Design, synthesis and biological Evaluation of Dual acetyl cholinesterase and beta-secretase inhibitors in treatment for alzheimer's Disease

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Abstract: With the recent research advances in molecular biology and technology multiple credible hypotheses about the progress of Alzheimer's disease (AD) have been proposed, among which the amyloid and cholinergic hypotheses are commonly used to develop reliable therapeutic agents. The multitarget-directed ligand (MTDL) approach was taken in this work to develop muilti-functional agents, which can mainly serve as dual beta-secretase (BACE 1) and Acetylcholinesterase (AChE) inhibitors. Series of new compounds were designed, synthesized and evaluated in this work, from which we identified 2-((4-(1,3-dioxoisoindolin-2-yl)benzyl)amino)-2-oxoethyl-2-(4-methoxyphenyl)acetate (**1h**) as a new dual cholinesterase and beta-secretase inhibitor without toxicity.

Keywords: Beta-Secretase; Acetylcholinesterase; Inhibitor; Alzheimer's Disease.

INTRODUCTION

Alzheimer's disease (AD), one of the most common form of dementia, is a progressive and neurodegenerative brain disorder that affects cholinergic neurons of central nervous system (CNS) accompanied by loss of spatial awareness and cognitive ability (Mc Khann *et al.*, 1984). At present, there are many credible hypotheses (Kumar and Singh, 2015) about the progress of AD, including the cholinergic, amyloid and tau hypothesis. Based on the amyloid and cholinergic hypothesis (Digiacomo *et al.*, 2015), many reliable therapeutic agents targeting AD were developed.

Considering the multifactor interacting property of AD pathogenesis, the multitarget-directed ligand (MTDL) approach (Melchiorre *et al.*, 2010) has been increasingly applied in this field by many researchers. As the developed multiple drugs, with the guideline of MTDL approach, have complementary mechanisms of action which could help to realize one designed compound targeting more than two of the pathogenic mechanisms causing Alzheimer's. In order to design effective dual inhibitors targeting to BACE 1 and AChE for the treatment of AD, the MTDL approach was also applied in this study.

Among the BACE 1 inhibitors reported in the literature, we found the works investigated by Gerritz *et al* (Gerritz *et al.*, 2012) are very interesting and promising, and give lots of important inspirations to our works. Compound 1 (fig. 1), a potent BACE 1 inhibitor developed by Gerritz *at el*, was used as a lead compound to extract the pharmacophore of BACE 1 inhibitor. Donepezil, a potent AChE inhibitor and commercial available drug in treatment with AD, was also used to extract the pharmacophore of AChE inhibitor. By combining the extracted pharmacophores of both compound 1 and donepezil, series of dual BACE 1 and AChE inhibitors were developed in this work.

As reported (Gerritz *et al.*, 2012), compound 1 (fig. 1) is a potent BACE 1 inhibitor. Analyzing the interactions between compound 1 and BACE 1 (PDB 1M4H), we found that there exist hydrogen bonds between N-1 and Phe 108, N-2 and Asp 32, N-2 and Asp 228, and N-3 and Asp 228; hydrophobic interaction between C-1 and Tyr 71, and Ar-2 and Asn 73; π–π stack interaction between Ar-1 and Phe 108, and Ar-1 and Tyr 71; and π-cation stack interaction between Ar-2 and Arg 235 (fig. 1). The BACE 1 inhibition related pharmacophores used in this work was extracted from compound 1. Although we had made slight changes on these extracted structures, the slightly changed structures still maintain the important pharmacophores, like N-1, N-2, C-1, Ar-1 and Ar-2 (fig. 2).

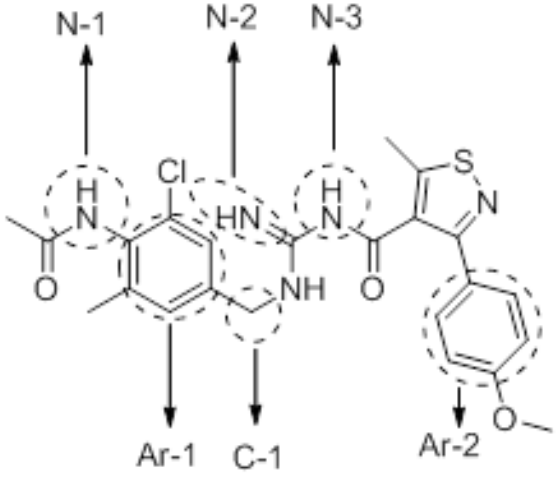

Fig. 1: The structure of compound **1** and its important pharmacophores to BACE 1 inhibition.

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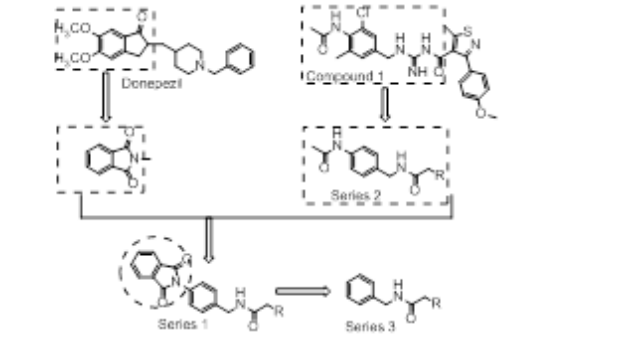
Donepezil, a potent AChE inhibitor and commercial available drug in treatment with AD, has been widely used in both clinic and academic field. Through studying the related literature of donepezil, we found its indanone moiety was often used in many dual BACE 1 and AChE inhibitors to improve the AChE inhibition.

The main reason is that the indanone moiety of donepezil can interact with the peripheral site at the entrance of AChE. On the basis of this inspiration, the indanone moiety of donepezil was also used to improve the AChE inhibition of our compounds in this work. In order to keep a balance between the AChE inhibition and the difficulties of synthesis, we changed the indanone moiety to a phthalimide group as they have similar important pharmacophores, like benzene ring, benzene conjunct five-membered ring and carbonyl. At the last, the extracted important pharmacophores from both compound 1 and donepezil were combined which resulted in the generation of compounds in series 1 (fig. 2).



R / Series	OH								
Series 1	1a	1b	1d	1e	—	1h	1i	1j	1k
Series 2	—	2b	2d	2e	—	2h	2i	2j	2k
Series 3	—	3b	3d	3e	3f	3h	3i	—	3k

Fig. 2: Design of target compounds.

From the bio-evaluation results of compounds in series 1, we find that most compounds in series 1 exhibit potent AChE inhibition, while have moderate BACE 1 inhibitory activity. In order to find the causes of the moderate BACE 1 inhibition of the compounds in series 1, another two groups of compounds, series 2 and series 3, were designed. In series 2 (fig. 2), the compounds bear an acetamide moiety instead of an N-phenylacetamide moiety in their main structure comparing to compounds in series 1. Therefore, the compounds in series 2 have a more similar structure with compound 1 (fig. 1) than that in series 1. In series 3 (fig. 2), the N-phenylacetamide moiety in the structure of the compounds in series 1 is cut off. Hence, the compounds in series 3 have a simple structure which is obviously different from both series 1 and series 2.

MATERIALS AND METHODS

Procedure for synthesis of 2-chloro-N-(hydroxylmethyl) acetamide

In a 50ml round-bottled flask, 2-chloroacetamide (9.3g, 0.1mol) and anhydrous potassium carbonate (0.5g, 3.6mmol) were added to an aqueous 36–38% solution of formaldehyde (8.1g, 0.1mol). Then the reaction system was stirred and heated under 75°C for five minutes, after that the reaction system was kept stirring overnight under room temperature. Next, pouring the reaction solution into a watch glass which was quickly cooled to 0°C. After the crystal solid was presented, the reaction mixture was removed to room temperature and dried for two days. At the end, almost 2.0g crystal solid was obtained and not given further purification, m.p. 101-104°C (literature, 102°C (Schraufstaetter and Goennert, 1962). Procedure for synthesis of 2-phenylisoindoline-1,3-dione (Sar *et al.*, 2010)

o-Phthalic anhydride (1.48g, 0.01mol) and aniline (0.93g, 10mmol) were stirred under reflux in acetic acid for 4h. Next pouring the reaction solution into prepared ice water and a white solid was obtained by filtering off the solution. The purified 2-phenylisoindoline-1,3-dione (1.80g, yield 81%) was obtained by recrystallization in ethonal, m.p. 139-141°C.

Procedure for synthesis of 2-chloro-N-(4-(1,3-dioxoisoindolin-2-yl)benzyl) acetamide

To a solution of 2-phenylisoindoline-1,3-dione (2.23g, 10mmol) in concentrate sulfuric acid (5ml) cooled to -10°C, 2-chloro-N-hydroxymethyl acetamide (1.85g, 15mmol) was slowly added. After the reaction solution was stirred for 12h, the reaction solution was slowly poured into prepared crushed ice, neutralized with potassium carbonate and then filtered. The crude product 2-chloro-N-(4-(1,3-dioxoisoindolin-2-yl) benzyl) acetamide (80% in yields) was a white solid and in good purity (m.p. 214-215°C), so without giving further purification when used in the next step. ESI-MS (m/z): 329 ($M + H^+$). ^{1}H NMR (500 MHz, $CDCl_3$) δ7.98 (dd, J = 5.4, 3.1 Hz, 2H), 7.82 (dd, J = 5.4, 3.0 Hz, 2H), 7.46 (s, 4H), 6.97 (s, 1H), 4.57 (d, J = 5.9 Hz, 2H), 4.14 (s, 2H). Procedure for synthesis of N-(4-(1,3-dioxoisoindolin-2-yl)benzyl)- 2-hydroxyacetamide (1a) (Dicholkar *et al.*, 2013)

To a cold solution (-5°C) of 2-chloro-N-(4-(1,3-dioxoisoindolin-2-yl) benzyl) acetamide (0.33g, 1.0mmol) in methanol (6ml), a solution of sodium hydroxide (44mg, 1.1mmol) in water (0.5ml) was slowly added during 15 minutes, then keeping the entire solution stirring under the same temperature for 1h. Methanol was then distilled out from the reaction mass at 27−30°C under reduced pressure, and the product was extracted with benzene (20ml). Washing the organic layer with 0.5 N HCl (10ml), 5% $NaHCO_3$, and brine (10ml) and then dring the solutionby using sodium sulfate. A crystalline product 1a (yield 57%) was produced by removing of benzene under

reduced pressure. Purification of crude product 1a was carried out through recrystallization using ethyl acetate and hexane. ESI-MS (m/z): 311 ($M + H^+$), m.p.180-181°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.71 (t, J = 7.6 Hz, 2H), 7.65 (d, J = 8.4 Hz, 2H), 7.42 (q, J = 6.5, 5.7 Hz, 2H), 7.21 (d, J = 8.4 Hz, 2H), 5.65 (s, 1H), 4.27 (d, J = 6.2 Hz, 2H), 3.86 (s, 2H), 1.06 (t, J = 7.0 Hz, 1H).

General procedure for synthesis the derivates of series 1, series 2 and series 3

To a solution of N-benzyl-2-chloroacetamide (15mmol) in DMF, various anhydrous carboxylate (17mmol) was added respectively at 27-30°C. Then the reaction solution was stirred under 65°C for 6h. After DMF was distilled out from the reaction mass at 100°C under reduced pressure, ethyl acetate (50ml) was added to extract the product, leaving behind unreacted carboxylate and salt, which were separated by filtration (Dicholkar *et al.*, 2013). Removal of ethyl acetate at room temperature and reduced pressures produced the crude products. Column purification of the crude products was processed using 50% ethyl acetate: 50% n-hexane. Procedure for synthesis of N-(4-acetamidolbenzyl)-2-chloroacetamide (Stokker *et al.*, 1981).

To a solution of acetanilide (1.35g, 10mmol) in concentrate sulfuric acid (5ml) cooled to -10°C, 2-chloro-N-hydroxymethyl acetamide (1.85g, 15mmol) was slowly added. After the reaction solution was stirred for 12h, the reaction solution was slowly poured into prepared crushed ice, neutralized with potassium carbonate and then filtered. The crude product N-(4-acetamidolbenzyl)-2-chloroacetamide (2.0g, yield 85%) was white and in good purity (m.p. 195-197^{o}C), so without giving further purification when used in the next step.

ESI-MS (m/z): 241($M + H^+$). ^{1}H NMR (500MHz, DMSO-d_6) δ 9.92(s, 1H), 8.68(t,J=5.5 Hz,1H), 7.52(d,J=8.5 Hz,2H), 7.18(d, J=8.5Hz,2H), 4.24(d,J=5.9Hz,2H), 4.11(s,2H), 2.03(s, 3H). Procedure for synthesis of N-benzyl-2-chloro-acetamide (Kumar and Nanjan, 2010)

To a mixture of benzylamine (2.14g, 20mmol) and Et3N (3.3ml, 24mmol) in anhydrous $CHCl_3$ (20ml), 2-chloroacety chloride (1.79ml, 24mmol) was slowly added under -5°C. Then the mixture was stirred for an additional 4h under -5°C. Removal of the solvent under reduced pressure, ice water (3×20ml) was added to wash the residue and then the solid was separated by filtration. The purity of the product was improved by recrystallization from a mixture solvent of Et_2O/petroleum, giving 1.77g N-benzyl-2-chloro-acetamide. Yield: 83%, m.p. 93-95°C (literature (Harte and Gunnlaugsson, 2006), 93°C.

In vitro AChE inhibitory activity screening

To test the inhibitory activity of all synthesized compounds against AChE, the spectrophotometric method of Ellman was taken by using purified AchE purchased from Sigma Chemical and donepezil from Dalian Meilun Biotech (China). All compounds were examined in 100mM phosphate buffer (pH 8.0) at 30°C, choosing acetylthiocholine (0.4mM) as substrates. Meanwhile, 5,5-Dithio-bis(2-nitrobenzoic) acid (DTNB, 0.2mM) was taken as color agent and the values of IC_{50} were calculated through UV spectroscopy based on the absorbance changes at 412nm as one concentration of compounds that develops 50% AChE activity inhibition. Data are displayedas means±SD. depending on three different experimentsat least (Ellman *et al.*, 1961).

In vitro BACE 1 inhibit activity screening

All synthesized compounds were tested for their BACE 1 inhibitor activities, using fluorescence resonance energy transfer (FRET) assay, which uses active recombinant human beta-secretase 1 (BACE 1, purchased from BioVision) and a specific substrate (H-RE(EDANS) EVNLDAEFK (Dabcyl) R-OH). The FRET-based enzymatic kits were purchased from Shanghai Haling Biological Technological CO., LTD., China. And the assay was carried out by following the manufacturers' instructions. The instructions are as follows: 2.5μL of test compound in known concentrations or DMSO (control), and enzyme (15μL) were preincubated in 10mM Tris-HCl (pH 8.0) at 25°C. Then, the substrate (15μL, 400μM) was added to initiate the reaction and the mixture was remained under 25°C for 60min. To evaluate the hydrolysis of substrate, the excitation wavelength and emission wavelength was set as 340nm and 490nm respectively. Data are displayed as means±SD. depending on three different experiments at least (Huang *et al.*, 2011).

Cytotoxicity assay (Liu et al., 2014)

The test was processed on the Human Embryonic Kidney 293 cells (HEK293) and its cell line was cultured by using the DMEM medium. The media was supplemented with penicillin (100units/ml), 10% fetal bovine serum (FBS) and streptomycin (100units/ml). The cells were sustained in a humidified environment at 37°C with 5% CO_2. Cells were sub-cultured and grown in collagen-coated tissue culture flasks. And when cells were 70% confluent, they were used for the experiments. By measuring the cells' ability to reduce WST-8 to WST-8 formazan, the cells cytotoxicity of the chosen compounds could be reflected. At the exponential growth phase of the cells, they were seeded to a 96-well culture plate in a proper concentration (5×10^3) and then incubated in a CO_2 incubator overnight. Next, 5μl compounds with a final concentration of 1, 10, 50, 100 and 200μM were added to each well and incubated for 4h in a CO_2 incubator. Then, 10μl WSK-8 (at final concentration 0.3mg/ml in DMEM without phenol red) was added to each well and incubated for another 2h. At the last, the absorbance values of each well were obtained by a microplate reader at 450nm.

Table 2: Physicochemical and spectral data of target compounds

Compound[a]	M.P.(°C)	ESI-MS (m/z)	^{1}H NMRδ(ppm)
1a	180-181	311	7.71 (t, *J*=7.6 Hz, 2H), 7.65 (d, *J*=8.4 Hz, 2H), 7.42 (q, *J*=6.5, 5.7 Hz, 2H), 7.21 (d, *J*=8.4 Hz, 2H), 5.65 (s, 1H), 4.27 (d, *J*=6.2 Hz, 2H), 3.86 (s, 2H), 1.06 (t, *J*=7.0 Hz, 1H).
1b	167-169	353	8.10-7.94 (m, 2H), 7.83 (dd, *J*=4.4, 2.4 Hz, 2H), 7.50-7.43 (m, 4H), 6.50 (s, 1H), 4.65 (s, 2H), 4.59 (d, *J* = 4.6 Hz, 2H), 2.19 (d, *J* = 0.6 Hz, 3H).
1d	202-205	429	8.76 (t, *J*=6.0 Hz, 1H), 7.98 (dd, *J*=5.6, 2.9 Hz, 2H), 7.92 (dd, *J*= .6, 3.0 Hz, 2H), 7.87 (d, *J*=8.3 Hz, 2H), 7.51 (d, *J*=7.5 Hz, 1H), 7.46 (d, *J*=7.8 Hz, 2H), 7.42 (d, *J*=2.7 Hz, 3H), 4.82 (s, 2H), 4.41 (d, *J*=5.9 Hz, 2H), 2.40 (s, 3H).
1e	205-207	449	8.06 (s, 1H), 8.00 – 7.94 (m, 3H), 7.82 (dd, *J* = 4.4, 2.4 Hz, 2H), 7.60 (dd, *J* = 6.4, 1.7, 0.9 Hz, 1H), 7.49 – 7.42 (m, 5H), 6.48 (s, 1H), 4.92 (s, 2H), 4.62 (d, *J* = 4.8 Hz, 2H).
1h	120-123	459	7.98 (dd, *J*=4.5, 2.4 Hz, 2H), 7.83 (dd, *J*=4.4, 2.3, 1.2 Hz, 2H), 7.46 – 7.41 (m, 2H), 7.36 – 7.30 (m, 2H), 7.22 – 7.17 (m, 2H), 6.86 – 6.76 (m, 2H), 6.19 (s, 1H), 4.69 – 4.62 (m, 2H), 4.42 (d, *J* = 4.4 Hz, 2H), 3.79 – 3.72 (m, 3H), 3.70 – 3.49 (m, 2H).
1i	109-110	463	7.98 (dd, *J* = 4.5, 2.4 Hz, 2H), 7.83 (dd, *J* =4.4, 2.4, 1.2 Hz, 2H), 7.43 (d, *J* = 6.6 Hz, 2H), 7.33 (d, *J* = 6.2 Hz, 2H), 7.19 (d, *J* = 6.7 Hz, 2H), 7.12 (d, *J* = 6.8 Hz, 2H), 6.19 (s, 1H), 4.66 (d, *J* = 4.7 Hz, 2H), 4.42 (d, *J* = 4.6 Hz, 2H), 3.67 (d, *J* = 2.9 Hz, 2H).
1j	106-108	443	7.99 (dd, *J* = 5.4, 3.0 Hz, 2H), 7.83 (dd, *J* = 5.5, 3.0 Hz, 2H), 7.53 – 7.34 (m, 4H), 7.29 – 7.19 (m, 8H), 6.08 (s, 1H), 4.63 (s, 2H), 4.47 (d, *J* = 5.9 Hz, 2H), 3.02 (t, *J* = 7.5 Hz, 2H), 2.80 (t, *J* = 7.4 Hz, 2H).
1k	215-218	416	9.29 (dd, *J* = 1.8, 0.7 Hz, 1H), 8.84 (dd, *J* = 3.9, 1.4 Hz, 1H), 8.36 (dt, *J* = 6.4, 1.6 Hz, 1H), 7.97 (dd, *J* = 4.4, 2.5 Hz, 2H), 7.82 (dd, *J* = 4.4, 2.4 Hz, 2H), 7.49 – 7.43 (m, 5H), 6.54 (s, 1H), 4.94 (s, 2H), 4.62 (d, *J* = 4.8 Hz, 2H).
2b	184-186	265	9.91 (s, 1H), 8.49 (t, *J* = 5.9 Hz, 1H), 7.51 (d, *J* = 8.5 Hz, 2H), 7.16 (d, *J* = 8.5 Hz, 2H), 4.50 (s, 2H), 4.24 (d, *J* = 6.0 Hz, 2H), 2.10 (s, 3H), 2.03 (s, 3H).
2d	153-155	341	7.89 – 7.83 (m, 2H), 7.48 (d, *J* = 6.7 Hz, 2H), 7.44 (d, *J* = 6.0 Hz, 1H), 7.37 (t, *J* = 6.1 Hz, 1H), 7.28 – 7.22 (m, 3H), 6.45 (s, 1H), 4.89 (s, 2H), 4.52 (d, *J* = 4.7 Hz, 2H), 2.43 (s, 3H), 2.19 (s, 3H).
2e	181-182	361	8.65 (t, *J* = 6.1 Hz, 1H), 8.07 – 7.96 (m, 2H), 7.79 (dd, *J* = 8.0, 2.2, 1.1 Hz, 1H), 7.61 (t, *J* = 7.9 Hz, 2H), 7.52 (d, *J* = 8.5 Hz, 2H), 7.19 (d, *J* = 8.5 Hz, 2H), 4.80 (s, 2H), 4.28 (d, *J* = 5.9 Hz, 2H), 2.02 (d, *J* = 8.0 Hz, 3H).
2h	97-99	371	7.81 (s, 1H), 7.43 (d, *J* = 6.5 Hz, 2H), 7.16 (dd, *J* = 6.9, 2.1 Hz, 2H), 7.08 (t, *J* = 6.1 Hz, 2H), 6.80 (dd, *J* = 6.9, 1.4 Hz, 2H), 6.24 (s, 1H), 4.67 – 4.54 (m, 2H), 4.29 (d, *J* = 4.5 Hz, 2H), 3.80 – 3.73 (m, 3H), 3.64 (d, *J* = 1.8 Hz, 2H), 2.20 – 2.08 (m, 3H).
2i	131-133	375	7.70 (s, 1H), 7.47 (d, *J* = 8.3 Hz, 2H), 7.30 (d, *J* = 3.1 Hz, 1H), 7.27 (d, *J* = 2.8 Hz, 1H), 7.24 – 7.18 (m, 2H), 7.15 (t, J= 8.3 Hz, 2H), 6.32 (s, 1H), 4.64 (s, 2H), 4.36 (s, 2H), 3.86 (s, 2H), 2.21 – 2.12 (m, 3H).
2j	126-129	355	7.64 (s, 1H), 7.45 (d, *J* = 6.5 Hz, 2H), 7.25 (dd, *J* = 9.0, 5.6 Hz, 2H), 7.20 (d, *J* = 5.1 Hz, 3H), 7.15 (d, *J* = 6.2 Hz, 2H), 6.18 (s, 1H), 4.59 (s, 2H), 4.34 (d, *J* = 4.7 Hz, 2H), 2.99 (t, *J* = 5.9 Hz, 2H), 2.77 (t, *J* = 6.0 Hz, 2H), 2.17 (s, 3H).
2k	185-187	328	9.24 (d, *J* = 2.1 Hz, 1H), 8.82 (dd, *J* = 4.9, 1.7 Hz, 1H), 8.33 (dt, *J* = 8.0, 1.9 Hz, 1H), 7.44 (d, *J* = 6.2 Hz, 3H), 7.24 (d, *J* = 8.4 Hz, 2H), 6.55 (s, 1H), 4.91 (s, 2H), 4.49 (d, *J* = 5.9 Hz, 2H), 2.17 (s, 3H).
3b	79-81	208	7.37 (t, *J* = 7.0 Hz, 2H), 7.32 (t, *J* = 8.1 Hz, 3H), 6.51 (s, 1H), 4.62 (d, *J* = 4.5 Hz, 2H), 4.56 – 4.48 (m, 2H), 2.15 (s, 3H).
3d	136-137	284	8.66 (t, *J*=5.9 Hz, 1H), 7.85 (d, *J*=7.8 Hz, 2H), 7.51 (d, *J*=7.3 Hz, 1H), 7.44 (t, *J*=7.7 Hz, 1H), 7.37-7.30 (m, 2H), 7.30-7.25 (m, 2H), 4.79 (s, 2H), 4.35 (s, 2H), 2.38 (d, J= 10.2 Hz, 3H).
3e	152-153	304	8.55 (t, *J* = 5.9 Hz, 1H), 7.43 (td, *J* = 6.7, 4.0 Hz, 2H), 7.33 – 7.25 (m, 4H), 7.22 (d, *J* = 7.0 Hz, 3H), 4.58 (s, 2H), 4.26 (d, *J* = 6.0 Hz, 2H).
3f	74-75	300	7.85 (d, *J*=9.4 Hz, 1H), 7.51 (t, *J*=8.7 Hz, 1H), 7.35 (q, *J*=8.5, 7.5 Hz, 6H), 7.04 (t, *J* =7.5 Hz, 1H), 6.92 (d, *J*=8.4 Hz, 1H), 4.91 (s, 2H), 4.56 (d, *J*=5.5 Hz, 2H), 3.43 (s, 3H).
3h	84-85	314	7.34 (q, *J* = 9.9, 8.3 Hz, 3H), 7.21 – 7.11 (m, 4H), 6.78 (d, *J* = 8.7 Hz, 2H), 6.21 (s, 1H), 4.62 (s, 2H), 4.37 (d, *J* = 5.8 Hz, 2H), 3.76 (s, 3H), 3.64 (s, 2H).
3i	77-79	318	8.56 (t, *J* = 5.9 Hz, 1H), 7.46 (td, *J* = 6.7, 4.0 Hz, 2H), 7.38 – 7.29 (m, 4H), 7.26 (d, *J* = 7.0 Hz, 3H), 4.59 (s, 2H), 4.32 (d, *J* = 6.0 Hz, 2H), 3.94 (s, 2H).
3k	115-117	271	9.20 (d, *J* = 10.1 Hz, 1H), 8.77 (d, *J* = 13.0 Hz, 1H), 8.30 (t, *J* = 7.6 Hz, 1H), 7.48 – 7.39 (m, 1H), 7.39 – 7.29 (m, 5H), 6.68 (s, 1H), 5.00 – 4.80 (m, 2H), 4.52 (dd, *J* = 10.6, 6.0 Hz, 2H).

[a]All synthesized compounds in this work are white or nearly white, and the mobile phases of the TLC are mainly composed with ethyl acetate and n-hexane in different ratios.

Cells without the test compounds acted as positive control and its assay values were set to 100%. The test compounds were dissolved in DMSO at 10mM concentration firstly and then diluted to above-mentioned concentration in phosphate buffer (10mM, pH 7.4). Data of this test was recorded depending on three independent experiments and values of each well were read in triplicate.

RESULTS

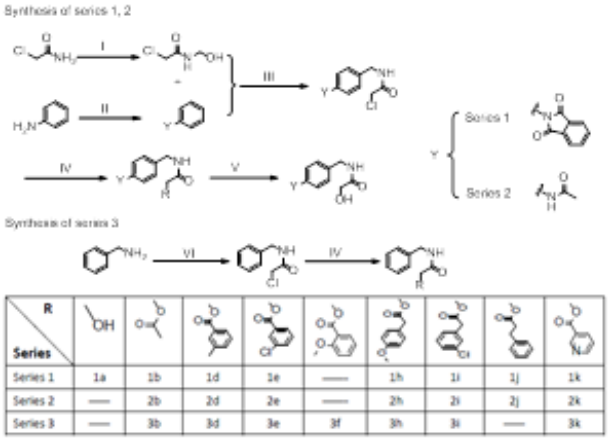
In this work, we had designed 3 series of compounds by combining the important pharmacohpores of compound 1 and donepezil which related to BACE 1 and AChE inhibition respectively. As shown in table 1, several synthesized compounds exhibit good BACE 1 and AChE inhibitory activity. In addition, the cytotoxicity assay of compound 1d, 1h and 1j showed low cell toxicity to HEK 293 cells *in vitro*. Among all these synthesized compounds, 1h exhibits potent AChE inhibition and moderate BACE 1 inhibition, as well as lower cell toxicity, which should be considered as a promising anti-AD candidate.

DISCUSSION

Chemistry

The synthesis of all compounds in 3 series is described in Scheme 1 and the relevant substitute group of each product is also described in Scheme 1. In the first step, chloroacetamide was mixed with an aqueous 36–38% solution of formaldehyde to generate 2-chloro-N-(hydroxylmethyl) acetamide. In the second step, aniline reacted with o-phthalic anhydride or acetic anhydride to obtain 2-phenylisoindoline-1,3-dione or acetanilide. In the third step, 2-chloro-N-(hydroxylmethyl) acetamide was slowly added to a solution of 2-phenylisoindoline-1,3-dione or acetanilide in concentrate sulfuric acid under 0°C, then 2-chloro-N-(4-(1,3-dioxoisoindolin-2-yl)benzyl) acetamide or N-(4-acetamidolbenzyl)-2-chloroacetamide was generated in good yield after the end of this reaction. While compound N-benzyl-2-chloro-acetamide was obtained from the reaction between benzylamine and 2-chloroacety chloride in anhydrous $CHCl_3$ under -5°C. In the fourth step, the derivates in each series was generally generated from the reaction between various anhydrous carboxylate and the relevant compounds obtained from the third step. And the general reaction of the fourth step is processed in dimethyl formamide (DMF) under 65°C. To get N-(4-(1,3-dioxoisoindolin-2-yl)benzyl)- 2-hydroxyacetamide, a solution of sodium hydroxide in water was slowly added to a cold solution of 2-chloro-N-(4-(1,3-dioxoisoindolin-2-yl) benzyl) acetamide in methanol under -5°C.

AChE and beta-secretase Inhibition. From table 1, it is obvious that there are three compounds, 1h, 1i and 1j have IC_{50} values of AChE inhibition less than 100nM and compound 1h is a little more potent AChE inhibitor than donepezil. As for BACE 1 inhibition, all synthesized compounds only exhibit moderate inhibitory activity and their IC_{50} values of BACE 1 inhibition lay between 1μM and 200μM. The docking studies had been performed to help us understand the force interactions between the enzyme and the ligand. In fig. 3 compound 1h docked with AChE (PDB 4EY7) was taken as an example of synthesized compounds to display their bind model, it was obvious to find that Trp 286 and Tyr 341 have π-π forces with 1h.



R / Series	OH								
Series 1	1a	1b	1d	1e	——	1h	1i	1j	1k
Series 2	——	2b	2d	2e	——	2h	2i	2j	2k
Series 3	——	3b	3d	3e	3f	3h	3i	——	3k

Scheme 1. Reagents and conditions: (I) 36–38% solution of formaldehyde, anhydrous potassium carbonate, 75°C for five minutes and stirring overnight at room temperature; (II) o-phthalic anhydride or aceticanhydride, acetic acid, reflux and stirring for 4h; (III) concentrated sulfuric acid, -10°C and stirring for 12h; (IV) various carboxylate, anhydrous NaI, DMF, 65°C and stirring for 6h; (V) NaOH solution, methanol, -5°C and stirring for 1h; (VI) chloroacetyl chloride, triethylamine, trichloromethane, -5°C and stirring for 4h.

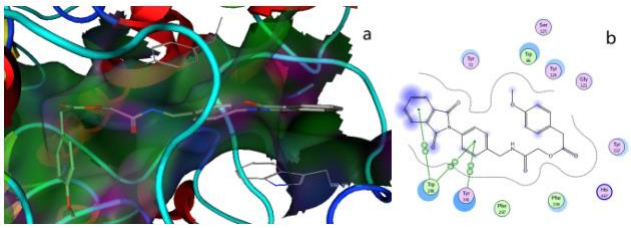


Fig. 3: In the left panel (a), compound 1h docked with the pocket of AChE, the 3D model of this complex. In the right panel (b), the force interactions between compound 1h and the residues of AChE in a 2D model.

As reported (Zhu *et al.*, 2009), Trp 286 is located at the entrance of AChE and served as a peripheral site of AChE. Meanwhile, the indanone moiety of the donepezil has π-π interaction with Trp 286, which makes a great contribution to its AChE inhibitory activity. Therefore, the π-π stack interaction between 1h and Trp 286 and Tyr 341 may also make important contributions to higher the AChE inhibition potency of 1h in a similar way.

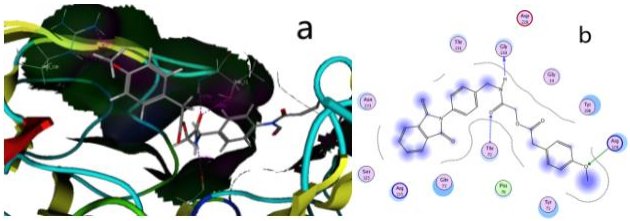


Fig. 4: In the left panel (a), a representation of molecular docking derived binding pose (3D) of compound 1h in the active site of BACE 1. In the right panel (b), docking simulated binding mode (2D) of compound 1h bound in the active site of BACE 1.

From fig. 4, we could find that, although compound 1h has interactions with the residues Thr 72, Arg 128 and Gly230 of BACE 1, the N-phenylphthalimide moiety of compound 1h did not show obvious interactions with the residues of BACE 1. So, we wondered whether the existing of N-phenylphthalimide moiety in our synthesized compounds decreased the potency of their BACE 1 inhibition, as the volume of N-phenylphthalimide moiety is large and the conformation is highly strained which can make these compounds difficult to cross the narrow gorge of the catalytic site of BACE 1.

In order to find the causes of the moderate BACE 1 inhibition of the compounds in series 1, another two groups of compounds, series 2 and series 3, were designed, synthesized and bio-evaluated. There are intended structure differences between series 2, series 3 and the former designed series 1. As for the compounds in series 2, they contain the N-phenylacetamide moiety in their main structure instead of the N-phenylphthalimide moiety, which is different from the core structure of compounds in series 1. While the core structure of compounds in series 3 is that the phthalimide group was cut away from the main structure of compounds in series 1 (fig. 2).

The bioassay results of these new designed compounds were displayed in table 1. As shown in table 1, it is evident that almost all these new designed compounds are less potent in AChE inhibition comparing to the most compounds in series 1, which might demonstrate that N-phenylphthalimide moiety plays an important role in maintain the AChE inhibition potency of the compounds. Although the AChE inhibition of the compounds in series 2 and 3 were all not potent, it was still obvious that compounds in series 2 are more potent AChE inhibitors than those in series 3, which may be mainly due to the existence of acetamide moiety in compounds of series 2 could form hydrogen bonds with the residues of the AChE.

As for the BACE 1 inhibition of the new designed compounds, compounds in series 2 are almost equal potent with those in series 1, but compounds in series 3 are much less potent than those in series 1. These results might demonstrate that changing the N-phenylphthalimide moiety with an N-phenyl acetamide group in the main structure of the compounds might decrease the π-π forces with the residues, but this negative effect could be compensated by the small space volume of N-phenylacetamide group as it is helpful for compounds to across the narrow gorge of the BACE 1. While cutting off the phthalimide group from the compounds might lead to the loss of both carbanyl group and benzene ring, hence the compounds might have less hydrogen bond and π-π forces with the residues of BACE 1. Although the BACE 1 inhibition of the new synthesized compounds was moderate, we can get useful information about how to optimize the BACE 1 inhibitors by combining and analyzing the bioassay results of all these synthesized compounds of three independent experiments.

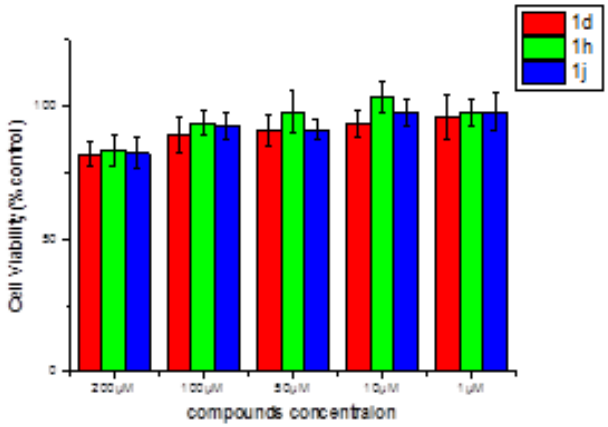


Fig. 5: The cell viability of compound 1d, 1h and 1j on HEK 293 cells using CCK-8 assay kits. The results reported are the mean±SD of three independent experiments.

Cytotoxicity assay

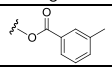
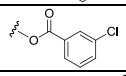
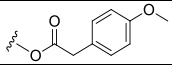
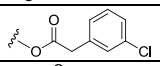
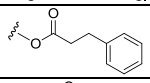
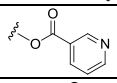
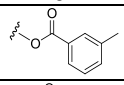
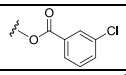
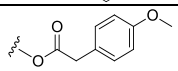
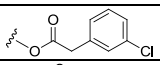
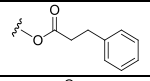
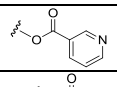
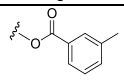
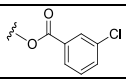
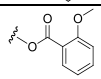
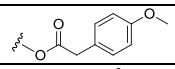
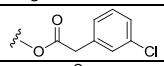
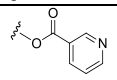
As cytotoxicity can reflect the toxicity of the compounds which may cause heavy damage to body, cytotoxicity assay has been widely used to test the compounds toxicity, especially in the process of developing new drugs. In this work, the effect of compound 1d, 1h and 1j on the viability of HEK 293 cells was studied by the CCK-8 assay (Liu *et al.*, 2014). The compounds were added to the cells in five different concentrations (1, 10, 50, 100, and 200μM) respectively and incubated for 4h at 37°C. The values (fig. 5) indicated that there are not great cytotoxicity differences among 1d, 1h and 1j at different concentrations.

Hence, these results demonstrate that all these tested compounds do not show obvious toxicity in the CCK-8 assay, suggesting that 1d, 1h and 1j lack general toxicity.

CONCLUSIONS

In this work, three series of new compounds were designed by using donepezil and compound 1 as template

Table 1: AChE and BACE 1 inhibition activity for all synthesized compounds.

Compound	R	AChE±SD[a]	BACE 1±SD[a]
		IC_{50}(nM)[a]	IC_{50}(μM)[a]
1a		391.62±27.58	43.41±1.17
1b		208.55±12.93	58.72±1.67
1d		121.43±4.07	46.96±1.13
1e		105.97±3.80	52.03±1.18
1h		11.89±0.95	8.82±0.14
1i		59.12±1.48	12.57±0.19
1j		27.78±1.89	17.46±0.28
1k		122.78±4.84	16.61±0.36
2b		338.51±12.63	56.75±1.63
2d		358.39±13.12	25.62±0.37
2e		224.7±5.73	33.31±0.58
2h		341.2±8.56	12.95±0.16
2i		378.55±11.43	45.58±1.36
2j		408.1±13.41	22.39±0.17
2k		475.39±15.77	13.23±0.25
3b		382.39±14.12	170.57±5.63
3d		507.47±17.37	111.08±3.67
3e		516.23±15.81	107.9±3.48
3f		546.7±17.34	134.62±2.52
3h		434.42±15.72	103.59±2.25
3i		431.83±18.59	105.89±3.37
3k		488.4±17.42	115.27±2.77
Donepezil		15.27±0.78	122.93±3.27

[a] The in vitro test compound concentration required to produce 50% inhibition of AChE and BACE 1. The result (IC_{50}) was the mean of three independent experiments.

molecular. Next, the designed compounds were successfully prepared by routine synthetic methods. Then, the inhibition activities of these compounds were evaluated against AchE and BACE-1. Among these compounds, 2-((4-(1,3-dioxoisoindolin-2-yl)benzyl)amino)-2-oxoethyl-2-(4-methoxyphenyl)acetate (1h) exhibits as a dual cholinesterase and beta-secretase inhibitor without toxicity. Considering the novel structure of compound 1h, it would be very useful to discover more potent dual AchE and BACE-1 inhibitors for treating Alzheimer's disease.

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REFERENCES

Dicholkar DD, Kumar P, Heer PK, Gaikar VG, Kumar S and Natarajan R (2013). Synthesis of n, n, n′, n′-tetraoctyl-3-oxapentane-1, 5-diamide (todga) and its steam thermolysis-nitrolysis as a nuclear waste solvent minimization method. *Ind. Eng. Chem. Res.*, **52**(7): 2457-2469.

Digiacomo M, Chen Z, Wang S, Lapucci A, Macchia M, Yang X, Chu J, Han Y, Pi R and Rapposelli S (2015). Synthesis and pharmacological evaluation of multifunctional tacrine derivatives against several disease pathways of AD. *Bioorg. Med. Chem. Lett.*, **25**(4): 807-810.

Ellman GL, Courtney KD, Andres V and Featherstone RM (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7**(2): 88-95.

Gerritz SW, Zhai W, Shi S, Zhu S, Toyn JH, Meredith Jr JE, Iben LG, Burton CR, Albright CF and Good AC (2012). Acyl guanidine inhibitors of β-secretase (BACE-1): Optimization of a micromolar hit to a nanomolar lead via iterative solid-and solution-phase library synthesis. *J. Med. Chem.*, **55**(21): 9208-9223.

Harte AJ and Gunnlaugsson T (2006). Synthesis of α-chloroamides in water. *Tetrahedron Lett.*, **47**(35): 6321-6324.

Huang W, Tang L, Shi Y, Huang S, Xu L, Sheng R, Wu P, Li J, Zhou N and Hu Y (2011). Searching for the multi-target-directed ligands against Alzheimer's disease: Discovery of quinoxaline-based hybrid compounds with AChE, H3R and BACE 1 inhibitory activities. *Bioorg. Med. Chem. Lett.*, **19**(23): 7158-7167.

Kumar A and Singh A (2015). A review on Alzheimer's disease pathophysiology and its management: An update. *Pharmacol. Rep.*, **67**(2): 195-203.

Kumar BP and Nanjan M (2010). Novel glitazones: Design, synthesis, glucose uptake and structure-activity relationships. *Bioorg. Med. Chem. Lett.*, **20**(6): 1953-1956.

Liu P, Niu Y, Wang C, Sun Q, Zhai Y, Yu J, Sun J, Xu F, Yan G and Huang W (2014). 4-Oxo-1, 4-dihydro-quinoline-3-carboxamides as BACE-1 inhibitors: Synthesis, biological evaluation and docking studies. *Eur. J. Med. Chem.*, **9**: 413-421.

McKhann G, Drachman D, Folstein M, Katzman R, Price D and Stadlan EM (1984). Clinical diagnosis of Alzheimer's disease report of the nincds-adrda work group under the auspices of department of health and human services task force on Alzheimer's disease. *Neurology*, **34**(7): 939-939.

Melchiorre C, Bolognesi ML, Minarini A, Rosini M and Tumiatti V (2010). Polyamines in drug discovery: From the universal template approach to the multitarget-directed ligand design strategy. *J. Med. Chem.*, **53**(16): 5906-5914.

Sar A, Lindeman S and Donaldson WA (2010). De novo synthesis of polyhydroxyl aminocyclohexanes. *Org. Biomol. Chem.*, **8**(17): 3908-3917.

Schraufstaetter E and Goennert R (1962). Alkylidene-and arylidene-bis-chloroacetamides, a new group of compounds active against bilharziasis. *Z. Naturforsch. B.*, **17**: 505.

Stokker G, Deana A, DeSolms S, Schultz E, Smith R, Cragoe Jr E, Baer J, Russo H and Watson L (1981). 2-(Aminomethyl) phenols, a new class of saluretic agents. 3. Effects of functional group reorientation and modification. *J. Med. Chem.*, **24**(9): 1063-1067.

Zhu Y, Xiao K, Ma L, Xiong B, Fu Y, Yu H, Wang W, Wang X, Hu D and Peng H (2009). Design, synthesis and biological evaluation of novel dual inhibitors of acetylcholinesterase and β-secretase. *Bioorg. Med. Chem. Lett.*, **17**(4): 1600-1613.