

Design and synthesis of tacrine-phenothiazine hybrids as multitarget drugs for Alzheimer's disease

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Abstract Tacrine is well-known drug for Alzheimer's disease as an acetylcholinesterase inhibitor. Rember is a bright and promising AD drugs targeting tau protein, and it is currently in Phase III clinical trials. Phenothiazine, the key pharmacophore of Rember, can prevent tau filament formation. In this work, several tacrine-phenothiazine hybrids (T1–T26) were designed for inhibiting acetylcholinesterase and tau protein involved in Alzheimer's disease. After initial screening with the help of computational chemistry software and Molegro Virtual Docker, three molecules (T5, T18, and T22) were selected for further synthesis and biological evaluation. Next, T5, T18, and T22 were synthesized and evaluated for their acetylcholinesterase and tau hyperphosphorylation inhibition. All the tested compounds had better acetylcholinesterase inhibitory activity compared with tacrine. Among them, compound T5 was found to be the most potent compound with IC_{50} 89 nM. Meanwhile, T5 markedly prevented tau hyperphosphorylation induced by okadaic acid in N2 α cell. Its P-tau level was decreased with 39.5 % inhibition when tested at 10^{-5} M, lower than that Rember (55.7 %). Besides acetylcholinesterase and tau hyperphosphorylation inhibition, T5 can also interact with

fibrill beta amyloid using surface plasmon resonance, the data of K_D were 5.51×10^{-8} M. All the above results indicated that tacrine-phenothiazine hybrids are potential multitarget directed ligands targeting acetylcholinesterase, tau protein, and beta amyloid.

Keywords Alzheimer's disease · Acetylcholinesterase inhibitor (AChEI) · Tau phosphorylation · Beta amyloid · Tacrine-phenothiazine hybrids

Introduction

Alzheimer's disease (AD), the most common dementia in elderly people, is a complex neurodegenerative disorder of the central nervous system (CNS). It is characterized by the progressive deterioration of cognition and the emergence of behavioral and psychological symptoms. In AD brains, the cholinergic system is the most dramatically affected. Besides cholinergic deficiency, the abnormal formation of extracellular senile plaques (SP) consisting of aggregated beta amyloid (A β) peptide deposits and intracellular neurofibrillary tangles (NFTs) consisting of hyper-phosphorylated forms of the microtubule-associated tau protein is the most characteristic pathological hallmarks present in AD brain.

Current clinical therapy for AD is mainly palliative treatment targeting acetylcholinesterase (AChE) or *N*-methyl-D-aspartate (NMDA) receptor. AChE inhibitors (AChEIs) can increase the level of neurotransmitter in synaptic cleft, thereby improving cognition level of mild to moderate AD patients (Massoud and Guathier 2010). So far, four AChEIs (tacrine, donepezil, galantamine, and rivastigmine) and one NMDA receptor antagonist (memantine) have been approved by FDA. However, their role in the course of preventing AD is limited;

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none of them have proven effective against the progression of AD. Given that AChEIs and NMDA receptor antagonist have still been questioned and multifactorial nature of AD, the classic “one molecule, one target” solution may not be effective enough. Researchers are focusing on the development of new therapy strategies for arresting and reverting its progression. One general concept is multitarget directed ligand (MTDL) approach (Cavalli *et al.*, 2008; Morphy and Rankovic, 2009), in which drugs are designed to address selected activities toward selected targets involved in the AD.

In line with MTDL paradigm, several multifunctional agents capable of hitting different biological targets have been developed and their biological profiles seem to be promising (Fernández-Bachiller *et al.*, 2010; Bajda *et al.*, 2011; Minarini *et al.*, 2012; Rizzo *et al.*, 2012).

It is the case of tacrine dimeric derivatives paved the way for the development of very interesting homodimer and heterodimer to prevent AD. An heptylene-tethered dimer of tacrine named bis (7)-tacrine (Fig. 1) is able to simultaneously contact the central anionic site (CAS) and the peripheral anionic site (PAS) (Pang *et al.*, 1996), prevent glutamate-induced neuronal apoptosis by blocking NMDA receptor (Li *et al.*, 2005; Luo *et al.*, 2007), and reduce A β generation by directly inhibiting beta-site APP cleavage enzyme (BACE1) activity (Fu *et al.*, 2008). As for the hybrid structure, it would reduce individual toxicity by special metabolic pathway compared with the combinational drugs. The recent studies following this strategy have led to the synthesis of several chemically diverse structures with dual or multiple biological profiles of AD, including AChE and monoamine oxidase B (MAO-B) dual inhibitors (Sterling *et al.*, 2002); AChE and A β aggregation inhibitors (Kapkov \acute{c} *et al.*, 2006; Tumiatti *et al.*, 2008; Ouberai *et al.*, 2011); A β aggregation and peroxidase activity inhibitors (Ghadami *et al.*, 2013); AChE and BACE1 dual inhibitors (Zhu *et al.*, 2009); AChE, A β aggregation inhibiting, and antioxidant multiple functional compounds (Cavalli *et al.*, 2007); and AChE, A β aggregation, and NMDA receptor inhibitor (Rosini *et al.*, 2008). The biological activities of hybrids mentioned above have been improved by the introduction of the second or the third pharmacophores targeting A β , BACE1, NMDA, or some oxidases and others.

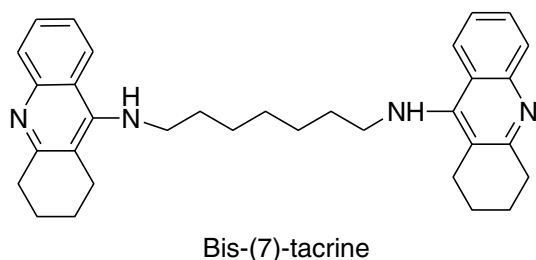


Fig. 1 Chemical structure of bis-(7)-tacrine

Nevertheless, many recent clinical studies showed that the treatment specially targeting A β had no effects on AD. Many drugs failed in phase III clinical trials, for example, Tarenflurbil developed by Myriad Genetics Company and Tramiprosate developed by Neurochem Company. On the other hand, the treatment targeting tau protein shows a bright future, a representative example is Rember developed by Taurx Company. 321 patients had participated in phase II clinical trials, it showed better efficacy. Phase III clinical trials are ongoing.

The aim of this study was to develop novel tacrine hybrids for their mainly inhibiting AChE and tau protein involved in the AD. It is reported that Rember (methylene blue, MB) can reduce soluble tau and regenerate cognition (O’Leary *et al.*, 2010). Phenothiazine, the key pharmacophore of Rember also can prevent tau filament formation (Taniguchi *et al.*, 2005). Herein, a series of tacrine-phenothiazine hybrids via an alkylenediamine-type spacer will be designed according to the principle of multitarget drugs design. These hybrids are expected to inhibit AChE and tau hyperphosphorylation and other targets involved in AD.

Results and discussion

Design and screen

On the basis of tacrine and phenothiazine, two series of new hybrids (T1–T26) are designed, as shown in Fig. 2.

ADME properties of the designed 26 molecules were predicted using the web-based PreADMET (v2.0) (<http://preadmet.bmdrc.org/>) (Chitranshi *et al.*, 2013; Lee *et al.*, 2003). This gave an estimate of physiochemical properties and the bioavailability of the molecule. Fundamental physiochemical features of CNS drugs are related to their ability

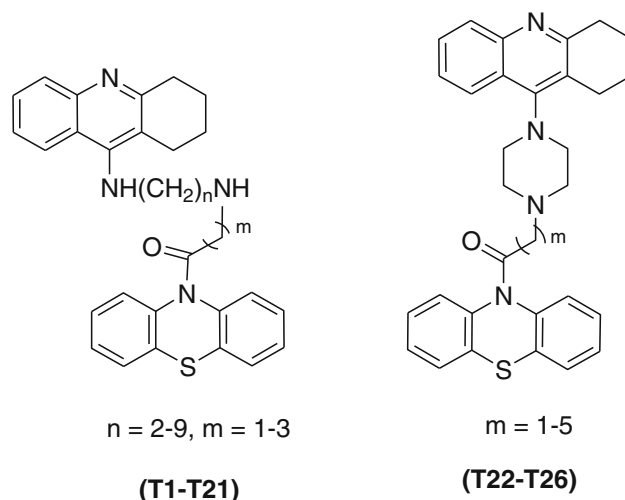


Fig. 2 General structure of the designed molecules

to penetrate the blood–brain barrier (BBB) and exhibit CNS activity. The molecule would regard as CNS penetrant with $C_{\text{brain}}/C_{\text{blood}} > 0.1$. The drug-likeness prediction of the 26 molecules is tabulated in Table 1. As could be seen from Table 1, all the molecules (T1–T26) exhibited general drug likeness. Their $C_{\text{brain}}/C_{\text{blood}}$ values are > 0.1 besides T23 and T24, which reveal their potential BBB penetrability.

After initial ADME screening, the molecular docking technique was undertaken for the best tacrine-phenothiazine hybrids inhibiting AChE and tau hyperphosphorylation among 26 molecules. In addition to AChE as a target protein in the process of molecular docking, another target protein which affects tau hyperphosphorylation must be found. It has been recognized that an imbalanced regulation between protein kinases and protein phosphatases is a direct result of AD-like tau hyperphosphorylation (Hanger *et al.*, 2009a). Among various kinases and phosphatases,

glycogen synthase kinase-3 β (GSK-3 β) is the most implicated (Hanger *et al.*, 2009b; Takashima, 2006). Herein, GSK-3 β was selected as another target protein in the present work.

Once the two mainly target protein was established, MolegroVirtual Docker (MVD) 2009 trial was applied for further molecular docking studies. Docking procedures basically aim to identify the correct conformation of the molecule ligands in the binding pocket of the protein and to predict the affinity between the molecule ligand and the protein (Su, 2011).

The crystal structure of AChE complex with donepezil (Aricept) was retrieved from the Protein Data Bank (PDB ID: 1EVE). Aricept and water molecule were firstly removed followed with enzyme optimization. Similarly, the crystal structure of GSK-3 β (PDB ID: 3GB2) was also retrieved. Finally, the minimized poses were recorded

Table 1 Physiochemical descriptors calculated for the selected compounds using PreADMET

| Compound name | n, m | Human intestinal absorption (HIA, %) | In vivo skin permeability (Log Kp, cm/h) | In vitro plasma protein binding (%) | In vivo blood–brain barrier penetration (C. brain/C. blood) |
|----------------|----------------|--------------------------------------|--|-------------------------------------|---|
| T1 | $n = 2, m = 1$ | 97.072429 | −2.64907 | 85.503982 | 0.403176 |
| T2 | $n = 3, m = 1$ | 97.141426 | −2.57576 | 86.078610 | 0.412231 |
| T3 | $n = 4, m = 1$ | 97.205072 | −2.3987 | 86.104074 | 1.00739 |
| T4 | $n = 5, m = 1$ | 97.263624 | −2.26372 | 85.985029 | 1.7899 |
| T5 | $n = 6, m = 1$ | 97.317235 | −2.14617 | 86.743558 | 2.8854 |
| T6 | $n = 7, m = 1$ | 97.366064 | −2.04459 | 86.241982 | 4.12672 |
| T7 | $n = 8, m = 1$ | 97.410275 | −1.95696 | 86.613634 | 5.31387 |
| T8 | $n = 9, m = 1$ | 97.450030 | −1.88099 | 87.126810 | 6.41301 |
| T9 | $n = 2, m = 2$ | 97.141834 | −2.5321 | 86.414089 | 0.55582 |
| T10 | $n = 3, m = 2$ | 97.205610 | −2.46422 | 84.459587 | 0.609947 |
| T11 | $n = 4, m = 2$ | 97.264175 | −2.30185 | 84.940830 | 1.38431 |
| T12 | $n = 5, m = 2$ | 97.317798 | −2.17936 | 84.864895 | 2.2915 |
| T13 | $n = 6, m = 2$ | 97.366640 | −2.07338 | 85.788631 | 3.59994 |
| T14 | $n = 7, m = 2$ | 97.410864 | −1.98203 | 85.189595 | 4.79546 |
| T15 | $n = 8, m = 2$ | 97.450634 | −1.9031 | 85.594753 | 5.95378 |
| T16 | $n = 2, m = 3$ | 97.205497 | −2.4124 | 84.687633 | 0.902598 |
| T17 | $n = 3, m = 3$ | 97.264182 | −2.35089 | 84.599574 | 0.973773 |
| T18 | $n = 4, m = 3$ | 97.317808 | −2.20486 | 85.659313 | 1.98348 |
| T19 | $n = 5, m = 3$ | 97.366650 | −2.09553 | 85.826236 | 3.16381 |
| T20 | $n = 6, m = 3$ | 97.410875 | −2.00129 | 86.997540 | 4.52704 |
| T21 | $n = 7, m = 3$ | 97.450645 | −1.91998 | 86.583695 | 5.68517 |
| T22 | $m = 1$ | 98.168642 | −2.65986 | 85.131086 | 0.482569 |
| T23 | $m = 2$ | 98.206526 | −2.54326 | 86.590993 | 0.0688374 |
| T24 | $m = 3$ | 98.242005 | −2.42453 | 84.645431 | 0.0847133 |
| T25 | $m = 4$ | 98.275096 | −2.29217 | 84.477508 | 0.130199 |
| T26 | $m = 5$ | 98.305831 | −2.17302 | 87.828355 | 0.237945 |
| Tacrine | | 96.517346 | −3.04445 | 95.393497 | 0.866481 |
| Bis(7)-tacrine | | 95.742616 | −2.08538 | 98.064683 | 15.6351 |
| Donepezil | | 97.951353 | −3.04165 | 84.615816 | 0.187923 |

using MVD scoring function. Only top scoring molecule ligand was taken for further synthesis and biological evaluation.

The molecular docking studies of the 26 ligands are illustrated as representative case in Table 2. The present molecular docking studies showed relatively low MolDock Score (−82.259 kJ/mol) for tacrine, which indicates low affinity toward AChE. In general, the affinity of the ligands (T1–T26) toward AChE or GSK-3 β was better than tacrine or donepezil. Among the 26 molecules, T5 had a significant affinity toward GSK-3 β and a moderate affinity toward AChE. The MolDock Score of T5 toward GSK-3 β was −148.821 kJ/mol; also its MolDock Score toward AChE (−183.585 kJ/mol) was slightly lower than bis (7)-tacrine (−190.66 kJ/mol). It was reported that bis (7)-tacrine had much better AChE inhibitory activity ($IC_{50} = 1.5$ nM) than tacrine ($IC_{50} = 223$ nM) (Carlier *et al.*, 1997). Meanwhile, T18 was also found as a potential molecule, which had a significant affinity toward AChE (−202.166 kJ/mol) and good affinity toward GSK-3 β . As for the affinity with GSK-3 β , the MolDock Score of T18 (−143.581 kJ/mol) was slightly lower than the best T5 (−148.821 kJ/mol).

On the other hand, it is also found from Table 2 that the affinity of the hybrid molecules via piperazine connection (T22–T26) was generally worse than those of molecules via diamine connection (T1–T21). For example, T22 binded to AChE and GSK-3 β with lower MolDock Score (−170.388 and −126.538 kJ/mol); even so, its MolDock Score was greater than tacrine or donepezil. Therefore, T5, T18, and T22 were selected as representative case for further synthesis and biological evaluation.

Chemistry

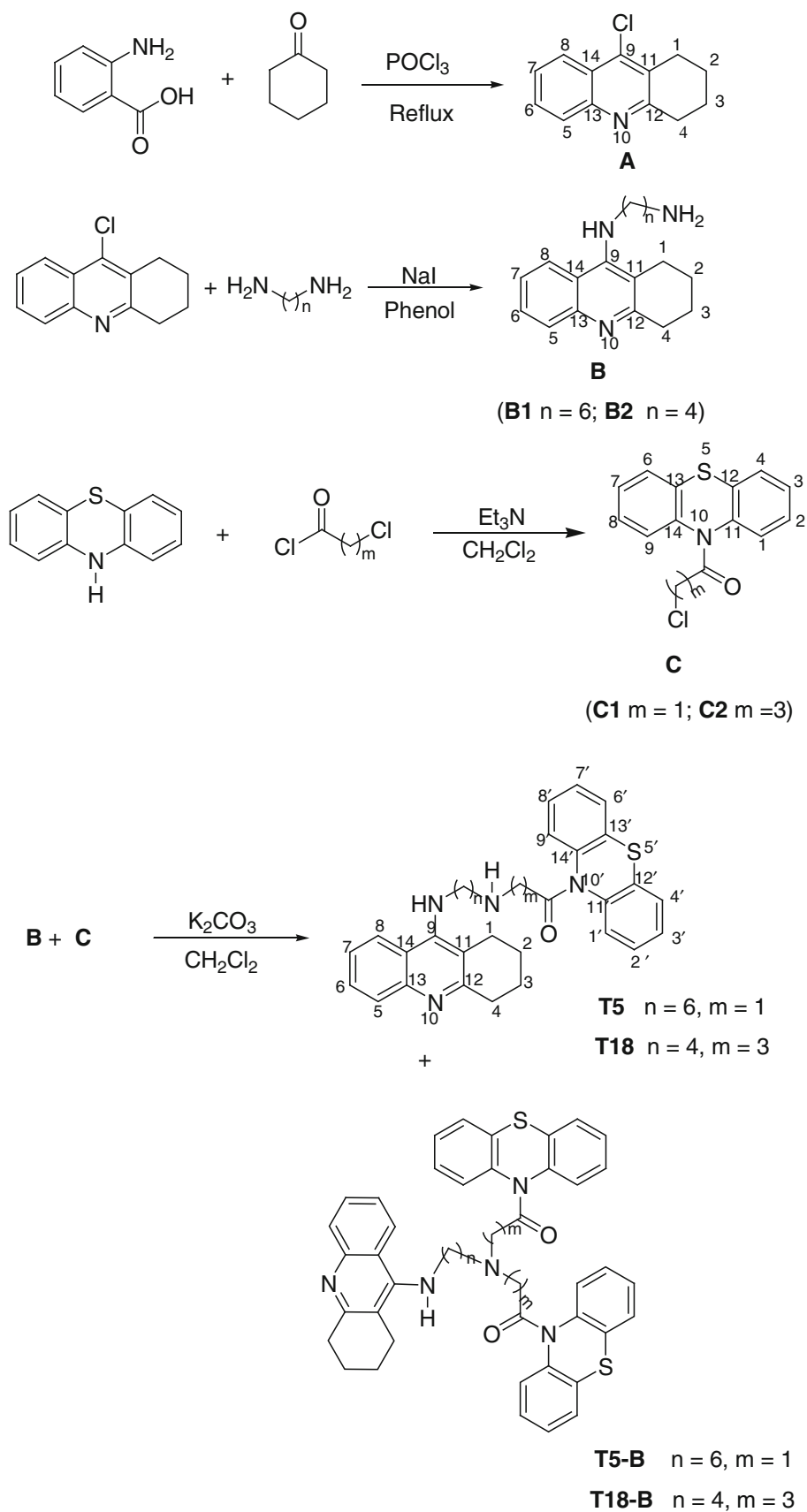
The synthesis route of T5 or T18 is shown in Scheme 1. Intermediates A, B, and C were prepared according to the literature (Hu *et al.*, 2002; Tang *et al.*, 2011; Darvesh *et al.*, 2005) and slightly modified, respectively. Once the intermediate was available, the resulting diamine (B1 or B2) was reacted with C1 or C2 in the presence of base (K_2CO_3 or Et_3N) to yield the desired T5 and T18. However, another new product (T5-B or T18-B) was also obtained, respectively, when we use CH_2Cl_2 or CH_3COCH_3 as reaction solvent under reflux condition. The two new products (T5/T5-B, T18/T18-B) were characterized as one or two molar amine displacement with slightly difference in molar ratio. Subsequently, the reactant ratio of B and C, reaction temperature, and reaction time were investigated for the optimal conditions of T5 or T18. Reducing temperature or shortening time was negative on the conversion of reactant and product yields. With the reaction processing and reactant consuming, the amount of T5-B or T18-B was on

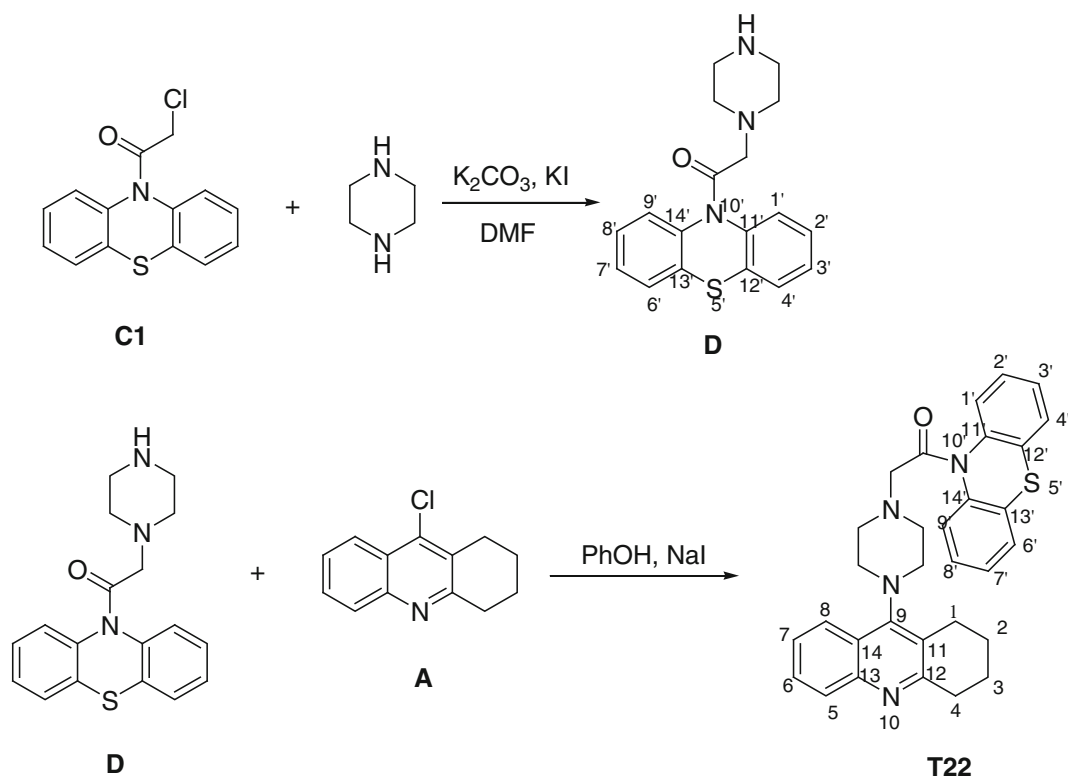
Table 2 MolDock scores of putative AChE and GSK-3 β binders for the selected compounds using MVD

| Compound name | AChE (1EVE) MolDock score (kJ/mol) | GSK-3 β (3GB2) MolDock score (kJ/mol) |
|----------------|--|---|
| T1 | −192.250 | −123.573 |
| T2 | −180.754 | −138.015 |
| T3 | −199.500 | −143.087 |
| T4 | −179.776 | −133.536 |
| T5 | −183.585 | −148.821 |
| T6 | −195.865 | −145.270 |
| T7 | −183.213 | −147.834 |
| T8 | −192.805 | −139.603 |
| T9 | −195.721 | −133.434 |
| T10 | −180.123 | −142.973 |
| T11 | −179.422 | −135.664 |
| T12 | −168.312 | −144.246 |
| T13 | −164.267 | −124.311 |
| T14 | −188.631 | −146.16 |
| T15 | −157.321 | −138.955 |
| T16 | −169.631 | −124.935 |
| T17 | −166.319 | −114.792 |
| T18 | −202.166 | −143.581 |
| T19 | −178.387 | −145.071 |
| T20 | −184.302 | −146.763 |
| T21 | −161.103 | −146.817 |
| T22 | −170.388 | −126.539 |
| T23 | −180.631 | −122.621 |
| T24 | −179.188 | −131.680 |
| T25 | −190.774 | −133.290 |
| T26 | −189.001 | −139.057 |
| Tacrine | −82.259 | −71.497 |
| Bis(7)-tacrine | −190.660 | −161.823 |
| Donepezil | −158.715 | −111.367 |

the increase and the proportion of the two new products was gradually reduced. T5 or T18 was not finally realized as unique product or main product even if the ratio of B and C was also changed. In addition, the type of solvents and bases had certain impact on the composition of the products. There are other by-products in the reaction mixture when we use triethylamine and CH_3COCH_3 instead of CH_2Cl_2 and K_2CO_3 .

The synthesis procedure of T22 is outlined in Scheme 2. Chloroacetyl phenothiazine (C1) was reacted with excessive piperazine in the presence of K_2CO_3/KI , 1-(10H-phenothiazin-10-yl)-2-(piperazin-1-yl) ethanone (D) was obtained as the unique product. Next, the intermediate D was converted to the desired T22 using similar synthesis method of B1 or B2.

Scheme 1 Synthesis of T5 and T18

**Scheme 2** Synthesis of T22**Table 3** Inhibitory activity on rain brain homogenate AChE

| Compound name | IC ₅₀ (nM) |
|---------------|-----------------------|
| T5 | 89 ± 19 |
| T5-B | 305 ± 29 |
| T18 | 109 ± 21 |
| T18-B | 283 ± 41 |
| T22 | 217 ± 30 |
| Tacrine | 275 ± 32 |
| Donepezil | 33 ± 8 |

Biological evaluation

The preliminary anti-acetylcholinesterase activity for the synthesized tacrine derivatives (T5, T5-B, T18, T18-B, T22) was performed according to AChE enzyme-linked immunosorbent assay (ELISA) Kit using tacrine and donepezil as reference compound. AChE was obtained from homogenates of rat brain. Results of anti-AChE activity of the compounds are illustrated in Table 3. It is easily seen that all the tacrine derivatives exhibited better inhibitory activity against AChE than tacrine. From the biological activity studies, we also found that T5-B and T18-B demonstrated weaker AChE inhibition as compared to T5 or T18. Their bulk structure (T5-B, T18-B) was partially responsible for the lower AChE inhibition, which cannot

Table 4 Inhibition of P-Tau for test compounds in OA-treated N2α cells

| Group | Concentration | P-Tau content (ng/L) | Inhibition (%) |
|---------|--------------------|--------------------------|----------------|
| Control | – | 22.8 ± 2.5 | – |
| OA | 60 nM | 68.6 ± 4.1 ^{##} | – |
| T5 | 10 ⁻⁵ M | 41.5 ± 0.9 ^{**} | 39.5 ± 1.1 |
| | 10 ⁻⁶ M | 44.8 ± 1.3 ^{**} | 34.7 ± 2.0 |
| | 10 ⁻⁷ M | 50.9 ± 1.5 ^{**} | 25.8 ± 1.9 |
| T18 | 10 ⁻⁵ M | 45.3 ± 0.8 ^{**} | 34.0 ± 0.7 |
| | 10 ⁻⁶ M | 49.3 ± 1.3 ^{**} | 26.7 ± 1.0 |
| | 10 ⁻⁷ M | 54.1 ± 0.6 ^{**} | 21.1 ± 1.3 |
| T22 | 10 ⁻⁵ M | 50.5 ± 4.3 ^{**} | 26.4 ± 2.1 |
| | 10 ⁻⁶ M | 56.6 ± 2.7 [*] | 17.5 ± 1.3 |
| | 10 ⁻⁷ M | 61.8 ± 2.0 | 9.9 ± 0.8 |
| Rember | 10 ⁻⁵ M | 30.4 ± 3.5 ^{**} | 55.7 ± 2.3 |
| | 10 ⁻⁶ M | 36.8 ± 4.2 ^{**} | 46.4 ± 1.0 |
| | 10 ⁻⁷ M | 44.3 ± 1.0 ^{**} | 35.4 ± 1.7 |
| Tacrine | 10 ⁻⁸ M | 53.4 ± 1.6 ^{**} | 22.2 ± 1.4 |
| | 20 μM | 61.3 ± 4.7 | 10.6 ± 1.1 |

^{##} $p < 0.01$ versus Control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ versus OA group

efficiently enter into binding pocket of AChE. And as for the three desired tacrine hybrids (T5, T18, and T22), T5 was found to be the most potent compounds with IC₅₀ 89

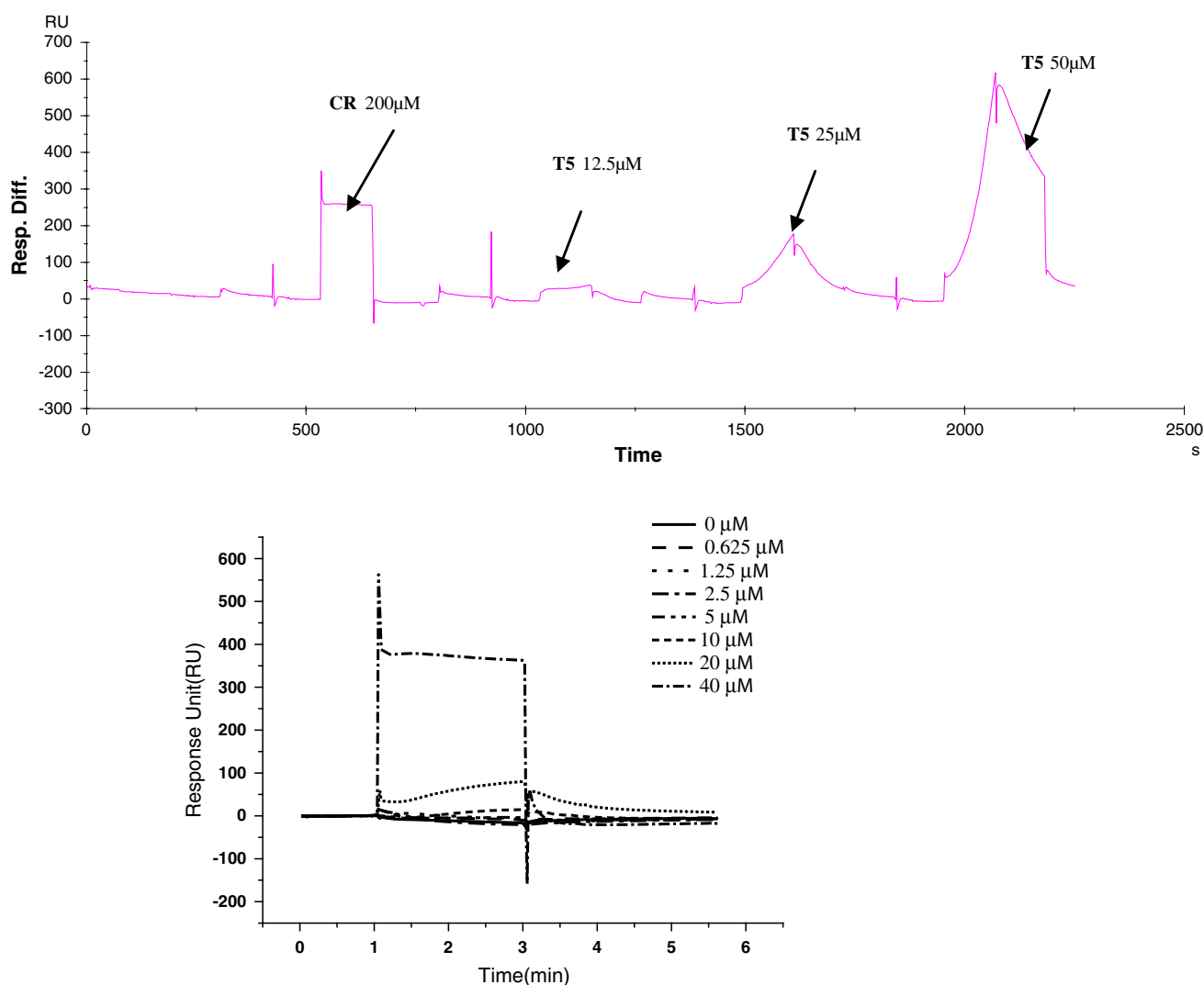


Fig. 3 Analysis of the affinities of T5 for fA β (1–40) by SPR

nM, lower than donepezil with IC₅₀ 33 nM. T22 showed the lowest activity in the three compounds, its IC₅₀ was 217 nM, slightly greater than tacrine (275 nM). The above-mentioned AChE inhibition results are consistent with the inhibition sequence (Table 2) predicted by MVD.

The effect of the tacrine hybrids (T5, T18, T22) on OA-induced P-Tau accumulation is shown in Table 4. The results were given by measuring the sum of P-Tau in OA-treated N2 α cells with an ELISA assay. 12 h exposure of N2 α cells to OA resulted in a significant increase in intracellular P-Tau generation, the P-Tau level reached to 68.6 ng/L compared with control group (22.8 ng/L). As shown in Table 4, a treatment with tacrine hybrids obviously prevented OA induced increase in the P-Tau level, which displayed inhibition higher than 25 % when tested at 10⁻⁵ M. Among the three hybrids, T5 was the most potent, which inhibited P-tau level by 39.5 % at 10⁻⁵ M. Even if

the concentration of T5 was reduced to 10⁻⁷ concentration of T5 was 10⁻⁷ M, the P-Tau inhibition (25.8 %) was almost equal to that of T22 at 10⁻⁵ M. At the same conditions, Rember displayed a percentage of inhibition ranged from 22.2 to 55.7 % when tested at 10⁻⁸ M–10⁻⁵ M.

On the basis of these promising results, T5 was also tested for its ability to bind with A β fibril (fA β). Interaction of T5 with fA β (1–40) was investigated by surface plasmon resonance (SPR) in grade concentrations at 0.625–40 μ M for generation of binding curves. As shown in Fig. 3, distinct association and dissociation reactions were observed after the addition of freshly prepared T5 to immobilized fA β (1–40), indicating that T5 could react with the immobilized fA β . The responses of T5 reached equilibrium (Req) quickly and returned to baseline rapidly after dissociation. The binding response was calculated as the Req value divided by the molecular weight. The dose–response

curve for T5 appeared to be monophasic, and it reached a saturation level at higher concentrations. The dissociation constant (K_D) was calculated from data at dose ranging from 0.625 to 40 μM by steady-state analysis using BIA evaluation software (version 4.1). The K_D of T5 to fA β (1–40) was 5.51×10^{-8} M, lower than Congo Red (CR, positive control) with K_D 6.78×10^{-7} M, while tacrine have no binding with fA β (1–40) under the same condition.

Conclusion

The tacrine-phenothiazine hybrid has already been successful in the design of MTDL for combating the multifactorial nature of AD. In addition to its classic anti-AChE effects, tacrine-phenothiazine hybrid has been proven to be effective in inhibiting P-Tau and binding with A β fibrils when presenting in a multimeric form. These results expand the pharmacological profile of tacrine hybrids and may also help to design new chemical entities targeting tau protein.

Experimental section

Chemistry

The reagents used in the synthesis were purchased from Sinopharm Chemical Reagent Co. Ltd., and were used without further purification unless otherwise indicated. All the reactions were monitored using TLC. Column chromatography was performed using silica gel (200–300 mesh). All yields of the products refer to isolated yield.

Melting points were determined on YRT-3 apparatus and uncorrected. IR spectra were obtained on a Thermo Scientific Nicolet 8700 system. ^1H NMR spectra were obtained on a Bruker Avance 300 (300 MHz) spectra or Agilent VNMRS 600 (600 MHz) with TMS as the internal standard, and chemical shifts were recorded in ppm along with coupling constants (J values) in Hz. Multiplicities were designated as singlet (s), broad singlet (br), doublet (d), triplet (t), and multiplet (m). Mass spectra (MS) were obtained by electrospray ionization (ESI) at 70 eV in a Finnigan LCQ Advantage MAX spectrometer with direct insertion probe. Elemental analysis (EA) was performed on an Elementar Analysensysteme GmbH Vario EL.

Synthesis of starting materials (compound A, B, C in Scheme 1 and compound D in Scheme 2)

9-chloro-1, 2, 3, 4-tetrahydroacridine (A, Scheme 1): pale yellow solid (This compound was prepared as follows: To a

mixture of *o*-aminobenzoic acid (22 mmol, 3 g) and cyclohexanone (20 mmol, 2 g) 15 ml POCl₃ at ice bath was carefully added. The mixture was heated under reflux for 4 h, then cooled to room temperature, and concentrated to give the slurry. The residue was diluted with ethyl acetate, neutralized with aqueous K₂CO₃, and washed with brine. The organic layer was dried over anhydrous K₂CO₃ and evaporated. The crude product was purified by flash chromatography to give the desired product. It was obtained as pale yellow solid, 3.4 g, 78 % yield; mp 69.0–69.8 °C; IR (KBr) ν_{max} 2,936.1, 1,577.7, 1,552.6, 1,481.2, 754.0 cm⁻¹; ^1H NMR (CDCl₃, 300 MHz): δ = 8.16 (1H, d, J = 8.4 Hz, H-8), 7.97 (1H, d, J = 8.4 Hz, H-5), 7.66 (1H, dd, J = 8.4, 9.6 Hz, H-6), 7.53 (1H, dd, J = 8.3, 9.6 Hz, H-7), 3.13 (2H, t, J = 6.0 Hz, H-4), 3.02 (2H, t, J = 5.4 Hz, H-1), 1.92–1.97 (4H, m, H-2 and H-3); ^{13}C (CDCl₃, 75 MHz): δ = 159.4 (C, C-12), 146.6 (C, C-13), 141.4 (C, C-9), 129.2 (C, C-11), 128.8 (CH, C-6), 128.5 (CH, C-5), 126.4 (CH, C-7), 125.3 (C, C-14), 123.6 (CH, C-8), 34.1 (CH₂, C-4), 27.4 (CH₂, C-1), 22.6 (CH₂, C-3, C-2). ESI-MS: m/z [M + 1]⁺ 218.25.

N-(1, 2, 3, 4-tetrahydroacridin-9-yl) hexane-1, 6-diamine (B1, Scheme 1): brown oily liquid (This compound was prepared as follows: In a round-bottomed flask (100 ml) equipped with a magnetic stirrer, 9-chloro-1, 2, 3, 4-tetrahydroacridine (9 mmol, 1.95 g), sodium iodide (1.8 mmol, 300 mg), and phenol (54 mmol, 5.1 g) were stirred at 90 °C for 2 h and then hexane-1, 6-diamine (63 mmol, 7.32 g) was added. The reaction temperature was raised to 120 °C and maintained 4 h. The mixture was cooled to room temperature and diluted with CHCl₃ and made basic with 10 % NaOH solution. The organic layer was washed with water and brine and dried over anhydrous MgSO₄. The filtrate was evaporated and the residue was purified on silica gel column chromatography (CH₂Cl₂/MeOH/NH₃·H₂O = 9:1:0.1) to give the brown oily product, 1.86 g, 70 % yield; IR (film) ν_{max} 3,359.0, 2,929.6, 1,563.3, 1,131.8, 764.4 cm⁻¹; ^1H NMR (CDCl₃, 300 MHz): δ = 7.90–7.97 (2H, m, H-5, H-8), 7.52–7.58 (1H, m, H-6), 7.32–7.37 (1H, m, H-7), 3.98 (2H, br, NH₂), 3.45–3.52 (2H, m, CH₂NH), 3.07 (2H, m, H-4), 2.66–2.72 (4H, m, H-1, CH₂NH₂), 1.91–1.95 (4H, m, H-2, H-3), 1.62–1.72 (2H, m, CH₂CH₂NH₂), 1.48–1.69 (6H, m, (CH₂)₃CH₂CH₂NH₂); ^{13}C (CDCl₃, 75 MHz): δ = 158.4 (C, C-12), 150.7 (C, C-9), 147.1 (C, C-13), 128.6 (CH, C-6), 128.3 (CH, C-5), 123.6 (CH, C-7), 122.7 (CH, C-8), 120.1 (C, C-14), 115.9 (C, C-11), 51.8 (CH₂, CH₂NH), 49.1 (CH₂, CH₂NH₂), 33.9 (CH₂, C-4), 32.1 (CH₂, CH₂CH₂NH₂), 31.2 (CH₂, CH₂CH₂NH), 26.8 (CH₂, CH₂CH₂CH₂NH₂), 25.6 (CH₂, CH₂CH₂CH₂NH), 24.5 (CH₂, C-1), 22.9 (CH₂, C-3, C-2).

N-(1, 2, 3, 4-tetrahydroacridin-9-yl) butane-1, 4-diamine (B2, Scheme 1): brown oily liquid (Prepared following the general procedure of B1 and starting from butane-1, 4-diamine (63 mmol, 5.55 g). The brown oily product B2

was obtained by chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_3\cdot\text{H}_2\text{O} = 10:1:0.1$), 1.62 g, 67 % yield); IR (film) ν_{max} 3,358.1, 2,931.2, 1,561.5, 1,129.8, 768.4 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz): $\delta = 7.89\text{--}7.96$ (2H, m, H-5, H-8), 7.53–7.57 (1H, m, H-6), 7.32–7.36 (1H, m, H-7), 4.01 (2H, br, NH_2), 3.46–3.50 (2H, m, CH_2NH), 3.11 (2H, m, H-4), 2.73–2.79 (4H, m, H-1, CH_2NH_2), 1.97–1.99 (4H, m, H-2, H-3), 1.39–1.63 (4H, m, $(\text{CH}_2)_2\text{CH}_2\text{NH}_2$); ^{13}C (CDCl_3 , 75 MHz): $\delta = 158.0$ (C, C-12), 150.1 (C, C-9), 147.1 (C, C-13), 128.7 (CH, C-6), 128.1 (CH, C-5), 123.6 (CH, C-7), 122.3 (CH, C-8), 120.1 (C, C-14), 115.9 (C, C-11), 51.5 (CH_2 , CH_2NH), 48.9 (CH_2 , CH_2NH_2), 33.6 (CH_2 , C-4), 31.0 (CH_2 , $\text{CH}_2\text{CH}_2\text{NH}_2$), 29.8 (CH_2 , $\text{CH}_2\text{CH}_2\text{NH}$), 24.8 (CH_2 , C-1), 22.8 (CH_2 , C-3, C-2).

2-chloro-1-(10H-phenothiazin-10-yl) ethanone (C1, Scheme 1): off-white solid (This compound was prepared as follows: A solution containing phenothiazine (26 mmol, 5.248 g), 2-chloroacetyl chloride (66 mmol, 7.45 g, 5.5 ml), and triethylamine (26 mmol, 4 ml) in dichloromethane (100 ml) was refluxed with stirring. After about 2–3 h reflux period, TLC analysis revealed that all phenothiazine were consumed. The reaction mixture was cooled to room temperature and then washed successively with 5 % aqueous sodium bicarbonate, 5 % hydrochloric acid, and water. The solution was dried over MgSO_4 , filtered, and evaporated. The crude product was purified by flash column chromatography with dichloromethane as elute and recrystallized from petroleum ether/dichloromethane (2:1). Finally, the desired product C1 was obtained as off-white solid, 3.6 g, 50 % yield.); mp 110.3–111.0 °C; IR (KBr) ν_{max} 1,693.1, 1,671.7, 1,335.1, 1,247.9, 1,170.3, 1,125.6, 761.6 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz): $\delta = 7.60$ (2H, d, $J = 7.8$ Hz, H-1, H-9), 7.48 (2H, d, $J = 7.8$ Hz, H-4, H-6), 7.33–7.38 (2H, m, H-2, H-8), 7.24–7.30 (2H, m, H-3, H-7), 4.18 (2H, s, CH_2Cl); ^{13}C (CDCl_3 , 75 MHz): $\delta = 165.4$ (C, CON), 137.8 (C, CH, C-11, C-14, C-4, C-6), 128.1 (CH, C-2, C-8), 127.3 (CH, C-1, C-9, C-3, C-7), 126.5 (C, C-12, C-13), 41.8 (CH_2 , CH_2Cl).

4-chloro-1-(10H-phenothiazin-10-yl) butan-1-one (C2, Scheme 1): off-white solid (Prepared following the general procedure of C1 and starting from 4-chlorobutanoyl chloride (66 mmol, 9.3 g). The crude product was purified by column chromatography with petroleum ether/ethyl acetate (4:1) as elute, C2 was obtained as off-white solid, 3.3 g, 42 % yield); mp 118.0–118.8 °C; IR (KBr) ν_{max} 1,693.4, 1,672.0, 1,336.1, 1,248.5, 1,170.3, 1,126.8, 762.0 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz): $\delta = 7.58$ (2H, d, $J = 7.6$ Hz, H-1, H-9), 7.47 (2H, d, $J = 7.6$ Hz, H-4, H-6), 7.36–7.38 (2H, m, H-2, H-8), 7.26–7.30 (2H, m, H-3, H-7), 3.43 (2H, t, $J = 7.5$ Hz, CH_2Cl), 2.26 (2H, t, $J = 4.8$ Hz, COCH_2), 2.01 (2H, m, $\text{CH}_2\text{CH}_2\text{Cl}$); ^{13}C (CDCl_3 , 75 MHz): $\delta = 172.5$ (C, CON), 135.9 (C, CH, C-11, C-14, C-4, C-6), 128.2 (CH, C-2, C-8), 126.1 (CH, C-1, C-9, C-3, C-7),

124.9 (C, C-12, C-13), 42.9 (CH_2 , CH_2Cl), 31.3 (CH_2 , COCH_2), 27.3 (CH_2 , $\text{CH}_2\text{CH}_2\text{Cl}$).

1-(10H-phenothiazin-10-yl)-2-(piperazin-1-yl) ethanone (D, Scheme 2): white solid (This compound was prepared as follows: To a solution containing chloroacetyl phenothiazine (7.2 mmol, 2.0 g), piperazine (36 mmol, 3.1 g), and potassium iodine (7.2 mmol, 1.2 g) in *N,N*-dimethyl formamide (100 ml) was carefully added anhydrous K_2CO_3 (7.2 mmol, 0.995 g) at room temperature. After about 10 h reaction period with stirring, TLC analysis revealed that chloroacetyl phenothiazine was consumed. *N,N*-dimethyl formamide and excess piperazine were removed under vacuum condition. The residue was poured into water and extracted with ethyl acetate; the organic layer was combined and washed with water and brine, dried over anhydrous Na_2SO_4 , filtered, and evaporated. The desired product D was obtained as white solid, 1.99 g, 85 % yield); mp 140.8–141.5 °C; IR (KBr) ν_{max} 3,316.6, 1,668.6, 1,460.0, 1,325.1, 1,261.2, 1,132.3, 773.7 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz): $\delta = 7.51\text{--}7.56$ (2H, m, H-1', H-9'), 7.41–7.46 (2H, m, H-4', H-6'), 7.19–7.34 (4H, m, H-2', H-3', H-7', H-8'), 3.29 (2H, s, CH_2CO), 2.71–2.74 (4H, m, CH_2NCH_2), 2.39–2.32 (4H, m, CH_2NHCH_2), 1.99 (1H, br, NH); ^{13}C NMR (CDCl_3 , 75 MHz): $\delta = 168.6$ (C, CON), 138.7 (C, CH, C-11', C-14', C-4', C-6'), 127.8 (CH, C-2', C-8', C-1', C-9'), 126.9 (CH, C-3', C-7'), 126.7 (C, C-12', C-13'), 60.9 (CH_2 , COCH_2), 54.0 (CH_2 , NCH_2), 45.9 (CH_2 , NHCH_2). ESI-MS: m/z [$\text{M} + 1$] $^+$ 326.1. Anal. Calcd. for $\text{C}_{18}\text{H}_{19}\text{N}_3\text{OS}$: C, 66.43; H, 5.88; N, 12.91. Found: C, 66.40; H, 5.78; N, 12.87.

Synthesis of the desired products (T5, T5-B, T18, T18-B in Scheme 1 and T22 in Scheme 2)

1-(10H-phenothiazin-10-yl)-2-(6-(5, 6, 7, 8-tetrahydroacridin-9-ylamino) hexylamino) ethanone (T5, Scheme 1): yellow foam (This compound was prepared as follows: To a solution of B1 (3 mmol, 891 mg) in dry dichloromethane, potassium carbonate (3 mmol, 416 mg) and potassium iodine (0.3 mmol, 50 mg) were added, the resulting mixture was stirred at room temperature for 1 h. Then, C1 (3 mmol, 828 mg) was added and the reaction mixture was refluxed for 10–12 h. Then, the mixture was cooled to room temperature and water was poured, the organic layer was combined and washed with water and brine, dried over Na_2SO_4 , filtered, and evaporated. The crude product was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_3\cdot\text{H}_2\text{O} = 15:1:0.1$). The desired product T5 was obtained as yellow foam, 338 mg, 21 % yield); IR (KBr) ν_{max} 3,352.9, 3,061.3, 1,679.8, 1,457.8, 763.1 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz): $\delta = 8.09\text{--}8.21$ (1H, m, H-5), 7.93–7.96 (1H, m, H-8), 7.54–7.63 (1H, m, H-6), 7.14–7.48 (9H, m, H-7, Ar-H in phenothiazine), 4.30 (1H, br, NH), 3.63 (2H, s, COCH_2), 3.45–3.49 (2H, m, CH_2NHAr), 3.11 (2H, m,

H-4), 2.53–2.64 (4H, m, H-1, CH_2NH), 1.89–1.91 (4H, m, H-2, H-3), 1.62–1.73 (2H, m, $\text{CH}_2\text{CH}_2\text{NH}$), 1.18–1.26 (6H, m, $(\text{CH}_2)_3\text{CH}_2\text{CH}_2\text{NH}$); ^{13}C NMR (CDCl_3 , 75 MHz): $\delta = 164.7$ (C, C-12), 163.5 (C, CON), 148.3 (C, C-9), 145.2 (C, C-13), 133.1 (C, C-11', C-14'), 132.0 (CH, C-4', C6') 128.1 (C, Ar), 124.3 (C, Ar), 118.5 (C, Ar), 114.9 (C, Ar), 50.9 (CH_2 , COCH_2), 48.6 (CH_2 , CH_2NHAr), 45.7 (CH_2 , CH_2NH), 35.2 (CH_2 , C-4), 31.2 (CH_2 , $\text{CH}_2\text{CH}_2\text{NHAr}$), 30.3 (CH_2 , $\text{CH}_2\text{CH}_2\text{NH}$), 26.9 (CH_2 , $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), 24.0 (CH_2 , C-1), 22.5 (CH_2 , C-2, C-3); ESI-MS: m/z [$\text{M} + 1$] $^+$ 537.3; Anal. Calcd. for $\text{C}_{33}\text{H}_{36}\text{N}_4\text{O}_5$: C, 73.85; H, 6.76; N, 10.44. Found C, 73.80; H, 6.65; N, 10.43.

2,2'-(6-(5,6,7,8-tetrahydroacridin-9-ylamino)hexylazanediy) bis(1-(10H-phenothiazin-10-yl)ethanone) (T5-B, Scheme 1): yellow foam (In the preparation process of T5, another product T5-B was obtained as a yellow foam by column chromatography, 628 mg, 27 % yield); IR (KBr) ν_{max} 3,356.9, 3,059.7, 2,929.7, 1,678.3, 1,460.6, 760.8 cm^{-1} ; ^1H NMR (CDCl_3 , 600 MHz): $\delta = 8.63$ (1H, d, $J = 8.5$ Hz, H-5), 8.11 (1H, d, $J = 8.6$ Hz, H-8), 7.68–7.72 (1H, m, H-6), 7.37–7.47 (9H, m, H-7, Ar-H in phenothiazine), 7.16–7.29 (8H, m, Ar-H in phenothiazine), 5.57 (1H, m, NHAr), 3.61–3.75 (6H, m, COCH_2 , COCH_2 , CH_2NH), 3.26–3.38 (2H, m, H-4), 2.48–2.60 (4H, m, H-1, CH_2N), 1.86–1.89 (4H, m, H-2, H-3), 1.66–1.70 (2H, m, $\text{CH}_2\text{CH}_2\text{NH}$), 1.29–1.35 (2H, m, $\text{CH}_2\text{CH}_2\text{N}$), 1.17–1.22 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$); ^{13}C NMR (CDCl_3 , 150 MHz): $\delta = 169.8$ (C, C-12, CON), 138.3 (C, Ar), 127.9 (C, Ar), 126.9 (C, Ar), 126.8 (C, Ar), 124.0 (C, Ar), 123.4 (C, Ar), 55.0 (CH_2 , COCH_2), 53.9 (CH_2 , NCH_2), 48.9 (CH_2 , NHCH_2), 31.2 (CH_2 , C-4), 29.6 (CH_2 , NHCH_2CH_2), 28.9 (CH_2 , $\text{NHCH}_2\text{CH}_2\text{CH}_2$), 27.6 (CH_2 , NCH_2CH_2), 26.6 (CH_2 , $\text{NCH}_2\text{CH}_2\text{CH}_2$), 24.3 (CH_2 , C-1), 22.5 (CH_2 , C-3), 21.9 (CH_2 , C-2); ESI-MS: m/z [$\text{M} + 1$] $^+$ 776.4; Anal. Calcd. for $\text{C}_{47}\text{H}_{45}\text{N}_5\text{O}_2\text{S}_2$: C, 72.74; H, 5.84; N, 9.02. Found C, 72.70; H, 5.73; N, 9.00.

1-(10H-phenothiazin-10-yl)-4-(4-(5, 6, 7, 8-tetrahydroacridin-9-ylamino) butylamino)butan-1-one (T18, Scheme 1): yellow foam (Prepared following the general procedure of T5 and starting from B2 (3 mmol, 807 mg) and C2 (3 mmol, 914 mg). The crude products were purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_3 \cdot \text{H}_2\text{O} = 15:1:0.1$), the desired product T18 was obtained as yellow foam, 306 mg, 19 % yield); IR (KBr) ν_{max} 3,357.7, 3,058.2, 1,680.7, 1,460.0, 760.1 cm^{-1} ; ^1H NMR (CDCl_3 , 600 MHz): $\delta = 8.07$ –8.15 (1H, m, H-5), 7.95–7.99 (1H, m, H-8), 7.60–7.68 (2H, m, H-6, H-7), 7.11–7.51 (8H, m, Ar-H in phenothiazine), 4.36 (1H, br, ArNH), 3.35–3.43 (2H, m, CH_2NHAr), 2.98–3.11 (2H, m, H-4), 2.54–2.63 (6H, m, CH_2NHCH_2 , H-1), 2.21–2.35 (2H, m, COCH_2), 1.75–1.91 (6H, m, H-2, H-3, $\text{CH}_2\text{CH}_2\text{CO}$), 1.42–1.61 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NHAr}$); ^{13}C NMR (CDCl_3 , 150 MHz): $\delta = 174.5$ (C, CON), 164.0 (C, C-12), 147.0 (C, C-9), 145.2 (C, C-13), 133.1 (C, C-11', C-14'), 132.1 (CH, C-4', C-6'),

128.1 (C, Ar), 124.5 (C, Ar), 118.9 (C, Ar), 115.3 (C, Ar), 50.9 (CH_2 , NHCH_2), 49.3 (CH_2 , CH_2NH), 45.0 (CH_2 , CH_2NHAr), 36.9 (CH_2 , C-4), 31.7 (CH_2 , COCH_2), 27.9 ($\text{COCH}_2\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2$), 24.9 (CH_2 , C-1), 23.0 (CH_2 , C-2, C-3); ESI-MS: m/z [$\text{M} + \text{H}$] $^+$ 537.3. Anal. Calcd. for $\text{C}_{33}\text{H}_{36}\text{N}_4\text{O}_5$: C, 73.85; H, 6.76; N, 10.44. Found C, 73.83; H, 6.71; N, 10.39.

4,4'-(4-(5,6,7,8-tetrahydroacridin-9-ylamino)butylazanediy) bis(1-(10H-phenothiazin-10-yl)butan-1-one) (T18-B, Scheme 1): yellow foam (In the preparation process of T18, another product T18-B was obtained as yellow foam by column chromatography, 675 mg, 28 % yield); IR (KBr) ν_{max} 3,360.5, 3,058.2, 1,675.7, 1,463.5, 765.1 cm^{-1} ; ^1H NMR (CDCl_3 , 600 MHz): $\delta = 8.58$ (1H, d, $J = 8.5$ Hz, H-5), 8.09 (1H, d, $J = 8.6$ Hz, H-8), 7.68–7.72 (1H, m, H-6), 7.33–7.48 (9H, m, H-7, Ar-H in phenothiazine), 7.16–7.28 (8H, m, Ar-H in phenothiazine), 5.48 (1H, m, NHAr), 3.45–3.60 (2H, m, CH_2NHAr), 3.11–3.19 (2H, m, H-4), 2.48–2.60 (8H, m, H-1, $(\text{CH}_2)_3\text{N}$), 2.25–2.34 (4H, m, CH_2CO), 1.66–1.70 (8H, m, H-2, H-3, $\text{CH}_2\text{CH}_2\text{CO}$), 1.35–1.57 (m, 4H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$); ^{13}C NMR (CDCl_3 , 150 MHz): $\delta = 175.0$ (C, CON), 164.9 (C, C-12), 146.3 (C, C-9, C-13), 130.9 (C, Ar), 129.1 (C, Ar), 125.3 (C, Ar), 119.5 (C, Ar), 54.9 (CH_2 , CH_2N), 53.1 (CH_2 , CH_2N), 46.8 (CH_2 , CH_2NH), 34.2 (CH_2 , C-4), 30.8 (CH_2 , CH_2CO), 27.6 (CH_2 , $\text{CH}_2\text{CH}_2\text{NHAr}$), 25.9 (CH_2 , $\text{CH}_2\text{CH}_2\text{N}$), 24.3 (CH_2 , C-1), 21.9 (CH_2 , C-2, C-3); ESI-MS: m/z [$\text{M} + 1$] $^+$ 804.3. Anal. Calcd. for $\text{C}_{49}\text{H}_{49}\text{N}_5\text{O}_2\text{S}_2$: C, 73.19; H, 6.14; N, 8.71. Found C, 73.15; H, 6.11; N, 8.70.

1-(10H-phenothiazin-10-yl)-2-(4-(5, 6, 7, 8-tetrahydroacridin-9-yl) piperazin-1-yl) ethanone (T22, Scheme 2): brown oily liquid (Prepared following the general procedure for the synthesis of B1 or B2. The starting material of the reaction included 9-chloro-1, 2, 3, 4-tetrahydroacridine (2 mmol, 435 mg), sodium iodine (0.3 mmol, 45 mg), phenol (8 mmol, 752 mg), and Compound D (2.5 mmol, 814 mg). The brown oily product T22 was obtained by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NH}_3 \cdot \text{H}_2\text{O} = 15:1:0.1$), 679 mg, 67 % yield); IR (KBr) ν_{max} 2,925.6, 1,682.6, 1,460.1, 1,262.6, 1,126.6, 761.3 cm^{-1} ; ^1H NMR (CDCl_3 , 600 MHz): $\delta = 8.05$ –8.09 (1H, m, H-5), 7.93–7.99 (1H, m, H-8), 7.48–7.60 (6H, m, Ar-H), 7.25–7.38 (4H, m, Ar-H in phenothiazine), 3.47 (2H, s, CH_2CO), 3.19–3.27 (4H, m, CH_2NAr), 3.09–3.11 (2H, t, $J = 6.6$ Hz, H-4), 2.86–2.89 (2H, t, $J = 6.6$ Hz, H-1), 2.58–2.69 (4H, m, CH_2N), 1.91–1.93 (2H, m, H-2), 1.82–1.85 (2H, m, H-3); ^{13}C NMR (CDCl_3 , 150 MHz): $\delta = 168.9$ (C, C-12), 160.5 (C, CON), 153.0 (C, C-9), 147.9 (C, C-13), 138.9 (C, C-11', C-14'), 128.8 (C, Ar), 128.5 (C, Ar), 128.1 (C, Ar), 127.9 (C, Ar), 127.2 (C, Ar), 127.0 (C, Ar), 126.0 (C, Ar), 125.0 (C, Ar), 124.1 (C, Ar), 60.8 (CH_2 , CH_2CO), 53.9 (CH_2 , CH_2N), 50.5 (CH_2 , CH_2N), 34.2 (CH_2 , C-4), 32.1 (CH_2 , C-1), 22.9 (CH_2 , C-2, C-3); ESI-MS: m/z [$\text{M} + 1$] $^+$

507.3. Anal. Calcd. for $C_{31}H_{30}N_4OS$: C, 73.49; H, 5.97; N, 11.06. Found: C, 73.40; H, 5.86; N, 11.05.

Biological evaluation

In vitro estimation of acetylcholinesterase inhibitory activity

The preliminary anti-acetylcholinesterase activity for the synthesized compounds was performed according to AChE enzyme-linked immunosorbent assay (ELISA) Kit (96T, R&D system) using tacrine and donepezil as reference compound. AChE was obtained from homogenates of rat brain. Test compounds (T5, T5-B, T18, T18-B, T22) were dissolved in DMSO and diluted with DMSO-H₂O and rat brain homogenates, the final concentration ranged from 0.01 to 100 μ M. Blank was prepared using rat brain homogenates instead of the test sample, and all other procedures were similar to those used in the case of the sample mixture. Absorbance (OD) was measured at 450 nm using microplate reader (DG5033A, Nanjing Huadong Electronics Group Co. Ltd.). The AChE content in sample is positively correlated with OD. AChE content in the negative control hole as 100 %, the reduction of AChE content in test samples hole is the enzyme inhibition rate. Experiments were done in triplicate. Data are expressed as mean \pm standard deviation (SD) and were analyzed using SPSS 19.0 statistical software.

In vitro estimation of P-Tau inhibitory activity

The N2 α cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (GIBCO). Cells were grown in a 100 % humidified incubator at 37 °C with 5 % CO₂ and passaged 2–3 days prior to use. Logarithmic phase's N2 α cells were seeded in 96-well plates at a density of 10⁴ cells per well. After growing for 12 h, the cells were incubated in DMEM containing 60 nM okadaic acid (OA, Sigma Chemical Co.) for 12 h. Then, test compound (or other compound) with final concentration of 10⁻⁴ M, 10⁻⁵ M, 10⁻⁶ M, 10⁻⁷ M, or 20 μ M methylene blue (Rember) were added to the culture medium and incubated with the cells for 24 h. Cells were washed twice with ice-cold PBS, homogenized in RIPA Lysis buffer (Solarbio Science & Technology Co.) for 5 min on ice, and the lysate was centrifugated at 14,000g for 5 min at 4 °C prior to ELISA detection. For quantitative analysis of P-Tau in cell lysates, the assay was performed as described in the instructions of the P-Tau ELISA Kit (Innogenetics NV Co.). Experiments were done in triplicate. Data are expressed as mean \pm standard deviation (SD) and were analyzed using SPSS 19.0 statistical software.

A β fibril (fA β) binding study by surface plasmon resonance (SPR)

A BIAcore 3,000 (Biacore Inc., USA) equipped with four flow cell channels on sensor chip was used for real-time binding studies. Carboxymethylated dextran 5 (CM5) sensor chip was used in this experiment for fA β (1–40). fA β (1–40) stored at 4 °C was centrifuged at 4 °C for 2 h at 16,000 g. fA β (1–40) precipitated completely, and the pellet was resuspended in water, sonicated, diluted with 10 mM sodium acetate (pH 4.0), and immobilized immediately on CM5 sensor chip by amine coupling. fA β (1–40) was immobilized on the CM5 chip in the second channel, and the first channel was used as a reference (without immobilization of fA β (1–40)). The carboxymethyl dextran surface was activated using 70 μ L of a mixture of 0.4 M *N*-ethyl-*N'*-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDC) and 0.1 M *N*-hydroxysuccinimide (NHS) at an injection rate of 10 μ L/min, followed by the injection of fA β (1–40) into channel 2 (70 μ L of fA β diluted with 600 μ L of PH 4.0 sodium acetate buffer and injected at a rate of 5 μ L/min).

After fA β immobilization, the remaining activated groups were blocked with 70 μ L of 1.0 M ethanolamine (pH 9.0). The reference cell was prepared by the above described amine coupling without the addition of fA β . The running buffer solution contained 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, 0.3 mM EDTA, and 0.05 % Tween 20, and the washing solvent contained 1 % DMSO.

After immobilization, test compounds with different concentrations were injected into the cell, and binding was observed. Each binding experiment was repeated three times, and the reaction was performed 25 °C using running buffer solution mentioned above. Test compound (T5) dissolved in DMSO was diluted with running buffer to a final concentration of 0.625–40 μ M and injected over the reference and fA β -immobilized flow cells at a flow rate of 30 μ L/min. The surface of sensor chip immobilized with fA β was regenerated with a mixture of 0.025 % sodium laurel sulfated (SDS) and 25 mM NaOH. Data from the reference cell without fA β immobilization were subtracted from raw data. The running buffer injection using the same method was also subtracted as a double reference (Yan *et al.*, 2007).

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