Design, synthesis and cytotoxicity of cell death mechanism of rotundic acid derivatives

Yu-Fang He a,b,†, Min-Lun Nan b,†, Jia-Ming Sun e, Zhao-Jie Meng d, Wei Li c,*, Ming Zhang d,f,*

a School of Pharmaceutical Sciences, Jilin University, Changchun 130021, China
b Jilin Academy of Chinese Medicine Sciences, Changchun 130012, China
c College of Chinese Medicinal Materials, Jilin Agricultural University, Changchun 130118, China
d Norman Bethune College of Medicine, Jilin University, Changchun 130021, China
e Development Center of Traditional Chinese Medicine and Bioengineering, Changchun University of Chinese Medicine, Changchun 130117, China
f Research and Development Center, Xiuzheng Pharmaceutical Group, Changchun 130012, China

A R T I C L E   I N F O

Article history:
Received 12 December 2012
Revised 22 February 2013
Accepted 1 March 2013
Available online 13 March 2013

Keywords:
Rotundic acid
Derivative
Cytotoxicity
Apoptosis

A B S T R A C T

In the present investigation, 16 new rotundic acid (RA) derivatives modified at the C-3, C-23 and C-28 positions were synthesized. The cytotoxicities of the derivatives were evaluated against HeLa, A375, HepG2, SPC-A1 and NCI-H446 human tumor cell lines by MTT assay. Among these derivatives, compounds 4–7 exhibited stronger cell growth inhibitory than RA and compound 4 was found to be the best inhibition activity on five human tumor cell lines with IC50 <10 μM. The apoptosis mechanism of compound 4 in HeLa cells was investigated by western blot analysis. The results indicated that compound 4 could induce apoptosis through increasing protein expression of cleaved caspase-3 and Bax, and decreasing protein expression of Bcl-2. In summary, the present work suggests that compound 4 might serve as an effective chemotherapeutic candidate.

In the history of Traditional Chinese Medicine (TCM), medicinal plants and their extracts were used to treat various diseases. Nowadays, compounds derived from natural medicines with the unique and diverse chemical entities still constituted a considerable resource for developing novel medicinal agents. Over the recent years, a variety of biologically active constituents, including ginsenoside Rg3, paclitaxel, platycodin D, triptolide and flavone eupato- rin, have been isolated from these sources and confirmed to have anti-cancer activity in both experimental and epidemiologic investigations. The bark of Ilex rotunda Thunb., was early recorded in ‘Ling Nan Cai Yao Lu’, has been used for a long time as a TCM for the treatment of colds, tonsillitis, pharyngitis, bone pain, acute gastroenteritis, and dysentery so on. Nowadays, this medicinal plant is listed in Pharmacopoeia of the People’s Republic of China, 2010.2

Rotundic acid (3β,19α,23-trihydroxy-urs-12-en-28-oic acid, RA), a pentacyclic triterpene acid, is the major component isolated from the dry bark of I. rotunda. In addition to existence in aquifoliaceae plants, RA also was isolated from Mussaenda Pubescens, Guettarda platypoda, Olea europaea, Planchonella ducilian, Nauclea officinalis. Although RA could be obtained from the above plant resources, there were little reports on its bioactivity. To date, in addition to the anti-tumor and lowering blood pressure activity, no reports on other activities related to RA were published. Our studies have proved that RA had the activity of prevention and treatment of cardiovascular disease and we have applied two patents (one of the patent had been authorized). More activities of RA need to be studied in the future.

In our previous study, the results showed that a few of new amino acid derivatives showed stronger cytotoxicities than RA. In order to find the compounds with better cytotoxicities, we continued to carry out chemical modification and antitumor activity of RA. Since the nitrogen-containing organic compounds had high biological activities, which play an important role on the chemical research, so many researchers carried out research in this area. In the work described herein, we focus on increasing the cytotoxicity of RA. As shown in Figure 1, due to higher steric hindrance, 19-OH is difficult to modified, Therefore, RA can be modified easily at C-3, C-23 and C-28 position. In the present study, we intend to modify RA to obtain better derivatives, their cytotoxicities are determined on the five human tumor cell lines including A-375 (human malignant melanoma cells), SPC-A1 (human lung adenocarcinoma), Hela (cervical cancer cells), HepG2 (hepatoma cells) and NCI-H446 (small cell lung cancer). The derivatives with low IC50 value will be further evaluated to explore its mechanism on the traditional apoptosis signal pathway.
Recently, the antitumor mechanism mainly focused on their effect on trigger apoptosis. Apoptosis, known as programmed cell death, is closely related to many anticancer reagents. It has been broadly accepted that mitochondria play an important role during drug-induced apoptosis in cancer cells. Many of the stimulus including anticancer drug that triggered apoptosis on the mitochondria, which respond to proapoptotic signals by releasing cytochrome C, which belongs to an effective catalyst of apoptosis. Members of the Bcl-2 family include either proapoptotic (Bax) or antiapoptotic (Bcl-2) function, could lead to mitochondrial death signaling through cytochrome C release. Normally, Bcl-2 is local-
izes to the mitochondria and blocks the activation of proapoptotic proteins, such as Bax, to the mitochondria. When the cancer underwent chemotherapy, proapoptotic Bax can be upregulated in response to sensing DNA damage, Bax in turn stimulates mitochondria to release cytochrome C. Cytochrome C is the major inducer of caspase activation downstream of mitochondria. Finally, it will activate caspase-3 to be cleaved and execute apoptosis.

As reported in our previous investigation, RA was isolated and purified from L. rotunda,26 its purity was >98% (HPLC assay) and the extraction yield of RA was much higher up to 100 mg/g. In this Letter, the synthetic routes of RA derivatives are outlined in Scheme 1. RA (1) was first converted to its 3,23-O-diacete (2), which was then treated with oxalyl chloride to give the 28-acetyl chloride (3).30 This intermediate was then reacted with the appropriate amino alcohols (2-amino ethanol, 2-amino-1-propanol, 3-amino-1-propanol, 2-amino-benzyl alcohol) in the presence of triethylamine to give the compound (4–7). The all 16 compounds obtained here were synthesized by hydrolyzation of compounds 4–7 gave the corresponding N-[3β,23-diacetoxy-19α-hydroxy-urs-12-en-28-oyl]-2-amino alcohols (compounds 8–11). Compounds N-[3β,23-diacetoxy-19α-hydroxy-urs-12-en-28-oyl]-2-amino alcohol acetates (compounds 12–15) were obtained by re-acetylation compounds 4–7 in the presence of 4-dimethyl aminopyridine (4-DMAP), respectively. Compound 3 was treated with phenylalanine or benzylamine in the presence of triethylamine to give the N-[3β,23-diacetoxy-19α-hydroxy-urs-12-en-28-oyl]-2-arylamino amine (compounds 16–17), hydrolyzation of compounds 16–17 to give the corresponding N-[3β,19α,23-trihydroxy-urs-12-en-28-oyl]-2-aromatic amine (18–19). The structures of these synthesized compounds were confirmed by Infrared (IR), Mass spectra (HR-ESI-MS),1H NMR and 13C NMR assay.31–34 The all 16 compounds obtained here were synthesized and reported for the first time, the purity of all these 16 compounds were above 98% with higher yield (HPLC).

In the present study, five kinds of human tumor cell lines including A-375, SPC-A1, Hela, HepG2 and NCI-H446 were the model chosen to determine the cytotoxicity of RA (as a positive control) and its derivatives 4–19. Antiproliferative effects were determined with MTT assay. Each experiment was repeated at least three times. The results are shown in Table 1.

As shown in Table 1, compound 2 (esterification of the 3-OH, 23-OH of RA) had stronger inhibitory activity against the five tumor cell lines with IC50 values of 11.41, 23.24, 13.09, 6.85 and 7.11 μM, respectively. Significant further improvement of the cell growth inhibition on the five cell lines was achieved when the selected amino alcohols was coupled to the C-28 to yield compounds 4–7. However, compounds 8–11, hydrolyzation product of corresponding compounds 4–7, were found to exhibit lower anti-tumor effect than compounds 4–7, and the anti-tumor activity of compounds 8–11 were even weaker than it’s lead compound RA. Though compounds 12–15 (esterification product of corresponding compounds 4–7) showed higher anti-tumor activity than compounds 8–11, they showed slightly lower anti-tumor activity compared to compounds 4–7, the activity on A375 and Hela, even as same as compounds 4–7. The results suggested that 3,23-O-diacete group might be important to the inhibitory activity of tumor cell growth and free 3,23-OH might be detrimental to the inhibitory activity. In addition, from the above results we can speculate the reason that the activity of compounds 4–7 higher than 12–15 might be that compounds 4–7 has free hydroxyl group at the C-28 amide side chain. Because of the existence of free hydroxyl groups, together with the 3,23-O-diacete group, may change water partition coefficient of compounds 4–7. As for compounds 16–19, the anti-tumor activity was not satisfactory as expected. Especially compounds 18–19, there was almost no inhibitory activity on the five tumor cell lines at all. However, the inhibitory activity of 16–17 was significantly higher than its hydrolysis product compounds 18–19. The result also gave the evidence that 3,23-O-diacete group should be the essential group to increase the activity of RA.

From the Table 1, we can also notice that the anti-tumor activity of compound 4 is the highest among these compounds. The IC50 value of compound 4 was significantly less than RA treatment group (<10 μM) on the five cell lines with IC50 values of 5.49, 3.61, 2.83, 4.40, and 6.67 μM, respectively. These results indicated that compound 4 can be the candidate for the development of new anticancer drug. To explore its elementary anticancer effect, the apoptosis signal related protein level will be tested in the following experiment.

As seen in above experiment, result about the cytotoxicity of compound 4 was excited. The IC50 is much lower in compound 4 treatment for 24 h compared to RA group and other derivatives. In this experiment, we treated Hela cell with compound 4 at 10, 20 and 40 μM for 24 h, Cisplatin (10 μg/ml) was used as positive control for 24 h.

Table 1
The IC50 values of RA and its derivatives on human cancer cell lines (μM)

<table>
<thead>
<tr>
<th>Compound</th>
<th>A375</th>
<th>SPC-A1</th>
<th>Hela</th>
<th>HepG2</th>
<th>NCI-H446</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.58 ± 1.22</td>
<td>83.95 ± 3.28</td>
<td>31.92 ± 2.01</td>
<td>7.33 ± 0.68</td>
<td>11.40 ± 2.32</td>
</tr>
<tr>
<td>2</td>
<td>11.41 ± 0.99</td>
<td>23.24 ± 1.26</td>
<td>13.09 ± 0.88</td>
<td>6.85 ± 0.63</td>
<td>7.11 ± 0.45</td>
</tr>
<tr>
<td>4</td>
<td>5.49 ± 1.02</td>
<td>3.61 ± 1.59</td>
<td>2.83 ± 1.01</td>
<td>4.40 ± 0.97</td>
<td>6.67 ± 1.12</td>
</tr>
<tr>
<td>5</td>
<td>4.77 ± 3.00</td>
<td>12.88 ± 3.55</td>
<td>11.35 ± 3.26</td>
<td>7.30 ± 0.89</td>
<td>5.51 ± 0.78</td>
</tr>
<tr>
<td>6</td>
<td>3.28 ± 0.88</td>
<td>13.36 ± 2.11</td>
<td>6.97 ± 1.97</td>
<td>5.49 ± 1.23</td>
<td>5.08 ± 1.26</td>
</tr>
<tr>
<td>7</td>
<td>3.71 ± 0.75</td>
<td>8.09 ± 1.46</td>
<td>3.32 ± 0.48</td>
<td>4.45 ± 1.03</td>
<td>7.03 ± 0.92</td>
</tr>
<tr>
<td>8</td>
<td>21.43 ± 2.01</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>45.60 ± 2.03</td>
<td>24.72 ± 1.87</td>
</tr>
<tr>
<td>9</td>
<td>35.48 ± 1.96</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>55.09 ± 2.02</td>
<td>39.81 ± 2.45</td>
</tr>
<tr>
<td>10</td>
<td>12.47 ± 2.01</td>
<td>83.18 ± 1.89</td>
<td>17.74 ± 1.55</td>
<td>77.27 ± 2.13</td>
<td>20.89 ± 2.26</td>
</tr>
<tr>
<td>11</td>
<td>18.97 ± 0.69</td>
<td>&gt;100</td>
<td>12.27 ± 0.86</td>
<td>&gt;100</td>
<td>13.96 ± 1.12</td>
</tr>
<tr>
<td>12</td>
<td>4.17 ± 0.12</td>
<td>9.37 ± 0.69</td>
<td>5.39 ± 0.37</td>
<td>4.44 ± 0.48</td>
<td>18.62 ± 0.66</td>
</tr>
<tr>
<td>13</td>
<td>5.10 ± 0.89</td>
<td>18.85 ± 1.77</td>
<td>5.30 ± 0.36</td>
<td>9.12 ± 1.25</td>
<td>14.69 ± 1.33</td>
</tr>
<tr>
<td>14</td>
<td>10.06 ± 1.61</td>
<td>11.96 ± 1.93</td>
<td>4.31 ± 0.91</td>
<td>11.96 ± 1.28</td>
<td>12.82 ± 1.83</td>
</tr>
<tr>
<td>15</td>
<td>9.12 ± 0.66</td>
<td>13.21 ± 1.55</td>
<td>4.27 ± 2.33</td>
<td>12.35 ± 2.78</td>
<td>69.69 ± 2.01</td>
</tr>
<tr>
<td>16</td>
<td>17.74 ± 1.03</td>
<td>16.21 ± 1.08</td>
<td>15.78 ± 2.01</td>
<td>22.23 ± 2.26</td>
<td>24.83 ± 0.75</td>
</tr>
<tr>
<td>17</td>
<td>13.71 ± 1.00</td>
<td>12.10 ± 2.06</td>
<td>19.18 ± 2.23</td>
<td>19.50 ± 0.99</td>
<td>26.92 ± 1.09</td>
</tr>
<tr>
<td>18</td>
<td>70.79 ± 0.83</td>
<td>&gt;100</td>
<td>23.17 ± 0.76</td>
<td>64.05 ± 0.69</td>
<td>70.79 ± 0.78</td>
</tr>
<tr>
<td>19</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Notes: Data are represented in mean ± SD; n = 3.

* IC50 values more than 100 μM are indicated as >100.

P < 0.05 versus RA and IC50 value <10 μM.
treatment group. The protein expression of Bax, Bcl-2, cytochrome C and caspase-3 were detected by western blot. As shown in Figure 2, the antiapoptosis effect of compound 4 presented dose dependent manners after 24 h treatment, Compound 4 could decrease the protein expression of Bcl-2, a antiapoptotic member of Bcl family, and increase a proapoptotic protein Bax level, the ratio of Bax to Bcl-2 was remarkable increased in a dose dependent manner. The key protein of mitochondrial related apoptosis signal, cytochrome C in the cytoplasm also dramatically increased following the compound 4 treatment. All these result indicated that compound 4 might block the expression of Bcl-2, which could localize to the mitochondria and block the activation of proapoptotic proteins, such as Bax, to the mitochondria. The ratio of Bax to Bcl-2 has been used in many studies as the evaluation the level of apoptosis. The present study, compound 4 increased this ration by elevated Bax expression and depressed Bcl-2 expression finaly trigger the cytochrome C release from mitochondrial. It has been broadly accepted that the alteration on Bcl-2, Bax was closely related to

Figure 2. Alteration of protein expression of Bax, Bcl-2, cytochrome C in Hela cells after 24 h compound 4 treatment. (A) Protein expression of Bcl-2; (B) protein expression of Bax; (C) the ratio of Bax to Bcl-2; (D) protein expression of cytochrome C; (E) presented compound 4, C presented cisplatin, *P<0.05 compared to control group, **P<0.01 compared to control group.

Figure 3. Alteration of protein expression of caspase-3 and cleaved caspase-3 in Hela cells after 24 h compound 4 treatment. (A) Protein expression of caspase-3; (B) protein expression of cleaved caspase-3, E presented compound 4; (C) presented cisplatin, *P<0.05 compared to control group, **P<0.01 compared to control group.
stimulation of apoptosis, the effector of their alteration presented as the cytochrome C releasing, which will trigger the cleave of executor caspase, caspase-3. As shown in Figure 3, the protein expression of caspase-3 was significantly elevated by compound 4 treatment, the cleaved protein expression of caspase-3 remarkably increased as well. It has been broadly reported that caspases are widely expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases. Caspase-3 among the caspase family is considered to be the most important of the executioner caspases. Caspases-3 is cysteine proteases, using the sulfur atom in cysteine to cleave polypeptide chains. And the cleaved caspase-3 is the active form when the apoptosis was triggered. The cleaved caspase-3 will finally induce the DNA fragmentation in cancer cell. Therefore, the elevation of cleaved caspase-3 is very important for the compound which affects cancer growth inhibition in cancer cell. Therefore, the elevation of cleaved caspase-3 and Bax, and decreasing the protein expression of Bcl-2. We can conclude that 3,23-OH of the executioner caspases. Caspases-3 is cysteine proteases, using the sulfur atom in cysteine to cleave polypeptide chains. And the cleaved caspase-3 is the active form when the apoptosis was triggered. The cleaved caspase-3 will finally induce the DNA fragmentation in cancer cell. Therefore, the elevation of cleaved caspase-3 is very important for the compound which affects cancer growth inhibition in cancer cell. Therefore, the elevation of cleaved caspase-3 and Bax, and decreasing the protein expression of Bcl-2.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.005. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes