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BACE1 inhibitory effects of lavandulyl flavanones from *Sophora flavescens*

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ABSTRACT

In order to access β-secretase (BACE1), and enzyme strongly implicated in the cause of Alzheimer's disease, inhibitors must possess sufficient lipophilicity to traverse two lipid bilayers. Current drug candidates, which are almost totally peptide-derived, are thus inefficient because cell permeability presents a serious limiting factor. In this study, lipophilic alkylated (C_{10}–C_5) flavanones from *Sophora flavescens* were examined for their inhibitory effects against β-secretase. Lavandulyl flavanones (**1**, **2**, **5**, **6**, and **8**) showed potent β-secretase inhibitory activities with IC_{50}s of 5.2, 3.3, 8.4, 2.6, and 6.7 μM, respectively, while no significant activity was observed in the corresponding hydrated lavandulyl flavanones (**4** and **7**) and prenylated flavanone (**3**). As we expected, lavandulyl flavanones reduced Aβ secretion dose-dependently in transfected human embryonic kidney (HEK-293) cells. In kinetic studies, all compounds screened were shown to be noncompetitive inhibitor.

1. Introduction

β-Amyloid (Aβ) peptide has been known to play a crucial role in the development of Alzheimer's disease (AD) because Aβ peptides can form insoluble plaques resulting in severe memory loss and neuronal cell death.[1] Aβ peptides are derived from a sequential proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases.[2] The late limiting step in this process is cleavage of membrane-bound APP by β-secretase 1 (BACE1, memapsin-2, Asp-2) to form soluble APP (sAPP β) and a 12 kDa peptide, C99.[3–6] One way to retard or prevent Alzheimer's disease may be to block these secretases, thus preventing proliferation of plaques. However, this approach itself is complicated as the inhibition of γ-secretase may elicit unwanted side effects as it is involved in the processing of other proteins (e.g., Notch).[7,8] For these reasons, BACE1 and its closely related homolog, BACE2 (memapsin-1, Asp-1), which has 50% sequence identity to BACE1 and also cleaves APP at the β-secretase site, have become important drug targets.[9–11] Perhaps, the most promising link between BACE1 and AD is the fact that both the expression and activity of BACE1 were found to be elevated in the brains of AD patients.[12] Interestingly, Aβ generation is so completely abolished in mice deficient BACE1 that BACE2 becomes the primary source of Aβ in the brain.[13,14]

Previous work in designing inhibitors for BACE1 has centered on peptide-derived structures, which act as transition state analogs based on the amino acid sequences at the cleavage site of APP by BACE1.[15] These species showed nanomolar IC_{50} values or better, but their viability as drug candidates in this case is minimal because of their high hydrophilicity. This is because the inhibitor must possess sufficient lipophilicity to traverse two lipid bilayers to reach BACE1, as it is localized in the TGN/endosomal lumen.[11] Thus, we elected to address the problem by developing lipophilic plant-derived nonpeptidic inhibitors against β-secretase which would be more likely to be able to reach the target.

With the goal of obtaining inhibitors with enhanced cell penetration, we decided to investigate BACE1 inhibitors from *Sophora flavescens* involving alkylated (C_{10}–C_5) flavanones. *S. flavescens* is one of the most ubiquitous traditional herbal medicines in East Asia, with an array of biological activities such as anticancer,[16] anti-inflammatory,[17] and tyrosinase inhibitory properties.[18] However, to the best of our knowledge, its application to β-secretase inhibition has never been reported. In this paper, we describe the development of low molecular weight BACE1 inhibitors containing a lavandulyl unit appended to a flavanone skeleton through both the cellular and enzymatic assays.

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2. Results

2.1. Isolation of BACE1 inhibitory flavanones

The chloroform fraction of the root of *S. flavescens* was selected for purification through activity (against BACE1)-guided fractionation. The flavanones-containing fractions could be further identified as they exhibit a characteristic dark brown spot on TLC. Repeated silica gel chromatography of this extract furnished five lavandulyl flavanones (**1**, **2**, **5**, **6**, and **8**), two hydrated lavandulyl flavanones (**4** and **7**), and one isoprenyl flavanone (**3**) (Fig. 1A). The spectroscopic data of compounds

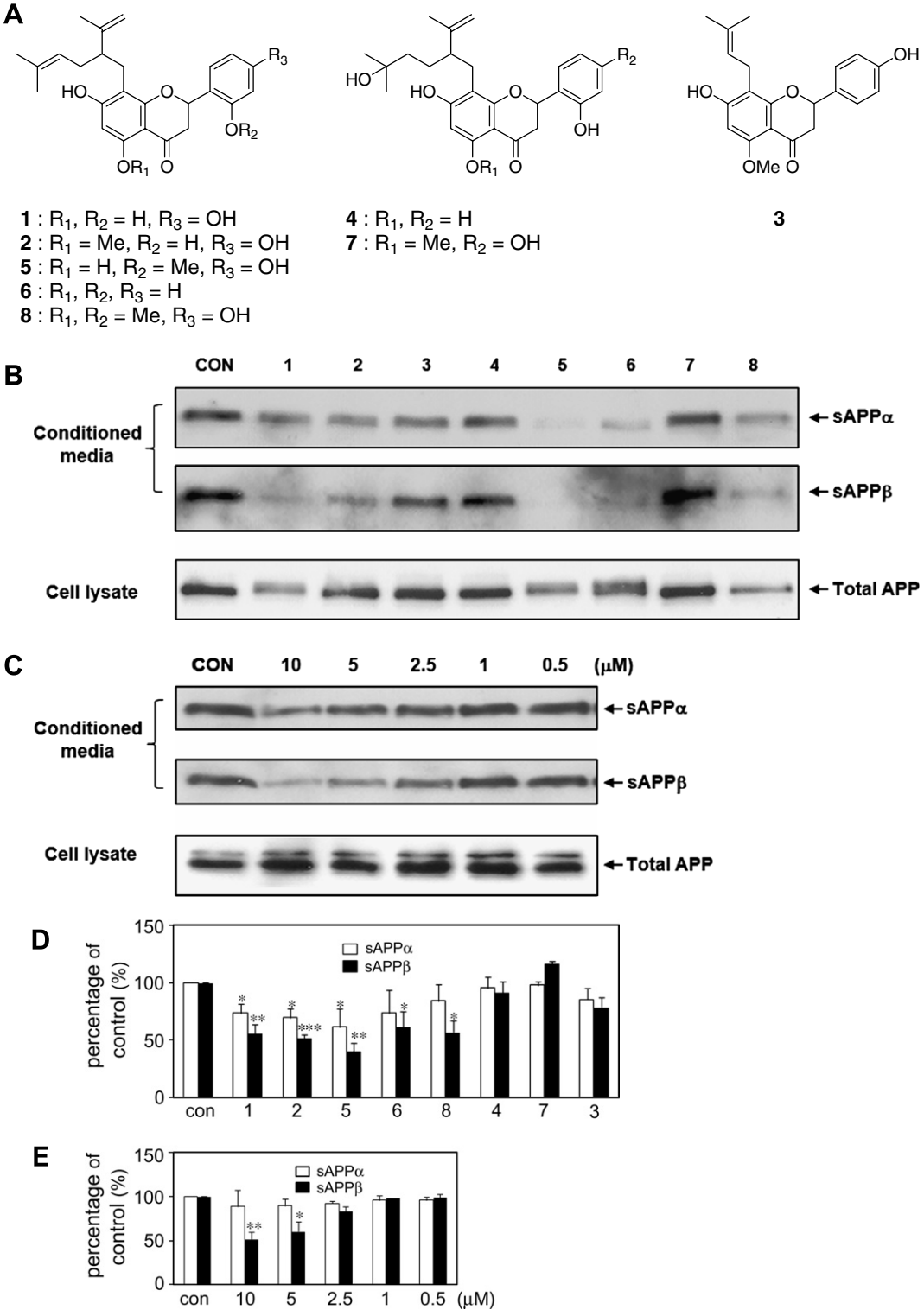

Figure 1. Chemical structures of isolated compounds **1**–**8** from the root of *Sophora flavescens* (A). Detection of sAPPβ and sAPPα in conditioned media using antibodies 6E10 and HB-1 and total APP in cell lysate using antibody MAB343 (B). The dose dependence for secretion of sAPPβ and sAPPα in the indicated concentrations of compound **5** (C). Quantification of multiple immunoblots by densitometric analysis (D,E). The results are means ± SD of three independent experiments. Statistical significance was estimated by ANOVA ($^{*}p < 0.05$, $^{**}p < 0.01$).

(**1**–**8**) agree with those previously published for sophoraflavanone G (**1**), kurarinone (**2**), isoxanthohumol (**3**), kushenol T (**4**), leachianone A (**5**), kushenol A (**6**), kurarinol (**7**), and (*2S*)-2′-methoxy kurarinone (**8**).[16,19–22] All isolated compounds (**1**–**8**) are highly lipophilic as can be expected from inspection of their chemical structures. Computer's calculation of AlogP98[23,24] predicts lavandulyl flavanones (**1**, **2**, **5**, **6**, and **8**) to have values of 5.7, 5.9, 5.9, 6.1, and 6.1, respectively. Prenylated flavanone (**3**) and hydrated flavanones (**4**, **7**) have lower lipophilicities (4.5 to 4.8). The isolated compounds (**1**–**8**) were evaluated for their inhibitory activity on toward BACE1 using both cell culture systems and in vitro assays.

2.2. Effects of isolated flavanone on APP processing in vitro

We initially examined each compound (**1**–**8**) for its inhibition of β-secretase activity using a simple *in vitro* assay involving free BACE1 (Fig. 2). This showed that all compounds are in fact relatively good inhibitors of the enzyme, with lavandulyl flavanones (**1**, **2**, **5**, **6**, and **8**) showing 10-fold greater potency than the other three inhibitors studied. Thus, under conditions where membrane diffusion is not as issue, the side chain on the inhibitor has only a small effect upon the inhibitory capacities of the enzymes.

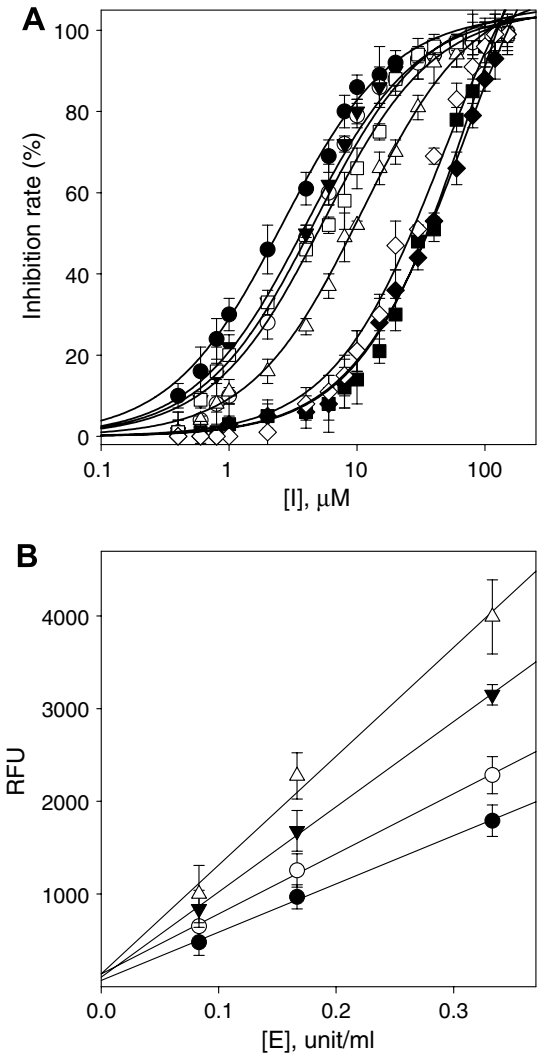

Figure 2. Effect of compounds **1**–**8** on the inhibitory activity of BACE1 (A). Conditions were as follows: 250 nM substrate, 1.0 U/ml β-secretase, 50 mM sodium acetate buffer (pH 4.5), at room temperature. Sophoraflavanone G (○), kurarinone (▼), isoxanthohumol (◇), kushenol T (■), leachianone A (△), Kushenol A (●), kurarinol (♦), and (2*S*)-2′-methoxy kurarinone (□). Relationship of the proteolytic activity of β-secretase with enzyme concentrations at different concentrations of compound **1**. Concentrations of compound **1** for curve from top to bottom: 0, 6, 15, and 30 μM (B).

2.3. Inhibitory activity of isolated flavanone on BACE1 enzyme

We proceeded to examine the efficacy of our inhibitors by monitoring sAPPβ and -α levels in conditioned media by Western blotting, using specific antibodies HB-1 and 6E10, respectively (Fig. 1B). Lavandulyl flavanones (**1**, **2**, **5**, **6**, and **8**) emerged to be the best inhibitors, showing markedly reduced sAPPβ and -α levels. The most potent inhibitor, **5**, was shown to inhibit APP production in a dose-dependent manner in conditioned media (Fig. 1C). Importantly, nonlavandulyl flavanones (**3**, **4**, and **7**) did not show the significant inhibitory effect (Fig. 1B and D). Quantitative analysis of the results of the immunoblot experiments on lavandulylated species showed that secretion of sAPPβ was reduced by approximately 50% relative to the control (**1**; 56.02%, **2**; 51.92%, **5**; 40.66%, **6**; 61.76%, **8**; 51.12%). However, the corresponding analysis on cell lysates showed that the levels of cellular APP did not change significantly under the same conditions. This effect was also independent of dosage (Fig. 1C and E). From these results, it is apparent that the lavandulyl group located at C-8 position in the flavanone B-ring plays an important role in BACE1 inhibitory activity in cells.

2.4. Mechanistic analysis

The inhibition mechanisms displayed by isolated flavanones (**1**, **2**, **5**, **6**, and **8**) were then studied. All inhibitors manifested the same relationship of enzyme activity and enzyme concentration. The inhibition of BACE1 by compound **1** is illustrated in Figure 2B, respectively. Plots of the initial velocity versus enzyme concentrations in the presence of different concentrations of compound **1** gave a family of straight lines, all of which passed through the origin. Increasing the inhibitor concentration lowered the line gradient, indicating that these compounds were reversible inhibitors.

Finally, the kinetic behavior of BACE1 at differing concentrations of compounds (**1**, **2**, **5**, **6**, and **8**) was studied. As illustrated in Figure 3A–E, the inhibition kinetics analyzed by Dixon plots show that compounds **1**, **2**, **5**, **6**, and **8** (K_i = 1.1, 7.2, 3.9, 3.0, and 10.6 μM) are noncompetitive inhibitors because increasing concentration of substrate resulted in family lines which declined with a common intercept on the *x*-axis, it means $-K_i$.

3. Discussion

In this paper, we have attempted to develop nonpeptidic small molecular BACE1 inhibitors from *S. flavescens*. We isolated eight lipophilic compounds consisting of alkylated (C_{10}–C_5) flavanones and examined their inhibitory effects against BACE1 using stable HEK293 cells. Interestingly, compounds **1**, **2**, **5**, **6**, and **8** did efficiently reduce secretion of sAPPβ but did not induced any significant changes in intracellular full-length APP levels, indicating that the latter was largely unaffected by the presence of the inhibitor. Compounds **3**, **4**, and **7** did not significantly reduce secretion of APPβ under the same cells.

As shown in Table 1, all flavanones exhibited a significant degree of β-secretase inhibition (IC_{50} 2.6–39.2 μM) in vitro BACE activity assays. These activities were significantly affected by subtle structural changes. The least active flavanone **7** (IC_{50} = 39.2 μM) differed from one of the most effective inhibitors screened, flava-

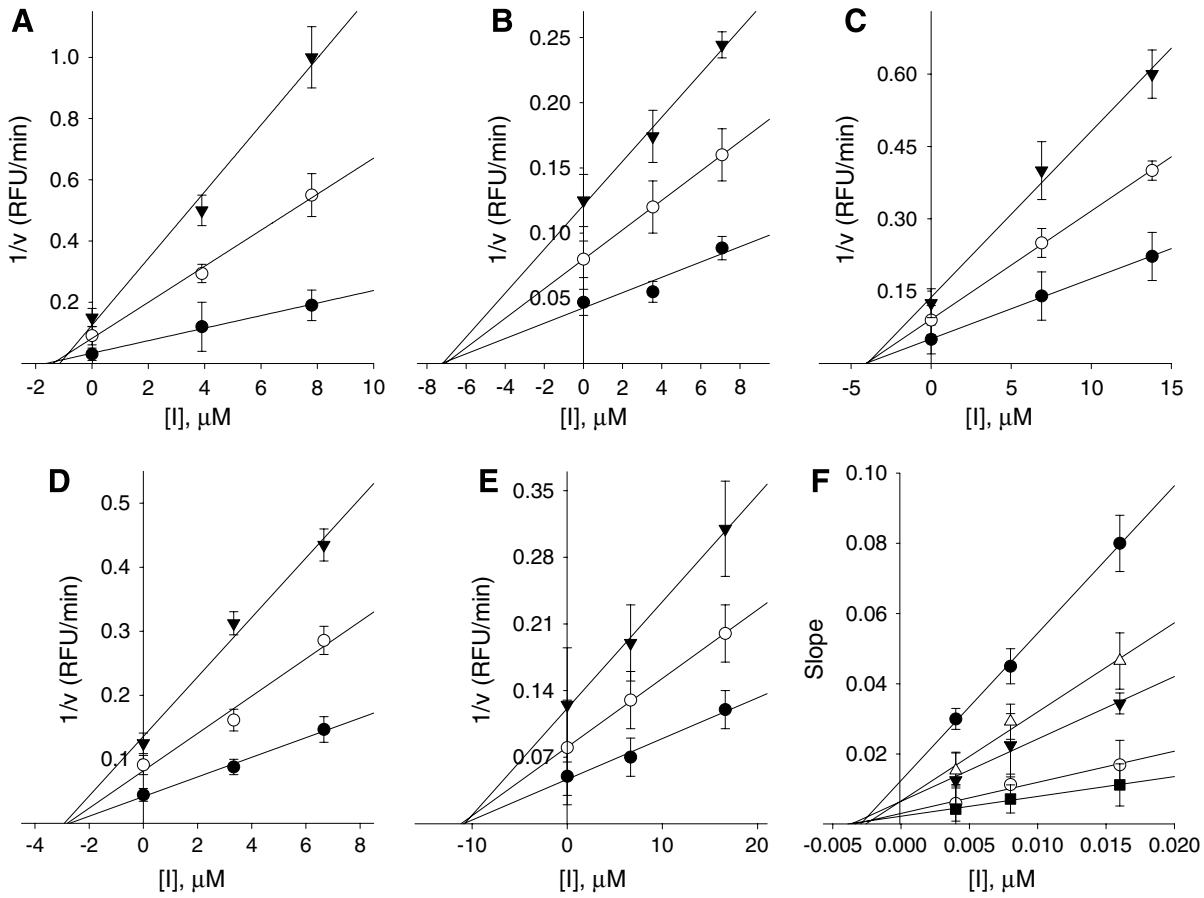

Figure 3. Dixon plots for inhibition of compounds **1**, **2**, **5**, **6**, and **8** on β-secretase for the proteolysis of substrate: compound **1** (A), compound **2** (B), compound **5** (C), compound **6** (D), and compound **8** (E). In the presence of different concentrations of substrate: 250 nM (●), 125 nM (○), and 62.5 nM (▼). (F) Slope replots: compound **1** (●), compound **2** (○), compound **5** (▼), compound **6** (△), and compound **8** (■).

Table 1
Inhibitory effects of isolated compounds **1**–**8** on human β-secretase activities

Compound	IC_{50} (μM) values[a]	Inhibition type (K_i)
1	5.2 ± 1.8	Noncompetitive (1.1 ± 0.23)
2	3.3 ± 2.0	Noncompetitive (7.2 ± 0.3)
3	27.7 ± 0.8	ND[b]
4	36.8 ± 7.5	ND
5	8.4 ± 3.1	Noncompetitive (3.9 ± 0.49)
6	2.6 ± 1.2	Noncompetitive (3.0 ± 0.11)
7	39.2 ± 3.6	ND
8	6.7 ± 2.2	Noncompetitive (10.6 ± 0.52)

[a] All compounds were examined in a set of experiments repeated three times.
[b] Not determined.

none **2** (IC_{50} = 3.3 μM), only by the fact that its lavandulyl appendage was hydrated. On the other hand, the set of lavandulyl flavanones (**1**, **2**, **5**, **6**, and **8**) were of almost equal efficiency with IC_{50} values lower than 10 μM. Furthermore, the inhibitory activity of prenyl flavanone **3** was ten times lower when compared to lavandulyl flavanones **1**, **2**, **5**, **6**, and **8**. Thus, it can be said that with the exception of the lavandulyl group at C-4″, other functionalities within the flavanone skeleton show significant tolerance. Thus, the lavandulyl appendage appears to play a pivotal role in the inhibition, which seems to be more subtle than simply functioning as a hydrophobic tag.

When we moved on to investigate the inhibition of β-secretase in cells we garnered further interesting data. The lavandulyl moiety within the flavones became even more paramount to inhibitor function as hydrated flavanones (**4** and **7**) and the prenylated analog (**3**) showed no reduction in APP levels in conditioned media. On the other hand, all lavandulylated analogs were able to reduce extracellular APP levels significantly. This accordingly gives good evidence that the lavandulyl motif will make a good basis for drug development.

In general there was a reasonable correlation between experiments performed in vitro and in cells, especially when error is taken into account. Any discrepancies may be ascribed to the increased importance of lipophilicity in cell-based assays. For instance compound **5** was found to be most effective in the cell-based assay, even though it was relatively less potent in in vitro experiments.

Kinetic analysis of the inhibitors, as depicted in Figures 2 and 3, elucidated a typical progress curve for reversible, noncompetitive behavior. A replot of slope versus the corresponding 1/[S] (Fig. 3F) is a straight line with a slope of $K_m/V_{max}K_i$ and an intercept of $1/V_{max}K_i$ on the *y*-axis, this result provides further evidence that lavandulyl flavanones (**1**, **2**, **5**, **6**, and **8**) exhibit noncompetitive inhibition.

In conclusion, our data suggest that lavandulyl flavanones can penetrate cells and effectively reach their target, BACE1, eliciting site-specific inhibition. The IC_{50} values of our inhibitors, although higher than peptide-derived transition state analog-based BACE1 inhibitors are none-the-less still in the low micromolar range. Given their enhanced lipophilic potency in cell, we believe that our compounds **1**, **2**, **5**, **6**, and **8** may be effective therapeutic reagents for further drug development in Alzheimer's disease.

4. Materials and methods

4.1. Materials

Fluorescence was measured with a HIDEX microplate fluorescence reader Plate CHAMELEON™V (Finland). BACE1 (recombinant human BACE1) assay kit was purchased from the PanVera Co., USA. AlogP98 was calculated by Discovery Studio 2.0 (Accelrys Co.). ^{1}H and ^{13}C NMR spectra were measured downfield relative to TMS in either $CDCl_3$, CD_3OD, or acetone-d_6 as indicated. 2D NMR data were obtained on a Varian Inova-500 spectrometer and Bruker DRX-500.

4.2. Isolation of BACE1 inhibitors from *S. flavescens*

Sophora flavescens was collected in Hamyang (Korea) and identified by Prof. Myong Gi Chung. A voucher specimen (Park, K.H. 112) of this raw material is deposited at Herbarium of Gyeongsang National University (GNU). Dried roots of *S. flavescens* (2 kg) were repeatedly extracted with chloroform at room temperature. The extract was then further purified via activity-guided purification and repeated column chromatography on silica.

4.3. Analyses of sAPPα, sAPPβ, and full-length APP

Western blot analysis of sAPPα, sAPPβ, and full-length APP were performed as previously described with slight modification.[25] Human embryonic kidney (HEK 293) cells, which stably express human wild type APP695 and BACE1 (BA-3), were used for the analyses of APP metabolites, sAPPα (a soluble and extracellular N-terminal fragment of APP cleaved by α-secretase), sAPPβ (a soluble and extracellular N-terminal fragment of APP cleaved by β-secretase), and full-length APP. The cells were maintained in DMEM supplemented with 10% FBS. The cells were grown on 35-mm tissue culture dishes in a 5% CO_2 incubator at 37 °C, until they became 80–100% confluent. The cells were then washed once with serum-free DMEM, and then serum-free DMEM with or without the isolated compounds (**1**–**8**) then was added. This mixture was then cultured for 4 h. To analyze sAPPα and -β in the conditioned medium, the medium was collected, and precipitated with 20% Trichloroacetic acid (TCA). Cell extracts were prepared for full-length APP. The cells were harvested in cold PBS, re-suspended in a lysis buffer (50 mM Tris–HCl, pH 7.6, 180 mM NaCl, 2 mM EDTA, 1% Triton X-100) and kept on ice for 30 min in the presence of protease inhibitor cocktail. After brief sonication, extracts were clarified by centrifugation at 14,000 rpm at 4 °C. The supernatant was recovered and protein concentration was determined by BCA assay. Cell extracts were subjected to Western blot analysis. sAPPα and -β were detected using primary antibodies, 6E10 (CHEMICON International Inc., CA, USA) and HB-1[26] which recognize the C-terminus of sAPPα and -β, respectively. The detection of full-length APP was performed using MAB343 (CHEMICON International Inc., CA, USA) as a primary antibody. All blots were probed with either anti-rabbit or anti-mouse horseradish peroxidase-conjugated (Amersham Bioscience, UK) secondary antibodies as required and detected by chemiluminescence (ECL, Amersham Bioscience, UK).

4.4. BACE1 enzyme assay

The assay was carried out according to the supplied manual with modifications.[27] 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 30% DMSO was incubated for 60 min at room temperature in the dark. The mixture was irradiated at 545 nm and the emission intensity at 590 nm was recorded. The inhibition ratio was obtained by the following equation:

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

where C was the fluorescence of the control (enzyme, buffer, and substrate) after 60 min of incubation, C_0 was the fluorescence of control at zero time, S was the fluorescence of the tested samples (enzyme, sample solution, and substrate) after incubation, and S_0 was the fluorescence of the tested samples at zero time. To allow for the quenching effect of the samples, the sample solution was added to the reaction mixture C, and any reduction in fluorescence by the sample was then investigated. All data are the mean of three experiments.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.05.080.

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