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Synthesis and Evaluation of Donepezil-Ferulic Acid Hybrids as Multi-Target-Directed Ligands against Alzheimer's Disease†

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Abstract

A novel family of donepezil-ferulic acid hybrids were designed, synthesized and biologically evaluated as multi-target-directed ligands against Alzheimer's disease by fusing a fragment of donepezil and ferulic acid. The *in vitro* assay indicated that some of these molecules exhibited potent cholinesterase inhibitory activities, outstanding radical scavenging activities, good neuroprotective effects on PC12 cells and could penetrate into the central nervous system. Compound **5c** especially showed excellent acetylcholinesterase inhibitor activity (IC_{50} values of 0.398 μM for electric eel acetylcholinesterase) and strong butyrylcholinesterase inhibitory activity (IC_{50} = 0.976 μM for equine serum butyrylcholinesterase). It also showed significant antioxidant activity (1.78 trolox equivalents by ABTS method, IC_{50} 24.9 μM by the DPPH method). Kinetic study and molecular docking indicated that compound **5c** interacted with both the peripheral anionic site and the catalytic binding site of acetylcholinesterase. Overall, these results indicated that compound **5c** is a promising drug candidate with balanced properties for the treatment of Alzheimer's disease.

Keywords

Alzheimer's disease; Donepezil; Ferulic acid; Cholinesterase; Antioxidant.

Abbreviations

AD, Alzheimer's disease; MTDL, multitarget-directed ligand; FA, ferulic acid; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; CNS, central nervous system; BBB, blood-brain barrier; MTT, methyl thiazolyl tetrazolium. DPPH 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2-azino-bis(3-ethylbenzthiazo-line-6-sulfonicacid).

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by memory loss and cognitive decline and affects over 46 million people worldwide.[1] Although considerable efforts have been made to investigate the pathophysiology of AD, the exact etiology of AD remains a mystery. Several hypotheses, including low levels of acetylcholine, dyshomeostasis of biometals, neuroinflammation and oxidative stress, are demonstrated to play significant roles in the pathogenesis of AD.[2-5] Currently, the primary therapeutic options for treatment of this disease are limited to three acetylcholinesterase (AChE) inhibitors (rivastigmine, donepezil and galantamine) and one *N*-methyl-$_D$-aspartate (NMDA) receptor antagonist (memantine).[6, 7] However, these drugs are effective in improving the symptoms for only a short period of time and cannot cure the disease. Recent studies have pointed out that drugs impacted on multiple targets can provide a more effective treatment strategy for AD than single target directed drugs.[8] Thus, the multi-target-directed ligand (MTDL) approach has been the major focus of attention, and a variety of compounds acting on various targets were developed.[9-11]

Based on the cholinergic hypothesis, degeneration of cholinergic neurons, reduced cholinergic neurotransmission and the deterioration of cognitive function of patients were major symptoms for AD.[12] Therefore, sustaining or recovering cholinergic function was supposed to be clinically beneficial. It was shown that cholinesterase (ChE) inhibitors were the most effective treatment for AD hitherto.[13] Acturally, AChE and butyrylcholinesterase (BuChE), two types of ChEs, are all able to hydrolyze

acetylcholine (Ach).[14] It was demonstrated by X-ray crystallography that AChE has a 20 Å deep narrow gorge which consists of two binding sites: the catalytic active site (CAS) at the bottom and the peripheral anionic site (PAS) near the entrance of the gorge.[15, 16] In addition, PAS was closely interacted with hydrolysis of Ach.[17] Besides, BuChE is another target of interest in the research and development for anti-Alzheimer drugs, since this enzyme exerts a compensatory effect in response to a large decrease in AChE activity when AD progresses.[18] Donepezil (**2**, Figure 1), an AChE inhibitor, is the second drug approved by the U.S. Federal Drug Agency (FDA) for the treatment of mild-to-moderate AD. It shows high selectivity for AChE as opposed to BuChE.[19] The safety and efficacy of donepezil in the treatment of AD has encouraged active research in the development of donepezil-like agents for AD therapy.[20, 21]

On the other hand, oxidative stress also plays a critical role in the AD pathological cascade.[22] Converging lines of evidence demonstrated that oxidative damage in cellular structures is augmented during aging and it occurs in the early stages of AD and promotes the formation of other pathological hallmarks of the disease, such as amyloid plaques and neurofibrillary tangles.[23] Moreover, a multinational study involving 23 developed countries suggested that higher consumption of dietary flavonoids, is associated with lower population rates of dementia.[24] Therefore, drugs which can prevent the oxygen free radical damage are helpful for the treatment of AD.

Due to the important effects of cholinesterase inhibitors and antioxidants in AD treatment, some agents were designed and synthesized, which could not only inhibit

AChE but also exhibit neuroprotective effect by decreasing the oxidative damage in brain.[25] A series of tacrine-ferulic acid hybrids designed by Benchekroun *et al*. exhibited excellent inhibition towards ChEs and strong antioxidant activity.[26] Besides, 2*H*-Chromen-2-one derivatives and donepezil–ferulic acid hybrids also showed good ChEs inhibitory activities and potent antioxidant effects.[27, 28]

Ferulic acid (FA, **1**, Figure 1) is one of the ubiquitous compounds in nature, especially abundant in cereals. FA, which belongs to the class of phenylpropanoid derivatives, is also identified as one of the main effective component of several Chinese medicine such as Ferulic, *Angelica Sinensis* and *RhizomaLigustici* (Chuanxiong). It was revealed that FA protected the progression of variety of age-related diseases due to its antioxidant properties.[29] FA can greatly attenuate neuronal cell death caused by reactive oxygen species (ROS) and protect the brain from amyloid-beta peptide (Aβ) neurotoxicity.[30, 31] A recent isolated study on rat hepatocytes suggested that FA and similar structures are effective in inhibiting or decreasing glyoxal or methylglyoxal-induced cytotoxicity and oxidative stress.[32] Additionally, FA protected PC12 cells against 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress by increasing catalase and superoxide dismutase activity and reducing cellular lactate dehydrogenase release and malondialdehyde levels.[33] Consequently, FA could act as the beneficial antioxidant fragment in the designed multi-target donepezil-antioxidant hybrids.

Considering these criteria, we report the design and synthesis of hybrids of ferulic acid and donepezil as a valued strategy to develop effective neuroprotective

compounds as potential AD drugs (Figure 1). The significant pharmacophores of donepezil and FA were fused to obtain novel compounds acting on multiple targets. We then report the biological activity studies of these compounds including the inhibition of ChEs activity, antioxidant properties, neuroprotection, metal chelation and blood–brain barrier permeation.

2. Results and discussion

2.1 Chemistry

The synthetic routes for designed compounds **4a-4i** and **5a-5n** were shown in Scheme 1. Commercially available ferulic acid derivatives **1a-1g** as starting materials were reacted with (1-benzylpiperidin-4-yl)amine (**3a**) or 4-(2-aminoe-thyl)-1-benzylpiperidine (**3b**) in a mixture of dichloromethane (DCM) and *N*, *N*-dime thylform-amide (DMF) to give the target compounds in good yields.[34] To further evaluate the role of the double bond between the phenyl ring and the amide, compound **6** and **7** were synthesized (Scheme 2) by Pd-C reduction process.[35] The syntheses of the target compounds **11a-11c** were presented in Scheme 3 to explore the effect of conjugated chains. Interestingly, the FA moiety possessing a dicarbonyl happened to be part of the curcumin, which is another natural product and exhibits great potential as a therapeutic agent for AD.[36] Briefly, ethyl 4-chloroace-toacetate, **8**, was reacted with Ph_3P to give the ylide **9** in good yield, upon a Wittig reaction with the corresponding aldehyde in tetrahydrofuran (THF) in the presence of NaH at 0 ^{o}C.[37] Finally, condensation of **10a-10c** with **3a** or **3b** in xylene under refluxing conditions yielded **11a-11c**.[35] All compounds were purified by column

chromatography. The structures were verified by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry as cited in the experimental section.

2.2 In vitro inhibition of ChEs

From clinical practice, it is well known that ChE inhibitors are effective in improving behavior and well-being and slowing down cognitive decline in patients with dementia. Thus, the inhibitory activities of the novel donepezil-ferulic acid hybrids and the reference compounds against AChE and BuChE were evaluated by the method of Ellman *et al*..[38]

Considering their lower cost and the high degree of sequence identity, AChE from electric eel and BuChE from equine serum were initially used. The results shown in Table 1 indicated that all the tested compounds showed moderate to good inhibitory activities towards AChE and BuChE with IC_{50} values ranging from micromolar to sub-micromolar and all target compounds were more potent than the parent compounds **3a**, **3b** and FA. The length of the alkyl spacer between the amide and pyridine ring could significantly influence both ChEs inhibitory activity. The compounds **5** series which bear a longer alkyl linker between the *N*-benzylpiperidine moiety and FA demonstrated better activities than the series of compounds **4**. Compounds without a hydroxyl group (**5g**-**5k**) on the phenylpropanoid moiety showed better inhibition of both ChEs than compounds (**5d**-**5f**) with hydroxyl groups. For example, **5k** (IC_{50} = 0.130 μM for AChE; IC_{50} = 0.416 μM for BuChE) possessing three methoxy groups was better than **5e** (IC_{50} = 5.17 μM for AChE; IC_{50} = 165.1 μM for BuChE) with two hydroxyl groups. The IC_{50} values of compounds (**5a**-**5c**) bearing

both methoxy group and hydroxyl group was between the IC_{50} value of the other two series of compounds, and the location of the hydroxyl and the methoxy (*e.g.*, **5a**: IC_{50} = 0.651μM; **5b**: IC_{50} = 1.06 μM) could significantly influence both ChEs inhibitory activity. Compound **5c** bearing two methoxys on the R_2 and R_4 position and one hydroxyl on the R_3 position has better ChEs inhibitory activity(IC_{50} = 0.398 μM for AChE; IC_{50} = 0.976 μM for BuChE).

To further evaluate the role of the double bond between the phenyl ring and the amide, compound **6** and **7** were synthesized (Scheme 2) and evaluated. Notably, **6** and **7** exhibited lower inhibitory activity for both ChEs, suggesting that the double bond and the conjugation system with the phenyl ring is essential to produce inhibitory effects for these analogues.

To further explore the effect of conjugated chains on ChEs inhibitory activities and mimic the structure of curcumin, the other series of compounds (**11a**-**11c**) which contain β-diketone were synthesized (Scheme 3) and evaluated. Obviously, **11a**-**11c** exhibited moderate inhibitory activity of both ChEs (11a, IC_{50} = 2.60 μM for AChE; IC_{50} = 1.08 μM for BuChE), suggesting that the β-diketone bond is not needed to produce inhibition for these analogues. Then, a selection of the compounds (**5a**-**5n** and **7**) were evaluated as inhibitors of human ChEs with aim to further evaluate them and the results were listed in Table 2. From the table, it can be seen that all the compounds are also potent inhibitors of human ChEs.

2.3 *In vitro* antioxidant activities

ABTS radical cation scavenging method

The antioxidant activities of all target compounds were evaluated using a radical scavenging assay ABTS (2, 2-azino-bis(3-ethylbenzthiazo-line-6-sulfonicacid)) with water-soluble Vitamin E analog-trolox as reference compound (Table 1).[39] Ferulic acid and curcumin were also analysized for comparison. The antioxidant activities of the compounds were provided as a trolox equivalent, with their relative potency at 25 μM compared with trolox. As shown in Table 1, some of these selected compounds demonstrated outstanding antioxidant activities. Compounds **4a**, **4d**, **4i**, **5a**, **5c**, **5e**, **6** and **7** had the ability to scavenge the ABTS radical with 1.41, 1.81, 1.65, 1.39, 1.78, 2.10, 0.76, 1.28 trolox equivalents, respectively.

DPPH radical scavenging method

DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals can be used in preliminary screening of scavenging reactive oxygen species, since these nitrogen radicals are much more stable and easier to handle than oxygen free radicals.[40] The IC_{50} of all the compounds in Table 1 indicated that the compounds (**4b**, **4c**, **4f**, **4g**, **4h**, **5g-5n**) without a phenolic hydroxyl had less radical scavenging activities. Obviously, compounds **4e** and **5d**, which contain one phenolic hydroxyl at the R_3 position of the phenylpropanoid moiety showed low radical scavenging activities. Compounds **4a**, **4d**, **5a** and **5e** which bear a phenolic hydroxyl group or methoxy group on R_2 demonstrated that the group at the ortho position of the phenolic hydroxyl is crucial. Moreover, the IC_{50} of compound **5c** (IC_{50} = 24.9 μM) showed that the location of the phenolic hydroxyl and methoxy group is nonadjustable. After all these biological evaluations, **5c** was chosen as the most promising compound for further study because

of its strong and balanced inhibition for both ChEs and antioxidant activity close to trolox.

2.4 Kinetic study of ChEs inhibition

To further uncover the mechanism of inhibition and the binding site of target compounds on ChEs, a kinetic study was performed with the most promising compound **5c**.[9] The Lineweaver-Burk plots of **5c** (Figure 2) showed both increasing slopes and increasing intercepts at increasing inhibitor concentration, which suggested that **5c** was a mixed-type inhibitor for both enzymes. This pattern indicated that compound **5c** might be a dual binding site inhibitor of AChE.

2.5 Molecular docking study with AChE

In order to further demonstrate the dual-site binding mode and get an insight into the interaction mechanism of **5c** with AChE, molecular docking study based on X-ray crystal structure of recombinant human acetylcholinesterase in complex with donepezil (hAChE, PDB code 1EVE) was carried out using the Molecular Operating Environment (MOE) software.[41, 42] These results shown in Figure 3 indicated that compound **5c** covered the binding gorge in a satisfactory orientation and conformation thus generate high inhibitory activity. The donepezil moiety occupied the CAS interacting by a π-π stacking with Trp84 and Phe330 of 4.37 Å and 4.36 Å, respectively. The phenylpropanoid moiety established a polar contact with Phe288 and Ser286 in the PAS (3.10 Å and 2.65 Å). After analyzing the docking results and taking the kinetic study into consideration, it was confirmed that compound **5c** was a dual binding site inhibitor that could interact simultaneously with PAS and CAS of

AChE.

2.6 Neuroprotection study

Motivated by the promising *in vitro* results of the antioxidant assay, target compounds **4a**-**4i**, **5a**-**5n, 6** and **7** were tested using the hydrogen peroxide (H_2O_2) model on PC12 cells to further confirm the antioxidant properties in neural cells. Toxic free radical formed from H_2O_2 results in oxidative damages on lipid, protein, and DNA, which finally cause mitochrondrial dysfunction, calcium imbalance, and apoptosis in neuronal cells.[43] PC12 cells were used as a screening model for studying neurodegenerative diseases and trolox was selected as positive control in this test.[44]

Firstly, the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazo-liumbromi-de] assay was carried out to evaluate activities of the target compounds at higher concentrations without the risk of inducing cytotoxicity in normal cells. As indicated in Figure 4, all compounds except **5m** did not show potential cytotoxic effects on PC12 cells at 20 μM afer incubation for 24 h.

The protective efficacy of target compounds against H_2O_2 at 5 μM was reported in Figure 5. Some of target compounds significantly showed protective effects against damage induced by H_2O_2 at 5 μM as shown in Figure 5. On the basis of the screening results above, compounds with good antioxidant activity were selected and tested to further evaluate the neuroprotective effect at 1, 5 and 10 μM (Figure 6). Notably, compounds bearing a phenolic hydroxyl group on the phenylpropanoid moiety exhibited much higher activies than compounds without a hydroxyl group, which is consistent with the result of the DPPH test.

2.7 Metal-Chelating Properties of 5c

The destruction of the balance of metal ions in CNS could result in neurodegenerative disorders. Studies have shown that the levels of biometals such as Cu^{2+}, Zn^{2+}, Fe^{2+}, and Fe^{3+} are higher in AD brain compared to healthy brain.[3, 45] Compounds with metal chelating effect might provide an additional and therapeutic strategy for the treatment of AD.

To evaluate the chelation ability of compound **5c**, a UV−vis spectroscopy assay was carried out with wavelength ranging from 200 to 400 nm (Figure 7).[46] The absorption spectra of **5c** (75 μM) alone or in the presence of $CuSO_4$, $FeSO_4$, $FeCl_3$, or $ZnCl_2$ (150 μM) for 30 min in methanol were recorded. It can be seen that new optical bands were detected at 272 nm after the addition of $CuSO_4$ to the solution of compound **5c**, which demonstrated the production of the corresponding complex via metal chelation. The chelating ability of **5c** was attributed to the presence of the dimethoxy group and hydroxyl group on the phenylpropanoid moiety and amide moiety in the core of the compound.[47, 48] However, with the addition of $FeCl_3$, $FeSO_4$ and $ZnCl_2$, there was no significant change.

2.8 *In vitro* blood–brain barrier permeation assay

In AD treatment, the ability of a drug to permeate the blood–brain barrier (BBB) is a critical process for central nervous system (CNS) drugs. The parallel artificial membrane permeability assay for BBB (PAMPA-BBB), which was described by Di *et al.*, was used to determine the BBB penetration of the target compounds.[49] Assay validation was performed by comparing experimental permeabilities of 9 reference

drugs with their reported values (Table 3), which gave a good linear correlation: P_e(exp) = 1.0416 P_e(Bibl.) – 0.8567 (R^2 = 0.9443). For blood–brain barrier permeation, we classified compounds as follows: compounds with P_e (10^{-6} cm s^{-1}) > 4.5 for high BBB permeation (CNS+), compounds with P_e (10^{-6} cm s^{-1}) < 2.1 for low BBB permeation (CNS-), and compounds with 4.5 > P_e (10^{-6} m s^{-1}) > 2.1 for uncertain BBB permeation (CNS±). The P_e values of these selected compounds are summarized in Table 4. It can be seen that compounds **5a**, **5c**, **5e** and **5k** might be able to cross the BBB.

3. Conclusion

In summary, this study involved the design, synthesis and biological evaluation of a novel series of MTDLs against AD by fusing the pharmacophores of ferulic acid and donepezil. The biological screening results indicated that most of the derivitives showed potent ChEs inhibitory activity. Specifically, target compounds displayed excellent potency in scavenging reactive free radicals. The optimal candidate compound, **5c**, exhibited potent ChEs inhibitory activities (0.398 μM for eeAChE, 0.321 μM for hAChE and 0.976 μM for eqBuChE, 1.22 μM for hBuChE), good biometal-chelating ability and antioxidant activity (1.78 trolox equivalent). Kinetic and molecular modeling studies indicated that **5c** was a mixed-type inhibitor, binding simultaneously to the active and peripheral sites of AChE. Above all, due to improvement of the activity, and BBB permeability, **5c** could thus be considered as potential multifunctional neuroprotective agent and serve as new a lead candidate in the treatment of AD.

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Legends

Scheme 1. Synthesis of **4a-4i** and **5a-5n**.

Scheme 2. Synthesis of **6** and **7**.

Scheme 3. Synthesis of **11a–11c.**

Table 1. Inhibition of eeAChE, eqBuChE, DPPH and ABTS of the synthesized compounds.

Table 2. Inhibition of human ChEs activities.

Table 3. Permeability ($P_e \times 10^{-6}$cm s^{-1}) in the PAMPA-BBB assay for 9 commercial drugs, used in the experiment validation.

Table 4. Permeability ($P_e \times 10^{-6}$cm s^{-1}) in the PAMPA-BBB assay for donepezil-ferulic acid hybrids and their predictive penetration in the CNS.

Figure 1. Drug design strategy for multi-target-directed ligands.

Figure 2. Kinetic study on the mechanism of eeChEs.

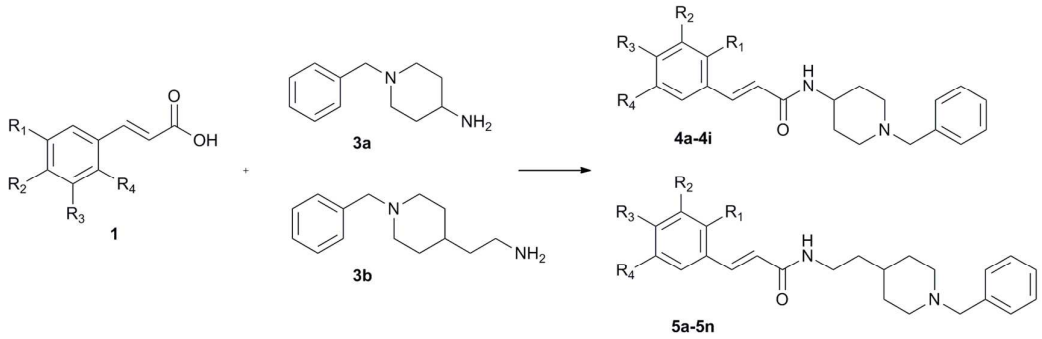
Figure 3. (A) 3D docking model of compound **5c** with hAChE. (B) 2D schematic diagram of docking model of compound **5c** with hAChE.

Figure 4. Effects of compounds on cell viability in PC12 cells.

Figure 5. Protective effects of **4a-4i**, and **5a-5n** against H_2O_2-induced injury in PC12 cells at 5 μM.

Figure 6. Protective effects of target compounds with prominent activities at 5 μM **4d**, **5a**, **5c**, **5e** and **7** against H_2O_2-induced injury in PC12 cells at 1, 5, and 10 μM.

Figure 7. UV absosobance spectrum of **5c** (75 μM) alone or in the presence of $CuSO_4$ (150 μM), $ZnCl_2$ (150 μM), $FeSO_4$ (150 μM), or $FeCl_3$ (150 μM) in MeOH.

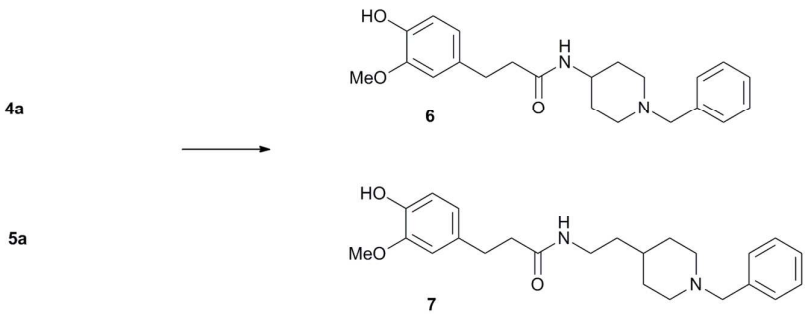


4a, R_1=OMe, R_2=OH, R_3=H
4b, R_1=OMe, R_2=OAc, R_3=H
4c, R_1=R_2=R_3=OMe
4d, R_1=R_2=OH, R_3=H
4e, R_1=R_3=H, R_2=OH
4f, R_1=R_3=H, R_2=F
4g, R_1=R_3=H, R_2=Cl
4h, R_1=R_3=H, R_2=NO_2
4i, R_1=R_3=OMe, R_2=OH

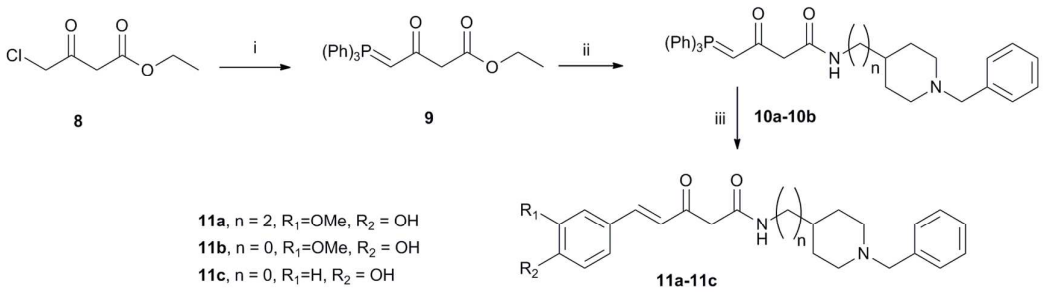
5a, R_1=R_4=H, R_2=OMe, R_3=OH
5b, R_1=R_4=H, R_2=OH, R_3=OMe
5c, R_1=H, R_2=R_4=OMe, R_3=OH
5d, R_1=R_2=R_4=H, R_3=OH
5e, R_1=R_4=H, R2=R3=OH
5f, R_1=OH, R_2=R_4=R_3=H
5g, R_1=R_2=R_4=R_3=H

5h, R_1=R_4=H, R_2=OMe, R_3=OAc
5i, R_1=R_4=H, R_2=R_3=OMe
5j, R_1=R_2=R_4=H, R_3=OMe
5k, R_1=H, R_2=R_4=R_3=OMe
5l, R_1=R_2=R_4=H, R_3=F
5m, R_1=R_2=R_4=H, R_3=Cl
5n, R_1=R_2=R_4=H, R_3=NO_2

Scheme 1. Synthesis of **4a-4i** and **5a-5n**. Reagents and conditions: EDCI, HOBt, DMF-DCM (1:1), rt, 12 h.



Scheme 2. Synthesis of **6** and **7**. Reagents and conditions: H_2, Pd/C, MeOH, rt, 8h.



Scheme 3. Synthesis of **11a–11c.** Reagents and conditions: (i) Ph_3P, toluene, 50°C, 24 h. (ii) xylene, reflux; (iii) NaH, THF, 40 °C, 3h, rt, overnight.

Table 1. Inhibition of eeAChE, eqBuChE, DPPH and ABTS of the synthesized compounds.

Compounds	IC_{50} (μM)[a]		Selectivity	DPPH assay	ABTS assay[c]
	eeAChE	eqBuChE	Index[b]	IC_{50} (μM)	
4a	29.3 ± 1.1	49.2 ± 1.5	1.68	32.5 ± 0.9	1.41
4b	22.0 ± 1.6	17.3 ± 0.8	0.786	N	0.35
4c	31.6 ± 1.3	1.25 ± 0.20	0.038	N	-
4d	62.1 ± 2.1	15.0 ± 0.89	0.241	10.6 ± 0.8	1.81
4e	70.8 ± 1.0	145.8 ± 3.0	2.06	980 ± 20	0.10
4f	59.3 ± 2.1	90.8 ± 1.9	1.53	N	-
4g	67.7 ± 2.5	51.4 ± 0.8	0.759	N	-
4h	7.12 ± 0.55	9.01 ± 0.87	1.26	N	-
4i	28.2 ± 1.2	7.34 ± 0.30	0.260	22.3 ± 1.1	1.65
5a	0.651 ± 0.036	1.22 ± 0.15	1.87	34.1 ± 1.8	1.39
5b	1.06 ± 0.05	2.47 ± 0.22	2.33	764 ± 18	0.27
5c	0.398 ± 0.028	0.976 ± 0.102	2.20	24.9 ± 1.4	1.78
5d	0.874 ± 0.07	1.24 ± 0.20	1.42	936 ± 31	
5e	5.17 ± 1.04	165.1 ± 2.8	31.9	10.7 ± 1.2	2.10
5f	2.16 ± 0.667	1.06 ± 0.17	0.491	977 ± 28	0.09
5g	0.543 ± 0.079	0.390 ± 0.091	0.718	N	-
5h	0.290 ± 0.031	0.670 ± 0.063	2.31	N	0.34
5i	0.285 ± 0.026	1.55 ± 0.18	5.43	N	-
5j	0.383 ± 0.032	0.244 ± 0.034	0.642	N	-
5k	0.130 ± 0.017	0.416 ± 0.061	3.20	N	-
5l	0.815 ± 0.080	1.98 ± 0.26	2.43	N	-
5m	0.521 ± 0.039	2.77 ± 0.27	5.32	N	-
5n	0.444 ± 0.034	3.10 ± 0.43	6.98	N	-
6	33.6 ± 1.7	31.9 ± 0.9	0.949	55.1 ± 2.1	0.76
7	7.48 ± 0.96	4.40 ± 0.48	0.588	48.3 ± 2.3	1.28
11a	2.60 ± 0.37	1.08 ± 0.16	0.415	76.8 ± 4.0	0.65
11b	26.3 ± 0.8	53.2 ± 0.9	1.99	88.7 ± 3.8	0.46
11c	58.3 ± 1.2	3.10 ± 0.54	0.053	890 ± 26	0.07
3a	N	N	-	n.t	n.t
3b	158.6 ± 1.8	N	-	n.t	n.t
Donepezil	0.035 ± 0.002	4.17 ± 0.27	0.251	n.t	n.t
Ferulic acid	N[d]	N	4.95	30.6 ± 1.6	1.21
Curcumin	N	N	4.43	23.3 ± 1.5	1.53
Trolox	n.t[e]	n.t.	-	n.t	1

[a] IC_{50}: 50% inhibitory concentration (means ± SD of three experiments).

[b] Selectivity Index = IC_{50} (eqBuChE)/IC_{50} (eeAChE).

[c] Data are expressed as (mmol trolox)/(mmol tested compound).

[d] Inactive at 1000 μM (highest concentration tested), at higher concentrations the compounds precipitate.

[e] n.t.= not tested

Table 2. Inhibition of human ChEs activities[a]

Compounds	IC_{50} (μM)[b]		Selectivity Index[c]
	hAChE	hBuChE	
5a	0.729 ± 0.133	1.12 ± 0.15	1.54
5b	1.66 ± 0.29	3.24 ± 0.50	1.95
5c	0.321 ± 0.012	1.22 ± 0.20	3.80
5d	2.20 ± 0.28	0.784 ± 0.112	0.36
5e	1.45 ± 0.18	145.1 ± 2.6	100
5f	2.68 ± 0.30	0.988 ± 0.069	0.37
5g	0.411 ± 0.018	1.41 ± 0.32	3.43
5h	0.339 ± 0.032	0.245 ± 0.048	0.72
5i	0.526 ± 0.028	2.54 ± 0.27	4.83
5j	0.690 ± 0.039	1.44 ± 0.20	2.09
5k	0.234 ± 0.015	0.669 ± 0.101	2.86
5l	3.30 ± 0.55	7.51 ± 0.64	2.28
5m	0.362 ± 0.027	2.07 ± 0.23	5.72
5n	0.337 ± 0.032	4.23 ± 0.48	12.5
7	5.32 ± 0.41	0.286 ± 0.078	0.054
donepezil	0.0308 ± 0.0025	8.28 ± 0.91	268

[a] AChE from human erythrocytes and BuChE from human serum were used.
[b] IC_{50}: 50% inhibitory concentration (means ± SD of three experiments).
[c] Selectivity Index = IC_{50} (hBuChE)/IC_{50} (hAChE).

Table 3. Permeability ($P_e \times 10^{-6}$cm s^{-1}) in the PAMPA-BBB assay for 9 commercial drugs, used in the experiment validation.

Commercial drugs	Bibl[a]	PBS/EtOH (70:30)[b]
Doptamine	0.2	0.24
hydrocortisone	1.9	1.89
piroxlcam	2.5	1.39
corticosterone	5.1	4.26
cloidine	5.3	4.56
progesterone	9.3	5.91
β-estradiol	12	12.02
verapamil	16	18.6
testoserme	17	15.6

[a] Taken from Ref.49.

[b] Data are the mean ± SD of three independent experiments.

Table 4. Permeability ($P_e \times 10^{-6}$cm s^{-1}) in the PAMPA-BBB assay for donepezil-ferulic acid hybrids and their predictive penetration in the CNS.

Compound	Pe×10^{-6}cm s^{-1}	Prediction
5a	5.16 ± 0.32	CNS+
5c	7.68 ± 0.59	CNS+
5e	5.38 ± 0.35	CNS+
5k	7.43 ± 0.60	CNS+

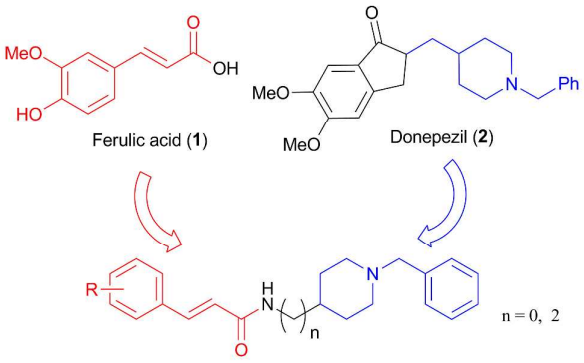


Figure 1. Drug design strategy for multi-target-directed ligands.

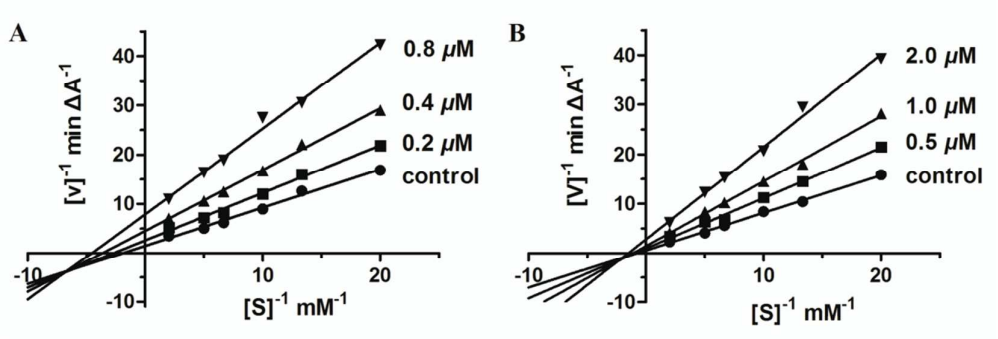


Figure 2. Kinetic study on the mechanism of eeChEs. (A) Kinetic study on the mechanism of *Ee*AChE inhibition by compound **5c**. Overlaid Lineweaver–Burk reciprocal plots of AChE initial velocity at increasing substrate concentration (0.05–0.50 mM) in the absence of inhibitor and in the presence of **5c** are shown. Lines were derived from a weighted least-squares analysis of the data points. (B) Kinetic study on the mechanism of eqBuChE inhibition by compound **5c**. Overlaid Lineweaver–Burk reciprocal plots of BuChE initial velocity at increasing substrate concentration (0.05–0.50 mM) in the absence of inhibitor and in the presence of **5c** are shown. Lines were derived from a weighted least-squares analysis of the data points.

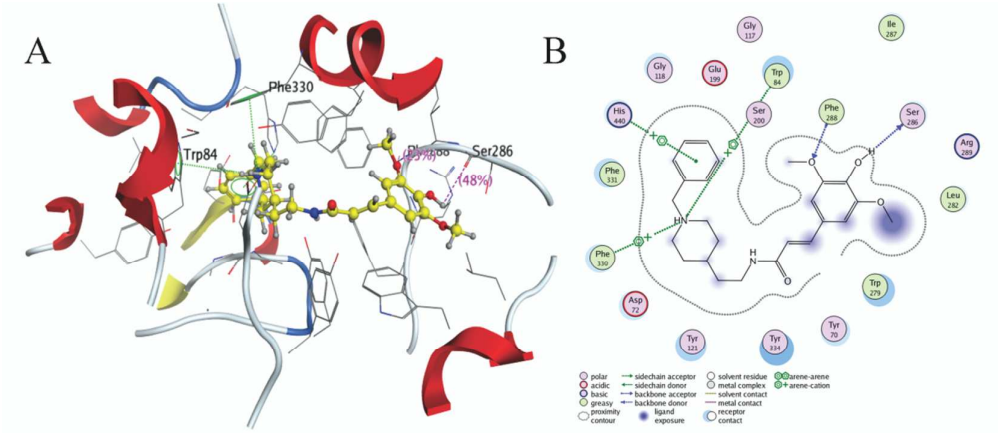


Figure 3. (A) 3D docking model of compound **5c** with hAChE. Atom colors: yellow-carbon atoms of **5c**, gray-carbon atoms of residues of hAChE, dark blue-nitrogen atoms, red-oxygen atoms. (B) 2D schematic diagram of docking model of compound **5c** with hAChE. The figure was prepared using the ligand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

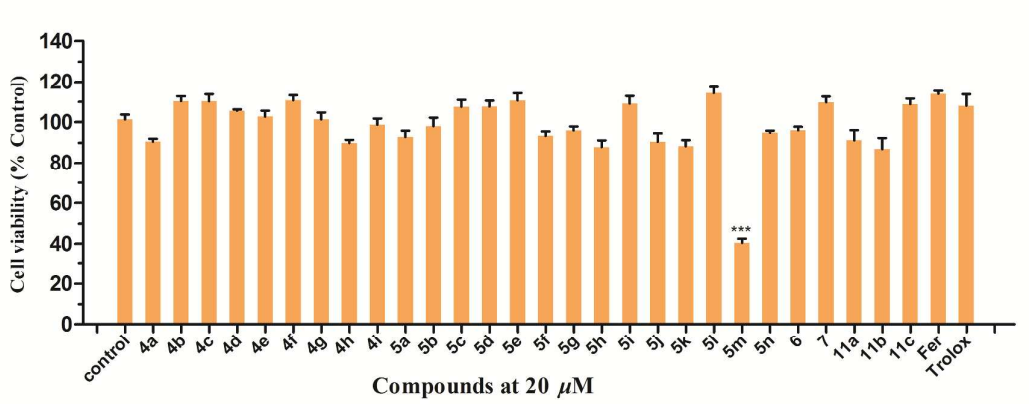


Figure 4. Effects of compounds on cell viability in PC12 cells. The cell viability was determined by the MTT assay after 24 h. The viability of untreated cells is defined as 100%. Data are expressed as the mean ± SD, n = 3. Statistical significance was analyzed by ANOVA: ***p< 0.001.

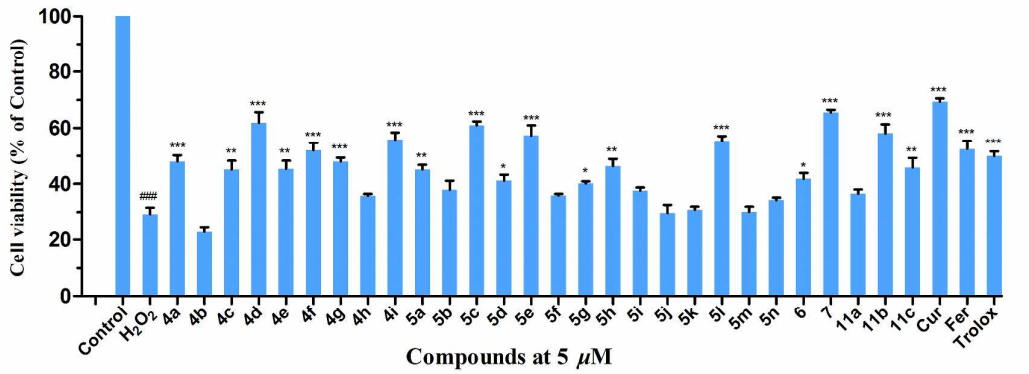


Figure 5. Protective effects of **4a-4i**, and **5a-5n** against H_2O_2-induced injury in PC12 cells at 5 μM. PC12 cells were pretreated by the tested compounds for 4 h. Then the cells were treated with 100 μM H_2O_2 for 20 h.Cell viability was determined by the MTT assay. The viability of untreated cells is defined as 100%. Data are expressed as the mean ± SD, n = 3. Statistical significance was analyzed by ANOVA: $^{###}P$<0.001 compared to control, *P < 0.05, **P < 0.01, ***P< 0.001 compared to H_2O_2.

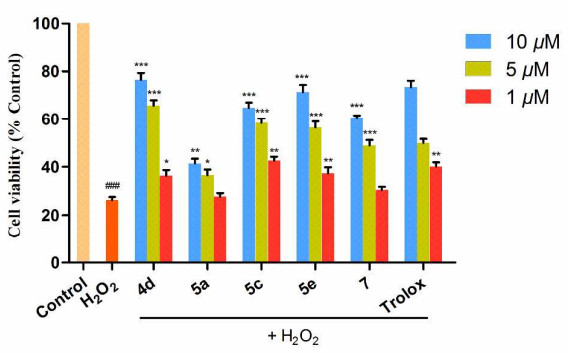


Figure 6. Protective effects of target compounds with prominent activities at 5 μM **4d**, **5a**, **5c**, **5e** and **7** against H_2O_2-induced injury in PC12 cells at 1, 5, and 10 μM. PC12 cells were pretreated by the tested compounds for 4 h. Then the cells were treated with 100 μM H_2O_2 for 20 h. Cell viability was determined using the MTT assay. The viability of untreated cells is defined as 100%. Data are expressed as the mean ± SD, n = 3. Statistical significance was analyzed by ANOVA: $^{\#\#\#}P<0.001$ compared to control, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P< 0.001$ compared to H_2O_2.

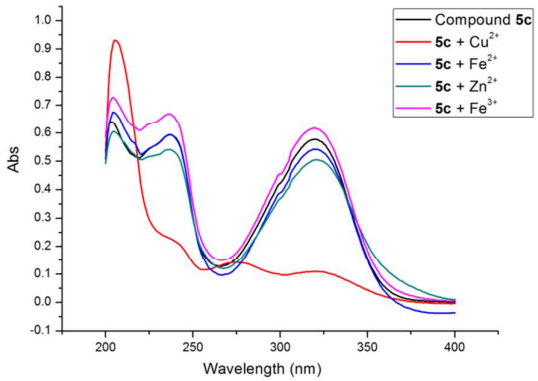


Figure 7. UV absosobance spectrum of **5c** (75 μM) alone or in the presence of $CuSO_4$ (150 μM), $ZnCl_2$ (150 μM), $FeSO_4$ (150 μM), or $FeCl_3$ (150 μM) in MeOH.

Graphical Abstract