The Resveratrol Trimer Miyabenol C Inhibits β-Secretase Activity and β-Amyloid Generation

Jin Hu1,2†, Ting Lin1‡, Yuehong Gao2, Junyue Xu1, Chao Jiang1, Guanghui Wang1, Guojun Bu2, Huaxi Xu1,2, Haifeng Chen1,* Yun-wu Zhang2*

1 Fujian Provincial Key Laboratory of Neurodegenerative Disease and Aging Research, School of Pharmaceutical Sciences, Xiamen University, Xiamen, 361102, China, 2 Institute of Neuroscience, College of Medicine, Xiamen University, Xiamen, 361102, China

‡ These authors contributed equally to this work.
* yunzhang@xmu.edu.cn (Y-wZ); haifeng@xmu.edu.cn (HC)

Abstract

Accumulation and deposition of amyloid-β peptide (Aβ) in the brain is a primary cause of the pathogenesis of Alzheimer’s disease (AD). Aβ is generated from amyloid-β precursor protein (APP) through sequential cleavages first by β-secretase and then by γ-secretase. Inhibiting β-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 (Vitis thunbergii var. taiwaniana), can markedly reduce Aβ and sAPPβ 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 α-secretases ADAM10 and TACE, and the γ-secretase component Presenilin 1, nor γ-secretase-mediated Notch processing and TACE activity. In contrast, although miyabenol C has no effect on altering protein levels of the β-secretase BACE1, it can inhibit both in vitro and in vivo β-secretase activity. Together, our results indicate that miyabenol C is a prominent β-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-β (Aβ) peptides. Ample evidence indicates that accumulation of Aβ peptides in vulnerable brain regions plays a central role in AD pathogenesis: Aβ 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]. Aβ is a proteolytic product of the amyloid-β precursor protein (APP) and is generated through sequential cleavages by enzymes called β- and γ-secretases. During this amyloidogenic processing, β-secretase first cleaves the type I transmembrane APP protein...
to generate an extracellular fragment known as sAPPβ and a membrane-associated carboxyl-terminal fragment known as APP β-CTF. APP β-CTF is then cleaved by γ-secretase to release Aβ. Alternatively, APP can be subjected to a non-amyloidogenic processing and cleaved by α-secretase within the Aβ domain. α-secretase-mediated cleavage precludes Aβ generation and generates an extracellular domain of APP known as sAPPα instead [3, 4].

β-cleavage of APP is the first and rate-limiting step in Aβ production. The transmembranous aspartic protease β-site APP cleaving enzyme 1 (BACE1) has been identified as the essential β-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 β-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 Aβ deposition in AD mice [16, 17]. Therefore, moderate inhibition of β-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 Vitis thunbergii 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 β-secretase inhibitor and can reduce Aβ 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 × 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-H2O as mobile phase, into three fractions (A-C). Fraction B (60% EtOH-H2O eluent, 130.0 g) was chromatographed on silica gel column using stepwise gradient elution with CHCl3-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 CHCl3-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-H2O (3:7, 4:6 and 1:1). The elution of MeOH-H2O (4:6) was further repeated by RP-18 and purified through preparative HPLC (RestekPrinnacle DB C18, 5 µm, 250×20 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 β-secretase inhibitor II (Millipore) were dissolved in DMSO (Sigma) as 20 mM stock solution. For treatment, cells grown to confluence were switched to be
incubated in FBS-free media that were added with indicated concentrations of miyabenol C, β-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 sAPPα and sAPPβ by Western blot. Treated cells were lysed in ice-cold lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1x 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 sAPPα and β-CTF, 1:1000) and anti-sAPPβ antibodies (1:500) from Covance; anti-α-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.

**NotchΔE cDNA construct and cell based γ-secretase activity assay**

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

**α-secretase activity assay**

The activity of α-secretase in cells was measured by using InnoZyme TACE Activity Kit (Millipore), following the manufacturer’s protocols.

**β-secretase activity assays**

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

**Aβ40 and Aβ42 ELISA assays**

Human Aβ40 and Aβ42 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 (50μg/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 CaCl2, 0.75 mM MgCl2, 0.8 mM Na2HPO4, 0.2 mM NaH2PO4) or miyabenol C (0.6μg/g). Vehicle and miyabenol C solution were injected at 4μL into the lateral ventricle using a 5μL-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 0.4μL/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 sAPPβ. 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 Aβ 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×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×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 ± 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]-. 1H-NMR (BrukerAvance spectrometer, DMSO-d6, 400MHz) δ: 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 I (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). 13C-NMR (BrukerAvance spectrometer, DMSO-d6,100MHz) δ: 162.3 (C-11b), 160.7 (C-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
Miyabenol C treatment reduces Aβ secretion

To study whether miyabenol C can affect Aβ 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, 100μM) for 10h and cell viability was evaluated by CCK-8 assay. The results showed that miyabenol C had no cytotoxicity at lower doses (5–20μM) but showed a dose-dependent cytotoxicity at higher doses (40–100μM) (Fig. 1B). Therefore, we used doses of 0–20μM in the following studies. When N2a695 cells were treated with miyabenol C (5, 10, 20μM) for 10 h, levels of Aβ40 (Fig. 1C) and Aβ42 (Fig. 1D) in conditioned media were markedly decreased in a dose-dependent manner.

Figure 1. Miyabenol C treatment inhibits Aβ 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), β-secretase inhibitor II (positive control, 2μM) or indicated doses of miyabenol C for 10h. Extracellular Aβ40 (C) and Aβ42 (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 does not affect γ-secretase activity

Because γ-secretase-mediated cleavage of APP is the final step for Aβ generation, it is possible that miyabenol C may inhibit γ-secretase activity and reduce Aβ production. To study this possibility, we transfected N2aWT cells with NotchΔE that can be directly cleaved by γ-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 γ-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 γ-secretase component (Fig. 2). These results suggest that miyabenol C does not inhibit γ-secretase activity.

Miyabenol C inhibits APP β-cleavage and promotes APP α-cleavage

Because β-secretase-mediated APP processing is the first step leading to Aβ generation, we studied whether miyabenol C affects β-secretase. In N2a695 cells, miyabenol C treatment dose-dependently decreased the secreted level of sAPPβ, an amino-terminal fragment of APP generated by β-secretase cleavage (Fig. 3A). Consistently, the level of APP β-CTF (a carboxyl-terminal fragment of APP generated by β-secretase cleavage) was also decreased upon miyabenol C treatment (Fig. 3B). These results suggest that miyabenol C inhibits β-cleavage of APP. In addition, miyabenol C treatments increased the level of secreted sAPPα, the major extracellular fragment of APP released by α-secretase cleavage (Fig. 3A). Moreover, we found that miyabenol C treatment did not affect protein levels of APP, the major β-secretase BACE1 and two major α-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 α- and β-secretase protein levels.

Miyabenol C inhibits β- but not α-secretase activity

Since miyabenol C inhibits β-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 β-secretase activity. The results revealed that miyabenol C indeed dramatically inhibited β-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
to that of β-secretase inhibitor II in vitro (Fig. 4C). Since there is another possibility that miyabenol C inhibits APP β-processing and Aβ generation through promoting α-secretase activity, we also assayed the activity of TACE, a major α-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 α-secretase activity.

Miyabenol C treatment reduces sAPPβ and soluble Aβ levels in the brains of APP/PS1 transgenic AD mice

We also examined the in vivo inhibitory effects of miyabenol C on β-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
then sacrificed for analyses. We found that mice that received miyabenol C treatment had markedly reduced sAPPβ levels in both cortex and hippocampus when compared with mice treated with vehicle (Fig. 5A). Moreover, miyabenol C treatment significantly reduced levels of Aβ42 and Aβ40 in TBSX-soluble fractions (Fig. 5B, D). However, neither Aβ42 nor Aβ40 levels in the TBSX-insoluble fractions were affected by miyabenol C treatment (Fig. 5C, E).
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 kinas 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 Aβ fibril formation [39].

In the present study, we isolated miyabenol C from the small-leaf grape (Vitis thunbergii var. taiwania). Importantly, we found that miyabenol C treatment can dramatically reduce Aβ secretion (Fig. 1) and increase sAPPα release (Fig. 3) in cell culture. Since miyabenol C does not affect total APP levels (Fig. 3B), we studied whether miyabenol C affects α-, β- or γ-secretases. Miyabenol C treatment did not affect the protein level of PS1, a major component of the γ-secretase complex, and γ-secretase-mediated Notch processing for NICD generation, suggesting that miyabenol C does not affect γ-secretase. In addition, miyabenol C treatment did not affect protein levels of two major α-secretases, ADAM10 and ADAM17/TACE, and cell-based TACE activity, implying that miyabenol C does not affect α-secretase. In contrast, although miyabenol C treatment did not affect protein levels of the essential β-secretase BACE1, it reduced cell-based β-secretase activity and in vitro BACE1 activity, as well as both sAPPβ and APP β–CTF levels, indicating that miyabenol C reduces Aβ generation through inhibiting β-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 sAPPβ and TBSX-soluble Aβ that represent newly generated portion through β-cleavage, whereas miyabenol C treatment did not affect the levels of TBSX-insoluble Aβ that are from aggregated species (Fig. 5).

Together, our results demonstrate that miyabenol C is a prominent β-secretase inhibitor and can reduce Aβ generation both in vitro and in vivo. Since β-secretase-mediated APP processing is a rate-limiting step for Aβ generation and inhibition of β-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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