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Inhibition and structural reliability of prenylated flavones from the stem bark of *Morus lhou* on β-secretase (BACE-1)

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ABSTRACT

The action of β-secretase is strongly tied to the onset of Alzheimer's disease. The development of inhibitors of β-secretase is thus critical to combating this disease, which threatens an ever increasing number of the population and grows in importance as the population ages. Herein we show that flavones from *Morus lhou* potently inhibit β-secretase. Our aim in this manuscript is to explore the inhibitory kinetics of natural compounds and develop a phamacophore model which details the critical features responsible for inhibitory activity. The IC_{50} values of compounds for β-secretase inhibition were determined to range between 3.4 and 146.1 μM. Prenylated flavone **2** (IC_{50} = 3.4 μM) was 20 times more effective than its parent compound, noratocarpetin **1** (IC_{50} = 60.6 μM). The stronger activity was related with resorcinol moiety on B-ring and isoprenyl functionality at C-3. Kinetic analysis shows that the four effective compounds (**1**–**4**) have a noncompetitive mode of action. The binding affinity of flavones for β-secretase calculated using in silico docking experiments correlated well with their IC_{50} values and noncompetitive inhibition modes.

Herein, we report inhibition and structure/activity analysis of flavones from stem bark of *Mous lhou* on β-secretase, an enzyme strongly implicated in the onset of Alzheimer's disease. Alzheimer's disease (AD) is a progressive, neurodegenerative disorder with no current cure and is the leading cause of dementia and death in elderly people.[1] As the average age of the population increases, the impact of this disease will continue to grow. It has been proposed that pathogenesis of this disease can be ascribed to the formation of amyloid plaques.[1,2] This hallmark signature of AD is formed by proteolysis of amyloid precursor protein (APP) by secretase enzymes (α, β, and γ) generating peptide fragments. The most relevant of these to AD are Aβ40 and Aβ42. Therapeutic efforts to target AD have focused on disruption of this cascade via γ- and more recently β-secretase inhibition.[3] Encouragingly, in 2001 β-secretase knockout mice were shown to be devoid of β-secretase activity.[4] Most importantly, these mice did not generate Aβ, and displayed a relatively normal phenotype.

β-Secretase (β-site APP cleaving enzyme or BACE-1) has been shown to be a membrane-bound aspartyl protease. The cleavage of APP by BACE-1 occurs in the lumina and is considered to be the rate-limiting step in the processing of APP to Aβ peptide.[3,5]

A strategy frequently employed in designing BACE-1 inhibitors is replacement of the scissile amide bond of APP with noncleavable transition state isosteres such as statine, hydroxyethylene, norstatine, or hydroxyl-ethylamine (HEA), all of which showed nanomolar affinity for BACE-1.[6–8] However, all drugs considered for Alzhemier's disease treatment must cross the blood–brain–barrier (BBB) and the plasma membrane.[9] Accordingly, enzyme inhibitors

Table 1
Inhibitory effects of tested compounds **1**–**8** on BACE-1 activities

Compd	IC_{50}[a] (μM)	Inhibition type (K_i, μM)
1	60.6 ± 2.7	Noncompetitive (82.6)
2	3.4 ± 1.3	Noncompetitive (2.2)
3	59.4 ± 12.7	Noncompetitive (64.1)
4	5.3 ± 0.2	Noncompetitive (10.6)
5	101.2 ± 5.3	ND[b]
6	135.9 ± 22.8	ND
7	146.1 ± 7.1	ND
8	103.5 ± 17.4	ND
Morin[c]	12.3 ± 1.4	ND

[a] All compounds were examined in a set of duplicated experiments; IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss.
[b] Not determined.
[c] This compound was used as a positive control.

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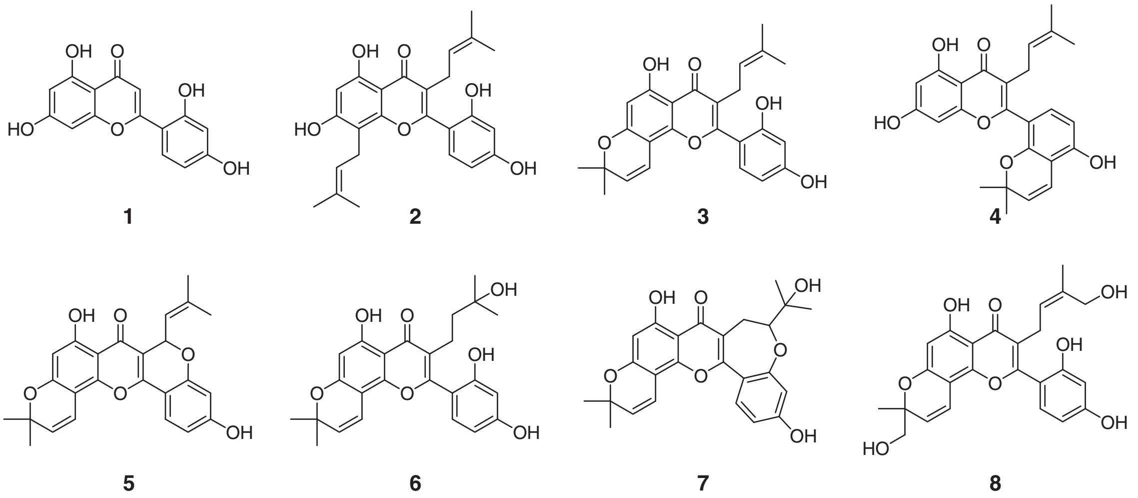

Figure 1. Chemical structures of isolated compounds (**1**–**8**) from the stem bark of *Morus lhou*.

with good therapeutic potential are preferably smaller than 700 Da, rendering large/hydrophilic peptide-based inhibitors of low viability in vivo. Thus, the secondary metabolites of plants and microbes which have relatively low-molecular weight and high lipophilicity may be good drug candidates for BACE-1 inhibitors.[10]

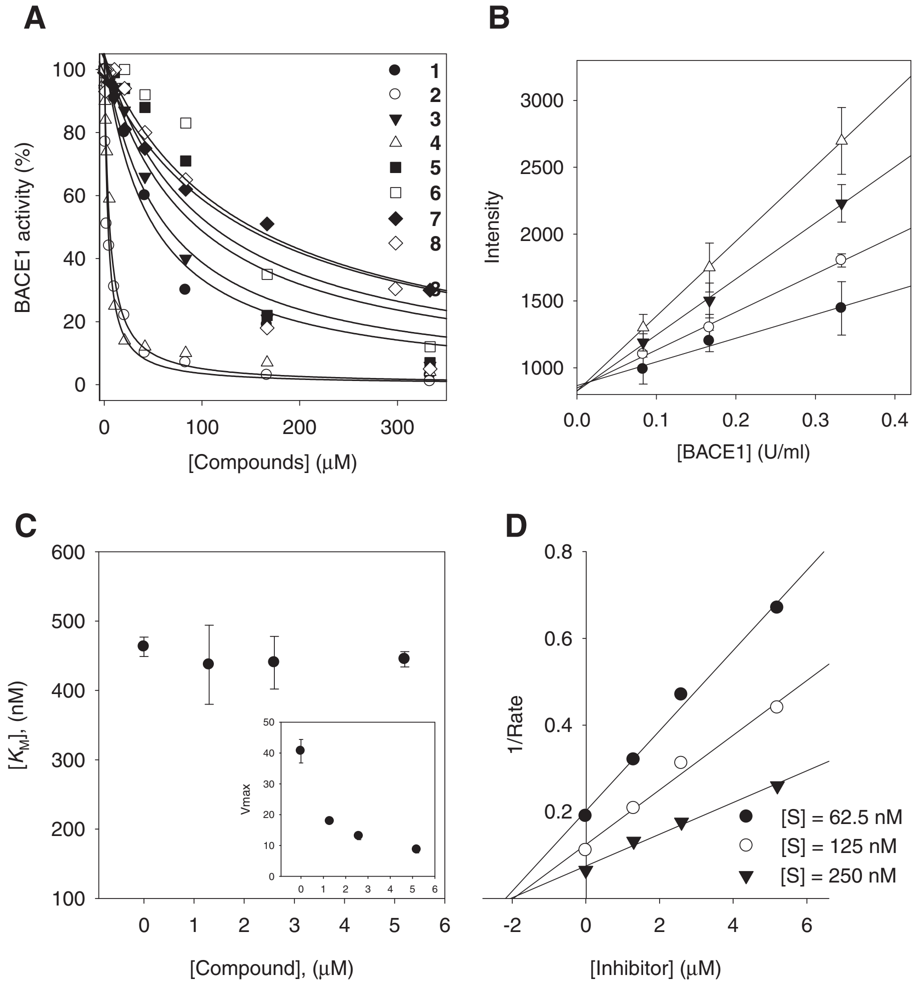

Figure 2. (A) Effect of flavones **1**–**7** on the activity of aspartyl protease BACE-1. Concentration-dependent inhibition of BACE-1 by flavones. (B) The hydrolytic activity of BACE-1 as function of enzyme concentrations at different concentrations of compound **2**. (C and D) Mechanistic analysis of prenylated flavone **2** on BACE-1. (C) The K_M values as a function of the concentration of prenylated flavone **2**. (Inset) Dependence of the values of V_{max} on the concentration of prenylated flavone **2**. (D) Dixon plots for the inhibition of prenylated flavone **2** on the aspartyl protease activity of BACE-1.

In recent years, we have become aware of the potency of alkyl substituted flavonoids as β-secretase inhibitors. Lavanduluylated flavanones achieved excellent BACE-1 inhibitory activity in both in vitro and in a cell based assay.[11]

We thus started to explore β-secretase inhibitors from *M. lhou* because it is a rich sourse of prenylated flavones and a ubiquitous traditional herbal medicine. It is also permitted as an ingredient of functional foods in many countries. For example, its young leaves and twigs have been classified as edible by KFDA (Korea Food Drug Administration). Its main bioactive constituents are flavonoids, cumarins, and terpenoids. Extracts of this plant have been reported to exhibit a broad spectum of pharmaceutical effects including neuroprotection and hypoglycemic, hypertension, antimicrobial, as well as anti-inflammatory properties. Recently, the flavonoids in this plant have been reported to inhibit the enzymatic oxidation of tyrosine to melanin.[12]

Methanol extracts of *M. lhou* stem bark exhibited significant BACE-1 inhibitory activity with an IC_{50} of 78.4 μg/mL. Through activity guided-fractionation of the stem bark of *M. lhou*, we isolated eight BACE-1 inhibiting flavones, the majority of which were prenylated. These compounds were identified as norartocarpetin (**1**), kuwanon C (**2**), morusin (**3**), kuwanon A (**4**), cyclomorusin (**5**), morusinol (**6**), neocyclomorusin (**7**), and mormin (**8**) using spectroscopic data including 2D-NMR (Fig. 1).[11,12]

The ability of compounds **1**–**8** to inhibit the cleavage of APP to Aβ was assessed using a fluorescent resonance energy transfer (FRET) peptide cleavage assay.[10,11] Herein BACE-1 proteolysis of a synthetic oligopeptide substituted with a fluorescent dye and a quencher leads to an increase in fluorescence by breaking the tether between the dye and the quencher. The FRET oligopeptide substrate, Rh-EVNLDAEFK-Quencher, and recombinant human BACE-1 were purchased from RD System, Inc. Authentic morin was used as a positive control (IC_{50} = 12.3 μM). The assay was carried out according to the supplied manual with small modifications. The inhibitory concentration (IC_{50}) values (μM) are presented in Table 1.

All isolated compounds showed dose-dependent, reversible inhibition of BACE-1 activity. As shown representatively in Figure 2A and B as the concentration of compound **2** was increased, the enzyme activity rapidly diminished and resulted in lowering of the slope of the lines.

Efforts to understand the structure–activity relationships of the lead molecule started with the resorcinol moiety. As shown in Table 1, the efficacies of these compounds varied across almost two orders of magnitude (3.4 μM < IC_{50} < 146.1 μM). Consistent with our previous observations and predictions, prenylated species were more effective than unsubstituted analogues (**2** and **4** vs **1**). Compound **2**, bearing prenyl groups at C3 and -8, showed the strongest inhibitory activity (IC_{50} = 3.4 μM) of BACE-1, whereas the corresponding parent compound **1** (IC_{50} = 60.6 μM) was much less effective. Importantly increasing the hydrophilicity of the pendant prenyl groups by hydroxylation (**3**–**6**) led to a decrease in potency.

It further seems apparent that compounds in which both hydroxyl functions in the resorcinol ring (ring B) are unsubstituted (**2** and **4**) exhibited a significant level of activity, with IC_{50} values of 3.4 and 5.3 μM, respectively. Whereas compounds bearing alkyl substitution therein (**3**, **5**–**8**) showed lower activities (IC_{50} values of 59.4, 101.2, 135.9, 146.1, and 103.5 μM, respectively).

Furthermore, ring-closure of the prenyl group onto the C7-OH to form pentacyclic structures (**5** and **7**) resulted in lower potency, indicating that a free C7-hydroxyl group is essential for the activity and/or that rings A and C adopt a conformation in the active site which cannot be obtained in the pentacyclic analogues.

In sum the best inhibitors possessed both a free resorcinol group and at least one prenyl motif at C3 in the A-ring. We thus predict that these motifs will interact strongly with the enzyme, which we investigate below.

All of the reported inhibitors from natural products possess aromatic rings, including biphenyl, stilbene, and benzopyran/furan, together with alkylated flavonoids are noncompetitive. We also tested the effect of prenylated flavones (**1**–**8**) on the kinetics of BACE-1 hydrolysis of the FRET substrate. As shown representatively in Figure 2C and D, the kinetic plots show that compound **2** has a noncompetitive inhibition profile. Because the Lineweaver–Burk plot ($1/V$ vs $1/[S]$) shows a family of straight lines with the same *x*-axis intercept (K_M and decreasing V_{max} as the concentration of **2** increases (Fig 2C). The inhibition kinetics were also analyzed by Dixon plots, ($1/V$ vs $[I]$ with varying concentrations of substrate). These gave a family of straight lines passing through the same point at the second quadrant (Fig. 2D). The K_i values in Table 1 were calculated from the Dixon plots.

Encouraged by the above results, we employed in silico docking simulations for the interaction of human BACE-1 with the isolated flavones. Molecular docking with BACE-1 was simulated and analyzed using AUTODOCK 3.0.5 software.[13–15] Our modeling calculations unveiled that each effective compound (**1**–**4**) had a preferred binding site which was not the active site (consistent with the noncompetitive inhibition mode, see Supplementary data). Figure 3 represents the binding pattern of the most potent compound, **2**. In this analysis, the OH present in the resorcinol moiety of the B-ring formed hydrogen bonds with the OH of THR231 (4.1023 Å), THR329 (3.525 Å) and ASP228 (2.640 Å) (Fig 3A).

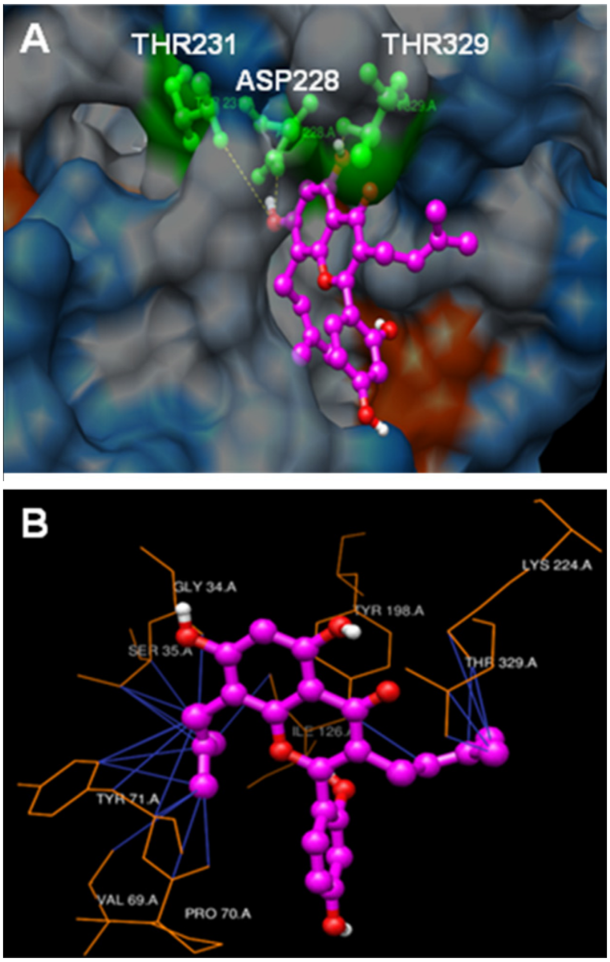

Figure 3. (A) Kuwanon C (**2**, IC_{50} = 3.4 μM) in the binding subsite of BACE-1. (B) Interaction of prenyl groups of (**2**) with surrounding amino acid residues in BACE-1.

Importantly these key interactions would be broken in analogues in which the hydroxyl groups of the resorcinol moiety were alkylated (**3**, **5**–**8**) and would lead to a reduction in binding energy. This is consisten with our SAR analysis. Moreover, the two prenyl groups in kuwanon C (**2**), which we identified as crucial for high activity, make hydrophobic interactions with surrounding amino acids; GLY34, SER35, VAL69, PRO70, TYR71, ILE126, TYR198, LYS224, and THR320, respectively (Fig. 3B). The potencies of the inhibitors norartocarpetin (**1**) and kuwanon C (**2**) correlated well with their binding energies: norartocarpetin (**1**) = −8.38 kcal/mol; kuwanon C (**2**) = −10.63 kcal/mol.

These differences in binding orientation and energy apparently explain the 40-fold smaller K_i value of kuwanon C (**2**) toward human BACE-1 compared with norartocarpetin (**1**). Thus, this docking experiment supports the inferences drawn from the enzymatic assay, revealing an important inhibitory action of prenylated flavones on human BACE-1.

The need to find new small molecules that have good BACE-1 inhibitory activities is ever growing, as this enzyme is a key player in the onset of Alzheimer's disease. Importantly, small hydrophobic inhibitors of BACE-1 <700 Da are expected to perform better in vivo than peptide/substrate analogues since to be effective in the body, the compound must cross the blood brain barrier. We expected that *M. lhou* would be a rich source of hydrophobic BACE-1 inhibitors since it is rich in prenylated compounds, and we have recently shown that prenylation of small molecule BACE-1 inhibitors can improve their potency.[11] Pleasingly, our research validates this hypothesis and has uncovered some low micromolar inhibitors of BACE-1. We further were able to uncover some important aspects of the SAR of these compounds, including the need for an A ring resorcinol moiety bearing two free hydroxyl groups, and the positive effect of prenylation upon inhibition.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.060.

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