Design, synthesis, biological evaluation and docking study of 4-isochromanone hybrids bearing *N*-benzyl pyridinium moiety as dual binding site acetylcholinesterase inhibitors (part Ⅱ)

Jia Wang[a], Chaolei Wang[a], Zheng Wu[a], Xinnan Li[a], Shengtao Xu[a,*], Jie Liu[b,*], Qinying Lan[c], Zheying Zhu[d] and Jinyi Xu[a,*]

[a]*State Key Laboratory of Natural Medicines and Department of Medicinal Chemistry, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing 210009, PR China*

[b]*Department of Organic Chemistry, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing, 210009, PR China*

[c]*Life Science and Technique Base Department of Life Science, Nanjing Agricultural University, Wei Gang, Nanjing 210018, China*

[d]*Division of Molecular Therapeutics & Formulation, School of Pharmacy, the University of Nottingham, University Park Campus, Nottingham NG7 2RD, U.K.*

Abstract:

A series of novel 4-isochromanone compounds bearing *N*-benzyl pyridinium moiety were designed and synthesized as acetylcholinesterase (AChE) inhibitors. The biological evaluation showed that most of the target compounds exhibited potent inhibitory activities against AChE. Among them, compound **1q** possessed the strongest anti-AChE activity with an IC_{50} value of 0.15 nM and high AChE/BuChE selectivity (SI >5000). Moreover, compound **1q** had low toxicity in normal nerve cells and was relatively stable in rat plasma. Together, the current finding may provide a new approach for the discovery of novel anti-Alzheimer's disease agents.

Key words:

Alzheimer's disease, acetylcholinesterase inhibitors, 4-isochromanone skeleton,

[*]Corresponding authors. Tel.: +86 025 83271299; Fax. +86 025 83302827 (J.X.);

E-mail: jinyixu@china.com (J. Xu); cpuxst@163.com (S. Xu); cpu-jill@163.com (J. Liu).

benzyl pyridine

Alzheimer's disease (AD) is a progressive and neurodegenerative disease characterized by dementia, memory loss and other cognitive impairments, which affects millions of elder people around the world[1,2]. Although many factors contribute to this disease, its etiology and pathogenesis remain unclear until now. Studies have shown that several factors, including obvious reduction in cholinergic neurons[3], accumulation and aggregation of β-amyloid peptide[4] (Aβ) and oxidative stress[5] play significant roles in the development of AD[6]. These physiological changes in turn lead to the current strategies for the treatment and prevention of AD. Among them, increasing levels of acetylcholine (Ach) through inhibition of cholinesterase (ChEs) has been used most frequently to treat AD[7]. Up to now, several acetylcholinesterase inhibitors such as donepezil, rivastigmine and galantamine have been approved and widely used for the treatment of AD[8]. There is evidence that two binding sites exist in AChE according to the crystal structure of the AChE of the eel, including the catalytic active site (CAS) and the peripheral anionic site (PAS)[9]. Therefore, it is meaningful to design dual-site inhibitors, which can interact with both the CAS and PAS to prevent AD.

It has been reported that *N*-benzyl pyridinium moiety was introduced into a series of aromatic scaffolds and emerged as an essential structure for some inhibitors of AchE[10] (Figure 1). In our previous studies, using donepezil as a template[11], a series of dual-site AChE inhibitors were synthesized by introducing *N*-benzyl pyridinium moiety into the natural product XJP (Figure 1). Among these compounds, the most potent compound **1** showed good AChE inhibitory activity (IC_{50} value : 8.9 nM) and excellent AChE/BuChE selectivity (SI >230)[11]. The structure-activity relationships (SARs) studies demonstrated that benzyl pyridine, 4-isochromanone skeleton and nitrogen ion were essential for the activity against AChE. Moreover, the activity was maintained or increased by introducing the fluorine or chlorine to the benzyl group. Other groups introduced to the para-position of the benzyl group diminished the inhibitory activity remarkably. However, the effect of methoxy group at

4-isochromanone skeleton on the AChE inhibitory activity was undefined. Besides, the preferred position of fluorine or chlorine on benzyl group and the importance of *α,β*-unsaturated ketone to the AChE inhibitory activity should be explored logically.

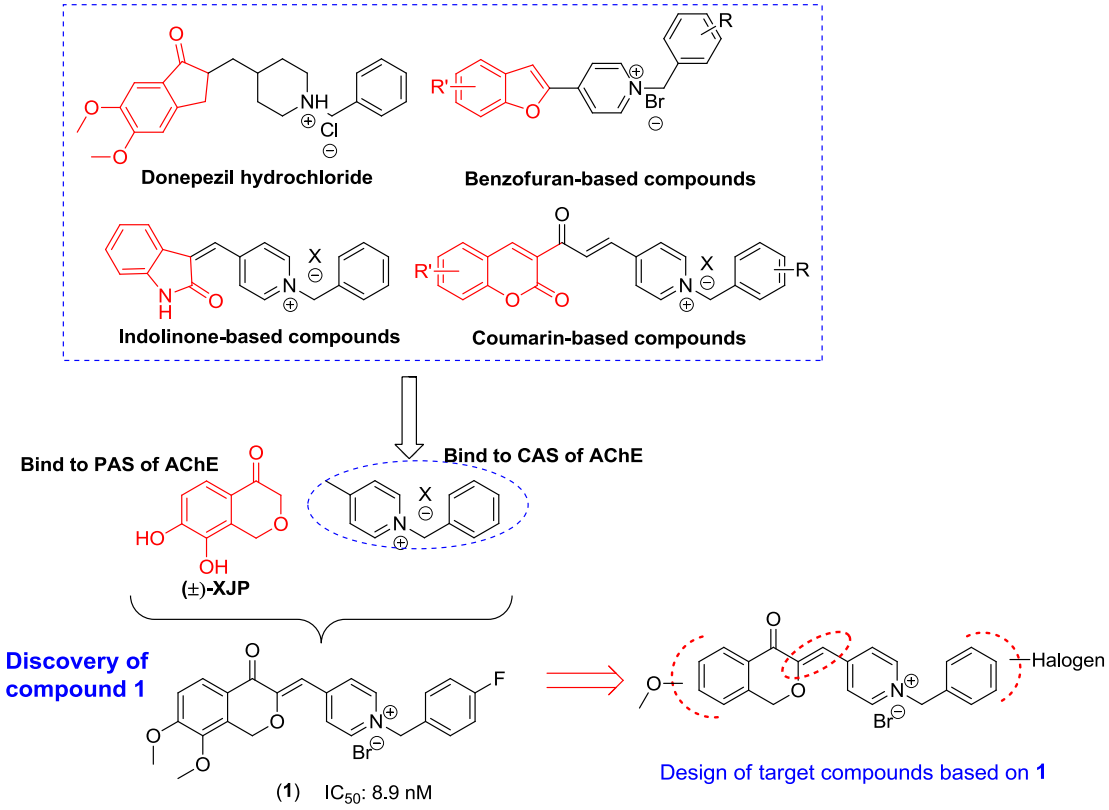
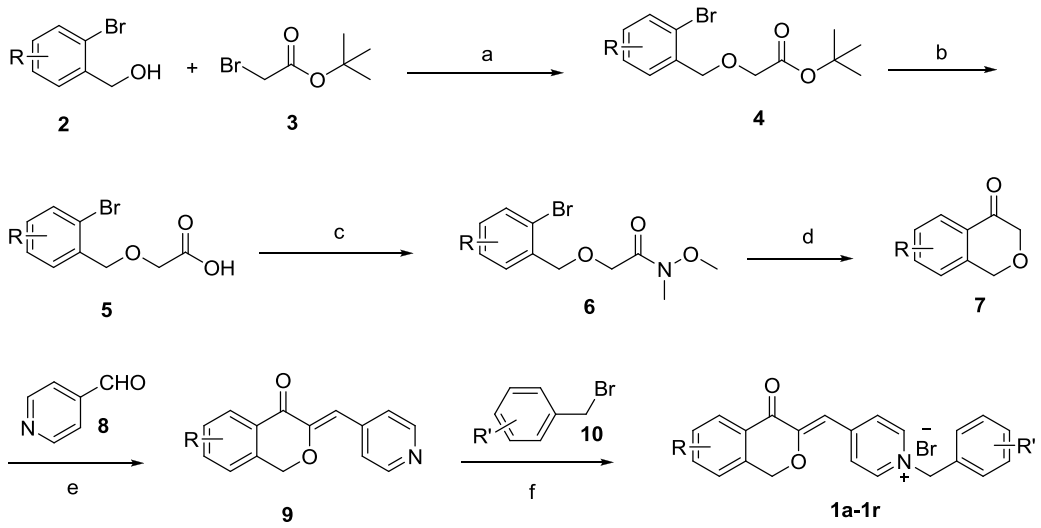


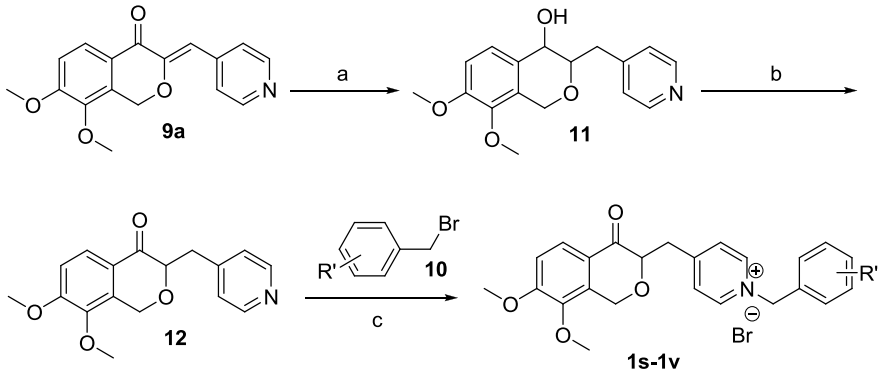
Figure 1. Discovery of potent AchE inhibitor **1** and the design of its derivatives

Inspired by our previous promising study and preliminary SARs, we embarked on the second-round design to accomplish the SARs and find more potent AChE inhibitors. Herein, a series of novel AChE inhibitors bearing 4-isochromanone moiety were further designed and synthesized using compound **1** as the lead compound. The ChEs inhibitory activities and selectivity of target compounds were also evaluated. Moreover, the neurotoxicity and plasma stability studies of potent compounds were disclosed in this research.



Scheme 1 *Reagents and conditions*: (a) K_2CO_3, H_2O/toluene, r.t., 30 min; (b) NaOMe, MeOH, r.t. then H_2O, 95% yield over two steps; (c) Oxalyl chloride, then N, O-Dimethyl hydroxylamine hydrochloride, 80% yield; (d) *t*-BuLi, THF, -78 °C, 1-5 min, 70% yield; (e) K_2CO_3, MeOH, r.t., 4 h, 60% yield; (f) CH_3CN, reflux, 1 h, 60%-80% yields.

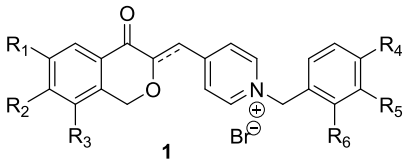
The synthetic route of target compounds was shown in Schemes 1-2. Based on our previously developed method for the construction of 4-isochromanone with Weinreb amide[12], we successfully completed the synthesis of 4-isochromanone skeleton to get the compound **7**, which reacted with pyridine formaldehyde to yield the intermediate **9** in the presence of K_2CO_3. Target compounds **1a-1r** were obtained after the nucleophilic substitution of compound **9**.



Scheme 2 *Reagents and conditions*: (a) H_2/Pd-C, 0.7 Mpa, r.t., overnight, 70% yield; (b) Dess-Martin reagent, r.t. 85% yield; (c) CH_3CN, reflux, 1 h, 60%-80% yields.

The intermediate **9** was hydrogenated with Pd-C as a catalyst at the pressure of 0.7 MPa to afford compound **11**, which was oxidized with Dess-Martin oxidizing reagent to yield compound **12**. Compounds **1s-1v** were obtained by the reaction of compound **12** with various substituted benzyl bromide.

Table 1. Inhibition of ChEs by target compounds, and their selectivity index



1

compound	R_1	R_2	R_3		R_4	R_5	R_6	IC_{50} (nM)		Selectivity Index
								AChE	BuChE	
1								8.93±0.4	2.07±0.06	232
1a	H	OCH_3	OCH_3	=	F	F	H	91.80±3.92	902.13±37.92	9.8
1b	H	OCH_3	OCH_3	=	F	H	F	25.63±1.09	438.92±20.14	17.1
1c	H	OCH_3	OCH_3	=	F	Cl	H	97.27±4.82	5580±21	57.4
1d	H	OCH_3	OCH_3	=	F	H	Cl	23.44±1.12	728.61±35.43	31.1
1e	H	OCH_3	OCH_3	=	H	CF_3	H	48.69±2.31	4280±12	87.9
1f	H	OCH_3	OCH_3	=	H	H	CF_3	70.03±3.19	592.36±28.18	8.5
1g	H	OCH_3	H	=	F	F	H	1023±45	59120±2580	57.8
1h	H	OCH_3	H	=	F	H	F	5.42±0.25	950±4.5	175.3
1i	H	OCH_3	H	=	F	Cl	H	96.82±4.32	3860±130	39.9
1j	H	OCH_3	H	=	F	H	Cl	5.09±0.23	1210±48	237.7
1k	H	H	H	=	H	H	H	31.92±1.52	2020±90	63.3
1l	H	H	H	=	F	H	H	30.57±1.32	1130±43	36.9
1m	H	H	H	=	H	F	H	28.22±1.28	2540±90	90.0
1n	H	H	H	=	H	H	F	33.32±1.48	2410±80	72.3
1o	OCH_3	OCH_3	H	=	H	H	H	0.29±0.013	968.68±48.21	3340.3
1p	OCH_3	OCH_3	H	=	H	F	H	0.34±0.015	329.04±15.12	967.8
1q	OCH_3	OCH_3	H	=	F	H	H	0.15±0.007	885.53±43.17	5903.5
1r	OCH_3	OCH_3	H	=	H	H	F	0.44±0.021	798.35±37.29	1814.4
1s	H	OCH_3	OCH_3	-	H	H	H	27.13±1.29	4306±25	158.7
1t	H	OCH_3	OCH_3	-	F	H	H	161.28±8.00	4920±18	30.6
1u	H	OCH_3	OCH_3	-	H	F	H	88.95±4.23	3210±15	36.1
1v	H	OCH_3	OCH_3	-	H	H	F	44.38±2.33	4560±18	102.7
donepezil								37.54±1.92	3410±150	90.8

MATERIALS AND METHODS

Chemistry

NMR experiments were run on a Bruker-ACF-300 and calibrated using TMS or residual deuterated solvent as an internal reference ($CDCl_3$:7.26 ppm for ^1H NMR and 77.16 ppm for ^{13}C NMR or DMSO-d_6: 2.50 ppm for ^1H NMR and 39.5 ppm for ^{13}C NMR). HRMS was performed on an Agilent 6530 Q-TOF mass spectrometer, and ESI-MS was carried out on an Agilent 6120 mass spectrometer. All commercial reagents and anhydrous solvents were obtained from commercial sources and were used without further purification unless otherwise specified.

General procedure for preparation of compounds

Compound **9** or **12** was dissolved in acetonitrile, then substituted bromobenzene was added. The mixture was refluxed at 80 ^oC for 1 h. The yellow solid precipitated and the target compound were obtained by filtration.

(*Z*)-1-(2,4-difluorobenzyl)-4-((7,8-dimethoxy-4-oxoisochroman-3-ylidene)methyl)pyridinium bromide (**1b**)

Yellow solid; ^1H NMR (300 MHz, DMSO-d_6) δ: 9.047 (d, J = 5.64 Hz, 2H), 8.45 (d, J = 5.64 Hz, 2H), 7.80 (m, 2H), 7.40 (m, 3H), 6.99 (s, 1H), 5.91 (s, 2H), 5.61 (s, 2H), 3.98 (s, 3H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.80, 157.54, 156.04, 150.28, 144.17, 142.32, 133.03, 132.46, 126.69, 124.44, 121.07, 117.84, 113.25, 112.52, 112.23, 105.05, 104.85, 104.71, 104.37, 63.75, 60.31, 56.31; MS-ESI: (ESI, pos.ion) m/z: 503.05. HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{19}F_2NO_4$: 424.1355, found: 424.1362.

(*Z*)-1-(3-chloro-4-fluorobenzyl)-4-((7,8-dimethoxy-4-oxoisochroman-3-ylidene)methyl)pyridinium (**1c**)

Yellow solid; ^1H NMR (300 MHz, DMSO-d_6) δ: 9.08 (d, J = 6.78 Hz, 2H), 8.43 (d, J = 6.78 Hz, 2H), 7.94 (dd, J_1= 1.98 Hz, J_2= 7.08 Hz, 1H), 7.85 (d, J = 8.76 Hz, 1H), 7.76 (m, 1H), 7.54 (t, J = 8.74 Hz, 1H), 7.32 (d, J = 8.79 Hz, 2H), 6.99 (s, 1H), 5.77 (s, 2H), 5.61 (s, 2H), 3.97 (s, 3H) , 3.82 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ:176.87,

157.57, 156.00, 150.16, 144.04, 132.49, 132.11, 130.07, 129.97, 126.81, 125.29, 124.46, 121.11, 117.79, 117.51, 113.28, 104.91, 63.74, 60.87, 60.33, 56.32; MS-ESI: (ESI, pos.ion): 440.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{21}ClFNO_4$: 440.1059, found: 442.1217.

(*Z*)-1-(2-chloro-4-fluorobenzyl)-4-((7,8-dimethoxy-4-oxoisochroman-3-ylidene)methyl)pyridinium bromide (**1d**)

Yellow solid; 1H NMR (300 MHz, DMSO-d_6) δ:8.95 (d, *J* = 6.90 Hz, 2H), 8.44 (d, *J* = 6.90 Hz, 2H), 7.86 (d, *J* = 8.76 Hz, 1H), 7.64 (m, 2H), 7.40 (m, 2H), 7.01 (s, 1H), 5.89 (s, 2H), 5.61 (s, 2H), 3.97 (s, 3H) , 3.82 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.83, 157.58, 156.16, 150.40, 144.33, 133.49, 133.37, 132.45, 126.60, 124.47, 121.09, 117.78, 117.44, 115.43, 115.15, 113.28, 104.83, 63.77, 60.32, 59.67, 56.32; MS-ESI: (ESI, pos.ion): 519.02; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{19}ClFNO_4$: 440.1059, found: 440.1055.

(*Z*)-4-((7,8-dimethoxy-4-oxoisochroman-3-ylidene)methyl)-1-(3-(trifluoromethyl)benzyl)pyridinium bromide (**1e**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.21 (d, *J* = 6.51 Hz, 2H), 8.46 (d, *J* = 6.51 Hz, 2H), 8.09 (s, 1H), 7.93-7.81 (m, 3H), 7.72 (t, *J* = 7.71 Hz, 1H), 7.33 (d, *J* = 8.79 Hz, 1H), 6.99 (s, 1H), 5.97 (s, 2H), 5.63, (s, 2H), 3.98 (s, 3H) , 3.83 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.82,157.5, 155.98, 150.15, 144.14, 142.29, 135.76, 133.00, 132.49, 130.33, 129.85, 129.43, 126.84, 126.02, 125.78, 125.73, 124.45, 121.06, 113.23, 104.88, 63.74, 61.30, 60.30, 56.31; MS-ESI: (ESI, pos.ion): 535.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{25}H_{20}F_3NO_4$: 456.1417, found:456.1415.

(*Z*)-4-((7,8-dimethoxy-4-oxoisochroman-3-ylidene)methyl)-1-(2-(trifluoromethyl)benzyl)pyridinium bromide (**1f**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.99 (d, *J* = 6.36 Hz, 2H), 8.50 (d, *J* = 6.36 Hz, 2H), 7.94-7.68 (m, 4H), 7.32 (m, 2H), 7.03 (s, 1H), 6.09 (s, 2H), 5.64 (s, 2H), 3.98 (s, 3H), 3.83 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.80, 157.57, 156.24, 150.56, 144.69, 142.29, 133.66, 132.51, 131.76, 130.32, 129.77, 126.88, 126.81, 126.69, 125.78, 124.49, 121.06, 113.26, 104.76, 63.79, 60.32, 59.05, 56.33;

MS-ESI: (ESI, pos.ion): 535.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{25}H_{20}F_3NO_4$: 456.1417, found: 456.1420.

(*Z*)-1-(2,4-difluorobenzyl)-4-((7-methoxy-4-oxoisochroman-3-ylidene)methyl)pyridinium bromide (**1g**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.02 (d, *J* = 6.60 Hz, 2H), 8.43 (d, *J* = 6.60 Hz, 2H), 8.00 (d, *J* = 9.30 Hz, 1H), 7.75 (q, *J* = 8.58 Hz, 1H), 7.45 (m, 1H), 7.26 (m, 1H), 7.13 (m, 2H), 6.99 (s, 1H), 5.59 (s, 2H), 5.53 (s, 2H), 3.91 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.75, 164.47, 162.61, 156.61, 150.34, 144.15, 141.90, 133.05, 132.97, 129.28, 126.70, 121.09, 117.99, 117.81, 115.88, 112.49, 112.25, 108.78, 105.07, 104.74, 104.39, 67.39, 56.37, 56.01; MS-ESI: (ESI, pos.ion): 473.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{23}H_{17}F_2NO_3$:394.1249, found: 394.1247

(Z)-1-(2-chloro-4-fluorobenzyl)-4-((7-methoxy-4-oxoisochroman-3-ylidene)methyl)pyridinium (**1j**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.98 (d, *J* = 6.15 Hz, 2H), 8.44 (d, *J* = 6.15 Hz, 2H), 7.99 (m, 1H), 7.68 (m, 2H), 7.41 (m, 1H), 7.14 (s, 2H), 7.01 (s, 1H), 5.95 (s, 2H), 5.60 (s, 2H), 3.91 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.71, 164.46, 164.12, 160.79, 156.66, 150.38, 144.30, 141.90, 134.49, 133.65, 133.52, 129.26, 128.05, 126.56, 121.08, 117.75, 117.42, 115.89, 115.42, 115.14, 108.76, 104.71, 67.39, 59.56, 56.03; MS-ESI: (ESI, pos.ion): 489.0; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{23}H_{17}ClFNO_3$: 410.0954, found: 410.0952.

(*Z*)-1-benzyl-4-((4-oxoisochroman-3-ylidene)methyl)pyridinium bromide (**1k**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.14 (d, *J* = 6.63 Hz, 2H), 8.44 (d, *J* = 6.63 Hz, 2H), 8.01 (d, *J* = 7.59 Hz, 1H), 7.78 (t, *J* = 7.20 Hz, 1H), 7.56 (m, 4H), 7.46 (m, 3H), 7.01 (s, 1H), 5.84 (s, 2H), 5.64 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.45, 156.24, 149.91, 144.11, 139.15, 135.03, 134.55, 129.19, 128.77, 128.66, 128.25, 127.88, 126.83, 126.41, 124.96, 105.13, 67.39, 62.29; MS-ESI: (ESI, pos.ion) : 407.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{22}H_{17}NO_2$: 328.1332, found: 328.1332.

(*Z*)-1-(2-fluorobenzyl)-4-((4-oxoisochroman-3-ylidene)methyl)pyridinium bromide（**1l**）

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.06 (d, *J* = 6.66 Hz, 2H), 8.46 (d, *J* = 6.66 Hz, 2H), 8.01 (d, *J* = 7.65 Hz, 1H), 7.81 (t, *J* = 7.20 Hz, 1H), 7.56 (m, 4H), 7.34 (m, 2H), 7.02 (s, 1H), 5.95 (s, 2H), 5.65 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.38, 162.05, 156.35, 150.15, 144.28, 139.11, 135.01, 132.01, 131.89, 131.33, 128.75, 127.83, 126.76, 126.38, 125.26, 124.95, 121,51, 121.33, 116.08, 115.81, 105.05, 67.40, 56.96; MS-ESI: (ESI, pos.ion):425.0; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{22}H_{16}FNO_2$: 346.1238, found: 346.1236.

(Z)-1-(3-fluorobenzyl)-4-((4-oxoisochroman-3-ylidene)methyl)pyridinium bromide (**1m**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.20 (d, *J* = 6.72 Hz, 2H), 8.46 (d, *J* = 6.72 Hz, 2H), 8.01 (d, *J* = 7.68 Hz, 1H), 7.80 (t, *J* = 7.47 Hz, 1H), 7.56 (m, 5H), 7.30 (m, 1H), 7.02 (s, 1H), 5.90 (s, 2H), 5.65 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.41, 163.76, 160.51, 156.28, 150.00, 144.18, 139.13, 136.99, 136.88, 135.01, 131.35, 131.24, 128.75, 127.85, 126.84, 126.39, 124.95, 116.29, 116.01, 115.97, 115.68, 105.12, 67.41, 61.36; MS-ESI: (ESI, pos.ion): 425.1. HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{22}H_{16}FNO_2$: 346.1238, found: 346.1237.

(*Z*)-1-(4-fluorobenzyl)-4-((4-oxoisochroman-3-ylidene)methyl)pyridinium bromide (**1n**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.14 (d, *J* = 6.78 Hz, 2H), 8.44 (d, *J* = 6.78 Hz, 2H), 8.01 (d, *J* = 7.59 Hz, 1H), 7.83 (t, *J* = 6.63 Hz, 1H), 7.71-7.54 (m, 4H), 7.32 (t, *J* = 8.82 Hz, 2H), 7.01 (s, 1H), 5.84 (s, 2H), 5.64 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.42, 164.11, 160.84, 156.25, 149.93, 144.02, 139.14, 135.03, 131.38, 131.26, 130.77, 128.77, 127.87, 126.83, 126.41, 124.96, 116.22, 115.93, 105.12, 67.40, 61.40; MS-ESI: (ESI, pos.ion):425.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{22}H_{16}FNO_2$: 346.1238, found: 346.1238.

(*Z*)-1-benzyl-4-((6,7-dimethoxy-4-oxoisochroman-3-ylidene)methyl)pyridinium bromide (**1o**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.12 (d, *J* = 5.91 Hz, 2H), 8.42 (d, *J* = 5.91 Hz, 2H), 7.58-7.40 (m, 6H), 7.15 (s, 1H), 6.96 (s, 1H), 5.85 (s, 2H), 5.55 (s, 2H), 3.91 (s, 3H), 3.85 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.34, 156.21, 154.93, 150.08, 149.11, 143.96, 134.66, 134.60, 129.18, 128.65, 126.65, 120.63, 107.25, 107.02, 104.55, 67.11, 62.16, 56.26, 55.66; MS-ESI: (ESI, pos.ion): 467.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{21}NO_4$: 388.1543, found: 388.1544.

(*Z*)-4-((6,7-dimethoxy-4-oxoisochroman-3-ylidene)methyl)-1-(3-fluorobenzyl)pyridinium bromide (**1p**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.13 (d, *J* = 6.27 Hz, 2H), 8.44 (d, *J* = 6.27 Hz, 2H), 7.54-7.30 (m, 5H), 7.15 (s, 1H), 6.98 (s, 1H), 5.86 (s, 2H), 5.56 (s, 2H), 3.91 (s, 3H), 3.86 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.37, 163.77, 160.53, 156.31, 154.96, 150.24, 149.15, 144.06, 136.92, 134.68, 131.36, 131.25, 126.69, 124.89, 120.67, 116.28, 115.99, 115.91, 115.62, 107.31, 107.04, 104.55, 67.13, 61.38, 56.27, 55.68; MS-ESI: (ESI, pos.ion):485.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{20}FNO_4$:406.1449, found: 406.1450.

(*Z*)-4-((6,7-dimethoxy-4-oxoisochroman-3-ylidene)methyl)-1-(4-fluorobenzyl)pyridinium bromide (**1q**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.11 (d, *J* = 6.81 Hz, 2H), 8.42 (d, *J* = 6.27 Hz, 2H), 7.67 (m, 2H), 7.41 (s, 1H), 7.32 (t, *J* = 8.88 Hz, 2H), 7.15 (s, 1H), 6.97 (s, 1H), 5.83 (s, 2H), 5.56 (s, 2H),3.91 (s, 3H), 3.86 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.37, 164.09, 160.83, 156.25, 154.95, 150.13, 149.13, 143.88, 134.66, 131.35, 131.23, 130.78, 126.66, 120.66, 116.21, 115.93, 107.29, 107.04, 104.55, 67.12, 61.29, 56.26, 55.68; MS-ESI: (ESI, pos.ion): 485.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{20}FNO_4$: 406.1449, found: 406.1452.

(*Z*)-4-((6,7-dimethoxy-4-oxoisochroman-3-ylidene)methyl)-1-(2-fluorobenzyl)pyridinium bromide (**1r**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.06 (s, 2H), 8.45 (s, 2H), 7.67-6.97 (m, 7H), 5.97 (s, 2H), 5.57 (s, 2H), 3.91 (s, 3H), 3.86 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.27, 162.07, 158.79, 156.33, 154.93, 150.33, 149.09, 144.16, 134.66, 131.99, 131.89, 131.34, 126.58, 125.23, 121.59, 121.41, 120.59, 116.08, 115.81, 107.24, 107.07, 104.49, 67.14, 56.79, 56.29, 55.66; MS-ESI: (ESI, pos.ion):485.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{20}FNO_4$: 406.1449, found: 406.1456.

1-benzyl-4-((7,8-dimethoxy-4-oxoisochroman-3-yl)methyl)pyridinium bromide（**1s**）

Yellow solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.54 (d, *J* = 6.36 Hz, 2H), 7.94 (d, *J* = 6.36 Hz, 2H), 7.75 (m, 3H), 7.33 (m, 3H), 6.94 (d, *J* = 8.76 Hz, 1H), 6.25 (s, 2H), 5.07 (d, *J* = 15.81 Hz, 1H), 4.74 (d, *J* = 15.81 Hz, 1H), 4.44 (m, 1H), 3.94 (s, 3H), 3.82 (s, 3H), 3.60(m, 1H), 2.29 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 192.18, 159.23, 157.00, 143.79, 142.62, 135.42, 134.43, 129.34, 129.21, 128.87, 123.62, 122.14, 112.10, 79.09, 62.43, 60.14, 56.12, 35.07; MS-ESI: (ESI, pos.ion): 469.1. HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{23}NO_4$: 390.0996, found: 390.0994.

4-((7,8-dimethoxy-4-oxoisochroman-3-yl)methyl)-1-(4-fluorobenzyl)pyridinium bromide (**1t**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.20 (d, *J* = 6.51 Hz, 2H), 8.15 (d, *J* = 6.51 Hz, 2H), 7.72 (m, 3H), 7.32 (t, *J* = 8.79 Hz, 1H), 7.20 (d, *J* = 8.76 Hz, 1H), 5.86 (s, 2H), 5.04-4.79 (m, 3H), 3.93 (s, 3H), 3.77 (s, 3H), 3.60(m, 1H), 2.29 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 192.18, 164.17, 160.91, 159.25, 157.00, 143.71, 142.62, 135.41, 131.58, 131.46, 130.64, 128.88, 123.63, 122.14, 116.27, 115.98, 112.09, 79.09, 62.43, 61.57, 60.13, 56.11, 35.06; MS-ESI: (ESI, pos.ion): 487.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{22}FNO_4$: 408.1606, found: 408.1672.

4-((7,8-dimethoxy-4-oxoisochroman-3-yl)methyl)-1-(3-fluorobenzyl)pyridinium bromide (**1u**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.25 (s, 2H), 8.17 (s, 2H), 7.76-7.20 (m, 6H), 5.91 (s, 2H), 4.91 (m, 3H), 3.92 (s, 3H), 3.74 (s, 3H), 3.63 (m, 1H), 2.27 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 192.18, 163.73, 160.48, 159.39, 157.00, 143.88, 142.61, 136.80, 136.70, 135.42, 131.40, 131.29, 128.90, 125.12,

123.63, 122.13, 116.42, 116.15, 115.85, 112.09, 79.07, 62.43, 61.58, 60.13, 56.11, 35.09; MS-ESI: (ESI, pos.ion): 487.1; HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{22}FNO_4$: 408.1606, found: 408.1603.

4-((7,8-dimethoxy-4-oxoisochroman-3-yl)methyl)-1-(2-fluorobenzyl)pyridinium bromide (**1v**)

Yellow solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.18 (d, J = 6.30 Hz, 2H), 8.20 (d, J = 6.30 Hz, 2H), 7.75 (d, J = 6.27 Hz, 2H), 7.58 (m, 1H), 7.53 (m, 2H), 7.22 (d, J = 8.76 Hz, 1H), 6.04 (s, 2H), 505-4.85 (m, 3H), 3.93 (s, 3H), 3.75 (s, 3H), 3.63 (m, 1H), 2.53 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 192.18, 162.13, 159.57, 144.25, 142.59, 135.42, 132.15, 131.60, 128.88, 126.69, 125.28, 124.47, 123.62, 122.13, 116.12, 115.85, 112.09, 79.06, 62.45, 60.33, 60.14, 57.03, 56.13, 35.12; MS-ESI: (ESI, pos.ion): 487.1. HRMS-ESI: $[M\text{-}Br^-]^+$ calcd. For $C_{24}H_{22}FNO_4$: 408.1606, found: 408.1604.

(*Z*)-6,7-dimethoxy-3-(pyridin-4-ylmethylene)isochroman-4-one

White solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.61 (m, 2H), 7.66 (m, 2H), 7.57 (s, 1H), 6.89 (s, 1H), 6.67 (s, 1H), 5.30 (s, 2H), 3.99 (s, 3H) , 3.98 (s, 3H); MS-ESI: (ESI, pos.ion): 298.1.

(*Z*)-3-(pyridin-4-ylmethylene)isochroman-4-one

Yellow solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.63 (m, 2H), 8.12 (d, J = 7.65 Hz, 2H), 7.65 (m, 3H), 7.51 (t, J = 7.32 Hz, 1H), 7.30 (m, 1H), 6.25 (s, 2H), 6.91 (s, 1H), 5.35 (s, 2H); MS-ESI: (ESI, pos.ion): 238.1.

AChE and BuChE inhibitory activity test assay

The compound was dissolved in DMSO, diluted with buffer A to the desired concentration, and the DMSO content in the solution was controlled to be less than 1%. To the 96-well plates were added: 160 μL of 1.5 mM DTNB, 50 μL of AChE (0.22 U/mL, prepared with Buffer B) and 10 μL of different concentrations of

inhibitor, incubated at 37 °C for 10 min, and then 30 μL of iodoacetylcholine (15 mM) was added rapidly. The absorbance changes of 0, 60, 120 and 180 s were measured at 405 nM wavelengths. BuchE was tested in an assay similar to AChE, which needed to replace AChE with BuChE (0.12 U/mL, prepared with Buffer B) and replace substrate iodoacetylcholine with thioiodobenzoyl chloride (15 mM), other conditions remain unchanged.

Cell viability

SH-SY5Y cells were planted in a 25 mL culture flask containing 1: 1 mixture of Eagle's minimum essential medium (EMEM) and ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, placed in a 5% carbon dioxide incubator at 37 °C. The cells were then grown at a density of 10,000 per well in 96-well plates. When the cells were fused, the cells were placed in serum-free medium and cultured with different concentrations of compound **1q**, **1r** (1, 5, 10, 25, 50 μM) for 24 h. After completion of the incubation, 20 μL of MTT was added at 37 °C for 4 h, and 200 μL of DMSO was added to dissolve methanine crystals at the end, and the absorbance was measured at 570 nM.

Plasma stability test

A certain amount of compound 1q was dissolved in DMSO, which was adjusted to the equivalent of free base 1.67 mg/mL of the mother liquor according to the purity and salinity, and then using 50% MeOH diluted it to 100 μg/mL of the INT solution. 2 μL of INT solution was took and mixed with 998 μL plasma (n = 2, the ratio of organic phase in the system does not exceed 0.5%), and the concentration of compound in plasma is 200 mg/mL, it was placed at 37 °C water bath incubation. The sampling point was set at 0 min, 10 min, 30 min, 1 h, 2 h. each sampling point took 100 μL of sample and was added 300 μL acetonitrile to precipitate, then centrifuged at 12,000 rpm for 5 min, supernatant 100 μL was added 100 μL 0.1% formic acid and took to LC-MS/MS analysis.

Determination of pharmacokinetic parameters

Three male Sprague-Dawley (SD) rats (200-220 g) were dosed with **1q** intravenously (i.v.) at dose of 1 mg/kg following Institutional Animal Care and Use Committee guidelines. The compound was dissolved in a vehicle of 5% DMA, 10% Solutol HS-15 and 85% saline. Blood samples (0.3 mL) were obtained via orbital sinus puncture at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 24 h time points and collected into heparinized tubes. Blood samples were centrifuged for cell removal, and the plasma supernatant was then transferred to a clean vial and subsequently stored in 4 ^{o}C prior to analysis. Test sample concentrations were determined by LC-MS/MS (Agela Technologies Venusil XBP C8(L) 2.1*5mm, 5μm, 150A) using propranolol as an internal standard. Pharmacokinetic parameters were calculated using winnonlin 5.2. software.

Results and discussion

To determine the inhibitory activities of these target compounds against AChE and BuChE[13], IC_{50} of all compounds were obtained. Lead compound **1** and donepezil were used as the reference compounds, and the results were shown in Table 1. It was found that the inhibitory activities of most compounds reached the nanomolar level against AChE and the micromolar level against BuChE. Among them, compound **1q** showed the most potent activity against AChE with IC_{50} of 0.15 nM, which was 250 and 160 times higher compared to the positive drug donepezil and the lead compound **1**, respectively. Furthermore, its selectivity index of AChE/BuChE reached up to 5903.

The SARs studies showed that when keeping fluorine group at the *para*-position of the benzyl group, the anti-AChE activity was enhanced or maintained by introducing halogens to the *ortho*-position. On the contrast, introducing halogens to the *meta*-position decreased the activity dramatically. As to compounds **1o-1r**, the number and position of the methoxy group on 4-isochromanone skeleton had a significant influence on the anticholinesterase activity. The compounds bearing methoxy group at both R_1 and R_2 positions exhibited more potent anticholinesterase

activity than those with methoxy group at both R_2 and R_3 positions. Besides, those compounds with reduced double bond also have good anticholinesterase activity (**1s-1v**).

Molecular docking study

In order to investigate the interaction mode of compounds **1** and **1q** with AChE, a molecular modeling study[14] was carried out by using Molecular Operating Environment (MOE) software and the PDB structure of 1EVE was taken from the RCSB Protein Data Bank for the docking procedure. Previous studies showed that two methoxy groups of donepezil interacted with Trp286 of acetylcholinesterase through hydrogen bond[15,16]. In the present study, the methoxy group at the C7 position of compound **1** interacted with Trp286 to form a hydrogen bond, while the methoxy group at the C8 position could not form a hydrogen bond with Trp286 due to steric hindrance (Figure 2b). However, both methoxy groups of compound **1q** could interacted with Trp286 through hydrogen bond, which was similar to that of donepezil, and at the same time, according to previous study, phenyl pyridine interacted with the CAS of AChE and the 4-isochromanone moiety interacted with the PAS of the AChE simultaneously. This interaction mode might explain why the activity of compound **1q** was significantly increased.

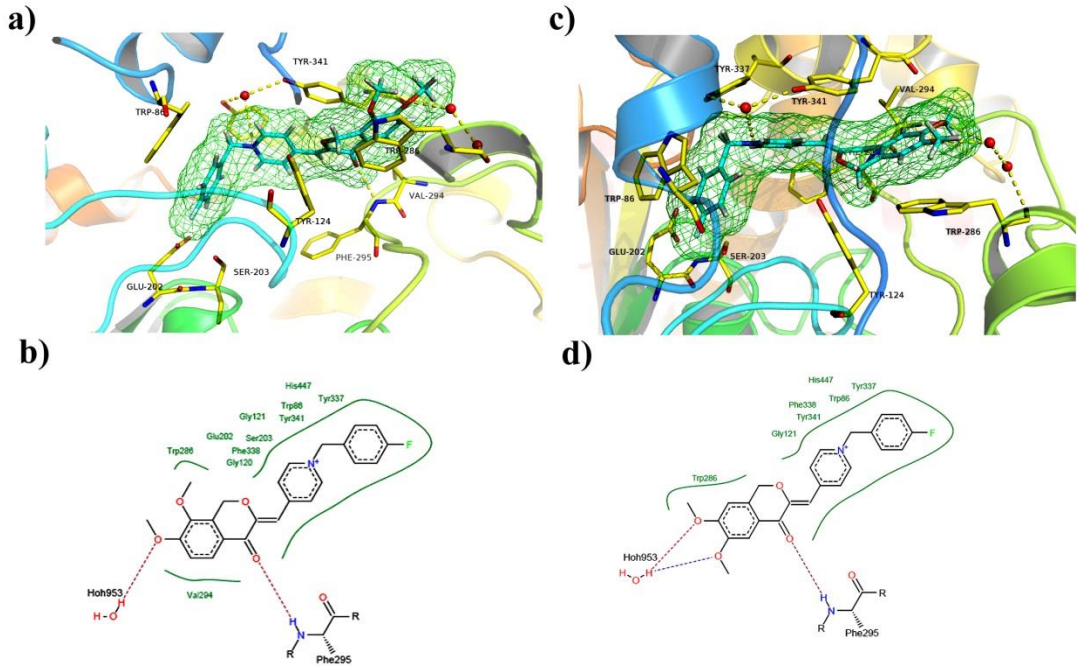


Figure 2. (a) 3D docking graph of compound **1**; (b) 2D docking graph of compound **1**; (c) 3D docking graph of compound **1q**; (d) 2D docking graph of compound **1q**.

Cell viability test of compounds 1q and 1r

To measure the toxicity of target compounds against nerve cells[17], the viability of SH-SY5Y cells treated with representative compounds **1q** and **1r** was determined and the result was shown in Table 2. When SH-SY5Y cells were exposed to compounds **1q** and **1r** at concentrations of 5 and 50 μM for 24 h, the survival rates of nerve cells did not decrease obviously. Even at the concentration of 100 μM, the survival rate just decreased slightly (cell viability >90%) and the morphology of the cells was not affected, indicating the low toxicity of these compounds.

Table 2. Cell viability of compounds **1q** and **1r** on nerve cells SH-SY5Y

Compound	Concentrations (μM)	Cell viability (%)
1q	5	98.3 ± 12.1
	50	97.8 ± 15.2
	100	93.4 ± 10.2
1r	5	97.8 ± 11.5
	50	97.6 ± 15.2

	100	91.4 ± 13.1

Plasma stability test and pharmacokinetic data of compound 1q

Compound **1q** has a good inhibitory activity on enzyme, in order to provide information for pharmacokinetic evaluation, representative compound **1q** was selected for preliminary plasma stability test. The results showed the concentration of compound **1q** in plasma decreased with time, but changed slightly in the period of 0 to 2 h, indicating that compound **1q** was relatively stable in rat plasma (Table 3).

Table 3. Plasma stability test of compound **1q**

Time	Analyte Peak Area (counts)	IS Peak Area (counts)	Area Ratio
0 min	3.46E+05	3.12E+05	1.11E+00
10min	3.41E+05	2.79E+05	1.22E+00
30min	3.34E+05	2.76E+05	1.21E+00
1 h	3.04E+05	2.58E+05	1.18E+00
2 h	2.84E+05	2.55E+05	1.11E+00

The further pharmacokinetic evaluation showed that compound **1q** had undesirable pharmacokinetic properties and its average half-life was only 1.94 hours (Table 4). Therefore, compound **1q** might be considered as a promising lead for the next round structural modification.

Table 4. Pharmacokinetic parameters (mean ± S.D) of **1q**（1.0 mg/kg, i.v.）in rats (n=3)

HL.Lambda_z(h)	Tmax (h)	Cmax (ng/ml)	AUClast (h*ng/ml)	AUCinf (h*ng/ml)	Vz_obs (ml/kg)	Cl_obs (ml/h/kg)	MRTlast (h)
1.94 ± 0.05	0.083 ±0.0	3947 ±311	2460 ±182	2465 ±182	1139 ±116	407 ±31	0.70 ±0.03

Note: The pharmacokinetic parameters were calculated using winnonlin 5.2.

HL: half life of the drug

Tmax: the time to reach the peak concentration of drug

Cmax: peak concentration of drug

AUC: area under the curve, it is an important indicator of the degree of drug absorption

Vz: apparent distribution volume

Cl: drug internal clearance rate

MRT: average dwell time

Conclusions

In conclusion, a series of novel AChE inhibitors derived from natural product XJP were further designed and synthesized based on our previous studies. The biological evaluation showed that these compounds had potent activities against AChE and good selectivity against BuChE. Among them, compound **1q** showed the strongest anti-AChE activity (IC_{50} = 0.15 nM), high AChE/BuChE selectivity (SI >5903) and low neurotoxicity. Additionally, compound **1q** possessed high plasma stability. As a result, compound **1q** might be considered as a promising lead compound for further research to develop new anti-AD agents.

Acknowledgments

This study was financially supported by the National Natural Science Foundation of China (No. 81302635), the Project Program of the State Key Laboratory of Natural Medicines, China Pharmaceutical University (No.SKLNMKF201710), China Postdoctoral Science Foundation (No. 2015M581903).

The authors declare no competing financial interest.

References

1. Luo, W.; Li, Y. P.; He, Y.; Huang, S. L.; Li, D.; Gu, L. Q.; Huang, Z. S., *Eur J Med Chem.* **2011,** *46* (6), 2609-16.

2. Ulrich, J. D.; Holtzman, D. M., *ACS Chem Neurosci.* **2016,** *7* (4), 420-7.

3. Caroline Doucet-Personeni, P. D. B., Rodney J. Fletcher, Adrian Kinkaid, Gitay Kryger, Bernard Pirard, Anne Taylor, Robin Taylor, John Taylor, Russell Viner Israel Silman, Joel L. Sussman, Harry M. Greenblatt, and Terence Lewis, *J. Med. Chem.* **2001,** *44*, 3203-3215.

4. Gessel, M. M.; Bernstein, S.; Kemper, M.; Teplow, D. B.; Bowers, M. T., *ACS Chem Neurosci.* **2012,** *3* (11), 909-18.

5. Lawrence M. Sayre, G. P., and Mark A. Smith, *Res. Toxicol.* **2008,** *21*, 172-188.

6. Qian, S.; He, L.; Mak, M.; Han, Y.; Ho, C. Y.; Zuo, Z., *Int J Pharm.* **2014,** *477* (1-2), 442-53.

7. Tommonaro, G.; Garcia-Font, N.; Vitale, R. M.; Pejin, B.; Iodice, C.; Canadas, S.; Marco-Contelles, J.; Oset-Gasque, M. J., *Eur J Med Chem.* **2016,** *122*, 326-38.

8. Digiacomo, M.; Chen, Z.; Wang, S.; Lapucci, A.; Macchia, M.; Yang, X.; Chu, J.; Han, Y.; Pi, R.; Rapposelli, S., *Bioorg Med Chem Lett.* **2015,** *25* (4), 807-10.

9. Lan, J. S.; Xie, S. S.; Li, S. Y.; Pan, L. F.; Wang, X. B.; Kong, L. Y., *Bioorg Med Chem.* **2014,** *22* (21), 6089-104.

10. Atukeren, P.; Cengiz, M.; Yavuzer, H.; Gelisgen, R.; Altunoglu, E.; Oner, S.; Erdenen, F.; Yuceakin, D.; Derici, H.; Cakatay, U.; Uzun, H., *Biomed Pharmacother.* **2017,** *90*, 786-795.

11. Wang, C.; Wu, Z.; Cai, H.; Xu, S.; Liu, J.; Jiang, J.; Yao, H.; Wu, X.; Xu, J., *Bioorg Med Chem Lett.* **2015,** *25* (22), 5212-6.

12. Wang, C.; Wu, Z.; Wang, J.; Liu, J.; Yao, H.; Lin, A.; Xu, J., *Tetrahedron.* **2015,** *71* (42), 8172-8177.

13. Huang, L.; Miao, H.; Sun, Y.; Meng, F.; Li, X., *Eur J Med Chem.* **2014,** *87*, 429-39.

14. Keri, R. S.; Quintanova, C.; Marques, S. M.; Esteves, A. R.; Cardoso, S. M.; Santos, M. A., *Bioorg Med Chem.* **2013,** *21* (15), 4559-69.

15. Cheung, J.; Rudolph, M. J.; Burshteyn, F.; Cassidy, M. S.; Gary, E. N.; Love, J.; Franklin, M. C.; Height, J. J., *J Med Chem.* **2012,** *55* (22), 10282-6.

16. van Greunen, D. G.; Cordier, W.; Nell, M.; van der Westhuyzen, C.; Steenkamp, V.; Panayides, J. L.; Riley, D. L., *Eur J Med Chem.* **2017,** *127*, 671-690.

17. Stefano Rizzo, C. l. R. r., Lorna Piazzi, Alessandra Bisi, Silvia Gobbi, Manuela Bartolini, Vincenza Andrisano, Fabiana Morroni, Andrea Tarozzi, Jean-Pierre Monti, and Angela Rampa,.*J Med Chem.* **2008,** *2008* (51), 2883-2886.