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Inhibitory Effects of Flavonoids Isolated from Leaves of *Petasites japonicus* on β-Secretase (BACE1)

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Abstract The deposition of the amyloid β (Aβ)-peptide following proteolytic processing of amyloid precursor protein (APP) by β-secretase (BACE1) and γ-secretase is critical feature in the progress of Alzheimer's disease (AD). Consequently, BACE1, a key enzyme in the production of Aβ, is a prime target for therapeutic intervention in AD. In the course of searching for BACE1 inhibitors from natural sources, the ethyl acetate fraction of *Petasites japonicus* showed potent inhibitory activity. Two BACE1 inhibitors quercetin (QC) and kaempferol 3-*O*-(6"-acetyl)-β-glucopyranoside (KAG) were isolated from *P. japonicus* by activity-guided purification. QC, in particular, non-competitively attenuated BACE1 activity with IC_{50} value of 2.1×10^{-6} M and K_i value of 3.7×10^{-6} M. Both compounds exhibited less inhibition of α-secreatase (TACE) and other serine proteases including chymotrypsin, trypsin, and elastase, suggesting that they were relatively specific and selective inhibitors to BACE1. Furthermore, both compounds significantly reduced the extracellular Aβ secretion in APP_{695}-transfected B103 cells.

Keywords: Alzheimer's disease, β-amyloid peptide, flavonoid, *Petasites japonicus*, β-secretase

Introduction

Alzheimer's disease (AD) is a major progressive neurodegenerative disorder histopathologically characterized by the presence of senile plaques (SPs) and neurofibirillary tangles (NFTs), followed by oxidative damage to neurons that ultimately results in dementia (1,2). SPs are primarily composed of aggregated amyloid β-peptide (Aβ), which is a 40-42 residue internal peptide segment of amyloid precursor protein (APP) (1). Although the mechanism of AD remains largely unknown, a large body of evidence suggests that Aβ production is a critical feature in the deterioration of the brain during AD (3).

The Aβ peptide is liberated by the action of 2 proteases, β-secretase and γ-secretase (4). β-Secretase initially cleaves the APP to form the *N*-terminus of Aβ at the Asp+1 residue of the Aβ sequence. Following β-secretase cleavage, C99 is the substrate of a second protease γ-secretase, which cleaves the APP to generate the *C*-terminus of Aβ, and the mature peptide is secreted from the cell. A third protease, α-secretase (TACE), non-pathologically cleaves the APP in the middle of the Aβ domain, thus precluding the formation of Aβ (5).

A number of studies provide strong evidence that aspartyl protease, BACE1 (β-site APP cleaving enzyme) as the major β-secretase in neurons responsible for Aβ generation in brain (6). The targeted deletion of BACE1 in APP transgenic mice completely abolishes the production and deposition of Aβ and clearly indicated that BACE1 is the β-secretase and that BACE1 inhibitors should reduce Aβ levels (7). Thus, as a rate-limiting enzyme that initiates Aβ formation *in vivo*, inhibition of BACE1 is one of the plausible ways for the prevention and/or treatment of AD.

Butterbur, *Petasites japonicus*, is a culinary vegetable in eastern Asia including Japan, Taiwan, and Korea. The root was used in treatment of migraines, asthma, and gastric ulcers in European countries (8). Some carcinogenic pyrrolizidine alkaloids, such as petasitenine and fukinotoxin were identified from the root of *Petasites* species (9-11). On the other hands, several antioxidants such as petasinophenol, phenylprophenoyl sulfonic acid, and fukinolic acid from the aerial part of *P. japonicus* have been identified (12,13). Recent research has proved the neuroprotective effect of a furfuran lignan from the butanol extract of *P. japonicus* on the oxidative damage in the brain of mice induced by kainic acid (14).

In our preliminary study, quercetin (QC) and kaempferol 3-*O*-(6"-acetyl)-β-glucopyranoside (KAG) isolated from the ethyl acetate fraction of *P. japonicus* showed novel protective properties on B103 cells against neurotoxicity induced by β-amyloid peptide. Our interest in *P. japonicus* has been augmented when finding that the plant also exhibited strong suppressive effect on β-secretase. Therefore, the present study was designed to extend the further possible protective effects of quercetin and kaempferol 3-*O*-(6"-acetyl)-β-glucopyranoside, the flavonoids derived from *P. japonicus*, on BACE1.

Materials and Methods

Chemicals All solvents, such as hexane, chloroform,

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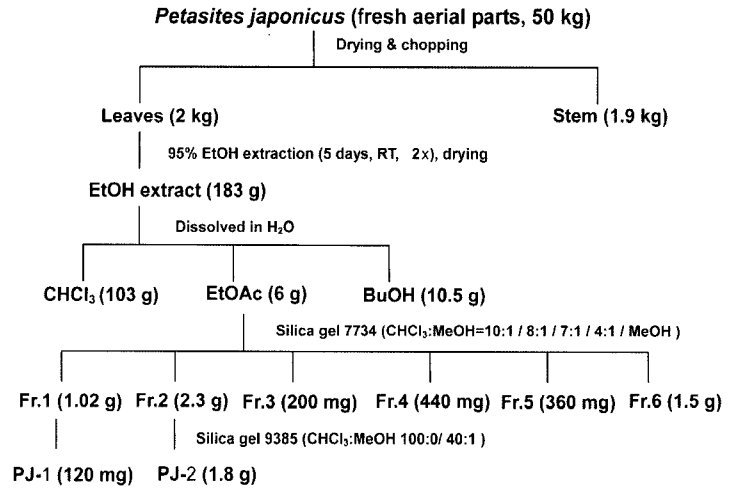

Fig. 1. Isolation scheme of PJ-1 and PJ-2 from the leaves of *Petasites japonicus*.

ethyl acetate, *n*-butanol, methanol, and water, used for extraction and chromatographic isolation were of analytical grade and purchased from Duksan Chemical (Anseong, Korea). Dulbelcco's modified Eagle medium (DMEM) and fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA) were purchased from Gibco Co. (Grand Island, NY, USA). G418 and trypan blue were obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant source, extraction, and solvent fractionation The fresh aerial parts of *P. japonicus* (50 kg) were purchased from agricultural farm in Uleumgol, Milyang, Korea in May, 2006. The voucher specimen (No. NPC-PJ 06001) is deposited at Natural Products Chemistry Lab., Kyungpook National University, Daegu, Korea. The leaves and stems were washed, separated, and dried at room temperature. The dried leaves of *P. japonicus* (2 kg) were chopped and extracted with 95% ethanol (10 L) for 1 week in a dark place (3×). The ethanol extract was concentrated to dryness under reduced pressure by a rotary evaporator. The ethanol extract (183 g) was suspended in water and consecutively partitioned with hexane (500 mL×3), chloroform (500 mL ×3), ethyl acetate (500 mL×3), and *n*-butanol (500 mL×3). Each fraction was separately collected and dried by a rotary evaporator.

Isolation and structural identification of PJ-1 and PJ-2 The most active ethyl acetate soluble fraction (more than 85% inhibition of BACE1 activity at 3 ppm, 6 g) was subjected to open column chromatography with silica gel eluted with chloroform and methanol (10:1→8:1→7:1 →4:1→1:1→100% methanol, v/v) (Fig. 1). A total of 6 fractions were collected. Fraction 2 was rechromatographed with silica gel eluted with chloroform and methanol (100% chloroform 80:1→70:1→60:1→40:1, v/v) to yield compound PJ-**2** (1.8 g). After removal of solvent, fraction 1 gave compound PJ-**1** (120 mg). The fractions were monitored by thin-layered chromatography (TLC) using development solution [EtOAc:MeOH:H_2O =60:1:1] and visualized by 10% sulfuric acid. ^1H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance Digital 400 spectrometer (Karlsruhe, Germany) at 400 and 100 MHz, respectively. Proton chemical shifts were referenced using tetramethylsilane (TMS) as an internal reference, and carbon chemical shifts were referenced to the solvent.

Compound PJ-**1** (yellow powder) was identified as quercetin by TLC comparison and high performance liquid chromatography-photodiode array (HPLC-PDA) analysis (retention time and on-line UV spectrum) with authentic standard. Compound PJ-**2** (white powder) was determined to be KAG. ^1H- and ^{13}C-NMR data were consistent with previously published data (15). The structures of identified compounds are presented in Fig. 2.

Cell lines, human APP (hAPP) construct, transfection, and culture cDNAs encoding wild-type human APP_{695} ($hAPP_{695}wt$) was subcloned into a cytomegalovirous (CMV) promoter/enhancer-driven expression vector (16). The resulting constructs were transfected transiently into B103 cells by using Lipofectamine^{TM} 2000 (Invitrogen). Cell lines stably expressing the construct hAPP695wt were selected by G418-containing medium (400 μg/mL) and were donated by Dr. Inhee Mook-Jung at Department of Biochemistry, College of Medicine, Seoul National University. B103-APP_{695} cells were cultured in DMEM supplemented with 10% FBS and 400 μg/mL genesticin at 37°C with 5% CO_2.

Determination of BACE1 activity A BACE1 (recombinant human BACE1) assay kit was purchased from PanVera (Madison, WI, USA). The assay was carried out according to the manufacturer's instructions with minor modifications. Briefly, a mixture of 10 μL of assay buffer (50 mM sodium acetate, pH 4.5), 10 μL of BACE1 (1.0 U/mL), 10 μL of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μL of sample dissolved in MeOH or dimethylsulfoxide (DMSO) were incubated for 60 min at 18°C. The mixture was excited at 545 nm and the light emitted at 585 nm was collected. The inhibition ratio was obtained using the following equation:

$$\text{Inhibition } (\%)=[1-(S-S_0)/(C-C_0)]\times 100$$

where C was the fluorescence of control (enzyme, assay buffer, and substrate) after 60 min of incubation, C_0 was the fluorescence of control at time 0, S was the fluorescence

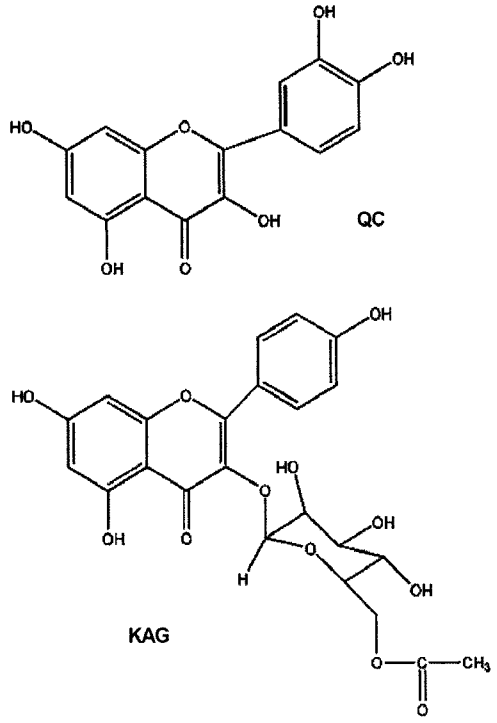

Fig. 2. The chemical structures of quercetin (QC) and kaempferol 3-*O*-(6''-acetyl)-β-glucopyranoside (KAG).

of tested samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S_0 was the fluorescence of the tested samples at time 0.

Determination of inhibitory activity on TACE, chymotrypsin, trypsin, and elastase α-Secretase activity was measured using an α-secretase assay kit with TACE according to the manual from R&D Systems (Minneapolis, MN, USA). Fluorescence was measured with a Bio-TEK enzyme-linked immunosorbent assay (ELISA) microplate fluorescence reader ELx 800 (Winooski, VT, USA). Chymotrypsin, trypsin, and elastase were assayed according to the protocols described in the Sigma-Aldrich catalog using *N*-benzoyl-L-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA respectively as substrates. Optical density (OD) was measured by Bio-Tek ELx 808 spectrophotometer (Winooski).

Determination of Aβ (1-42) generation For quantification of Aβ secretion, B103-APP_{695} cells were plated at a density of 2×10^6 cells/well in 6-well plates in medium lacking geneticin and treated for 72 hr with BACE1 inhibitors, QC and KAG derived from *P. japonicus*. Both compounds were dissolved in DMSO and added to cells at the designed concentration. The final concentration of DMSO was always adjusted to less than 0.01%, a concentration found to have no effect on the cell viability (data not shown) and DMSO solvent control was included in each experiment. Aβ (1-42) secreted into the medium was quantitatively detected with commercially available β-amyloid (1-42) immunoassay kit (Biosource, Camarillo, CA, USA). The assay procedure followed in accordance with the protocol supplied by the manufacturer with minor modification. Aβ was quantified from a standard curve

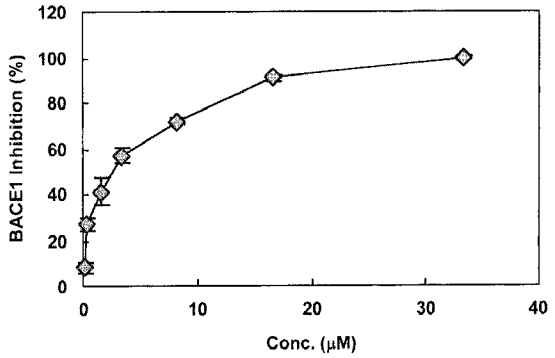

Fig. 3. Concentration dependent inhibition of BACE1 by quercetin (IC_{50}, 2.1×10^{-6} M). The activities (%) are expressed as mean±SE of 3 independent experiments.

using freshly prepared Aβ (1-42) (Sigma-Aldrich) and normalized to total cellular protein.

Statistical analysis All experiments were performed in triplicate. For each experiment, data are expressed as the mean±SE (n=3). Differences were considered significant when the p values were <0.05.

Results and Discussion

QC and KAG selectively inhibited BACE1 activity Due to the crucial role of Aβ found in pathogenesis of AD, inhibiting Aβ formation by targeting the secretase that cleave APP to generate Aβ is one of the approaches for the treatment and/or prevention of AD. Here, the present study determined 2 compounds QC and KAG from *P. japonicus* possessing BACE1 inhibiting properties. The recombinant BACE1 inhibitory activity of QC was shown in Fig. 3. QC inhibited the activity of BACE1 in a dose-dependent manner showing almost complete suppression of BACE1 activity at a concentration of 17 μM. The IC_{50} value was 2.1×10^{-6} M and that of the positive control, (-)-epigallocatechin gallate was 1.6×10^{-6} M) (17). Dixon plot of QC demonstrated that the BACE1 inhibition was non-competitive with a substrate, thus strongly indicating that they might bind either to the β-secretase subsite or to another regulatory site with the inhibition constant value (K_i) of 3.7×10^{-6} M (Fig. 4). KAG also mitigated the activity of BACE1 in a concentration dependent mode, however, much lower than that of QC, suggesting that acetyl glucoside moiety of KAG may impeded its inhibitory activity against BACE1 (Fig. 5).

The inhibitory activity of QC and KAG against TACE an α-secretase candidate involved in the normal amyloidogenic process, was compared with that of BACE1 to check the enzyme specificity. Both compounds did not exhibit significant inhibition of α-secreatase (TACE) (Table 1). Moreover, the compounds exhibited no significant attenuation against other serine proteases including chymotrypsin, trypsin, and elastase, demonstrating that they were relatively selective and specific inhibitors to BACE1, as is the case of other natural inhibitors (18).

Several synthetic inhibitors of BACE1 with relatively low nanomolar IC_{50} concentrations such as octapeptide, OM99-1, OM99-2, OM00-3, and amino-ethylenes have

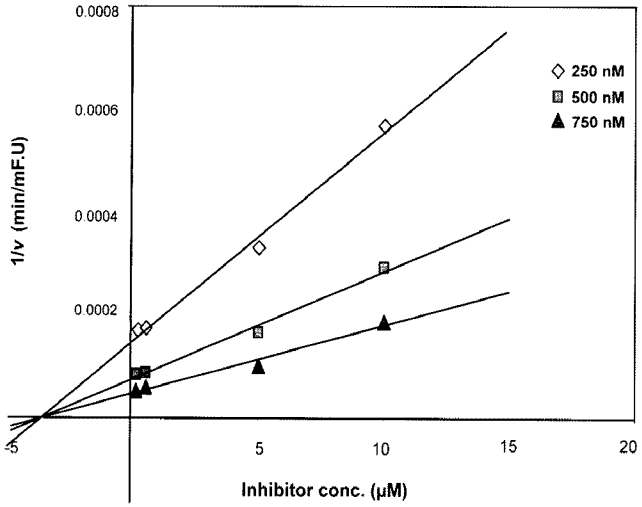

Fig. 4. Dixon plot of quercetin from the leaves of *Petasites japonicus* (K_i, 3.7×10^{-6} M).

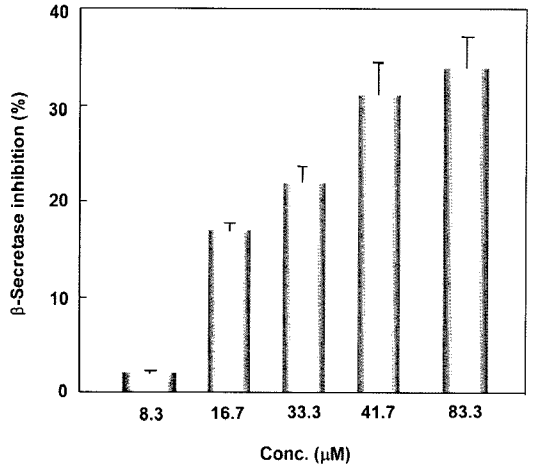

Fig. 5. β-Secretase (BACE1) inhibitory property of kaempferol 3-*O*-(6''-acetyl)-β-glucopyranoside (KAG). The activities (%) are expressed as mean±SE of 3 independent experiments.

been reported (19-22). In spite of their high inhibitory properties, these synthetic peptidomimetic BACE1 inhibitors are required to reduce their molecular sizes to overcome metabolic instability, since BACE1 inhibitors with therapeutic potential preferably need to be smaller than 700 Da (23). However, limited attention has been focused on identifying non-peptidic phytochemicals that are able to attenuate the activity of BACE1. Previously reported naturally occurring BACE1 inhibitors were from green tea, *Smilacis Chinae*, *Dalbergia sissoo*, *Sanguisorbae radix*, pomegranate, crab shells, and etc (17,24-27). BACE1 inhibitors from green tea exhibited an IC_{50} value of 1.6-4.5×10^{-6} M (17). Ellagic acid (IC_{50}, 3.9×10^{-6} M), tellimagrandin II (IC_{50}, 3.1×10^{-6} M), 1, 2, 3, 4, 6-penta-galloylglucopyranoside (IC_{50}, 3.8×10^{-6} M), and chitosan derivatives (24-27) have been shown to have weaker BACE1 suppression activities than QC tested in the present study.

Table 1. Inhibitory activity of quercetin (QC) and 3-*O*-(6''-acetyl)-β-gluco-pyranoside (KAG) against α-secretase (TACE) and other serine proteases[1)]

Conc. (μM)	Chymotrypsin	Trypsin	Elastase	TACE
QC				
50	21.3±0.9	39.1±1.3	26.8±1.4	15±1.1
100	9.7±2.4	36.7±2.4	19.6±2.0	18±1.4
KAG				
250	10.2±0.5	36.8±2.3	27.4±2.8	28±3.2
500	6.9±0.8	36.3±1.4	28.1±1.1	21±1.4

[1)]The activities (%) are expressed as mean±SE of 3 independent experiments.

QC and KAG suppressed Aβ production in APP_{695}-transfected B103 cells The inhibitory activity of QC and KAG on cellular BACE1 was determined using B103 cells stably expressing APP_{695} wild type incubated in the absence or presence of an increasing concentration of QC and KAG for 72 hr. Aβ (1-42) secretion into the medium from these cells consequently detected. The control B103-APP_{695} cells with no treatment of inhibitors exhibited Aβ (1-42) secretion level of 245.7 pg/mL after 72 hr incubation. Both QC and KAG significantly reduced levels of Aβ secretion in APP_{695}-transfected B103 cells at the concentration range over 10 to 100 μM, which might be resulting from the impediment of β-cleavage of APP by inhibiting BACE1 activity (Fig. 6). QC, in particular, exerted marked suppression of Aβ (1-42) secretion in B103-APP_{695} cells even at 1 μM level.

Both *in vitro* and *in vivo* studies have indicated that the generation and deposition of Aβ initiate a pathogenic cascade that ultimately leads to neuronal loss and dementia. However, the underlying mechanism relating β-amyloid-peptide are complex, but may involve *N*-methyl-D-aspartate (NMDA) receptor, a glutamate receptor subtype, modulation induced by glutamate release, increment of intracellular Ca^{2+} concentration and oxidative stresses (2,4,28). In light of the antioxidant properties of flavonoids, particularly QC is anticipated to have cytoprotective property via regulation of cellular homeostasis that is vulnerable to oxidative stress. Therefore, further study is needed to verify that the decrease in Aβ (1-42) peptides in the present study is due to specific inhibition of β-cleavage of APP by determining the levels of the BACE1-derived cleavage products the extracellular soluble APPβ (sAPPβ) and C-terminal fragment (CTFβ) (29).

All the drugs considered for treatment of AD are required to possess sufficient lipophilicity to traverse the blood-brain barrier and the plasma membrane efficiently, thus achieving high concentration in the brain with high potency (23). For BACE1 inhibitors reported so far, these factors might be difficult to meet as these BACE1 inhibitors are synthetic peptidomimetics of the β-cleavage site in APP. QC exerted less inhibitory property than those synthetic inhibitors, however, it has been verified that QC possesses sufficient lipophilicity to flux into brain regions (30). Hence, it is very meaningful in finding natural

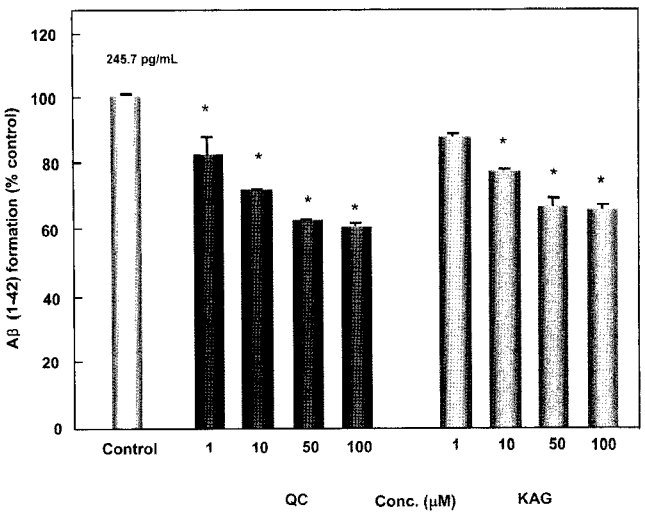

Fig. 6. Inhibition of quercetin (QC) and kaempferol 3-*O*-(6''-acetyl)-β-gluco pyranoside (KAG) against Aβ(1-42) formation in APP_{695}-transfected B103 cell. The activities (%) are expressed as mean±SE of 3 independent experiments.

BACE1-selective inhibitors with high potency, thus acting as a starting point for rational non-peptidyl drug design and be useful for researching the enzyme properties of BACE1.

QC is one of the most frequently researched flavonoids for its anti-proliferative, anticarcinogenic, and antioxidative activities, but the study on its neuroprotective property is limited (31-33). Previous study proved that QC exhibited protective effect on hydroxyperoxide-induced neurodegeneration in PC 12 cells (34). QC inhibited glutamate-induced cytotoxicity in the mouse hippocampal HT-22 cell line by suppressing oxidative stress, preventing loss of intracellular glutathione (GSH), and maintaining Ca^{2+} homeostasis (35).

From these results together with our previous results, QC and KAG exhibited multiple cytoprotective effects to suppress the progress of AD by reduction of Aβ production resulting from inhibition of BACE1 and detoxification of Aβ through several structural benefits. In conclusion, our study demonstrated that inhibition of the activity of BACE1 was achieved by QC which is a small potent, BACE1-selective, non-peptidomimetic with high brain penetration, thus preventing neuronal degeneration.

Acknowledgments

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