RESEARCH ARTICLE

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Cite this: *Med. Chem. Commun.*,
2017, 8, 1459

Synthesis and pharmacological evaluation of multi-functional homoisoflavonoid derivatives as potent inhibitors of monoamine oxidase B and cholinesterase for the treatment of Alzheimer's disease†‡

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A series of homoisoflavonoid derivatives was designed, synthesized and evaluated as potential multi-functional anti-Alzheimer's agents for their inhibitory activity on cholinesterase and monoamine oxidase. Among them, compound **16** showed moderate acetylcholinesterase (AChE) inhibitory activity (eeAChE IC_{50} = 0.89 ± 0.02 μ M; hAChE IC_{50} = 0.657 ± 0.002 μ M) and significant monoamine oxidase B (MAO-B) inhibitory activity (hMAO-B IC_{50} = 0.0372 ± 0.0002 μ M). Kinetic analysis of AChE, MAO-B inhibition and molecular modeling studies revealed that compound **16** is a dual binding site inhibitor of AChE and noncompetitive inhibitor of MAO-B. Furthermore, **16** could penetrate through the blood-brain barrier (BBB) *in vitro*. Most importantly, oral administration of **16** demonstrated no marked signs of acute toxicity and it could significantly reverse scopolamine-induced memory impairment in mice. These results suggested that compound **16** is a promising multifunctional drug candidate with potential effect for the treatment of Alzheimer's disease.

Received 19th April 2017,
Accepted 23rd May 2017

DOI: 10.1039/c7md00199a

rsc.li/medchemcomm

Introduction

With the development of society, mankind begins to focus on his health, and dementia is one of the most serious health issues.¹ Alzheimer's disease (AD), a form of dementia, is marked mainly by progressive memory loss, cognitive decline and behavior disorder.^{2,3} Senile plaques and neurofibrillary tangles are the two leading pathologic hallmarks of AD.⁴

Due to the complexity of the causative factors, several hypotheses were put forward to illuminate AD, including cholinergic system dysfunction, β -amyloid peptide (A β) deposits, tau protein aggregation, and calcium mishandling.⁵ From 1984 to 2014, considerable investments have been devoted to clinical trials and drug development against AD; only a few drugs were approved for AD, which include cholinesterase inhibitors (donepezil, galantamine, rivastigmine and tacrine) and an *N*-methyl- *D*-aspartate (NMDA) receptor antagonist (memantine).⁶ Apart from memory and cognitive improve-

ment, these marketed drugs have shown only little efficacy for preventing the progression of the disease.⁷ Thus, the pattern of 'one-molecule-one-target' might not be applicable to the complex nature of AD. Accordingly, the 'multi-target-directed ligand' (MTDL) strategy has been developed in recent years.^{8,9} The combination of pharmacophore molecules with different biological activity is one of the most common design strategies. Several researches have described the underlying therapeutic applications of multimodal cholinesterase inhibitors with additional activities, such as amyloid plaques inhibition,^{10,11} antioxidant,^{12,13} and monoamine oxidase (MAO) inhibition.^{14,15}

Cholinesterase (ChE), a glycoprotein, is classified into two types: AChE and butyrylcholinesterase (BuChE).¹⁶ AChE (EC 3.1.1.7) is responsible for the hydrolysis of acetylcholine (ACh) in the central and peripheral nervous systems,⁵ and the inactivation of AChE induced by diverse inhibitors can result in an increasing in the ACh levels.¹⁷ AChE can hydrolyze the natural neurotransmitter, ACh, faster than the synthetic substrate butyrylcholine (BuCh). Conversely, BuChE demonstrates similar activity towards the two substrates.¹⁸ The cholinergic hypothesis suggests that cognitive impairment is mainly caused by the deficit of ACh.¹⁹ Similarly, alterations in dopaminergic and monoaminergic neurotransmitter system are also thought to be responsible for the abnormal behavior of AD.^{15,20} This

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† The authors declare no competing interests.

‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c7md00199a

evidence has led to the suggestion that MAO is also an effective target for AD therapy.^{21,22} Monoamine oxidase (MAO, EC 1.4.3.4) is a flavoenzyme that catalyzes the oxidative deamination of biogenic and dietary amines. Owing to differences in substrate and inhibitor selectivity, MAO exists as two isoforms, MAO-A and MAO-B. Furthermore, 5-hydroxytryptamine (5-HT) and β -phenylethylamine (PEA) are preferentially catabolized by MAO-A and MAO-B, respectively.^{23,24} Except for the increased amine neurotransmission, the beneficial properties of MAO inhibitors (IMAOs) are associated with a reduction in the levels of end products like hydrogen peroxide and aldehydes, which promote the formation of reactive oxygen species (ROS) and may contribute to neuronal damage.²⁵ MAO-A inhibitors are used in depressive disorder, while the MAO-B inhibitors are employed to treat neurodegenerative diseases, such as AD.²⁶ Furthermore, MAO-B activity is significantly increased in the brain region associated with AD.²⁷ Overall, these observations suggest that the inhibition of MAO-B seems to be beneficial for AD.

Homoisoflavanoids are specific flavonoids found in a variety of roots, bulbs and leaves of the Fabaceae and Asparagaceae families.²⁸ Considerable attention has been paid to their diverse biological activities, including antioxidant,^{28,29} antifungal,³⁰ and anti-inflammatory activities.³¹ Recent research indicates that natural homoisoflavanoids could significantly inhibit MAO-B activity.³² Moreover, increasing

evidence suggests that homoisoflavanoid derivatives, modified at the *para* hydroxyl position, exhibit improved activity against AD.^{33,34}

Based on the homoisoflavanoid potential against AD, we designed, synthesized and evaluated a new series of suitably substituted compounds. Starting from a multitarget directed ligand, a new molecular framework was projected following a hybridization strategy (Fig. 1).³⁵ A variety of hybrids was designed by connecting the 3-benzylidenechroman-ones to different alkylamine, alkylbenzylamine or alkylpiperazine groups and were characterized *via* a number of AChE inhibitors (like donepezil) through a flexible spacer. The 3-benzylidenechroman-one was chosen as a molecular fragment because it could inhibit MAO-B by fitting into the MAO-B enzymatic cleft,³² and this scaffold was also presumably necessary for interaction at the peripheral anionic site (PAS) of AChE. Considering the important functions of the nitrogen atom from the amine side chain, which interacts with the catalytic site (CAS) of AChE,^{14,36,37} we introduced these groups to acquire a dual AChE-MAO B inhibitor. In order to diversify the compounds and analyze the structure-activity relationship (SAR), the inhibitors were designed so that position of the amine chain varied from the 2'-OH to 3'-OH and 4'-OH of 3-benzylidenechroman-ones. The designed compounds were evaluated for their ChEs and MAOs inhibitory activities *in vitro*. The kinetics of the enzyme inhibition

Fig. 1 The rational design strategy for the multi-functional potential anti-Alzheimer's agents.

Scheme 1 Reagents and conditions: (i) piperidine, EtOH, room temperature, overnight; (ii) K₂CO₃, KI, CH₃CN, 65 °C, overnight; (iii) K₂CO₃, KI, acetone, 56 °C, overnight.

activity and blood–brain barrier (BBB) permeation were evaluated. Moreover, molecular docking was performed to obtain an insight into the mechanism of the selected compound. Finally, the optimal compound **16** was further assessed for acute toxicity *in vivo* as well as for memory improvement in the mouse model of scopolamine-induced impairment.

Results and discussion

Chemistry

The synthesis of target compounds **16–45** was accomplished, and is summarized in Scheme 1. The base-catalyzed condensation of compound **1** with substituted benzaldehydes **2–4** was carried out to afford the key intermediates, (*E*)-3-benzylidenechroman-4-ones **5–7**.³⁸ Subsequently, treatment of **5–7** with different α,ω -dibromoalkanes in acetonitrile produced compounds **8–15**.³⁹ These compounds were then treated with dimethylamine and other secondary amines in the presence of potassium carbonate to afford the target products, **16–45**, in 15–82% yield.

Cholinesterase inhibitory activity

The AChE inhibitory activities of all the target compounds, **16–45**, are listed in Table 1. Donepezil and compound **5** were used as reference compounds. The results showed that all the target compounds showed moderate activities, with IC₅₀ values ranging from submicromolar to micromolar concentrations. Compound **16** was most efficacious against eeAChE (IC₅₀ = 0.89 ± 0.02 μM). Most of the synthetic compounds had better inhibitory activities against eeAChE compared to

precursor **5**, which indicated that the amine chain is necessary for AChE inhibition. The results also showed that the general trend for eeAChE inhibition was 4'-*O*-modified > 2'-*O*-modified > 3'-*O*-modified compounds. To optimize the inhibitory activities of the compounds, the linker length and the terminal amines groups were introduced into the lead compound. In contrast, the compound with a two-carbon spacer between the amine groups and hydroxyl of compound **5** exhibited better inhibition than those with a three-carbon, four-carbon or six-carbon linker. For example, the eeAChE inhibitory activity of compound **16** (IC₅₀ = 0.89 ± 0.02 μM) was five-fold higher than that of **17** (IC₅₀ = 5.21 ± 0.18 μM), which has a three-carbon linker, and twelve-fold higher than that of **18** (IC₅₀ = 12.29 ± 0.12 μM) and **19** (IC₅₀ = 12.81 ± 0.11 μM), which have four-carbon and six-carbon linkers, respectively. As an exception, the eeAChE inhibitory activity of **27**, which has a 6-methylene chain, was better than that of **24**. The results revealed that linker length played an important role in eeAChE inhibitory activity. The terminal amine group on the side chain also was crucial for AChE inhibition. Most of the compounds possessing an *N*-methyl or *N*-ethyl group (**16–23**, **42–45**) displayed better eeAChE inhibitory activity than those possessing benzylamine (**24–30**) or piperazine group (**32–37**). This was because the alkylamino side chain can interact with the catalytic active site (CAS) of AChE,⁴⁰ and this was also supported by the molecular docking study.

Almost all the compounds exhibited moderate or very weak eqBuChE inhibitory activity and showed higher selectivity for eeAChE over eqBuChE. This selectivity profile may be beneficial in lowering the peripheral cholinergic side effects

Table 1 MAOs and ChEs inhibition data of the synthesized compounds

Compound	R	Position	<i>n</i>	IC ₅₀ ^a (μM) ± SD		Selectivity index eqBuChE/eeAChE	IC ₅₀ (μM) ± SD		Selectivity index hMAO-A/hMAO-B
				eeAChE	eqBuChE		hMAO-B	hMAO-A	
16	A	<i>p</i> -OH	2	0.89 ± 0.02	28.71 ± 1.21	32.3	0.0372 ± 0.0002	38.1 ± 0.14	1024
17	A	<i>p</i> -OH	3	5.21 ± 0.18	20.34 ± 0.89	3.9	0.0438 ± 0.0003	N ^b	>2283
18	A	<i>p</i> -OH	4	12.29 ± 0.12	18.91 ± 0.23	1.54	0.756 ± 0.002	N	>132
19	A	<i>p</i> -OH	6	12.81 ± 0.11	9.12 ± 0.14	0.712	3.22 ± 0.11	39.1 ± 0.17	12.1
20	B	<i>p</i> -OH	2	1.03 ± 0.01	28.88 ± 0.73	28	0.0381 ± 0.001	46.6 ± 0.08	1223
21	B	<i>p</i> -OH	3	3.20 ± 0.06	8.27 ± 0.15	2.58	0.166 ± 0.010	62.9 ± 0.20	379
22	B	<i>p</i> -OH	4	6.10 ± 0.10	4.61 ± 0.14	0.756	2.86 ± 0.22	52.9 ± 0.32	18.5
23	B	<i>p</i> -OH	6	3.75 ± 0.24	4.83 ± 0.33	1.29	2.90 ± 0.17	37.8 ± 0.21	13.1
24	C	<i>p</i> -OH	2	19.33 ± 1.30	41.46 ± 0.14	2.14	2.28 ± 0.14	N	>43.8
25	C	<i>p</i> -OH	3	52.26 ± 0.31	19.29 ± 0.80	0.369	0.880 ± 0.005	N	>114
26	C	<i>p</i> -OH	4	19.72 ± 0.22	18.41 ± 0.35	0.934	8.61 ± 0.04	N	>11.6
27	C	<i>p</i> -OH	6	1.77 ± 0.09	50.74 ± 1.11	28.7	>100	N	>1
28	D	<i>p</i> -OH	2	1.29 ± 0.07	49.39 ± 0.28	38.3	4.29 ± 0.03	N	>23.3
29	D	<i>p</i> -OH	3	29.42 ± 0.15	17.16 ± 0.07	0.583	1.59 ± 0.04	—	—
30	D	<i>p</i> -OH	4	43.34 ± 0.59	10.85 ± 0.16	0.25	3.68 ± 0.13	N	>27.2
31	D	<i>p</i> -OH	6	16.96 ± 0.25	40.00 ± 0.60	2.36	>100	N	>1
32	E	<i>p</i> -OH	3	10.11 ± 0.06	15.45 ± 0.10	1.53	22.7 ± 1.3	N	>4.41
33	E	<i>p</i> -OH	4	13.02 ± 0.11	10.40 ± 0.27	0.799	16.4 ± 0.2	N	>6.1
34	E	<i>p</i> -OH	6	32.34 ± 0.19	27.27 ± 0.18	0.843	6.24 ± 0.4	N	>16.1
35	F	<i>p</i> -OH	2	21.02 ± 0.17	31.45 ± 0.13	1.5	97.5 ± 2.9	N	>1.03
36	F	<i>p</i> -OH	3	1.02 ± 0.07	89.55 ± 0.99	87.8	38.6 ± 0.54	N	>2.59
37	F	<i>p</i> -OH	6	60.78 ± 0.14	N	>1.65	>100	N	>1
38	A	<i>o</i> -OH	2	65.26 ± 0.82	10.03 ± 0.17	0.154	>100	N	>1
39	A	<i>o</i> -OH	3	75.08 ± 1.21	8.74 ± 0.18	0.116	>100	N	>1
40	B	<i>o</i> -OH	2	65.30 ± 1.25	11.50 ± 0.34	0.176	43.8 ± 2.1	N	>2.64
41	B	<i>o</i> -OH	3	54.79 ± 0.26	4.89 ± 0.35	0.0892	N	N	—
42	A	<i>m</i> -OH	2	1.04 ± 0.02	8.37 ± 0.19	8.05	0.571 ± 0.022	38.0 ± 0.44	66.5
43	A	<i>m</i> -OH	3	4.97 ± 0.06	2.16 ± 0.01	0.435	0.0484 ± 0.0002	93.7 ± 1.11	1935
44	B	<i>m</i> -OH	2	4.16 ± 0.17	1.68 ± 0.06	0.403	0.824 ± 0.021	80.0 ± 0.46	97.1
45	B	<i>m</i> -OH	3	14.07 ± 0.28	1.03 ± 0.03	0.0732	18.2 ± 0.21	64.7 ± 0.05	3.55
5	—	—	—	44.4 ± 0.12	N	>2.25	0.242 ± 0.002	N	>413
Donepezil	—	—	—	0.0163 ± 0.0003	1.96 ± 0.10	120	n.t.	n.t.	n.t.
Pargyline	—	—	—	n.t. ^c	n.t.	n.t.	0.0752 ± 0.0004	1.10 ± 0.01	14.6

^a IC₅₀: 50% inhibitory concentration (means ± SD of three experiments). ^b Inactive at 100 μM (highest concentration tested), at higher concentrations the compounds precipitate. ^c n.t. = not tested.

and providing lower toxicity; *e.g.* tacrine, an AChE inhibitor, exhibited some side effects, which may be attributed to its poor selectivity.⁴¹

Table 2 Inhibition of human AChE activity

Compound	IC ₅₀ ^a (μM)		Selectivity index hBuChE/hAChE
	hAChE	hBuChE	
16	0.657 ± 0.002	9.31 ± 0.02	14.2
20	3.29 ± 0.01	23.6 ± 1.1	7.17
22	14.1 ± 0.4	11.3 ± 0.3	0.801
23	1.85 ± 0.05	4.25 ± 0.06	2.30
27	40.9 ± 1.6	20.0 ± 0.3	0.489
28	22.3 ± 0.7	74.24 ± 0.2	33.3
36	4.72 ± 0.08	N ^b	>21.2
42	1.41 ± 0.01	4.44 ± 0.03	3.15
43	25.72 ± 0.10	39.3 ± 0.6	1.53
44	30.3 ± 0.1	10.3 ± 0.4	0.340
5	99.9 ± 2.12	N	1.01
Donepezil	0.0247 ± 0.0003	5.58 ± 0.04	226

^a IC₅₀: 50% inhibitory concentration (means ± SD of three experiments). ^b Inactive at 100 μM (highest concentration tested), at higher concentrations the compounds precipitate.

The good biological profiles of many of the inhibitors (16, 20, 22, 23, 27, 28, 36, 42, 43 and 44) against eeAChE encouraged us to extend our inhibition assays to the human enzymes, AChE (hAChE) and BuChE (hBuChE). The bioactivities of selected compounds are listed in Table 2. All the test compounds showed moderate inhibition against hAChE, which

Fig. 2 Kinetic study on the mechanism of eeAChE inhibition by compound 16. Overlaid Lineweaver-Burk reciprocal plots of AChE initial velocity at increasing substrate concentration (0.05–0.25 mM) in the absence of inhibitor and in the presence of 16 are shown. Lines were derived from a weighted least-squares analysis of the data points.

Fig. 3 (A) Reversibility studies of hMAO-B inhibition by compound **16**. Compound **16** and pargyline were pre-incubated for various periods of time (0–60 min) with hMAO-B at concentrations equal to twofold the IC_{50} values for the inhibition of the enzyme. After dilution to concentrations of **16** and pargyline equal to IC_{50} , the inhibitory rates were recorded. (B) Kinetic study on the mechanism of hMAO-B inhibition by **16**. Overlaid Lineweaver–Burk reciprocal plots of hMAO-B initial velocity at increasing *p*-tyramine concentration (0.05–0.25 mM) in the absence of inhibitor and in the presence of **16** are shown. Lines were derived from a weighted least-squares analysis of the data points.

was relatively low compared to that against eeAChE. Unexpectedly, compound **16** (hAChE $IC_{50} = 0.657 \pm 0.002 \mu\text{M}$) and **23** (hAChE $IC_{50} = 1.85 \pm 0.05 \mu\text{M}$), bearing respective *N*-methyl and *N*-ethyl groups, exhibited better inhibitory activity against eeAChE. This phenomenon may have resulted from the conformational difference between hAChE and eeAChE.⁴² Taking the aforementioned results into account, compound **16** was selected for further studies, due to its relatively potent ChEs inhibitory activity compared to the other synthesized compounds.

Kinetic study of eeAChE inhibition

To explore in-depth the mechanism of eeAChE inhibition, compound **16** was selected for enzyme kinetic study. The results displayed that both slopes and intercepts were increased accordingly with the concentration of compound **16** (Fig. 2). This pattern indicated that **16** was a mixed-type inhibitor of eeAChE, thus supporting dual binding site AChE inhibition. Replots of the slope *versus* different concentration of compound **16** gave an inhibition constant, K_i , of $0.404 \mu\text{M}$.

Monoamine oxidase inhibitory activity

To elucidate the multitarget profile of the synthetic compounds, the inhibitory activity against hMAO-B and hMAO-A was determined and compared with the lead compound **5** and pargyline by the Amplex Red MAO assay.⁴³ As seen in Table 2, compounds **16–18**, **20**, **21**, **25**, and **42–44**, showed potent hMAO-B inhibitory activity, acting in the nanomolar (IC_{50} ranging from 0.0372 ± 0.0002 to $0.880 \pm 0.005 \mu\text{M}$). On the contrary, they were less potent against hMAO-A. The most potent hMAO-B inhibitor (**16**; hMAO-B $IC_{50} = 0.0372 \pm 0.0002$

μM) also showed good selectivity towards hMAO-B and hMAO-A ($SI = 1024$). Compounds **32–37**, bearing a piperazine group, and **26–31**, bearing a benzylamine group, both exhibited relatively moderate or poor hMAO-B and hMAO-A inhibitory activity. Similarly, the 2'-*O*-modified compounds **38–41** and the 3'-*O*-modified compound **45** also showed moderate hMAO-B and hMAO-A inhibitory activities. Altogether, these results showed that compounds bearing *N*-methyl or *N*-ethyl groups were relatively better hMAO-B inhibitors. Among them, compound **16** was the most potent inhibitor, even better than pargyline and the lead compound **5**.

Reversibility and kinetic study of MAO-B inhibition

To explore the reversibility/irreversibility toward hMAO-B inhibitor, the most potent hMAO-B inhibitor compound **16** was selected for further study by a time-dependent inhibition assay.¹⁴ Compound **16** was pre-incubated with hMAO-B over different time periods (0, 15, 30, 45, 60 min) at a concentration of twofold IC_{50} , $0.003 \mu\text{M}$. The irreversible hMAO-B inhibitor pargyline was used as a reference (Fig. 3A). Our findings demonstrated that **16** was not a time-dependent inhibitor but was a reversible hMAO-B inhibitor.

To further study the type of hMAO-B inhibition, the kinetic behavior of **16** toward hMAO-B was determined by generating a Lineweaver–Burk plot (Fig. 3B). The catalytic rates were measured at five different *p*-tyramine concentrations (0.05–0.25 mM) with different concentrations of **16**. The results showed that the V_{max} decreased as the concentration of **16** increased, whereas the K_m value kept constant. Therefore, compound **16** was determined to be a noncompetitive inhibitor, with a K_i value of $0.551 \mu\text{M}$.

In vitro blood–brain barrier permeation study

The ability to cross the blood–brain barrier (BBB) is a significant criterion for successful central nervous system (CNS) drugs. To evaluate whether the synthesized compounds could penetrate this barrier, a parallel artificial membrane permeation assay (PAMPA) was adopted.⁴⁴ Compared with the permeability of 8 commercial drugs reported in the literature (ESI,† Table S1), a plot of the experimental data *versus* reported values generated a

Table 3 Permeability results ($P_e \times 10^{-6} \text{ cm s}^{-1}$) from the PAMPA-BBB assay for selected compounds with their predicted penetration into the CNS

Compounds	Permeability ^a ($P_e \times 10^{-6} \text{ cm s}^{-1}$)	Prediction
16	10.56 ± 0.41	CNS+

^a Data are the mean $\pm$ SD of three independent experiments.

Fig. 4 (A) 3D docking model of compound **16** with hAChE. Atom colors: yellow-carbon atoms of **16**, gray-carbon atoms of residues of hAChE, dark blue-nitrogen atoms, red-oxygen atoms. The dashed lines represent the interactions between the protein and the ligand. (B) 2D schematic diagram of docking model of compound **16** with AChE. The figure was prepared using the ligand interactions application in MOE.

Fig. 5 (A) 3D docking model of compound **16** with hMAO-B. Atom colors: yellow-carbon atoms of **16**, gray-carbon atoms of residues of hMAO-B, green-carbon atoms of FAD, dark blue-nitrogen atoms, red-oxygen atoms. The dashed lines represent the interactions between the protein and the ligand. (B) 2D schematic diagram of docking model of compound **16** with hMAO-B. The figure was prepared using the ligand interactions application in MOE.

good linear correlation, $P_e(\text{exp.}) = 0.6022 P_e(\text{Bibl.}) + 0.8073$ ($R^2 = 0.9749$) (Fig. S1†). From this equation and considering the limitation established by Di *et al.* for BBB permeation, the following ranges of permeability were established: compounds with $P_e(10^{-6} \text{ cm s}^{-1}) > 3.22$ for high BBB permeation (CNS+), compound with $P_e(10^{-6} \text{ cm s}^{-1}) < 2.31$ for low BBB permeation (CNS-), and compound with $2.31 < P_e(10^{-6} \text{ cm s}^{-1}) < 3.22$ for uncertain BBB permeation (CNS±). From the results shown in Table 3, compound **16** showed a higher P_e value than 3.22 ($P_e = 10.56 \times 10^{-6} \text{ cm s}^{-1}$), which indicated that **16** can cross the BBB and may reach the therapeutic targets in the CNS.

Molecular modeling studies

To study the possible interaction mode of compound **16** with AChE and hMAO-B, molecular modeling was performed

using the Molecular Operating Environment (MOE) software. The X-ray crystal structures of hAChE and hMAO-B were acquired from the PDB. As shown in Fig. 4A and B, compound **16** can simultaneously interact with the PAS and CAS of hAChE (PDB 4EY7). The phenyl group of chroman-4-one interacted with the PAS of hAChE through a π - π stacking interaction with Trp 286 (3.76 Å). In the bottom of the gorge, the protonated nitrogen of *N*-dimethylamino can bind to the CAS at the indole ring of Trp 86 (4.07 Å) *via* a cation- π interaction. The docking results implied that **16** is a dual site binding inhibitor of hAChE. All these results were in accordance with our molecular design for AChE inhibition.

For our MAO-B studies, the X-ray crystal structure with FAD of hMAO-B (PDB 2V60) was used. We docked compound **16** into the binding site of hMAO-B and observed that **16** can suitably fit into the pocket. In close proximity to the FAD

Fig. 6 Effects of compound **16** on scopolamine-induced memory deficit in the step-down passive avoidance test. Compound **16** (at 3, 9, 27 mg kg⁻¹, p.o.), or donepezil (5 mg kg⁻¹, p.o.) were orally given 1 h before treatment with scopolamine. After 30 min, the mice were treated with scopolamine (3 mg kg⁻¹, i.p.) and test in the step-down passive avoidance. Values are expressed as the mean SEM (*n* = 10). #*p* < 0.05 vs. normal group. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 vs. scopolamine-treated control group.

cofactor, a π - π stacking was seen between Tyr 398 (3.97 Å) and the phenyl ring of chroman-4-one. Moreover, another π - π interaction was established between the substituted phenyl ring and Tyr 326 (4.45 Å) (Fig. 5A and B). Besides, a hydrogen bond was also formed between the oxygen of chroman-4-one and Tyr 435-OH (3.06 Å). All the above-mentioned results further confirmed that **16** is a potent hMAO-B inhibitor.

Acute toxicity studies

Single oral doses of 0, 677, 1333 or 2000 mg kg⁻¹ were administered in order to investigate the acute toxicity of compound **16** *in vivo*. Kunming mice were randomly divided into 4 groups (5 mice per group): 0, 677, 1333, and 2000. Mice were observed the first 4 h after oral administration of the compound for fatality and abnormal behavior. The abnormal effect was monitored for the next 14 days, including body weight changes, fatality changes and mental behavior. All mice were executed on the 14th day and examined for possible damages to the heart, liver and kidneys. No acute toxicity was observed during the experimental period. This observation indicated that compound **16** was safe at doses up to 2000 mg kg⁻¹ within two weeks.

Cognitive and memory assays *in vivo*

Scopolamine induces memory impairment and has been used widely to assess potential agents for AD therapy.^{3,37,45} To determine whether the treatment of **16** improves memory in scopolamine-induced mice, the passive avoidance test was performed.⁴⁶

As the results show (Fig. 6), the latency of mice treated with scopolamine alone (3 mg kg⁻¹) was significantly shorter (*p* < 0.05) than that of vehicle-treated mice (normal group). Treatment with compound **16** (3, 9, 27 mg kg⁻¹) increased the latency time in a dose-dependent manner, and the maximum dose group (27 mg kg⁻¹) exhibited longer latency (241.2 s) (*p* < 0.001) than the other dose groups, even better than the donepezil group (5 mg kg⁻¹) (215.3 s). Therefore, the opti-

mal dose of **16** might be 27 mg kg⁻¹. These results revealed that compound **16** may induce reversed scopolamine-induced memory impairment, based on the step-down passive avoidance test, by increasing brain cholinergic activity due to the inhibition of AChE.

Conclusion

In conclusion, our study involved the synthesis of a new series of homoisoflavonoid derivatives as multitarget anti-Alzheimer agents. These compounds demonstrated inhibitory activity of AChE and MAO *in vitro*. Among them, compound **16** exhibited moderate inhibitory activity toward AChE and significant hMAO-B inhibitory activity. Kinetic and molecular docking studies demonstrated that compound **16** was a dual binding site AChE inhibitor and noncompetitive inhibitor of MAO-B. Moreover, compound **16** could cross the blood-brain barrier, based on the PAMPA-BBB assay. *In vivo* assays demonstrated that compound **16** did not show signs of acute toxicity at doses of up to 2000 mg kg⁻¹, and could result in reverse cognitive deficit, according to the step-down passive avoidance test. Above all, compound **16** might be a promising lead as a multitarget agent for further study in the treatment of AD.

Abbreviations

AD	Alzheimer's disease
AChE	Acetylcholinesterase
A β	β -Amyloid protein
NMDA	N-Methyl-D-aspartate
MTDL	Multi-target-directed ligand
ChE	Cholinesterase
BuChE	Butyrylcholinesterase
ACh	Acetylcholine
BuCh	Butyrylcholine
MAO	Monoamine oxidase
5-HT	5-Hydroxytryptamine
PEA	β -Phenylethylamine

IMAOs	MAO inhibitors
ROS	Reactive oxygen species
PAS	Peripheral anionic site
CAS	Catalytic site
SAR	Structure–activity relationship
BBB	Blood–brain barrier
SI	Selectivity index
CNS	Central nervous system
PAMPA	Parallel artificial membrane permeation assay

Acknowledgements

We gratefully acknowledge the financial support of the National Natural Science Foundation of China (81573313), the Program for Changjiang Scholars and Innovative Research Team in University (IRT_15R63), the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). All procedures were approved by the China Pharmaceutical University Animal Care and Use Committee (IACUC) and were in compliance with the National Institute of Health (NIH) guidelines.

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