
Wen Luo*, Jiang Wang, Xin Zhang, Chen Hong and Chao-Jie Wang*

Key Laboratory of Natural Medicine and Immuno-Engineering, Henan University, Kaifeng 475004, P.R. China

Abstract

A series of genistein-polyamine conjugates (5a-5h) were designed, synthesized and evaluated as multi-functional anti-Alzheimer agents. The results showed that these compounds had significant cholinesterases (ChEs) inhibitory activity, compound 5b exhibited the strongest inhibition to acetylcholinesterase (AChE) with an IC₅₀ value of 2.75 µM, which was better than that of rivastigmine (5.60 µM). A lineweaver-burk plot and molecular modeling study showed that compound 5b targeted both the catalytic active site (CAS) and the peripheral anionic site (PAS) of AChE. Besides, compound 5b showed potent metal chelating ability. In addition, it was found that 5a-5h did not affect HepG-2 cell viability at the concentration of 10 µM.

Eight genistein-polyamine conjugates were synthesized as anti-Alzheimer agents with cholinesterases inhibition activity and potent metal chelating ability, and they showed low cytotoxicity against HepG-2 cell at 10 µM.

Keywords: Alzheimer’s disease; Genistein; Polyamine; Cholinesterase; Metal-chelating

Introduction

Alzheimer’s disease (AD), the most common form of neurodegenerative senile dementia, is associated with selective loss of cholinergic neurons and reduced level of acetylcholine neurotransmitter and it is characterized by memory deficit and progressive impairment of cognitive functions [1]. It affects millions of elderly people, and the number of patients is expected to increase in the next 20 years. Many factors have been found to be implicated in AD, such as low levels of acetylcholine, β-amyloid deposits, oxidative damage and metal ions, which seem to play significant roles in the disease [2]. Current treatment of AD focuses on increasing cholinergic neurotransmission in the brain by inhibiting cholinesterases (ChEs) with medicines including tacrine, donepezil, rivastigmine and galantamine [3]. Unfortunately, the potential effectiveness offered by the above inhibitors is often limited by the side effects. For example, clinical studies have shown that tacrine has hepatotoxic liability [4]. Due to the multi-pathogenesis of AD, one of the current strategies is to develop novel anti-AD agents with multiple potencies [5].

Genistein is a polyphenolic compound and belongs to the category of isoflavones, which is isolated from the dyer’s broom and Genista tinctoria [6]. It expresses a wide range of biological activities, such as antioxidant, anti-cancer and antimicrobial [7-9]. Recent years, it was reported that genistein showed neuroprotective effect and ameliorated learning and memory deficits in AD rat model [10,11]. Besides, a number of genistein derivatives have been reported as anti-AD agents in the past years (B-D Figure 1) [12,13]. These results indicate that genistein could be used as leading compound for the treatment of AD.

Polyamines are aliphatic molecules with amine groups distributed along their structure [14]. They have always been the concern of medicinal chemists as a universal template [15]. Our group has been involved in the development of polyamine conjugates as potential drugs for many years [16-19], and it was found that quinoline-polyamine conjugates exhibited potent ChEs inhibition activity, and it was shown that polyamine occupied the gorge of AChE [20]. Therefore, in the present study, in order to enhance the pharmacological potential of genistein, a series of genistein conjugates modified by polyamine were designed, synthesized as anti-Alzheimer agents.
Materials and Methods

Materials

$^1$H NMR spectra were recorded using TMS as the internal standard in DMSO or D$_2$O with a Bruker AV-400 spectrometer at 400 MHz. MS spectra were recorded on a Shimadzu LCMS-2010A instrument with an ESI mass selective detector. Elemental analyses were performed on a Gmbe VarioEL Elemental Instrument. Flash column chromatography was performed with silica gel (200-300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd.

Chemistry

The synthetic routes to target compounds are summarized in Scheme 1. The starting material genistein 1 was treated with ethyl 2-chloroacetate in acetonitrile to give intermediate 2, which was heated with K$_2$CO$_3$ in water and then acid by HCl yielded compound 3.

a) Synthesis of intermediate 2: Genistein (2.70 g, 10 mmol), ethyl 2-chloroacetate (1.47 g, 12 mmol), anhydrous K$_2$CO$_3$ (0.69 g, 5 mmol) and catalytic amount KI (0.05 g) was added in anhydrous acetonitrile (100 mL), and the mixture was reflux for 6 h. The solution was filtered and then acid by HCl to give light yellow solid (2.60 g, yield 73%), MS (ESI) m/z: 357.1 (M+H$^+$). White solid, m.p. 143–145 ºC, yield 49%. $^1$H NMR (400 MHz, D$_2$O) δ 7.99-7.89 (m, 1H, 2-H), 7.22 (s, 2H, 2'-H, 6'-H), 6.81 (s, 2H, 3'-H, 5'-H), 6.41-6.34 (m, 1H, 8-H), 6.23 (s, 1H, 6-H), 4.36 (s, 2H, 10-H), 3.52 (s, 2H, 13-H), 3.19 (s, 2H, 14-H), 2.58 (s, 6H, 16-H, 17-H). ESI-MS m/z: 384.4 (M+1$^+$). Anal. calcd for C$_{21}$H$_{21}$NO$_6$·0.1C$_2$H$_5$OH: C 65.52%, H 5.76%, N 3.60%; found C 65.44%; H 5.82%; N 3.43%.

b) Synthesis of intermediate 3: Compound 2 (0.71 g, 2 mmol), 5% Na$_2$CO$_3$, and DMSO (40 mL) was heated at 85 ºC for 10 h. Then the reaction mixture was poured into 10% HCl (300 mL) and stand for overnight, filtered and then acid by HCl to give light yellow solid (2.30 g, yield 70%).

c) General procedure for the synthesis of intermediates 4a-4h: Intermediate 3 (0.36 g, 1 mmol), 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 0.29 g, 1.5 mmol) and N-hydroxysuccinimide (NHS, 0.17 g, 1.5 mmol) were stirred at room temperature for 1.5 h, and then amines or Boc protected amines (1.2 mmol) were added, the mixture were stirred at room temperature overnight. The solvent was poured into water and extracted with ethyl acetate (20 mL×3), the solution was dried over anhydrous Na$_2$SO$_4$ and concentrated, the intermediates 4a-4h were purified by flash chromatography with chloroform/ methanol/ammonia (20:1:0.5%) elution.

d) General procedure for the synthesis of target compounds 5a-5h: The intermediates 4a-4h were dissolved in EtOH (10 mL) and stirred at 0 ºC for 10 min. Then 4 M HCl was added dropwise at 0 ºC. The reaction mixture was stirred at room temperature overnight. The solution typically gave a white solid as a precipitate. The solid was concentrated and washed several times with absolute ethanol and ether, and dried under vacuum to give the pure target compounds 5a-5h.

$N$-butyl-2-(5-hydroxy-3-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl)acetamide (5a). White solid, m.p. 180–183 ºC, yield 63%. $^1$H NMR (400 MHz, DMSO) δ 8.99 (s, 1H, 5-OH), 9.63 (s, 1H, 4'-OH), 8.43 (s, 1H, 2-H), 8.16 (s, 1H, 12-NH), 7.44-7.36 (m, 2H, 2'-H, 6'-H), 6.87-6.79 (m, 2H, 3'-H, 5'-H), 6.66 (d, J=2.3 Hz, 1H, 8-H), 6.45 (d, J=2.3 Hz, 1H, 6-H), 4.61 (s, 2H, CH$_2$CO-H), 3.13 (dd, J=13.0, 6.8 Hz, 2H, 14-H), 1.46-1.37 (m, 2H, 15-H), 1.26 (dd, J=15.1, 7.5 Hz, 2H, 13-H), 0.86 (t, J=7.3 Hz, 3H, 16-H). ESI-MS m/z: 399.4 (M+1$^+$). Anal: calcd for C$_{21}$H$_{21}$NO$_6$·HCl·0.1H$_2$O: C 56.52%, H 5.76%, N 5.79%; found C 56.44%; H 5.82%; N 5.58%.

$N$-(2-(dimethylamino)ethyl)-2-(5-hydroxy-3-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl)acetamide hydrochloride (5b). White solid, m.p. 143–145 ºC, yield 49%. $^1$H NMR (400 MHz, D$_2$O) δ 7.99-7.89 (m, 1H, 2-H), 7.22 (s, 2H, 2'-H, 6'-H), 6.81 (s, 2H, 3'-H, 5'-H), 6.41-6.34 (m, 1H, 8-H), 6.23 (s, 1H, 6-H), 4.36 (s, 2H, 10-H), 3.52 (s, 2H, 13-H), 3.19 (s, 2H, 14-H), 2.58 (s, 6H, 16-H, 17-H). ESI-MS m/z: 399.4 (M+1$^+$). Anal: calcd for C$_{33}$H$_{34}$N$_2$O$_4$·HCl: C 57.05%, H 5.43%, N 6.34%; found C 57.03%; H 5.52%; N 6.73%.

$N$-(3-aminopropyl)-2-(5-hydroxy-3-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yloxy)acetamide hydrochloride (5c). White solid, m.p. 187–189 ºC, yield 50%. $^1$H NMR (400 MHz, D$_2$O) δ 7.98 (s, 1H, 2-H), 7.27 (d, J=8.5 Hz, 2H, 2'-H, 6'-H), 6.87 (d, J=8.4 Hz, 2H, 3'-H, 5'-H), 6.38 (s, 1H, 8-H), 6.23 (d, J=2.0 Hz, 1H, 6-H), 4.31 (s, 10-H), 3.30 (t, J=6.8 Hz, 2H, 14-H), 3.00-2.93 (m, 2H, 13-H), 1.90-1.82 (m, 2H, 15-H). ESI-MS m/z: 385.4 (M+1$^+$). Anal: calcd for C$_{21}$H$_{21}$N$_2$O$_4$·HCl·0.5H$_2$O: C 49.64%, H 5.83%, N 5.79%; found C 49.69%; H 5.60%; N 5.58%.

$N$-(4-aminobutyl)-2-(5-hydroxy-3-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yloxy)acetamide hydrochloride (5d). White solid, m.p. 214–216 ºC, yield 48%. $^1$H NMR (400 MHz, D$_2$O) δ 7.83 (s, 1H, 2-H), 7.14 (s, 2H, 2'-H, 6'-H), 6.76 (d, J=7.9 Hz, 2H, 3'-H, 5'-H), 6.21 (s, 1H, 8-H), 6.08 (s, 1H, 6-H), 4.14 (s, 2H, 10-H), 3.12 (t, J=6.8 Hz, 2H, 13-H), 2.95-2.80 (m, 2H, 15-H), 1.90-1.82 (m, 2H, 14-H), 1.80-1.72 (m, 2H, 16-H). ESI-MS m/z: 399.4 (M+1$^+$). Anal: calcd for C$_{33}$H$_{34}$N$_2$O$_4$·HCl·0.5H$_2$O: C 49.64%, H 5.83%, N 5.79%; found C 49.69%; H 5.60%; N 5.58%.

Figure 1: Chemical structures of genistein and genistein derivative.
Enzyme inhibition assays

All the assays were under 0.1 M KHPO$_4$ 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), 50 µL of 0.01 M DTNB, 10 µL of enzyme, and 50 µL of 0.01 M substrate (Acetylthiocholine chloride). The substrate was added to the assay medium containing enzyme, buffer, and DTNB with inhibitor (0, 5, 10, 20, 35, 50 µM) 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. [21]. In vitro BChE assay used the similar method described above. The concentration of compound that effected 50% inhibition of ChEs activities (IC$_{50}$) were calculated by nonlinear regression of the inhibition ratio-concentration curve, using Origin 7.5 program.

Kinetic characterization of AChE inhibition

Kinetic characterization of AChE was performed using a reported method. Six different concentrations of substrate were mixed in the 1 mL 0.1 M KHPO$_4$ 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.

Molecular modeling

The crystal structure of the torpedo AChE (code ID: 1ZGB) was obtained in the Protein Data Bank after eliminating the inhibitor and water molecules. The 3D structure of compound 5b was prepared as similar as previously described [20].

Docking studies were carried out using the AUTODOCK 4.0 program using ADT. The enzyme structure was used as an input for the AUTOGGRID program. AUTOGGRID performed a calculated atomic affinity grid maps for each atom type in the ligand plus an electrostatics water molecules. The 3D structure of compound 5b was prepared as similar as previously described [20].

Flexible ligand docking was performed for the compounds. Docking calculations were carried out using the Lamarckian genetic algorithm (LGA) and all parameters were the same for each docking.

Metal-chelating study

The chelating studies were made in water using a UV-vis spectrophotometer (SHIMADZU UV-2450PC). The absorption spectral of compound 5b (20 µM), alone or in the presence of FeCl$_3$, CuSO$_4$ or ZnCl$_2$ (20 µM) was recorded with wavelength ranging from 200 to 800 nm.
200 to 500 nm after incubating for 30 min at room temperature. The final volume of reaction mixture was 1 mL, and the final concentrations of tested compound and metals were 20 µM.

**MTT assay of HepG-2 cell viability**

Cells were cultured at 37 °C under a 5% CO₂ atmosphere. The antiproliferative ability of compounds was evaluated in HepG-2 cells by the conversion of MTT to a purple formazan precipitate as previously described [19]. Cells were seeded into 96-well plates at 5×10³ cells/well. After 12 h, 10 µM of compounds were subsequently added and incubated for 48 h. The inhibition rate was calculated from plotted results using untreated cells as 100%.

**Results and Discussion**

**Enzyme inhibition assays**

All the newly synthesized compounds (5a-5h) were screened against AChE and BChE in vitro according to the modified Ellman method. Rivastigmine was used as control. The ChEs inhibition results were listed in Table 1 as the inhibition ratio at a tested concentration of 50 µM (Table 1). We also tested the IC₅₀ value of compounds 5b and 5h (Table 2).

The results showed that all of the target compounds possessed ChEs inhibition activity, and compound 5b exhibited the strongest inhibition to AChE with an IC₅₀ value of 2.75 µM which was better than rivastigmine (5.60 µM), compound 5h also showed good activity with IC₅₀ values of 46.59 µM. Genistein, the parent molecular, inhibited the AChE activity to less than 50% at the concentration of 100 µM (Table 2), it indicated that conjugation polyamines with genistein could increase the inhibition activity of AChE. Besides, it seems that AChE inhibitory potency of conjugates was closely related to the length and the end group of the polyamine chain. Compounds (5b, 5c and 5d) modified by diamine were more active than compounds conjugated with monoamine and triamine.

In the assay of BChE inhibtion studies, compound 5h showed the most potent inhibition for BChE with 39.20% inhibition rate at the concentration of 50 μM. These compounds showed quite weaker inhibitory effect than AChE. The result indicated that these genistein derivatives maybe favor binding to AChE, which was in agreement with the literature report [12].

<table>
<thead>
<tr>
<th>Compd.</th>
<th>R</th>
<th>X</th>
<th>Inhibition ratios for AChE (%) a</th>
<th>Inhibition ratios for BChE (%) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td></td>
<td>0</td>
<td>7.88 ± 3.58</td>
<td>1.19 ± 0.59</td>
</tr>
<tr>
<td>5b</td>
<td></td>
<td>1</td>
<td>90.40 ± 1.23</td>
<td>26.05 ± 2.52</td>
</tr>
<tr>
<td>5c</td>
<td></td>
<td>1</td>
<td>37.02 ± 2.07</td>
<td>13.77 ± 1.05</td>
</tr>
<tr>
<td>5d</td>
<td></td>
<td>1</td>
<td>34.49 ± 2.76</td>
<td>9.02 ± 0.23</td>
</tr>
<tr>
<td>5e</td>
<td></td>
<td>2</td>
<td>19.28 ± 1.87</td>
<td>11.72 ± 0.82</td>
</tr>
<tr>
<td>5f</td>
<td></td>
<td>2</td>
<td>10.19 ± 0.44</td>
<td>9.52 ± 1.48</td>
</tr>
<tr>
<td>5g</td>
<td></td>
<td>2</td>
<td>16.54 ± 1.36</td>
<td>17.88 ± 2.83</td>
</tr>
<tr>
<td>5h</td>
<td></td>
<td>2</td>
<td>51.04 ± 0.55</td>
<td>39.20 ± 5.64</td>
</tr>
<tr>
<td>Rivastigmine</td>
<td>-</td>
<td>-</td>
<td>86.45 ± 0.71</td>
<td>94.60 ± 3.19</td>
</tr>
</tbody>
</table>

Table 1: Inhibitory activity of target compounds for AChE and BChE.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>IC₅₀ AChE (µM) a</th>
<th>IC₅₀ BChE (µM) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>5b</td>
<td>2.75 ± 0.28</td>
<td>&gt;50</td>
</tr>
<tr>
<td>5h</td>
<td>46.59 ± 3.87</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Genistein</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Rivastigmine</td>
<td>5.60 ± 1.50</td>
<td>1.65 ± 0.05</td>
</tr>
</tbody>
</table>

IC₅₀ (µM), 50% inhibitory concentration (means ± SEM of three experiments) of AChE or BChE.

Table 2: IC₅₀ of some target compounds for AChE and BChE.
Kinetic characterization of AChE inhibition

The inhibition type of AChE was investigated by graphical analysis of steady state inhibition data (Figure 3, left) using compound 5b as a typical example. The Lineweaver-Burk plots describing 5b inhibition showed both increasing slopes and increasing intercepts with higher inhibitor concentration, indicating a mixed-type inhibition. These results revealed that compound 5b bound to both catalytic active site (CAS) and the peripheral anionic site (PAS) of AChE, which is in agreement with the results of our molecular modeling studies.

Molecular modeling

To investigate the interaction mode of compound 5b with TcAChE (PDB code: 1ZGB), molecular modeling was carried out by AUTODOCK 4.0 package with PyMOL program (Figure 3, right) [22,23]. The docking result demonstrated that compound 5b exhibited multiple binding modes with AChE. In the 5b-TcAChE complex, compound 5b occupied the entire enzymatic CAS, mid-gorge and PAS. The charged nitrogen made a cation-π interaction with the Trp84. At the midgorge recognition site, the chromone moiety displayed classic π-π stacking with the phenyl ring of Trp334, with the ring-to-ring distance being 4.4 and 4.7 Å respectively. At the PAS, the benzene of genistein moiety stacked against the Trp279 through π-π interaction with the distance of 4.4 Å. The result showed that compound 5b was able to bind both CAS and PAS of AChE which was in agreement with the result of kinetic study.

Metal-chelating study

The abnormally high levels of biometals in affected areas of the brain catalyze the formation of reactive oxygen species, which further aggravates oxidative stress contributing to β-amyloid formation. These effects have rendered metal chelators as very promising drugs for AD. So, the chelation abilities of compound 5b towards biometal Fe³⁺, Cu²⁺ and Zn²⁺ in water were studied by UV-vis spectrometry. The results in Figure 4 showed that the absorbance spectra of 5b exhibited an apparent increase after the addition of Fe³⁺ or Zn²⁺, and a red shift in the result of kinetic study.

In conclusion, a series novel of genistein-polyamine conjugates (5a-5h) was designed, synthesized and evaluated for cholinesterase inhibition, metal-chelating activities and human hepatoma cell viability. Results indicated that these compounds had significant ChEs inhibitory activity. Compound 5b exhibited the strongest inhibition to AChE with an IC₅₀ value of 2.75 µM. A linearweaver-burk plot and molecular modeling study showed that compound 5b targeted both the CAS and PAS of AChE. Besides, compound 5b showed potent metal chelating ability. In addition, these compounds showed low cytotoxicity by MTT assay in vitro. Compound 5b may be considered to be a novel multi-potent, low toxicity drug candidate for the treatment of AD.

MTT assay of cell viability

The toxicity of synthesized compounds was determined in human hepatoma cell line HepG-2. Results indicated that the most potent three inhibitors, 5b and 5h, showed no obvious effect on cell viability at concentrations of 10 µM, as shown in Table 3. Compared with tacrine, they had a lower toxicity on cell viability.

In conclusion, a series novel of genistein-polyamine conjugates (5a-5h) was designed, synthesized and evaluated for cholinesterase inhibition, metal-chelating activities and human hepatoma cell viability. Results indicated that these compounds had significant ChEs inhibitory activity. Compound 5b exhibited the strongest inhibition to AChE with an IC₅₀ value of 2.75 µM. A linearweaver-burk plot and molecular modeling study showed that compound 5b targeted both the CAS and PAS of AChE. Besides, compound 5b showed potent metal chelating ability. In addition, these compounds showed low cytotoxicity by MTT assay in vitro. Compound 5b may be considered to be a novel multi-potent, low toxicity drug candidate for the treatment of AD.

Acknowledgements

We thank the Natural Science Foundation of China (No. 21172053, 21302041), the Postdoctoral Science Foundation of China (No. 2012MS21391), the Postdoctoral Science Foundation of Henan Province (No. 2011015), and the Foundation of Henan Educational Committee (No. 14A350008) for financial support of this study.

References
