaque burden, and reverse cognitive deficits in murine models of AD [14]. However, the beneficial effects of BEX on A β metabolism and plaque burden are still controversial [15], although recent evidence demonstrated that BEX affected signaling pathways related to neuronal differentiation and neuron projections [16], and rescued the age-related decline of synaptic proteins PSD95, GluR1, and NR1 which regulate synaptic plasticity [17], suppress inflammation and astrogliosis, and activate microglial phagocytosis [8]. Taken together, BEX seems to have neuroprotective effects mediated via multifaceted mechanisms. However, serious side effects following BEX treatment have been reported, including hyperlipidemia [18], hypothyroidism [19], hepatomegaly [20], and anemia [21], which limit the usage of BEX for the treatment of cutaneous T-cell lymphoma. In this context, alternative RXR agonists with a gene expression profile, bioavailability, and BBB permeability distinct from BEX are required for AD treatment or prophylaxis.

In our previous study, two prenylated flavonoids referred to as SPF1 and SPF2 were isolated from the roots of *Sophora*

tonkinensis Gagnep., a Chinese folk medicine. SPF1 and SPF2 showed selective RXR agonist activities with EC₅₀ values of 0.77 μ M and 0.78 μ M, respectively [22]. SPFs activate PPARs/RXR and RXR/LXR heterodimers, which are classified as permissive heterodimers, alone or in combination with the ligands for PPARs or LXR. They also increase ATP-binding cassette transporter 1 (ABCA1) and apoE mRNA expression similarly to BEX. However, the gene expression profiles induced by BEX and SPF1 are not identical and only a limited number of genes overlap (unpublished data). Three isoforms of RXRs have been identified and these are implicated in the diverse regulation of gene transcription based on their ability to dimerize with various partner NRs and to form homodimers or tetramers [23]. In addition, the recruitment of various RXR ligand-dependent cofactors increases the diversity of RXR-regulated gene transcription [24]. Thus, considering the pleiotropic effects of RXR with different properties from other NRs, alternative RXR activators with neuroprotective effects, but not adverse effects, will be potential candidates for therapeutic agents to prevent or treat AD. Therefore, we investigated the neuroprotective effects of SPF1 and SPF2 and found that they protected PC12 cells from A β -induced neurotoxicity by a mechanism dependent on RXR/LXR heterodimers.

Materials and methods

Reagents

2-[[3-Hydroxy-2',2-dimethyl-8-(3-methyl-2-butenyl)]chroman-6-yl]-7-hydroxy-8-(3-methyl-2-butenyl)-chroman-4-one (SPF1) and 2-[[2-(1-hydroxy-1-methylethyl)-7-(3-methyl-2-butenyl)-2',3-dihydrobenzofuran]-5-yl]-7-hydroxy-8-(3-methyl-2-butenyl)-chroman-4-one (SPF2) were isolated from the root of *Sophora tonkinensis* Gagnep. as described in our previous paper [22]. The RXR agonist bexarotene (BEX) and LXR agonist T0901317 (T090) were purchased from Cayman Chemicals (Denver, CO, USA). All chemicals were dissolved in dimethyl sulfoxide (DMSO) (Fujifilm Wako Pure Chemical, Osaka, Japan) and stored at -20°C until use. Amyloid- β peptide (A β _{25–35}) was obtained from Peptide Institute, Inc. (Osaka, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI1640 medium, penicillin, streptomycin, and poly-L-lysine (PLL) were obtained from Fujifilm Wako Pure Chemical. Fetal bovine serum (FBS), horse serum (HS), and nerve growth factor (NGF) were purchased from Nichirei Biosciences Inc. (Tokyo, Japan), Gibco (Grand Island, NY, USA), and PeproTech, Inc. (Rocky Hill, NJ, USA), respectively. Cyclosporine A (CsA) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Cell culture

The rat pheochromocytoma cell line PC12 and hepatocellular carcinoma cell line HepG2 were obtained from the JCRB Cell Bank (Osaka, Japan). PC12 cells were maintained in RPMI1640 medium supplemented with 10% HS, 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ in air. PC12 cells were differentiated with 50 ng/ml NGF for 3 days into sympathetic neuron-like cells and the differentiated PC12 cells were then used in the following experiments. HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Cell viability assay

Cell viability was determined by MTT assay. Briefly, PC12 cells were seeded into PLL-coated 96-well microplates at a density of 3×10^5 cells/ml. The cells were treated with SPF1 (1 µM), SPF2 (1 µM), and BEX (1 µM) for 12 h, followed by exposure to Aβ_{25–35} (0.5 µM). Aβ_{25–35} was dissolved in DMSO, diluted to 5% DMSO solution with sterile distilled H₂O, and stored until use. For all cell cultures, the concentration of DMSO was less than 0.025%. After 48 h incubation, the cells were thoroughly washed twice with phosphate-buffered saline (PBS), and MTT solution was added to the culture at a final concentration of 0.5 mg/ml and incubated for another 6 h at 37 °C. Sodium dodecyl sulfate (SDS) was then added for 18 h to dissolve formazan crystals. The absorbance of each well was determined at 570 nm by microplate reader (Beckman Coulter®, Brea, CA, USA). The cell viability was calculated as the percentage of the control group.

Detection of apoptotic cells by Hoechst 33342 dye staining

PC12 cells were seeded onto PLL-coated cover glasses at a density of 5×10^4 cells/ml. After treatment with the compounds, then Aβ_{25–35}, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature and washed with PBS 3 times. Thereafter, the cells were stained with 2 µg/ml Hoechst 33342 dye for 1 h at room temperature. The cells were then thoroughly washed with PBS 3 times before observation with a BZ-9000 fluorescence microscope (Keyence Corp., USA). The dye was excited at 360 nm emission and filtered at 460 nm. Apoptotic cells were determined as cells with condensed nuclei and strong bright Hoechst 33342 staining. Total cells and apoptotic cells in 12 randomly selected fields

were counted by two persons. The result was expressed as the percentage of apoptotic cells to the total cells.

Total RNA extraction and real-time qPCR

PC12 cells were seeded into 24-well PLL-coated plates at a density of 2×10^5 cells/ml. After treatment with the compounds, total RNA was extracted from cells using RNAiso Plus (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions. A total of 250 ng RNA was reverse-transcribed using ReverTra Ace (Toyobo, Japan). Quantitative real-time PCR (RT-qPCR) with SYBR green (Thermo Fisher Scientific, K.K., Tokyo, Japan) was performed using the TP800 Thermal Cycler Dice® Real-time System (Takara Bio. Inc., Shiga, Japan). qPCR consisted of the following conditions: 50 cycles of 5 s at 95 °C and 30 s at 63 °C. The relative expression of target gene mRNA was calculated by the ΔΔCt method and normalized to the amount of β-actin expression (Table 1).

Total protein extraction and Western blot analysis

PC12 cells were seeded into 6-well PLL-coated plates at a density of 2×10^5 cells/ml. After treatment with the compounds, total protein was extracted from the cells with RIPA lysis buffer, and then the protein concentration was determined by Bradford assay (Bio-Rad, USA). Protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Immobilon®, USA). The membranes were blocked with 3% (w/v) BSA in TBS-T buffer (Tris-buffered saline containing 0.1% Tween 20) at room temperature for 2 h and incubated with anti-ABCA1 antibody (1:1000, Abcam) and anti-β-actin antibody (1:1000, BioLegend) at 4 °C overnight. The next day, the membranes were washed with TBS-T 3 times and incubated with horseradish peroxidase-linked anti-mouse IgG antibody (1:5000, Cell Signaling Technology) at room temperature for 1 h. The membranes were then washed with TBS-T 3 times and bands were detected by using an enhanced chemiluminescence reagent (ImmunoStar®, Fuji-film Wako Pure Chemical), and imaged by ImageQuant LAS-4000mini (GE Healthcare, USA).

Table 1 List of primers used in the present study

Gene	Forward primer	Reverse primer
Rat ABCA1	TGC GGC GTC AAG	ACG ACC AGT GTA
Rat β-actin	CCT GTC ATC T	GCA GGG ACC A
	CCC ATC TAT GAG	TTT AAT GTC ACG
	GGT TAC GC	CAC GAT TTC
Human SREBC-1c	TCA GCG AGG CGG	CAT GTC TTC GAT
Human GAPDH	CTT TGG AGC AG	GTC GGT CAG
	CGA GAT CCC TCC	GGT GCT AAG CAG
	AAA ATC AA	TTG GTG GT

Statistical analysis

Data are presented as the mean \pm SD. The significance of differences was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's *t* test. *p* values less than 0.05 were considered statistically significant.

Results

Effect of SPFs on A β_{25-35} -induced cytotoxicity in PC12 cells

To explore the neuroprotective effects of the naturally occurring RXR agonists shown in Fig. 1 on A β_{25-35} -induced neuronal cell death, SPF1, SPF2, and BEX were incubated with NGF-differentiated PC12 cells with characteristics of sympathetic neuron-like cells and cell viability was evaluated using the MTT method (Fig. 2). In this experiment, the concentration of A β_{25-35} peptide was set at 0.5 μ M, where about 50% of cells were killed according to the results of a preliminary experiment. The concentration of SPFs was also set at 1 μ M, where SPFs had the most potent effects in preliminary experiments. SPF1 and SPF2 exhibited no cytotoxicity against PC12 cells, similarly to BEX. Following a 48-h incubation with A β_{25-35} at a concentration of 0.5 μ M, cell viability was reduced to approximately 50% of the control group, while SPFs and BEX pretreatment for 12 h significantly reduced A β_{25-35} -induced cytotoxicity in PC12 cells.

Fig. 1 Chemical structures of SPF1, SPF2, and bexarotene

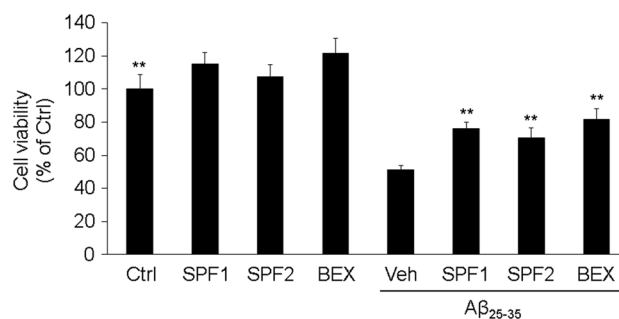
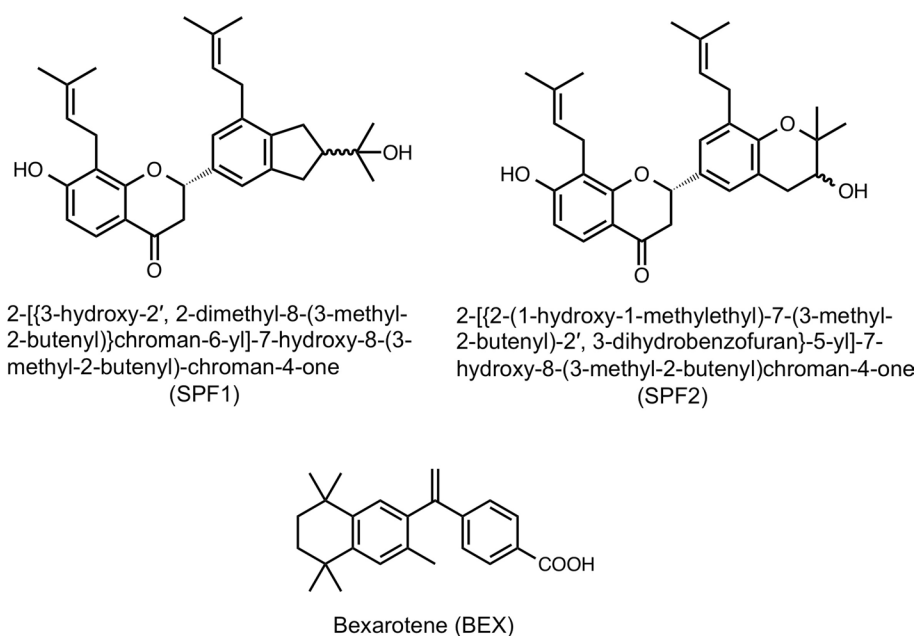


Fig. 2 Effect of SPFs on the cell viability of A β_{25-35} -treated PC12 cells. NGF-differentiated PC12 cells were treated with 1 μ M SPF1, SPF2, or BEX for 12 h, followed by 48-h exposure to 0.5 μ M A β_{25-35} . Cell viability was measured by the MTT method. Data represent the mean \pm SD of six determinants of a representative experiment from two independent experiments with similar results. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's *t* test. *p* values less than 0.05 were considered significant. ***p* < 0.01 vs. A β_{25-35} -treated vehicle group (*Veh*)

Effect of SPFs on A β_{25-35} -induced apoptosis in PC12 cells

To further study the protective effect of SPFs on A β_{25-35} -induced cell death, PC12 cells were stained with Hoechst 33342 to assess the characteristics of pyknotic nuclei in apoptotic cells as previously reported [25]. A β_{25-35} treatment increased the percentage of apoptotic cells with cell shrinkage, chromatin condensation, and strong bright fluorescent nuclei, whereas SPFs or BEX reduced the apoptotic cell population in PC12 cells treated with A β_{25-35} (Fig. 3a, b).

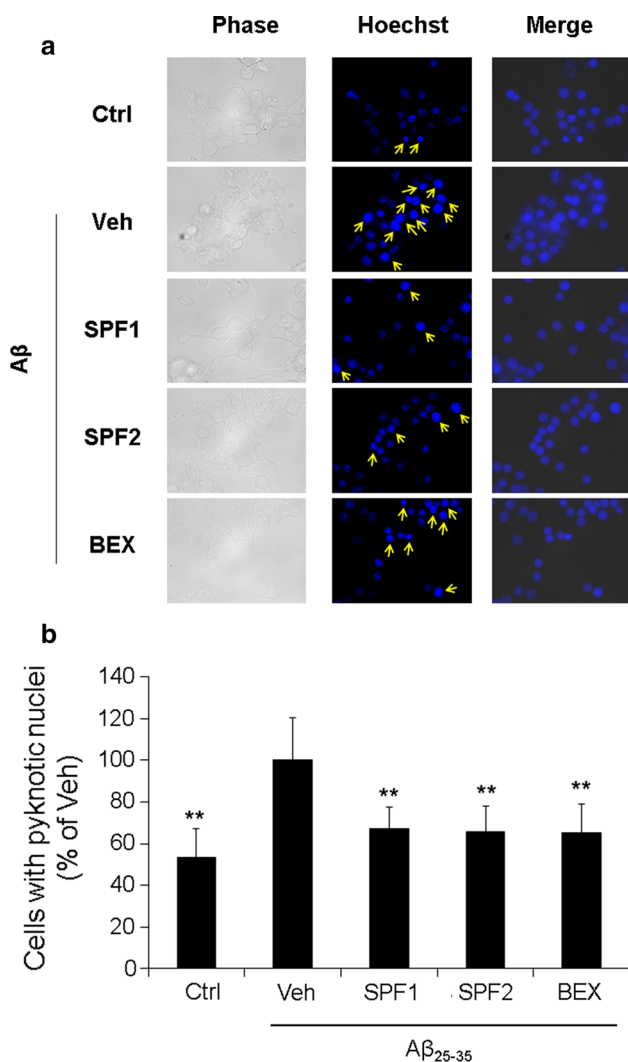


Fig. 3 Protective effects of SPFs on $A\beta_{25-35}$ -induced apoptosis in PC12 cells. NGF-differentiated PC12 cells were treated with 1 μ M SPF1, SPF2, or BEX for 12 h, followed by 48-h exposure to 0.5 μ M $A\beta_{25-35}$. Cells were stained with Hoechst 33342 and then apoptotic cells with pyknotic nuclei were examined under a fluorescence microscope as described in Materials and Methods (Fig. 3a). Data represent the mean \pm SD of three wells of a representative experiment from two independent experiments with similar results. The y-axis indicates the percentage of apoptotic cells to total cells (Fig. 3b). Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's *t* test. *p* values less than 0.05 were considered significant. ***p* < 0.01 vs. $A\beta_{25-35}$ -treated vehicle group (*Veh*)

Involvement of RXR in the protective effects of SPFs on $A\beta_{25-35}$ -induced apoptosis

To investigate whether RXR is involved in the protective effect of SPFs, the RXR-specific pan-antagonist PA452 was used. SPFs significantly inhibited $A\beta_{25-35}$ -induced apoptosis in PC12 cells, and the inhibitory effects were reversed by PA452 treatment (Fig. 4). BEX also exerted an inhibitory effect on $A\beta_{25-35}$ -induced apoptosis, and its effect was

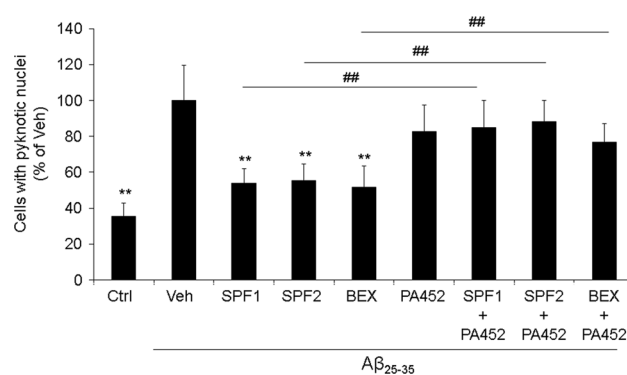


Fig. 4 Effects of RXR antagonist on protective effect of SPFs on $A\beta_{25-35}$ -induced apoptosis in PC12 cells. NGF-differentiated PC12 cells were treated with 1 μ M SPF1, SPF2, or BEX for 12 h in the presence or absence of the RXR antagonist PA452 (1 μ M), followed by 48-h exposure to 0.5 μ M $A\beta_{25-35}$. Data represent the mean \pm SD of three wells of a representative experiment from two independent experiments with similar results. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's *t* test. *p* values less than 0.05 were considered significant. ***p* < 0.01 vs. $A\beta_{25-35}$ -treated vehicle group (*Veh*), ###*p* < 0.01 between the indicated groups in the presence or absence of PA452

suppressed by PA452. Similar results were obtained when another RXR antagonist, HX531, was used instead of PA452 (data not shown).

Effect of SPFs in combination with an LXR agonist on $A\beta_{25-35}$ -induced apoptosis

In a previous study, we demonstrated that SPFs activated RXR/LXR heterodimers more effectively than PPAR γ /RXR heterodimers in the presence of an agonist for partner NR [22]. Therefore, we examined the effects of SPFs in combination with the LXR agonist T0901317 on $A\beta_{25-35}$ -induced apoptosis in PC12 cells. Interestingly, T0901317 alone had a protective effect on $A\beta_{25-35}$ -induced apoptosis in PC12 cells, and the effects of SPFs were significantly potentiated in the presence of T0901317 (Fig. 5).

Involvement of ABCA1 in the protective effects of SPFs and LXR agonist on $A\beta_{25-35}$ -induced apoptosis in PC12 cells

ATP-binding cassette transporter A1 (ABCA1), a well-known target gene regulated by LXR and RXR, is involved in cholesterol efflux for HDL generation or the regulation of signal transduction by modulating plasma membrane lipid composition [26]. Therefore, the mRNA and protein levels of ABCA1 were determined after treating PC12 cells for 24 h with SPFs at a concentration of 1 μ M. As shown in Fig. 6, SPFs induced ABCA1 mRNA

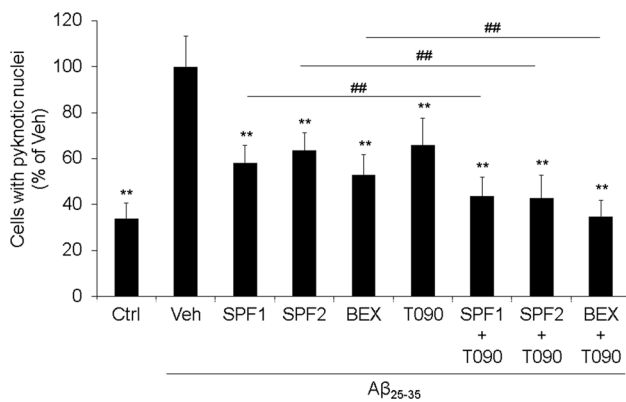


Fig. 5 Protective effects of SPFs in combination with T0901317 on A β_{25-35} -induced apoptosis in PC12 cells. NGF-differentiated PC12 cells were treated with 1 μ M SPF1, SPF2, or BEX for 12 h in the presence or absence of the LXR agonist T0901317 (T090) (1 μ M), followed by 48-h exposure to 0.5 μ M A β_{25-35} . Data represent the mean \pm SD of three wells. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's *t* test. *p* values less than 0.05 were considered significant. ***p* < 0.01 vs. A β_{25-35} -treated vehicle group (*Veh*), ###*p* < 0.01 between the indicated groups in the presence or absence of T0901317

and protein expression, and their induction was potentiated by the LXR agonist T0901317. Similar results were obtained with BEX. These results indicated that SPFs exert synergistic effects on ABCA1 expression via the RXR/LXR heterodimer. To further elucidate whether the protective effects of SPFs were associated with ABCA1 function, cyclosporine A (CsA), which inhibits the function of ABCA1 cholesterol efflux by blocking ABCA1 and ApoA-1 binding ability [27], was used. As shown in Fig. 7, CsA partially, but significantly, reversed the protective effects of SPFs on A β_{25-35} -induced apoptosis in PC12 cells, as well as those of BEX and T0901317. These results suggest that the protective effects of SPFs on A β_{25-35} -induced apoptosis in PC12 cells are partially dependent on ABCA1 function.

Effects of SPFs and BEX on sterol regulatory element-binding protein 1c (SREBP-1c) mRNA expression in HepG2 cells

BEX causes some serious side effects [18–21]. Therefore, we used HepG2 cells to determine whether SPFs induced the mRNA expression of SREBP-1c, which is considered a causal gene for hyperlipidemia. BEX and the LXR agonist T0901317 significantly increased the mRNA levels of SREBP-1c, whereas SPFs failed to show such an effect (Fig. 8).

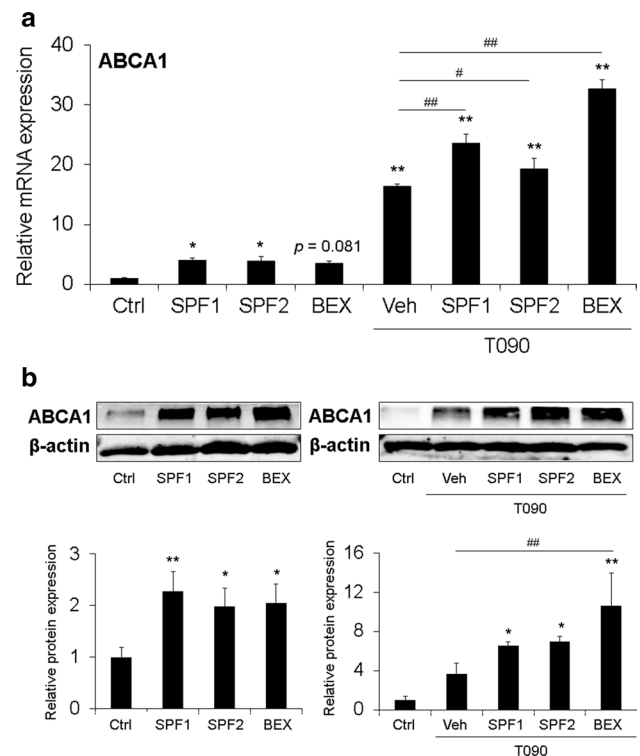


Fig. 6 ABCA1 **a** mRNA or **b** protein levels after treatment of PC12 cells with SPFs and BEX in the presence or absence of T0901317. NGF-differentiated PC12 cells were treated with 1 μ M SPF1, SPF2, or BEX for 24 h in the presence or absence of T0901317 (T090) (0.1 μ M). The mRNA and protein levels of ABCA1 were assessed using RT-qPCR and Western blotting, respectively. mRNA levels were normalized relative to β -actin mRNA levels and then represented as a fold change from the control group (*Ctrl*). The data represent the mean \pm SD of a representative experiment from two independent experiments with similar results, and were evaluated for statistical significance by one-way analysis of variance (ANOVA) followed by Bonferroni's *t* test. **p* < 0.05, ***p* < 0.01 vs. **a** T0901317-treated vehicle group (*Veh*) (*n* = 3), **b** vehicle group (*Ctrl*) or T0901317-treated vehicle group (*Veh*) (*n* = 3)

Discussion

Accumulating evidence has demonstrated that A β , especially A β -oligomers, transmit or block a variety of signals to neuronal cells via various receptors or channels, finally resulting in neuronal cell death [3]. Given the urgent need for the development of effective therapeutic agents for AD, we focused on A β oligomer-induced neurotoxicity as a therapeutic target and sought to evaluate the potential of RXR agonist SPFs. We used NGF-differentiated PC12 cells as an *in vitro* model to study A β oligomer-induced neurotoxicity. We used A β_{25-35} instead of A β_{1-40} or A β_{1-42} because the A β_{25-35} fragment is a functional neurotoxic domain that is detected in AD patients [28, 29]. In this study, the pretreatment of PC12 cells with SPFs effectively reduced cell death (apoptosis) induced by A β_{25-35} and the

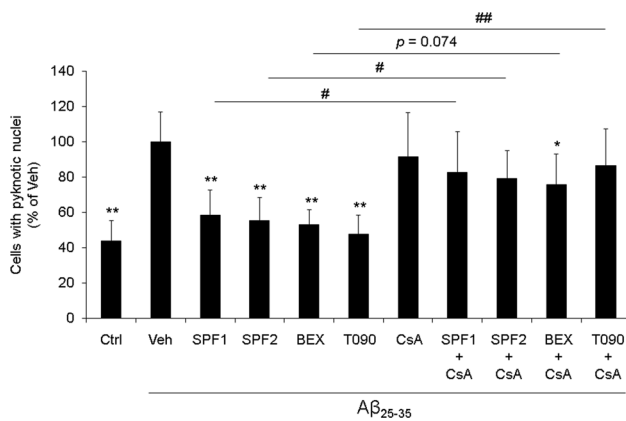


Fig. 7 Effects of cyclosporine A on protective effect of SPFs on $A\beta_{25-35}$ -induced apoptosis in PC12 cells. NGF-differentiated PC12 cells were treated with 1 μ M SPF1, SPF2, or BEX for 12 h in the presence or absence of cyclosporine A (CsA) (2.5 μ M), followed by 48-h exposure to 0.5 μ M $A\beta_{25-35}$. Data represent the mean \pm SD of three wells of a representative experiment from two independent experiments with similar results. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's *t* test. p values less than 0.05 were considered significant. ** $p < 0.01$ vs. $A\beta_{25-35}$ -treated vehicle group (*Veh*), * $p < 0.05$, ** $p < 0.01$ between the indicated groups in the presence or absence of CsA treatment

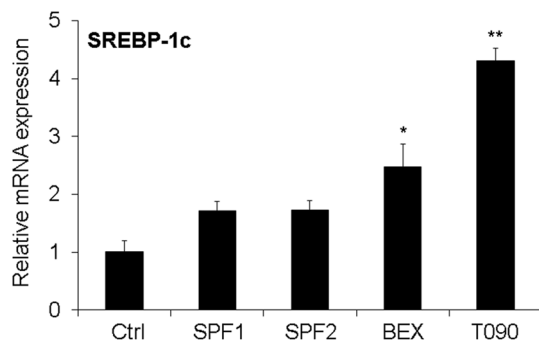


Fig. 8 Effects of SPFs and BEX on SREBP-1c mRNA expression in HepG2 cells. HepG2 cells were treated with 10 μ M SPF1, SPF2, BEX, or T0901317 for 12 h. The mRNA levels of SREBP-1c were assessed using RT-qPCR. mRNA levels were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels and then represented as a fold change from the control group (*Ctrl*). The data represent the mean \pm SD, and were evaluated for statistical significance by one-way analysis of variance (ANOVA) followed by Bonferroni's *t* test. * $p < 0.05$, ** $p < 0.01$ vs. vehicle group (*Ctrl*)

RXR antagonist PA452 abolished the protective effects of SPFs. The synthetic RXR agonist BEX also had a similar effect on $A\beta_{25-35}$ -induced apoptosis, which indicated that SPFs exert protective effects on $A\beta_{25-35}$ -induced neurotoxicity via RXR. On the basis of our previous study showing that SPFs activated RXR/LXR heterodimers more effectively than PPAR γ /RXR heterodimers [22], the impact of the LXR agonist T0901317 on $A\beta_{25-35}$ -induced apoptosis was studied. T0901317 alone decreased $A\beta_{25-35}$ -induced

apoptosis and potentiated the anti-apoptotic effect in the presence of SPFs. These results strongly suggest that SPFs activate RXR/LXR heterodimers to protect PC12 cells from $A\beta_{25-35}$ -induced neurotoxicity, and the protective effect was enhanced in combination with T0901317. To examine the mechanism underlying the protective effect on $A\beta_{25-35}$ -induced neurotoxicity, the expressions of ABCA1 mRNA and protein were assessed by RT-qPCR and Western blot analysis, respectively. ABCA1 is transcriptionally regulated by LXR and RXR, and a loss-of-function mutation in ABCA1 or polymorphisms in ABCA1 are associated with a high risk of AD [30, 31], which suggests that ABCA1 has an important role in protection against AD development and the protection of neuronal cells from $A\beta_{25-35}$ -induced neurotoxicity. Treatment of PC12 cells with SPFs alone increased ABCA1 expression and this was synergistically increased in the presence of T0901317. Intriguingly, the ABCA1 protein levels correlated with the degree of the SPFs' protective effect with or without T0901317. Therefore, CsA, a functional inhibitor of ABCA1, was added to PC12 cells, followed by $A\beta_{25-35}$ treatment. CsA significantly reduced the protective effects of SPFs, BEX, and T0901317 on $A\beta_{25-35}$ -induced neurotoxicity, although CsA alone did not affect $A\beta_{25-35}$ -induced apoptosis. Thus, SPFs might exert protective effects on $A\beta_{25-35}$ -induced neurotoxicity by inducing the production of ABCA1 protein via RXR/LXR heterodimers.

ABCA1 plays critical roles not only in cholesterol efflux to lipid-free apoA1 and apoE for HDL generation, but also in the regulation of plasma membrane lipid composition, especially the decrease of cholesterol level in lipid rafts, specialized membrane lipid domains implicated in signal transduction, neurotransmission, and ligand–receptor interaction [26]. T0901317 and platicodin D were reported to inhibit lipopolysaccharide-induced inflammatory responses in mammary or lung epithelial cells by activating the LXR–ABCA1 signaling pathway to reduce the cholesterol levels of lipid rafts and the translocation of Toll-like receptor 4 (TLR4) into the lipid rafts [32, 33]. $A\beta$ triggers signals to induce inflammation, ER stress, oxidative stress, and elevated levels of intracellular Ca^{2+} [3, 4], which cause neuronal cell death. Many potential $A\beta$ -oligomer binding proteins or receptors have been proposed to date, including RAGE, NMDAR, CaSRs, and TLR4, which are most likely to evoke inflammatory-related signals in neuronal cells [34–37]. In particular, RAGE is expressed on neurons, and is markedly increased in AD [38]. Furthermore, the activation of RAGE by $A\beta$ generates reactive oxygen species (ROS) and induces inflammatory signals via mitogen-activated protein kinase or nuclear factor- κ B activation, finally resulting in neuronal cell death [39]. However, whether ABCA1 is implicated in the regulation of RAGE function by altering cholesterol levels in plasma membranes remains

to be determined to further elucidate the effects of SPFs. In addition, A β activates TLR4 expressed on neurons to induce inflammatory signals. TLR4 is widely expressed on microglia, astrocytes, and neurons in rodents, but not on neurons in humans [40]. In this study, PC12 cells derived from rat pheochromocytoma were used, suggesting that the effects of SPFs might be explained by the modulation of TLR4 function by increased ABCA1. However, the species difference in the expression of TLR4 among cell types indicates the necessity of more detailed studies to evaluate the efficacy of SPFs in AD patients.

Numerous naturally occurring plant-derived products have been reported to protect neuronal cells from A β oligomer-induced neurotoxicity via diverse mechanisms. For example, cyanidin suppressed A β oligomer-induced inflammation and ROS production via the TLR4/NADPH oxidase pathway [41, 42]. Epigallocatechin gallate (EGCG) inhibited ER stress [43], hesperidin reversed A β -induced mitochondrial dysfunction and ROS production [44], and icariin attenuated tau protein hyperphosphorylation [45]. Thus, there are many naturally occurring products with the potential to treat AD patients, although further detailed studies are required to elucidate their efficacy in vivo or in clinical studies.

In the present study, we demonstrated that SPFs protected PC12 cells from A β _{25–35}-induced neurotoxicity in a similar manner to BEX. However, BEX or T0901317, but not SPFs, increased the mRNA levels of SREBP-1c, a causal gene for hyperlipidemia, in HepG2 cells, the most common side effect of BEX or LXR agonists. Thus, SPFs might be RXR agonists that induce fewer side effects compared with BEX.

In conclusion, we demonstrated that the RXR agonists SPF1 and SPF2 protected PC12 cells from A β _{25–35}-induced neurotoxicity in an RXR-dependent manner, that their effects were markedly enhanced by the LXR agonist T0901317, and were in part related to ABCA1 function. Thus, it becomes evident that SPF1 and SPF2 possess properties distinct from other naturally occurring products reported to have beneficial effects on neurotoxicity. Therefore, the results of this study may provide a novel approach to the future treatment or prevention of AD. However, the precise mechanism involved and their efficacy in vivo using model animals remain to be determined.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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