



3'-O-Acetyl-24-Epi-7,8-Didehydrocimigenol-3-O-β-D-Xylopyranoside Decreases Amyloid Beta Production in Amyloid Precursor Protein-Transfected HeLa Cells

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Abstract

Extracellular beta amyloid (A β) plaques are the neuropathological hallmarks of Alzheimer's disease (AD). Accordingly, reducing A β levels is considered a promising strategy for AD prevention. 3'-O-acetyl-24-epi-7,8-didehydrocimigenol-3-O-β-D-xylopyranoside significantly decreased the A β production and this effect was accompanied with reduced sAPP β production known as a soluble ectodomain APP fragment through β -secretases in HeLa cells overexpressing amyloid precursor proteins (APPs). This compound also increased the level of sAPP α , which is a proteolytic fragment of APP by α -secretases. In addition, 3'-O-acetyl-24-epi-7,8-didehydrocimigenol-3-O-β-D-xylopyranoside decreased the protein level of β -secretases, but the protein levels of A disintegrin and metalloproteinase (ADAM) family, especially ADAM10 and ADAM17, are increased. Thus, 3'-O-acetyl-24-epi-7,8-didehydrocimigenol-3-O-β-D-xylopyranoside could be useful in the development of AD treatment in the aspect of amyloid pathology.

Key Words: Alzheimer's disease, Anti-amyloidogenic effect, Secretases

INTRODUCTION

AD is a progressive neurodegenerative disorder which is recognized as the most common form of dementia among the elderly characterized by progressive dysfunction of cognition and memory (Scheltens *et al.*, 2016). It affects the millions of the elderly and the number of AD patients has dramatically increased. In the worldwide, approximately 35.6 million people are currently affected from AD, but it is expected that the number of people living with AD will reach 135 million people in 2050 (Prince *et al.*, 2013; Kim *et al.*, 2015a). Although the cause of AD has not yet been fully understood, but several studies investigated that A β deposition is one of the major causes of AD pathology in the early onset familial AD (Hardy and Orr, 2006). Senile plaques formed by extracellular A β accumulation are one of the AD pathologic hallmarks which believed to not only interfere the synapses neu-

ron communication but also lead to cell death (Karran and De Strooper, 2016). The A β is known to be produced from APP (Gu *et al.*, 2018). The shedding of APP generates sAPP β and CTF β through β -secretase, and consequential CTF β cleavage produces A β 40 as well as A β 42 by γ -secretase (Park, 2010; Prince *et al.*, 2013). However, α -secretase cleaves APP and then generates sAPP α and CTF α precluding the A β formation (Park, 2010). Eventually, the A β production will directly be related with the sAPP β generation and contrary correlated with the proportion of sAPP α (Kim *et al.*, 2015b). Considering such previous findings, reduction of A β or delaying the A β deposition in the brain would be the possible therapeutic targets in the treatment or prevention of AD (Hardy and Orr, 2006; Kim *et al.*, 2015b; Karran and De Strooper, 2016). Furthermore, various secretases would be the key factors to modulate to deliver such beneficial effects. The ADAM family are known as α -secretases such as ADAM9, ADAM10, and ADAM17,

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which catalyze the APP ectodomain shedding. ADAM10 is the major ADAM family member which is responsible for the constitutive activity, while other ADAM family such as ADAM9 and ADAM17 are accountable for the cleavage regulation (Vassar *et al.*, 2009; Kuhn *et al.*, 2010; Kim *et al.*, 2015a).

Several drugs are used for slowing down the progression of AD, but they do not significantly delay symptom development or cure the disease. However naturally derived therapeutics showed advantages in slowing down AD development and delaying the onset of symptoms (Kim *et al.*, 2015b; Gu *et al.*, 2018; Chen *et al.*, 2019; Lee *et al.*, 2019). Considering the facts that natural products usually show less side effects compared to the synthetic chemicals and exhibit diversified beneficial effects, natural resources are gaining much attentions in the field of neurodegenerative diseases. *Cimicifuga dahurica* (Turcz.) Maxim. (*C. dahurica*) is traditionally used as an antipyretic and analgesic in East Asia region such as Korea, China, Japan, and Russia. Several studies have been conducted to determine the activity of specific flavonoid compounds isolated from *C. dahurica*. Distinguishable compounds such as cycloartane-type triterpenoids, indolinone alkaloids, phenolics contained in this medicinal plant are believed to have antioxidative effects, anti-cancer, and anti-inflammatory (Tian *et al.*, 2007; Qin *et al.*, 2016; Zhang *et al.*, 2016; Lv *et al.*, 2017; Nguyen Phuong Thao *et al.*, 2018). However, the A β inhibition effects of compounds isolated from *C. dahurica* have not been studied. In this study, we hypothesized that a compound isolated from *C. dahurica* roots might exhibit anti-A β effects in APP overexpressing HeLa cells. To test this hypothesis, we examined the effect of the compound on A β production and its underlying mechanisms by investigating the formation of sAPP α and sAPP β as well as the activities of both α - and β -secretases.

MATERIALS AND METHODS

Chemicals and reagents

Rabbit anti-APP antibodies to detection the C-terminal of APP were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). FBS was purchased from ATCC Company (Manassas, VA, USA). DMEM, penicillin/streptomycin, G418, and 0.25% trypsin-EDTA were purchased from GIBCO-BRL Company (Carlsbad, CA, USA). Zeocin were purchased from Invitrogen Company (Carlsbad, CA, USA). Rabbit anti-GAPDH, anti-rabbit horseradish peroxidase linked IgG, anti-ADAM9 antibodies and lysis buffer were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-BACE1 antibody, and anti-APP antibody to detection both mAPP and imAPP were obtained from Abcam Company (Cambridge, UK). Anti-ADAM10 antibody was obtained from Calbiochem Company (San Diego, CA, USA). Anti-TACE and anti-ADAM17 antibodies were obtained from Chemicon Company (Billerica, MA, USA). All other chemicals were of analytical grade obtained from Sigma-Aldrich Co.

Plant materials

The roots of *C. dahurica* were obtained from Naemome Dah (Ulsan, Korea) which is the herbal resource company on February 2016. Prof. Young Ho Kim from Chungnam National University (Daejeon, Korea) identified and the herbarium of the College of Pharmacy, Chungnam National University de-

posited a voucher specimen (CNU-16003).

Plant extraction and isolation of compounds

The 95% ethanol extract of *C. dahurica* (65.3 g) was prepared by extracting dried roots of *C. dahurica* (2.5 kg) with ethanol and concentrating. Then, the extract was successively partitioned with n-hexane, CH₂Cl₂, and H₂O. From the CH₂Cl₂ fraction and water layer, 52 compounds were isolated by several rounds of column chromatography and their structures were elucidated by spectroscopic methods. These results have been previously reported elsewhere (Thao *et al.*, 2017a, 2017b, 2017c). Among the 52 compounds isolated from *C. dahurica*, 3'-O-acetyl-24-epi-7,8-didehydrocimigenol-3-O- β -D-xylopyranoside (Comp 27) was selected for further studies on its beneficial effects against the cytotoxicity of amyloidogenic HeLa cell line.

Cell culture and viability assay

HeLa cells stably transfected with an APP carrying Swedish mutation (APP^{sw}) using BioT (Bioland Scientific LLC, Paramount, CA, USA) according to the manufacturer's instructions. APP^{sw} transfected HeLa cells were grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 260 μ g/mL Zeocin and 400 μ g/mL G418. The cells were maintained 37°C under a humidified atmosphere of 5% CO₂ : 95% air. The stock sample solution was dissolved in DMSO and kept at -20°C, and diluted to the final concentration in fresh media before each experiment. The final DMSO concentration of the sample solutions did not exceed 0.5% in all experiments.

For measuring the cell viability, we used the EZ-Cytox kit according to the manufacturer's instruction obtained from DAEILLAB Co (Cheongwon, Korea). HeLa cells were seeded in 96-well plates for 24 h and then various concentrations of comp 27 (0-10 μ M) were treated for 12 h. The microplate reader (BIO-TEK® Dower-Wave; BioTek, Winooski, VT, USA) used to measured absorbance at 450 nm. The results were expressed as the percentage of MTT reduction, assigning the 100% value to the absorbance of the control group.

Western blot analysis

APP^{sw}-transfected HeLa cells were collected after they were treated with the indicated concentrations of samples and chemicals for the indicated times. After harvested the cells using PBS (pH 7.2), the proteins from cell pellets were lysed in a cold lysis buffer containing protease inhibitor cocktail obtained from Sigma-Aldrich Co. The lysates were centrifuged at 13,000 rpm for 20 min at 4°C. The protein content of the supernatant was determined by the Bradford assay obtained from Bio-Rad Laboratories (Hercules, CA, USA) and used in the subsequent experiments. Protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride membranes. After blocked membranes with 5% non-fat milk for 1 h at room temperature, the membranes incubated overnight at 4°C with primary antibodies including anti-APP, ADAM9, ADAM10, ADAM17, and BACE1. The membranes were washed three times using tris-buffered saline buffer with tween 20 (TBST). The membranes were incubated with horseradish peroxidase-conjugate anti-rabbit IgG antibodies for 1 h. Immunoreactive bounds were visualized using enhanced chemiluminescence (ECL) Advance western blotting detection reagents (34095, Bio-Rad Laboratories). Lu-

minescent Image Analyzer (LAS-4000, Fujifilm, Minato City, Tokyo, Japan) performed for the Imaging and quantitative densitometry analyses. All the protein levels were normalized to that of GAPDH.

Aβ and sAPPα peptide assay

APPsw-transfected HeLa cells were cultured with the compound or DMSO in DMEM for 8 h and then the medium was harvested for subsequent analyses. For the secreted Aβ detection, the kits for Aβ42 (KHB3442) and Aβ40 (KHB3482) were obtained from Invitrogen Company and used according to the supplier's instructions. For sAPPα detection, sAPPα (27734) ELISA kit obtained from IBL Company was used in this study according to the supplier's instructions as well.

Statistical analysis

Data were analyzed with Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA) using one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test. Statistical significance was set at $p < 0.05$ and the results are expressed as the mean \pm SEM.

RESULTS

The structure of comp 27 isolated from *C. dahurica* is shown in Fig. 1A. To test the effect of comp 27 on cell viability, HeLa cells transfected with APPsw were treated with comp 27 (1.25, 2.5, 5, 7.5, and 10 μ M) for 8 h. It did not affect cell viability except for 10 μ M concentration (Fig. 1B). Therefore, we

used these concentrations except 10 μ M in this study.

Then, we examined the effect of comp 27 on Aβ secretion. Cells were incubated with 2.5, 5, and 7.5 μ M comp 27 for 8 h, and we measured the levels of Aβ42 and Aβ40 used specific ELISA kits from the conditioned media. The production of both were decreased in a dose-dependent manner. The Aβ42 level was reduced by 66.7%, 56.1%, and 46.2% at 2.5, 5, and 7.5 μ M of comp 27, respectively (Fig. 1C). The Aβ40 level was also reduced by 75.7%, 64.5%, 52.5% at 2.5, 5, and 7.5 μ M of comp 27, respectively (Fig. 1D).

β-Secretase and γ-secretase generated Aβ through sequential cleavage of APP. On the other hand, α-secretase and γ-secretase generated precluding Aβ by cleavage within the Aβ domain. Thus, we further tested the effects of comp 27 on the production of APP proteolytic fragments, sAPPβ and sAPPα, as well as the APP expressions to investigate the two pathways. The secreted level of sAPPα was increased by 116.2%, 121.1%, and 131.2% at 2.5, 5, and 7.5 μ M of comp 27, respectively (Fig. 2A). In addition, treatment of 7.5 μ M comp 27 significantly decreased the level of sAPPβ to 50.6% (Fig. 2B, 2C). On the other hand, comp 27 did not change the levels of both mature and immature APP (mAPP and imAPP) (Fig. 2C, 2D).

Comp 27 increased sAPPα secretion and decreased the secretion of Aβ and sAPPβ. However, it did not affect total APP expression. Therefore, we expected that comp 27 may affect either ADAM family or BACE1 which are respectively acting as α- and β-secretases.

Next, we investigated whether comp 27 affect ADAM family expressions and activities. Cells were incubated with 2.5,

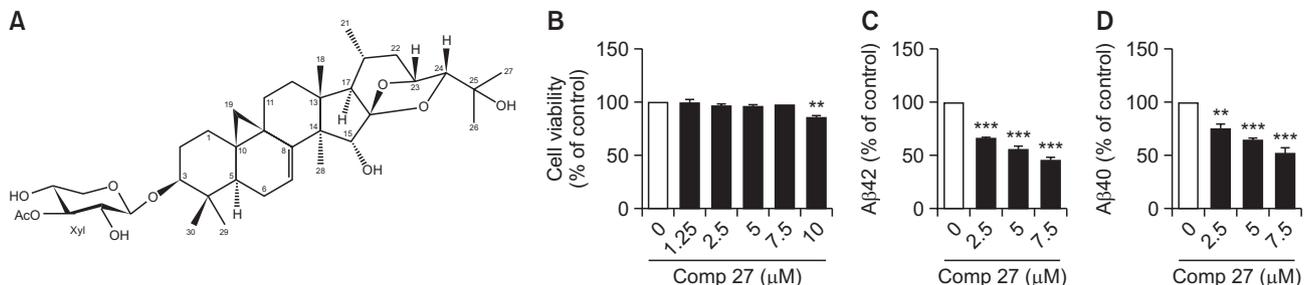


Fig. 1. Effect of comp 27 on cell viability and Aβ secretion. (A) Chemical structure of 3'-O-acetyl-24-epi-7,8-didehydrocimigenol-3-O-β-D-xylopyranoside (comp 27). (B) The effect of comp 27 on cell viability. (C, D) ELISA result of Aβ42 and Aβ40 secretion after comp 27 treatment. ** $p < 0.01$, *** $p < 0.001$ compared to the control group.

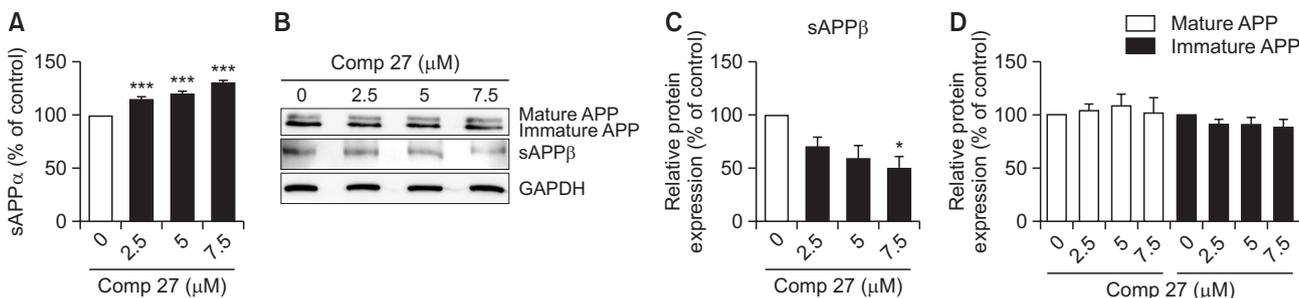


Fig. 2. Effect of comp 27 on APP processing. (A) ELISA result of sAPPα after comp 27 treatment. (B) Western blot analysis of sAPPβ, mature APP and immature APP after comp 27 treatment. (C) Comp 27 dose-dependently decreased the level of sAPPβ. (D) Comp 27 did not change the levels of both mature and immature forms of APP. * $p < 0.05$, *** $p < 0.001$ compared to the control group.

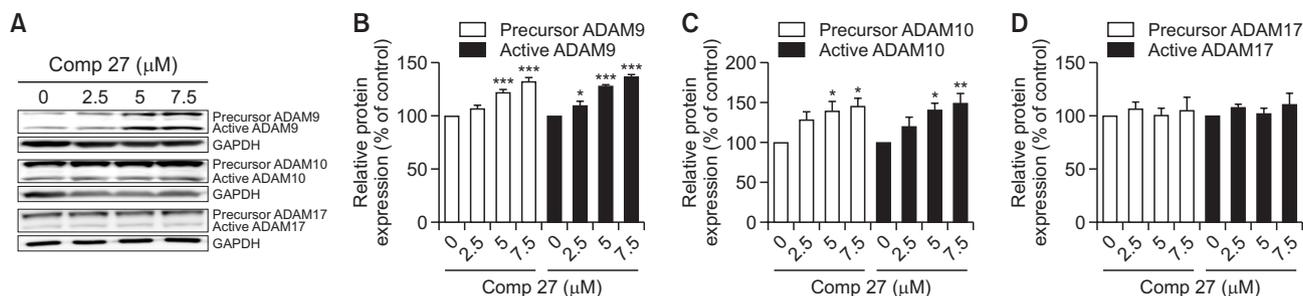


Fig. 3. Effect of comp 27 on the Expression of ADAM family. (A) Western blot analysis of ADAM9, 10 and 17 after comp 27 treatment. (B) Comp 27 dose-dependently increased the levels of precursor and active ADAM9. (C) Comp 27 dose-dependently increased the levels of precursor and active ADAM10. (D) Comp 27 did not change the levels of both precursor and active ADAM17. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control group.

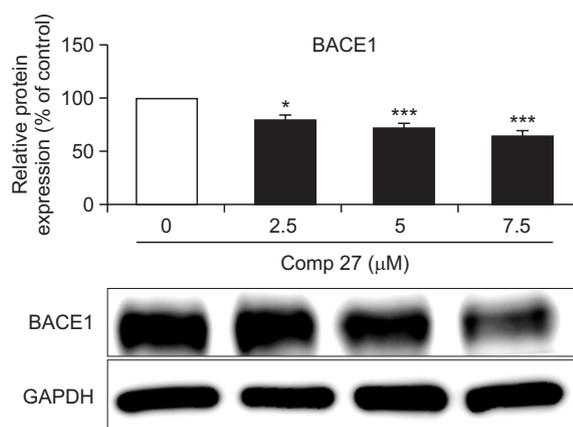


Fig. 4. Effect of comp 27 on BACE1 Expression. comp 27 dose-dependently reduced the expression of BACE1. * $p < 0.05$ and *** $p < 0.001$ compared to the control group.

5, and 7.5 μ M of comp 27 for 8 h, and the levels of ADAMs in lysates were measured using Western blot analysis. ADAM9 and ADAM10 exist as a pro-enzyme state, which are converted to mature form to cleavage of the APP (Lammich *et al.*, 1999). We found that both precursor and active ADAM9 and ADAM10 were increased in a dose-dependent manner (Fig. 3A). Treatment with 7.5 μ M of the compound increased ADAM9 levels to 136.5%, and ADAM10 levels to 150.3%, respectively (Fig. 3B, 3C). On the other hand, comp 27 did not change the expression of any forms of ADAM17, precursor and active ADAM17 (Fig. 3D).

We tried to determine whether comp 27 influences BACE1 protein expression. As we expected, compound 27 dose-dependently decreased BACE1 expression. The level of BACE1 was decreased by 77.2%, 68.5%, and 55.5% at 2.5, 5, and 7.5 μ M comp 27, respectively (Fig. 4).

DISCUSSION

Cimicifuga dahurica (Turcz.) Maxim. is commonly called 'shengma'. It is one of the ancient herbal medicines that has been subject of extensive studies. It is distributed widely in Northeast Asia and Russia, and traditionally used as an anti-

pyretic and analgesic agent (Thao *et al.*, 2018). In this study, we first showed that comp 27, one of the isolated compounds from *C. dahurica*, significantly decreased both A β 42 and A β 40 secretion in HeLa cells overexpressing mutant APPs within the range of no cytotoxicity. We further investigated the possible underlying mechanism of comp 27. Comp 27 increases sAPP α secretion which may be attributed to the expression of α -secretases. As expected, comp 27 increased ADAM9 and ADAM10 expression without affecting ADAM 17 activity. This result indicates that the comp 27-induced A β reduction occurs through increasing the level of α -secretase, especially ADAM9 and ADAM10. In addition, we found that comp 27 decreases sAPP β formation concomitantly. Reduction of secreted sAPP β might be due to either decreasing APP expression itself or inhibiting the expression level of BACE1, which is a β -secretase responsible for the cleavage generating the A β peptides in the amyloidogenic pathology. According to our observation, comp 27 did not affect the expressions of both mature and immature APPs. Instead, we detected that comp 27 decreased BACE1 expression which can be the main cause of reduced expression of sAPP β . BACE1 expression, however, can be regulated at the amount of transcription, translation, or protein degradation. So, further studies need to be performed to investigate the specific mechanisms underlying the comp 27 in such processes.

A β oligomers stimulate the kind of biological signaling pathway involving oxidative stress and neuroinflammation (Agostinho *et al.*, 2010). This process leads not only a neuronal synapses and dendrites impairment but also disintegration of the neural circuits and neuronal loss eventually (Vargas *et al.*, 2018). Accordingly, reducing the A β generation using any substance is considered to likely a good approach for treatment or prevention of AD. To date, it was not successful to develop an effective drug to stop or modify the progression of AD. Natural products could be an excellent source to reveal a hint for the therapeutic candidates against chronic and complexed disorders including neurodegenerative diseases. Since APP is cleaved within its extracellular domain by α -secretase or β -secretase, the promoting effect of comp 27 on α -secretase expression could decrease amyloidogenic process of APP by β -secretase. It also suggests that inhibitory effect of comp 27 on β -secretase expression could result in the same effect. Thus, our data suggest that comp 27 decreases A β production *in vitro* via modulation of two kinds of enzymes directly involved in APP cleavage. Confirmation of such significant anti-

A β effects in the future animal studies would promote comp 27 to be a possible therapeutic candidate for the AD pathology.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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