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Original article

Multifunctional tacrine–trolox hybrids for the treatment of Alzheimer's disease with cholinergic, antioxidant, neuroprotective and hepatoprotective properties

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ABSTRACT

Combining tacrine with trolox in a single molecule, novel multifunctional hybrids have been designed and synthesized. All these hybrids showed ChE inhibitory activity in nanomolar range and strong antioxidant activity close to the parent compound trolox. Among them, compound **6d** was the most potent inhibitor against AChE (IC_{50} value of 9.8 nM for eeAChE and 23.5 nM for hAChE), and it was also a strong inhibitor to BuChE (IC_{50} value of 22.2 nM for eqBuChE and 20.5 nM for hBuChE). Molecular modeling and kinetic studies suggested that **6d** was a mixed-type inhibitor, binding simultaneously to CAS and PAS of AChE. In vivo hepatotoxicity assays indicated that **6d** was much less toxic than tacrine. In addition, it showed neuroprotective effect and good ability to penetrate the BBB. Overall, all these results highlighted **6d** a promising multifunctional agent for AD treatment.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease characterized by progressive memory loss, decline in language skills and other cognitive impairments [1,2]. Despite considerable efforts devoted to investigate the pathophysiology of AD, the causes and mechanisms of the disease are still not fully understood [3]. Currently, the predominant class of therapeutic agent in AD treatment is acetylcholinesterase (AChE) inhibitors, which can increase the cholinergic neurotransmission in the synaptic cleft by inhibiting degradation of acetylcholine (ACh) [4]. However, due to the multifactorial nature of AD, modulation of such a single target can only enable a palliative treatment instead of slowing or halting the neurodegeneration [5]. Thus, a more appropriate strategy to face this disease is proposed by development of multifunctional molecules that can simultaneously modulate different targets or mechanisms involved in the neurodegenerative AD cascade [6,7]. Since AChE inhibitors are still clinical effective for improving symptoms of AD, the common approach for designing multifunctional molecules is to modify a known AChE inhibitor, providing it with additional activity for AD treatment [8].

Tacrine (Fig. 1), the first AChE inhibitor approved for the treatment of AD, emerge as a popular structure in design of multifunctional molecules in recent years, because it can inhibit AChE in high potency and have a low molecular weight suitable for modification [9,10]. A lot of multifunctional molecules by connecting tacrine with another fragment having additional activities have been designed and synthesized [10–14]. However, due to the serious hepatotoxicity of tacrine, further use of these compounds was limited [15]. To date, the exact mechanisms of tacrine-induced hepatotoxicity are largely unknown [16]. However, evidences indicated that oxidative stress seemed to play a role. It was demonstrated that tacrine could decrease intracellular glutathione concentration, which lead to the generation of reactive oxygen species (ROS) and lipid peroxidation in the human liver cell [17]. Meanwhile, several studies also proved that tacrine-induced hepatotoxicity could be prevented by antioxidants [18]. .

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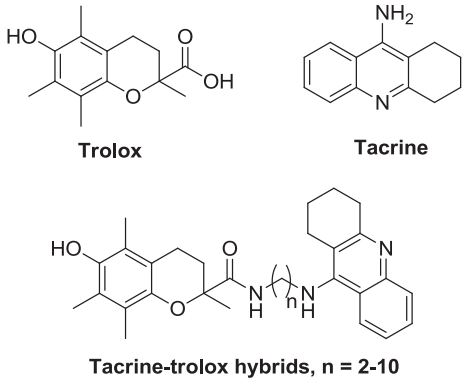


Fig. 1. Structures of trolox, tacrine and tacrine–trolox hybrids.

In fact, besides the ability to conquer the hepatotoxicity of tacrine, antioxidants are also beneficial for AD treatment [19]. Compared with other tissues, the brain is more sensitive to free radical damage [20]. The antioxidant system in brain progressively decays during aging. Especially in AD brain, it decline more rapidly than normal. An increasing body of evidence indicates that the oxidative damage occurs in earliest stage of AD pathogenesis and promotes the formation of other pathological hallmarks of the disease, such as amyloid plaques and neurofibrillary tangles [21]. Therefore, given all the above facts, researchers hypothesized that combining tacrine with an antioxidant fragment might afford more effective multifunctional molecules, which could not only inhibit AChE and reduce the hepatotoxicity of tacrine but also exhibit neuroprotective effect by decreasing the oxidant damage in brain. To test this hypothesis, some tacrine–antioxidant hybrids have been synthesized such as tacrine–silibinin hybrid [22], tacrine–ferulic acid hybrid [23] and mercapto–tacrine hybrids [24]. All these compounds showed improved pharmacological properties and low hepatotoxicity compared to tacrine. Driven by these positive results, recently we focused our work on searching new tacrine–antioxidant hybrids.

Trolox (Fig. 1) is a powerful antioxidant widely used in biological or biochemical applications to reduce oxidative stress [25]. Numerous reports indicate that trolox can protect liver from the damage induced by chemical insults both in vitro and in vivo [26–28]. In addition, it showed neuroprotective effect through scavenging ROS and attenuating the neurotoxicity mediated by Aβ and H_2O_2 on hippocampal neurons [29,30]. Furthermore, as a lipid-soluble analog of trolox, Vitamin E has been already used to prevent the tacrine-induced hepatotoxicity in clinic and delay the AD progression in patients with moderately severe AD [18,31]. Therefore, all these results suggested that trolox might be a useful antioxidant fragment for designing multifunctional tacrine–antioxidant hybrids.

In this study, a series of new multifunctional compounds by hybridizing tacrine with trolox have been designed and synthesized (Fig. 1). Tacrine was selected to inhibit AChE, and trolox was used to overcome the hepatotoxicity of tacrine and prevent oxidant damage in brain. The AChE has a nearly 20 Å deep narrow gorge which consists of two binding sites: a catalytic active site (CAS) at the bottom of the gorge and a peripheral anionic site (PAS) near the entry of the gorge. Considering compounds binding simultaneously to PAS and CAS were more promising for AD treatment, thus, alkylene linkers of different lengths were used to tether these two fragments [32,33]. Here, we report the design, synthesis and biological evaluation of a series of new tacrine–trolox hybrids as multifunctional agents for the treatment of AD.

2. Results and discussion

2.1. Chemistry

The synthesis of target and intermediate compounds is represented in Scheme 1. As a previously described protocol [34,35], heating anthranilic acid with cyclohexanone in the presence of $POCl_3$ for 2 h gave 9-chloro-1, 2, 3, 4-tetrahydroacridine **3**. The obtained **3** was then engaged with different aliphatic diamines to produce the intermediate 9-aminoalkylamino-1, 2, 3, 4-tetrahydroacridines **4a**–**g** [12,34]. Finally, activation of trolox with 1, 1′-carbonyldiimidazole (CDI) and subsequent coupling to **4a**–**g** afforded target compounds **6a**–**g**.

2.2. In vitro inhibition of ChEs

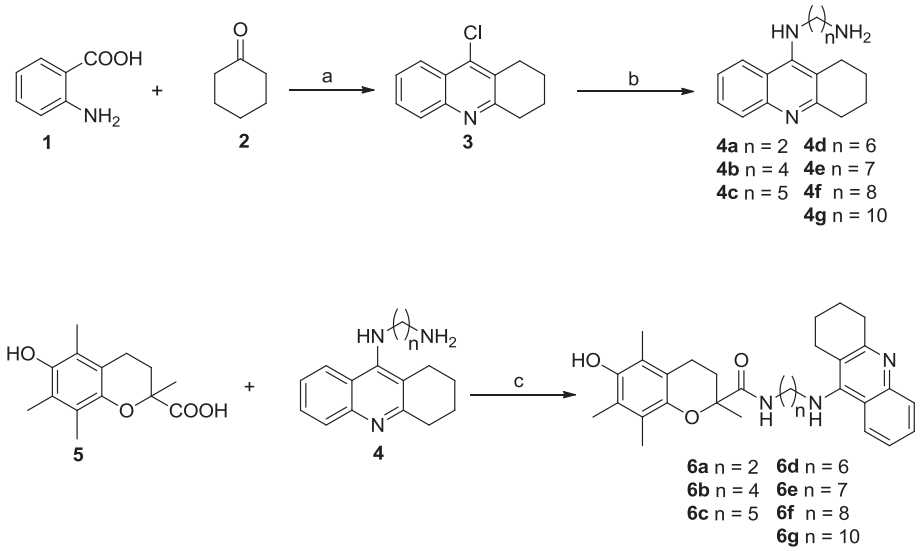
There are two types of cholinesterases (ChEs) in human body, namely, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). In normal conditions, AChE hydrolyzes about 80% of acetylcholine while BuChE plays only a supportive role [36]. However, recent studies indicate that, as AD progresses, the activity of AChE gradually decreases while that of BuChE significantly increases. In later stage of the disease, BuChE can compensate for the deficit of AChE in brain [37]. Therefore, simultaneous inhibition of both AChE and BuChE would be more valuable for AD treatment.

The ChE inhibitory activity of **6a**–**g** was measured by Ellman's assay using tacrine as reference compound [38]. Because of their lower cost and the high degree of sequence identity to the human enzymes, acetylcholinesterase (AChE) from electric eel and butyrylcholinesterase (BuChE) from equine serum were initially used. The results shown in Table 1 indicate that all compounds are potent inhibitors of both ChEs. Similar to the tacrine, most of them showed more potent inhibitory activity for BuChE than for AChE. The length of the alkyl spacer between trolox and tacrine moiety could significantly influence the AChE inhibitory activity. Compound **6d** with a six-carbon spacer exhibited the most potent AChE inhibitory activity with IC_{50} value of 9.8 nM, which was 11-fold stronger than that of tacrine. Lengthening or shortening the length of alkyl spacer reduced AChE inhibition. In contrast, the length of the alkyl spacer did not show a clear trend for BuChE inhibition. Compound **6b** with a four-carbon spacer gave the most potent BuChE inhibitory activity (IC_{50} = 15.6 nM). However, the long spacer length was in general not beneficial for BuChE inhibition (e.g., **6f**: IC_{50} = 150.5 nM; **6g**: IC_{50} = 262.8 nM).

All compounds having potent inhibitory activity for ChEs of animal origin were then tested on human ChEs with aim to better evaluate them and the results are summarized in Table 1. From the table, it can be seen that all compounds are also potent inhibitors of human ChEs. For BuChE inhibition, most of these compounds showed improved inhibitory activity for hBuChE compared to eqBuChE. Compound **6b** displayed the highest inhibitory activity for hBuChE with the IC_{50} value of 5.2 nM. However, no significant change between hAChE and eeAChE was observed. Although compound **6d** exhibited the inhibitory activity for hAChE was slightly lower than for eeAChE (9.8 nM versus 23.5 nM), it was still the most potent inhibitor of hAChE. Such an activity change made **6d** more balanced for both ChE inhibition (IC_{50} = 23.5 nM for hAChE; IC_{50} = 20.5 nM for hBuChE), which would be more beneficial for AD treatment.

2.3. In vitro antioxidant activities

The antioxidant activities of target compounds **6a**–**g** were evaluated using a radical scavenging assay (DPPH assay) with trolox as reference compound (Table 1) [39]. The results indicated that all



Scheme 1. Synthesis of tacrine–trolox hybrids. Reagents and conditions: (a) $POCl_3$, reflux, 4 h; (b) $NH_2(CH_2)_nNH_2$, KI, 1-pentanol, reflux, 12 h; (c) 1, 1′-carbonyldiimidazole (CDI), r.t. overnight.

compounds retained the antioxidant activity of the parent compound trolox. Connecting the trolox with tacrine moiety and variation of linker length between the two fragments did not significantly affect the antioxidant activity of trolox, which indicated that hybridizing these two scaffolds was rational. After all these biological evaluation, **6d** 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 AChE inhibition

To investigate whether **6d** is a dual binding site inhibitor, an enzyme kinetic study was performed. By plotting the 1/velocity against 1/[substrate], a set of Lineweaver–Burk double reciprocal plots were constructed and shown in Fig. 2. Form the figure, it can be seen that both increasing slopes and intercepts with increasing inhibitor concentrations, which suggested that **6d** was a mixed-type inhibitor for AChE. On basis of the previous reports [35,40], this result meant that compound **6d** 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 insight into the interaction mechanism of **6d** with AChE, molecular docking study based on X-ray crystal structure of recombinant human acetylcholinesterase in complex with donepezil (hAChE, PDB code 4EY7) was carried out using Molecular Operating Environment (MOE) software. As shown in Fig. 3, trolox moiety was located into PAS of AChE through a π–π stacking interaction with Trp 286 (3.55 Å). The tacrine moiety occupied the CAS and stacked against the indole ring of Trp 86 with ring to ring distance of 4.53 Å. After analyzing the docking results and taking the kinetic study into consideration, we could confirm that compound **6d** was a dual binding site inhibitor that could interact simultaneously with PAS and CAS of AChE.

2.6. In vitro neuroprotection study

Since **6d** was a potent antioxidant comparable to the trolox, we investigated its neuroprotective capacity against oxidative stress in PC 12 cells. H_2O_2 was used as toxic insult to induce oxidative damage, and the parent compound trolox was selected as positive control. After treating the cells with different concentrations of compound **6d** for 2 h, 100 μM of H_2O_2 was added and incubated for 24 h. Cell viability was measured by MTT assay [41]. As reported in Fig. 4A, **6d** could significantly inhibit cell death at 3.125 μM and 6.25 μM. However, to our surprise, compound showed decreased cell viability as the concentration increased. At 12.5 μM, the compound even exhibited much lower viability than that of H_2O_2 only treated group. These results made us to test potential cytotoxicity of **6d** on PC 12 cells. However, it can be seen from Fig. 4B that the compound do not lead to cell death at concentration ranging from 1.563 to 12.5 μM. After studying the previous reports [24,42], we found that, in fact, similar phenomena were also observed in reported compounds and marketed drugs (e.g., mercapro-tacrine derivatives, galanthamine and donepezil). The increased cell death at high concentration might arise from additional interactions between compound and other biological targets.

2.7. In vivo hepatotoxicity study

To determine whether introduction of trolox could reduce hepatotoxicity, compound **6d** was selected for the assay with male rats [24,43]. After treating rats with highest tolerated dose of tacrine (6 μmol/100 g body weight) or equimolar dose of **6d**, the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum obtained at different times (0, 24, 36 and 72 h) were measured. As shown in Fig. 5, tacrine caused significant hepatotoxicity, showing increased activity of both AST and ALT at 24 and 36 h, while compound **6d** did not alter these parameters from 0 to 72 h, which suggested that **6d** had little hepatotoxicity.

For histological study, the Fig. 6B indicated that, after tacrine administration, an increase in the number of inflammatory cells in portal fields and a large area of necrosis and distinct fatty degeneration of the hepatocytes were observed. In contrast, only minor morphological changes were found after the treatment with compound **6d** (Fig. 6C). All these results strongly demonstrated that introduction of trolox could reduce the hepatotoxicity of tacrine.

Table 1
Inhibitory activities of eeAChE, eqBuChE, hAChE, hBuChE and DPPH.

Compd.	IC_{50} (nM)[a]		SI[d]	IC_{50} (nM)[a]		SI[g]	IC_{50} (μM)[a]
	AChE[b]	BuChE[c]		hAChE[e]	hBuChE[f]		DPPH
6a	149.7 ± 2.4	41.3 ± 1.9	0.28	283.8 ± 4.5	56.3 ± 2.5	0.20	30.4 ± 2.3
6b	558.4 ± 9.7	15.6 ± 1.4	0.03	535.0 ± 10.1	5.2 ± 1.1	0.01	44.2 ± 2.1
6c	249.5 ± 2.3	35.4 ± 2.1	0.14	215.3 ± 2.4	10.2 ± 1.8	0.05	40.7 ± 1.8
6d	9.8 ± 0.7	22.2 ± 1.3	2.27	23.5 ± 1.7	20.5 ± 1.2	0.87	48.7 ± 2.8
6e	92.4 ± 1.4	61.7 ± 2.2	0.67	143.7 ± 3.6	19.2 ± 1.9	0.13	46.9 ± 2.2
6f	228.1 ± 2.1	150.5 ± 7.3	0.66	226.5 ± 7.1	34.7 ± 1.1	0.15	47.3 ± 1.9
6g	386.9 ± 4.2	262.8 ± 5.4	0.68	453.3 ± 6.2	110.1 ± 3.1	0.24	43.2 ± 2.2
Tacrine	112.1 ± 4.2	35.2 ± 4.2	0.31	435.1 ± 5.9	23.2 ± 2.3	0.05	
Trolox							35.6 ± 3.2

[a] Results are the mean of three independent experiments (n = 3) ± SD.
[b] AChE (EC 3.1.1.7) from electric eel.
[c] BuChE (EC 3.1.1.8) from horse serum.
[d] AChE selectivity index = IC_{50}(BuChE)/IC_{50}(AChE).
[e] AChE (EC 3.1.1.7) from human erythrocytes.
[f] BuChE (EC 3.1.1.8) from human serum.
[g] hAChE selectivity index = IC_{50}(hBuChE)/IC_{50}(hAChE).

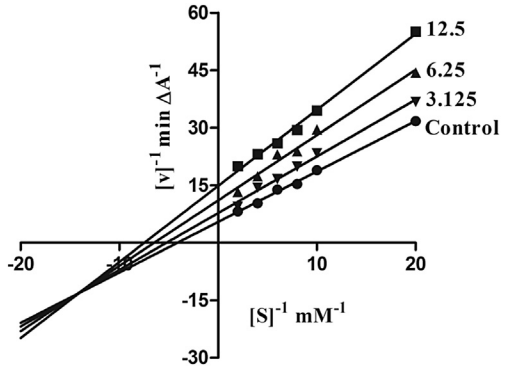


Fig. 2. Kinetic study on the mechanism of eeAChE inhibition by compound **6d**. Overlaid Lineweaver–Burk reciprocal plots of AChE initial velocity at increasing substrate concentration (0.05–0.50 mM) in the absence of inhibitor and in the presences of different concentrations of **6d** are shown.

2.8. In vitro blood–brain barrier permeation study

A success CNS drug should be able to cross the Blood–Brain Barrier (BBB) [44]. Thus, to determine whether compound **6d** could penetrate the BBB, a parallel artificial membrane permeation assay (PAMPA) of blood–brain barrier was used. This model described by Di et al. was widely used to predict BBB permeation with high success [45]. The in vitro permeabilities (P_e) of compound **6d** and reference drugs were determined through a lipid extract of porcine brain with a mixture of PBS and EtOH (70:30). Assay validation was performed by comparing experimental permeabilities of 10 reference drugs with their reported values (Supporting information, Table S1), which gave a good linear correlation: P_e (exp.) = 0.8597 P_e (Bibl.) − 0.276 (R_2 = 0.9726). From this equation and considering the limits established by Di et al. for BBB permeation, we established that compound with P_e value above 3.16×10^{-6} cm s^{-1}could penetrate into the CNS. Compound **6d** showed a P_e value of 8.87×10^{-6} cm s^{-1}, indicating that it could easily cross the BBB.

3. Conclusion

In conclusion, a series of novel tacrine–trolox hybrids have been designed and synthesized as multifunctional agents for the treatment of AD. The biological screening results indicated that all of them showed potent ChE inhibitory activity with IC_{50} value in nanomolar range, and also gave strong antioxidant activity close to parent compound trolox. Among these compounds, **6d** possessing the highest inhibitory activity for eeAChE and hAChE as well as strong inhibition for eqBuChE and hBuChE stood out as the most promising compound for further study. Kinetic and molecular docking studies revealed that **6d** was a mixed-type inhibitor that could simultaneously interact with PAS and CAS of AChE. In vivo hepatotoxicity assays indicated that **6d** did not show signs of hepatotoxicity, which was much safer than tacrine. In addition, it displayed neuroprotective effect against H_2O_2-induced PC 12 cell injury and good ability to penetrate the CNS. Overall, all these results qualified **6d** as a potential multifunctional agent for the treatment of AD.

4. Experimental section

4.1. Chemistry

All chemicals (reagent grade) used were purchased from Sino pharm Chemical Reagent Co., Ltd. (China). Reaction progress was monitored using analytical thin layer chromatography (TLC) on precoated silica gel GF254 (Qingdao Haiyang Chemical Plant, Qing-Dao, China) plates and the spots were detected under UV light (254 nm). IR (KBr-disc) spectra were recorded by Bruker Tensor 27 spectrometer. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker ACF-500 spectrometer at 25 °C and referenced to TMS. Chemical shifts are reported in ppm (δ) using the residual solvent line as internal standard. Splitting patterns are designed as s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were obtained on a MS Agilent 1100 Series LC/MSD Trap mass spectrometer (ESI-MS) and a Mariner ESI-TOF spectrometer (HRESIMS), respectively. Column chromatography was performed on silica gel (90–150 μm; Qingdao Marine Chemical Inc.). Compounds **3** and **4** were prepared following previously published methods [12,34,35,46].

4.1.1. General procedures for the preparation of compounds *6a–g*

A solution of **5** (2.0 mmol) and 1, 1′-carbonyldiimidazole (2.2 mmol) in 25 mL of anhydrous THF was stirred at room temperature for 1 h. The **4** (2.0 mmol) was added to the solution, and stirring was continued overnight. The reaction mixture was diluted with H_2O and extracted with EtOAc. The organic extracts were combined, washed with brine, and dried with anhydrous Na_2SO_4, and the solvent was evaporated *in vacuo* to give the crude product,

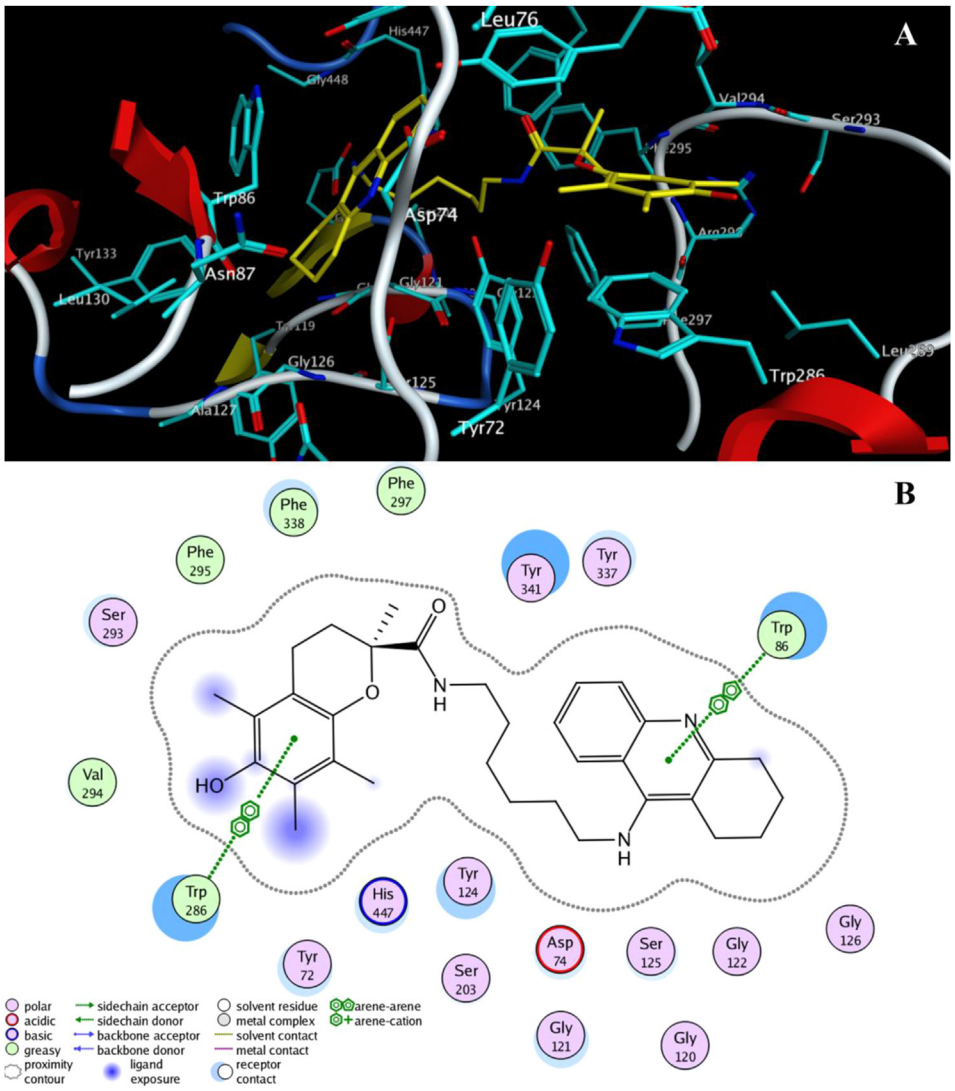


Fig. 3. (A) 3D docking model of compound **6d** with AChE. Atom colors: yellow–carbon atoms of **6d**, cyan–carbon atoms of residues of AChE, dark blue–nitrogen atoms, red–oxygen atoms. (B) 2D schematic diagram of docking model of compound **6d** with AChE. The figure was prepared using the ligand interactions application in MOE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which was purified by silica gel chromatography with CH_2Cl_2–MeOH = 20:1 as an eluent to afford corresponding target compound as yellow oil.

*4.1.1.1. 6-Hydroxy-2,5,7,8-tetramethyl-N-(2-((1, 2, 3, 4-tetrahydroacridin-9-yl)amino)ethyl)chroman-2-carboxamide (**6a**).* Intermediate **4a** was reacted with 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid following the general procedure to give the desired product **6a** with a yield of 64%. IR (KBr) ν 3422, 3235, 2932, 1701, 1586, 1488, 1427, 1260, 767, 705, 678 cm^{-1}; ESI/MS *m/z*: 474.2 $[M+H]^+$; ^1H NMR (500 MHz, DMSO) δ 8.18 (d, J = 8.5 Hz, 1H), 7.75 (d, J = 8.5 Hz, 1H), 7.66–7.57 (m, 2H), 7.39 (t, J = 7.6 Hz, 1H), 6.18 (s, 1H), 3.64 (s, 2H), 3.48–3.32 (m, 2H), 2.91 (t, J = 5.9 Hz, 2H), 2.71–2.54 (m, 2H), 2.48–2.41 (m, 1H), 2.37–2.28 (m, 1H), 2.07–1.98 (m, 7H), 1.93 (s, 3H), 1.72–1.65 (m, 4H), 1.72–1.63 (m, 1H), 1.24 (s, 3H). ^{13}C NMR (125 MHz, DMSO) δ 174.82, 163.93, 155.84, 152.79, 146.35, 144.17, 130.02, 125.55, 124.32, 124.25, 123.12, 121.65, 120.60, 118.92, 117.48, 114.72, 77.63, 55.36, 48.47, 32.01, 29.95, 24.99, 24.01, 22.73, 22.14, 20.39, 13.20, 12.46, 12.19. HRMS: calcd for $C_{29}H_{36}N_3O_3$ $[M+H]^+$ 474.2751, found 474.2755.

*4.1.1.2. 6-Hydroxy-2, 5, 7, 8-tetramethyl-N-(4-((1, 2, 3, 4-tetrahydroacridin-9-yl)amino)butyl) chroman-2-carboxamide (**6b**).* Intermediate **4b** was reacted with 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid following the general procedure to give the desired product **6b** with a yield of 60%. IR (KBr) ν 3425, 2930, 1647, 1589, 1522, 1441, 1257, 1088, 756, 678 cm^{-1}; ESI/MS *m/z*: 502.2 $[M+H]^+$; ^1H NMR (500 MHz, DMSO) δ 8.18 (d, J = 8.5 Hz, 1H), 7.76 (d, J = 8.5 Hz, 1H), 7.64 (t, J = 7.5 Hz, 1H), 7.41 (t, J = 7.5 Hz, 1H), 7.24 (t, J = 6.0 Hz, 1H), 6.27 (s, 1H), 3.50 (t, J = 6.5 Hz, 2H), 3.11–3.00 (m, 2H), 2.93 (t, J = 5.5 Hz, 2H), 2.66 (t, J = 5.5 Hz, 2H), 2.47 (d, J = 6.0 Hz, 1H), 2.42–2.33 (m, 1H), 2.12–2.07 (m, 1H), 2.05 (d, J = 2.0 Hz, 6H), 1.96 (s, 3H), 1.86–1.75 (m, 4H), 1.72–1.64 (m, 1H), 1.49–1.34 (m, 4H), 1.32 (s, 3H). ^{13}C NMR (125 MHz, DMSO) δ 173.73, 164.01, 155.54, 152.83, 146.35, 144.34, 130.20, 125.17, 124.42, 124.24, 123.12, 121.63, 120.69, 118.91, 117.58, 114.54, 77.68, 47.80, 38.52, 31.79, 29.98, 27.93, 26.92, 25.06, 24.42, 22.72, 22.07, 20.55, 13.19, 12.48, 12.21. HRMS: calcd for $C_{31}H_{40}N_3O_3$ $[M+H]^+$ 502.3064, found 502.3060.

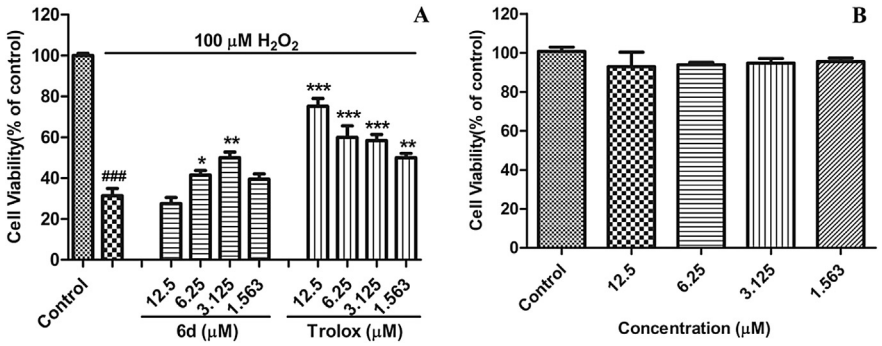


Fig. 4. (A) Protective effects of compound **6d** on H_2O_2-induced cell death in PC 12 cells. Cells were incubated with different concentration of **6d** for 2 h and then treated with 100 μM H_2O_2 for 24 h. Trolox was used as positive control. (B) Effects of various concentrations of compound **6d** on cell viability in PC 12 cells after treatment for 48 h. Cell viability was measured by MTT assay. Data were shown as mean ± SD of three independent experiments (n = 6). *P*### < 0.001 compared to control, *P*** < 0.01, *P**** < 0.001 compared to H_2O_2-treated cells.

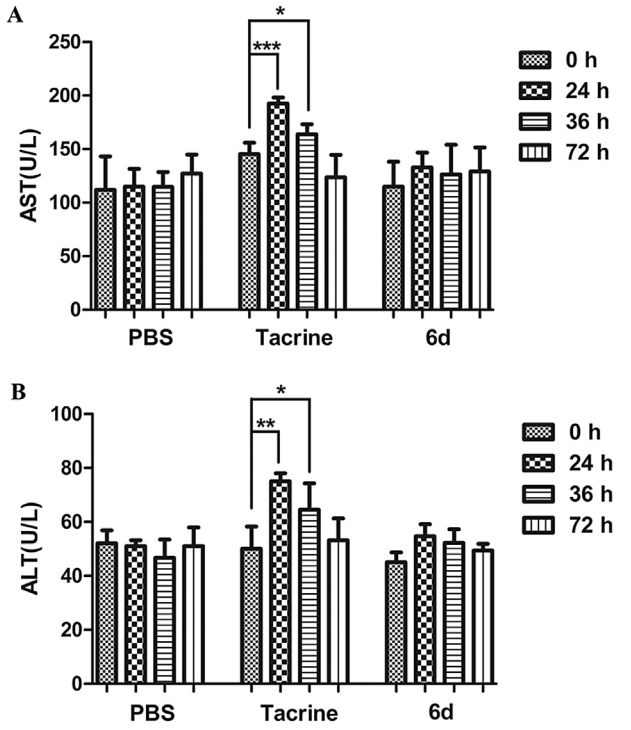


Fig. 5. The AST and ALT activity after administration of equimolar doses (6 μmol/100 g b. wt.) of tacrine or compound **6d**. Results are expressed as mean ± SD (n = 8–9, *t* test, statistical significant difference compared to control values before administration, $P^* \leq 0.05$, $P^{**} \leq 0.01$, $P^{***} \leq 0.001$).

*4.1.1.3. 6-Hydroxy-2, 5, 7, 8-tetramethyl-N-(5-((1, 2, 3, 4-tetrahydroacridin-9-yl) amino) pentyl)chroman-2-carboxamide (**6c**).* Intermediate **4c** was reacted with 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid following the general procedure to give the desired product **6c** with a yield of 66%. IR (KBr) *ν* 3425, 2930, 2860, 1651, 1589, 1523, 1449, 1348, 1256, 1088, 756, 678 cm^{-1}; ESI/MS *m/z*: 516.3 $[M+H]^+$; ^1H NMR (500 MHz, DMSO) *δ* 8.18 (d, *J* = 8.5 Hz, 1H), 7.75 (d, *J* = 8.5 Hz, 1H), 7.63 (t, *J* = 7.5 Hz, 1H), 7.46–7.31 (m, 1H), 7.18 (t, *J* = 6.0 Hz, 1H), 6.13 (s, 1H), 3.47 (t, *J* = 7.0 Hz, 2H), 3.12–2.97 (m, 2H), 2.92 (t, *J* = 6.0 Hz, 2H), 2.67 (t, *J* = 6.0 Hz, 2H), 2.47 (d, *J* = 6.0 Hz, 1H), 2.42–2.35 (m, 1H), 2.17–2.09 (m, 1H), 2.05 (d, *J* = 8.0 Hz, 6H), 1.96 (s, 3H), 1.85–1.76 (m, 4H), 1.72–1.66 (m, 1H), 1.58–1.48 (m, 2H), 1.38–1.27 (m, 5H), 1.15–1.07 (m, 2H). ^{13}C NMR (125 MHz, DMSO) *δ* 173.63, 163.85, 155.75, 152.82, 146.63, 144.53, 130.13, 125.27, 124.29, 124.34, 123.31, 121.39, 120.74, 118.83, 117.69, 114.57, 77.69, 48.23, 38.66, 31.72, 30.68, 29.61, 29.58, 26.52, 26.21, 25.02, 24.58, 22.22, 20.57, 13.25, 12.39, 12.31. HRMS: calcd for $C_{32}H_{42}N_3O_3$ $[M+H]^+$ 516.3221, found 516.3225.

*4.1.1.4. 6-Hydroxy-2, 5, 7, 8-tetramethyl-N-(6-((1, 2, 3, 4-tetrahydroacridin-9-yl)amino)hexyl) chroman-2-carboxamide (**6d**).* Intermediate **4d** was reacted with 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid following the general procedure to give the desired product **6b** with a yield of 61%. IR (KBr) *ν* 3428, 2928, 2857, 1648, 1589, 1524, 1452, 1415, 1256, 1088, 757, 678 cm^{-1}; ESI/MS *m/z*: 530.3 $[M+H]^+$; ^1H NMR (500 MHz, DMSO) *δ* 8.21 (s, 1H), 7.76 (d, *J* = 8.5 Hz, 1H), 7.65 (t, *J* = 7.5 Hz, 1H), 7.42 (t, *J* = 7.5 Hz, 1H), 7.15 (t, *J* = 6.0 Hz, 1H), 6.27 (s, 1H), 3.52 (t, *J* = 7.0 Hz, 2H), 3.07 (dq, *J* = 13.0, 6.5 Hz, 1H), 3.01–2.95 (m, 1H), 2.93 (t, *J* = 6.0 Hz, 2H), 2.69 (d, *J* = 6.0 Hz, 2H), 2.47 (t, *J* = 6.0 Hz, 1H), 2.42–2.34 (m, 1H), 2.19–2.10 (m, 1H), 2.07 (s, 3H), 2.04 (s, 3H), 1.96 (s, 3H), 1.87–1.75 (m, 4H), 1.72–1.64 (m, 1H), 1.57–1.47 (m, 2H), 1.35 (s, 3H), 1.31–1.26 (m, 2H), 1.23–1.16 (m, 2H), 1.07–0.99 (m, 2H). ^{13}C

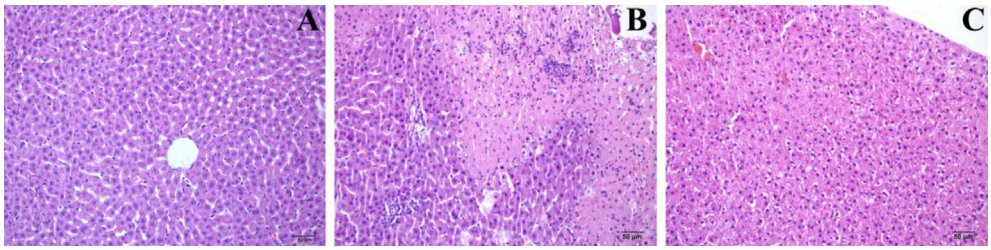


Fig. 6. Histomorphological appearance of livers of male rats after treatment with the solvent only (control) (A) and 24 h after administration of tacrine (B) and **6d** (C). H&E, original magnification: ×200.

NMR (125 MHz, DMSO) δ 173.58, 163.91, 155.57, 152.87, 146.34, 144.37, 130.17, 125.22, 124.37, 124.29, 123.12, 121.56, 120.72, 118.93, 117.61, 114.55, 77.74, 48.18, 38.61, 31.84, 30.74, 29.97, 29.34, 26.33, 26.07, 25.05, 24.69, 22.73, 22.11, 20.60, 13.19, 12.47, 12.22. HRMS: calcd for $C_{33}H_{44}N_3O_3$ $[M+H]^+$ 530.3377, found 530.3379.

*4.1.1.5. 6-Hydroxy-2, 5, 7, 8-tetramethyl-N-(7-((1, 2, 3, 4-tetrahydroacridin-9-yl)amino)heptyl)chroman-2-carboxamide (**6e**).* Intermediate **4e** was reacted with 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid following the general procedure to give the desired product **6e** with a yield of 58%. IR (KBr) ν 3425, 2930, 2856, 1649, 1588, 1524, 1447, 1349, 1256, 1088, 756, 678 cm^{-1}; ESI/MS *m/z*: 544.3 $[M+H]^+$; ^1H NMR (500 MHz, DMSO) δ 8.23 (d, *J* = 8.5 Hz, 1H), 7.78 (d, *J* = 8.5 Hz, 1H), 7.67 (t, *J* = 7.5 Hz, 1H), 7.44 (t, *J* = 7.5 Hz, 1H), 7.14 (t, *J* = 6.0 Hz, 1H), 6.46 (s, 1H), 3.58 (t, *J* = 7.0 Hz, 2H), 3.11–3.03 (m, 1H), 3.01–2.95 (m, 1H), 2.94 (t, *J* = 6.0 Hz, 2H), 2.68 (d, *J* = 5.5 Hz, 2H), 2.47 (d, *J* = 6.0 Hz, 1H), 2.43–2.34 (m, 1H), 2.20–2.12 (m, 1H), 2.08 (s, 3H), 2.05 (s, 3H),1.97 (s, 3H), 1.82 (s, 4H), 1.73–1.65 (m, 1H), 1.62–1.54 (m, 2H), 1.35 (s, 3H), 1.30–1.12 (m, 6H), 1.06–0.96 (m, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.42, 163.63, 155.27, 153.37, 146.41, 144.27, 130.36, 124.72, 124.59, 124.61, 123.27, 121.57, 120.72, 118.52, 117.71, 114.38, 77.69, 48.24, 38.68, 31.61, 30.74, 29.91, 29.58, 29.13, 29.05, 26.59, 26.41, 25.07, 22.71, 22.05, 20.68, 13.26, 12.25, 12.28. HRMS: calcd for $C_{34}H_{46}N_3O_3$ $[M+H]^+$ 544.3534, found 544.3536.

*4.1.1.6. 6-Hydroxy-2, 5, 7, 8-tetramethyl-N-(8-((1, 2, 3, 4-tetrahydroacridin-9-yl)amino)octyl) chroman-2-carboxamide (**6f**).* Intermediate **4f** was reacted with 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid following the general procedure to give the desired product **6f** with a yield of 63%. IR (KBr) ν 3428, 2927, 2854, 1652, 1589, 1524, 1451, 1415, 1256, 1088, 756, 678 cm^{-1}; ESI/MS *m/z*: 558.3 $[M+H]^+$; ^1H NMR (500 MHz, DMSO) δ 8.23 (d, *J* = 8.5 Hz, 1H), 7.78 (d, *J* = 8.5 Hz, 1H), 7.67 (t, *J* = 7.5 Hz, 1H), 7.44 (t, *J* = 7.5 Hz, 1H), 7.13 (t, *J* = 6.0 Hz, 1H), 6.45 (s, 1H), 3.59 (t, *J* = 7.0 Hz, 2H), 3.45 (q, *J* = 7.0 Hz, 1H), 3.07 (td, *J* = 13.0, 6.5 Hz, 1H), 3.01–2.95 (m, 1H), 2.93 (d, *J* = 5.5 Hz, 2H), 2.68 (d, *J* = 5.5 Hz, 2H), 2.43–2.34 (m, 1H), 2.20–2.13 (m, 1H), 2.08 (s, 3H), 2.08 (s, 3H), 1.97 (s, 3H), 1.82 (s, 4H), 1.73–1.65 (m, 1H), 1.64–1.58 (m, 2H), 1.36 (s, 3H), 1.31–1.20 (m, 4H), 1.19–1.09 (m, 4H), 1.03–0.96 (m, 2H). ^{13}C NMR (125 MHz, DMSO) δ 173.57, 163.95, 155.21, 153.22, 146.38, 144.35, 130.42, 124.76, 124.45, 124.38, 123.08, 121.52, 120.69, 118.69, 117.62, 114.27, 77.76, 48.19, 38.72, 31.53, 30.73, 29.98, 29.37, 29.04, 29.00, 26.61, 26.28, 25.00, 24.74, 22.66, 22.00, 20.60, 13.18, 12.45, 12.21. HRMS: calcd for $C_{35}H_{48}N_3O_3$ $[M+H]^+$ 558.3690, found 558.3691.

*4.1.1.7. 6-Hydroxy-2, 5, 7, 8-tetramethyl-N-(10-((1, 2, 3, 4-tetrahydroacridin-9-yl)amino)decyl)chroman-2-carboxamide (**6g**).* Intermediate **4g** was reacted with 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid following the general procedure to give the desired product **6g** with a yield of 65%. IR (KBr) ν 3428, 2927, 2856, 1652, 1589, 1524, 1451, 1413, 1256, 1088, 758, 678 cm^{-1}; ESI/MS *m/z*: 586.3 $[M+H]^+$; ^1H NMR (500 MHz, DMSO) δ 8.24 (d, *J* = 8.5 Hz, 1H), 7.78 (d, *J* = 8.5 Hz, 1H), 7.67 (t, *J* = 7.5 Hz, 1H), 7.45 (dd, *J* = 11.5, 4.0 Hz, 1H), 7.16 (t, *J* = 6.0 Hz, 1H), 6.37 (s, 1H), 3.60 (t, *J* = 7.0 Hz, 2H), 3.12–3.06 (m, 1H), 3.03–2.97 (m, 1H), 2.95 (t, *J* = 5.8 Hz, 2H), 2.71 (d, *J* = 5.8 Hz, 2H), 2.46–2.35 (m, 2H), 2.23–2.15 (m, 2H), 2.10 (s, 3H), 2.08 (s, 3H), 2.00 (s, 3H), 1.88–1.78 (m, 4H), 1.75–1.67 (m, 1H), 1.65–1.58 (m, 2H), 1.38 (s, 3H), 1.35–1.08 (m, 10H), 1.07–0.96 (m, 3H). ^{13}C NMR (125 MHz, DMSO) δ 173.59, 164.03, 155.07, 152.84, 146.38, 144.34, 130.54, 125.29, 124.49, 124.42, 123.07, 121.52, 120.67, 118.83, 117.61, 114.19, 77.76, 48.14, 38.73, 30.71, 29.97, 29.38, 29.29, 29.25, 29.07, 26.63, 26.36, 24.99, 24.75, 22.75, 22.63, 22.16, 21.91, 20.60, 13.19, 12.46, 12.22. HRMS: calcd for $C_{37}H_{52}N_3O_3$ $[M+H]^+$ 586.4003, found 586.4002.

4.1.2. In vitro inhibition experiments of ChEs

Acetylcholinesterase (AChE, E.C. 3.1.1.7) from electric eel and human erythrocytes, butyrylcholinesterase (BuChE, E.C. 3.1.1.8) from equine serum and human serum, S-butyrylthiocholine iodide (BTCI), acetylthiocholine iodide (ATCI), 5, 5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB) and tarcine hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). The inhibitory activities of test compounds **6a**–**g** was evaluated by Ellman's method [38]. The compounds were dissolved in DMSO and diluted with the buffer solution (50 mM Tris–HCl, pH = 8.0, 0.1 M NaCl, 0.02 M $MgCl_2 \cdot 6H_2O$) to yield corresponding test concentrations (DMSO less than 0.01%). In each well of the plate, 160 μL of 1.5 mM DTNB, 50 μL of AChE (0.22 U/mL eeAChE or 0.05 U/mL hAChE) or 50 μL of BuChE (0.12 U/mL eqBuChE or 0.024 U/mL hBuChE) were incubated with 10 μL of different concentrations of test compounds (0.001–100 μM) at 37 °C for 6 min. After this period, acetylthiocholine iodide (15 mM) or S-butyrylthiocholine iodide (15 mM) as the substrate (30 μL) was added and the absorbance was measured with a wavelength of 405 nm at different time intervals (0, 60, 120, and 180 s). IC_{50} values were calculated as concentration of compound that produces 50% enzyme activity inhibition, using the Graph Pad Prism 4.03 software (San Diego, CA, USA). Results are expressed as the mean ± SD of at least three different experiments performed in triplicate.

4.1.3. In vitro evaluation of antioxidant activity

The antioxidant activities of compounds **6a**–**g** were evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging assay according to the method of Lee et al. with slightly modifications [39]. Briefly, 95 μL of DPPH radical solution (300 μM) was added in a 96-well plate containing 5 μL of different concentrations of test compound dissolved in MeOH, and incubated for 30 min at 37 °C in the dark. The absorbance of each well was measured at 517 nm using a microplate reader. The IC_{50} values of test compounds were determined using the Graph Pad Prism 4.03 software (San Diego, CA, USA). Results are expressed as the mean ± SD of at least three different experiments performed in triplicate.

4.1.4. Kinetic study of AChE inhibition

The kinetic study of AChE was performed by Ellman's method with three different concentrations (12.5, 6.25 and 3.125 nM) of compound **6d**. Lineweaver–Burk reciprocal plots were constructed by plotting 1/velocity against 1/[substrate] at varying concentrations of the substrate acetylthiocholine (0.05–0.5 mM). The plots were assessed by a weighted least-squares analysis that assumed the variance of velocity (*v*) to be a constant percentage of *v* for the entire data set. Data analysis was performed with Graph Pad Prism 4.03 software (San Diego, CA, USA).

4.1.5. Docking study

Molecular docking studies were performed using Molecular Operating Environment (MOE) software version 2008.10 (Chemical Compouting Group, Montreal, Canada). The X-ray crystal structure of recombinant human acetylcholinesterase in complex with donepezil (hAChE, PDB code 4EY7) was obtained from the Protein Data Base (PDB). Hydrogens and partial charges were added using protonate 3D application in MOE. The compound **6d** was constructed using the MOE builder module and energy minimized using Merck Molecular force field (MMFF94x, RMSD gradient: 0.05 kcal mol^{-1} $Å^{-1}$) [47]. The MOE Dock application was used for docking **6d** into the active site of the protein. The poses were generated by the Triangle Matcher placement method and then were rescored using ASE scoring function. The Forcefiled was

selected as the refinement method. The retained best poses were visually inspected and the interactions with binding pocket residues were analyzed.

4.1.6. Cell culture and measurement of cell viability

PC 12 cells was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and grown in RPMI-1640 medium containing 10% (v/v) foetal bovine serum, 100 U penicillin/mL and 100 mg streptomycin/mL under 5% CO_2 at 37 °C. The culture media were changed every other day. After pretreatment with different concentrations of test compound (1.563–12.5 μM) for 2 h, PC 12 cells were incubated with 100 μM of H_2O_2 for 24 h. The cell viability was evaluated using MTT assay [41]. Briefly, 10 μL of 5 mg/ml MTT was added to each well and incubated for 4 h at 37 °C. Then, 200 μL of dimethylsulfoxide (DMSO) was added to dissolve the dark blue formazan crystals formed in intact cells, and the absorbance at 570 nm was detected by a microplate reader. PC 12 cells were cultured without test compound or H_2O_2 as control group and the results were expressed by percentage of control.

4.1.7. In vivo hepatotoxicity study

Investigations were performed on adult male Sprague Dawley (SD) rat [24,43]. Rats were housed in a room that was automatically maintained on a 12-h light/dark cycle at 25 °C and proper humidity. Rats were given food and tap water ad libitum. Tacrine hydrochloride was dissolved in PBS (pH = 7.4) and 6 μmol/100 g b. wt. were administered i.p. Compound **6d** was dissolved in acidic saline and the equimolar dose corresponding to tacrine was administered i. p. Heparinized serum was obtained 0 h, 24 h, 36 h and 72 h after administration from the retrobulbar plexus to determine aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity by using the Beckman Coulter UniCel DxC 800 Synchron Clinical System.

For histological analysis, the rats were sacrificed in ether anesthesia 24 h after administration, and the livers were harvested. A 3 mm section of each liver was immediately placed in 10% buffered formaldehyde, fixed for three days and embedded in paraffin. Subsequently, 4 μm sections were prepared from the paraffin blocks. The sections of each block were deparaffinated and stained with hematoxylin and eosin (HE) for histopathological examinations.

4.1.8. In vitro blood–brain barrier permeation assay

The ability of test compounds that penetrate into brain was evaluated using a parallel artificial membrane permeation assay (PAMPA) for blood–brain barrier according to the method established by Di et al. [45]. Commercial drugs, PBS (pH = 7.4), DMSO and dodecane were obtained from Sigma and Aladdin. Porcine brain lipid (PBL) was purchased from Avanti Polar Lipids. The donor microplate (96-well filter plate, PVDF membrane, pore size is 0.45 μm) and the acceptor microplate (indented 96-well plate) were both from Millipore. The 96-well UV plate (COSTAR) was acquired from Corning Inc. The compound was firstly dissolved in DMSO at a concentration of 5 mg/mL. Then, it was diluted 200-fold with a mixture of PBS/EtOH (70:30) to give a final concentration of 25 μg/mL. The filter membrane in donor microplate was coated with PBL dissolved in dodecane (4 μL, 20 mg/mL). After that, 200 μL of diluted solution and 300 μL of PBS/EtOH (70: 30) were added to the donor wells and the acceptor wells, respectively. The donor filter plate was carefully placed on the acceptor plate to make the underside of filter membrane can contact with buffer solution. After leaving this sandwich assembly undisturbedly for 16 h at 25 °C, the donor plate was carefully removed, and the concentrations of test compound in the acceptor, donor and reference wells were measured with a UV plate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA). Each sample was analyzed at least three independent runs in four wells, and the results are given as the means ± SD. A plot of the experimental P_e values of 10 standard drugs versus their bibliographic values provided a good linear correlation, P_e (exp.) = 0.8597 P_e (Bibl.) − 0.276 (R_2 = 0.9726) (Supporting information, Fig. S1).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2015.01.058. These data include MOL files and InChiKeys of the most important compounds described in this article.

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