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Synthesis and antitumor activities of piperazine- and cyclen-conjugated dehydroabietylamine derivatives

DOI 10.1515/hc-2015-0025
Received March 7, 2015; accepted June 29, 2015; previously published online July 25, 2015

Abstract: A series of piperazine- and cyclen-conjugated dehydroabietylamine derivatives were synthesized and characterized by $^1$H NMR, $^{13}$C NMR, and HRMS. The in vitro antitumor activities of conjugates 10–13 against MCF-7 and HepG-2 tumor cell lines were evaluated using CCK-8 assay. The results show that the synthesized compounds cause a dose-dependent inhibition of cell proliferation and display different antitumor activities with the IC$_{50}$ values ranging from 23.56 to 78.92 μM. Moreover, the antitumor activity of conjugate 10 against the MCF-7 cell line is superior to that of the positive control 5-fluorouracil. In addition, flow cytometric assay revealed that the representative conjugate 10 could induce apoptosis in MCF-7 tumor cells in a dose-dependent manner.

Keywords: antitumor activity; conjugate; cyclen; dehydroabietylamine; piperazine.

Introduction

Cancer remains the primary cause of death due to the lack of effective drugs [1]. Natural compounds have played an important role in anticancer drug discovery, where the fraction of the drugs derived from natural products amounts to 60% [2]. Dehydroabietylamine is an abietane diterpenic amine that is obtained as a part of a mixture of amines derived from rosin. Recent studies have found that dehydroabietylamine derivatives demonstrate broad biological activities, such as antibacterial, antiinflammatory, antioxidative, and antitumor activities [3–12]. These results arouse our interest in screening for new potential antitumor drugs by the introduction of various functional groups to the dehydroabietylamine skeleton.

A number of piperazine and 1,4,7,10-tetraazacyclododecane (cyclen) heterocyclic derivatives as chemotherapeutic drugs have attracted considerable attention during the past decade. Studies have shown that the introduction of piperazine and cyclen moieties can modulate the physicochemical properties and enhance the bioactivity of the compounds [13–21]. However, the studies on the synthesis and antitumor activities of heterocyclic derivatives derived from dehydroabietylamine have not been reported. Our present work is to design and synthesize a series of piperazine- and cyclen-conjugated dehydroabietylamine derivatives and to evaluate in vitro antitumor activities of these conjugates against HepG-2 and MCF-7 cells. Furthermore, the apoptotic effect induced by the representative conjugates is also investigated by flow cytometry.

Results and discussion

The synthetic route to the target conjugates 10–13 is shown in Scheme 1. The reaction between tri-Boc-protected cyclen 1 or Boc-protected piperazine 2 and chloracetyl chloride afforded the respective products 3 and 4. Compound 6 was obtained by the reaction between 3 and 5a in the presence of KOH. Compounds 7–9 were synthesized using a similar methodology. The target compounds 10–13 were obtained by deprotection of Boc group in HCl-ethanol solution. All these products exhibit good water solubility. The structures of all synthesized conjugates were confirmed by $^1$H NMR, $^{13}$C NMR, and HRMS.

The in vitro antitumor activities of the conjugates 10–13 were evaluated by means of CCK-8 assay against MCF-7 and HepG-2 tumor cell lines. The inhibition rates of cell viability with different concentrations of conjugates are shown in Figure 1, and the IC$_{50}$ values are given in Table 1. It can be seen that the treatment with increasing doses of all conjugates causes a dose-dependent inhibition of cell proliferation. As
Inhibition (%)

Figure 1. Antiproliferation effect of conjugates 10, 11, 12, and 13 against MCF-7 (i) and HepG-2 (ii) tumor cells. Cells were plated and incubated with the indicated concentrations of 10, 11, 12, and 13 (5, 10, 20, 40, and 80 μM). After 24 h of treatment, cell proliferation was measured by the CCK-8 assay. Data represent the means±SD of triplicate experiments.

Table 1. The IC50 values of the conjugates 10–13 and 5-FU against tumor cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>25.4±2.04</td>
<td>49.6±3.26</td>
<td>62.5±4.01</td>
<td>78.9±2.68</td>
<td>27.1±1.95</td>
</tr>
<tr>
<td>HepG-2</td>
<td>27.0±1.88</td>
<td>52.6±2.94</td>
<td>23.5±1.45</td>
<td>71.9±2.33</td>
<td>6.8±0.65</td>
</tr>
</tbody>
</table>
indicated by the IC\textsubscript{50} values. In addition, conjugate prior to that of the positive control 5-fluorouracil (5-FU), as gate 45.65, and 53.75% by the treatment with 10, 20, and 40 The percentage of apoptotic cells were increased to 10.46, 4.10% of the total number of cells in the control group. in MCF-7 cell lines were determined by flow cytometry. as part of the R group exhibit only mild cytotoxic activities against MCF-7 and HepG-2 cells. Meanwhile, conjugates 11 and 13 containing benzene ring as part of the R group exhibit only mild cytotoxic activities against MCF-7 and HepG-2 cells. The effect of conjugate 10 on apoptosis in MCF-7 cell was investigated. Apoptosis assay may provide preliminary information about the mechanism of growth inhibition of tumor cells. The apoptosis ratios (including the early and late apoptosis rates) induced by conjugate 10 in MCF-7 cell lines were determined by flow cytometry. The results are given in Figure 2. The apoptotic rate was 4.10% of the total number of cells in the control group. The percentage of apoptotic cells were increased to 10.46, 45.65, and 53.75% by the treatment with 10, 20, and 40 \textmu M conjugate 10, respectively. The results indicate that apoptosis induction of the conjugate 10 in MCF-7 tumor cells changes in a dose-dependent manner.

**Conclusion**

A series of cyclic polyamine-dehydroabietylamine conjugates 10–13 were synthesized and characterized. The \textit{in vitro} antitumor activities of these compounds against HepG-2 and MCF-7 cells were evaluated. The effect of conjugate 10 against the MCF-7 cells was slightly superior to that of the positive control 5-FU. In addition, flow cytometric assay indicated that the representative conjugate 10 induces apoptosis in MCF-7 cells in a dose-dependent manner. These results encourage us to synthesize additional new dehydroabietylamine derivatives with the expected more potent antitumor activity.

**Experimental**

All reagents were purchased from commercial sources and used without further purification. The HepG-2 (liver hepatocellular carcinoma cell) and MCF-7 (human breast adenocarcinoma cell) were obtained from ATCC. High resolution mass spectrometry (HRMS) data were recorded on a Bruker Daltonics Bio TOF instrument. \textsuperscript{1}H NMR (400 MHz) and \textsuperscript{13}C NMR (100 MHz) spectra were measured on a Varian INOVA-400 spectrometer. Flow cytometry was performed using a BD FASaria Cell Sorter.

**Preparation of compound 5b**

The ethanol solution (30 mL) of dehydroabietylamine (5a, 145 g, 5 mmol) and \textit{p}-hydroxybenzaldehyde (0.61 g, 5 mmol) was heated under reflux for 4 h. After cooling, sodium borohydride (0.185 g, 5 mmol) was added and the mixture was stirred at room temperature for 12 h. Then, the mixture was concentrated under reduced pressure and quenched with water (10 mL). The aqueous phase was extracted with ethyl acetate (3×30 mL). The solvent was removed under reduced pressure and the residue was purified by column chromatography eluting with petroleum ether/ethyl acetate, 3:1, to give product 5b as a white solid: yield 72%; mp 143–145°C; \textsuperscript{1}H NMR (CDCl\textsubscript{3}): \(\delta\) 0.95 (s, 3H), 1.21 (s, 3H), 1.26 (d, 6H, \(J = 12\) Hz), 1.35–1.79 (m, 8H), 2.24–2.31 (m, 2H), 2.69 (d, H, \(J = 10.8\) Hz), 2.80–2.84 (m, 3H), 3.63–3.72 (m, 2H), 6.72 (d, 2H, \(J = 8.0\) Hz), 6.88 (s, 1H), 6.98 (d, 1H, \(J = 8.0\) Hz), 7.14 (d, 2H, \(J = 7.0\) Hz), 7.17 (d, H, \(J = 7.0\) Hz); \textsuperscript{13}C NMR (CDCl\textsubscript{3}): \(\delta\) 154.8, 147.5, 165.4, 134.8, 132.3, 129.4, 126.8, 124.3, 123.8, 115.3, 60.8, 54.1, 45.5, 38.4, 374, 36.9, 36.2, 33.4, 30.3, 25.4, 19.3, 18.8, 18.7. ESI-HRMS. Anal. Calcd for C\textsubscript{27}H\textsubscript{38}NO ([M+H]+): m/z 392.2953. Found: m/z 392.2956.
Preparation of compounds 3 and 4

Compound 3 was prepared according to the literature [22] by the reaction of I with chloracetyl chloride in the presence of Et,N at 0°C. Purification by silica gel column chromatography eluting with petroleum ether/ethyl acetate, 1:1, gave product 3 as a white solid; yield 65%; 1H NMR (CDCl3): δ 1.46–1.49 (s, 27H, Boc-H), 1.38–1.36 (m, 16H, CHJ), 0.06 (s, 2H), MS-ESI: m/z 548 (M+).

Compound 4 was synthesized using a similar procedure: yield 77%; 1H NMR (CDCl3): δ 1.47 (s, 9H, Boc-H), 3.43–3.61 (m, 8H, CH), 4.05 (s, 2H), ESIMS: m/z 262 (M+).

General procedure for the preparation of compounds 6–9

The tetrahydrofuran (THF) solution (30 mL) of 3 or 4 (0.5 mmol), 5a or 5b (0.5 mmol), and KOH (0.084 g, 1.5 mmol) was stirred at 60°C for 10 h. After cooling, the mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography eluting with petroleum ether/ethyl acetate, 1:1, to give desired product 6–9 as a white solid.

General procedure for the preparation of compounds 10–13

To a stirred solution of 6, 7, 8, or 9 (0.3 mmol) in ethanol (10 mL) at room temperature was slowly added 5 mL of 3 M HCl in ethanol solution. After stirring overnight, the reaction mixture was concentrated under reduced pressure. The residue was washed by anhydrous ether to furnish a hydrochloride salt of 10, 11, 12, or 13 as a white powder.

Compound 10 Yield 87%; mp 224–226°C; 1H NMR (DMSO-d6): δ 0.09 (s, 3H), 1.14 (s, 3H), 1.21 (d, 6H, J = 6.8 Hz), 1.50–1.72 (m, 8H), 2.17–2.31 (m, 2H), 2.50 (d, H, J = 10.8 Hz), 2.79–2.87 (m, 3H), 3.02–3.10 (m, 16H), 3.43–3.51 (m, 2H), 6.75 (s, 1H), 6.98 (d, 1H, J = 7.6 Hz), 7.17 (d, J, H, J = 8.0 Hz); 13C NMR (DMSO-d6): δ 166.9, 164.7, 165.1, 134.2, 126.3, 132.8, 123.5, 58.5, 50.8, 48.2, 46.2, 54.3, 44.4, 38.7, 36.2, 36.9, 36.0, 34.6, 32.8, 28.7, 24.9, 23.9, 18.2, 18.0. ESIMS. Calcd for C59H78N7O13 ([M+H]+): m/z 848.4712. Found: m/z 848.4714.

Compound 11 Yield 73%; mp 244–246°C; 1H NMR (DMSO-d6): δ 0.89 (s, 3H), 1.13 (s, 3H), 1.20 (d, 6H, J = 1.2 Hz), 1.38–1.61 (m, 8H), 2.24–2.28 (m, 2H), 2.51 (d, H, J = 12 Hz), 2.73–2.80 (m, 3H), 3.05–3.16 (m, 16H), 3.43–3.58 (m, 2H), 4.91 (s, 2H), 6.85 (s, 1H), 6.98 (d, 1H, J = 6.0 Hz), 7.03 (d, 2H, J = 8.0 Hz), 7.13 (d, J, H, J = 8.0 Hz), 7.46 (d, 2H, J = 8.0 Hz); 13C NMR (DMSO-d6): δ 168.8, 158.8, 158.6, 156.5, 154.1, 131.8, 129.0, 126.2, 123.8, 123.4, 115.0, 65.7, 56.1, 49.4, 45.6, 44.5, 42.0, 42.5, 41.7, 37.7, 28.8, 23.9, 18.6, 18.2. ESIMS. Calcd for C38H56N3O4 ([M+H]+): m/z 564.4591. Found: m/z 564.4593.

Compound 12 Yield 75%; mp 194–196°C; 1H NMR (DMSO-d6): δ 0.99 (s, 3H), 1.14 (s, 3H), 1.21 (d, 6H, J = 12 Hz), 1.47–1.72 (m, 8H), 2.27–2.31 (m, 2H), 2.76 (d, H, J = 8.0 Hz), 2.81–2.90 (m, 3H), 3.05–3.16 (m, 8H), 3.43–3.60 (m, 2H), 4.90 (s, 2H), 6.78 (s, 1H), 6.95 (d, 1H, J = 7.2 Hz), 7.17 (d, J, H, J = 8.0 Hz); 13C NMR (DMSO-d6): δ 163.7, 146.5, 145.1, 134.1, 126.3, 123.8, 123.5, 58.7, 47.5, 44.2, 42.1, 41.1, 38.1, 37.3, 37.0, 36.1, 34.8, 32.9, 28.8, 25.3, 23.9, 18.6, 18.2. ESIMS. Calcd for C59H78N7O13 ([M+H]+): m/z 848.4712. Found: m/z 848.4714.

In vitro cytotoxicity assay

Cytotoxicities of all compounds against MCF7 and HepG2 cell lines were determined using a cell counting kit-8 (CCK-8) assay. The cells were plated in 96-well culture plates at density of 1×104 cells per well and incubated for 24 h at 37°C in a wet atmosphere containing 5% CO2.
The tested compound was dissolved in PBS and then the diluted solution was treated with the cells for 24 h at 37°C in a 5% CO2 incubator; 5-FU was used as a positive control. Then, 10 μL of a freshly diluted CCK-8 solution [5 mg/mL in phosphate buffer saline (PBS)] was added to each well for 2 h. The cell survival was evaluated by measuring the absorbance at 450 nm. The IC50 value, which indicates the inhibition growth of 50% of cells relative to non-treated control cells, was calculated as the concentration of tested compound by best fit curving estimation. All experiments were carried out in triplicate.

**Assessment of cell apoptosis**

The MCF-7 cells were plated in 6-well culture plates at density of 3×104 cells per well. The cells were incubated with different concentrations of compound 10 for 24 h. The cultured MCF-7 cells were washed twice with PBS (pH 7.4) and then resuspended gently in 400 μL of binding buffer. The cell solution was then stained with Annexin V-APC/PI (Ex. 488 nm, Em. 585 nm) using a Becton-Dickinson BD FACSAria II flow cytometer. The data were analyzed using BD FACSDiva software. The percentage of cells in early and late apoptosis was calculated.

**Acknowledgments:** This study was financially supported by the National Science Foundation of China (No. 21172182 and 21362026).

**References**


