Tertiary amine derivatives of chlorochalcone as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitors: the influence of chlorine, alkyl amine side chain and α,β-unsaturated ketone group

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ABSTRACT

A new series of tertiary amine derivatives of chlorochalcone (4a–4l) were designed, synthesized and evaluated for the effect on acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). The results indicated that all compounds revealed moderate or potent inhibitory activity against AChE, and some possessed high selectivity for AChE over BuChE. The structure–activity investigation showed that the substituted position of chlorine significantly influenced the activity and selectivity. The alteration of tertiary amine group also leads to obvious change in bioactivity. Among them, IC50 of compound 4l against AChE was 0.17 ± 0.06 μmol/L, and the selectivity was 667.2 fold for AChE over BuChE. Molecular docking and enzyme kinetic study on compound 4l suggested that it simultaneously binds to the catalytic active site (CAS) and peripheral anionic site (PAS) of AChE. Further study showed that the pyrazoline derivatives synthesized from chlorochalcones had weaker activity and lower selectivity in inhibiting AChE compared to that of chlorochalcone derivatives.

Introduction

Alzheimer’s disease (AD), as one of most common diseases in the elderly population, is a chronic and progressive neurodegenerative disorder. Although the precise etiology of AD is not elucidated enough, acetylcholinesterase (AChE) inhibitors were confirmed to be as the primary drugs to slow down the progression of AD in the present.

In recent, a lot of tertiary amine derivatives originated from natural products were synthesized and evaluated as AChE inhibitors. The previous investigations in our laboratory suggested that chlorochalcone with tertiary amine side chain, such as dimethylamine, diethy lamine, dihydropyrrole or piperidine had more potent effects than other nitrogen-containing chalcones on inhibiting AChE10–12. In order to explore the influence of substituent on inhibiting AChE in chalcone derivatives, halogen atoms were considered to introduce into the chalcone scaffold. Chlorine was selected to modify the chalcone derivatives, considering chlorine exist in a lot of drugs in clinic application, such as chlorphenamine, chlorpro mazine, chloroquine, etc. In the investigations searching for AChE inhibitors, some chlorine-containing compounds revealed AChE inhibition properties.

In this study, a series of chlorochalcones with tertiary amine side chain were synthesized and structure–activity relationship investigation was performed to explore the influence of chlorine in inhibiting AChE. In addition, four pyrazoline compounds were synthesized from chlorochalcones and evaluated for the bioactivity to explore the influence of α,β-unsaturated ketone group. Compound 4l, which had the most potent inhibitory activity against AChE, was selected to perform the kinetic and molecular docking studies for exploring the binding mode or mechanism to AChE.

Materials and methods

Chemistry

All chemicals and reagents were of analytical reagent grade and used without further purification. The melting points were measured on a WRS-IA melting point detector. 1H NMR spectra were recorded on a Bruker 400 MHz instrument in CDCl3 with TMS as the internal reference. Mass spectra were obtained on Finnigan LCQ advantage MAX by electrospray ionization (ESI-MS). Infrared spectrum (IR) was obtained on Shimadzu Infinity-1 infrared spectrometer. The purity of compounds was checked by Shimadzu LC-20A high-performance liquid chromatography. Elemental analysis was performed by elemental analyzer.

General method for synthesis of 4-hydroxy-chlorochalcones (3a–3c)

Compounds 3a–3c were synthesized according to the relative references. 4-hydroxyacetophenone (1.36 g, 10 mmol) and ethanol (20 mL) were mixed and stirred in the flask for 10 min until the solid was dissolved. Then 30% NaOH (3 mL) was dropped into the mixture, followed by stirring for 30 min in ice bath. Then chloride benzaldehyde (11 mmol) dissolving in ethanol (5 mL) was dropped into the mixture, and the mixture continued to stir for about 36 h at 25°C monitoring by TLC until the reaction was completed. Then...
the pH of the mixture was adjusted to 2–3 by HCl (1 mol/L). The mixture was kept in ice bath for 2 h, followed by the appearance of precipitation. The precipitation was filtered and washed by distilled water, and then dried at 50 °C, refined by the recrystallization in anhydrous ethanol. In result, compounds 3a–3c were gained.

**4'-Hydroxy-2-chlorochalcone (3a)**

Light yellow solid was gained with yield of 80.7%. It was a known compound which was reported to have antitumor activity.\(^\text{17}\)

**4'-Hydroxy-3-chlorochalcone (3b)**

Light yellow solid, yield: 73.5%; m.p: 1 2 0 ~ 122 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\), ppm): 6.96–6.98 (2H, d, J = 8.0 Hz, 3'-H and 5'-H), 7.26–7.34 (2H, m, 4-H and 5-H), 7.54–7.81 (3H, m, x-H, 2-H and 6-H), 8.10–8.16 (3H, m, β-H, 2'-H and 6'-H), 11.10 (1H, s, OH). MS m/z (ESI): [M + H]^+ 259. IR (KBr) ν/cm\(^{-1}\): 3426, 3016, 1667, 2, 1617, 1583, 1446, 1340, 1288, 1215, 1172, 839 and 732. Anal. calcd for C\(_{19}\)H\(_{18}\)ClNO\(_2\): C, 69.19; H, 6.11; N, 3.61; O, 8.72.

**4'-Hydroxy -4-chlorochalcone (3c)**

Light yellow solid was gained with yield of 85.6%. It is a known compound which was reported to inhibit monoamine oxidases.\(^\text{18}\)

**General method for synthesis of 4-amino alkyl-chalcones (4a–4l)**

Compounds 4a–4l were synthesized according to the relative references.\(^\text{19}\) 4'-hydroxy chalcones (0.2620 g, 1 mmol) and potassium carbonate (0.4140 g, 3 mmol) were mixed in acetone (25 mL) in oil bath with continuous stirring at 56 °C for 30 min, then chloroethyldimethylamine hydrochloride, chloroethylthiethylamine hydrochloride, chloroethylylpyrrolidino hydrochloride (3 mmol) and sodium iodide (0.075 g, 0.5 mmol) were added into it. The mixture was refluxed overnight, filtered, and concentrated. The concentrate was extracted with CH\(_2\)Cl\(_2\) (3 x 30 mL). The CH\(_2\)Cl\(_2\) phase was washed by saturated NaHCO\(_3\) (2 x 30 mL), saturated salt water (3 x 30 mL), dried with sodium sulfate anhydrous, followed by concentrating in vacuum and then the residue was purified by silica gel to gain the product with dichloromethane/methanol (80:1) as the eluent.

**4'-Hydroxy-2-chlorochalcone (3a)**

Light yellow solid, yield: 81.6%; m.p: 7 6 ~77 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\), δ (ppm): 1.09 (6H, s, 2 x NCH\(_3\)), 2.92 (2H, t, J = 12.0 Hz, OCH\(_2\)CH\(_3\)), 4.14 (2H, t, J = 12.0 Hz, OCH\(_2\)CH\(_3\)), 6.98–7.00 (2H, m, 3'-H and 5'-H), 7.27–7.34 (2H, m, 3-H and 6-H), 7.43–7.77 (3H, m, x-H and 4-H and 5-H), 8.02–8.05 (2H, m, 2'-H and 6'-H), 8.15–8.19 (1H, d, J = 16.0 Hz, β-H). MS m/z (ESI): [M + H]^+ 330. IR (KBr) ν/cm\(^{-1}\): 2806, 2789, 1661, 1611, 1576, 1458, 1261, 1227, 1176, 1020 and 730. Anal. calcd for C\(_{19}\)H\(_{18}\)ClNO\(_2\): C, 69.19; H, 6.11; N, 4.25; O, 9.70; found C, 69.52; H, 6.37; N, 4.29; O, 9.24.

**4'-Hydroxy-3-chlorochalcone (3b)**

Light yellow solid, yield: 85.7%; m.p: 2 3 3 ~ 234 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\), δ (ppm): 2.37 (6H, s, 2 x NCH\(_3\)), 2.79 (2H, t, J = 12.0 Hz, OCH\(_2\)CH\(_3\)), 4.14 (2H, t, J = 12.0 Hz, OCH\(_2\)CH\(_3\)), 7.00–7.02 (2H, m, 3'-H and 5'-H), 7.38–7.40 (2H, m, 2-H and 6-H), 7.51 (1H, d, J = 16.0 Hz, x-H), 7.55–7.59 (2H, m, 3-H and 5-H), 7.73–7.77 (1H, d, J = 16.0 Hz, β-H), 8.02–8.04 (2H, m, 2'-H and 6'-H), MS m/z (ESI): [M + H]^+ 330. IR (KBr) ν/cm\(^{-1}\): 2805, 2774, 2359, 1658, 1595, 1558, 1456, 1246, 1227, 1175, 1020 and 731. Anal. calcd for C\(_{19}\)H\(_{18}\)ClNO\(_2\): C, 69.19; H, 6.11; N, 4.25; O, 9.70; found C, 68.98; H, 6.07; N, 4.28; O, 9.72.

**4'-Hydroxy -4-chlorochalcone (3c)**

Light yellow solid, yield: 85.6%. It is a known compound which was reported to inhibit monoamine oxidases.\(^\text{18}\)

**4'-Hydroxy-2-chlorochalcone (3a)**

Light yellow solid, yield: 85.6%. It is a known compound which was reported to have antitumor activity.\(^\text{17}\)

**4'-Hydroxy-3-chlorochalcone (3b)**

Light yellow solid, yield: 73.5%; m.p: 1 2 0 ~ 122 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\), δ (ppm): 1.12 (6H, s, 2 x NCH\(_3\)), 2.68–2.74 (4H, m, 2 x NCH\(_2\)CH\(_3\)), 2.96 (2H, t, J = 12.0 Hz, OCH\(_2\)CH\(_3\)), 4.17 (2H, t, J = 12.0 Hz, OCH\(_2\)CH\(_3\)), 6.98–6.99 (2H, d, J = 8.0 Hz, 3'-H and 5'-H), 7.26–7.35 (2H, m, 4-H and 5-H), 7.54–7.81 (3H, m, x-H, 2-H and 6-H), 8.10–8.16 (3H, m, β-H, 2'-H and 6'-H), MS m/z (ESI): [M + H]^+ 358. IR (KBr) ν/cm\(^{-1}\): 2970, 2810, 1661, 1508, 1337, 1246, 1173, 1024, 833 and 735. Anal. calcd for C\(_{24}\)H\(_{20}\)ClNO\(_2\): C, 70.48; H, 6.76; N, 3.91; O, 8.94; found: C, 70.63; H, 6.62; N, 3.85; O, 8.78.

**4'-Hydroxy-2-chlorochalcone (3c)**

Light yellow solid, yield: 85.6%. It is a known compound which was reported to have antitumor activity.\(^\text{17}\)

**4'-Hydroxy-2-chlorochalcone (3a)**

Light yellow solid, yield: 85.6%. It is a known compound which was reported to inhibit monoamine oxidases.\(^\text{18}\)

**4'-Hydroxy-3-chlorochalcone (3b)**

Light yellow solid, yield: 85.6%. It is a known compound which was reported to have antitumor activity.\(^\text{17}\)

**4'-Hydroxy -4-chlorochalcone (3c)**

Light yellow solid, yield: 85.6%. It is a known compound which was reported to inhibit monoamine oxidases.\(^\text{18}\)
2.77 (2H, t, J=12.0 Hz, OCH2CH3), 4.15 (2H, t, J=12.0 Hz, OCH2CH3), 6.98–7.00 (2H, m, 3’-H and 5’-H), 7.27–7.34 (2H, m, 3’-H and 6-H), 7.43–7.47 (3H, m, x-H and 4-H and 5-H), 8.02–8.05 (2H, m, 2’-H and 6’-H), 8.15–8.19 (1H, d, J=16.0 Hz, β-H). MS m/z (ESI): [M + H]+ 370. IR (KBr) ν/cm−1: 3065, 2978, 2149, 1644, 1580, 1510, 1479, 1421, 1269, 1243, 1211, 1175, 1163, 1028, 988 and 814. Anal. calcd for C24H22ClN3O2: C, 67.67; H, 6.63; N, 9.87; O, 7.51; found C, 67.78; H, 6.52; N, 9.81; O, 7.46.

1-(5-(2-Chlorophenyl)-3-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)phenyl)-4,5-dihydro-1H-pyrazol-1-yl) ethan-1-one (5b)

Yellow viscous liquid, yield: 74.2%. 1H NMR (400 MHz, CDCl3) δ (ppm): 1.84–1.87 (6H, m, piperidine-H), 2.69–2.73 (3H, s, COCH3), 2.96–2.99 (4H, m, piperidine-H), 3.09 (2H, t, J=12.0 Hz, OCH2CH3), 3.21 (1H, dd, J=4.0 Hz, 16.0 Hz, CH2-H4), 3.76 (1H, dd, J=12.0 Hz, 16.0 Hz, CH2-H6), 4.20 (2H, t, J=12.0 Hz, OCH2CH3), 5.76–5.81 (1H, m, NCH), 6.90–6.93 (2H, m, 3’-H and 5’-H), 7.13–7.35 (4H, m, 3-H and 4’-H and 5-H and 6-H), 7.63–7.67 (2H, m, 2’-H and 6’-H). MS m/z (ESI): [M + H]+ 412. IR (KBr) ν/cm−1: 3446, 3421, 3063, 3032, 2935, 2858, 2779, 1642, 1590, 1512, 1339, 1243, 1219, 1173, 1020, 980 and 726. Anal. calcd for C25H22ClN3O2: C, 67.60; H, 6.36; N, 10.20; O, 7.77; found C, 66.91; H, 6.48; N, 10.09; O, 7.84.

1-(5-(3-Chlorophenyl)-3-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)phenyl)-4,5-dihydro-1H-pyrazol-1-yl) ethan-1-one (5c)

Yellow viscous liquid, yield: 77.6%. 1H NMR (400 MHz, CDCl3) δ (ppm): 1.84–1.87 (6H, m, piperidine-H), 2.69–2.73 (3H, s, COCH3), 2.96–2.99 (4H, m, piperidine-H), 3.09 (2H, t, J=12.0 Hz, OCH2CH3), 3.13 (1H, dd, J=4.0 Hz, 16.0 Hz, CH2-H6), 3.76 (1H, dd, J=12.0 Hz, 16.0 Hz, CH2-H6), 4.20 (2H, t, J=12.0 Hz, OCH2CH3), 5.76–5.80 (1H, m, NCH), 6.83–6.85 (2H, m, 3’-H and 5’-H), 7.04–7.07 (4H, m, 2’-H and 4’-H and 5-H and 6-H), 7.66–7.70 (2H, m, 2’-H and 6’-H). MS m/z (ESI): [M + H]+ 412. IR (KBr) ν/cm−1: 3443, 3417, 3062, 3032, 2936, 2881, 1645, 1583, 1510, 1479, 1339, 1269, 1243, 1211, 1175, 1020, 980 and 735. Anal. calcd for C25H22ClN3O2: C, 67.60; H, 6.36; N, 10.20; O, 7.77; found C, 66.89; H, 6.45; N, 10.34; O, 7.61.

1-(5-(4-Chlorophenyl)-3-(4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)phenyl)-4,5-dihydro-1H-pyrazol-1-yl) ethan-1-one (5d)

Yellow viscous liquid, yield: 67.3%. The reaction mixture was cooled to room temperature and 30 mL CH2Cl2 was added into it. When the reaction was completed, the mixture was cooled to room temperature and 30 mL CH2Cl2 was added into it. The CH2Cl2 phase was washed by saturated K2CO3 solution, and dried by anhydrous sodium sulfate. After the solution was filtered and the solvent was evaporated under reduced pressure, the crude product was gained. The refined product was achieved by the silica column chromatography with the eluent (methanol: methylene chloride = 1:20).

Log p measurement

Log p, defined as the logarithm of octanol-water partition coefficient is an important parameter to evaluate the lipophilicity of compounds. It can be calculated by determining the concentration of compound in octanol phase and water phase until the partition equilibrium was completed. In present investigation, log p of compounds 4a–4l was measured by the shake flask method with slight modification, and PBS (pH = 7.4) was used as the water phase. The mobile phase was methanol: 0.1% triethylamine.
(TEA)/80:20 (v/v), at a flow rate of 1.0 mL min⁻¹ through a C₁₈ column (250 nm × 4.6 mm, 5 μm) at 32 °C with detect wavelength 318 nm. Experiments were conducted in triplicate and log p values were calculated.

**AChE and BuChE inhibition assay**

AChE/BuChE activity assays were conducted by Ellman method with light modification. The brain and serum of Sprague–Dawley rat was used as the resource of AChE and BuChE, respectively. Each compound was dissolved in Tween 80 and diluted with water to various concentrations immediately before use. The reaction mixture containing 40 μL AChE/BuChE, 100 μL acetylthiocholine iodide/S-Butyrylthiocholine iodide, 2.76 mL Na₂HPO₄/NaH₂PO₄ buffer (pH 8.0, 0.1 mol/L), and 100 μL different concentrations of test compounds were incubated at 30 °C for 25 min. Then the reaction was terminated via adding 100 μL 20% sodium dodecyl sulfate (SDS), then 100 μL 10 mmol/L 5, 5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) was added to generate the yellow anion 5-thio-2-nitrobenzoic acid. The absorbance of each assay mixture was measured at 412 nm by UV spectroscopy. The IC₅₀ values were calculated by Bliss method and expressed as mean ± SD of the replicates. Rivastigmine was used as the control drug.

**Kinetic studies**

Kinetic experiment was performed by a reported method with some modifications. Compound 4l was added into the assay solution and pre-incubated with the enzyme at 30 °C, followed by the addition of 100 μL acetylthiocholine iodide including five concentrations. The assay solution contained 100 μL compound 4l, 100 μL DTNB, 2.76 mL 0.1 mol/L Na₂HPO₄/NaH₂PO₄ buffer (pH 8.0). Kinetic profile of AChE was determined by UV spectrophotometer at 412 nm. Additionally, the blank control experiment was conducted with the vehicle to replace compound 4l solution.

**Molecular docking**

Molecular docking was performed by molecular operating environment (MOE) software package (Chemical Computing Group Inc., Montréal, Canada). The X-ray crystallographic structures of AChE (PDB code: 1EVE) and BuChE (PDB code: 1P01) were gained from Protein Data Bank (PDB) and Electron Density Map compilation (EMD) database, respectively. The X-ray crystallographic structures of AChE and BuChE were used as the active site of the protein after energy being minimized. The dock scoring in MOE software was done by ASE scoring function.

**Results and discussion**

**Chemistry**

Three different chlorobenzaldehydes (compounds 2a–2c) reacted with 4-hydroxyl chalcone in ethanol with sodium hydroxide as catalyst to generate three 4'-hydroxy–chlorochalcones (compounds 3a–3c). Then compounds 3a–3c reacted with four different alkyl amines to gain a series of amine alkyl – substituted chlorochalcone derivatives (compounds 4a–4l) in the presence of potassium carbonate, acetone and sodium iodide. The total synthetic route is shown in **Scheme 1**.

For the synthesis of 4'-hydroxy–chlorochalcones, the concentration of sodium hydroxide dramatically influences the yield and purity of the products. As a result, 30% sodium hydroxide was chosen as the catalyst. For the synthesis of compounds 4a–4l, the solvent for the reaction is important. In this reaction, acetone was selected as the solvent for its easy operation and low toxicity. In addition, sodium iodide was used as the catalyst to enhance the reaction activity and accelerate the reaction process. For the synthesis of different tertiary amine substituted chlorochalcones, the reaction time ranged from 5 to 8h. Pyrazoline compounds were synthesized from chlorochalcones, hydrazine and acetic acid (Scheme 2). High-yield desired compounds were gained via this reaction.

New synthetic compounds were characterized by proton nuclear magnetic resonance spectroscopy (¹H NMR), IR and mass spectrometry (MS) and elemental analysis. The purities of all synthesized compounds were confirmed to be higher than 98% by HPLC.

**Bioactivity evaluation**

Tertiary amine group, which is thought as a versatile pharmacophore appeared in the structures of many drugs in clinic practice, such as local anesthetics lidocaine, estrogen receptor modulator, tamoxifen, antipsychotic drug chlorpromazine and antimalarial chloroquine. Interestingly, in this investigation, some of synthesized compounds are known compounds. Compounds 3a and 3c were reported to be as anticancer agent and monoamine oxidase Inhibitor, respectively. Compounds 4d and 4f were patent protected compounds three decades ago (US Patent 4342782, US Patent 2668813), but there are no reports on the bioactivity of

![Scheme 1](image-url)
Table 1. AChE and BuChE inhibitory activity and log p of chlorochalcone derivatives.

| Compound | R¹ | R² | AChE (µmol/L) | BuChE (µmol/L) | Selectivity | Log p |
|----------|----|----|---------------|----------------|-------------|-------|
| 3a       | 2-Cl | –  | >500          | >500           | –           | –     |
| 3b       | 3-Cl | –  | >500          | >500           | –           | –     |
| 3c       | 4-Cl | –  | >500          | >500           | –           | –     |
| 4a       | 2-Cl | –  | 2.11 ± 0.38   | 41.12 ± 2.31   | 19.5        | 1.75  |
| 4b       | 3-Cl | –  | 3.76 ± 0.26   | 32.14 ± 7.16   | 8.5         | 1.83  |
| 4c       | 4-Cl | –  | 1.55 ± 0.16   | 43.62 ± 6.94   | 28.1        | 1.61  |
| 4d       | 2-Cl | –  | 5.42 ± 0.20   | 63.83 ± 9.21   | 11.8        | 1.63  |
| 4e       | 3-Cl | –  | 5.83 ± 0.42   | 80.21 ± 7.18   | 13.8        | 1.62  |
| 4f       | 4-Cl | –  | 3.78 ± 0.39   | 101.03 ± 17.23 | 26.7        | 1.69  |
| 4g       | 2-Cl | –  | 1.80 ± 0.48   | 35.62 ± 7.31   | 19.8        | 1.75  |
| 4h       | 3-Cl | –  | 1.31 ± 0.17   | 24.51 ± 6.14   | 18.7        | 1.72  |
| 4i       | 4-Cl | –  | 0.91 ± 0.09   | 114.21 ± 26.15 | 125.5       | 1.79  |
| 4j       | 2-Cl | –  | 0.81 ± 0.03   | 32.08 ± 5.71   | 39.6        | 1.85  |
| 4k       | 3-Cl | –  | 0.28 ± 0.05   | 31.06 ± 3.41   | 110.9       | 1.81  |
| 4l       | 4-Cl | –  | 0.17 ± 0.06   | 113.43 ± 18.22 | 667.2       | 1.83  |
| 5a       | 4-Cl | –  | 5.94 ± 0.27   | 97.64 ± 3.78   | 16.4        | –     |
| 5b       | 2-Cl | –  | 6.31 ± 0.16   | 29.91 ± 1.25   | 4.74        | –     |
| 5c       | 3-Cl | –  | 2.41 ± 0.34   | 27.62 ± 2.58   | 11.5        | –     |
| 5d       | 4-Cl | –  | 2.04 ± 0.26   | 108.03 ± 5.56  | 52.96       | –     |
| Rivastigmine | –  | –  | 10.54 ± 0.86  | 0.26 ± 0.08    | 0.025       | –     |

IC50: 50% inhibitory concentration (means ± SD of three experiments).
Selectivity for AChE is defined as IC50 (BuChE)/IC50 (AChE).
*Used for positive control.

Based on the data in Table 1, it seemed that the variation of amino-alkyl side chain markedly influence the inhibitory potency of compounds against AChE. Generally, the order of the inhibitory potency of these derivatives was as followed: pyrrolidine group > piperidine group > dimethylamine group > diethylamine group. Compounds 4j, 4k and 4l, which were substituted by pyrrolidine group, exhibited potent inhibitory activity with IC50 values less than 1.0 µmol/L. On other hand, the substituted position of chlorine atom in chalcone scaffold was very important for the inhibitory and selectivity for AChE. For dimethylamine or diethylamine substituted chlorochalcone derivatives the order of inhibitory potency against AChE was: Para > Meta > Ortho, while for piperidine or pyrrolidine substituted chlorochalcone derivatives the order was: Para > Ortho > Meta. In addition, all Para-substituted chlorochalcone derivatives had the highest selectivity in inhibiting AChE over BuChE. Among them, compound 4l had the highest selectivity as 667 fold. Compared with the tertiary amine derivatives of fluoro-chalcone in our previous report, compound 4l showed higher selectivity against AChE than that of fluoro-chalcone derivatives.

Compound 4l was selected for enzyme kinetic studies. The linear Lineweaver–Burk equation was applied to evaluate the inhibition profile. The graphical analysis of the steady-state inhibition data of compound 4l was shown in Supplement data: Figure 1. According to the analysis, compound 4l presented a mixed-type inhibition for that Km increased and Vmax decreased with the increasing of the concentration of compound 4l. The competitive inhibition constant (Kc) and the noncompetitive constant (Knc) were 0.38 and 2.95 µmol/L, respectively (Supplement data: Table 2).

To explore the possible interacting mode between the chlorochalcone derivatives and AChE, molecular docking was performed for compound 4l with software MOE2008. As shown in Supplement data: Figure 2, this compound exhibited a multiple points-binding mode with AChE (Supplement data: Figure 2A). In the top of the gorge, the aromatic moiety adopted an appropriate orientation for its binding to peripheral anionic site (PAS), via the π–π stacking interaction with Trp279. The side chain interacted with Tyr334 in the mid-gorge zone. In the bottom of the gorge, the nitrogen of pyrrolidine ring binds to Trp84 via a cation–π interaction with Tyr334. According to the analysis, compound 4l can only bind with BuChE via Tyr257 and Gly311 that were not the important amino acids in the catalytic process of BuChE (Supplement data: Figure 2B). These results may partially explain its potent inhibition and high selectivity for AChE. As a potential compound for treatment of AD, log p was thought as an important physical chemistry parameter to evaluate or predict the ability to cross blood brain barrier (BBB). It was reported that the log p with
optimum central nervous system (CNS) penetration was around 2 ± 0.7. As shown in Table 1, log \( p \) values of new synthesized compounds ranged from 1.61 to 1.83, which indicated that all the compounds had sufficiently lipophilicity to pass the BBB \textit{in vivo}.

In further study to explore the influence of chalcone scaffold on bioactivity, pyrazoline derivatives were synthesized from chlorochalcones. The bioactivity evaluation showed that the inhibition potency of pyrazoline derivatives against AChE dramatically decreased compared to that of chlorochalcone derivatives (Table 1). It indicated that \( \alpha,\beta \)-unsaturated ketone group in chalcone possibly play an important role for the inhibitory activity against AChE. In addition, the selectivity of pyrazoline derivatives to inhibiting AChE over BuChE also decreased significantly.

**Conclusion**

In the present investigation, a series of chlorochalcones and pyrazoline derivatives were synthesized and evaluated in inhibiting AChE and BuChE. The result showed that the substituted position of chlorine significantly influenced the activity and selectivity of compounds in inhibiting AChE. For those compounds with the same amine alkyl side chain, \textit{Para}-substituted chlorochalcone had the highest activity and selectivity. The pyrazoline derivatives synthesized from chlorochalcones with the cyclization of \( \alpha,\beta \)-unsaturated ketone group had weaker activity and lower selectivity in inhibiting AChE compared to that of chlorochalcone derivatives, suggesting that \( \alpha,\beta \)-unsaturated ketone group was important for inhibiting AChE. Among them, compound 41 revealed the strongest AChE inhibitory activity (IC\( _{50} \): 0.17 \( \mu \)mol/L) and highest selectivity (Ratio: 667.2). Enzyme kinetic study suggested that compound 41 was a mixed-type inhibitor. Molecular docking supported the mixed-type inhibition mechanism. Compound 41 might serve as a potential agent for the treatment of AD.

**Disclosure statement**

The authors confirm that this article content has no conflict of interest.

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