Journal of Alzheimer's Disease 31 (2012) 1–6
IOS Press

Supplementary Data

Gintonin, a Ginseng-Derived Lysophosphatidic Acid Receptor Ligand, Attenuates Alzheimer's Disease-Related Neuropathies: Involvement of Non-Amyloidogenic Processing

Sung Hee Hwang[a,1], Eun-Joo Shin[b,1], Tae-Joon Shin[a], Byung-Hwan Lee[a], Sun-Hye Choi[a], Jiyeon Kang[a], Hyeon-Joong Kim[a], Seung-Hwan Kwon[c], Choon-Gon Jang[c], Jun-Ho Lee[a], Hyoung-Chun Kim[b,*] and Seung-Yeol Nah[a,*]
[a]*Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University, Seoul, Korea*
[b]*Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University, Chunchon, Korea*
[c]*Department of Pharmacology, College of Pharmacy, Sungkyunkwan University, Suwon, Korea*

Accepted 5 April 2012

EXPERIMENTAL DETAILS

A$\beta_{1\text{-}42}$ peptide preparation and cell protective effect of gintonin from A$\beta_{1\text{-}42}$ toxicity

A$\beta_{1\text{-}42}$ peptide was solubilized in sterile water and stored in small aliquots at –20°C. The stock solution was diluted in PBS (pH 7.4) at 100 μM and pre-aged at 37°C for 4 days to have aggregated A$\beta_{1\text{-}42}$ peptide. SH-SY5Y cells were seeded at 2×10^3 cells per well into 96-well plates. After 48 h, the culture medium was replaced with modified N2 medium and incubated with gintonin in N2 medium at indicated concentrations. After 1 h, the cells were overlaid with pre-aggregated A$\beta_{1\text{-}42}$ at indicated concentrations. After 48 h, the cell viability was assessed by XTT assay.

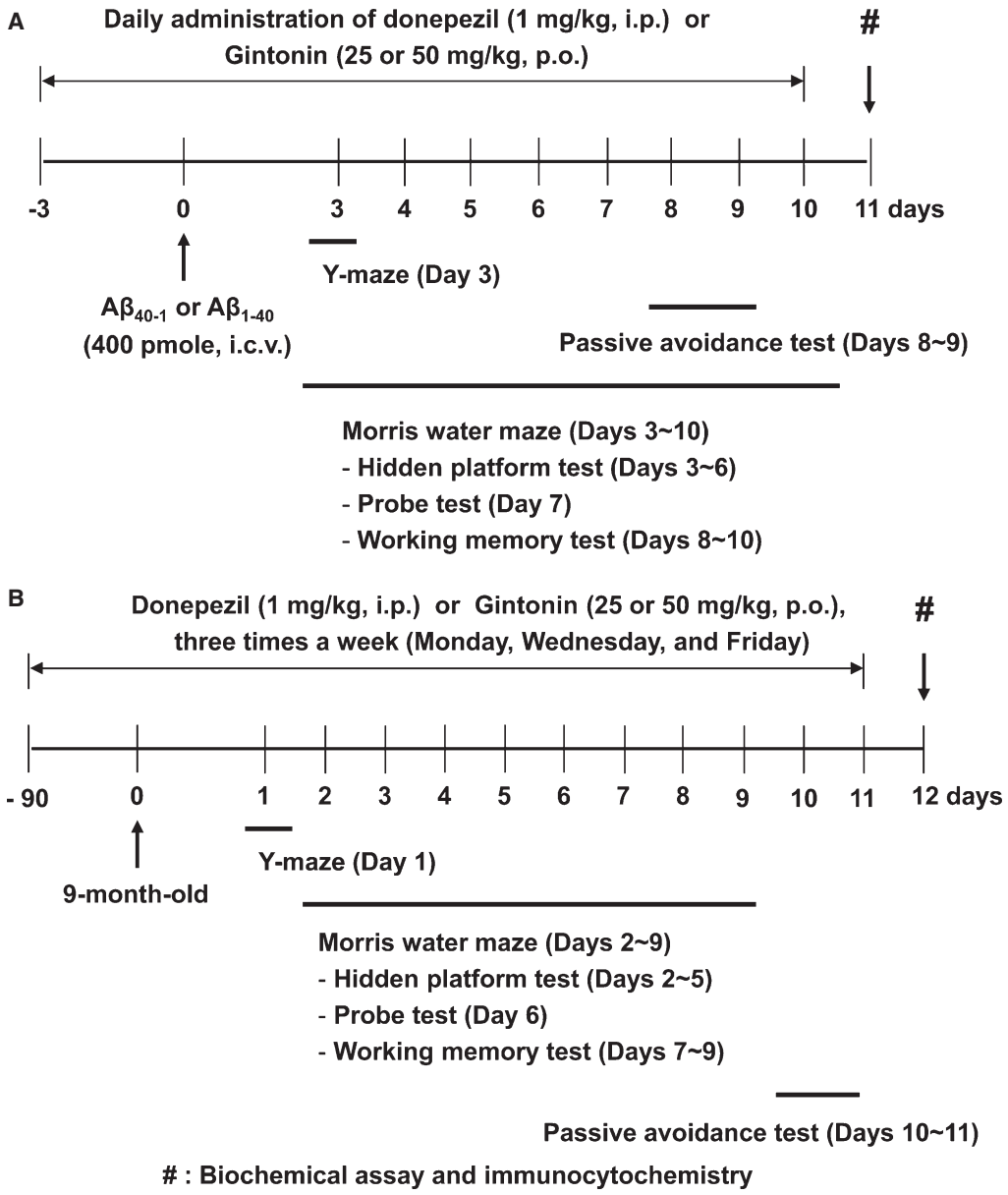
Measurement of ADAMs in SH-SY5Y cells

Cells treated with gintonin were washed with cold PBS, collected, and lysed with modified RIPA buffer to obtain whole cell lysate as described in the Materials and Methods section. To obtain the membrane fraction, cells treated with gintonin were washed with cold PBS, collected, and lysed in a lysis buffer containing 20 mM

[1]These authors contributed equally to this study.

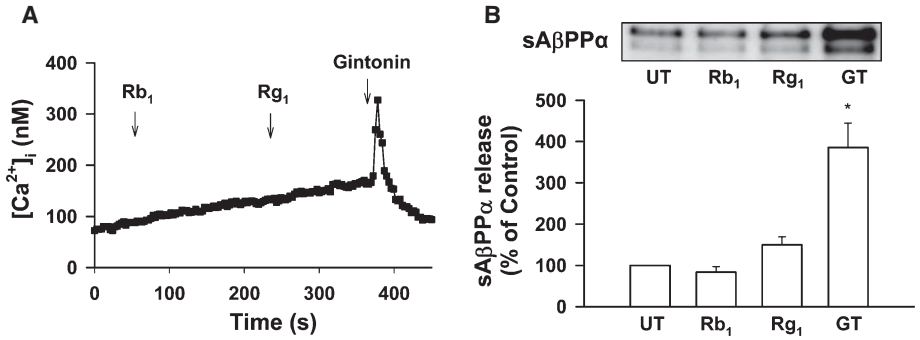
*Correspondence to: Hyoung-Chun Kim, PhD, Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University, Chunchon 200-701, Korea. Tel.: +82 33 250 6917; Fax: +82 33 255 7865; E-mail: kimhc@kangwon.ac.kr and Seung-Yeol Nah, PhD, Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea. Tel.: +82 2 450 4154; Fax: +82 2 450 2809; E-mail: synah@konkuk.ac.kr.

ISSN 1387-2877/12/$27.50

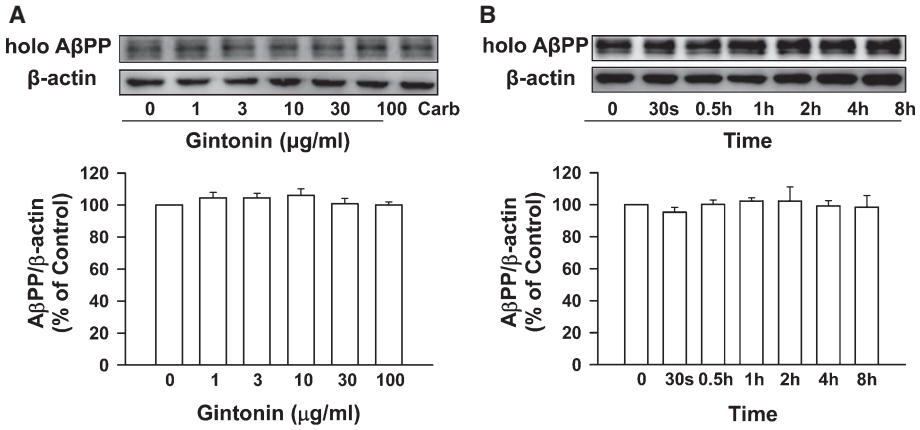


Supplementary Figure 1. Experimental schedule to evaluate the effect of gintonin on the memory impairment in the $A\beta_{1-40}$-treated (A) or the AβPPswe/PSEN-1 double Tg (B) mice. A) Each C57BL/6 mouse was administered $A\beta_{40-1}$ or $A\beta_{1-40}$ (400 pmol, i.c.v. injection). Gintonin (25 or 50 mg/kg, p.o. administration) or DPZ (1 mg/kg, i.p. injection) was administered to the Aβ-treated mice 3 days before the Aβ i.c.v. injection and then once a day. Three days after the Aβ i.c.v. injection, each group was divided into two subgroups. Mice in one subgroup were first subjected to the Y-maze test and then to the passive avoidance test. Mice in the other subgroup were subjected to the Morris water maze test. B) After chronic treatment of AβPPswe/PS1dE9 double Tg mice with gintonin (25 or 50 mg/kg, p.o. administration, three times a week) or DPZ (1 mg/kg, i.p. injection, three times a week) for three months, the behavioral study was started for mice at the age of nine months. The Y-maze, Morris water maze, and passive avoidance tests were performed sequentially. During the behavioral study, the treatment with gintonin and DPZ was continued but the drugs were administered 30 min after the behavioral test to avoid a direct effect on the performance. Animals were sacrificed 1 day after the final passive avoidance test for biochemical assay and immunocytochemistry.

Tris-HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol, 0.32 M sucrose, and a protease inhibitor cocktail [1]. The cell lysates were centrifuged at 12,000 g for 30 min at 4°C. The pellets were then resuspended in lysis buffer supplemented with 1.0% Triton X-100. The resuspended pellet was incubated on ice for 45 min and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was then collected



Supplementary Figure 2. Treatment of ginsenosides induced neither $[Ca^{2+}]_i$ transient (A) nor significant sAβPPα release in SH-SY5Y cells (B). A) Fura-2 AM (4 μM) loaded cells were treated with ginsenoside Rb_1 (10 μM), ginsenoside Rg_1 (10 μM), and gintonin (30 μg/ml), as described in Fig. 2. B) SH-SY5Y cells were treated with either untreated vehicle (UT), ginsenoside Rb_1 (10 μM), ginsenoside Rg_1 (10 μM), or gintonin (100 μg/ml) for 1 h, and the released sAβPPα into the cell culture media were measured as described in Fig. 1. The values represent the means ± SEM ($n = 4$-5) (*$p < 0.05$, compared with untreated control).



Supplementary Figure 3. Gintonin does not affect cellular full-length AβPP expression in SH-SY5Y cells. A) SH-SY5Y cells were treated for 1 h with different concentrations of gintonin or 1 mM carbachol (Carb) as a positive control. B) SH-SY5Y cells were treated with vehicle or 100 μg/ml gintonin at the indicated time intervals. Twenty-five micrograms of protein from each cell lysate were loaded onto SDS-PAGE gels for the detection of cellular full-length AβPPs (using anti-22C11 antibodies) and β-actin expression. Data are expressed as percentages of the control and represent the mean ± SEM ($n = 4$-5).

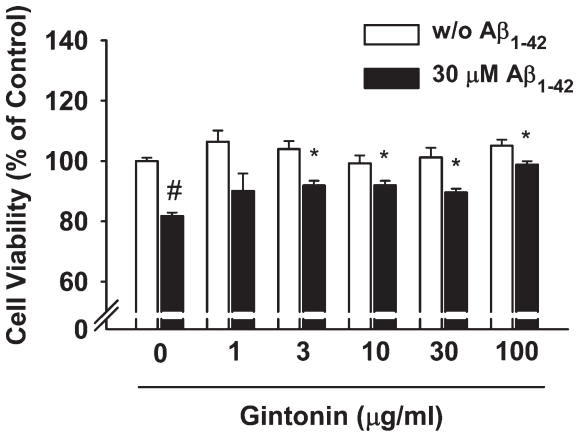
and used for western blotting analysis with the anti-ADAM10 polyclonal antibodies.

ANIMAL EXPERIMENTS

Drug treatment

$Aβ_{40\text{-}1}$ and $Aβ_{1\text{-}40}$ were dissolved in 0.1 M PBS at pH 7.4, and aliquots were stored at −20°C. The Aβs in each aliquot were aggregated by incubation in sterile distilled water at 37°C for 4 days. Two-month-old C57BL/6 mice were administered $Aβ_{40\text{-}1}$ or $Aβ_{1\text{-}40}$ (400 pmol, i.c.v. injection) according to the procedure established by Laursen and Belknap [2]. Each mouse was injected consciously in the bregma using a 10-μl microsyringe (Hamilton, Reno, Nevada, USA) fitted with a 26-gauge needle inserted at a depth of 2.4 mm. The injection volume was 5 μl. The injection placement and needle track were visible and could be verified during dissection.

Gintonin (25 or 50 mg/kg, p.o. administration) was dissolved in sterile water and administered to the Aβ-treated mice for 14 consecutive days. DPZ (1 mg/kg, i.p. injection) was applied as a reference drug. The experimental schedule is shown in supplementary Figure 1A. Gintonin or DPZ administration commenced 3 days before the Aβ i.c.v. injection, and the drug administration was continued once a day throughout the experimental period. The behavioral study commenced on day 3 after the Aβ i.c.v. injection and was performed sequentially. During the behavioral study, gintonin or DPZ was administered 30 min after



Supplementary Figure 4. Gintonin reduced cytotoxicity with concentration-dependent manner in SH-SY5Y cells. $A\beta_{1-42}$ (30 μM) was pre-aggregated for 4 days and was added to the wild-type SH-SY5Y cells with or without various concentrations of gintonin and incubated for 48 h. Cell viability was assayed using XTT assay. The data represent the the mean ± SEM ($n = 3$) ($^{\#}p < 0.05$, compared with untreated control cells; $^{*}p < 0.05$, compared with $A\beta_{1-42}$ treated cells in the absence of gintonin).

the behavioral test to avoid a direct effect on the performance.

MEMORY TESTS

Y-maze test

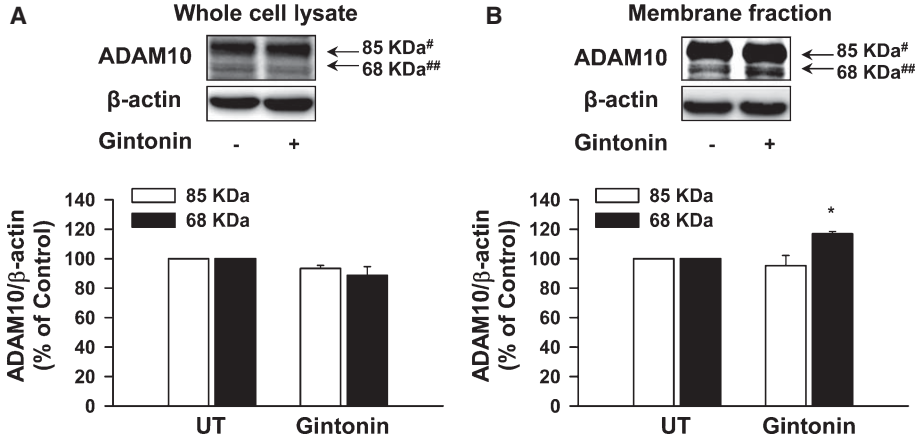
The Y-maze test was performed as described previously [3]. Briefly, the Y-shaped maze was constructed of black acrylic with three identical arms separated by 120°. Each arm was 40 cm long, 12 cm tall, and 10 cm wide. The mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The percent alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus two) multiplied by 100.

Passive avoidance test

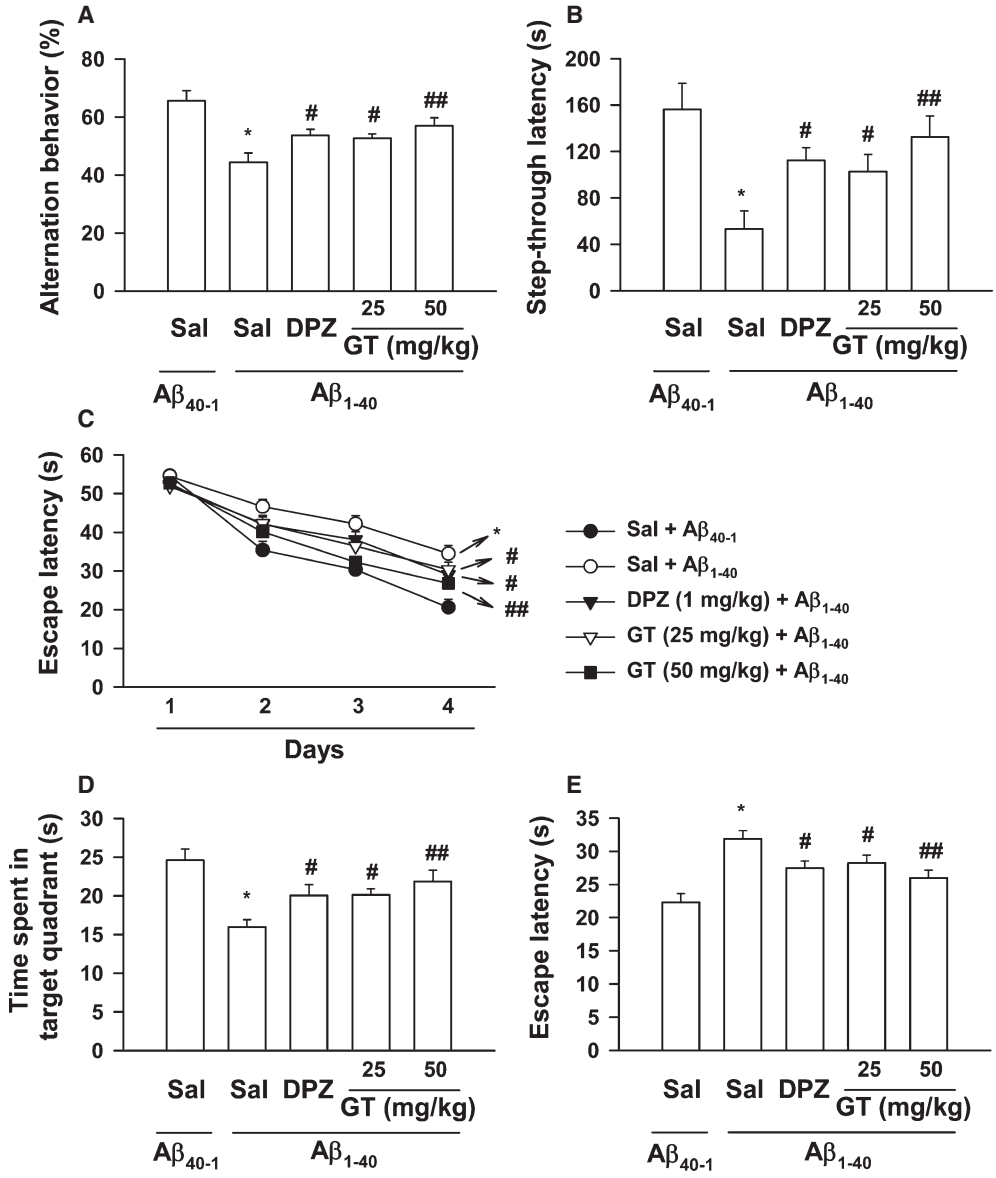
The passive avoidance test was conducted with a Gemini Avoidance System (San Diego Instruments, San Diego, CA, USA) that consisted of a two-compartment shuttle chamber and a constant current shock generator [3, 4]. During the acquisition trial, each mouse was placed into a dark compartment (the start chamber). After 20 s, this chamber was illuminated, and the door was opened to allow the mouse to freely move into the second dark chamber. Immediately after the mouse had entered the dark chamber, the door was closed, and one inescapable, scrambled electric shock (0.8 mA, 2 s) was delivered through the floor grid. The mouse was then returned to its home cage. Twenty-four hours later, each mouse was placed in the start chamber again (retention trial). The interval between the placement in the illuminated chamber and entry into the dark chamber was measured as the step-through latency in both the acquisition and the retention trials (maximum of 300 s).

Morris water maze test

The Morris water maze test apparatus was a cylindrical water pool, 97 cm in diameter and 60 cm in



Supplementary Figure 5. Gintonin treatment induces ADAM10 translocation from the cytosol to the membrane of SH-SY5Y cells. Cells were treated with either vehicle or gintonin (100 μg/ml) for 1 h, and whole cell lysates (A) or membrane fractions (B) were subjected to immunoblotting for detection of α-secretase expression. The data represent the mean ± SEM ($n = 4$-5). $^{*}p < 0.05$ compared with untreated (UT) cells. $^{\#}$immature form (precursor); $^{\#\#}$mature form (active).



Supplementary Figure 6. Effect of gintonin (GT, 25 or 50 mg/kg, p.o. administration) on $A\beta_{1\text{-}40}$-induced memory impairment as assessed by the Y-maze test (A), passive avoidance test (B), and Morris water maze test [hidden platform test (C), probe test (D), and working memory test (E)]. DPZ (1 mg/kg, i.p. injection) was used as a reference drug. Each value is the mean ± SEM ($n = 10$). (A, B), 4 daily trials ($n = 10$) (C), 2 daily trials ($n = 10$) (D), or 12 trials (4 daily trials for consecutive 3 days; $n = 10$) (E). $^{*}p < 0.01$, compared with saline (Sal) + $A\beta_{40\text{-}1}$, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared with Sal + $A\beta_{1\text{-}40}$ [one-way ANOVA (A, B, D, and E) or repeated measure one-way ANOVA (C), followed by Fisher's PLSD test].

height. During testing, the tank was filled with water (23 ± 2°C) clouded with powdered milk. A transparent platform was placed inside the tank; the top of the platform was 2 cm below the water surface in the center of one quadrant of the maze. The tank was located in a large room with numerous extra-maze cues that were constant throughout the study [3, 4]. The movements of the animal in the tank were recorded and analyzed with a video tracking system (EthoVision, Noldus, The Netherlands).

Hidden platform test

For each training trial in the hidden platform test, the mouse was placed into the pool at one of five positions; the sequence of the positions was selected

randomly. The platform was located a constant position throughout the test period in the middle of one quadrant, equidistant from the center and the edge of the pool. In each training session, the latency to escape onto the hidden platform was recorded. If the mouse found the platform, it was allowed to remain there for 10 s and was then returned to its home cage. If the mouse was unable to find the platform within 60 s the training was terminated and a maximum score of 60 s was assigned. The training was conducted four times a day for four consecutive days, and the results were the average values of four trials per day [3, 4].

Probe test

One day after the final trial of the hidden platform test, two probe test trials were performed. The platform was removed from the pool, and each mouse was allowed to swim for 60 s in the maze. The time spent in the target quadrant where the platform had been located was recorded and averaged across two trials [3, 4].

Working memory (repeated acquisition) test

The working memory test was started 1 day after the probe test and conducted five times a day for three consecutive days. The working memory test was procedurally similar to the reference memory test, except that the platform location was changed daily. The first trial of the day was an informative sample trial in which the mouse was allowed to swim to the platform in its new location. The spatial working memory was regarded as the mean escape latency from the second to fifth trials [3, 4].

REFERENCES

[1] Yang HQ, Pan J, Ba MW, Sun ZK, Ma GZ, Lu GQ, Xiao Q, Chen SD (2007) New protein kinase C activator regulates amyloid precursor protein processing *in vitro* by increasing alpha-secretase activity. *Eur J Neurosci* **26**, 381-391.

[2] Laursen SE, Belknap JK (1986) Intracerebroventricular injections in mice. Some methodological refinements. *J Pharmacol Meth* **16**, 355-357.

[3] Yamaguchi Y, Miyashita H, Tsunekawa H, Mouri A, Kim HC, Saito K, Matsuno T, Kawashima S, Nabeshima T (2006) Effects of a novel cognitive enhancer, spiro[imidazo-[1,2-α]pyridine-3,2-indan]-2(3H)-one (ZSET1446), on learning impairments induced by amyloid-$\beta_{1\text{-}40}$ in the rat. *J Pharmacol Exp Ther* **317**, 1079-1087.

[4] Jhoo JH, Kim HC, Nabeshima T, Yamada K, Shin EJ, Jhoo WK, Kim W, Kang KS, Jo SA, Woo JI (2004) β-Amyloid (1-42)-induced learning and memory deficits in mice: Involvement of oxidative burdens in the hippocampus and cerebral cortex. *Behav Brain Res* **155**, 185-196.