

RESEARCH ARTICLE

# The Resveratrol Trimer Miyabenol C Inhibits $\beta$ -Secretase Activity and $\beta$ -Amyloid Generation

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## Abstract

Accumulation and deposition of amyloid- $\beta$  peptide ( $A\beta$ ) in the brain is a primary cause of the pathogenesis of Alzheimer's disease (AD).  $A\beta$  is generated from amyloid- $\beta$  precursor protein (APP) through sequential cleavages first by  $\beta$ -secretase and then by  $\gamma$ -secretase. Inhibiting  $\beta$ -secretase activity is believed to be one of the most promising strategies for AD treatment. In the present study, we found that a resveratrol trimer, miyabenol C, isolated from stems and leaves of the small-leaf grape (*Vitisthunbergii* var. taiwaniana), can markedly reduce  $A\beta$  and sAPP $\beta$  levels in both cell cultures and the brain of AD model mice. Mechanistic studies revealed that miyabenol C affects neither protein levels of APP, the two major  $\alpha$ -secretases ADAM10 and TACE, and the  $\gamma$ -secretase component Presenilin 1, nor  $\gamma$ -secretase-mediated Notch processing and TACE activity. In contrast, although miyabenol C has no effect on altering protein levels of the  $\beta$ -secretase BACE1, it can inhibit both *in vitro* and *in vivo*  $\beta$ -secretase activity. Together, our results indicate that miyabenol C is a prominent  $\beta$ -secretase inhibitor and lead compound for AD drug development.

## Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder characterized by impaired memory and cognition. One of the major pathological hallmarks of AD in the brain is senile plaques, which are composed of heterozygous amyloid- $\beta$  ( $A\beta$ ) peptides. Ample evidence indicates that accumulation of  $A\beta$  peptides in vulnerable brain regions plays a central role in AD pathogenesis:  $A\beta$  is neurotoxic and can trigger a cascade of neurodegenerative steps including the formation of senile plaques and neurofibrillary tangles, synaptic dysfunction, and eventual neuronal loss [1, 2].  $A\beta$  is a proteolytic product of the amyloid- $\beta$  precursor protein (APP) and is generated through sequential cleavages by enzymes called  $\beta$ - and  $\gamma$ -secretases. During this amyloidogenic processing,  $\beta$ -secretase first cleaves the type I transmembrane APP protein

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to generate an extracellular fragment known as sAPP $\beta$  and a membrane-associated carboxyl-terminal fragment known as APP  $\beta$ -CTF. APP  $\beta$ -CTF is then cleaved by  $\gamma$ -secretase to release A $\beta$ . Alternatively, APP can be subjected to a non-amyloidogenic processing and cleaved by  $\alpha$ -secretase within the A $\beta$  domain.  $\alpha$ -secretase-mediated cleavage precludes A $\beta$  generation and generates an extracellular domain of APP known as sAPP $\alpha$  instead [3, 4].

$\beta$ -cleavage of APP is the first and rate-limiting step in A $\beta$  production. The transmembrane aspartic protease  $\beta$ -site APP cleaving enzyme 1 (BACE1) has been identified as the essential  $\beta$ -secretase *in vivo* [5–8]. The level and activity of BACE1 are found to be elevated in postmortem brain of sporadic AD patients [9–11], suggesting a causative role of BACE1 in AD. Although homozygous BACE1 knock-out mice develop certain phenotypic abnormalities including reduced body size, hyperactive behavior, decreased grip strength and elevated pain sensitivity [12–14], probably because the cleavage of other BACE1 substrates such as neuregulin 1 [13, 14] and  $\beta$ -subunits of voltage-gated sodium channels [15] is blocked, BACE1 heterozygous knockout mice do not develop such abnormal phenotypes and heterozygous knockout of BACE1 still can reduce A $\beta$  deposition in AD mice [16, 17]. Therefore, moderate inhibition of  $\beta$ -secretase activity is considered as a promising strategy for AD intervention.

Natural products have been recognized as sources of new lead compounds for the treatment of various diseases including AD [18, 19]. The small-leaf grape *Vitisthunbergii* var. taiwaniana (VTT) is a wild grape native to Taiwan where, along with other species of *Vitis* spp., is used as a folk medicine [20]. The extracts or purified compounds from VTT have been reported to have anti-microbial [21], anti-inflammatory [22], anti-hypertensive [23] and neuroprotective activities [20]. In the present study, we isolated a resveratrol trimer, miyabenol C, from the stem and leaf extracts of VTT. Importantly, we demonstrate that miyabenol C is a prominent  $\beta$ -secretase inhibitor and can reduce A $\beta$  levels both *in vitro* and *in vivo*, suggesting that miyabenol C may be a lead compound for AD drug development.

## Materials and Methods

### Isolation of miyabenol C from stem and leaf extracts of VTT

Dried stems and leaves of VTT (5.5 kg) were boiled and refluxed for 2.5 h with 60% of aqueous ethanol solution (10 L  $\times$  2 times). After filtration, the extracted solution was concentrated *in vacuo*. The extract (230.3 g) was then separated over a Diaion HP-20 column, using EtOH-H<sub>2</sub>O as mobile phase, into three fractions (A-C). Fraction B (60% EtOH-H<sub>2</sub>O eluent, 130.0 g) was chromatographed on silica gel column using stepwise gradient elution with CHCl<sub>3</sub>-MeOH (100:0 ~ 0:100) to obtain 11 fractions (Fr. 1-11). Fr.5 (5.0 g) was subjected to silica gel column chromatography and eluted with CHCl<sub>3</sub>-MeOH (95:5, 9:1, 85:15, 0:100) to generate 8 sub-fractions (Fr.5-1 ~ Fr.5-8). Fr. 5-5 (835 mg) was applied to ODS column chromatography and eluted with MeOH-H<sub>2</sub>O (3:7, 4:6 and 1:1). The elution of MeOH-H<sub>2</sub>O (4:6) was further repeated by RP-18 and purified through preparative HPLC (RestekPrinnacle DB C<sub>18</sub>, 5  $\mu$ m, 250 $\times$ 20 mm) with elution of 45% aqueous methanol solution to generate a pure compound (65mg), whose structure was then determined by means of NMR and MS.

### Cell culture and treatment

Maintenance of human neuroblastoma SH-SY5Y cells [24], mouse neuroblastoma N2a naive cells (N2aWT) [25] and N2a cells stably expressing human APP695 (N2a695) [26] was as previously reported [27, 28].

Purified miyabenol C and  $\beta$ -secretase inhibitor II (Millipore) were dissolved in DMSO (Sigma) as 20 mM stock solution. For treatment, cells grown to confluency were switched to be

incubated in FBS-free media that were added with indicated concentrations of miyabenol C,  $\beta$ -secretase inhibitor II or DMSO for 10h.

### Cell viability test

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay (Dojindo), following the manufacturer's protocol.

### Western blot and antibodies

Conditioned media from treated cells were assayed for sAPP $\alpha$  and sAPP $\beta$  by Western blot. Treated cells were lysed in ice-cold lysis buffer (10mM Tris pH7.5, 150mM NaCl, 1mM EDTA, 1% NP-40, 1 $\times$  Protease Inhibitor Cocktail), and equal protein amounts of cell lysates were analyzed by Western blot. Antibodies used for Western blot included: 369 (against the carboxyl terminus of APP, 1:1000) and ab14 (against PS1 amino-terminal fragment, 1:1000) developed in our laboratory; 6E10 (against sAPP $\alpha$  and  $\beta$ -CTF, 1:1000) and anti-sAPP $\beta$  antibodies (1:500) from Covance; anti- $\alpha$ -tubulin (1:10000) and anti-ADAM10 (1:1000) antibodies from Millipore; anti-ADAM17 (1:1000) antibody from Abcam; and anti-c-Myc (1:1000) and anti-NICD (1:1000) antibodies from CST. The mouse monoclonal antibody 3D5 (against BACE1, 1:1000) was a kind gift from Dr. Robert Vassar at Northwestern University.

### Notch $\Delta$ E cDNA construct and cell based $\gamma$ -secretase activity assay

Notch $\Delta$ E fragment contains the transmembrane and intracellular domains of Notch1 and is an immediate substrate of  $\gamma$ -secretase for generating Notch intracellular domain (NICD). For  $\gamma$ -secretase activity assay, N2aWT cells were transiently transfected with the Notch $\Delta$ E plasmid. After splitting equally, cells were treated with DMSO, the  $\gamma$ -secretase inhibitor compound E (Millipore) or miyabenol C for 10h. Cell lysates were analyzed by Western blot for Notch $\Delta$ E and NICD levels. Cellular  $\gamma$ -secretase activity was estimated by the generation of NICD.

### $\alpha$ -secretase activity assay

The activity of  $\alpha$ -secretase in cells was measured by using InnoZyme *TACE* Activity Kit (Millipore), following the manufacturer's protocols.

### $\beta$ -secretase activity assays

Cell-based  $\beta$ -secretase activity and cell-free BACE1 activity were measured by using commercial Kits from Millipore and Sigma, respectively, following the manufacturers' protocols.

### A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> ELISA assays

Human A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> in conditioned media from treated N2a695 cells were assayed by sandwich ELISA, following a previously described protocol [29].

### Ethics statement

All animal procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Xiamen University (IACUC #: XMULAC20120012).

## Miyabenol C treatment of APP/PS1 mice and sample analysis

C57BL6 mice co-expressing the Swedish mutant APP and the exon-9 deletion mutant PS1 (APP/PS1) were provided by Nanjing Biomedical Research Institute of Nanjing University, China. For treatment, 12-month-old APP/PS1 transgenic mice were anesthetized with sodium pentobarbital (50 $\mu$ g/g) and placed in a stereotaxic apparatus before intracerebroventricular injection of vehicle (45% DMSO in artificial cerebrospinal fluid: 148 mM NaCl, 3 mM KCl, 1.4 mM CaCl<sub>2</sub>, 0.75 mM MgCl<sub>2</sub>, 0.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.2 mM NaH<sub>2</sub>PO<sub>4</sub>) or miyabenol C (0.6 $\mu$ g/g). Vehicle and miyabenol C solution were injected at 4 $\mu$ L into the lateral ventricle using a 5 $\mu$ L-blunt needle equipped with asyringe pump (KD Scientific). The stereotaxic coordinates for the lateral ventricle were AP 0.5 (0.5 mm posterior to bregma), L 1 (1 mm left from mid-sagittal line) and H 2.2 (2.2 mm below bregma). Sixty seconds after insertion of the needle, vehicle or miyabenol C solution were injected at a constant flow rate of 0.4 $\mu$ L/min. The injection needle was kept in place for an additional 10 min to prevent reflux of fluid.

Three days after treatment, mice were anesthetized and euthanized by transcardial perfusion with ice-cold physiological saline. Brain cortex and hippocampus were dissected and lysed for Western blot analysis of APP and sAPP $\beta$ . Alternatively, samples were weighed and sequentially extracted into TBSX- (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100) soluble and GuHCl-soluble fractions for A $\beta$  sandwich ELISAs. This separation was carried out following a previously validated method [30] with some modification. Briefly, samples were placed in 2mL ice-cold glass dounce homogenizer containing TBSX homogenization buffer. Samples were homogenized on ice, transferred into pre-chilled 1.5 mL polyallomer ultracentrifuge tubes, and centrifuged at 100,000 $\times$ g for 1 h at 4°C. The supernatant was collected as TBSX-soluble fraction. The pellet was re-suspended in 5M GuHCl, mixed by rotation at room temperature for 6 h, and centrifuged at 16,000 $\times$ g for 30 min. The supernatant was collected as GuHCl soluble fraction.

## Statistical analysis

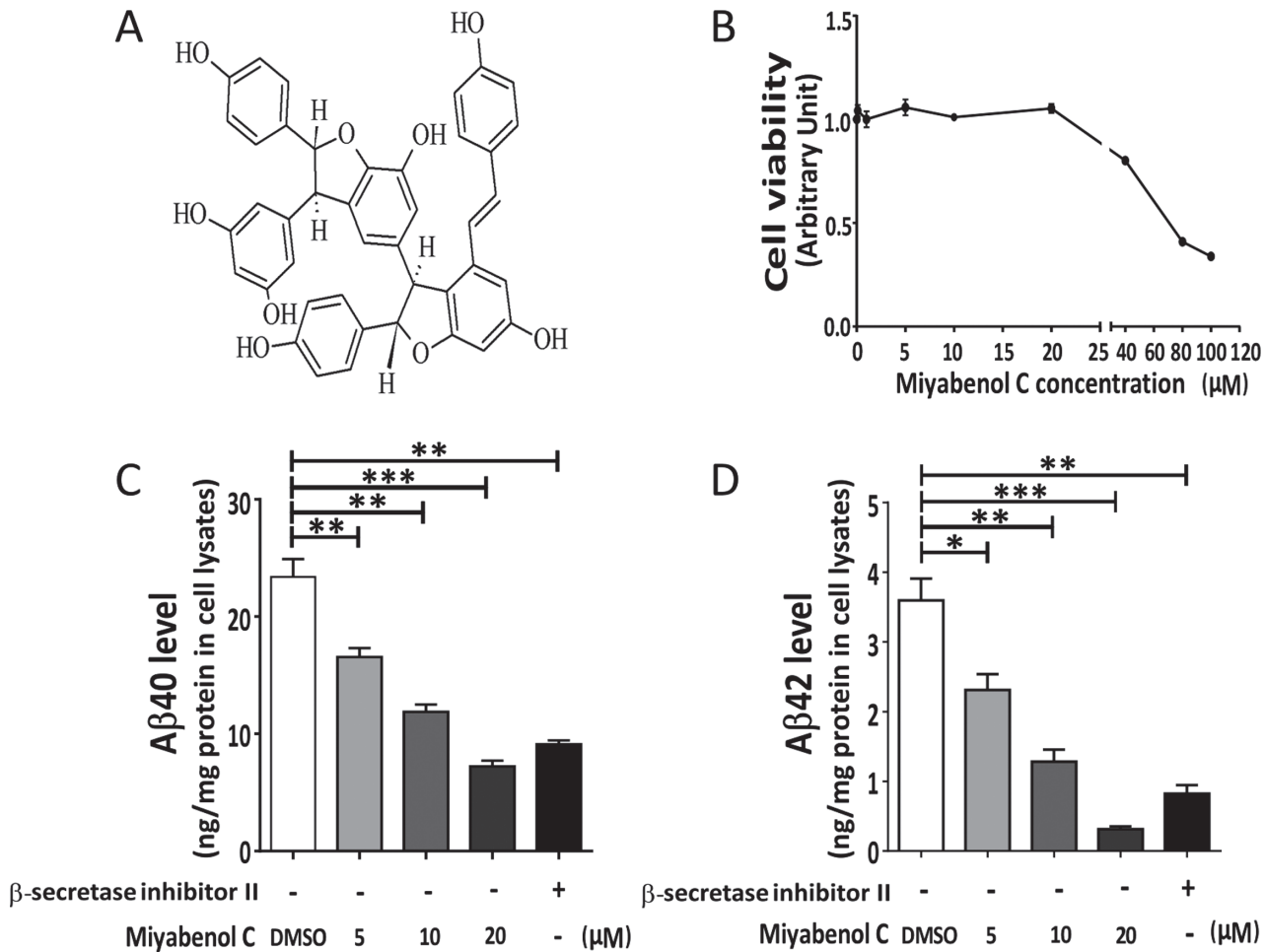
The statistical analysis was carried out by using GraphPad Prism Software 5.0. Data were analyzed by Student's t-test or One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, and presented as the mean  $\pm$  standard deviation.

## Results

### Isolation and identification of miyabenol C

We isolated a series of compounds from stems and leaves of VTT (unpublished data). One of the compounds was purified in a yellow and solid form. This compound was subjected to MS and NMR and the spectral data were:

ESI-MS (Bruker FTMS):  $m/z$  679 [M-H]<sup>-</sup>. <sup>1</sup>H-NMR (BrukerAvance spectrometer, DMSO-d<sub>6</sub>, 400MHz)  $\delta$ : 7.18 (2H, d,  $J$  = 8.8Hz, H-2a, H-6a), 7.13 (2H, d,  $J$  = 8.4Hz, H-2c, H-6c), 6.90 (1H, d,  $J$  = 16.4Hz, H-7c), 6.84 (2H, d,  $J$  = 8.8Hz, H-3a, H-5a), 6.75 (2H, d,  $J$  = 8.4Hz, H-3c, H-5c), 6.62 (1H, d,  $J$  = 16.4Hz, H-8c), 6.58 (2H, d,  $J$  = 8.8Hz, H-3b, H-5b), 6.52 (2H, d,  $J$  = 8.8Hz, H-2b, H-6b), 6.32 (1H, d,  $J$  = 2.0Hz, H-12b), 6.24 (1H, d,  $J$  = 2.0Hz, H-12a), 6.19 (2H, d,  $J$  = 2.0Hz, H-10a, H-14a), 6.09 (1H, d,  $J$  = 2.0Hz, H-14b), 5.39 (1H, d,  $J$  = 5.2Hz, H-7a), 5.22 (1H, d,  $J$  = 1.2Hz, H-7b), 4.64 (1H, d,  $J$  = 5.2Hz, H-8a), 4.33 (1H, d,  $J$  = 1.2Hz, H-8b). <sup>13</sup>C-NMR (BrukerAvance spectrometer, DMSO-d<sub>6</sub>, 100MHz)  $\delta$ : 162.3 (C-11b), 160.7 (H-11c), 160.3 (C-13b), 160.3 (C-13a), 160.3 (C-11a), 159.5 (C-13c), 158.2 (C-4c), 157.6 (C-4b), 157.0 (C-4a), 147.6 (C-9a), 143.5 (C-9b), 135.9 (C-9c), 133.4 (C-1a), 133.3 (C-1b), 131.2 (C-7c), 129.3 (C-1c), 128.7 (C-2c, C-6c), 127.8 (C-2a, C-6a), 127.4 (C-2b, C-6b), 122.8 (C-8c), 121.4



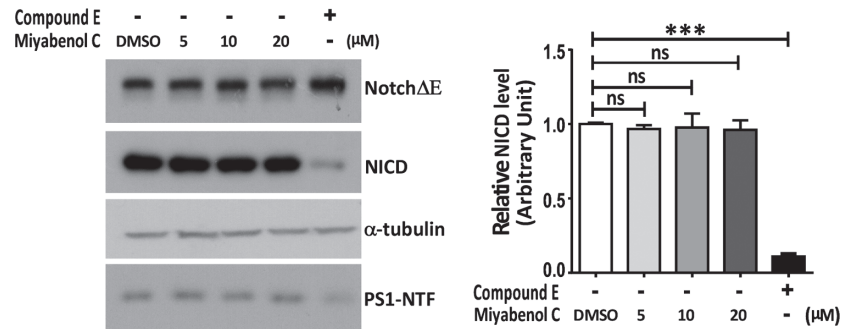
**Figure 1. Miyabenol C treatment inhibits A $\beta$  secretion.** (A) Chemical structure of miyabenol C. (B) N2a695 cells were treated with indicated doses of miyabenol C for 10h. Cell viability was measured using CCK-8 assay,  $n = 3$ . Error bars indicate standard deviation. (C, D) N2a695 cells were treated with DMSO (negative control),  $\beta$ -secretase inhibitor II (positive control, 2 $\mu$ M) or indicated doses of miyabenol C for 10h. Extracellular A $\beta$ 40 (C) and A $\beta$ 42 (D) levels were quantified by ELISA (One-way ANOVA followed by Tukey's post hoc test,  $n = 3$ , \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ).

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(C-10c), 118.6 (C-10b), 116.7 (C-3c, C-5c), 116.5 (C-3a, C-5a), 115.7 (C-3b, C-5b), 107.7 (C-14b), 107 (C-10a, C-14a), 104.4 (C-14c), 102.6 (C-12a), 97.2 (C-12c), 96.4 (C-12b), 94.6 (C-7a), 92.3 (C-7b), 57.2 (C-8a), 51.1 (C-8b). By comparison, we found that the spectral data were identical to that of miyabenol C (Fig. 1A), a previously reported resveratrol trimer [31], indicating that the compound we isolated is miyabenol C.

### Miyabenol C treatment reduces A $\beta$ secretion

To study whether miyabenol C can affect A $\beta$  generation, we first measured its cytotoxicity. N2a695 cells were treated with increasing doses of miyabenol C (0, 0.1, 1, 5, 10, 20, 40, 80, 100 $\mu$ M) for 10h and cell viability was evaluated by CCK-8 assay. The results showed that miyabenol C had no cytotoxicity at lower doses (5–20 $\mu$ M) but showed a dose-dependent cytotoxicity at higher doses (40–100 $\mu$ M) (Fig. 1B). Therefore, we used doses of 0–20 $\mu$ M in the following studies. When N2a695 cells were treated with miyabenol C (5, 10, 20 $\mu$ M) for 10 h, levels of A $\beta$ 40 (Fig. 1C) and A $\beta$ 42 (Fig. 1D) in conditioned media were markedly decreased in a dose-dependent manner.



**Figure 2. Miyabenol C does not inhibit  $\gamma$ -secretase activity.** N2aWT cells were first transfected with Notch $\Delta$ E. After splitting equally, cells were treated with DMSO (negative control), the  $\gamma$ -secretase inhibitor compound E (positive control, 0.5 $\mu$ M) or indicated doses of miyabenol C for 10h. Equal protein amounts of cell lysates were subjected to SDS-PAGE and Western blot. NICD levels were quantified by densitometry using image J for comparison (One-way ANOVA followed by Tukey's post hoc test, n = 3, ns:  $p > 0.05$ , \*\*\*:  $p < 0.001$ ).

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### Miyabenol C does not affect $\gamma$ -secretase activity

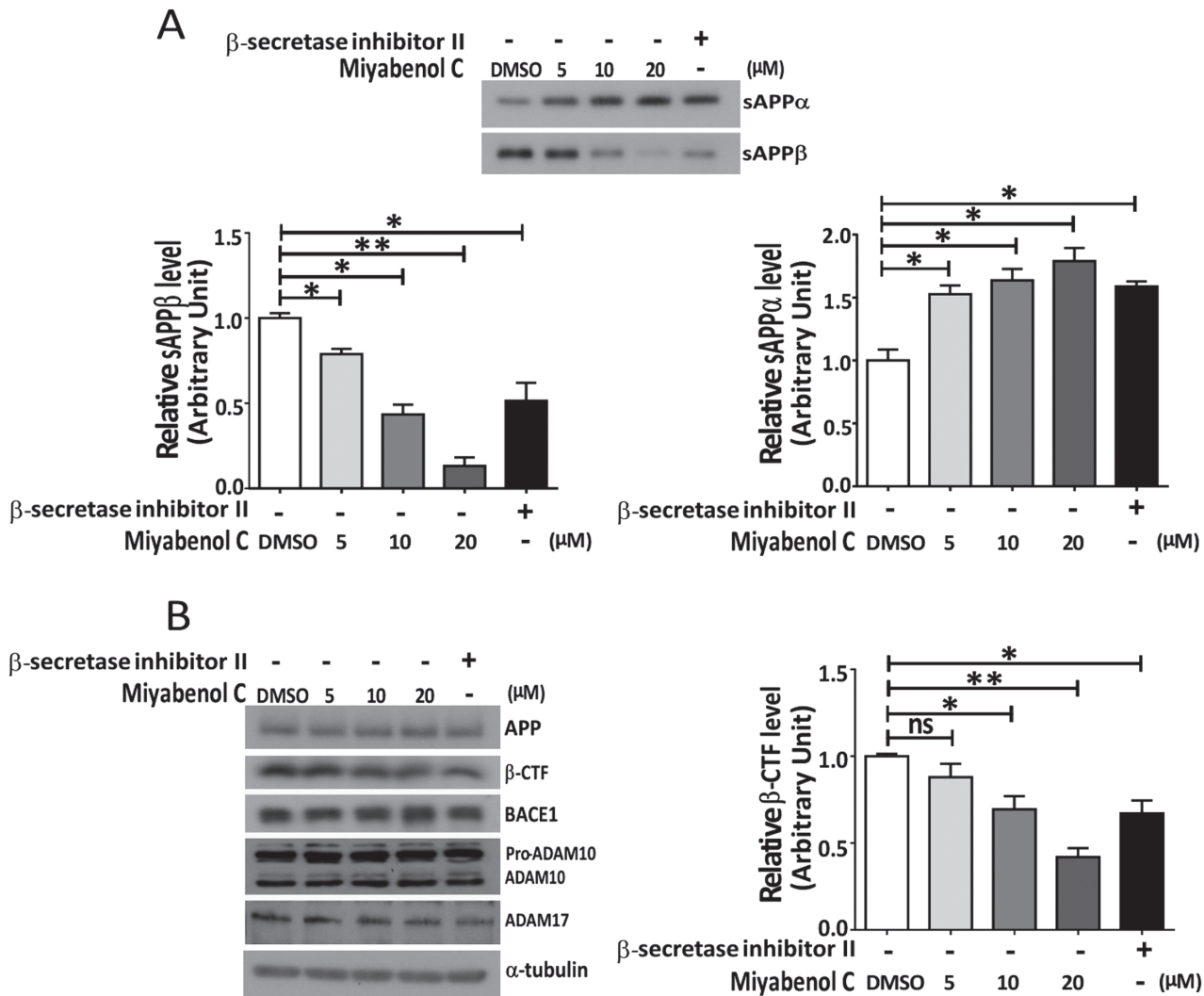
Because  $\gamma$ -secretase-mediated cleavage of APP is the final step for A $\beta$  generation, it is possible that miyabenol C may inhibit  $\gamma$ -secretase activity and reduce A $\beta$  production. To study this possibility, we transfected N2aWT cells with Notch $\Delta$ E that can be directly cleaved by  $\gamma$ -secretase for NICD generation [32, 33]. Cells were then treated with miyabenol C. The generation of NICD was measured by Western blot and the results showed that miyabenol C had no effect on inhibiting NICD generation, whereas the  $\gamma$ -secretase inhibitor compound E dramatically blocked NICD generation (Fig. 2). In addition, miyabenol C treatment did not affect the protein levels of PS1, a major  $\gamma$ -secretase component (Fig. 2). These results suggest that miyabenol C does not inhibit  $\gamma$ -secretase activity.

### Miyabenol C inhibits APP $\beta$ -cleavage and promotes APP $\alpha$ -cleavage

Because  $\beta$ -secretase-mediated APP processing is the first step leading to A $\beta$  generation, we studied whether miyabenol C affects  $\beta$ -secretase. In N2a695 cells, miyabenol C treatment dose-dependently decreased the secreted level of sAPP $\beta$ , an amino-terminal fragment of APP generated by  $\beta$ -secretase cleavage (Fig. 3A). Consistently, the level of APP  $\beta$ -CTF (a carboxyl-terminal fragment of APP generated by  $\beta$ -secretase cleavage) was also decreased upon miyabenol C treatment (Fig. 3B). These results suggest that miyabenol C inhibits  $\beta$ -cleavage of APP. In addition, miyabenol C treatments increased the level of secreted sAPP $\alpha$ , the major extracellular fragment of APP released by  $\alpha$ -secretase cleavage (Fig. 3A). Moreover, we found that miyabenol C treatment did not affect protein levels of APP, the major  $\beta$ -secretase BACE1 and two major  $\alpha$ -secretases ADAM10 and ADAM17 (i.e. Tumor necrosis factor alpha converting enzyme, TACE) [34–36] (Fig. 3B). These results suggest that miyabenol C reduces APP amyloidogenic processing not through affecting  $\alpha$ - and  $\beta$ -secretase protein levels.

### Miyabenol C inhibits $\beta$ - but not $\alpha$ -secretase activity

Since miyabenol C inhibits  $\beta$ -processing of APP without affecting BACE1 levels, miyabenol C probably directly inhibits BACE1 activity. To ascertain this possibility, we carried out a cell-based assay to measure the  $\beta$ -secretase activity. The results revealed that miyabenol C indeed dramatically inhibited  $\beta$ -secretase activity in both N2aWT and SY5Y cells (Fig. 4A, B). Moreover, miyabenol C markedly inhibited BACE1 activity *in vitro*, and its effect was comparable



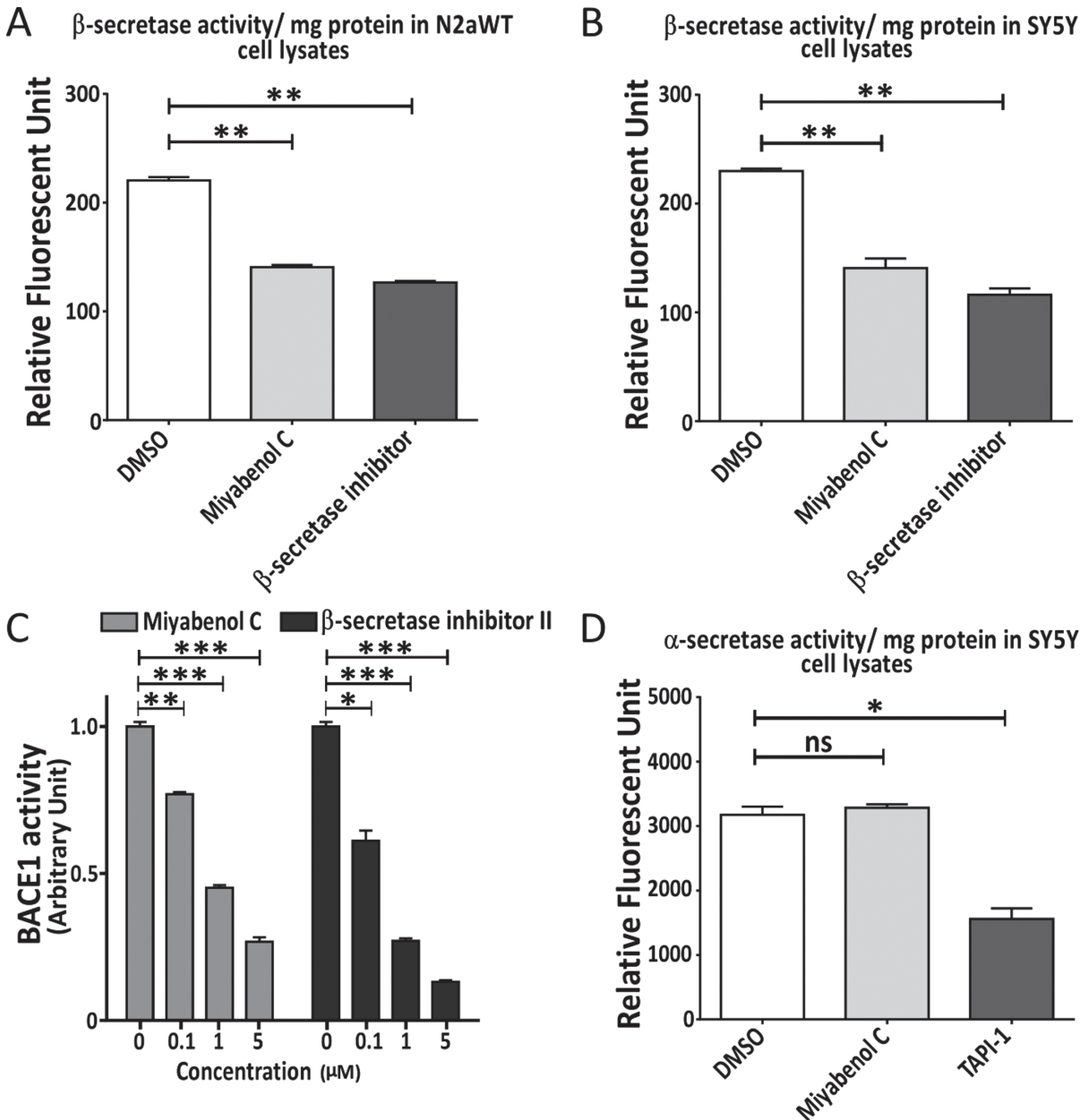
**Figure 3. Miyabenol C treatment reduces APP  $\beta$ -CTF and sAPP $\beta$  levels and increases sAPP $\alpha$  levels.** N2a695 cells were treated with DMSO (negative control),  $\beta$ -secretase inhibitor II (2 $\mu$ M) or indicated doses of miyabenol C for 10h. (A) Conditioned media and (B) cellular lysates were subjected to SDS-PAGE and Western blot. sAPP $\alpha$ , sAPP $\beta$  and  $\beta$ -CTF levels were quantified by densitometry using image J for comparison (One-way ANOVA followed by Tukey's post hoc test, n = 3, ns:  $p > 0.05$ , \*:  $p < 0.05$ , \*\*:  $p < 0.01$ ).

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to that of  $\beta$ -secretase inhibitor II *in vitro* (Fig. 4C). Since there is another possibility that miyabenol C inhibits APP  $\beta$ -processing and A $\beta$  generation through promoting  $\alpha$ -secretase activity, we also assayed the activity of TACE, a major  $\alpha$ -secretase in SY5Y cells treated with miyabenol C. We found that miyabenol C did not affect TACE activity (Fig. 4D), suggesting that miyabenol C does not affect  $\alpha$ -secretase activity.

### Miyabenol C treatment reduces sAPP $\beta$ and soluble A $\beta$ levels in the brains of APP/PS1 transgenic AD mice

We also examined the *in vivo* inhibitory effects of miyabenol C on  $\beta$ -secretase activity using APP/PS1 transgenic mice, a transgenic mouse model of AD that expresses APP Swedish mutant and PS1 exon-9 deletion mutant. We injected miyabenol C or vehicle control into the lateral ventricle of 12 month-old APP/PS1 mice for a short period of time (72h). Mice were

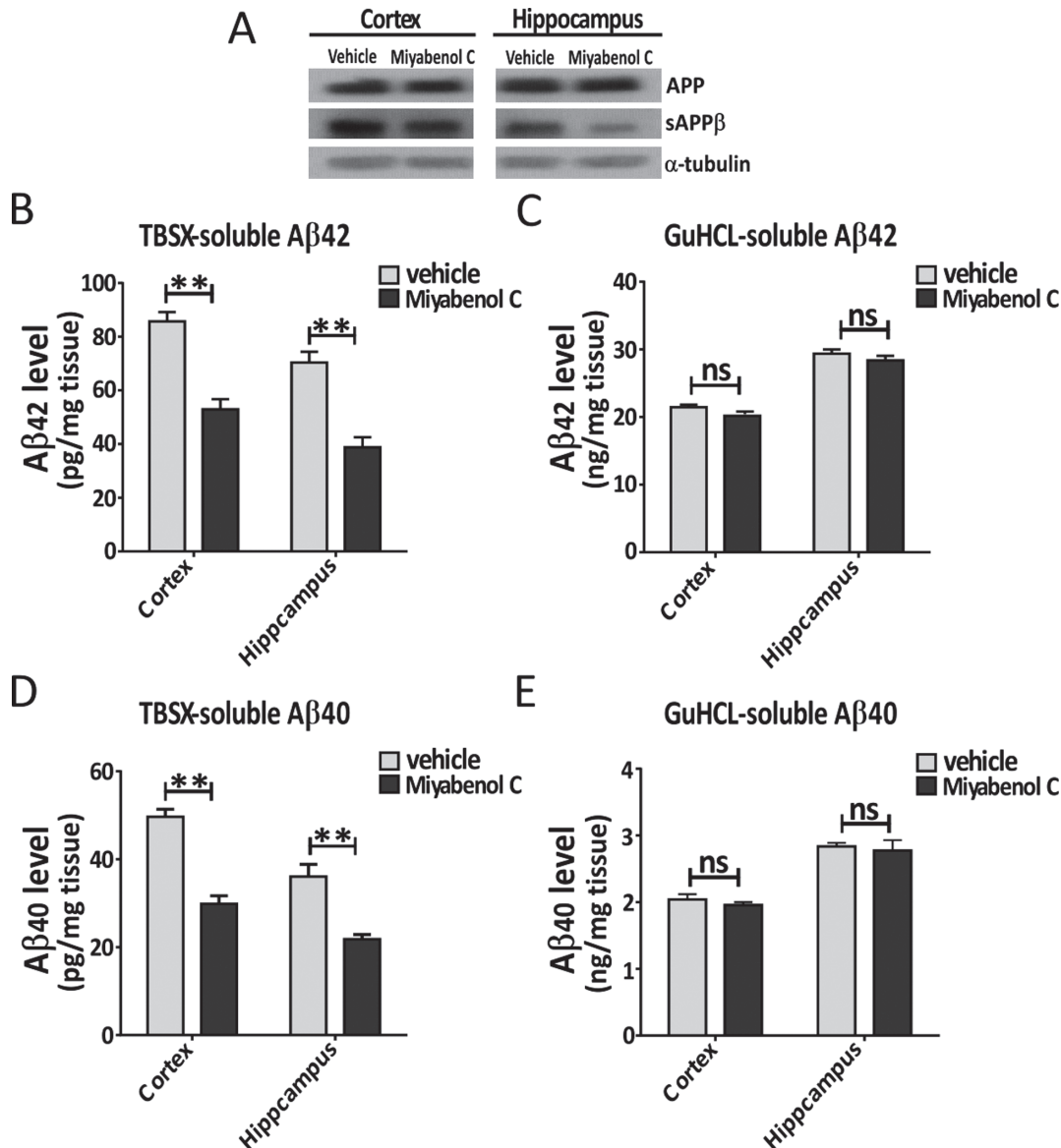


**Figure 4. Miyabenol C inhibits  $\beta$ -secretase activity.** (A) N2aWT and (B) SH-SY5Y cells were treated with DMSO (negative control), miyabenol C (10 $\mu$ M) or a  $\beta$ -secretase inhibitor (positive control, 2 $\mu$ M, provided in the kit) for 10h. Cell lysates were assayed for  $\beta$ -secretase activity by using a commercial kit from Millipore and subjected to comparison. (C) Indicated amounts of miyabenol C and  $\beta$ -secretase inhibitor II were incubated with BACE1 and its substrate provided by a commercial kit from Sigma, for 2h at 37°C. BACE1 activity was measured for comparison. (D) SY5Y cells were treated with DMSO (negative control), miyabenol C (10 $\mu$ M) or the  $\alpha$ -secretase inhibitor TAPI-1 (positive control, 10 $\mu$ M) for 10h. Cell lysates were assayed for the  $\alpha$ -secretase (TACE) activity for comparison (One-way ANOVA followed by Tukey's post hoc test, n = 3, ns:  $p > 0.05$ , \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ).

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then sacrificed for analyses. We found that mice that received miyabenol C treatment had markedly reduced sAPP $\beta$  levels in both cortex and hippocampus when compared with mice treated with vehicle (Fig. 5A). Moreover, miyabenol C treatment significantly reduced levels of A $\beta$ 42 and A $\beta$ 40 in TBSX-soluble fractions (Fig. 5B, D). However, neither A $\beta$ 42 nor A $\beta$ 40 levels in the TBSX-insoluble fractions were affected by miyabenol C treatment (Fig. 5C, E).





**Figure 5. Short term miyabenol C treatment reduces sAPP $\beta$  and soluble A $\beta$  levels in APP/PS1 AD mouse brain.** APP/PS1 AD mice (12 month old) were treated with miyabenol C (0.6 $\mu$ g/g) or vehicle (45% DMSO in artificial cerebrospinal fluid) through intracerebroventricular injection (4 $\mu$ l) for 72h. (A) Cortex and hippocampus of treated mice were dissected and lysed. Equal protein amounts of lysates were analyzed by Western blot. Levels of TBSX-soluble A $\beta$ 42 (B) and A $\beta$ 40 (D), as well as TBSX-insoluble (re-dissolved in GuHCl) A $\beta$ 42 (C) and A $\beta$ 40 (E) in the cortex and hippocampus of treated mice were measured by ELISA assays (Student's t test, n = 6, ns:  $p > 0.05$ , \*\*:  $p < 0.01$ ).

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## Discussion

Natural products provide excellent sources of new therapeutic compounds for disease intervention. Resveratrol and its derivatives are rich in grapes and possess strong anti-oxidant functions. Although resveratrol and its derivatives are considered to have therapeutic potential in AD [37, 38], detailed mechanism underlying their protective effects has yet to be determined.

Miyabenol C is a resveratrol trimer and has been found in plants including various *Vitis* species [39, 40], *Caragana* species [41–44], *Carex* species [45, 46], *Parthenocissus quinquefolia* [47], and *Sophora davidii* [48]. Previous studies have shown that miyabenol C possesses

various functions, such as being anti-proliferative and pro-apoptotic in tumor cells [45, 49], inhibiting the activity of protein kinases C [44, 50], antagonizing human 5-hydroxytryptamine (5-HT) receptor [51], having estrogenic activity through binding to estrogen receptor [52, 53], and having ecdysteroid antagonistic activity in *Drosophila* cell bioassays [46]. However, information on the role of miyabenol C in AD is scarce, with only one recent study showing that miyabenol C has marginal effect on inhibiting A $\beta$  fibril formation [39].

In the present study, we isolated miyabenol C from the small-leaf grape (*Vitisthunbergii* var. taiwaniana). Importantly, we found that miyabenol C treatment can dramatically reduce A $\beta$  secretion (Fig. 1) and increase sAPP $\alpha$  release (Fig. 3) in cell culture. Since miyabenol C does not affect total APP levels (Fig. 3B), we studied whether miyabenol C affects  $\alpha$ -,  $\beta$ - or  $\gamma$ -secretases. Miyabenol C treatment did not affect the protein level of PS1, a major component of the  $\gamma$ -secretase complex, and  $\gamma$ -secretase-mediated Notch processing for NICD generation, suggesting that miyabenol C does not affect  $\gamma$ -secretase. In addition, miyabenol C treatment did not affect protein levels of two major  $\alpha$ -secretases, ADAM10 and ADAM17/TACE, and cell-based TACE activity, implying that miyabenol C does not affect  $\alpha$ -secretase. In contrast, although miyabenol C treatment did not affect protein levels of the essential  $\beta$ -secretase BACE1, it reduced cell-based  $\beta$ -secretase activity and *in vitro* BACE1 activity, as well as both sAPP $\beta$  and APP  $\beta$ -CTF levels, indicating that miyabenol C reduces A $\beta$  generation through inhibiting  $\beta$ -secretase activity. Consistently, we found that short-term (72h) treatment with miyabenol C in the brain of APP/PS1 mice markedly reduced the levels of both sAPP $\beta$  and TBSX-soluble A $\beta$  that represent newly generated portion through  $\beta$ -cleavage, whereas miyabenol C treatment did not affect the levels of TBSX-insoluble A $\beta$  that are from aggregated species (Fig. 5).

Together, our results demonstrate that miyabenol C is a prominent  $\beta$ -secretase inhibitor and can reduce A $\beta$  generation both *in vitro* and *in vivo*. Since  $\beta$ -secretase-mediated APP processing is a rate-limiting step for A $\beta$  generation and inhibition of  $\beta$ -secretase activity is a promising strategy for AD intervention, miyabenol C may be a lead compound for AD drug development.

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## Author Contributions

Conceived and designed the experiments: HC Y-wZ. Performed the experiments: JH TL YG JX CJ GW. Analyzed the data: JH GB HX HC Y-wZ. Wrote the paper: JH HC Y-wZ.

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