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RESEARCH ARTICLE

Synthesis and biological evaluation of novel *N,N′*-bis-methylenedioxybenzyl-alkylenediamines as bivalent anti-Alzheimer disease ligands

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Abstract

A novel series of *N,N′*-bis-methylenedioxybenzyl-alkylenediamines **5a**–**5g** have been designed, synthesized and evaluated as bivalent anti-Alzheimer's disease ligands. The enzyme inhibition assay results indicated that compounds **5e**–**5g** inhibit both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in the micromolar range (IC_{50}, 2.76–4.24 μM for AChE and 3.02–5.14 μM for BuChE), which was in the same potential as the reference compound rivastigmine (IC_{50}, 5.50 μM for AChE and 1.60 μM for BuChE). It was found that compounds could bind simultaneously to the peripheral and catalytic sites of AChE. β-Amyloid (Aβ) aggregation inhibition assay results showed that compound **5e** exhibited highest self-mediated Aβ fibril aggregation inhibition activity (40.3%) with a similar potential as curcumin (41.6%). It was also found that **5e**–**5g** did not affect neuroblastoma cell viability at the concentration of 50 μM.

Keywords Alzheimer's disease, cholinesterase, β-amyloid aggregation, inhibitors, synthesis

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative brain disorder that affects millions among the aging population. The etiology of AD is not completely known, although there are diverse hallmarks such as low levels of acetylcholine and β-amyloid (Aβ) deposits that seem to play significant roles in the disease[1]. One of the most useful approaches involved in the design of new agents for the treatment of AD focuses on increasing cholinergic neurotransmission in the brain by inhibiting cholinesterases (ChE) such as donepezil, rivastigmine and galanthamine[2]. The main function of both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) is terminating the impulse transmission at cholinergic synapses in which ChE catalyzes the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid. But clinical experience shows that ChE inhibition is a palliative treatment, which does not address the etiology of AD[3]. In recent times, due to the multi-pathogenesis of AD, one of the current strategies to develop novel anti-AD agents focuses on compounds with multiple potencies[4].

On the other hand, the progressive deposition of Aβ in AD's brain is generally considered to be fundamental to the development of neurodegenerative pathology. And cell toxicity associated with Aβ fibril aggregation provides an explanation for the neuronal cell loss found in AD[5]. Therefore, preventing of Aβ fibril aggregation in the brain is currently another target as potential therapy for AD[6,7]. Several inhibitors, such as curcumin[8,9] and benzofuran analogues[5,10], were developed.

Schizadrins were main bioactive components in medicinal plants of schisandra that have exhibited many pharmacological effects[11]. Recently, it was reported that the schizadrins showed significant inhibition of the activity of AChE, and structure–activity relationship indicated that aromatic methylenedioxy group was necessary[12]. Moreover, it is interesting that γ-schizadrin containing methylenedioxybenzyl had inhibition activity on Aβ production[13]. In order to develop a novel type

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of difunctional anti-AD agents that could inhibit both cholinesterase and Aβ aggregation, a series of *N,N′*-bis-methylenedioxybenzyl-alkylenediamines (NBMA, **5a**–**5g**) were designed, synthesized, and assayed for their AChE, BuChE, and Aβ aggregation inhibitory activities.

Materials and methods

Materials

^{1}H and ^{13}C NMR spectra were recorded using TMS (Tetramethyl silicane) as the internal standard in $CDCl_3$ with a Bruker BioSpin GmbH spectrometer at 400 and 100 MHz, respectively; MS spectra were recorded on a Shimadzu LCMS-2010A instrument with an ESI mass selective detector, and high-resolution mass spectra (HRMS) on Shimadzu LCMS-IT-TOF (Liquid chromatography-mass spectrometry-ion trap-time of flight). The purities of compounds were confirmed to be higher than 95% by analytical HPLC performed with a dual pump Shimadzu LC-20AB system equipped with a Ultimate XB-C18 column (4.6×250 mm, 5 μm) and eluted with methanol/water (30:70 to 50:50) containing 0.1% TFA (Trifluoroacetic Acid) at a flow rate of 0.5 mL/min. All reagents used in this study were of AR grade and available commercially.

Chemistry

The synthetic routes to target compounds are summarized in Scheme 1. The starting material vanillin **1** was treated with bromine in acetic acid to give 5-bromovanillin **2**, which was heated with NaOH and copper powder in water yielded 3,4-dihydroxy-5- methoxybenzaldehyde **3**. Methylenation of compound **3** by a convenient procedure (CH_2Br_2–CuO–K_2CO_3–DMF) afforded the intermediate **4** in 72% yield, and conversion to the target compounds **5a**–**5g** by reaction with diamine and then reduction with $NaBH_4$ in methanol. All the target compounds were purified by flash chromatography with chloroform/methanol/ammonia (20:1:0.5%) elution.

There are some different methods in the preparation of intermediate **4** reported in many articles[14–16]. Most of them reacts under nitrogen, and the yield was between 50 and 70%. In our synthesis, CuO was used as catalyst, K_2CO_3, CH_2Br_2, and **3** were stirred in DMF (Dimethylformamide) at 140°C for 3 h without nitrogen, could get intermediate **4** in a satisfactory yield.

Synthesis of intermediate 4

CuO (0.13 g), anhydrous K_2CO_3 (1.75 g) and CH_2Br_2 (1.25 g) were added to a solution of compound **3** (1.0g) in anhydrous DMF (12.5 mL), and the mixture was heated at 140°C for 4 h. The reaction mixture was poured into water, and the product was extracted with ether. The ethereal extract was washed successively with 2% HCl, 2% NaOH and water. Evaporation of the solvent after drying over anhydrous K_2CO_3 gave light brown crystalline mass, which on recrystallization from MeOH, afforded colorless pillars[14,17], yield: 72%, m.p. 129–130°C (m.p. 131–132°C)[15].

General procedure for the synthesis of compounds 5a–5g

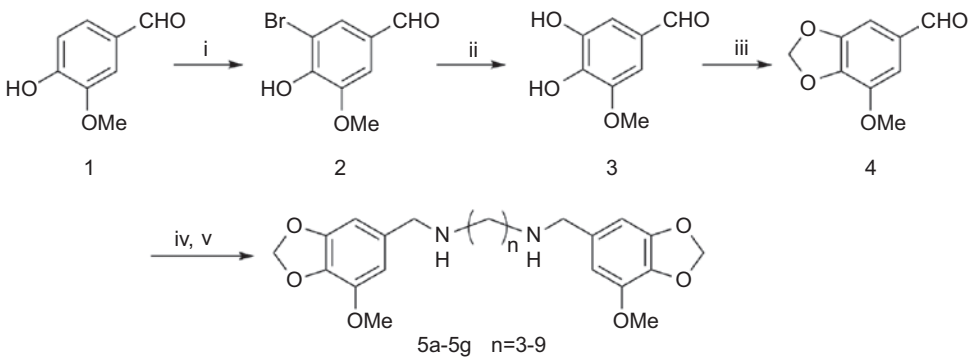
Aromatic aldehyde **4** (0.36 g, 2 mmol) and 1 mmol diamine were stirred in MeOH for 4 h and then were reduced directly by $NaBH_4$ (6 mmol) at room temperature for 4 h. The solvent was evaporated and the residue was poured into water and extracted with ethyl acetate, the solution was dried over anhydrous $MgSO_4$ and then concentrated, the target compounds were purified by flash chromatography with chloroform/methanol/ammonia (20:1:0.5%) elution[18].

N^1,N^3-bis((7-methoxybenzo[d][1,3]dioxol-5-yl)methyl) propane-1,3-diamine (5a)

Colorless oil. MS (ESI, *m/z*): $[M + H]^+$ 403.1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.50 (s, 4H, Ar–H), 5.94 (s, 4H,–OCH_2O–), 3.88 (s, 6H,–OCH_3), 3.68 (s, 4H,–CH_2–), 2.70 (t, J=6.7 Hz, 4H,–CH_2–), 2.40 (s, 2H,–CH_2–), 1.77–1.70 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.83, 143.53, 134.55, 134.14, 107.47, 102.20, 101.32, 56.54, 53.89, 47.79, 29.54; purity: 97.3% by HPLC. HRMS (ESI): calcd for $(M + H)^+$ ($C_{21}H_{26}N_2O_6$) requires *m/z* 403.1869, found 403.1856.

N^1,N^4-bis((7-methoxybenzo[d][1,3]dioxol-5-yl)methyl)butane-1,4-diamine (5b)

Colorless oil. MS (ESI, *m/z*): $[M+H]^+$ 417.1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.50 (s, 4H, Ar–H), 5.93 (s,



Scheme 1. Reagent and condition: (i) Br_2, AcOH, rt, 1h; 95% (ii) NaOH, Cu, H_2O, reflux, 18h; 60% (iii) CH_2Br_2,CuO, K_2CO_3, DMF, 140°C, 4h; 70% (iv) MeOH, diamine, rt, 4h (v) $NaBH_4$, rt, 4h, 80–90%.

4H,–OCH_2O–), 3.89 (s, 6H,–OCH_3), 3.67 (s, 4H,–CH_2–), 2.62 (t, J=6.5 Hz, 4H,–CH_2–), 1.82 (s, 2H,–CH_2–), 1.71–1.39 (m, 2H,–CH_2–); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.79, 143.51, 135.02, 134.05, 107.36, 102.18, 101.29, 56.54, 53.96, 49.07, 27.81; purity: 99.9% by HPLC. HRMS (ESI): calcd for $(M + H)^+$ ($C_{22}H_{28}N_2O_6$) requires m/z 417.2026, found 417.2017.

N^1,N^5-bis((7-methoxybenzo[d][1,3]dioxol-5-yl)methyl) pentane-1,5-diamine (5c)

Colorless oil. MS (ESI, m/z): $[M + H]^+$ 431.2; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.50 (s, 4H, Ar–H), 5.93 (s, 4H,–OCH_2O–), 3.89 (s, 6H,–OCH_3), 3.67 (s, 4H,–CH_2–), 2.60 (t, J=7.1 Hz, 4H,–CH_2–), 1.57–1.46 (m, 4H,–CH_2–), 1.45–1.32 (m, 4H,–CH_2–); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.79, 143.51, 135.27, 134.02, 107.34, 102.18, 101.28, 56.55, 54.11, 49.24, 30.00, 25.11; purity: 99.1% by HPLC. HRMS (ESI): calcd for $(M + H)^+$ ($C_{23}H_{30}N_2O_6$) requires m/z 431.2182, found 431.2178.

N^1,N^6-bis((7-methoxybenzo[d][1,3]dioxol-5-yl)methyl)hexane-1,6-diamine(5d)

Colorless oil. MS (ESI, m/z): $[M + H]^+$ 445.1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.51 (s, 4H Ar–H), 5.93 (s, 4H,–OCH_2O–), 3.89 (s, 6H,–OCH_3), 3.67 (s, 4H,–CH_2–), 2.60 (t, J=7.2 Hz, 4H,–CH_2–), 1.54–1.45 (m, 6H,–CH_2–), 1.37–1.30 (m, 4H,–CH_2–); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.81, 143.53, 135.29, 134.04, 107.39, 102.19, 101.27, 56.57, 54.09, 49.28, 30.05, 27.30; purity: 100% by HPLC. HRMS (ESI): calcd for $(M + H)^+$ ($C_{24}H_{32}N_2O_6$) requires m/z 445.2339, found 445.2340.

N^1,N^7-bis((7-methoxybenzo[d][1,3]dioxol-5-yl)methyl) heptane-1,7-diamine (5e)

Colorless oil. MS (ESI, m/z): $[M + H]^+$ 459.0; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.51 (s, 4H, Ar–H), 5.94 (s, 4H,–OCH_2O–), 3.90 (s, 6H,–OCH_3), 3.68 (s, 4H,–CH_2–), 2.59 (t, J=7.2 Hz, 4H,–CH_2–), 1.54–1.46 (m, 6H,–CH_2–), 1.37–1.30 (m, 6H,–CH_2–); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.79, 143.52, 135.27, 134.03, 107.34, 102.20, 101.28, 56.56, 54.10, 49.33, 30.03, 29.46, 27.30; purity: 99.8% by HPLC. HRMS (ESI): calcd for $(M + H)^+$ ($C_{25}H_{34}N_2O_6$) requires m/z 459.2495, found 459.2493.

N^1, N^8-bis((7-methoxybenzo[d][1,3]dioxol-5-yl)methyl)octane-1,8-diamine (5f)

Colorless oil. MS (ESI, m/z): $[M + H]^+$ 473.1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.51 (s, 4H, Ar–H), 5.93 (s, 4H,–OCH_2O–), 3.89 (s, 6H,–OCH_3), 3.67 (s, 4H,–CH_2–), 2.59 (t, J=7.2 Hz, 4H,–CH_2–), 1.61 (s, 2H,–CH_2–), 1.54–1.44 (m, 4H,–CH_2–), 1.33–1.25 (m, 8H,–CH_2–); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.77, 143.49, 135.24, 133.99, 107.32, 102.18, 101.26, 56.55, 56.48, 54.07, 49.32, 30.04, 29.47, 27.28; purity: 99.9% by HPLC. HRMS (ESI): calcd for $(M + H)^+$ ($C_{26}H_{36}N_2O_6$) requires m/z 473.2652, found 473.2652.

N^1,N^9-bis((7-methoxybenzo[d][1,3]dioxol-5-yl)methyl) nonane-1,9-diamine (5g)

Colorless oil. MS (ESI, m/z): $[M + H]^+$ 487.2; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.51 (s, 4H, Ar–H), 5.93 (s, 4H,–OCH_2O–), 3.89 (s, 6H,–OCH_3), 3.68 (s, 4H,–CH_2–), 2.59 (t, J=7.2 Hz, 4H,–CH_2–), 1.67 (s, 2H,–CH_2–), 1.56–1.40 (m, 4H,–CH_2–), 1.32–1.25 (s, 10H,–CH_2–); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.79, 143.51, 135.24, 134.02, 107.36, 102.21, 101.28, 56.55, 54.08, 49.34, 30.05, 29.52, 29.49, 27.34; purity: 99.7% by HPLC. HRMS (ESI): calcd for $(M + H)^+$ ($C_{27}H_{38}N_2O_6$) requires m/z 487.2808, found 487.2807.

Pharmacology

All the synthesized compounds were screened for AChE and BuChE inhibition activities. AChE (E.C. 3.1.1.7, from *electric eel*), BuChE (E.C. 3.1.1.8, from *equine serum*), 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB), butyrylthiocholine chloride and acetylthiocholine chloride (ATC) were purchased from Sigma–Aldrich and rivastigmine hydrochloride standard was purchased from Sunve (Shanghai) Pharmaceutical Co., Ltd. Curcumin used in this work were synthesized and characterized in our laboratory[19].

Enzyme inhibition assays

All the assays were under 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 8.0, using a Shimadzu 2450 Spectrophotometer. Enzyme solutions were prepared to give 2.0 units/mL in 2 mL aliquots. The assay medium contained phosphate buffer, pH 8.0 (1 mL), 50 µL of 0.01 M DTNB, 10 µL of enzyme, and 50 µL of 0.01 M substrate (ATC). The substrate was added to the assay medium containing enzyme, buffer, and DTNB with inhibitor after 15 min of incubation time. The activity was determined by measuring the increase in absorbance at 412 nm at 1 min intervals at 37°C. Calculations were performed according to the method of the equation in Ellman et al.[20]. *In vitro* BuChE assay use the similar method described above.

Kinetic characterization of AChE was performed using a reported method. Six different concentrations of substrate were mixed in the 1 mL 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.0), containing 50 µL of DTNB, 10 µL AChE, and 50 µL substrate. Test compound was added into the assay solution and pre-incubated with the enzyme at 37°C for 15 min, followed by the addition of substrate. Kinetic characterization of the hydrolysis of ATC catalyzed by AChE was done spectrometrically at 412 nm. A parallel control with no inhibitor in the mixture, allowed adjusting activities to be measured at various times.

Inhibition of self-mediated Aβ aggregation

The thioflavin-T fluorescence method was used[21]. Aβ42 peptide sodium (Anaspec Inc.) was dissolved in phosphate buffer (pH 7.40, 0.01 M) to obtain a 20 µM solution. Compounds were first prepared in dimethyl sulfoxide (DMSO) to obtain a 10 mM solution. The final concentration of Aβ42 and inhibitors was 20 µM. After incubated in 37°C for 24 h, thioflavin-T (5 µM in 50 mM

glycine–NaOH buffer, pH 8.00) was added. Fluorescence intensity method was carried out (excitation at 450 nm and emission at 485 nm). Each inhibitor was run in triplicate. The fluorescence intensities were recorded, and the percentage of inhibition on aggregation was calculated by the following expression: $(1 - I_{Fi}/I_{Fc}) \times 100\%$ in which I_{Fi} and I_{Fc} were the fluorescence intensities obtained for absorbance in the presence and absence of inhibitors, respectively, after subtracting the fluorescence of respective blanks.

MTT (methyl thiazolyl tetrazolium) assay of SH-SY5Y cell viability

SH-SY5Y cells, at passages between 3 and 16 after thawing, were maintained in Dulbecco's modified Eagle's medium containing 15 nonessential amino acids and supplemented with 10% fetal calf serum, 1 mM glutamine, 50 mg/μL penicillin, and 50 mg/μL streptomycin. Cultures were seeded into flasks containing supplemented medium and were maintained at 37°C in 5% CO_2 humidified air. For assays, SH-SY5Y cells were sub-cultured in 96-well plates at a seeding density of 10,000 cells per well. After 24 h, they were placed into serum-free medium and treated with compounds. After 48 h, the survival of cells was determined by MTT assay. Briefly, after incubated with 20 μL of MTT (2.5 mg/mL) at 37°C for 4 h, living cells containing MTT formazon crystals were solubilized in 100 μL of DMSO. The absorbance of each well was measured by a microculture plate reader at the wavelength of 570 nm.

Results and discussion

Enzyme inhibition assays

All the newly synthesized compounds (**5a**–**5g**) were tested for their inhibition activities toward AChE and BuChE *in vitro* according to the modified Ellman method with commercially available rivastigmine as the reference standard. The ChE inhibition results were summarized in Table 1.

The results showed that most of the target compounds possessed ChE inhibition activity, and the AChE inhibitory potency of derivatives was closely related to the length of the alkylene chain. Compared with rivastigmine (IC_{50} = 5.50), **5e**, **5f**, and **5g** (IC_{50} = 4.24, 2.79, and 2.76 μM, respectively) showed slightly better AChE inhibitory activity, **5c** and **5d** (IC_{50} = 13.25 and 9.78 μM) showed lower inhibition potential, **5b** showed a quite weak inhibitory effect with a IC_{50} value of 119.96 μM, and **5a** was not active at 125μM. The results indicated that NBMA derivatives required chains of suitable length to bind at both the catalytic site and the peripheral site of the AChE.

Table 1. Inhibition activity of AChE and BuChE.

Compound	Chain length (n)	IC_{50} (μM)±SEM AChE[1]	IC_{50} (μM)±SEM BuChE[2]
5a	3	>125	52.31 ± 1.52
5b	4	119.96 ± 5.04	12.13 ± 0.51
5c	5	13.25 ± 1.19	3.35 ± 0.10
5d	6	9.78 ± 0.22	2.07 ± 0.11
5e	7	4.24 ± 0.08	3.02 ± 0.18
5f	8	2.79 ± 0.02	3.11 ± 0.06
5g	9	2.76 ± 0.02	5.14 ± 0.31
Rivastigmine		5.50 ± 1.50	1.60 ± 0.03

[1]AChE from *electric eel*, IC_{50}, 50% inhibitory concentration (means ± SEM of three experiments) of AChE.
[2]BuChE from *equine serum*, IC_{50}, 50% inhibitory concentration (means ± SEM of three experiments) of BuChE.

Compared with AChE activity, the BuChE inhibitory potency was less impacted by the chain length. The majority of compounds (**5c**–**5f**) showed a similar inhibition activity with rivastigmine (IC_{50} = 1.60 μM), the highest potency (IC_{50} = 2.07 μM) was achieved in **5d** with six methylene groups between two aromatic units.

Most NBMA derivatives showed greater selectivity for BuChE. The reason seems to be the enzymic conformational difference that relatively small residues line the

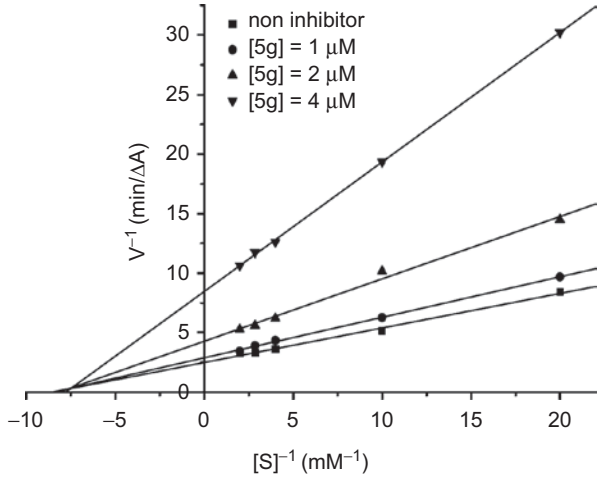


Figure 1. Lineweaver–Burk plot for the inhibition of acetylcholinesterase by **5g**.

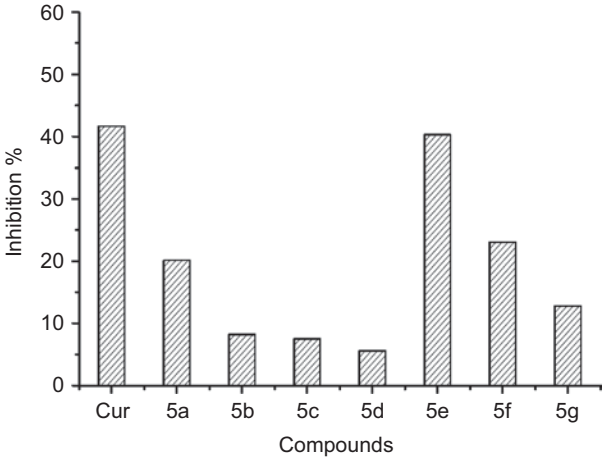


Figure 2. Compounds **5a**–**5g** $A\beta_{1-42}$ fibril inhibition compared with that of curcumin. The test was conducted in the presence of 20 μM compounds.

Table 2. MTT assay of SH-SY5Y cell viability.

Compound	5a	5b	5c	5d	5e	5f	5g	Curcumin
Chain length (n)	3	4	5	6	7	8	9	-
IC_{50} (μM)	19.38	12.79	13.44	9.16	>50	>50	>50	40.00

gorge in BuChE, this allow bulky group to better fit inside the gorge of BuChE[2]. And recently, BuChE was considered a potential target, because it also plays an important role in regulating acetylcholine levels[22]. The concurrent inhibition of both AChE and BuChE was shown to provide additional benefits in AD, such as rivastigmine[23]. In this study **5e**–**5g** had significant inhibition at similar lever with rivastigmine on both enzymes.

The nature of AChE inhibition caused by a representative compound **5g** was investigated by the graphical analysis of steady-state inhibition data (Figure 1). The Lineweaver–Burk plots showed both increasing slopes and increasing intercepts with higher inhibitor concentration. The pattern indicated the mixed-type inhibition that was similar to that of tacrine. The result revealed that compound **5g** was able to bind both of the catalytic site and the peripheral site of AChE.

Inhibition of self-mediated Aβ aggregation

All the target compounds were tested for their ability to inhibit self-mediated aggregation of Aβ(1-42) by using a thioflavin T fluorescence method[21]. Compared with the reference compound curcumin, the results showed that all the target compounds significantly inhibit Aβ aggregation at 20μM, with percentages of inhibition ranging from 5 to 40% (Figure 2). The compound **5e** was found to be most potent. And the activity of the compounds did not depend on the chain length of the connecting linker.

MTT assay of cell viability

The toxicity of synthesized compounds was determined in human neuroblastoma cell line SH-SY5Y. Results indicated that the most potent three inhibitors, **5e**, **5d**, and **5g**, showed no obvious effect on cell viability at concentrations of 50 μM, as shown in Table 2. Compared with curcumin, they had a lower toxicity on cell viability.

Conclusions

In summary, seven of NBMA compounds **5a**–**5g** were designed, synthesized and evaluated for cholinesterase, self-induced Aβ aggregation inhibition activities and human neuroblastoma cell viability. Results indicated that their AChE and BuChE inhibition activities were comparable with or stronger than that of rivastigmine. The inhibition activity of AChE was greatly influenced by the length of the alkylene chain. Besides, compounds **5e**, **5f**, and **5g** potently significantly inhibited self-induced Aβ aggregation. And compounds **5e**–**5g** did not show significant cell cytotoxicity for neuroblastoma cell at concentrations of 50 μM. Compound **5e** may be considered to be novel multipotent anti-Alzheimer lead drugs and further pharmacological evaluations are in progress.

Declaration of interest

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