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Rational design and synthesis of highly potent anti-acetylcholinesterase activity huperzine A derivatives

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ABSTRACT

By targeting multi-active sites of acetylcholinesterase (AChE), a series of huperzine A (Hup A) derivatives with various aromatic ring groups were designed and synthesized by Schiff reaction. They were evaluated as AChE and butyrylcholinesterase (BChE) inhibitors. Results showed very significant specificity that the group of imine derivatives cou ld inhibit *Tc*AChE and *h*AChE, but no inhibitory effect on *h*BChE was detected. The experiment was explained by a docking study. In the docking model, we confirmed that aromatic ring of Hup A derivatives played the π–π stacking against aminophenol residues of AChE, and the structure–activity relationship (SAR) was discussed.

1. Introduction

Alzheimer's disease (AD) is one of the most common age-related chronic neurodegenerative dementia affecting more than 20 million people worldwide.[1] As the world population ages, AD has become an urgent public health problem. Cholinesterase inhibition (especially AChE inhibition) is the current approach for the treatment of AD. For example, tacrine (trade name: Cognex^{R}),[2] donepezil (trade name: Aricept^{R}),[3] rivastigmine (trade name: Exelon^{R}),[4] galanthamine (trade name: Reminyl^{R})[5] are all typical AChE inhibitory drugs. Compared with the above four AChE inhibitors, Hup A (trade name: Shuangyiping) has better penetration through the blood–brain barrier, higher oral bioavailability and longer duration of AChE inhibitory action.[6,7]

Many attempts have been made to synthesize Hup A, Hup A analogs or derivatives since 1989,[8–19] however, only very few compounds have obvious and potent anti-AChE activity. For example, ZT-1 is being developed as a new anti-AD drug candidate in both China and Europe.[20]

The crystallographic structure of *Tc*AChE (*Tc* stands for Torpedo californica) and their complexes with various inhibitors (PDB ID: 1VOT, 1ZGC, 1ZGB, 1E66, 1EVE, 1W6R)[21–25] showed that the *Tc*AChE active sites contained an acylation site (catalytic anionic site) and a peripheral anionic site. Moreover, some dual-site ligands have been described recently as being highly potent inhibitors of AChE, such as bis(7)-tacrine,[26] bis-huperzine A,[27] bis-huperzine B,[28] huperzine A-tacrine,[29] huperzines series[30] and the galanthamine series.[31–33] So, it is reasonable to hypothesize that the dual-site ligands (such as Hup A derivatives) could simultaneously interact with the catalytic anionic site and the peripheral site, and that potency of the dual-site ligands could be greatly improved.

Based on the theory of the dual-site ligands approach, the crystal structure of *Tc*AChE and their inhibitions and the computational searching method, we synthesized a series of Hup A derivatives for improved bioactivity and selective index. In vitro investigation showed that nine compounds (**1**, **3**, **4**, **5**, **6**, **7**, **8**, **10**, **11**) were stronger than their parent compound Hup A in *Tc*AChE, and that all the compounds were more potent than positive control tacrine in *h*AChE, but absence of inhibition effect detected on *h*BChE. Thus, we report here the synthesis, biological activity, molecular modeling and SAR of Hup A derivatives.

2. Results and discussion

X-ray analysis of *Tc*AChE complex Hup A (PDB ID: 1VOT) led to the interesting results that only few direct interactions between the inhibitor and the active sites of enzyme were responsible for the strong affinity,[21] indicating that the lactam ring and ethylidene

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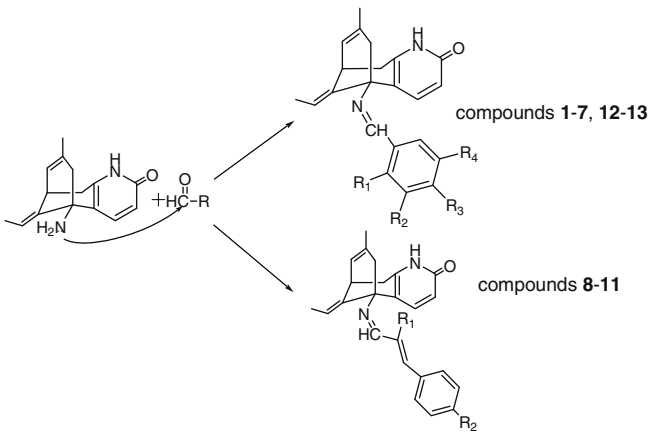
methyl were active functional groups,[13,15] suggesting that these groups must be preserved among structure modification. In addition, primary group of Hup A has a less interaction with Trp84 and Phe330 of aromatic amino acids, with distances between the nitrogen and the centroid of the ring of 4.8 and 4.7 Å, respectively,[21] implying that structure modification can be carried out on the NH_2 group in order to increase mutual affinity and reduce the distance. Further analysis showed that nearly 70% surface of active gorge is linked with rings of 14 conserved aromatic amino acids, called 'active aromatic gorge'.[22,34–36] The π–π stacking against those aminophenol residues would play a great role in the inhibitory activity of Hup A, and it is reasonable to introduce the aromatic ring group to the amino position of Hup A. In order to simultaneously match dual-site ligands and π–π stacking, the Hup A complex with aromatic ring (**1**,[37] **2**, **3**,[37] **4**–**7**, **8**,[19] **9**–**13**) were synthesized by a simple and useful Schiff reaction (Scheme 1 and Table 1). The compounds were further purified by column chromatography eluting with $CHCl_3/CH_3OH$ (1:0, 40:1, 20:1). All compounds were identified using spectroscopic techniques (IR, ^1H and ^{13}C NMR spectra and MS) (see Supplementary data about the specific experiment).

To determine the compounds **1**–**13** for the treatment of AD, their AChE inhibitory activity were assayed using method of Ellman.[38] As shown in Table 1, four potential compounds **3**, **5**, **6**, **7** were approximately 865-fold, 1065-fold, 477-fold and 517-fold stronger than the positive control, respectively, and were also more stronger than Hup A as *Tc*AChE inhibitor. The bioactivity of *h*AChE results revealed that all the Hup A derivatives with aromatic ring were potent than positive control tacrine, and were equivalent with their parent compound Hup A, but showed very high selected index (Table 1). Difference in bioactivities on *Tc*AChE and *h*AChE were observed, and mechanism of action is still worthy of further investigation. The aromatic compounds connected with Hup A are benzaldehyde or cinnamaldehyde derivatives, which are natural while some are widely used as food flavoring, essential oil and medicine, suggesting that these compounds themselves should belong to the category of low toxicity. Moreover, it has been proved that ZT-1 is less toxic in mice than Hup A.[39] We synthesized a series of derivatives having structural analogues like ZT-1. So, compounds **1**–**13** maybe less toxic in mice or human than Hup A. Further research on the toxicity profile is on-going.

In order to gain further insight to mechanism of inhibition, docking study was performed using GOLD 3.1 to generate binding model for the synthetic Hup A derivatives **1**–**11** on the basis of the existing X-ray crystal structure of *Tc*AChE (PDB ID: 1VOT). Firstly, the docking reliability was validated using the known X-ray structure of *Tc*AChE in complex with the molecular ligand



Scheme 1. A general Schiff reaction of formula synthesized Hup A derivatives.

Table 1
Chemical structure and activity of synthetic Hup A derivatives

	R_1	R_2	R_3	R_4	IC_{50} (nM)	
					*Tc*AChE	*h*AChE
1	H	OCH_3	OH	H	6.7	16
2*	H	H	$N(CH_3)_2$	H	23.3	31
3	H	H	OCH_3	H	0.0246	31
4*	H	OEt	OH	H	0.842	25
5*	H	OCH_3	H	OCH_3	0.02	29
6*	OCH_3	H	OCH_3	H	0.0446	84
7*	H	H	CN	H	0.0412	22
8	H	H	/	/	1.81	24
9*	$(CH_2)_4CH_3$	H	/	/	796	51
10*	CH_3	H	/	/	1.63	25
11*	H	$N(CH_3)_2$	/	/	0.121	40
12*	OSO_2Na	H	H	H	18.2	41
13*	H	H	OAll	H	13.9	31
P0407	/	/	/	/	21.3	/
Hup A	/	/	/	/	11.4	21
Tacrine	/	/	/	/	/	128

Note: Compounds **1**–**13** are synthetic compounds. Values are expressed as means (standard error of the mean of at least four experiments); P0407 is Physostigmine Salicylate (as a positive control); * stands for new compounds; BChE is not detect; All stands for β-D-allose.

HUP999 (Hup A) (PDB ID: 1VOT). The extracted and optimized HUP999 was re-docked to the binding sites of *Tc*AChE and the docked conformation corresponding to the highest Gscore was selected as the most probable binding conformation. The root-mean-square deviation (RMSD) of conformations between co-crystallized HUP999 and re-docked HUP999 is equal to 0.772 Å, suggesting that a high docking reliability of GOLD in reproducing the experimentally observed binding mode for Hup A and parameter set for the GOLD simulation was reasonable to reproduce the X-ray structure (see Supplementary data). Therefore, the GOLD method and the parameter set could be extended to search the *Tc*AChE binding conformations for compounds **1**–**11**.

From Figure 1A, it can be seen that compounds **1**–**11** exhibited a remarkable preference for binding to HUP999-binding cleft. Two different binding modes have been observed among those compounds (Fig. 1B). Compounds **1**, **3**, **4**, **5**, **6**, **7** and **8** located phenyl ring to the hemi-circle grooves formed by residues of Typ70, Val71, Asp72, Asn85, Gly123, Ser122, Tyr121 and Ser124 (Fig. 2A). Compounds **2**, **9**, **10** and **11** extend phenyl ring to the hydrophobic sub-site composed by residues of Tyr70, Trp279 and Tyr334 (Fig. 2B). In comparison with Hup A, the derivatives have a little interaction with active sites of *Tc*AChE (see Supplementary data), it is clear that the phenyl group substitution at NH_2 would favor the interaction between Hup A derivatives and *Tc*AChE. As a result, it is not surprised that our synthetic compounds **1**–**11** were more active than Hup A.

In our study, the binding mode of compounds **5** and **9** could be two representative cases of whole Hup A derivatives we synthesized (Fig. 1B). The docking conformation and corresponding complex analysis of compound **5** are depicted in Figure 2A. Compound **5** is located in a strong hydrophobic pocket of *Tc*AChE, in which a substituted-phenyl group is involved in hydrophobic interactions with Typ70, Val71, Asp72, Asn85, Gly123, Ser122, Tyr121 and Ser124. Hup A parent moiety is composed by two flexible rings and one rigid benzene ring. It can be seen that two flexible aliphatic ring and side chain engaged in hydrophobic contacting with Gly118, Tyr130, His440, Gly441, Gly115, Trp81 and His437, while benzene ring have strong π–π interactions with the residues of Phe290, Phe330 and Phe331. Moreover, two hydrogen bonds were formed by two methoxyl groups substituted at benzene ring with residues Asp72 and Gly123, with bond length of 3.14 and 2.67 Å, respectively. The hydrogen bond receptor at the benzene ring

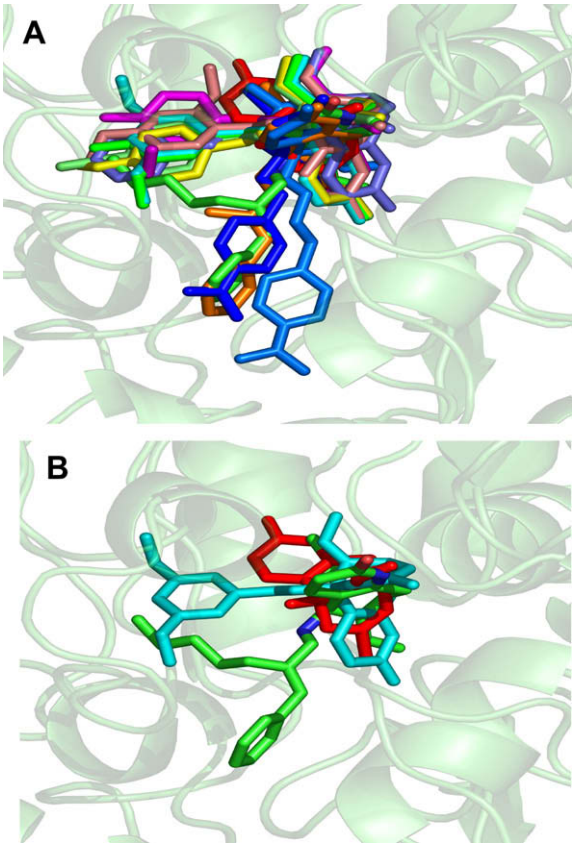


Figure 1. Docked conformations showing interactions with residues in the active site for Hup A and compounds **1**–**11** (Hup A: red, **1**: green, **2**: blue, **3**: yellow, **4**: magenta, **5**: cyan, **6**: salmon, **7**: lime, **8**: pink, **9**: green, **10**: orange, **11**: marine).

would increase the inhibitory activity (compounds **3**, **5**, **6** and **7**), and further analysis found that phenyl ring at C-3, 5 would increase the electron density due to imine bond, however, phenyl ring at C-2, 4, 6 would decrease the electron density, which maybe explain the relationship between the position of methoxy group and activity (IC_{50}: **5** < **3** < **7** < **6**). But hydrogen bond donor would decrease the inhibitory activity (compounds **1** and **4**). For compound **9**, the parent moiety had a very similar binding profile when compared with compound **5** except substituted phenyl ring and long aliphatic chain. Figure 2B represents the predicted conformation and schematic binding plot of compound **9** into the *Tc*AChE active site. Compound **9** had a hydrophobic interaction with *Tc*AChE, with long aliphatic chain occupying the hydrophobic semicircle groove of *Tc*AChE formed by residues of Tyr70, Asp72, Pro86, Tyr121 and Ser122, while the substituted phenyl ring extends to a hole which has no interaction with residues of active sites of *Tc*AChE. Moreover, there were two hydrogen bond formed by the polar H atom of Trp84 with carbonyl oxygen and the N atom of Hup A moiety with Tyr121, with bond lengths of 2.81 and 3.32 Å, respectively. This observation agreed with the experimental results that compounds **2**, **9**, **10** and **11** have a less potent activity than the other compounds, because the phenyl ring extends to a hole which has a little interaction with the residues of *Tc*AChE.

It is worthy of note the compound **13**. Compound **13** was synthesized basing on the recent studies that bipharmacophore or bimolecular (hybrid-molecular) strategy demonstrated to be powerful in enhancing the potency and selectivity relative to its monomeric lead.[40] Moreover, applying the strategy is especially useful to the enzyme AChE with the geometry active-site gorge, the specific sites at its two extremities.[22,26,41,42] Compound **13** could probably

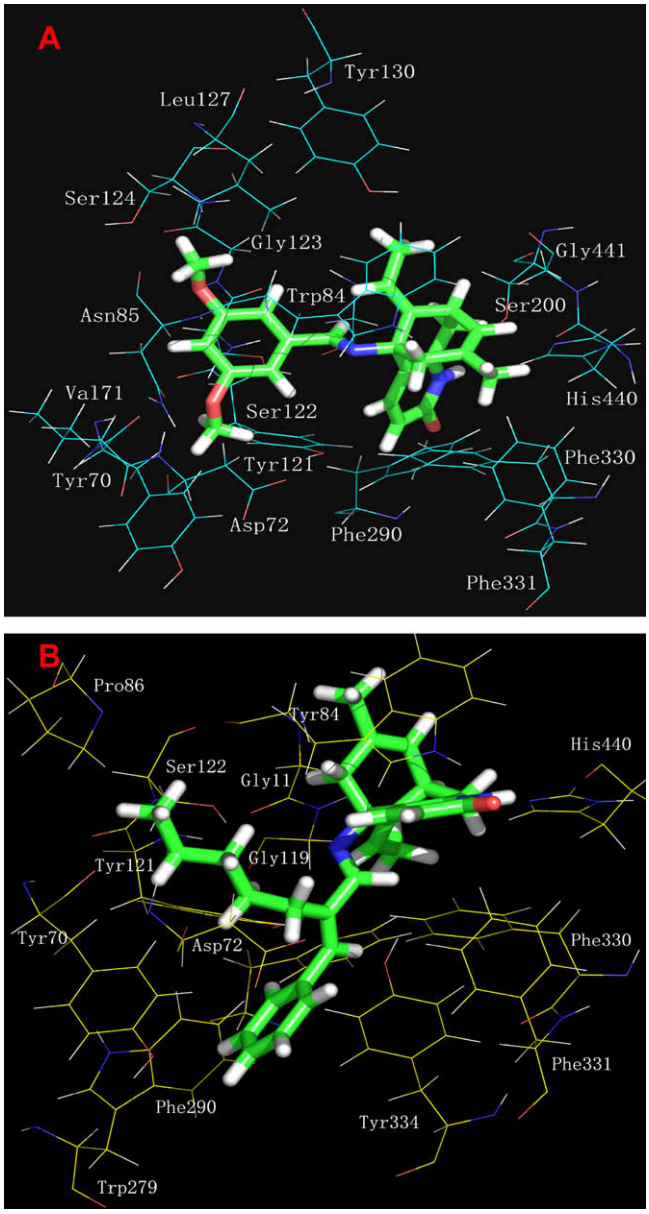


Figure 2. Compounds **5** (A) and **9** (B) interacted with the active site residues of the *Tc*AChE. 2D representation of ligand–protein interactions were analyzed between compounds **5**, **9** and *Tc*AChE see Supplementary data.

rapidly transform into the active metabolite Hup A, which could be a potent medicine.[20] Another bipharmacophore is helicid (i.e., 4-(β-D-allopyranosyloxy)-benzaldehyde), which was originally isolated as one of the main active constituents from *Helicid nilgrinica* Bedd. The helicid is the main ingredient of 'Shen Shuai Guo Su' tablets, used to treat symptoms of neurasthenia, neuralgia and insomnia, and no obvious side effect has been reported.[43] Recently, research reported that the helicid and its analogues showed novel relative neuro-bioactivity, such as acetylcholinesterase inhibitors, antidepressant activity.[44,45] It is responsible for combination helicid with Hup A by Schiff reaction.

3. Conclusion

From this study, the anti-AChE was improved due to Hup A NH_2 group connected to benzaldehyde, and aromatic rings position at C-2, C-3, C-4, C-5 with small group such as methoxyl, however, cin-

namaldehyde in α-position with the group leads to decrease anti-AChE activity. The results provided new insights into the factors affecting AChE–ligand interaction in the active gorge. But differences in the structures and conformations of these enzymes must take into consideration.

4. Experimental

4.1. General procedures

NMR spectra were recorded at 400 and 500 MHz using $CDCl_3$ as the solvent and chemical shifts were referenced to internal solvent peaks. All melting points were measured with *X-4* apparatus, uncorrected. Optical rotations were recorded at *Horiba SEAP-300* spectropolarimeter. IR spectra were recorded *Shimadzu IR-450* instrument, in cm^{-1}, KBr pellets. FAB-MS and HRMS were recorded at *VG-AUTOSPEC-3000* spectrometer; in *m/z* (rel. int.% of the base peak). Silica gel (200–300 mesh, Qingdao Marine Chemical, China) was used for column chromatography (CC). Fractions were monitored by TLC, and spots were visualized by heating TLC plates sprayed with 10% H_2SO_4. All materials were obtained from commercial suppliers and used without further purification.

4.2. Chemistry

All the compounds (**1**–**13**) were prepared from Hup A by Schiff reaction with benzaldehyde and cinnamaldehyde derivatives, see Scheme 1, detailed method and spectroscopic data see Supplementary data.

4.3. Bioactivity test

To determine the potential interest of compounds **1**–**13** for the treatment of AD, their AChE inhibitory activity were assayed by the method of Ellman. Five different concentrations of each compound were used in order to obtain inhibition of AChE activity comprised between 20% and 90%. The assay solution consisted of a 0.1 M phosphate buffer pH 8.0, with the addition of 340 μM 5,5′-dithiobis (2-nitrobenzoic acid), 0.02 unit/mL of *Tc*AChE, *h*AChE, and *h*BChE (Sigma Chemical), and 550 μM of substrate (acetylthiocholine iodide or butyrylthiocholine iodide). Test compounds were added to the assay solution and pre-incubated at 37 °C with the enzyme for 20 min followed by the addition of substrate. Assays were done with a blank containing all components except AChE in order to account for non-enzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analyzed in triplicate, and IC_{50} values were determined graphically from log concentration–inhibition curves.

4.4. Molecular modeling methods

All the molecular modeling studies were carried out using the molecular docking software GOLD 3.1 running on PC with AS4. In order to learn the interaction mode between Hup A derivatives and AChE, molecular docking simulations were carried out with the program GOLD 3.1 which used a genetic algorithm to explore the full range of ligand conformational flexibility with partial flexibility of protein. The structure of *Tc*AChE and the Hup A analogues were built using the SYBYL 7.1 molecular modeling software. The original ligand and water were removed from the coordinated set of the AChE (PDB ID: 1VOT). The following default genetic algorithm parameters were used: 100 population sizes, 1.1 for selection, 5 number of islands, 100,000 number of genetic operations and 2 for the niche size. The ligand-based was created at the center of the catalytic triad and the active site defined as 10 Å around it. The GoldScore (Gscore) was opted to rank order the docked conformations. Ligplot 4.4 3 was used to generate hydrogen bonds and hydrophobic interactions between the best-docked conformational pose of the ligand and the amino acid residues in the active site of the protein.

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Supplementary data

Supplementary data (experimental procedures and characterization of compounds **1**–**13**, activity results and docking study) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.017.

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