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Discovery of a series of selective and cell permeable beta-secretase (BACE1) inhibitors by fragment linking with the assistance of STD-NMR

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ABSTRACT

Two β-secreatase (BACE1) inhibitors from natural products (cinnamic acid and flavone) were linked to furnish potent, cell permeable BACE1 inhibitors with noncompetitive mode of inhibition, with the assistance of saturated transfer difference (STD)-NMR technique. Some of these conjugates also exhibited selective BACE1 inhibition over other aspartyl proteases such as BACE-2 and renin, as well as poor cytotoxicity. Taken together, conjugates **4** represent a new series of BACE inhibitors warrants further investigation for their potential in Alzheimier's disease therapy.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease which frequently occurs in the elderly people. It is pathologically characterized by the presence of extracellular senile plaques consisting of amyloid-beta (Aβ) deposits, and intracellular neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau protein aggregates, and the ultimate neuronal function impairment or cell death. The incidence of sporadic AD is continuously growing as the aging population increases, especially in the developed countries, thus making AD one of the most serious diseases to elderly people [1].

In the most widely accepted β-amyloid cascade hypothesis for AD pathogenesis, among many other mechanisms, a peptide consisting of 37–43 amino acids, Aβ, is proposed to aggregate into soluble oligomers and fibrils which are toxic to neurons and cause neuronal death. Another protein tau can also be a causative factor independently, presumably acting downstream of Aβ [2].

Aβ is generated by the sequential endoproteolytic cleavage of amyloid precursor protein (APP), a transmembrane protein expressed in many tissues and organs, by β-secretase [3] (BACE1) and γ-secretases in the brain. As BACE1 catalyzes the rate-limiting reaction in Aβ biogenesis, and BACE1 gene knockout mice lacking Aβ production are viable and fertile [4], this protein is regarded as a promising therapeutic target for AD. The expectation to halt or even prevent AD by targeting BACE1 and subsequent modulation on Aβ, makes it more attractive than some other symptom-modifying targets, such as acetylcholinesterase (AchE) and *N*-methyl D-aspartate (NMDA) receptor [5].

As such, BACE1 inhibitors have been actively pursued for more than a decade, and the availability of several crystal structures for ligand-BACE complex released since early 2000 s [6] has speeded up the discovery and optimization of BACE1 inhibitors, especially those active-site directed peptidomimetic inhibitors. Most of those peptide-based inhibitors are competitive inhibitors which also suffer from unfavorable pharmacokineics properties, particularly poor blood brain barrier (BBB) permeability and low oral bioavailability, preventing them to be promising drug candidates [7,8].

Consequently, many non-peptide BACE1 inhibitors were pursued, some of which have entered clinical trials at different stages, e.g. MK-8931 and AZD-3293 at phase III trials [9]. However, the diverse toxicity found in several clinical trials became a serious problem and thus

Abbreviations: AD, Alzheimer's disease; BACE1, beta site amyloid precursor protein cleaving enzyme 1 or beta-secretase; FRET, fluorescence resonance energy transfer; STD, Saturated Transfer Difference; NP, natural product

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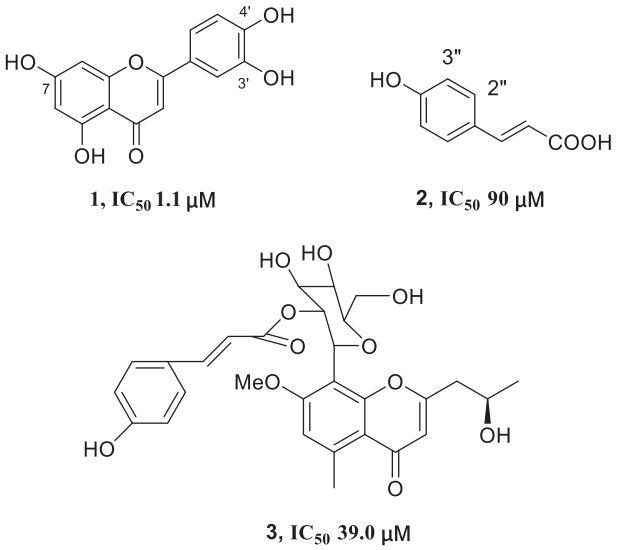


Fig. 1. Structures of BACE1 inhibitory natural products and their IC_{50} inhibitory BACE1 in references.

delayed the progress of drug development [10,11]. The toxicity has arisen from, at least for some of the drug candidates, the indiscriminate blocking of almost all BACE1 functions by the active site directed inhibitors [3].

Unlike the competitive inhibitors, non-competitive inhibitors binding to allosteric sites may selectively inhibit specific function of an enzyme. It is interesting to note that most natural product (NP) derived BACE1 inhibitors with known mechanisms exhibit non-competitive mode of inhibition, while a small portion of such inhibitors exhibit the mixed mode of inhibition [12]. Such NP-based BACE1 inhibitors with weak to moderate activity are of particular interest during our pursuit to novel non-competitive inhibitors.

The major hurdle for the rational improvement of non-competitive inhibitors is the structure for ligand-BACE1 complex is not available, so we sought the help from a NMR technique, saturation transfer difference (STD). STD-NMR has emerged as a powerful screening tool and a straightforward way to study the binding epitopes of small molecules [13,14].

Luteolin (**1**)[15], *p*-hydroxy-cinnamic acid (**2**)[16] and chromone glycoside AV-4-1 (**3**)[17] are several BACE1 inhibitory NPs found by our and other labs (Fig. 1). During our exploration of their binding epitopes in complex with BACE1 by STD-NMR, we found **1** and **2** could bind simultaneously to the BACE1 protein (Fig. 2), whereas **1** and **3** not. This observation gave us the hint that the binding of **3** to BACE1 is tolerated (although its activity is not only moderate, e.g., IC_{50} 10^{-5} M), whereas the linking of **1** and **2** as fragments at proper position (northern/northwestern part of the flavone **1**, based on the reduced but retained BACE1 inhibitory activity of the same core but more complex chromenone glycoside **3**) and with an appropriate linker may result in more potent and possible non-competitive BACE1 inhibitors.

(A) ^{1}H NMR of 1 (0.5 mM) with BACE1 (10 μM); (B) STD-NMR of **1** (0.5 mM) with BACE1 (10 μM); (C) ^{1}H NMR of **2** (2 mM) with BACE1 (10 μM), (D) STD-NMR of **2** (2 mM) with BACE1 (10 μM); (E) STD-NMR of **1** (0.8 mM) and **2** (2 mM) with BACE1 (10 μM)

In this work, we designed the conjugates (Fig. 3) through ω-amino acids and polyethyleneglycols of different length as linkers. Interestingly, some potent and cell permeable inhibitors with poor cytotoxicity and good enzyme selectivity were found, and their non-competitive mode of inhibition confirmed.

2. Results and discussion

To link two BACE1 binding segments, **1** and **2**, we first examined the STD effect of these two compounds. The effect is almost indistinguishable for different positions on **2**, suggesting the surrounding of

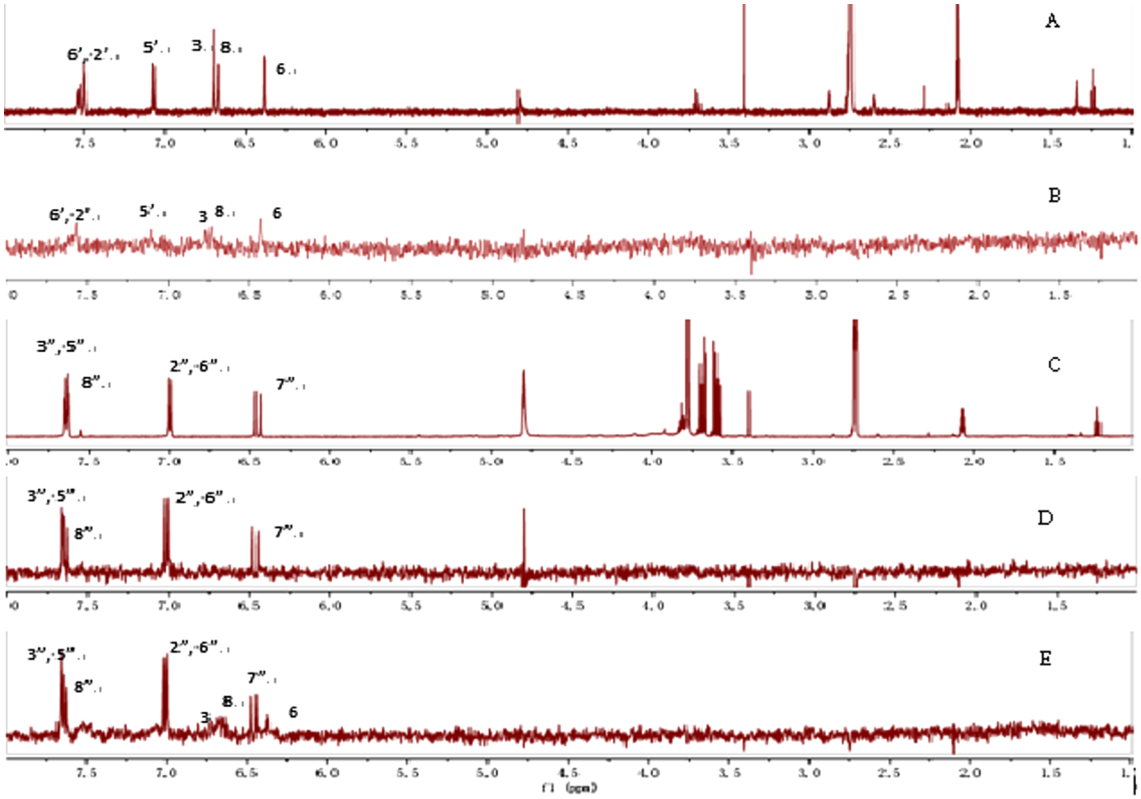


Fig. 2. ^{1}H NMR and STD-NMR spectra of **1** and **2**.

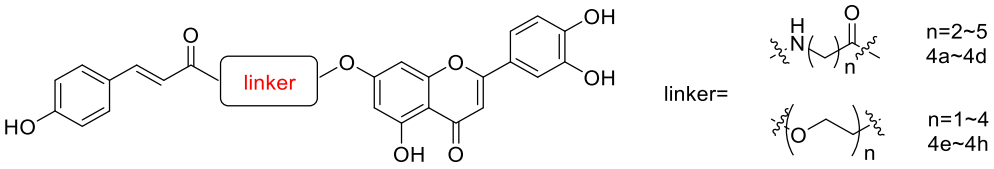


Fig. 3. The design of conjugates **4.**

this very small molecule by the protein. In contrast, stronger STD effect was observed for C-3 and C-6 hydrogens than other part of this molecule in **1**, implicating a shorter distance between these positions in the inhibitor and BACE1 protein. We thus chose to tether 7-OH in the upper part of **1** to the carboxyl group in **2** through the ω -amino acid linkers of different length.

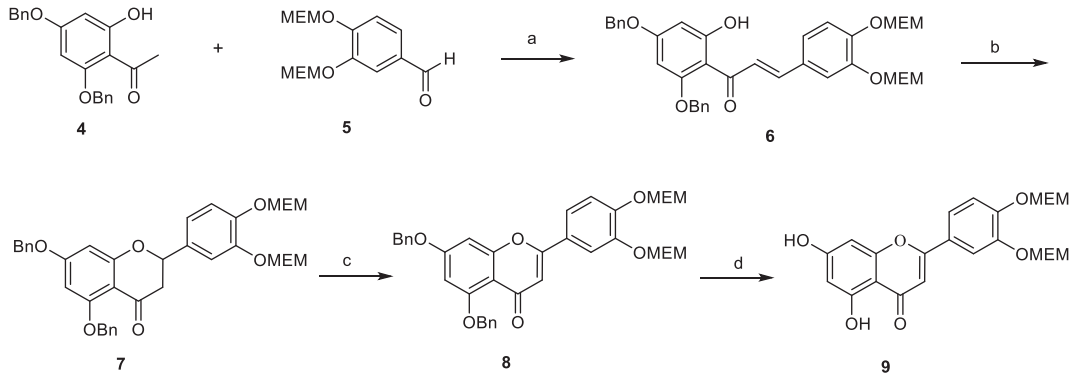
The synthetic routes for conjugates **4a-d** were outlined in schemes 1 and 2. One building block of the conjugate from **1**, 3′,4′-dimethoxyethyloxymethyl (MEM) luteolin (**9**), was prepared by a modified method of literature [18] (Scheme 1). The benzyl protected trihydroxybenzoacetophenone **4** and MEM protected dihydroxybenzaldehyde **5** were condensed to chalcone **6**, which was then cyclized, oxidized and deprotected to furnish **9**.

The other building block from **2**, 4-MEM-*O*-cinnamic acid (**10**), was synthesized over three steps. By the coupling of **9** with different ω -amino acid methyl esters, *N*-amido ω -amino acid methyl esters **12a-d** were furnished. Intermediates **12a-d** were then hydrolyzed to remove methyl ester without breaking the amide bond, coupled with **9** and deprotected by trimethylsilyl iodide (TMSI) to remove MEM protective group, affording desired conjugates **4a-d** (Scheme 2).

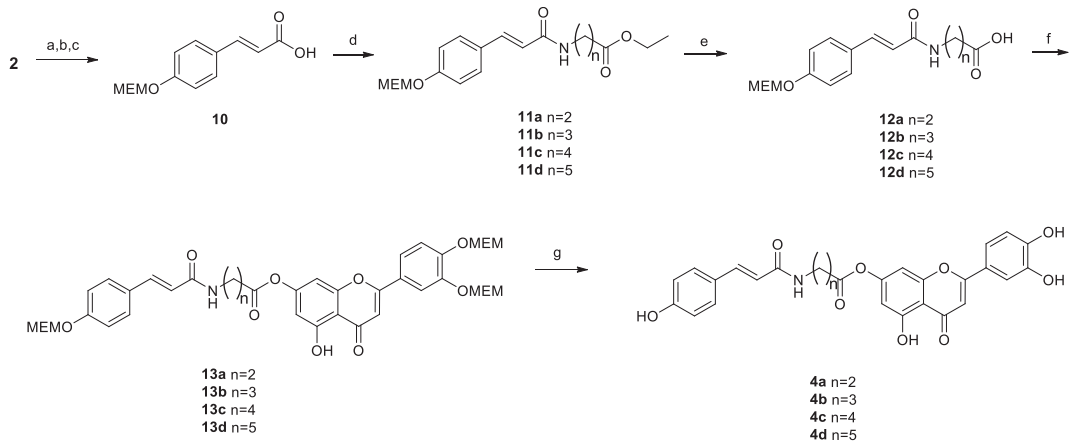
Conjugates **4a-d** were measured for their BACE1 inhibitory activity. Whereas the inhibition rate at 10 μM increased as the length of linker became longer, the inhibition rate of the conjugate **4d** with longest linker (n = 5) decreased to 1.9%. Among these conjugates, **4c** with 4-aminobutyric acid linker is most active.

Although all of the tested conjugates were weakly active, we wondered whether inhibitory activity of most active conjugate **4c** could be improved through the incorporation of other linkers. In addition, their poor water solubility prevented the IC_{50} measurement as those compounds of 100 μM were not soluble in the assay buffer. Hence, we designed and synthesized another series of conjugates by incorporating the ethyl glycol to reduce the rigidity of the linker and increase the water solubility, and also extend the length of linker to maximal four ethyleneglycols to explore the optimal distance between these two plausible binding sites occupied by **1** and **2**.

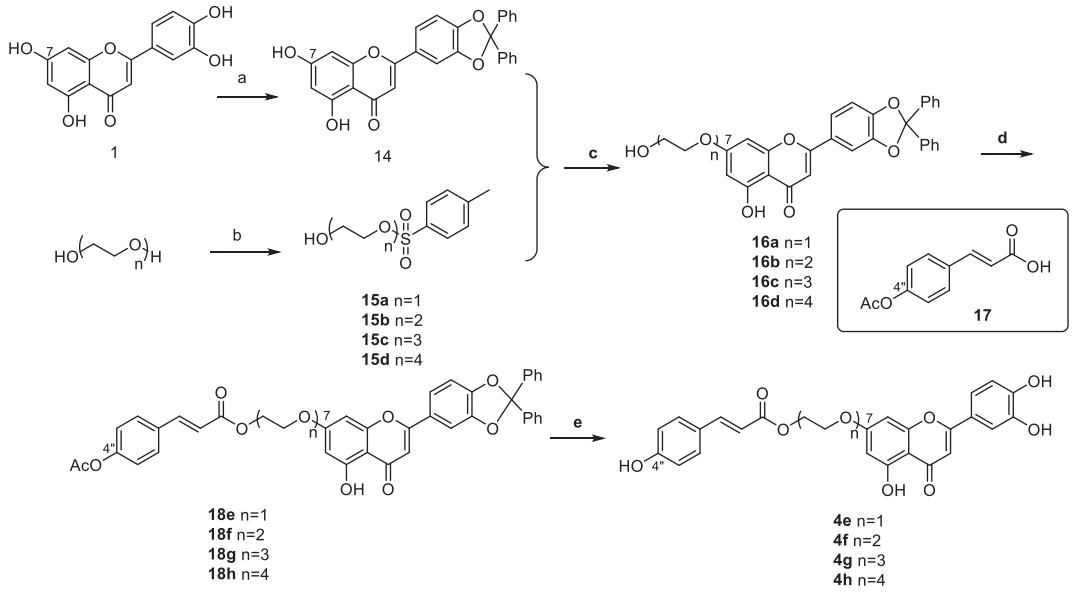
The diphenylmethylidene was chosen as the protective group for luteolin in **14**, which was treated with different glycol tosylate to introduce glycol into 7-position furnishing **15**. The terminal hydroxyl group in **16** was esterified with 4″-acetyl cinnamic acid (**17**), and then deprotection under acidic conditions to afford desired conjugates **4e-h**



Scheme 1. Synthesis of intermediate **9.** Reagents and conditions: a. 20% KOH aq. ethanol, room temperature, 54%; b. NaOAc, H_2O, ethanol, reflux, 46%; c. I_2, pyridine, 100 °C, 69%; d. H_2, Pd/C, EtOAc, room temperature, 85%.



Scheme 2. Synthesis of conjugates **4a-d.** Reagents and conditions: a. EtOH, conc.H_2SO_4, 95%. b. MEMCl, DIEA, DCM, rt, 91%. c. LiOH, EtOH, THF, H_2O, 95%. d. (i) $SOCl_2$, MeOH, reflux, (ii) 1-(3-DiMethylaMinopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), dimethylaminopyridine (DMAP), Et_3N, CH_2Cl_2, r.t., 56%-83%. e. LiOH, EtOH, tetrahydrofuran (THF), H_2O, 84%-98%. f. **9**, EDCI, DMAP, Et_3N, CH_2Cl_2, 40 °C, 30%-57%. g. Iodotrimethylsilane (TMSI), CH_3CN, −20 °C, 67%-88%.



Scheme 3. Synthesis of conjugates **4e-h.** Reagents and conditions: a. dichlorodiphenylmethane, diphenyl ether, 165 °C, 70%. b. TsCl, Et_3N, DCM, 40 °C, 21–23%. c. K_2CO_3, CH_3CN, reflux, 46%-79%. d. **17**, EDCI, DMAP, Et_3N, THF, 40 °C, 34%-60%. e. aq. 38% HCl in AcOH (v/v 1:15), r.t., 26–80%.

(Scheme 3).

The poly(ethyleneglycol) linked conjugates **4e-h** were first tested for their BACE1 inhibitory effects at 10 μM. It is found that the inhibitory activity of conjugates **4e-h** decreased when the length of linker increases. Moreover, **4e** bearing the single ethylene glycol linker showed most potent inhibitory activity among all tested conjugates with an IC_{50} of 0.25 μM, which was better than both fragments **1** and **2**.

To assess the cellular BACE1 inhibitory activity of the conjugates, **4b-d** and **4e-h** were incubated in APP overexpressing HEK293 cells comparing with compound **3** and the two fragments **1** and **2** in two groups and their inhibition on the three main Aβ species Aβ-38, Aβ-40 and Aβ-42, the main amyloidogenic peptides produced by BACE1 cleavage of APP peptide, was measured by an ELISA-like immunoassay method. Aβ levels were normalized to the protein concentration in the lysate in order to rule out differences in total cell number per well of the culture plate. Additionally, the normalized Aβ level was set to 100% for the DMSO-treated control. At the concentrations of 25 and 50 μM all conjugates with amino acid linkers, except **4c**, dose-dependently reduced Aβ levels, although only moderate to low activity was observed (ca. 55% Aβ remaining at 50 μM). Conjugate **4c** showed nearly no ability to reduce Aβ levels. In another series of conjugates with polyglycol linkers, the most potent enzyme inhibitor **4e** showed a slightly different profile: the increase in Aβ levels was very moderate (< 25%) for Aβ1-38 and Aβ1-42 and not observed for Aβ1-40 at lower compound concentration and inhibition of Aβ was already seen at 10 μM and was enhanced at higher concentrations. In parallel to their BACE1 inhibitory activity in enzyme assay, **4e** was the most potent and **4c** the least potent in the inhibition of the most toxic species Aβ-42 production (after normalization to protein). The reference compound **3** showed no cellular activity at all tested concentrations, **4e** had much obvious influence than luteolin (**1**) (see Fig. 4).

Luteolin (**1**), one segment of the conjugate, is a non-competitive BACE1 inhibitor. We are interested to know if this fragment of the conjugate will keep or change the mode of inhibition. The Dixon plot was used to determine the kinetics of **4e** inhibiting BACE1. As shown in **Fig. S3, 4e** is still found as a non-competitive inhibitor with a Ki value of 0.35 μM.

In addition, we hope to understand the interaction of the conjugate with BACE1, to validate our fragment based strategy and assist further structural optimization. However, all conjugates **4a-h** were not completely dissolved in the buffer we used to measure STD-NMR, even in the presence of 30% DMSO-d_6 (v/v). Thus we tried to improve the water solubility of the conjugate through the introduction of mono- and di-phosphoryl groups into **4e**, the most active compound in the series.

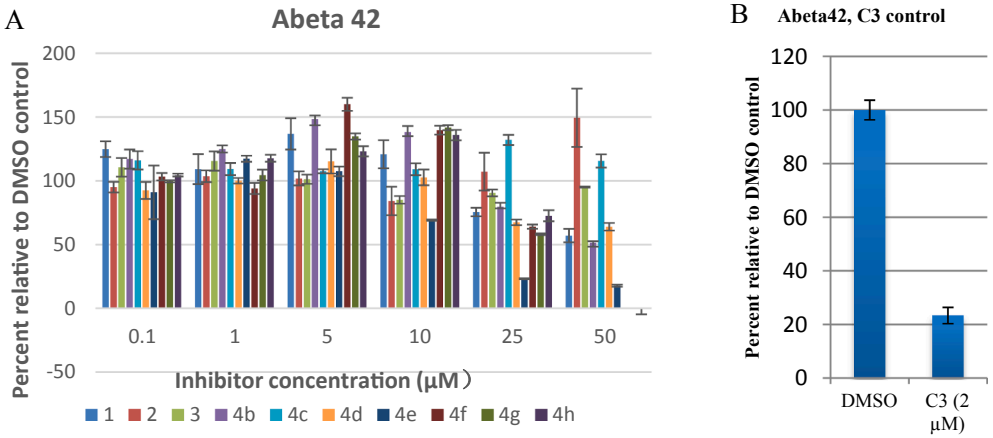
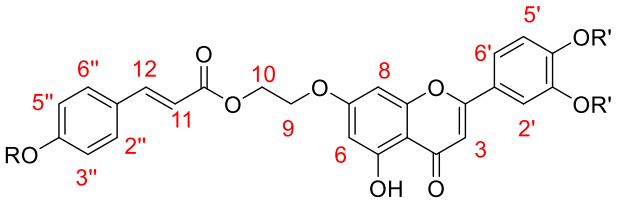


Fig. 4. BACE1 inhibitory activity in cells. (A) The normalized Aβ1-42 level in APP overexpressing HEK293 cells after the treatment of **1**, **2**, **3**, **4b ~ 4 h**, compared with DMSO (100%). (B) Aβ1-42 level for DMSO and the known inhibitor C3 [20].



4i R=$(HO)_2PO_2$, R'=H
4j R=H, R'=$(HO)_2PO_2$

Fig. 5. The structures of phosphates **4i** and **4j**.

The 4″-O-phosphate (**4i**) and 3′,4′-di-phosphate (**4j**) were prepared through a common intermediate **18e** (4″-Ac-3′,4′-diphenylmethylene derivative of **4e**) by phosphorylation and deprotection at proper position (Fig. 5, Schemes S1, S2).

The BACE1 inhibitory activities of **4i** and **4i** were retained, exhibiting IC_{50} values of 2.13 and 3.93 μM respectively, while enhancing the solubility in the buffer enabling STD-NMR experiments (Table 1). The solubility of **4j** was better than the compound **4i**, and its BACE1 inhibitory activity only a little weaker than **4e**. Thus, **4j** was chosen to record its STD-NMR in a pH 4.5 buffer (CD_3COOD/CD_3COONa), although 20% DMSO-d_6 (v/v) as additives are still needed to prepare a clear solution. We measured the enzymatic inhibition of our compounds in the presence and absence of DMSO in above buffer, and found their inhibitions were very similar. Such an observation suggested that the enzymatic activity was well preserved even in the presence of 20% DMSO.

The experimental STD spectra were shown in Fig. 6 and data in Table 2. The largest STD effects were observed for the two benzene rings moiety at the end of the molecule, demonstrated by the highest intensity enhancement of H-2′, H-3″, H-5″ (assigned as 100% intensity). The STD effects of carbon-carbon double bond (H-11, 12) and chromone core structure (H-3, 6, 8) were a little weaker than the terminal of two benzene rings, and those of the protons (H-9, 10) on the glycol linker not observed. Accordingly, it could be postulated that both terminal in the conjugate are closer to the protein (very possibly in two sub-pockets), and the 3′ or 4′-phenol and 4″-phosphate moiety (4″-phenol and 3′ or 4′-phosphate moiety) on both terminal phenyls may get involved in the interaction with BACE1 directly.

As BACE1 is a member of aspartyl protease family, its selective inhibition over other closely related proteases is required to avoid possible severe side effects. Thus, we measured the inhibition of **4e** on BACE-2 and renin, and found 27.6% and 25.9% inhibition rates at a concentration of 10 μM respectively, corresponding to IC_{50} over 10 μM (vs. 0.25 μM on BACE1).

It is also crucial for the BACE1 inhibitors to exert their activities at sub-toxic concentration, so their cytotoxicity in HEK293 cells were also assessed by MTT assays. The IC_{50} for all of the tested compounds are above 50 μM, among which **4a, b, e, g, i**, **j** and **1**, **3** are the least cytotoxic (IC_{50} > 100 μM).

BACE1 has been recognized as a promising therapeutic target for AD, although the safety of BACE inhibition was in dispute during the drug development over last decade [19]. Thus, BACE1 inhibitor with reasonable potency and with the selectivity on APP processing over other substrates have been actively pursued to avoid unendurable toxicity. Non-competitive inhibitors, which are able to enhance the selectivity and reach the reasonable potency by allosteric modulation of enzyme activity, should be given more attention, although in reality they are still underexplored.

As luteolin (**1**) are known to interact with many other enzymes, such as carbonic anhydrase [21], tyrosinase [22], α-glucosidase [23], etc., our BACE1 inhibitors, either defined as 7-modified analogs or luteolin-based conjugates, may arise the concern to interact with those proteins. However, it is worth pointing out that the selective inhibition on BACE1 over other structurally related aspartyl proteases has been assessed in this study. Although the possibility to interact with proteins other than BACE1 structurally related ones cannot be fully excluded, it is argued that our compounds, after attachment of a comparably large segment (cinnamoyl acid + linker, MW 110–310) to another segment/one of the starting compounds (luteolin, MW ~ 370) may change the way of luteolin interacting with proteins. Nonetheless, the selectivity to a wider spectrum of proteins could be assessed in further optimization of this series of conjugates.

Table 1
Enzymatic inhibition activity of conjugates **4a-4j.**

	Enzyme Inhibition	
Compounds	Inhibition rate at 10 μM	IC_{50} (μM)
4a	NA	–
4b	10.9% ± 3.1%	–
4c	14.7% ± 5.3%	–
4d	1.9% ± 2.8%	–
4e	92.0% ± 1.5%	0.25 ± 0.16
4f	73.0% ± 5.0%	2.22 ± 0.86
4 g	20.1% ± 3.1%	–
4 h	NA	–
4i	101.7% ± 3.7%	1.31 ± 0.28
4j	86.3% ± 14.5%	2.34 ± 0.28
luteolin (**1**)	62.5% ± 2.5% or 66.7% ± 7.25%	1.10 ± 1.34
p-hydroxy-cinnamic acid (**2**)		~100
AV-4-1 (**3**)		39.0*

* Data from Lv L. et al. *Planta Med.* 2008, 74: 540-5.

In conclusion, we discovered a series of conjugates as cell-permeable, low cytotoxic, highly selective non-competitive BACE1 inhibitors by linking two NP fragments with the assistance of STD-NMR. Such non-competitive inhibitors may provide an opportunity to decrease APP processing by BACE1 inhibition, whereas sparing other BACE1 functions, thus providing promising drug candidates.

3. Experimentals

3.1. In vitro BACE1 enzyme assay

The BACE1 FRET assay kit was purchased from the PanVera Co. (Invitrogen, USA). The assay was carried out according to the supplied manual with modifications. Briefly, assays were performed in triplicate in 96-well black plates with 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- Quencherin 50 mM, ammonium bicarbonate), and 10 μL of compound dissolved in 10% DMSO. The fluorescence intensity was measured with a TECAN infinite 200 microplate reader for 60 min at 25 °C in the dark. The mixture was irradiated at 544 nm and the emission intensity recorded at 590 nm. The percent inhibition (%) was obtained by the following equation: Inhibition % = (1 − SS/SC) × 100%, where SC is the slope of fluorescence change of the control (enzyme, buffer, and substrate) during 60 min, and SS is the slope of fluorescence change of the tested samples (enzyme, sample solution, and substrate) during 60 min of measurement. IC50 values were calculated from the nonlinear curve fitting of percentage inhibition against inhibitor concentration using Prism 3.0 software.

3.2. Saturation transfer difference (STD) -NMR

The sample solutions consisting of 10 μM BACE1 protein and 2 mM ligand, 50 mM acetate buffer, 150 mM NaCl, and 20% deuterated DMSO in D_2O at pH 4.0 were prepared. All STD-NMR experiments were performed at 298 K on a Bruker Avance III 500 MHz spectrometer equipped with a 1.7-mm NMR microprobe. ^{1}H-spectrum was obtained

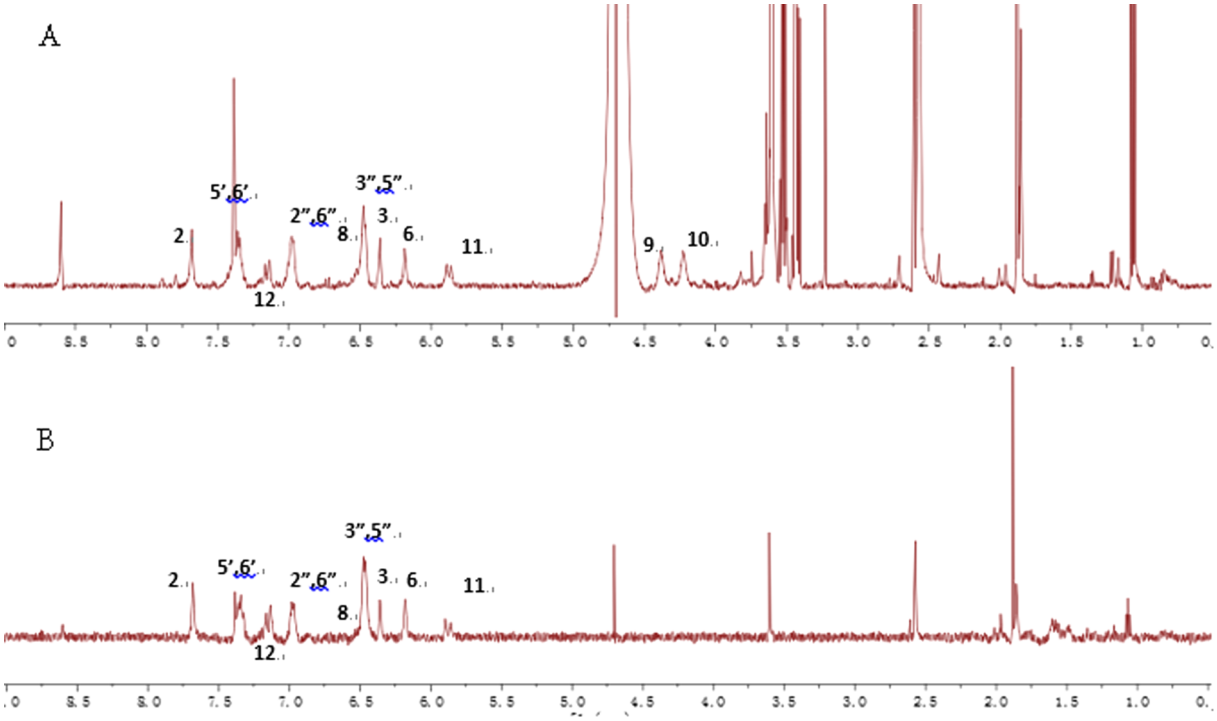


Fig. 6. NMR and STD-NMR spectra of **4j.** (A) ^{1}H NMR of **4j** (2 mM) with BACE1 (10 μM); (B) STD-NMR of **4j** (2 mM) with BACE1 (10 μM).

Table 2
Signal intensity enhancement in STD of **4j.**

Proton	Intensity enhancement (%)	Proton	Intensity enhancement (%)
2′	100%	10	0%
5′, 6′	90%	11	53%
3	60%	12	90%
6	74%	2″, 6″	58%
8	100%	3″, 5″	100%
9	0%		

first as reference spectrum, and subsequently, an STD spectrum was obtained. The protein was saturated on-resonance at 0 ppm and off-resonance at 29 ppm with a cascade of 40 selective Gaussian shaped pulses, of 50 ms duration with a 4 μs delay between each pulse in all STD NMR experiments. The total duration of the saturation time was set to 2 s. A total of 2048 scans per STD NMR experiment were acquired and a 3-9-19 pusle sequence was used to water suppression. A spin-lock filter with 9615.38 kHz strength and duration of 30 ms was applied to suppress protein background.

Other experimentals including the synthesis, enzyme kinetic assay, cellular activity and cytotoxicity please refer to supplemental information.

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Declaration of Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103253.

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