Discovery of novel isoflavone derivatives as AChE/BuChE dual-targeted inhibitors: synthesis, biological evaluation and molecular modelling

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ABSTRACT
AChE and BuChE are druggable targets for the discovery of anti-Alzheimer's disease drugs, while dual-inhibition of these two targets seems to be more effective. In this study, we synthesised a series of novel isoflavone derivatives based on our hit compound G from in silico high-throughput screening and then tested their activities by in vitro AChE and BuChE bioassays. Most of the isoflavone derivatives displayed moderate inhibition against both AChE and BuChE. Among them, compound 16 was identified as a potent AChE/BuChE dual-targeted inhibitor (IC50: 4.60 μM for AChE; 5.92 μM for BuChE). Molecular modelling study indicated compound 16 may possess better pharmacokinetic properties, e.g. absorption, blood–brain barrier penetration and CYP2D6 binding. Taken together, our study has identified compound 16 as an excellent lead compound for the treatment of Alzheimer's disease.

Introduction
Alzheimer's disease (AD) is one of the most common neurodegenerative diseases and accounts for more than 80% of dementia cases worldwide in elderly people. It is in particular characterised by a forebrain cholinergic neuronal loss, a progressive cognitive decline and neuropsychiatric cholinergic disturbances, which seems to be closely related to pathological formation of β-amyloid. Studies have demonstrated that memory impairment in patients with dementia is due to the selective and irreversible deficiency in the cholinergic functions. Cholinesterase is the enzyme which catalyses the hydrolysis of acetylcholine, and thus inhibition of cholinesterase can be an effective way for the treatment of AD.

Two types of the cholinesterase, namely, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) were discovered in the nervous system. However, AChE has a 107-fold higher acetylcholine hydrolytic activity than BuChE does under the same condition. Many AChE inhibitors were discovered, of which donepezil and galantamine (cf. Figure 1) have been approved by Food and Drug Administration (FDA). Nevertheless, the clinical use demonstrated they have modest memory and cognitive improving functions for patients with AD. Specifically, they only provide a symptomatic and palliative pharmacological effect instead of curing AD, and their effects would “wear off” after a certain period of treatment. Evidences suggested that additional inhibition of brain BuChE may become an important therapeutic strategy for AD treatment. It was reported that the BuChE had a key role that it could partly compensate for the action of AChE. In addition, studies indicated that a balanced inhibition of both AChE and BuChE may be beneficial for treating the cognitive deficits. Such multifunctional cholinesterase inhibitors have been identified so far and were showing positive clinical outcome for the treatment of AD. For instance, the AChE/BuChE dual-targeted inhibitor, i.e. a “blockbuster drug” rivastigmin (cf. Figure 1) displayed a more potent effect than donepezil.

In order to identify new classes of AChE/BuChE dual-targeted inhibitors, in silico high-throughput screening (HTS) was performed to screen our in-house chemical library that contained more than 30,000 compounds. On the top-ranked compound list, an isoflavone derivative G (cf. Figure 2) attracted our attention as it displayed favourable binding modes with both AChE and BuChE (cf. Supplementary Figure S1). To the best of our knowledge, the scaffolds of flavonoids and its closely related ones such as homoisolavonoids, trihydroxyflavone and trihydroxyflavone were privileged structures that showed beneficial effects on neurological disorders, e.g. anti-inflammatory, metal chelation, neuroprotection, Aβ fibril formation inhibition and free radical scavenging effect. In addition, a recent study revealed a few compounds structurally similar to G were AChE inhibitors. These evidences prompted us to test the activity of G against both AChE and BuChE. Interestingly, the compound G showed a dual-targeting effect, with an IC50 of 1.47 μM for AChE and 3.37 μM for BuChE. In this study, we explored its preliminary structure-activity relationship (SAR) by synthesising a series of novel isoflavone derivatives and testing their inhibitory effects on both AChE and BuChE. Then we carried out molecular docking to predict binding modes of isoflavone derivatives to AChE and BuChE. Moreover, we predicted the pharmacokinetic properties of this class of compounds in silico.
General procedure for the preparation of intermediates (a1–a4)
Formononetin (5 g, 18.6 mmol), finely grounded anhydrous K$_2$CO$_3$ (30 g, 270 mmol) and 1,2-dibromoethane (17.2 ml, 199 mmol) or 1,3-dibromopropane (20.1 ml, 199 mmol) or 1,4-dibromobutane (24 ml, 198 mmol) were added into 500 ml acetone solution. The mixture was refluxed for 10 h. The residues were then added into 100 ml water and stirred for 1 h and filtrated by suction. After that, the pastry was washed by 5 ml water for three times. The solid was finally dried in vacuum at 50 ℃ to give the desired product without further purification.

7-(2-Bromoethoxy)-3-(4-methoxyphenyl)-4H-chromen-4-one (a1)
The compound was obtained as white solid in 93.4% yield. Purity 98.5% (by HPLC); mp 177.7–179.6 ℃; $^1$H NMR (400 MHz, CDCl$_3$) δ 8.23 (d, J = 8.8 Hz, 1H), 7.92 (s, 1H), 7.50 (d, J = 8.8 Hz, 2H), 7.02–6.96 (m, 3H), 6.86 (d, J = 2.0 Hz, 1H), 4.39 (t, J = 6.4 Hz, 2H), 3.84 (s, 3H), 3.69 (t, J = 6.4 Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 175.9, 162.4, 159.8, 157.9, 152.2, 130.2, 128.2, 125.1, 124.2, 119.1, 114.7, 114.1, 101.2, 68.3, 55.5, 28.4. ESI-MS m/z 375.38 [M + H]$^+$.

7-(3-Bromopropoxy)-3-(4-methoxyphenyl)-4H-chromen-4-one (a2)
The compound was obtained as white solid in 91.5% yield. Purity 99.2% (by HPLC); mp 121.5–123.6 ℃; $^1$H NMR (400 MHz, CDCl$_3$) δ 8.22 (d, J = 8.8 Hz, 1H), 7.92 (s, 1H), 7.50 (d, J = 8.8 Hz, 2H), 7.02–6.91 (m, 3H), 6.87 (d, J = 2.4 Hz, 1H), 4.22 (t, J = 6.0 Hz, 2H), 3.84 (s, 3H), 3.63 (t, J = 6.0 Hz, 2H), 2.38 (m, J = 6.0 Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 179.6–179.4, 162.4, 159.8, 157.9, 152.2, 130.2, 128.0, 125.1, 124.3, 118.7, 114.8, 114.1, 100.9, 66.1, 55.5, 32.1, 29.7. ESI-MS m/z 389.34 [M + H]$^+$.

7-(4-Bromobutoxy)-3-(4-methoxyphenyl)-4H-chromen-4-one (a3)
The compound was obtained as white solid in 88.6% yield. Purity 98.7% (by HPLC); mp 152.8 ℃; $^1$H NMR (400 MHz, CDCl$_3$) δ 8.22 (d, J = 8.8 Hz, 1H), 7.91 (s, 1H), 7.50 (d, J = 8.8 Hz, 2H), 6.99–6.96 (m, 3H), 6.84 (d, J = 2.4 Hz, 1H), 4.10 (t, J = 6.0 Hz, 2H), 3.84 (s, 3H), 3.51 (t, J = 6.0 Hz, 2H), 2.14–1.98 (m, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 175.8, 163.2, 159.6, 157.9, 152.0, 130.1, 127.8, 124.9, 124.2, 118.5, 114.7, 114.0, 100.6, 66.7, 55.3, 33.2, 29.3, 27.6. ESI-MS m/z 403.31 [M + H]$^+$.

7-(4-Bromobutoxy)-5-hydroxy-3-(4-(2-bromoethoxy)phenyl)-4H-chromen-4-one (a4)
The compound was obtained as white solid in 91.5% yield. Purity 99.6% (by HPLC); mp 157.1 ℃; $^1$H NMR (400 MHz, CDCl$_3$) δ 12.87 (s, 1H), 7.88 (s, 1H), 7.46 (d, J = 8.8 Hz, 2H), 6.40 (dd, J = 2.0, 15.2 Hz, 2H), 4.36 (t, J = 6.0 Hz, 2H), 3.85 (s, 3H), 3.66 (t, J = 6.0 Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 181.0, 164.0, 163.1, 160.0, 158.0, 152.9, 130.2, 123.9, 123.0, 114.3, 106.8, 98.7, 93.2, 68.3, 55.5, 28.4. ESI-MS m/z 391.38 [M + H]$^+$.

7-(2-Bromoethoxy)-3-(4-(2-bromoethoxy)phenyl)-4H-chromen-4-one (a5)
The procedure for the preparation of a5 was the same with that for a1–a4, except that the formononetin was replaced with 5-hydroxy formononetin. The compound was obtained as white solid in 94.4% yield. Purity 99.6% (by HPLC); mp 157.1–159.2 ℃; $^1$H NMR (400 MHz, CDCl$_3$) δ 12.87 (s, 1H), 7.88 (s, 1H), 7.46 (d, J = 8.8 Hz, 2H), 6.98 (d, J = 8.8 Hz, 2H), 6.40 (dd, J = 2.0, 15.2 Hz, 2H), 4.36 (t, J = 6.0 Hz, 2H), 3.85 (s, 3H), 3.66 (t, J = 6.0 Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 124.9, 124.2, 118.5, 114.7, 114.0, 100.6, 66.7, 55.3, 33.2, 29.3, 27.6. ESI-MS (m/z) 467.44 [M + H]$^+$.
General procedure for the synthesis of compounds 1–15

Intermediate a1 (1.87 g, 5 mmol) or a2 (1.88 g, 5 mmol) or a3 (2.02 g, 5 mmol) or a4 (100 mg, 0.26 mmol) were dissolved in 150 ml acetonitrile or 70 ml DMF. Then K₂CO₃ (13.8 g, 100 mmol) and various substituted amino-group (16.4 mmol) were added. The mixture was stirred and refluxed for 3 h and monitored by TLC. When the reaction was finished, the reaction solution was poured into 600 ml water and stirred for 5 min. After that, the mixture was filtrated by suction and the pasty was washed by water, then, dried by vacuum at 50 °C to yield the desired products with chromatography, recrystallisation, or without any further purification.

5-Hydroxy-3-(4-methoxyphenyl)-7-(2-(piperidine-1-yl)ethoxy)-4H-chromen-4-one (1)
The compound was obtained as white solid in 85.7% yield. Purity 99.3% (by HPLC); mp 81.3–83.1 °C. ¹H NMR (400 MHz, CDCl₃) δ 12.38 (s, 1H), 7.86 (s, 1H), 7.46 (d, J = 8.4 Hz, 2H), 6.98 (d, J = 8.4 Hz, 2H), 6.40 (d, J = 13.2 Hz, 2H), 4.20 (brs, 2H), 3.84 (s, 3H), 2.83 (brs, 2H), 2.56 (brs, 4H), 1.65 (brs, 4H), 1.47 (brs, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 181.0, 164.9, 162.8, 159.9, 158.1, 152.8, 130.2, 123.8, 123.1, 114.2, 106.4, 983.9, 93.1, 66.8, 57.7, 55.5, 52.6, 26.0, 24.2. HRMS: calcd for C₂₃H₂₆NO₃[M + H]⁺, 396.1811; found: 396.1807.

3-(4-Methoxyphenyl)-7-(3-(piperidine-1-yl)propoxy)-4H-chromen-4-one (2)
The compound was obtained as white solid in 7% yield. Purity 98.8% (by HPLC); mp 152.8–154.0 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.19 (d, J = 8.8 Hz, 1H), 7.91 (s, 1H), 7.50 (d, J = 8.8 Hz, 2H), 6.99–6.96 (m, 3H), 2.86 (d, J = 2.0 Hz, 1H), 4.12 (t, J = 6.4 Hz, 2H), 3.84 (s, 3H), 2.50 (t, J = 7.2 Hz, 2H), 2.42 (brs, 4H), 2.07–2.00 (m, 2H), 1.64–1.58 (m, 3H), 1.48–1.44 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 175.9, 163.5, 159.6, 158.0, 152.0, 130.1, 127.7, 124.8, 124.3, 118.3, 114.9, 114.0, 100.6, 67.2, 55.7, 53.3, 54.7, 26.6, 26.0, 24.4. HRMS: calcd for C₂₃H₂₆NO₃[M + H]⁺, 394.2017; found: 394.2017.

7-(2-(5-Methoxy-1H-indol-3-yl)ethylamino)ethoxy)-4H-chromen-4-one (7)
The compound was obtained as yellow solid in 51.8% yield. Purity 99.9% (by HPLC); mp 236.0–238.3 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.14 (d, J = 0.8 Hz, 1H), 8.03 (d, J = 8.8 Hz, 1H), 7.46 (d, J = 8.8 Hz, 2H), 7.20 (d, J = 8.8 Hz, 1H), 7.05 (s, 1H), 7.01 (d, J = 2.4 Hz, 1H), 6.96 (d, J = 8.8 Hz, 2H), 6.89–6.85 (m, 2H), 6.72 (dd, J = 8.8, 2.4 Hz, 1H), 4.13 (t, J = 4.2 Hz, 2H), 3.81 (s, 3H), 3.75 (s, 3H), 3.03 (t, J = 4.2 Hz, 2H), 2.97 (t, J = 3.6 Hz, 4H); ¹³C NMR (100 MHz, CDCl₃) δ 177.9, 164.8, 161.1, 159.5, 154.9, 154.9, 133.1, 131.3, 128.9, 128.2, 125.9, 124.4, 119.2, 116.4, 113.0, 112.8, 112.6, 101.9, 101.2, 68.3, 56.2, 55.7, 50.3, 48.7, 26.0. HRMS: calcd for C₂₃H₂₃NO₅[M + H]⁺, 485.2076; found: 485.2077.

7-(2-(5-Methoxy-1H-indol-3-yl)ethylamino)propoxy)-3-(4-methoxyphenyl)-4H-chromen-4-one (8)
The compound was obtained as yellow solid in 51.8% yield. Purity 98.7% (by HPLC); mp 108.8–109.5 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.17 (d, J = 0.8 Hz, 1H), 8.04 (d, J = 8.8 Hz, 1H), 7.47 (d, J = 8.4 Hz, 2H), 7.21 (d, J = 8.4 Hz, 1H), 7.06–7.05 (m, 2H), 6.98 (d, J = 8.8 Hz, 2H), 6.90 (d, J = 2.0 Hz, 1H), 6.82 (dd, J = 8.8, 2.0 Hz, 1H), 6.75 (dd, J = 8.8, 2.0 Hz, 1H), 6.08 (t, J = 6.0 Hz, 2H), 3.82 (s, 3H), 3.80 (s, 3H), 2.98 (s, 4H), 2.87 (t, J = 6.8 Hz, 2H), 2.05–1.99 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 178.0, 165.0, 161.2, 159.6, 155.0, 154.9, 133.5, 131.4, 128.9, 128.2, 125.9, 125.4, 124.4, 119.1, 116.4, 114.9, 113.1, 112.7, 110.7, 101.7, 68.4, 56.3, 55.7, 50.7, 47.5, 29.3, 25.8. HRMS: calcd for C₂₃H₂₃NO₅[M + H]⁺, 499.2233; found: 499.2231.

7-(2-(5-Methoxy-1H-indol-3-yl)ethylamino)butoxy)-3-(4-methoxyphenyl)-4H-chromen-4-one (9)
The compound was purified by silica column (CH₂Cl₂:CH₃OH= 400: 9) and obtained as yellow solid in 26.1% yield. Purity 99.2% (by HPLC); mp 76.8–79.5 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.14 (s, 1H), 8.06 (d, J = 6.8 Hz, 1H), 7.45 (d, J = 8.4 Hz, 2H), 7.21
The compound was obtained as pale yellow solid in 54.1% yield. Purity 98.8% (by HPLC); mp 135.0, 174.6, 162.9, 159.0, 157.4, 153.5, 138.9, 130.1, 128.1, 126.9, 114.9, 113.0, 112.7, 101.8, 96.3, 65.3, 53.7, 50.7, 49.9, 27.7, 26.8, 25.9. HRMS calcld for C_{13}H_{13}N_{2}O_{4} [M + H]^+ 513.2389, found: 513.2380.

7-(2-Benzyl(methyl)amino)ethoxy)-3-(4-methoxyphenyl)-4H-chromen-4-one (10)

The compound was obtained as white solid in 76.1% yield. Purity 98.8% (by HPLC); mp 135.0–136.6 °C. 1^H NMR (400 MHz, DMSO-d$_6$) δ: 8.42 (s, 1H), 8.02 (d, J = 8.8 Hz, 1H), 7.53 (d, J = 8.4 Hz, 2H), 7.32–7.42 (m, 5H), 7.18 (s, 1H), 7.08 (d, J = 8.8 Hz, 1H), 7.00 (d, J = 8.4 Hz, 2H), 4.26 (t, J = 5.6 Hz, 2H), 3.79 (s, 3H), 3.59 (s, 2H), 2.79 (t, J = 5.6 Hz, 2H), 2.25 (s, 3H); 13^C NMR (100 MHz, DMSO-d$_6$) δ 174.6, 162.9, 159.0, 157.4, 153.5, 139.1, 130.1, 128.1, 126.9, 126.9, 124.1, 123.4, 117.6, 115.1, 113.6, 101.1, 66.8, 61.6, 55.1, 54.9, 42.3. HRMS: calcld for C$_{26}$H$_{28}$NO$_4$ [M + H]^+ 416.1862, found: 416.1855.

7-(2-Dibenzylaminethoxy)-3-(4-methoxyphenyl)-4H-chromen-4-one (11)

The crude product was recrystallised by ethyl acetate. The final product was obtained as white solid in 49.9% yield. Purity 98.4% (by HPLC); mp 118.9–120.0 °C. 1^H NMR (400 MHz, DMSO-d$_6$) δ: 8.42 (s, 1H), 8.02 (d, J = 8.8 Hz, 1H), 7.53 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 7.6 Hz, 4H), 7.32 (d, J = 7.6 Hz, 4H), 7.23 (t, J = 7.2 Hz, 2H), 7.09 (s, 1H), 7.05–6.99 (m, 3H), 4.25 (t, J = 5.6 Hz, 2H), 3.79 (s, 3H), 3.68 (s, 4H), 2.83 (t, J = 5.6 Hz, 2H); 13^C NMR (100 MHz, DMSO-d$_6$) δ 174.6, 162.8, 159.0, 157.4, 153.4, 139.2, 130.0, 128.5, 128.2, 126.9, 126.9, 124.1, 123.3, 117.5, 115.1, 113.6, 100.1, 66.7, 57.9, 55.1, 51.2. HRMS: calcld for C$_{22}$H$_{24}$NO$_4$ [M + H]^+ 352.1543, found: 352.1541.

7-(2-(Piperidin-1-yl)ethoxy)-3-(4-(2-piperidin-1-yl)ethoxy)phenyl)-4H-chromen-4-one (16)

Intermediate a5 (932 mg, 2 mmol) was dissolved in 50 ml acetonitrile. Then K$_2$CO$_3$ (4.60 g, 33.2 mmol) and piperidine (0.31 mmol) were added. The mixture was refluxed and stirred overnight. When the reaction ended, the reaction mixture was poured into 200 ml water and stirred for 30 min. After that, the mixture was filtered by suction and the pastry was washed by water (6 ml) for three times. The crude product was purified by 200–300 mesh silica column (CH$_2$Cl$_2$:CH$_3$OH = 20:3). The purified compound was obtained as white solid in 13.6% yield. Purity 99.6% (by HPLC); mp 148.7–152.2 °C. 1^H NMR (400 MHz, CDCl$_3$) δ 8.19 (d, J = 9.2 Hz, 1H), 7.91 (s, 1H), 7.48 (t, J = 8.8 Hz, 2H), 7.00–6.96 (m, 3H), 6.86 (d, J = 2.4 Hz, 1H), 4.20 (t, J = 6.0 Hz, 2H), 4.15 (t, J = 6.0 Hz, 2H), 2.84–2.79 (m, 4H), 2.53 (brs, 8H), 1.65–1.59 (m, 8H), 1.46 (brs, 4H); 13^C NMR (100 MHz, CDCl$_3$) δ 176.0, 163.3, 158.9, 158.0, 152.2, 130.3, 127.9, 125.0, 124.4, 118.6, 115.0, 114.1, 100.8, 69.5, 55.5, 52.4, 51.1, 49.2, 43.1, 43.4, 39.8, 32.6, 30.5, 30.4. HRMS: calcld for C$_{28}$H$_{27}$N$_2$O$_4$ [M + H]^+ 477.2735, found: 477.2743.

(3-Hydroxyphenyl)-7-(2-(piperidin-1-yl)ethoxy)-4H-chromen-4-one (17)

The piperidine (4 ml, 80 mmol) which was diluted in THF (5 ml) was added dropwise into the chloroacetyl chloride (3.1 ml, 40 mmol) dissolved in THF (10 ml) and stirred for 10h at room temperature, the mixture was condensed by reduced pressure to dryness and the residue was dissolved in 50 ml acetonitrile. Then, for mononetin (1 g, 3.73 mmol) and K$_2$CO$_3$ (4.60 g, 33.2 mmol) were added into the solution. The reaction was refluxed overnight. When the reaction finished, the reaction solution was poured into...
200 ml water, stirred and filtrated by suction. The pastry was washed by water and purified by 200–300 mush silica column (CH₂Cl₂:CH₂Cl:OH = 100:1). The compound was obtained as pale yellow solid in 45.3% yield. Purity 99.0% (by HPLC); mp 144.8–145.8 °C; 1H NMR (400 MHz, CDCl₃) δ 8.16 (d, J = 8.8 Hz, 1H), 7.87 (s, 1H), 7.45 (d, J = 8.8 Hz, 2H), 6.99 (d, J = 8.8 Hz, 1H), 6.93–6.89 (m, 3H), 4.75 (s, 2H), 3.79 (s, 3H), 3.53 (brs, 2H), 3.42 (brs, 2H), 1.62–1.51 (m, 6H); 13C NMR (100 MHz, CDCl₃) δ 175.7, 165.0, 162.3, 159.5, 157.7, 152.2, 130.1, 127.0, 124.8, 124.1, 118.9, 114.6, 113.9, 101.4, 67.5, 55.3, 46.3, 43.2, 26.5, 25.5, 24.3. HRMS calcd for C₂₅H₂₅NO₅ [M + H]⁺, 424.2124, found: 424.2124.

3-(4-Methoxyphenyl)-7-(3-oxo-3-(piperidin-1-yl)propoxy)-4H-chromen-4-one (18)

K₂CO₃ (7.5 g, 54.3 mmol), 3-bromopropionic acid (6 g, 39.2 mmol) and formononetin (2 g, 7.4 mmol) were dissolved in 100 ml acetone and refluxed for 2 days. After that, the reactant was condensed by reduced pressure to dryness and then the residue was poured into 400 ml water. In order to precipitate the product, the solution should be acidified with HCl (1 N) to pH 5. Then, the precipitated white solid was filtrated by suction and washed with water (5 ml) for three times. 3-(3-(4-Methoxyphenyl)-4-oxo-4H-chromen-7-yl)oxy)propionic acid crude product (2.465 g) was precipitated white solid was filtrated by suction and washed with saturated K₂CO₃ and brine respectively. The organic phase was taken, dried with Na₂SO₄, filtrated by suction and the filtrate was dissolved in DMF (70 ml), then, piperidine (0.302 ml, 3.30 mmol) and hydrochloro-1-(3-dimethylaminopropyl)-3-ethylcarbodiimide salt were added and stirred overnight at room temperature. When the reaction finished, the reactant was poured into 200 ml water and extracted with CH₂Cl₂, washed with saturated K₂CO₃ and brine respectively. The organic phase was taken, dried with Na₂SO₄, filtrated by suction and the filtrate was condensed by reduced pressure. The residues were purified by silica column (petroleum ether:ethanol acetate = 2:1).

The final compound was obtained in 50 ml DMF, then, N,N-diisopropylethylamine (1.02 ml, 5.85 mmol), piperidine (0.5 ml, 5.1 mmol) and hydrochloro-1-(3-dimethylaminopropyl)-3-ethylcarbodiimide salt were added and stirred overnight at room temperature. When the reaction finished, the reactant was poured into 200 ml water and extracted with CH₂Cl₂, washed with saturated K₂CO₃ and brine respectively. The organic phase was taken, dried with Na₂SO₄, filtrated by suction and the filtrate was condensed by reduced pressure. The residues were purified by silica column (petroleum ether:ethanol acetate = 2:1). The final compound was obtained as pale yellow solid in 26.9% yield. Purity 99.5% (by HPLC); mp 161.0–163.0 °C; 1H NMR (400 MHz, CDCl₃) δ 8.19 (d, J = 8.8 Hz, 1H), 7.91 (s, 1H), 7.50 (d, J = 8.4 Hz, 2H), 6.96 (brd, J = 8.4 Hz, 3H), 6.90 (s, 1H), 4.42 (t, J = 6.0 Hz, 2H), 3.84 (s, 3H), 3.54 (brd, 4H), 2.89 (t, J = 6.0 Hz, 2H), 1.70–1.60 (m, 6H); 13C NMR (100 MHz, DMSO-d₆) δ 174.6, 167.6, 162.9, 159.0, 157.4, 153.5, 130.1, 126.9, 124.1, 123.3, 117.5, 115.0, 113.6, 101.0, 65.1, 55.1, 45.9, 42.0, 31.8, 26.0, 25.3, 24.0. HRMS: calcd for C₂₄H₂₆NO₆ [M + H]⁺, 424.2181, found: 424.1805.

2-(3-(3-Methoxyphenyl)-4-oxo-4H-chromen-7-yl)oxy)ethyl piperidine-1-carboxylate (19)

Intermediate a1 (0.748 mg, 2 mmol), K₂CO₃ (4.60 g, 33.3 mmol) was dissolved in DMF (70 ml), then, piperidine (0.302 ml, 3.30 mmol) was poured into the reactant liquid, stirred and refluxed for 3 h. When the reaction finished, the mixture was poured into water (300 ml), stirred for 30 min and filtrated by suction. The pastry was washed by water (5 ml) for three times and purified by 200–300 mush silica column eluted by CH₂Cl₂. The compound was obtained as pale yellow solid in 12.6% yield. Purity 99.1% (by HPLC); mp 104.5–109.4 °C; 1H NMR (400 MHz, CDCl₃) δ 8.19 (d, J = 8.8 Hz, 1H), 7.90 (s, 1H), 7.48 (d, J = 8.8 Hz, 2H), 7.00 (dd, J = 8.8, 2.0 Hz, 1H), 6.95 (d, J = 8.8 Hz, 2H), 6.86 (d, J = 2.0 Hz, 1H), 4.46 (t, J = 6.0 Hz, 2H), 4.27 (t, J = 6.0 Hz, 2H), 3.82 (s, 3H), 3.41 (brs, 4H), 1.56–1.51 (m, 6H); 13C NMR (100 MHz, CDCl₃) δ 175.1, 163.0, 159.5, 157.8, 155.0, 152.1, 130.1, 127.8, 124.8, 124.2, 118.6, 114.8, 113.9, 100.9, 67.0, 63.1, 55.3, 44.9, 25.6, 24.3. HRMS: calcd for C₂₄H₂₆NO₆ [M + H]⁺, 424.1760, found: 424.1751.

AChE/BuChE bioassay

To measure in vitro AChE/BuChE activity, modified Ellman’s method was performed using a 96-well plate reader (BioTek ELx808). AChE (0.5 U/mg) was extracted from rat cortex while BuChE (3.4 U/mg) was obtained from human plasma. Each well contained 50 μl potassium phosphate buffer (KH₂PO₄/K₂HPO₄, 0.1 M, pH 8.0), 25 μl test compounds and 25 μl enzyme. Notably, the test compounds were firstly dissolved in the mixture of 50% methanol and 50% DMSO, and eventually diluted so that the final concentration of both solvents was less than 1% in the assay. They were pre-incubated for 60 min at 37 °C, and then 125 μl 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, 3 mM in buffer) was added. Characterisation of the hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride catalysed by AChE/BuChE was performed spectrometrically at 412 nm, followed by the addition of substrate (acetylthiocholine iodide or butyrylthiocholine chloride 3 mM in water, respectively). The activity was determined by measuring the increase in absorbance at 412 nm after 15 min. For those compounds with inhibition rate >50% at the concentration of 50 μM, the IC₅₀ values were further determined. A control experiment was performed under the same condition without any inhibitor and the blank contained buffer, water, DTNB and substrate. The described method was also performed for BuChE bioassay. In the bioassay, donepezil (an AChE inhibitor) and tetrasisopropyl pyrophosphoramide (Iso-OMPA, a BuChE inhibitor) were used as positive controls of AChE and BuChE, respectively.

Molecular docking

In order to understand the protein-ligand interactions between the synthesised isoflavone derivatives and AChE/BuChE, the most potent inhibitor as an example was docked against AChE and BuChE. The ligand structure was built and prepared by DS 2016 (Discovery Studio version 2016, San Diego, CA, USA). Hydrogen atoms were added to the structure and its ionisation states at pH 7.3 to 7.5 were generated. Besides, its lowest-energy conformation was generated prior to docking. The X-ray structures of AChE (PDB ID: 5EIH) and BuChE (PDB ID: 4BDS) were retrieved from the Protein Data Bank. Both of them were high-resolution structures of protein-ligand complexes and their organisms were consistent with those of the enzymes used for bioassay. After stripping the cognate ligand from each complex, molecular docking was carried...
out by GOLD 3.0.1 in which the target protein was kept rigid, while the ligands were left flexible to explore the conformational space inside the binding site. After that, the docking poses of the ligand were visually inspected.

In silico prediction of pharmacokinetic properties

Pharmacokinetic properties, in particular blood–brain barrier penetration (BBB), are quite important for the drugs for the treatment of AD. Therefore, the pharmacokinetic properties of AChE/ BuChE inhibitors were predicted by the “ADMET Descriptors” module implemented in DS2016. The pharmacokinetic properties available in this module were aqueous solubility, BBB, CYP2D6 binding, hepatotoxicity, intestinal absorption and plasma protein binding.

Results and discussion

Chemistry

In total, 20 isoflavone derivatives (cf. Table 1) were synthesised and their chemical structures were confirmed by melting point, HR MS, 1H NMR and 13C NMR.

The synthetic route of new isoflavone derivatives was designed and depicted in Scheme 1 according to earlier reports.33,34 Briefly, the bromo-substituted intermediates a1–a5 were prepared through commercially available isoflavones and dibromoalkane in acetone in the presence of potassium carbonate. Reaction between a1–a5 and corresponding amines in acetonitrile afforded compounds 1–16. At first, we synthesised compounds 1–9 and evaluated their biological activity. Structure-activity relationship (SAR) of these compounds demonstrated the optimal length of the linker between the amino group and isoflavone core structure seems to be 2. Previous reports indicated that various amino groups can form a cation–π interaction with the aromatic amino acid residues (i.e. Trp84 in AChE and Try82 in BuChE) located in the catalysis active site of the cholinesterases.35 This observation prompted us to design and synthesise compounds 10–20 by substitution of different amino groups.

It is worth noting that carbamate substituted compounds 19 and 20 can be produced by the reaction of amines and bromo-substituted isoflavone in the presence of DMF and K2CO3 rather than the reported reaction of acyl chloride with hydroxyl-substituted isoflavone in acetonitrile. Interestingly, compound 17 can be synthesised by step 1 and step 2 as shown in Scheme 1, however this synthetic route was not suitable for compound 18 because of the poor reactive activity of 3-chloro-1-(piperidin-1-yl)propan-1-one which has a longer distance between reactive site and amide compared to 2-chloro-1-(piperidin-1-yl) ethan-1-one. Therefore, the intermediate 3-{3-(4-methoxyphenyl)-4H-chromogen-4-one-7-yloxy}propanoic acid was obtained by the reaction of formononetin with 3-bromopropionic acid. Piperidine was then added to the intermediate to produce the final compound 18.

In vitro inhibition of AChE and BuChE

Both in vitro AChE and BuChE inhibitory activity were evaluated for all the synthesised isoflavone derivatives. The results are shown in Table 2. To be noted donepezil and Iso-OMPA were used as the positive drugs.

As shown in Table 2, five isoflavone derivatives, i.e. 1, 3, 14, 15 and 16, showed both AChE and BuChE inhibitory effect. Notably,
compound 16 displayed equivalent inhibitory activity to two single-targeting drugs (Donepezil and Iso-OMPA) as well as our hit compound G.

Based on the chemical structures and their biological activity, it is clear that AChE and BuChE inhibitory activities changed markedly because of the change of amino substitutes. Compounds would manifest inhibitory effect when C7 was substituted by piperidine or N-methylethanamide. In the meanwhile, the length of the side chain also played a significant role in maintaining the inhibitory activity. Generally, compounds with side chains of two or four carbon atoms can display a dual-targeting inhibition whereas those with three carbon atoms selectively inhibited BuChE. Besides, compounds with a side chain of four carbon atoms showed stronger inhibition for BuChE than those with two carbon atoms. However, ester-substituted compounds displayed no AChE inhibitory effect. The alternative isoflavone core changed the inhibition level for both AChE and BuChE. C5 hydroxyl substitution improved the inhibitory effect. When C4 was substituted for 2-piperidineethoxyl group, the most potent AChE inhibitor 16 was obtained. All the information provided us with clues for further lead optimisation.

**Binding modes of isoflavone derivatives**

As compound 16 was the most potent AChE/BuChE dual-targeted inhibitor, it was selected as an example and used in the docking simulation. As shown in Figure 3, compound 16 was able to bind both AChE and BuChE. Regarding AChE, the piperidine moiety in C4 position occupied the “entrance cavity” by forming π-cation interaction with the key amino acid residues TRP286 and TYR341, while the isoflavone core structure was well accommodated in the catalytic cleft through π−π T-shaped interactions with PHE297/TYR341 and hydrogen bonds with TYR124/TYR337. Furthermore, the π-cation interaction between the piperidine ring in C7 position with TRP86, the salt bridge with GLU202 and the hydrogen bond with HIS447 also contributed to the stabilisation of protein–ligand interaction.

Protein–ligand interactions between compound 16 with BuChE were similar to that for AChE (cf. Figure 4). It deserved to mention that the piperidine moiety in C7 position formed a hydrogen bond with the key amino acid residue TYR332 which can also interact with the isoflavone structure through π−π stacking and π−π T-shape. Protonated amino group in C4 position can stabilise the complex through forming a salt bridge with both TRP86.

Table 2. *In vitro* inhibition of AChE and BuChE for compounds 1–20.

| Name   | Inhibition % at 50 μM | IC<sub>50</sub> (μM) |
|--------|----------------------|----------------------|
|        | AChE | BuChE | AChE | BuChE |
| G      | n.d. | n.d.  | 1.47 | 3.37  |
| 1      | 71.10| 95.74 | 10.3 | 4.12  |
| 2      | 28.34| 97.00 | n.d. | 23.2  |
| 3      | 85.65| 98.11 | 46.39| 11.1  |
| 4      | 58.66| 84.13 | 100.4| 141.3 |
| 5      | 25.38| 91.32 | n.d. | 103.6 |
| 6      | 63.76| 94.77 | 106.6| 68.08 |
| 7      | 3.68 | 45.74 | n.d. | n.d.  |
| 8      | 2.42 | 60.63 | n.d. | 15.6  |
| 9      | 0.72 | 37.92 | n.d. | n.d.  |
| 10     | 3.34 | 2.72  | n.d. | n.d.  |
| 11     | −6.27| −18.48| n.d. | n.d.  |
| 12     | 5.47 | −16.87| n.d. | n.d.  |
| 13     | 17.61| −6.62 | n.d. | n.d.  |
| 14     | 79.38| 99.86 | 9.75 | 7.66  |
| 15     | 75.22| 99.88 | 57.74| 7.19  |
| 16     | 100.34| 99.74 | 4.60 | 5.92  |
| 17     | 0.54 | −2.83 | n.d. | n.d.  |
| 18     | −2.36| 5.59  | n.d. | n.d.  |
| 19     | −11.56| 53.03 | n.d. | 9.59  |
| 20     | −4.84| −5.49 | n.d. | n.d.  |
| Donepezil | 64.82 | 94.28 | 1.05 | 5.78  |
| Iso-OMPA | n.d. | 94.28 | n.d. | 5.78  |

*a.n.d.: not determined.*

Scheme 1. The general procedure for the synthesis of compounds 1–20. (i): K<sub>2</sub>CO<sub>3</sub>, acetone, Br(CH<sub>2</sub>)<sub>n</sub>Br, 60°C; (ii): RH (amines), K<sub>2</sub>CO<sub>3</sub>, DMF/Acetonitrile, 100°C, 3 h; (iii): 40% HBr, 120°C, 3 h; (iv): piperidine, THF r.t. 10 h; (v): formononetin, K<sub>2</sub>CO<sub>3</sub>, acetone; (vi): K<sub>2</sub>CO<sub>3</sub>, 3-bromopropionic acid, acetone, 60°C, 2 days; (vii) DMF, N, N-diisopropylethylamine, piperidine, 12 h, r.t.
Predicted pharmacokinetic properties

As shown in Table 3, all the AChE/BuChE dual-targeted inhibitors reported in this study appeared to have poor solubility in aqueous media whereas possess good absorption. Like compound G, compounds 3, 14 and 16 were predicted to penetrate the BBB, which was a favoured property for drugs to treat neurodegenerative diseases, e.g., AD. Encouragingly, compound 16 may not bind to CYP2D6, which was different from other compounds, including the hit compound G. This unique feature would be beneficial for ensuring the efficacy of compound 16 and avoiding the side-effect. These data prove our hit-to-lead optimisation strategy seems to be also effective in optimising pharmacokinetic property.

Conclusion

In this study, we report the synthesis of a series of novel isoflavone derivatives based on our hit compound G identified by in silico HTS. The in vitro AChE/BuChE bioassay has shown 5 out of 20 isoflavone derivatives were AChE/BuChE dual-targeted inhibitors. SAR analysis has demonstrated the length of the side chain and the type of amino-substituted group played an essential role in AChE inhibition, whereas both factors had little effect on the BuChE inhibition. Among these derivatives, compound 16 possessed the greatest AChE inhibition as well as strong BuChE inhibitory activity. The molecular docking study demonstrated that π-cation and π-π interactions were essential for compound 16 to display its dual-targeting effect. In addition, the in silico prediction of pharmacokinetic properties has indicated 1) compounds 3, 14 and 16 were able to penetrate the BBB and 2) compound 16 may be more effective and less toxic due to no binding to CYP2D6. Taken together, compound 16 may warrant further development as a potential drug-like AChE/BuChE dual-targeted inhibitor for the treatment of AD.
Disclosure statement
The authors declare no conflict of interest in this work.

Funding
This work was partly supported by National Major Scientific and Technological Special Project for Significant New Drugs Development [2012ZX09301002-001, 2013ZX09402103 and 2014ZX09507003-002] and National Natural Science Foundation of China [81603027].

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