Synthesis and cytotoxicity evaluation of 3-amino-2-hydroxypropoxygenistin derivatives

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[ABSTRACT] Soy isoflavones exhibit various biological activities, such as antioxidant, anti-tumor, anti-inflammatory, and cardiovascular protective effects. The present study was designed to investigate the effects of sixteen synthesized 3-amino-2-hydroxypropoxygenistin derivatives on cell proliferation and activation of Nrf2 (Nuclear factor erythroid 2-related factor 2)/ARE (antioxidant response elements) pathway in human cancer cell lines. Most of the tested compounds exerted greater cytotoxic activity than genistein, as measured by MTT assay. Moreover, compound 8c showed the highest ARE-luciferase reporter activity among the test compounds. It strongly promoted Nrf2 nuclear translocation and up-regulated the expression of total Nrf2 and downstream targets NQO-1 and HO-1 at protein level. The present study may provide a basis for the application of isoflavone derivatives as Nrf2/ARE pathway inducers for cancer therapy and cancer prevention.

[KEY WORDS] Genistein derivatives; Nrf2/ARE pathway; Cytotoxicity; Anti-cancer; Isoflavone


Introduction

Isoflavones are classified as “polyphenols”, with a basic skeleton of 3-phenyl chromone. Soy isoflavones are a series of nonnutritive compounds which possess different mechanisms of action for the control of numerous biological functions [1-2], including anti-tumor [3-6], osteoporosis [7] and cardiovascular preventive [8], and antioxidant [9] effects.

Genistein (4′,5,7-trihydroxyisoflavone) is the pre-dominant isoflavone in soybean, of which significant biological effects have been illustrated with respect to anti-cancer properties [10]. It is reported to be able to protect cells from reactive oxygen species (ROS) by scavenging free radicals and reducing the expression of stress-response related genes [11]. ROS may induce oxidative damage to cell structures, subsequently leading to chronic inflammation and eventually cancer and cardiovascular and neurodegenerative diseases [13]. The Kelch-like ECH associated protein 1 (Keap1)/Nuclear factor-erythroid 2-related factor 2 (Nrf2)/Antioxidant response element (ARE) is a major signaling pathway that responds to oxidative stress and regulates a battery of cytoprotective proteins at transcriptional level [13]. Following exposure of cells to electrophiles or oxidative stress, Nrf2 escapes from Keap1-mediated degradation and is translocated to the nucleus, activating ARE-dependent gene expression of a series of antioxidative and cytoprotective proteins. Therefore, Keap1-Nrf2-ARE pathway activators are becoming attractive hotspots in research and development of novel anticancer agents.

Most of known ARE inducers (or activators) are indirect inhibitors against Keap1-Nrf2 interaction, and Michael acceptor (olefins or acetylenes conjugated with electron-with-
drawing carbonyl groups) is a prominent member therein, which is believed to form covalent adducts with the sulphydryl groups of key cysteines in Keap1 (Fig. 1A). Structurally, natural and synthetic flavonoids are derivatives with $\alpha,\beta$-unsaturated ketone (a Michael acceptor) and are considered to be potential ARE inducers (Fig. 1B).

In our previous work, a series of daidzein derivatives were synthesized and their bioactivities evaluated. For further exploration of the ARE-inducing activation by isoflavone derivatives, sixteen genistein derivatives were synthesized with basic side chains at 7- and/or 4$'$-position to improve hydrophilic property and bioactivities of parent compound (Fig. 1C). And their cytotoxicity were tested with MTT assay and induction of ARE with ARE-luciferase reporter assay and activation effects on Nrf2/ARE pathway were evaluated with Western blot analysis. The present study would provide more effective candidate compounds for cancer chemotherapy and chemoprevention.

**Fig. 1 Structures of Michael acceptors and isoflavones. (A) The proposed reaction mechanism of Michael addition reactions between Michael acceptors and cysteine sulphydryl groups in Keap1. (B) Isoflavonoids that contain electrophilic Michael acceptors. and (C) Structures of target compounds**

**Results and Discussion**

**Chemistry**

The target compounds 3a-3g and 5a-5e were prepared as described in Figs. 2A and 2B. Treatment of genistein (1) with epichlorohydrin, through the control of different reaction conditions, gave 7,4$'$-bis-(2,3-epoxypropoxy)-5-hydroxy isoflavone (2) and 7-(2,3-epoxypropoxy)-5,4$'$-dihydroxyisoflavone (4) which were treated with substituted amines, respectively, to afford the respective 7,4$'$-bis-(3-amino-2-hydroxypropoxy)-5-hydroxyisoflavone derivatives 3a-3g and 7-(3-amino-2-hydroxypropoxy)-5,4$'$-dihydroxyisoflavone derivatives 5a-5e.

$4'$-(3-Amino-2-hydroxypropoxy)-5-hydroxyisoflavone derivatives were prepared as described in Fig. 2C. Selective protection of 7-hydroxyl groups of genistein was carried out via reaction with chloromethyl ether. The resulting compound 6 was then reacted with epichlorohydrin to produce compound 7, which was then treated with substituted amines respectively to afford the respective 7-methoxymethoxy -4$'$-(3-amino-2-hydroxypropoxy)-5-hydroxyisoflavone derivatives 8a-8d.

The much acidic 7-OH group in genistein can be available for selective mono-O-alkylations of these isoflavones. In previous studies, the exceeded use of halocarbon would increase the yield of 7-substituted derivatives, but 7,4$'$-bisubstitution would be obtained mainly when extra epichlorohydrin was used. To obtain 7-(2,3-epoxypropoxy)-5,4$'$-dihydroxyisoflavone, different alkali and weight ratio were tried. We found out that sodium hydride was relatively ideal, but the yield was still low, due to high impurity. Synthesis of compound 7 was started with protection of the 7-OH group with chloromethyl ether.

**Antitumor activity and effects on Nrf2/ARE pathway**

To investigate the cytotoxicities of the target compounds, all the synthesized genistein derivatives were dissolved in DMSO and were tested in five human cancer cell lines, including breast cancer cell lines MDA-MB-231 and MCF-7, colon cancer cell lines HCT116 and HT-29, and hepatoma cell line 7402, using MTT assay with cis-platin and 5-FU as positive controls. Their cytotoxic activity was presented as the concentration inhibiting 50% of cancer cell growth (IC$_{50}$).
As shown in Tables 1 and 2, apart from compound 3c and 5c that were with poor cytotoxicity, most of the synthesized compounds were more effective against five cell lines than the parent compound genistein, especially compound 3g, which showed the most significant cytotoxic activity against three cell lines (HT-29, HCT116, and 7402) with IC_{50} close to 20 μmol·L\(^{-1}\) and 7.2 μmol·L\(^{-1}\) against MCF-7 cell line. Hence, these compounds demonstrated significant anti-proliferative effects on different human cancer cell lines.

Table 1  The cytotoxicity of genistein and its synthetic derivatives on three cancer cell lines (means ± SD, n = 5)

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC_{50} (μmol·L(^{-1}))</th>
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<tbody>
<tr>
<td></td>
<td>HT-29</td>
</tr>
<tr>
<td>Genistein</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>34.40 ± 0.9</td>
</tr>
<tr>
<td>3a</td>
<td>36.08 ± 0.9</td>
</tr>
<tr>
<td>3b</td>
<td>68.85 ± 3.1</td>
</tr>
<tr>
<td>3c</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>3d</td>
<td>83.98 ± 3.4</td>
</tr>
<tr>
<td>3e</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>3f</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>3g</td>
<td>26.87 ± 2.8</td>
</tr>
<tr>
<td>3h</td>
<td>31.00 ± 1.5</td>
</tr>
<tr>
<td>5a</td>
<td>79.44 ± 2.8</td>
</tr>
<tr>
<td>5b</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>5c</td>
<td>110.59 ± 2.9</td>
</tr>
<tr>
<td>5d</td>
<td>79.48 ± 2.7</td>
</tr>
<tr>
<td>5e</td>
<td>24.96 ± 2.1</td>
</tr>
<tr>
<td>8a</td>
<td>81.19 ± 2.5</td>
</tr>
<tr>
<td>8b</td>
<td>76.59 ± 3.1</td>
</tr>
<tr>
<td>8c</td>
<td>85.26 ± 4.1</td>
</tr>
</tbody>
</table>
Table 2  The cytotoxicity of genistein and its synthetic derivatives on two cancer cell lines (means ± SD, n = 5)

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC50 (μmol·L−1)</th>
<th>MDA-MB-231</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td></td>
</tr>
<tr>
<td>5-FU</td>
<td>107.23 ± 2.1</td>
<td>104.11 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>46.08 ± 0.9</td>
<td>7.97 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>61.85 ± 3.1</td>
<td>87.13 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>3c</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td></td>
</tr>
<tr>
<td>3d</td>
<td>96.98 ± 3.4</td>
<td>43.67 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>3e</td>
<td>&gt; 200</td>
<td>65.57 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>3f</td>
<td>&gt; 200</td>
<td>167.09 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>3g</td>
<td>76.87 ± 2.8</td>
<td>7.24 ± 0.2</td>
<td></td>
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<tr>
<td>5a</td>
<td>36.00 ± 1.5</td>
<td>88.20 ± 2.4</td>
<td></td>
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<tr>
<td>5b</td>
<td>80.44 ± 2.8</td>
<td>87.76 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>5c</td>
<td>&gt; 200</td>
<td>&gt; 200</td>
<td></td>
</tr>
<tr>
<td>5d</td>
<td>107.59 ± 2.9</td>
<td>132.28 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>5e</td>
<td>77.48 ± 2.7</td>
<td>111.79 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>8a</td>
<td>44.96 ± 2.1</td>
<td>10.76 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>8b</td>
<td>80.19 ± 2.5</td>
<td>27.25 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>8c</td>
<td>106.59 ± 3.1</td>
<td>51.89 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>8d</td>
<td>115.26 ± 4.1</td>
<td>59.89 ± 2.6</td>
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</table>

We chose 50 μmol·L−1 as test concentration to ensure the following tests carried out in functional cancer cells.

To demonstrate the activation effects of these compounds at 50 μmol·L−1 on Nrf2/ARE pathway, ARE-luciferase reporter assay was performed [18]. t-BHQ (tertiary butyl-hydroquinone) was used as a positive control and 0.1% DMSO was used as a vehicle control. All the compounds alone induced ARE-luciferase activity in HepG2-C8 cells, with compounds 8c and 3g at 50 μmol·L−1 strongly induced ARE-luciferase reporter gene as compared to any other treatments (P < 0.05; Fig. 3). And ARE fold induction by compound 8c was over 6 times stronger than t-BHQ, and compound 3g was nearly twice than t-BHQ.

All these results suggested that compounds 8c and 3g had stronger cytotoxicity than genistein, and induced expression of ARE protein, which could be linked to the activation of Nrf2/ARE pathway.

**Effects of compound 8c on Nrf2 nuclear translocation and intracellular ROS level, and activation of the Nrf2/ARE pathway**

When cells are exposed to oxidative stress, Nrf2 escapes from Keap1 and transfers into the nucleus, binds to ARE, and eventually activates ARE-dependent gene expression of a series of antioxidative and cytoprotective proteins and decreases the level of ROS, which is a marker of oxidative stress. To test the possibility that 8c induced ARE through Nrf2/ARE pathway, the ability of 8c to induce the translocation of Nrf2 into nucleus and increase the down-stream proteins heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO-1) expressions were measured in MDA-MB-231 cell lines. As shown in Fig. 4, obviously, a dose-dependent translocation of Nrf2 into nucleus by 8c was observed in the tested cells as measured by Western blot analysis, and total Nrf2 protein was increased meanwhile. Additionally, the protein levels of the downstream genes HO-1 and NQO-1 were also increased after 8c treatment. The intracellular ROS level was decreased dose-dependently by 8c.
**Fig. 4** The Nrf2/ARE pathway activation induced by compound 8c. A (1): the cytoplasm Nrf2 and nucleus Nrf2 protein levels after 8c and t-BHQ (30 µmol·L\(^{-1}\)) treatment in MDA-MB-231 cell line at 12 h. B (1): the total protein level in MDA-MB-231 cell line. A (2), B (2): The relative expression of proteins was normalized to β-actin. The data are shown as means ± SD (n = 3). *P < 0.05, **P < 0.01 vs control. C(1): the relative fluorescence of ROS in MDA-MB-231 cell line after treatment by 8c with various concentrations (µmol·L\(^{-1}\)) for 12 h. C(2): the relative fluorescence of ROS in MDA-MB-231 cell line after treatment for different time by 8c (50 µmol·L\(^{-1}\))

**Experimental**

**Instruments and reagents**

All chemicals used in the present study were of reagent grade and commercially available. All the \(^1\)H NMR spectra were recorded on a Bruker AV-300 or AV-500 model spectrometer (Bruker, Billerica, USA). Chemical shifts for \(^1\)H NMR spectra were expressed in parts per million (δ) with tetramethysilane (TMS) as an internal standard. ESI-MS spectra were recorded on a LCMS-2010EV mass spectrometer (Shimadzu, Kyoto, Japan). Melting points were measured using a Beijing Biotech X-4 micro melting point apparatus (Beijing, China) or an open capillary tube with an electrothermal apparatus (self-made equipment) and the results were uncorrected. Infrared spectra were recorded on a FTIR-8400S spectrometer (Shimadzu, Kyoto, Japan) using KBr pellets. The elemental analyses were performed in the Vario EL III elemental analyzer (Elementar, Frankfurt, Germany).

Tert-butylhydroquinone (t-BHQ) was purchased from Sigma-Aldrich (St Louis, USA) and dissolved by DMSO.
7,4-Bis-(2,3-epoxypropanoyl)-5-hydroxyisoflavone (2)

Genistein (2.70 g, 10.0 mmol) was added to anhydrous ethanol (200 mL), followed by refluxing until the solution became clear. Then, anhydrous potassium carbonate (1.52 g, 11.0 mmol) and epichlorhydrin (93.10 g, 10.0 mmol) were added, followed by refluxing for 6 h and hot filtration. The liquid was cooled and filtered to obtain a yellowish solid and the intermediate was directly into the next reaction.

7,4-Bis-(2-hydroxy-3-isopropylaminopropoxy)-5-hydroxyisoflavone (3a)

Compound 2 (0.57 g, 1.5 mmol) was added to anhydrous ethanol (20 mL), followed by refluxing until the solution became clear. Then, isopropylamine (0.27 g, 4.5 mmol) was added, followed by refluxing for 6 h and hot filtration. The solvent was removed by rotary evaporation to yield a residue. The residue was purified through column chromatography eluted with chlorform-methanol (10 : 3) to give 3a (0.16 g, 21.8%) as yellow solid, mp 118–120 °C. 1H NMR (500 MHz, DMSO-d6): 1.03 (d, J = 5.9 Hz, 12H, 4CH3), 2.61–2.66 (m, 2H, 2CH2), 2.71–2.83 (m, 4H, 2CH2N), 3.88–4.13 (m, 6H, 2OCHCH2O), 6.42 (d, J = 2.2 Hz, 1H, 6-H), 6.67 (d, J = 2.2 Hz, 1H, 8-H), 7.02 (d, J = 8.7 Hz, 2H, 3′, 5′-H), 7.51 (d, J = 8.7 Hz, 2H, 2′, 6′-H), 8.44 (s, 1H, 2-H); ESI-MS m/z 501 [M + H]+; IR (KBr): 3 380, 2 931, 2 904, 1 665, 1 613, 1 578, 1 510, 1 242, 1 179, 827 cm−1; Anal. Calcd. for C27H36N2O7: C64.78, H7.63, N8.80.

Compounds 3b–3g were synthesized accordingly to the aforementioned method for synthesis of 3a.

7,4-Bis-(2-hydroxy-3-diethylaminopropoxy)-5-hydroxyisoflavone (3b)

Yellowish solid, yield 17.5%, mp 106–108 °C; 1H NMR (500 MHz, DMSO-d6): 0.96 (t, J = 5.9 Hz, 12H, 4CH3), 2.40–2.58 (m, 12H, 2CH2N(CH2)2), 3.90–3.93 (m, 4H, 2CH2O), 4.00–4.05 (m, 1H, OCH), 4.12–4.15 (m, 1H, OCH), 4.77 (br, 1H, OH), 4.84 (br, 1H, OH), 6.41 (d, J = 2.1 Hz, 1H, 6-H), 6.66 (d, J = 2.1 Hz, 1H, 8-H), 7.01 (d, J = 8.6 Hz, 2H, 3′, 5′-H), 7.51 (d, J = 8.6 Hz, 2H, 2′, 6′-H), 8.44 (s, 1H, 2-H), 12.91 (s, 1H, 5-OH); ESI-MS m/z 529 [M + H]+; IR (KBr): v: 3 433, 2 939, 1 659, 1 609, 1 574, 1 514, 1 443, 1 252, 1 180, 1 061, 836 cm−1; Anal. Calcd. for C35H38N2O7: C65.89, H7.63, N5.30; found C65.77, H7.68, N5.26.

7,4-Bis-(2-hydroxy-3-(4-methylpiperazin-1-yl)propoxy)-5-hydroxyisoflavone (3c)

Yellowish solid, yield 10.3%, mp 81–84 °C; 1H NMR (500 MHz, DMSO-d6): 2.31 (s, 6H, 2CH3), 2.45–2.56 (m, 20H, 2CH2N(CH2CH2)2N), 3.90–3.93 (m, 1H, OCH), 3.98–4.02 (m, 4H, 2CH2O), 4.10–4.13 (m, 1H, OCH), 4.97 (br, 2H, 2OH), 6.62 (d, J = 2.1 Hz, 1H, 6-H), 6.67 (d, J = 2.1 Hz, 1H, 8-H), 7.01 (d, J = 8.6 Hz, 2H, 3′, 5′-H), 7.51 (d, J = 8.6 Hz, 2H, 2′, 6′-H), 8.45 (s, 1H, 2-H), 12.91 (s, 1H, 5-OH); ESI-MS m/z 583 [M + H]+; IR (KBr): v: 3 380, 2 900, 2 881, 1 674, 1 613, 1 574, 1 514, 1 454, 1 292, 1 248, 1 182, 1 045, 824 cm−1; Anal. Calcd. for C39H46N4O7: C63.90, H7.27, N9.62; found C63.79, H7.38, N9.52.
2CH₃COCH₃), 6.43 (d, J = 2.0 Hz, 1H, 6-6-H), 6.67 (d, J = 2.0 Hz, 1H, 8-H), 7.02 (d, J = 8.3 Hz, 2H, 3′, 5′-H), 7.51 (d, J = 8.3 Hz, 2H, 2′, 6′-H), 8.45 (s, 1H, 2-H), 12.91 (s, 1H, 5-OH); ESI-MS m/z 553 [M + H]+; IR (KBr) ν: 3 384, 2 932, 1 657, 1 613, 1 512, 1 248, 1 182, 1 043, 831 cm⁻¹; Anal. Calcd. for C₁₃H₁₉N₂O₇: C 67.37, H 7.30, N 3.40; found C 67.19, H 7.39, N 3.43.

7-(2,3-Epoxypropanoxy)-5,4-dihydroxyisoflavone (4)

Sodium hydride (0.17g, 6.9 mmol) was added to a solution of genistin (0.81g, 3.0 mmol) in DMF (20 mL) at 5 °C. Then, epichlorohydrin (0.43 g, 4.5 mmol) was added and the mixture was stirred at 50 °C for 6 h. The liquid was filtered and the filtrate was poured into ice-water (20 mL) and the pH of the solution was adjusted to 6–7 by adding 3 mol·L⁻¹ of hydrochloric acid. The aqueous layer was extracted with ethyl acetate (3 × 40 mL). The combined organic layer was washed with water (2 × 20 mL), dried over anhydrous MgSO₄ and concentrated in vacuum. The residue was purified through column chromatography eluted with chloroform–methanol (100 : 1) to give 4 (0.43 g, 44.0%) as yellowish solid.

7-(2-Hydroxy-3-isopropylaminopropoxy)-5,4-dihydroxyisoflavone (5a)

To a mixture of 4 (0.43 g, 1.3 mmol) and anhydrous ethanol (20 mL) was added isopropylamine (0.24 g, 4.0 mmol). The mixture was refluxed for 6 h and the filtrate was evaporated. The residue was purified through column chromatography eluted with chloroform–methanol (100 : 1) to give 5a (0.15g, 29.6%) as yellowish solid, mp 211–213 °C.

1H NMR (300 MHz, DMSO-d₆) δ: 0.60 (d, J = 6.0 Hz, 6H, 2CH₃), 2.65–2.90 (m, 3H, CH₂NCH), 3.95–4.13 (m, 3H, OCHCH₂O), 6.42 (d, J = 1.8 Hz, 1H, 6-H), 6.67 (d, J = 1.8 Hz, 1H, 8-H), 6.83 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 7.39 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 8.41 (s, 1H, 2-H), 9.57 (s, 1H, 4-OH); ESI-MS m/z 386 [M + H]+; IR (KBr) ν: 2 974, 1 647, 1 609, 1 576, 1 559, 1 437, 1 252, 1 177, 837 cm⁻¹; Anal. Calcd. for C₁₃H₁₆N₂O₇: C 67.14, H 6.12, N 3.40; found C 66.93, H 6.08, N 3.43.

Compounds 5b–5d were synthesized according to the aforementioned method for synthesis of 5a.

7-(2-Hydroxy-3-tertbutylaminopropoxy)-5,4-dihydroxyisoflavone (5b)

Offwhite solid, yield 26.1%, mp 186–188 °C. 1H NMR (300 MHz, DMSO-d₆) δ: 1.06 (s, 9H, 3CH₃), 2.58–2.73 (m, 2H, 2H, CH₃N), 3.82–4.14 (m, 3H, OCHCH₂O), 5.02 (br, 1H, OH), 6.41 (s, 1H, 6-H), 6.66 (s, 1H, 8-H), 6.83 (d, J = 8.3 Hz, 2H, 3′, 5′-H), 7.39 (d, J = 8.3 Hz, 2H, 2′, 6′-H), 8.40 (s, 1H, 1-H), 9.53 (br, 1H, 4-OH); ESI-MS m/z 400 [M + H]+; IR (KBr) ν: 2 876, 1 663, 1 611, 1 574, 1 559, 1 516, 1 489, 1 317, 1 256, 1 184, 1 049, 835 cm⁻¹; Anal. Calcd. for C₁₃H₁₇N₂O₇: C 65.44, H 6.02, N 3.63; found C 65.59, H 6.09, N 3.58.

7-(2-Hydroxy-3-(4-ethylpiperazin-1-yl)propoxy)-5,4-dihydroxyisoflavone (5c)

Compound 5c was synthesized according to the aforementioned method for synthesis of 5a.

7-(2-Hydroxy-3-(4-ethylpiperazin-1-yl)propoxy)-5,4-dihydroxyisoflavone (5d)

Offwhite solid, yield 36.0%, mp 180–182 °C. 1H NMR (300 MHz, DMSO-d₆) δ: 1.35–1.39 (m, 2H, CH₂), 1.48–1.54 (m, 4H, 2CH₂), 2.30–2.45 (m, 6H, N(CH₂CH₃)₂), 3.98–4.02 (m, 2H, CH₂O), 4.11–4.13 (m, 1H, OCH), 6.42 (s, 1H, 6-H), 6.66 (s, 1H, 8-H), 6.83 (d, J = 8.4 Hz, 2H, 3′, 5′-H), 7.39 (d, J = 8.4 Hz, 2H, 2′, 6′-H), 8.40 (s, 1H, 2-H), 9.57 (s, 1H, 4-OH); ESI-MS m/z 441 [M + H]+; IR (KBr) ν: 2 818, 1 647, 1 609, 1 576, 1 520, 1 447, 1 252, 1 177, 837 cm⁻¹; Anal. Calcd. for C₁₃H₁₈N₂O₇: C 66.44, H 6.41, N 3.40; found C 66.29, H 6.51, N 3.36.

7-Methoxy-6-methyl-5,4-dihydroxyisoflavone (6)

Anhydrous potassium carbonate (3.00 g, 22.0 mmol) and few potassium iodide was added to a solution of 1 (5.40 g, 20.0 mol) in DMF (60 mL). Chloromethyl ether (1.80 g, 22.0 mmol) in DMF (60 mL) was added. The mixture was stirred in the room temperature for 5h. The liquid was filtered and the filtrate was poured into ice-water (200 mL) and the pH of the solution was adjusted to 6–7 by adding 3 mol·L⁻¹ of hydrochloric acid. The aqueous layer was extracted with ethyl acetate (3 × 60 mL). The combined organic layer was washed with water (2 × 15 mL), dried over anhydrous MgSO₄ and concentrated in vacuum. The residue was purified through column chromatography eluted with chloroform–methanol (100 : 3) to give 6 (2.60 g, 41.4%) as yellowish solid, mp 155–157 °C.

7-Hydroxy-5,4-dihydroxyisoflavone (7)

Compound 6 (1.26 g, 4.0 mmol) was added to anhydrous ethanol (30 mL), followed by refluxing until the solution became clear. Then, anhydrous potassium carbonate (0.55 g,
7-Methoxymethyl-4′-(2-hydroxy-3-isopropylaminopropoxy)-5-hydroxyisoflavone (8a)

Compound 7 (0.90 g, 2.4 mmol) was added to anhydrous ethanol (20 mL), followed by refluxing until the solution became clear. Then, isopropylamine (0.49 g, 7.8 mmol) was added, followed by refluxing for 6 h and hot filtration. The solvent was removed by rotary evaporation to yield a residue. The residue was purified through column chromatography eluted with chlororm–methanol (100 : 3) to give 8a (0.47 g, 44.7%) as pale yellow solid, mp 118 °C. 1H NMR (300 MHz, DMSO-d6) δ: 1.02 (d, J = 6.1 Hz, 6H, 2CH3), 2.64–2.66 (m, 1H, NCH), 2.78–2.82 (m, 2H, NCH2), 3.41 (s, 3H, OCH3), 3.87 (m, 2H, NCH2), 3.42 (s, 3H, CH3), 3.47 (t, J = 6.1 Hz, 2H, CH2O), 3.88–4.03 (m, 3H, OCHCH2O), 4.30 (t, J = 1.0 Hz, 1H, OCH), 4.80 (d, 1H, OH), 5.33 (s, 2H, OCH2O), 6.48 (d, J = 2.2 Hz, 1H, 6-H), 6.72 (d, J = 2.2 Hz, 1H, 8-H), 7.01 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 7.51 (d, J = 8.8 Hz, 2H, 2′, 6′-H), 8.46 (s, 1H, 2-H); ESI-MS m/z 443 [M + H]+; IR (KBr) ν: 3 447, 1 672, 1 557, 1 516, 1 443, 1 302, 1 250, 1 182, 1 146, 1 086, 1 042, 997, 820 cm−1; Anal. Calcd. for C24H29NO7: C 64.32, H 6.34, N 3.26; Found: C 64.23, H 6.42, N 3.19.

7-Methoxymethyl-4′-(2-hydroxy-3-tertbutylaminoproxy)-5-hydroxyisoflavone (8b)

White solid, yield 57.3%, mp 160–163 °C. 1H NMR (500 MHz, DMSO-d6) δ: 2.36–2.46 (m, 12H, CH2N(CH2CH2)2NCH3), 3.42 (s, 3H, CH3), 3.47 (t, J = 6.1 Hz, 2H, CH2O), 3.88–4.03 (m, 3H, OCHCH2O), 4.30 (t, J = 1.0 Hz, 1H, OCH), 4.80 (d, 1H, OH), 5.33 (s, 2H, OCH2O), 6.48 (d, J = 2.2 Hz, 1H, 6-H), 6.72 (d, J = 2.2 Hz, 1H, 8-H), 7.01 (d, J = 8.8 Hz, 2H, 3′, 5′-H), 7.51 (d, J = 8.8 Hz, 2H, 2′, 6′-H), 8.46 (s, 1H, 2-H); ESI-MS m/z 501 [M + H]+; IR (KBr) ν: 3 541, 3 470, 3 416, 2 924, 1 657, 1 576, 1 510, 1 250, 1 086, 1 042, 997, 820 cm−1; Anal. Calcd. for C26H32N2O7: C 62.39, H 6.44, N 5.60; Found: C 62.59, H 6.51, N 5.67.

Cell culture

Human breast cancer cell lines MDA-MB-231 and MCF-7, colon cell lines HT-29 and HCT116, and hepatoma cancer cell lines HepG2 and 7402 were obtained from the Cell Bank of Shanghai, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 and MCF-7 cells were grown in RPMI 1640 medium (KGM 31800-500) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) [19]. HT-29 and HCT116 cells were grown in RPMI 1640 medium (KGM 31800-500) supplemented with 10% fetal calf serum (CAS: F8240-100). HepG2 and 7402 cells were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal calf serum (CAS: F8240-100). The human hepatoma HepG2-C8 cell line was previously established by stable transfection with an ARE-luciferase construct. They were grown in RPMI 1640 medium (KGM 31800-500) supplemented with 10% heat-inactivated fetal bovine serum (Gibco).

Cell viability assay

The cell cytotoxicity of compounds was determined by the MTT assay [20]. The logarithmic cells were plated into 96-well plates (Corning, New York, NY, USA) at approximately 3 000/well in 100 μL of medium for 24 h at 37 °C, and then treated with various concentrations of compounds in 100 μL per well. After incubation, 20 μL of MTT (5 mg/mL in PBS) was added to each well and the plate was incubated for an additional 4 h at 37 °C. The culture medium was discarded and DMSO was added (100 μL/well). The control cells were treated with 0.1% DMSO. The suspension was placed on micro-vibrator for 5 min and the absorbance (A) was measured at 570 nm by the Universal Microplate Reader (EL800, BioTek Instruments Inc. Winooski, VT, USA). Cell inhibition ratio was calculated using 1 − Auntreated/Acontrol, where Auntreated and Acontrol are the absorbance from treated and control cells after 24 h incubation, respectively.

Western blotting analysis

To collect the total cellular protein, the cells were collected and re-suspended in 37 μL of Lysis buffer (1 mL of RAPI Lysis, 10 μL of PMSF) for 1 h, and then centrifuged at 12 000 r.min−1 for 25 min at 4 °C. Protein concentration was determined by the BCA assay (Thermo, Massachusetts, USA) at 570 nm.

To collect cytoplasm and nucleus protein, the cells were...
collected and re-suspended in 50 μL of buffer I (975.7 μL of ddH2O, 10 μL of 1 mmol·L−1 Hapes, 10 μL of 1 mmol·L−1 KCl, 1.5 μL of 1 mmol·L−1 MgCl2, 1 μL of 1 mmol·L−1 DTT, 10 μL of PMSF), after 15 min, 2.75 μL of 1% NP40 was added and mixed. The cells were centrifuged at 12 000 r min−1 20 min at 4 °C, the supernatant was cytoplasm protein, and the precipitation was re-suspended in 25 μL of buffer II (307 μL of ddH2O, 420 μL of 1 mmol·L−1 NaCl, 250 μL of glycerol, 20 μL of 1 mmol·L−1 Hapes, 1.5 μL of 1 mmol·L−1 MgCl2, 1 μL of 1 mmol·L−1 DTT, 0.4 μL of 0.5 mmol·L−1 EDTA, and 10 μL of PMSF). After 60 min, the cells were centrifuged at 12 000 r min−1 28 min at 4 °C, the supernatant was nuclear protein. The protein concentration was determined by the BCA assay at 570 nm. The proteins were separated by SDS-PAGE and then transferred onto PVDF membranes (Perkin Elmer, Northwalk, CT, USA) using a semi-dry transfer system (Bio-rad, Hercules, CA, USA). After blocking with 3% BSA for 90 min at 37 °C, and washing three-times by PBST (Phosphate buffered saline tween20), the membranes were incubated for 1 h at 37 °C and 4 °C overnight with a primary antibody. Second day, the membranes were washed three times by PBST and reacted with a secondary antibody for 1 h at 37 °C. Then, the membranes were developed by film[21].

**Luciferase reporter assay**

The logarithmic HepG2-ARE-C8 cells were plated in 24-cell plates at approximately 100 000 cells/well in 1 mL medium for 24 h at 37 °C, and then treated with various concentrations of compounds for 12 h at 37 °C. After 12 h, the cells were collected and harvested in the luciferase cell lysis reagent for 20 min. After centrifugation, 20 μL of supernatant was used for determining the luciferase activity according to the protocol provided by the manufacturer (Jiman, GM-040501A, China). The data were obtained in triplicates and expressed as fold induction over the control [22].

**Conclusion**

Based on rational approaches, we synthesized sixteen genistein derivatives, and identified several of them as potential Nrf2/ARE pathway inducers.

In terms of chemistry, we found that the use of K2CO3 as alkali received a good result when 7- and 4′- were both substituted with epichlorohydrin; while 7- is mono-substituted with epichlorohydrin, a more alkaline sodium hydride was relatively ideal. And 7-hydroxyl group was protected by chloromethyl ether and then reacted with epichlorohydrin to introduce 2,3-epoxypropanoyl to 4′-position. During the reaction, epichlorohydrin was easy to open loop, but there was no effect.

The biological studies demonstrated that most of the target compounds significantly inhibited the proliferation of human cancer cell lines, especially compound 3g. The study also revealed that genistein derivatives exhibited antioxidant effects. Compound 8c had the strongest ARE-inducing activity in HepG2-C8 cells, by increasing the total protein level of Nrf2 and the translocation of Nrf2 into nucleus. The increased level of Nrf2 in total cellular extract and nuclei extract demonstrated that the target compounds not only induced the total amount of Nrf2 protein, but also the activated Nrf2 level. Because the translocation of Nrf2 into nuclei is regarded as the beginning of Nrf2/ARE pathway activation, the increased expression of the downstream genes HO-1 and NQO-1 after 8c treatment also supported this view. Keap1 was isolated as an inhibitor protein of Nrf2 [23]. Exposure to a number of stressors and inducing agents leads to dissociation of Nrf2 from Keap1, thereby rescuing Nrf2 from proteasomal degradation and allowing for import into the nucleus [23]. Hence, these results indicated that the target compounds may activate the Nrf2/ARE pathway through interaction with Keap1. But the definite mechanisms should be further investigated.

Comparing with our previous work [14], genistein derivatives represented more effective cytotoxicity than daidzein derivatives on different human cancer cells with same modification on 7 and/or 4′ position, e.g., genistein derivative 8d exerted stronger cytotoxicity than daidzein derivative 8c [14] in HCT-116, HT-29, and 7402 cells. The only difference between these two compounds is the existence of hydroxy on 5 position. Therefore, we speculated that the 5-OH may be important to cytotoxicity of isoflavones.

Antiproliferative effects of isoflavone derivatives varied with the position of substitution and type of amine on side chain, typically, 4′-substituted or 7,4′-bisubstituted > 7-substituted and chain amine > cyclamine, especially the compounds with isopropylamine or tert-butylamine as side chain performed more effective cytotoxicity.

The 4′-substituted or 7, 4′-substituted compounds (e.g., 8c and 3g) presented stronger ARE inducing effects than 7-substituted compounds (e.g., 5e and 5d). This may be related to the interactions between target compounds with Keap1. The modification on 7 and/or 4′ position of isoflavone, especially 4′ position, may provide more binding sites to Keap1 and influence the space volume of target compounds, thus leading to better activity. However, this hypothesis needs further exploration.

In summary, 3-amino-2-hydroxypropoxysiloxane derivatives exhibited significant inhibitory effects on proliferation of human cancer cell lines and activation of Nrf2/ARE signaling pathway. Therefore, they are considered to be potential therapeutic and/or preventive agents against cancer.

**References**


