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Design, synthesis and evaluation of flavonoid derivatives as potential multifunctional acetylcholinesterase inhibitors against Alzheimer's disease

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ABSTRACT

A new series of flavonoid derivatives were designed, synthesized and evaluated as potential multifunctional AChE inhibitors against Alzheimer's disease. Most of them exhibited potent AChE inhibitory activity, high selectivity for AChE over BuChE, and moderate to good inhibitory potency toward Aβ aggregation. Specifically, compound **12c** was the strongest AChE inhibitor, being 20-fold more potent than galanthamine and twofold more potent than tacrine, and it also had ability to inhibit Aβ aggregation (close to the reference compound) and to function as a metal chelator. Molecular modeling and enzyme kinetic study revealed that it targeted both the catalytic active site and the peripheral anionic site of AChE. Consequently, this class of compounds deserved to be thoroughly and systematically studied for the treatment of Alzheimer's disease.

Alzheimer's disease (AD), characterized by memory loss, language impairment, personality changes and decline in intellectual ability, is a highly complex and progressive neurodegenerative disorder in the elderly population.[1] Many factors are considered as the key pathological hallmarks of AD, such as cholinergic system dysfunction, accelerated aggregation of β-amyloid (Aβ) peptides and the dyshomeostasis of biometals.[2–6] These factors provide a basis for the cholinergic, amyloid and biometal hypotheses for AD pathology, respectively.

According to the cholinergic hypothesis, the cognitive and memory symptoms of AD are caused by the drastic decline of acetylcholine,[7] and recent reports suggest that increasing acetylcholinesterase (AChE) inhibition and simultaneously improving selectivity for AChE over butyrylcholinesterase (BuChE) would be a promising direction for AD treatment.[8] Furthermore, the crystallographic structure of AChE reveals that it contains two separate ligand binding sites—a catalytic active site (CAS) at the bottom of deep narrow gorge and a peripheral cationic site (PAS) at the entrance.[9,10] Hence, the simultaneous binding to both the CAS and PAS has been advocated to design potent and selective AChE inhibitors.[11,12]

The amyloid cascade hypothesis attributes the pathogenesis of AD to the accelerated aggregation of Aβ in the brain resulting in the formation of senile plaques and then to neurofibrillary tangles, neuronal cell death, and ultimately dementia.[13,14] Aβ40 and Aβ42 are the most common peptides found in amyloid plaques. Though the amount of secreted Aβ42 is only 10% of Aβ40, Aβ42 is more prone to aggregation and more neurotoxic compared with Aβ40.[15,16] Therefore, preventing this peptide from aggregation is a potential therapy for AD.

It has been reported that biometals also play a very important role in many critical aspects of AD.[17] The gradual accumulation of Cu(II) and Fe(II) in the neuropil and plaques of the brain linked to the production of reactive oxygen species and oxidative stress contributes to AD pathology.[18] Direct evidences show that metal ion levels in AD individuals are three to sevenfolds higher than those in healthy people.[19] Thus, the modulation of these biometals in the brain is also a potential therapeutic strategy for AD patients.

Up to now, most of therapies for AD focus on increasing cholinergic neurotransmission by acetylcholinesterase inhibitors (AChEIs), including tacrine, donepezil, rivastigmine and galantamine.[20] These drugs can only improve symptoms for most patients but do not address the etiology of AD. Therefore, searching for

Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; CAS, catalytic anionic site; PAS, peripheral anionic site; AChEI, acetylcholinesterase inhibitor; ChEs, cholinesterases; MOE, Molecular Operating Environment; SI, selectivity index; MD, molecular dynamic.

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more efficient strategies to combat this disease is highly needed.[21] Due to the complex multifactorial nature of AD, molecules that modulate the activity of a single protein target are incapable of significantly altering the progression of the disease. In contrast, multifunctional molecules with two or more complementary biological activities might represent an important advance in the treatment of AD. Accordingly, we are devoted to the study of multifunctional AChEIs that not just inhibit AChE, but decrease Aβ42 aggregation and chelate metals.

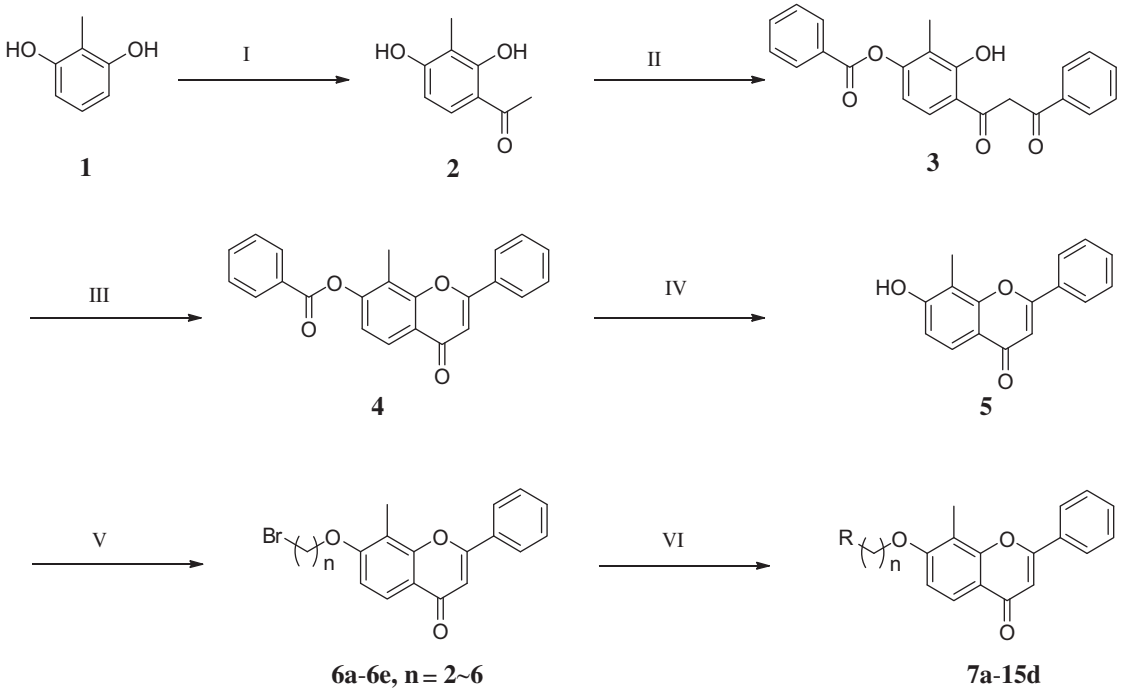
Flavonoids, ubiquitously presented in fruits and vegetables, are well-known natural compounds, and have attracted increasingly widespread attention in present-day society as they possess a wide range of pharmacological properties related to a variety of neurological disorders, like neuro-protective effect,[22] AChE inhibitory activity,[23] Aβ fibril formation inhibitory activity,[24] free radical scavenging effect,[25] and metal-chelating ability.[26] Thus, the design and synthesis of new effective flavonoid derivatives are an interesting strategy for the research on anti-AD drugs. According to the structure of AChE, in order to design dual binding site AChEIs, we decided to connect flavonoid scaffold with terminal amine groups through carbon spacers of different lengths. The terminal amine groups, protonated at physiological pH, could occupy the CAS via cation–π interaction, while flavonoid scaffold could interact with the PAS of AChE via aromatic stacking interactions. Flexible carbon spacer was lodged in the narrow mid-gorge,[27] and the length of carbon spacer was changed aiming to obtain optional conformation that could make the designed compounds interact with both the CAS and PAS of AChE.

Here, a series of flavonoid derivatives with different basic side-chains (n = 2–6) were designed, synthesized and evaluated for their cholinesterases (ChEs) inhibition, anti-Aβ42 aggregation and metal-chelating ability. The structure–activity relationships were discussed based on the pharmacological activities.

The synthetic pathways of the flavonoid derivatives were outlined in Scheme 1. At the first stage, 2-methylbenzene-1,3-diol **1** was acetylated by a Friedel–Crafts type reaction with acetic anhydride and boron fluoride ethyl ether ($BF_3{\cdot}Et_2O$) as Lewis acid, which led to a high yield of 1-(2,4-dihydroxy-3-methylphenyl)ethanone **2**.[28] Then, compound **2** was condensed with benzoyl chloride to produce β-diketone **3** through our modified Baker–Venkataraman transformation method by using K_2CO_3 in acetone.[29] The obtained β-diketone **3** was treated with NaOAc/HOAc to obtain 8-methyl-4-oxo-2-phenyl-4*H*-chromen-7-yl benzoate **4**. After removing the benzoyl group of **4**, flavonoid scaffold **5** was acquired in 93% yield. The alkylation of **5** with different α,ω-dibromoalkanes in DMF provided **6a**–**6e** in 68–80% yields. Finally, the target products **7a**–**15d** were gained by the reaction of **6a**–**6e** with commercially available secondary amines (e.g., pyrrolidine and diethylamine) in 66–85% yields. The purities of all final compounds were confirmed to be higher than 95% by HPLC (shown in Supplementary data).

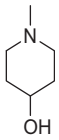
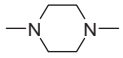
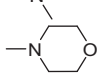
To determine the potential anti-AD effects of compounds **7a**–**15d**, the inhibition of AChE (from electric eel) and BuChE (from equine serum) were tested using the method of Ellman et al. with galanthamine and tacrine as reference compounds.[30,31] The IC_{50} values for ChEs inhibition and selectivity index (SI) for the inhibition of AChE over BuChE were summarized in Table 1. These results indicated that all of the compounds gave higher inhibitory activities against AChE than the precursor compound **5** (IC_{50} value: 87.05 μM). Specifically, compound **12c**, whose diethylamine group was linked to flavonoid scaffold by a four-carbon spacer, was the most potent inhibitor with an IC_{50} value of 0.13 μM, being 20-fold more potent than galanthamine (IC_{50} value: 2.67 μM) and twofold more potent than tacrine (IC_{50} value: 0.269 μM). Hence, the introduction of the aminoalkyl-substituted groups could significantly increase the inhibitory activities of derivatives.

Changing the length of the alkyl chain could affect their ability to contact both sites of AChE and thereby influence the AChE inhibitory potency. Based on the screening data (Fig. 1), compounds **7a**–**7e**, combining pyrrolidine group with flavonoid scaffold by a two- to six-carbon spacer, exhibited different levels of inhibitory activities against AChE (**7a**, n = 2, IC_{50} value: 2.04 μM; **7b**, n = 3, 0.952 μM; **7c**, n = 4, 0.238 μM; **7d**, n = 5, 0.243 μM; **7e**, n = 6,



Scheme 1. Reagents and conditions: (I) Ac_2O (1.1 equiv), $BF_3{\cdot}Et_2O$ (2.4 equiv), 80 °C, 6 h, 95%; (II) benzoyl chloride, K_2CO_3, acetone, reflux; (III) NaOAc/HOAc, reflux; (IV) K_2CO_3, $MeOH/CH_2Cl_2$, rt; (V) $Br(CH_2)_nBr$, K_2CO_3, DMF; (VI) NHR, K_2CO_3, DMF, KI. ('R-' were showed in Table 1)

Table 1
Inhibition of AChE, BuChE and self-induced Aβ42 aggregation activities of the synthesized compounds

Compound	R	*n*	IC50[a] (μM)		Selectivity Index[c]	Aβ42 aggregation inhibition[d] (%)
			AChE	BuChE		
5			87.05	>100		n.a.[b]
7a		2	2.04 ± 0.21	2.71 ± 0.36	1.3	42.03 ± 0.95
7b		3	0.952 ± 0.024	2.78 ± 0.41	2.9	27.12 ± 3.56
7c		4	0.238 ± 0.006	5.70 ± 0.63	23.8	21.83 ± 2.78
7d		5	0.243 ± 0.011	1.91 ± 0.12	7.8	39.29 ± 1.21
7e		6	0.764 ± 0.014	3.28 ± 0.39	4.3	38.12 ± 1.14
8a		2	2.10 ± 0.17	>100	>47.5	30.54 ± 2.35
8b		3	0.590 ± 0.022	5.84 ± 0.54	9.9	41.67 ± 1.01
8c		4	0.225 ± 0.017	>100	>442.9	37.32 ± 1.18
8d		5	0.216 ± 0.008	2.77 ± 0.22	12.8	36.74 ± 1.28
8e		6	1.89 ± 012	3.74 ± 0.27	2.0	43.23 ± 1.12
9a		2	1.83 ± 0.18	3.21 ± 0.19	1.7	26.89 ± 3.76
9b		3	2.94 ± 0.25	4.12 ± 0.31	1.4	21.35 ± 4.56
9c		4	0.607 ± 0.031	>100	>164.7	22.08 ± 3.96
9d		5	0.604 ± 0.034	4.85 ± 0.53	8.0	37.38 ± 1.58
9e		6	3.15 ± 0.31	3.93 ± 0.42	1.2	39.04 ± 1.36
10c		4	0.234 ± 0.005	>100	>427.2	37.48 ± 1.24
10d		5	0.329 ± 0.009	1.41 ± 0.11	4.3	41.67 ± 1.06
11c		4	1.66 ± 0.26	5.75 ± 0.38	3.5	31.14 ± 2.02
11d		5	1.87 ± 0.51	4.87 ± 0.61	2.6	39.94 ± 1.17
12c		4	0.130 ± 0.002	4.82 ± 0.32	37.1	38.95 ± 1.57
12d		5	0.264 ± 0.004	>100	>377.7	40.87 ± 1.09
13c		4	0.328 ± 0.008	>100	>304.7	34.29 ± 2.16
13d		5	0.210 ± 0.003	2.54 ± 0.20	12.1	37.86 ± 1.73
14c		4	2.88 ± 0.37	>100	>34.7	20.42 ± 6.56
14d		5	8.63 ± 0.84	>100	>4.8	24.59 ± 4.56
15c		4	25.3 ± 1.7	3.82 ± 0.39	0.2	n.a.b
15d		5	19.6 ± 1.1	3.93 ± 0.35	0.2	n.a.b
Tacrine			0.269 ± 0.023	0.054 ± 0.001	0.2	n.a.b
Galantamine			2.67 ± 0.15	12.7 ± 0.2	4.8	n.a.b
Curcumin			n.a.[b]	n.a.[b]		50.12 ± 0.88

[a] IC_{50}: 50% inhibitory concentration (means ± SEM of three experiments).
[b] Not available.
[c] Selectivity Index = IC_{50} (BuChE)/IC_{50} (AChE).
[d] Inhibition of self-mediated Aβ42 aggregation, the measurements were carried out in the presence of 20 μM compounds (means ± SEM of three experiments).

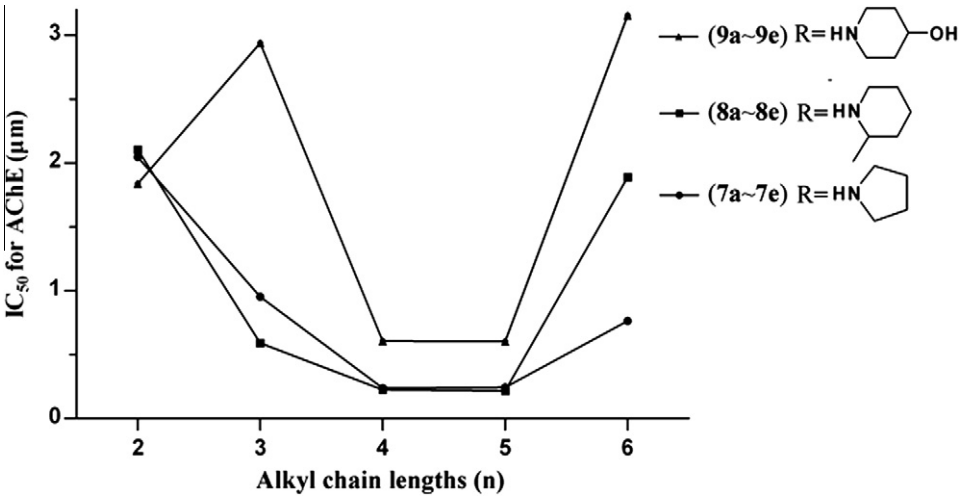


Figure 1. Effects of alkyl chain lengths on anti-AChE activities.

0.764 μM). The compounds with a four- or five-carbon spacer (**7c** and **7d**) displayed higher inhibitory potency. The same trend was observed for compounds linked with 2-methylpiperidine (**8a**–**8e**) and piperidin-4-ol (**9a**–**9e**). Clearly, the optimal chain lengths determined experimentally for inhibiting AChE were the four- and five-carbon spacers.

With the optimal length of the linker in hand, we introduced different terminal amine groups to flavonoid scaffold to further

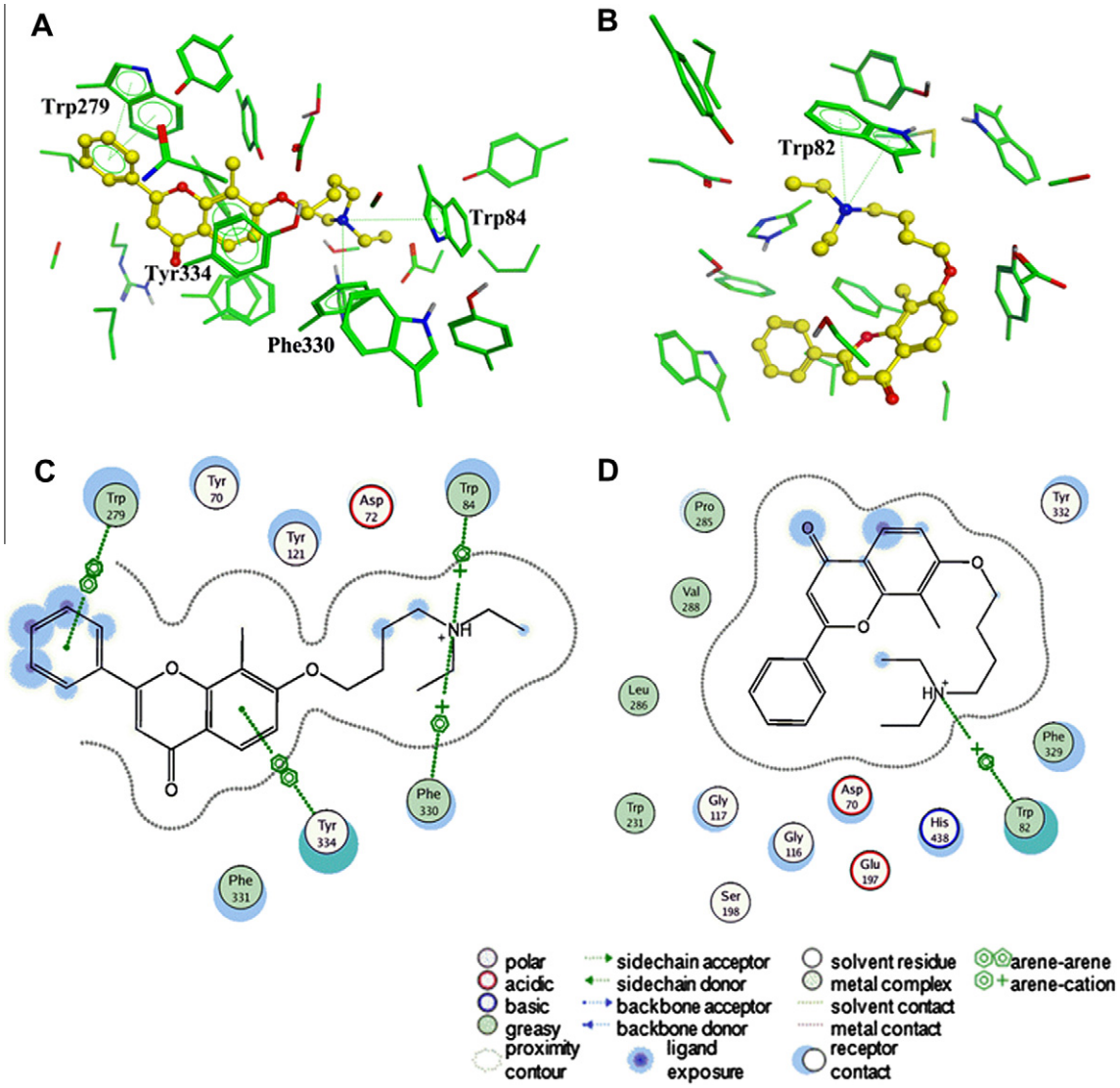


Figure 2. Molecular modeling of compound **12c** with AChE (A and C) and BuChE (B and D) generated with MOE.

explore structure–activity relationship. Obviously, flavonoid derivatives with cyclic monoamine groups (pyrrolidine, 2-methylpiperidine and piperidine) in the optimal side chains showed high inhibitory activity against AChE similar to those with chain monoamine groups (diethylamine and dimethylamine), indicating that both the open-loop and closed-loop groups had the ability to occupy the CAS of AChE. On the other hand, compounds **9c**, **9d**, **11c**, **11d** and **14c**–**15d**, possessing additional nitrogen or oxygen atom in the terminal group, exhibited much weaker AChE inhibitory potency than the compounds containing only one nitrogen atom (e.g., **10c** and **10d**). These results suggested electron-withdrawing effects of nitrogen or oxygen atom might reduce the electronic density of the terminal amine, thereby impacting on protonation at physiological pH, which could diminish the cation–π interaction between the terminal nitrogen and the CAS of AChE. Besides, the length of the alkyl chain could affect the selectivity for AChE. The compounds with a four-carbon spacer showed higher selectivity than the compounds with a five-carbon length (e.g., **7c**, SI: 23.8 > **7d**, SI: 7.8; **8c**, SI: 442.9 > **8d**, SI: 12.8).

To further investigate the interaction modes and selectivity to two ChEs, molecular modeling was carried out by Molecular Operating Environment (MOE) software package. The X-ray crystallographic structures of AChE (PDB code: 1EVE) and BuChE (PDB code: 1P0I) were obtained from protein data bank. 3D structure of the strongest AChE inhibitor **12c** was built using the builder interface of MOE program, and docked into the active site of the protein after energy minimized. The Dock scoring in MOE software was done using ASE scoring function. Finally, the geometries of resulting complex were displayed in Figure 2. Compound **12c** manifested multiple binding modes (Fig. 2, A and C) with AChE. The flavonoid moiety adopted an appropriate orientation for its binding to PAS via the π–π stacking interactions with Trp279 and Tyr334, and their ring-to-ring distance was between 4.12 and 4.16 Å. Moreover, the conformation of the side chain conformed to the shape of the mid-gorge, and in the bottom of the gorge, the charged nitrogen of diethylamine group was observed to bind to the CAS via a cation–π interaction between Trp84 (5.03 Å) and Phe330 (5.01 Å). In contrast, the binding only involved a cation–π interaction between Trp82 (4.03 Å) and the nitrogen of diethylamine with BuChE, and no other obvious interactions were observed (Fig. 2, B and D). These results might explain why compound **12c** had strong AChE inhibitory activity and high selectivity for AChE over BuChE.

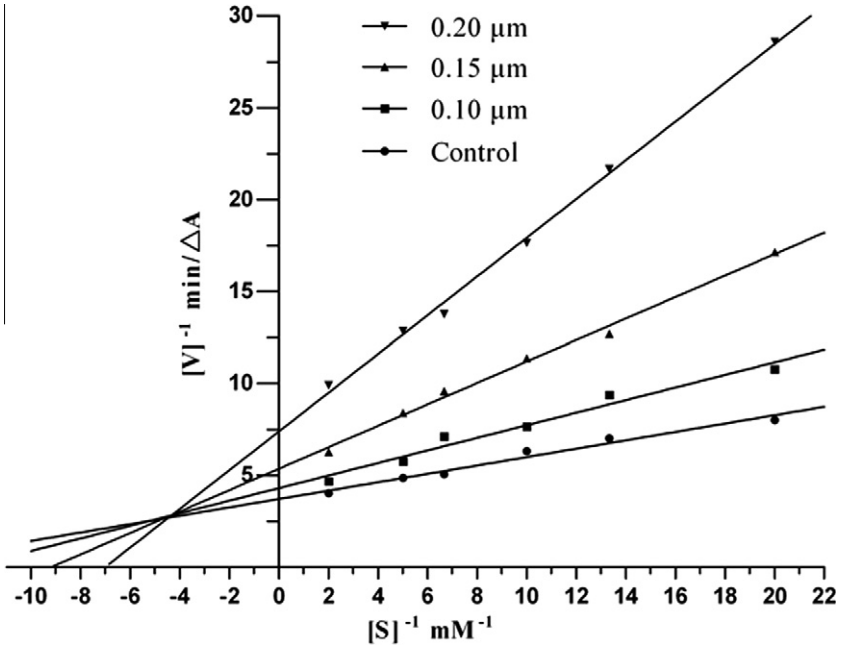


Figure 3. Lineweavere–Burk plot for the inhibition of AChE by compound **12c**.

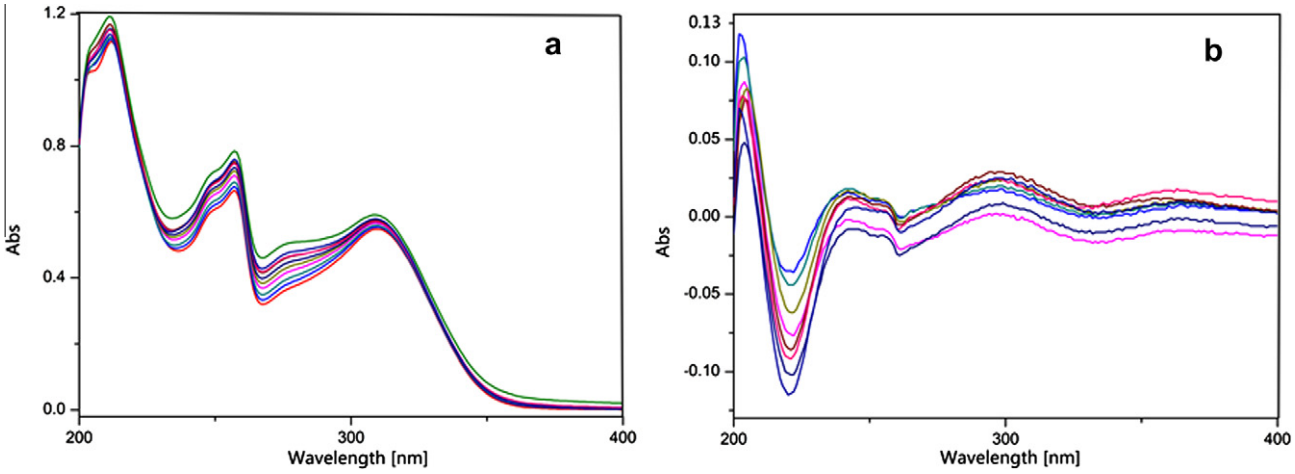


Figure 4. UV–vis (200–400 nm) absorption spectra of **12c** (35 μM) in methanol after addition of ascending amounts of $CuCl_2$ (1–50 μM) (a) and differential spectra due to **12c**–Cu^{2+} complex formation obtained by numerical subtraction from the above spectra of those of $CuCl_2$ and **12c** at the corresponding concentrations (b).

After molecular docking, molecular dynamic (MD) simulations were performed using MOE software to evaluate the stability of **12c**-AChE complex.[32,33] The root-mean-square deviation (RMSD) was observed and remained at about 1.055 Å, very stable and much lower, in the end of the MD. The results showed the complex was stable during the MD simulation.

Kinetics of compound **12c** was further examined to determine the nature of AChE inhibition by graphical analysis of steady-state inhibition data.[34] As shown in Figure 3, the Lineweavere–Burk plots showed both increasing slopes and increasing intercepts on the *y*-axis at higher inhibitor concentration, which revealed a mixed-type inhibition. Hence, the enzyme kinetic study also suggested that the inhibitor **12c** bound to both sites of AChE, consistent with the results of molecular modeling studies.

Subsequently, we evaluated these compounds for their potential to inhibit the self-induced Aβ42 aggregation using thioflavin *T* assay (Table 1).[35] Compared with the reference compound curcumin (50.12% at 20 μM), these flavonoid derivatives showed moderate to good potencies (20.42–43.23% at 20 μM). Interestingly, the anti-Aβ aggregation activity seemed to depend on the length of the side chain. The compounds with a five-carbon spacer (e.g., **12d**, 40.87%) showed better anti-Aβ aggregation activity than the compounds with a four-carbon length (e.g., **12c**, 38.95%). However, the current data were inadequate to derive a structure–activity relationship for predicting the activity toward anti-Aβ aggregation, and further research was required.

In addition, compound **12c** was also taken as an example to test for its metal-chelating effect using difference UV–vis spectra recorded in methanol at 298 K with wavelength ranging from 200 to 400 nm.[4,19] The absorption of **12c** (35 μM) decreased along with the increasing concentration of Cu^{2+} from 1 to 50 μM in Figure 4a. And the increase in absorbance, better estimated by an inspection of differential spectra in Figure 4b, implied that there was an interaction between copper ion and **12c**. The result using Fe^{2+} was similar to that using Cu^{2+}. These observations indicated that compound **12c** could chelate Cu^{2+} and Fe^{2+}. Therefore, from these

preliminary results, one can suggest that **12c** might also act against AD by a chelation mechanism.

In conclusion, a new series of flavonoid derivatives were designed, synthesized and subjected to biological evaluation. Most of these compounds were potent inhibitors of AChE, with IC_{50} values ranging from micromolar to submicromolar, and showed moderate to good potencies toward inhibition of self-mediated Aβ42 aggregation. Among them, compound **12c**, which contained a diethylamine group linked to flavonoid scaffold by a four-carbon spacer, exhibited the most potent AChE inhibitory activity, high selectivity for AChE over BuChE, anti-Aβ42 aggregation, as well as biometal-chelating effect. The molecular modeling study and inhibition kinetic analysis indicated that compound **12c** bound to both the CAS and PAS of AChE. Overall, compound **12c** had strong potential to serve as a multifunctional AChEI for the treatment of AD and this type of multifunctional flavonoid compounds might provide a useful template for the development of new anti-AD agents with multiple potencies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.095. These data include MOL files and InChiKeys of the most important compounds described in this article.

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