Design, synthesis, and biological evaluation of 5*H*-thiazolo[3,2-*a*]pyrimidine derivatives as a new type of acetylcholinesterase inhibitors

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Abstract

Acetylcholinesterase(AChE) inhibitors are important research topics because of their wide range of associated health implications, especially for the treatment of Alzheimer's disease. The finding of novel AChE inhibitors is presented here. A docking screening model of AChE inhibitor was used to evaluate a series of 5*H*-thiazolo[3,2-*a*] pyrimidine derivatives. The virtual screening hits were analyzed in drug likeness and physical-chemical features, therefore were focused to those compounds in the present article. To investigate the relationship between the bioactivities and the structures, 10 target compounds with the 5*H*-thiazolo[3,2-*a*]pyrimidine scaffold were synthesized as potential AChE inhibitors, and the pharmacological assay result also conformed, some compounds displayed considerable inhibitory effects with inhibition rates above 50% at 10 μM, and all their core structures are very different from those of known AChE inhibitors. The results demonstrate the effectiveness and validity of the virtual screening approach especially of the docking screening approach, and provide a starting point for the development of novel drugs to treat Alzheimer's disease.

Keywords: Acetylcholinesterase inhibitor, heterocycles, synthesis, docking screening, 5*H*-thiazolo[3,2-*a*]pyrimidines derivatives

Introduction

Alzheimer's disease (AD) is a chronic, slowly progressive neurodegenerative disorder, clinically characterized by an impairment of cognitive function.[1] AD is commonly believed to be associated with the dysfunction of the central cholinergic system. Acetylcholinesterase (AChE) plays a key role in the regulation of the cholinergic system.[2] And hence, inhibition of AChE has emerged as one of the most promising strategies for the treatment of AD.[3-5]

Molecular docking is an efficient tool for investigating receptor-ligand interactions and for virtual screening, which plays a key role in rational drug design, especially when the crystal structure of a receptor or enzyme is available.[6-8]

Therefore, in this study we first explored the AChE complex structures with dozens of known AChE inhibitors which are available from the Protein Data Bank (PDB). The result was used as the docking protocol to investigate their global binding mode of AChE. In addition, a small focused library with 5*H*-thiazolo[3,2-*a*]pyrimidine derivatives was built and screened virtually using the docking method.

The docking model built with Autodock 4.0 software was generated and checked for its reliability, we went through a self-compiled 3D database to get some preferable candidates for future synthesis. On the basis of the obtained hits, we undertook an exploratory study of their *in vitro* AChE inhibitory effects.

The aim of this study was to demonstrate the strategy of utilizing computer-aided drug discovery in search for AChE inhibitors in the field of new scaffolds. We believe that this procedure will be helpful in the design of novel AChE inhibitory compounds.

Results and Discussion

Generation of the docking model for AChE inhibitors. Virtual screening

Docking models have often been proven to be useful tools for rationalizing ligand-target interaction and for making this information available to virtual screening techniques. For this study the model was obtained starting from 3D structural information, of which those protein-ligand complexes are available as PDB files, using the AutoDock 4.0 software package.

Molecular modeling studies were performed using human AChE, since they represent the pharmacological target for the development of new drugs, also considering of *Torpedo californica* AChE complexes. Nevertheless, a comparison of their amino acid sequences revealed an overall identity of 59.09% (1B41 *vs* 1W6R). As to the binding site, which is to mean, the residues within 15nm of the ligand, the identity rose up to 75.6%(1B41 vs 1W6R), only small differences were found comparing the amino acid composition of the active sites, which could be seen clearly in Figure 1.

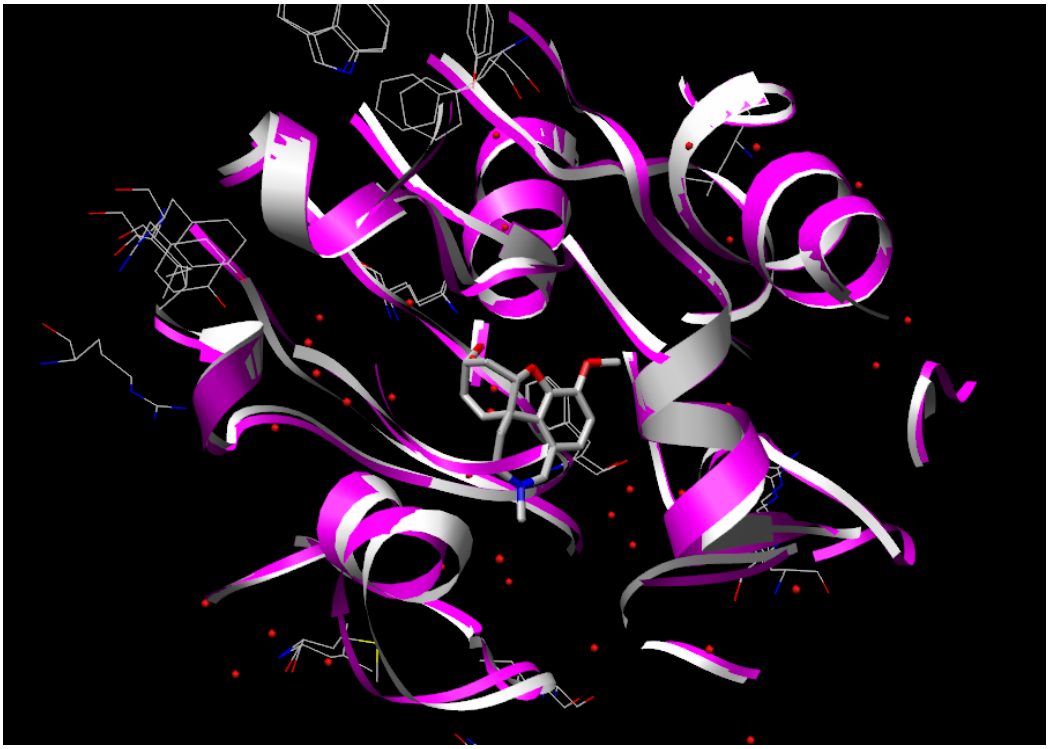


Figure 1. Human AChE (shown as ribbon in pink) aligned to *Torpedo californica* AChE (shown ribbon in silver) complex with galanthamine(Hydrogens are omitted for the sake of clarity, water molecules are represented as red balls).

On the basis of extensive X-ray crystallography and mutagenesis studies, AChE appears to have a binding domain that interacts with divergent enzyme inhibitors, which includes those residues as D74, W86, N87, G120, G121, G122, Y124, S125, G126, L130, E202, S203, F297, Y337, F338, Y341, H447, G448, and I451, showed in Figure 2. Their binding affinities are dependent, in part, on their 3D fitness within the substrate binding domain of the enzyme, and the chemical basis underpinning the interaction between inhibitor and binding domain.

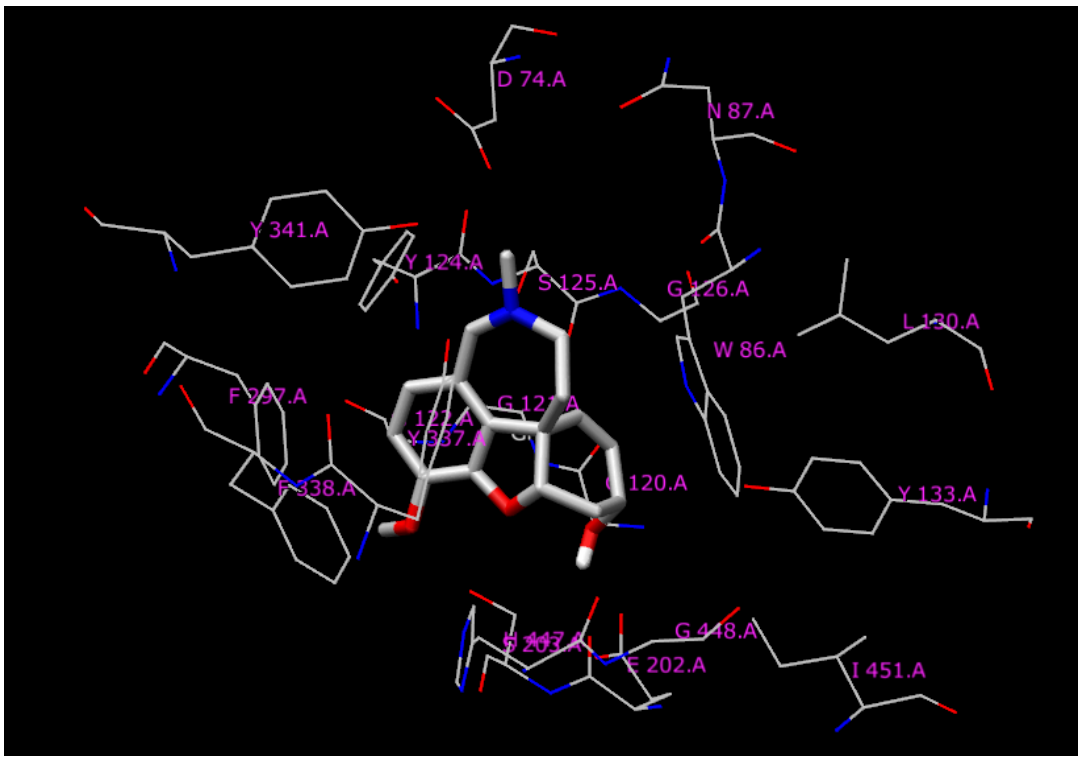


Figure 2. Docking of Galantamine in the active site of human AChE model. Galantamine is shown as stick models. The residues interacting with the ligand, are shown as line models. (Hydrogens and water molecules are omitted for the sake of clarity, all colored in atom type).

Therefore, the docking procedure which indicates the enzyme-inhibitor binding interactions was used for the virtual screening experiment, using a protocol including molecular mechanics, genetic algorithm, and Lamarckian GA calculations. On the basis of the obtained hits, 5*H*-thiazolo[3,2-a]pyrimidine derivatives emerged as promising candidates. Selected conformers of compounds were docked into the human AChE structure (PDB code 1B41), showed in Figure 3 and Figure 4.

As a validation of the docking procedure the structure of galantamine was docked back into the active site of the enzyme with all water molecules deleted. Eight out of the ten top scoring docking configurations resembled almost exactly as the crystallized complex of galanthamine-enzyme, the RMSD was below 1.0. This allowed to the conclusion that the results obtained with the applied docking procedure were meaningful.

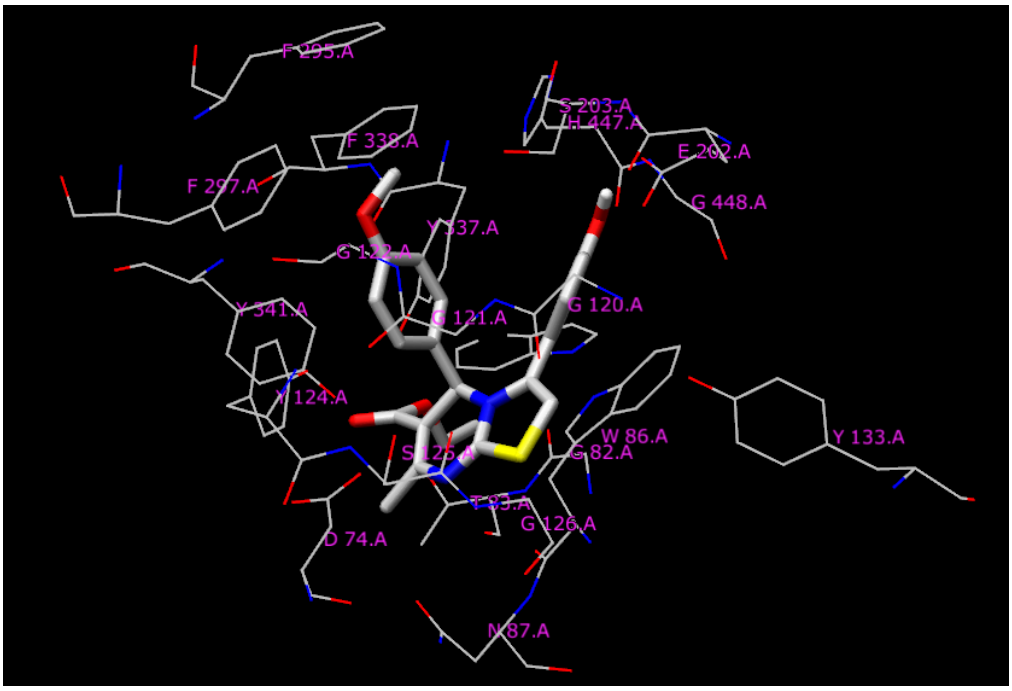


Figure 3. Docking of Compound 1 in the active site of human AChE model. Compound 1 is shown as stick models. The residues interacting with the ligand, are shown as line models. (Hydrogens and water molecules are omitted for the sake of clarity, all colored in atom type).

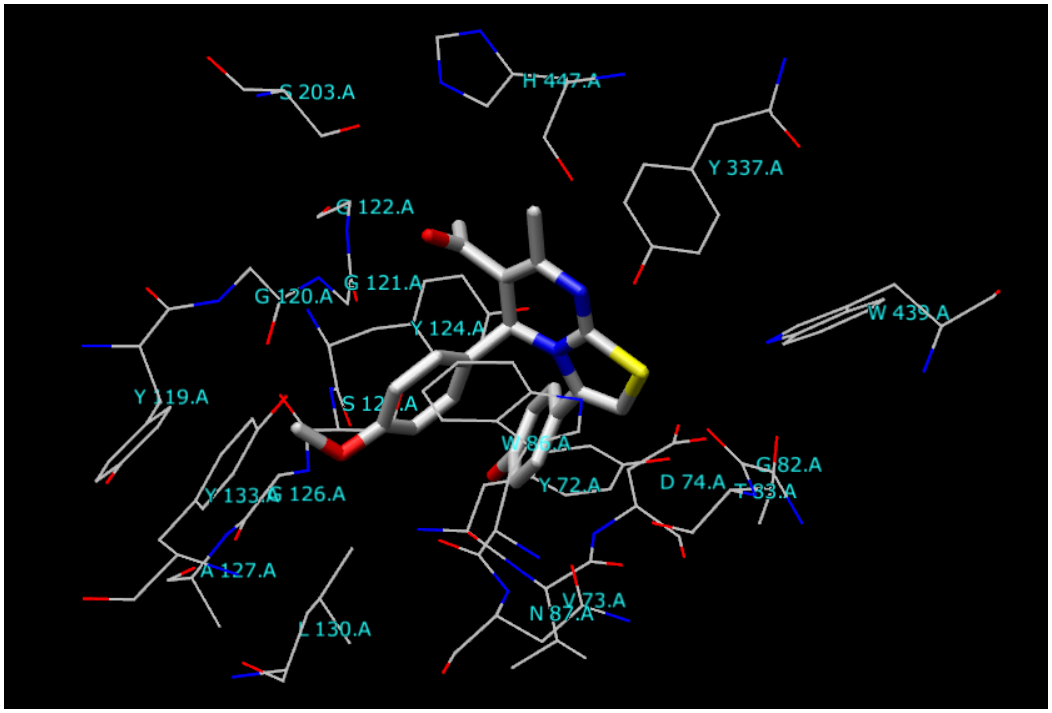
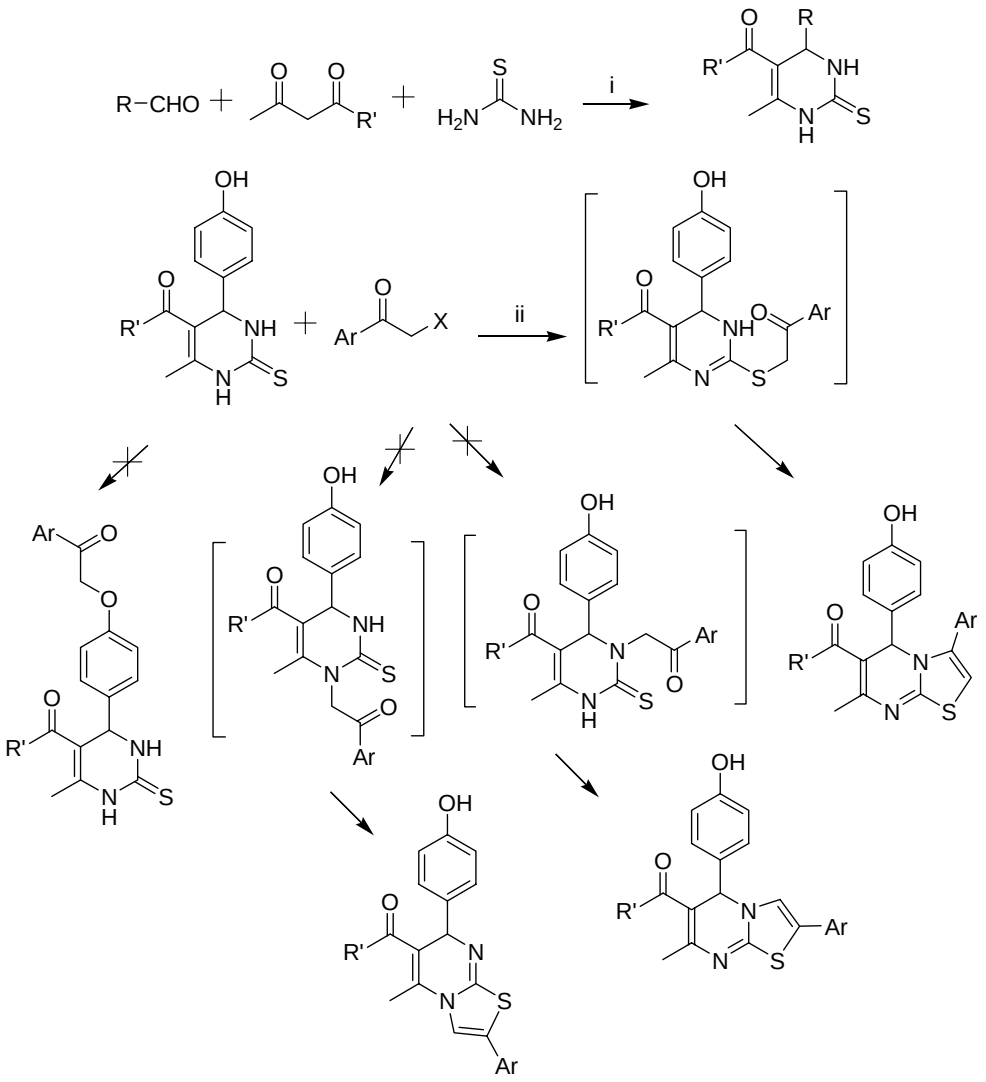


Figure 4. Docking of Compound 10 in the active site of human AChE model. Compound 10 is

shown as stick models. The residues interacting with the ligand, are shown as line models. (Hydrogens and water molecules are omitted for the sake of clarity, all colored in atom type).

Chemistry

Compound 1-10 were obtained in satisfactory yields(all above 80%), and the synthetic pathways to obtain the targets are described in Schemes 1.



Scheme 1. Reagents: (i) HCl-EtOH, reflux, 4h; (ii) AcONa/AcOH, reflux, 8-24h.

The synthesis of the dihydropyrimidines is a well known three component one pot reaction of a substituted aldehyde, 1,3-dicarbonyl compound and thiourea (Biginelli reaction).[9-10] The Hantzsch-type condensation of dihydropyrimidines with a substituted phenacyl chlorides led to the 3-substituted 5*H*-thiazolo[3,2-a]pyrimidine derivatives.[11,12] The Hantzsch-type condensation may yield several isomerisms. The yields could result either from initial formation of oxyalkyl,

aminoalkyl or isomeric sulfane, then condensed *via* elimination of water. However, the product made out to be in the last mechanism.

Analyzing the chemical shifts of the 5*H*-thiazolo[3,2-*a*]pyrimidine scaffold in ^{1}H-NMR spectra, we could find that they were all in the narrow range of 7.5-7.3 ppm, far from 5.5 ppm, which could be assigned as the proton of C2 in the 5*H*-thiazolo[3,2-*a*]pyrimidine scaffold. Therefore, the substitute should be at the C3 in the 5*H*-thiazolo[3,2-*a*]pyrimidine scaffold. Those assigned structures could be confirmed by NOE assay, HMBC, HSQC and COSY spectra. Taking compound 4 for example, showed in the Figure 5 below, in the 1D selective ROESY experiments with 500ms mixing time, when the proton at 6.22 ppm was irradiated, the protons at 7.32 ppm displayed the NOE effects; and vice versa. Thus, we could conclude that the substituting aryl groups of 5*H*-thiazolo[3,2-a]pyrimidine scaffold was spatially near to each other. The Hantzsch-type condensation in AcONa/AcOH buffer led to form 3-substituted 5*H*-thiazolo[3,2-a]pyrimidine derivatives confirmed in all respects (chemical shifts, NOE effects and so on).

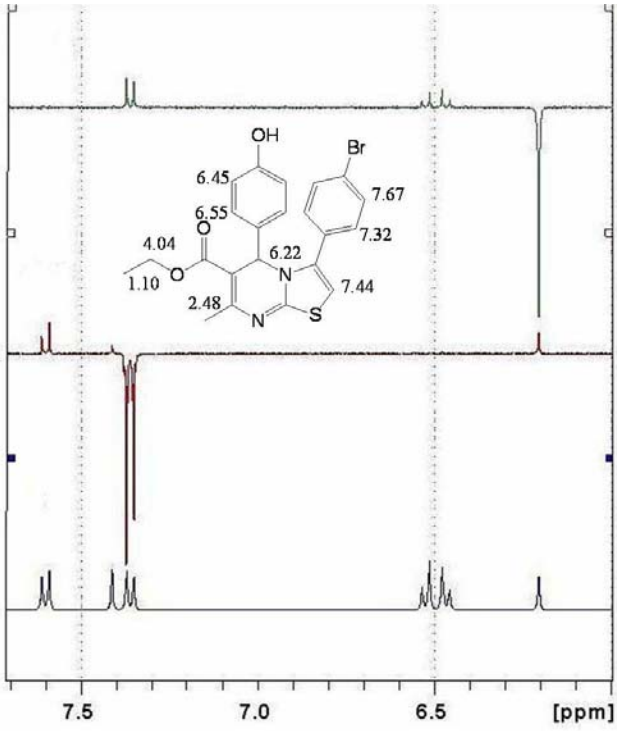
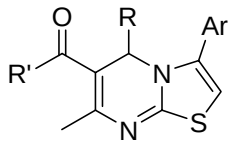


Figure 5. The 1D selective ROESY experiments with 500ms mixing time of compound 4.

Biology. Inhibition of AChE activities

Table 1 illustrates the biological activity of compounds against freshly prepared human AChE, in comparison to the neostigmine bromide.

Table 1. Inhibition of AChE Activities by the targets at 10 μM (n=2)



Compound	R	R'	Ar	Inhibition (%, mean)
1	4-methoxyphenyl	ethoxy	4-methoxyphenyl	55.32
2	4-hydroxylphenyl	ethoxy	4-methoxyphenyl	48.95
3	4-methoxylphenyl	ethoxy	4-hydroxyphenyl	60.32
4	4-hydroxylphenyl	ethoxy	4-bromophenyl	46.55
5	n-propyl	ethoxy	4-chlorophenyl	56.73
6	n-propyl	ethoxy	4-hydroxyphenyl	60.83
7	4-hydroxylphenyl	methyl	4-chlorophenyl	60.87
8	4-hydroxylphenyl	methyl	4-bromophenyl	51.97
9	4-hydroxylphenyl	methyl	4-methylphenyl	48.10
10	4-methoxyphenyl	methyl	4-hydroxyphenyl	60.25
Neostigmine bromide (0.1M)				100

Human AChE (SigmaC-1682) 0.5unit was used. Each performed in double. The incubation time was 20 min, with gentle shake.

Conclusions

In summary, we disclosed a rational design of novel series of potent 3-substituted 5*H*-thiazolo[3,2-*a*]pyrimidine derivatives as AChE inhibitors, binding to the active site of human AChE substrate domain. Molecular modeling studies led to the identification of 3-substituted 5*H*-thiazolo[3,2-*a*]pyrimidine derivatives, and the biological data were in full agreement with the proposed binding mode, confirming our hypothesis. 3-Substituted 5*H*-thiazolo[3,2-*a*]pyrimidine derivatives may represent a leading structure to generate enzyme inhibitors as novel therapeutical entities for severe neurodegenerative diseases. The pharmacological study of 3-substituted 5*H*-thiazolo[3,2-*a*]pyrimidine derivatives targeting Alzheimer's disease will be reported in due course.

Experimental Section

General Procedures. All reagents and solvents were purchased from common commercial suppliers and were used without further purification. All melting points were taken in open capillary tubes and are uncorrected. The nuclear magnetic resonance spectra were recorded in DMSO-d_6 solutions, using Bruker 300 MHz spectrometers, 400 MHz spectrometers and 600 MHz spectrometers, chemical shifts are reported in δ values (ppm) relative to internal TMS and *J*

values are reported in Hertz, other experiments such as HSQC (Heteronuclear Single-Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) were obtained in standard conditions. The mass spectra (MS) were obtained by electronic impact (EI) at 70 eV in an Agilent spectrometer (with direct insertion probe) or by electrospray (ESI) in a Waters spectrometer. The IR spectra were obtained using a Perkin-Elmer 298 spectrometer (Perkin-Elmer, Norwalk, CT, USA).

Molecular modeling

(1) Alignments study. The structure of human AChE and *Torpedo californica* AChE was compared using the skeleton retrieved from the Protein Database(PDB code 1B41 and 1W6R). The conformational analyses were carried out in UCSF Chimera(UCSF Chimera, Version 1, USA), with use of structure comparison tools MatchMaker and MatchAlign.

(2) Conformational analyses. The conformational analyses of human AChE were carried out by means of the structure editing tools DockPrep, using default settings.

(3) Docking simulations. To identify the possible binding mode of the compounds with the AChE, docking simulations were performed by means of the AutoDock 4.0 package software(UCSF AutoDock4.0, USA) and using the cocrystal between the AChE and Fasciculin (PDB code 1B41). Fasciculin was removed off, and the missing atoms were properly completed by means of the SPDB Viewer(GSK SPDB Viewer, Version3.7, Swiss). Hydrogen atoms were added to the protein amino acids, and the atomic partial charges were loaded in UCSF Chimera software package.

General procedures of synthesis 3-substituted 5*H*-thiazolo[3,2-*a*]pyrimidine derivatives

In a typical procedure a mixture of 5.00 mmol of substituted aldehyde, 7.50 mmol of ethyl acetoacetate or acetoacetone, 6.50 mmol thiourea, 5 mL of EtOH, and two drops of concentrated HCl was stirred at 50-55 °C for 10 h. One drop of concentrated HCl was added every 2 h. After the mixture was allowed to stand at 0 °C overnight, the precipitate was filtered to give 6-methyl-3, 4-dihydropyrimidine-2(1*H*)-thione in good yields. Then used to react with substituted phenacyl chloride respectively. In a typical procedure a mixture of 6-methyl-3,4-dihydropyrimidine-2(1*H*) -thione (10 mmol), substituted phenacyl chloride (10 mmol) and AcONa/AcOH (2g/20mL) was refluxed for 8-24 h. After completion of the reaction as indicated by TLC, the reaction liquid was cooled to room temperature, which crystallized as 3-substituted 5*H*-thiazolo[3,2-a]pyrimidine derivatives. The precipitate, in each case, was collected, and recrystallized from ethanol to give the target compounds **1-10**.

3,5-bis(4-methoxyphenyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylic acid ethyl ester(1). This compound was obtained as a white solid, 82% yield; mp: 205–206^{o}C. GC-MS: 51, 67, 77, 89, 103, 115, 132, 149, 175, 206, 231, 246, 275, 301, 319, 329, 347, 363, 365, 391, 403, 407, 421, 436; IR(KBr, cm^{-1}): 1176, 1252, 1383, 1629, 3441; ^{1}H-NMR(300MHz, DMSO-d_6)δ(ppm): 1.09(3H, t), 2.48(3H, s), 3.65(3H, s), 3.83(3H, s), 4.02(2H, q), 6.27(1H, s), 6.57(2H, d, *J* = 8.7 Hz), 6.70(2H,d, *J* = 8.7 Hz), 7.07(2H, d, *J* = 8.7 Hz), 7.23(2H,d, *J* = 8.7 Hz),

7.28(1H, s).

3-(4-methoxyphenyl)-5-(4-hydroxylphenyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylic acid ethyl ester(2). This compound was obtained as a white solid, 81% yield; mp: 236–237°C. ESI-MS (m/z): 423.6 ($(M+H)^+$, 100), 867.1 ($(2M+Na)^+$, 42); IR (KBr, cm^{-1}): 848, 1178, 1251, 1384, 1528, 1610, 3413; ^{1}H-NMR (400 MHz, DMSO-d_6)δ(ppm): 1.08 (3H, t), 2.50 (3H, s), 3.82 (3H, s), 4.03 (2H, q), 6.25 (1H, s), 6.45 (2H, d, *J* = 8.0 Hz), 6.56 (2H, d, *J* = 8.0 Hz), 7.05 (2H, d, *J* = 8.4 Hz), 7.27 (2H, d, *J* = 8.4 Hz), 7.43 (1H, s), 9.77 (1H, br, s); ^{13}C-NMR (100 MHz, DMSO-d_6): δ(ppm) 13.9, 17.6, 55.5, 58.3, 60.4, 103.1, 111.0, 114.2, 114.6, 114.7, 115.3, 119.6, 127.9, 129.9, 131.2, 139.7, 142.0, 158.0, 160.2, 160.8, 164.2.

3-(4-hydroxyphenyl)-5-(4-methoxylphenyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylic acid ethyl ester(3). This compound was obtained as a white solid, 83% yield; mp: 210–211°C. GC-MS: 51, 67, 77, 89, 105, 118, 128, 150, 158, 176, 192, 203, 217, 231, 241, 258, 273, 287, 305, 315, 334, 349, 351, 377, 391, 393, 407, 422; IR(KBr, cm^{-1}): 835, 1253, 1383, 1526, 1611, 3430; ^{1}H-NMR(300MHz, DMSO-d_6)δ(ppm): 1.08(3H, t), 2.50(3H, s), 3.72(3H, s), 4.03(2H, q), 6.30(1H, s), 6.60(2H, d, *J* = 8.7 Hz), 6.73(2H, d, *J* = 8.7 Hz), 6.88(2H, d, *J* = 8.7 Hz), 7.15(2H, d, *J* = 8.7 Hz), 7.30(1H, s), 10.02(1H, s).

3-(4-bromophenyl)-5-(4-hydroxylphenyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylic acid ethyl ester(4). This compound was obtained as a white solid, 84% yield; mp: 214–215°C. ESI-MS (m/z): 473.5 ($(M+3H)^+$, 100), 964.9 ($(2M+H+Na)^+$, 30); IR (KBr, cm^{-1}): 822, 1090, 1261, 1384, 1528, 1710, 2716, 3430; ^{1}H-NMR (400 MHz, DMSO-d_6)δ(ppm): 1.10 (3H, t), 2.48 (3H, s), 4.04 (2H, q), 6.22 (1H, s), 6.45 (2H, d, *J* = 8.4 Hz), 6.55 (2H, d, *J* = 8.4 Hz), 7.32 (2H, d, *J* = 8 Hz), 7.44 (1H, s), 7.67 (2H, d, *J* = 8 Hz), 9.74 (1H, br, s); ^{13}C-NMR (100 MHz, DMSO-d_6)δ(ppm): 13.9, 17.6, 58.1, 58.6, 60.4, 103.2, 112.1, 114.6, 115.2, 115.4, 124.6, 126.8, 128.0, 129.8, 131.7, 131.8, 138.8, 141.8, 158.0, 160.5, 164.0.

3-(4-chlorophenyl)-5-(n-propyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylic acid ethyl ester(5). This compound was obtained as a white solid, 80% yield; mp: 130–131°C. ESI-MS (m/z): 376.5($(M+H)^+$, 100); IR(KBr, cm^{-1}): 1275, 1384, 1527, 1685, 2552, 2962, 3430; ^{1}H-NMR(300MHz, DMSO-d_6)δ(ppm): 0.55(3H, t), 0.73-1.07(2H, m), 1.23(3H, t), 1.32-1.34(2H, m), 2.43(3H, s), 4.13-4.15(2H, m), 5.39(1H, t), 7.54(1H, s), 7.64(2H, d, *J* = 8.7 Hz), 7.68(2H, d, *J* = 8.7 Hz).

3-(4-hydroxyphenyl)-5-(n-propyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylic acid ethyl ester(6). This compound was obtained as a white solid, 84% yield; mp: 133–134°C. MS(API-ES): 359.3; IR(KBr, cm^{-1}): 836, 1085, 1271, 1384, 1522, 2934, 3433; ^{1}H-NMR(300MHz, DMSO-d_6)δ(ppm): 0.56(3H, t), 0.59-1.02(2H, m), 1.21(3H, t), 1.28-1.30(2H, m), 2.43(3H, s), 4.13-4.14(2H, m), 5.44(1H, t), 6.94(2H, d, *J* = 8.4 Hz), 7.40(3H, d, *J* = 8.4 Hz).

3-(4-chlorophenyl)-5-(4-hydroxyphenyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-ethanone(7) This compound was obtained as a yellowish solid, 87% yield; mp: 229–230°C. ESI-MS (m/z): 397.3($(M+H)^+$, 100); IR (KBr, cm^{-1}): 821, 1093, 1250, 1290, 1384, 1515, 1635, 2668, 3089, 3433; ^{1}H-NMR (400 MHz, DMSO-d_6)δ(ppm): 2.27 (3H, s), 2.54 (3H, s), 6.32 (1H, s), 6.45 (2H, d, *J* = 8.8 Hz), 6.54 (2H, d, *J* = 8.8 Hz), 7.43 (2H, d, *J* = 8.4 Hz), 7.46 (1H, s), 7.62 (2H, d, *J* = 8.4

Hz), 9.78 (1H, br, s); ^{13}C NMR (100 MHz, DMSO-d_6)δ(ppm): 18.6, 30.9, 58.5, 112.4, 112.6, 114.6, 115.5, 120.4, 126.4, 128.0, 129.4, 131.5, 135.5, 138.7, 141.1, 158.1, 160.2,194.6.

3-(4-bromophenyl)-5-(4-hydroxyphenyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-ethanone(8) This compound was obtained as a yellowish solid, 86% yield; mp: 215–216^{O}C. ESI-MS (m/z): 443.4 ((M+3H)$^+$, 100), 904.7 ((2M+H+Na)$^+$, 51); IR (KBr, cm^{-1}): 820, 1291, 1384, 1512, 1610, 2600, 3422; ^{1}H-NMR (400 MHz, DMSO-d_6)δ(ppm): 2.27 (3H, s), 2.53 (3H, s), 6.32 (1H, s), 6.45 (2H, d, *J* = 8.4 Hz), 6.53 (2H, d, *J* = 8.4 Hz), 7.35 (2H, d, *J* = 8 Hz), 7.45 (1H, s), 7.76 (2H, d, *J* = 8 Hz), 9.78 (1H, br, s); ^{13}C-NMR (100 MHz, DMSO-d_6)δ(ppm): 18.5, 21.0, 30.7, 56.0, 58.0, 58.1, 112.2, 112.5, 115.4, 124.2, 126.7, 127.9, 129.3, 131.6, 131.9, 138.7, 141.0, 158.0, 160.2, 171.9, 194.6.

3-(4-methylphenyl)-5-(4-hydroxyphenyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-ethanone(9). This compound was obtained as a yellowish solid, 87% yield; mp: 230–231^{o}C. ESI-MS (m/z): 377.6 ((M+H)$^+$, 100), 775.2 ((2M+Na)$^+$, 38); IR (KBr, cm^{-1}): 830, 1170, 1248, 1384, 1608, 3414; ^{1}H-NMR(400 MHz, DMSO-d_6)δ(ppm): 2.27 (3H, s), 2.42 (3H, s), 2.54 (3H, s), 6.38 (1H, s), 6.42 (2H, d, *J* = 8.4 Hz), 6.52 (2H, d, *J* = 8.4 Hz), 7.28 (2H, d, *J* = 8.0 Hz), 7.35-7.37 (3H, m), 9.70 (1H, br, s); ^{13}C NMR (100 MHz, DMSO-d_6)δ(ppm): 18.6, 21.0, 21.1, 30.8, 57.7, 111.1, 112.5, 115.4, 124.6, 127.9, 129.4, 129.5, 140.0, 140.4, 158.0, 194.6.

3-(4-hydroxyphenyl)-5-(4-methoxyphenyl)-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidine-6-ethanone(10). This compound was obtained as a yellowish solid, 85% yield; mp: 217–218^{o}C. GC-MS: 51, 67, 77, 89, 103, 115, 132, 149, 175, 206, 231, 246, 275, 301, 319, 329, 347, 363, 365, 391, 403, 407, 421, 436; IR(KBr, cm^{-1}): 1176, 1252, 1384, 1629, 3441; ^{1}H-NMR(300MHz, DMSO-d_6)δ(ppm): 1.09(3H, t), 2.48(3H, s), 3.65(3H, s), 3.83(3H, s), 4.02(2H, q), 6.27(1H, s), 6.57(2H, d, *J* = 8.7 Hz), 6.70(2H,d, *J* = 8.7 Hz), 7.07(2H, d, *J* = 8.7 Hz), 7.23(2H,d, *J* = 8.7 Hz), 7.28(1H, s).

NMR Analysis. To confirm the structure of 5*H*-thiazolo[3,2-*a*] pyrimidine derivaitives, the structures were determined by ^{1}H, ^{1}H-^{1}H COSY, HMBC, HMQC, ROESY and ^{13}C NMR spectroscopy. A positive nuclear Overhauser effect(NOE) confirmed that the structure was of 3-substituted 5*H*-thiazolo[3,2-a]pyrimidine derivatives.

Biology

Inhibition of AChE. The method of Ellman et al. was followed[13]. AChE stock solution was prepared by dissolving human AchE 0.5unit in 100 mM PBS buffer (pH 7.4). Tested target compounds (10 μM) were prepared in DMSO. The assay solution consisted of 100 mM PBS buffer (pH 7.4), with the addition of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB, Ellman's reagent), AChE(5 μl), drug(1 μl), and 12.5 mM acetylthiocholine iodide(ATCh) water solution. The final assay volume was 900 μl. Incubate the reaction at 37ºC for 15 min with continuous gentle shake. Add 50 ml ATCh and 50 ml DTNB. Incubate at 37ºC for about 20 min with continuous gentle shake, Wait until the yellow color developed. Measure at 412 nm. Calculate the specific inhibition rates.

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