Apoptosis-Inducing Effects of Two Anthraquinones from *Hedyotis diffusa* WILLD.

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Two anthraquinones which inhibit activity of the Src tyrosine kinase were isolated from a water extract of *Hedyotis diffusa* WILLD. and identified as 2-hydroxy-3-methylanthraquinone (compound 1) and 1-methoxy-2-hydroxyanthraquinone (compound 2). Both compounds showed inhibitory activity against protein tyrosine kinases v-src and pp60src and arrested the growth of SPC-1-A, Bcap37 and HepG2 cancer cells. Observation of mitochondrial membrane potential collapse and caspase-3 activation following treatment with the compounds indicates that their apoptotic induction activity may act via the mitochondrial apoptotic pathway. Compared with compound 2, compound 1 is more active as an antagonist of Src kinase, which might account for its higher potency to induce growth arrest and apoptosis. These results provide a deeper insight into the functions of these two simple anthraquinones and the anti-tumour pathway of *Hedyotis diffusa* WILLD.

**Key words** *Hedyotis diffusa*; anthraquinone; apoptosis; c-src protein tyrosine kinase; mitochondrial membrane potential; caspase-3

*Hedyotis diffusa* WILLD. is a traditional herb commonly used in cancer therapy in China. It is also found to have other pharmacological activities, including anti-inflammatory, antibacterial and central nervous system regulatory actions. In clinical application, *Hedyotis diffusa* WILLD. is widely used in the therapies against liver, lung, colon, brain, pancreas and other cancers.1–5 Previous studies have shown that the triterpenes and polysaccharide from the herb have the ability to inhibit the proliferation of tumor cells, and that the primary extract of the herb could induce apoptosis in human breast cancer cell line SPC-A-1.4–6 However, few reports have documented the active anticancer compounds and the therapeutic pathway of the herb.

In the process of cancer development, some proteins exhibit different expression and activity levels compared with normal cells, such as c-src, c-yes, Fyn and EGFR.7,8 For example, overexpression and increased specific kinase activity of c-src is associated with the development of many types of cancers, including colon, breast, gastric, lung, pancreatic, neural, and ovarian cancers.11,12 Based on this activity they are very attractive targets for anti-cancer and herbal therapeutic research. Up to now many herbal compounds have been explored as the potential inhibitors of these target proteins for application in the development of new anti-tumor drugs.13,14

In this study, we extracted two anthraquinones from a water extract of *Hedyotis diffusa* WILLD. that can inhibit c-src kinase activity, and studied their anti-tumor effects and underlying molecular mechanisms in cancer cells. The results may provide a new understanding of the function of these two simple anthraquinones and the anti-tumor pathway of *Hedyotis diffusa* WILLD.

**MATERIALS AND METHODS**

**Structure Identification** The molecules were identified by NMR, mass, IR and UV spectra. NMR spectra were recorded in DMSO for 1H- and 13C-NMR at 500 MHz on a Bruker Avance 500 MHz spectrometer. Mass spectra were run on an esquire3000plus mass spectrometer. The IR spectra were taken on a Perkin-Elmer FT-IR spectrometer. UV spectra were taken on a Perkin-Elmer UV/VIS spectrometer.

**MTT Assay of Cell Viability** Cells were trypsinized in log phase growth, counted, and seeded in 96-well culture plates at a concentration of 10^4 cells per well. After incubation overnight, the medium was aspirated and drugs administered in complete medium at different dilutions in a total volume of 200 µl were added into 96-well plates. After incubation for an additional 48 h, the supernatant was aspirated, 20 µl of 5 mg/ml MTT solution and 80 µl complete medium were added to each well and the plates incubated for 4 h. The resultant MTT-formazan crystals were dissolved in 150 µl/well DMSO and the plates were read at 550 nm. All measurements were performed in triplicate and each experiment was repeated 3 times. The IC<sub>50</sub> was calculated from the 50% formazan formation compared with the control without treatment by compounds.

**In Vitro Kinase Assay** The tyrosine kinase activities of c-src and total cell protein were determined by ELISA using a Tyrosine Kinase Activity Kit (Chemicon) according to the manufacturer’s instructions.

**Cell Culture and Drug Treatment** Human hepatocellular carcinoma HepG2 cells were cultured on RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in 25-cm<sup>2</sup> culture flasks in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. For the drug treatment experiments, cells were harvested during the exponential growth phase and seeded at a density of 5×10⁴—1×10⁵ cells/well into multi-well culture plates. After overnight growth, the cells were treated with compounds (predissolved in DMSO at a concentration of 50 mM) at selected concentrations for a period of 1—2 d.

**Hoechst 33258 Staining and Annexin V-FITC Analysis**

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At the end of drug treatment, the cells in each well were washed once with PBS and fixed with 4% formaldehyde in PBS for 20 min. The cells were then washed with PBS and stained with 1 μg/ml Hoechst 33258 in PBS at 37°C for 15 min, and then viewed under a fluorescent microscope for nuclear morphological changes. For Annexin V-FITC Analysis, treated cells were stained with Annexin V-FITC/PI, and the apoptotic cell population was analyzed immediately by flow cytometry (FACSCalibur, BD Bioscience).

Caspase-3 Activity Assay After treatment by the compounds, the caspase-3 activity of the cell lysate was determined using a caspase colorimetric assay kit (Keygen Biotech Co., Ltd., Nanjing, China) according to the supplier’s manual. The optical density of the assay solutions was measured using a spectrophotometer at 405 nm.

Measurement of Mitochondrial Membrane Potential (ΔΨm) Mitochondrial membrane potential was measured using the cationic lipophilic green fluorochrome rhodamine-123 (Rh 123) by flow cytometry. Cells were washed twice with PBS and incubated with 1 μg/ml Rh 123 at 37°C for 30 min. The cells were then washed twice by PBS and fluorescence was determined by flow cytometry. Disruption of ΔΨm is associated with a lack of Rh 123 retention and a decrease in fluorescence.

Statistical Analysis The results were expressed as mean ± S.D. (standard deviation) and the differences between groups were analysis by ANOVA and Student’s t test. p<0.05 was considered statistically significant.

RESULTS

Structure Identification of Two Bioactive Compounds Two yellow compounds with inhibitory activity against c-src tyrosine kinase were separately isolated from a water extract of Hedyotis diffusa WILLD. Their structures were confirmed as two known anthraquinones, 2-hydroxy-3-methylanthraquinone (compound 1) and 1-methoxy-2-hydroxyanthraquinone (compound 2) by IR, UV, MS, 1H-NMR, 13C-NMR and HMBC analysis and comparison with the reported data (Fig. 1). Compound 1 was previously reported by Tai et al. from Hedyotis diffusa WILLD. Compound 2 from Hedyotis diffusa WILLD. was first reported in this paper.

Inhibitory Activity against pp60c-src and Cell Proliferation The two compounds showed inhibitory activity against protein tyrosine kinases pp60src (Table 1). The activities of pp60c-src, active GST-v-src protein and natural SPC-A-1 cell lysate, prepared as target proteins, were inhibited by both compounds 1 and 2, though compound 1 showed a higher inhibitory activity against all three kinase preparations than compound 2.

We next determined the effects of these two compounds on cell viability. Human lung cancer cell line, SPC-A-1, human breast cancer cell line, Bcap37, human liver cancer cell line, HepG2, and human colon cancer cell line, SW480, were treated with different doses of the compounds to examine the influence of the compounds on cell viability assessed by the MTT assay. As shown in Fig. 2 and Table 2, in these cell lines, compound 1 was more effective in inhibiting cell growth than compound 2, corresponding with their different Src inhibition activities. Of special interest, we observed SW480 cells were much less sensitive to the compounds, compared with SPC-A-1, Bcap37 and HepG2 cells. It was previously shown that c-src protein is expressed at a relatively low level in SW480. These data shows that these two compounds may arrest cell growth through their direct inhibition of the activity of c-src tyrosine kinase.

Effect of the Compounds on Apoptosis in HepG2 Cells To identify whether these two hydroxyanthraquinones induce apoptosis, the cells treated with the compounds (10 μM) for 48 h were observed by Hoechst 33342 staining. As shown in Fig. 3A, compared with the control cells, the treated cells exhibited condensed nuclei. The treated cells were also stained by Annexin V-FITC and PI, and the population of apoptotic cells was analyzed by flow cytometry. As seen in Fig. 3B, the drug treatment significantly increased the proportion of apoptotic cells. These results demonstrate the ability of these two compounds to induce apoptosis in HepG2 cells.

Role of the Mitochondrial Pathway in Compound-Induced Apoptosis in HepG2 Cells Apoptosis is often accompanied by a loss of mitochondrial transmembrane potential (ΔΨm). To identify whether the two hydroxyanthraquinones induce apoptosis, the cells treated with the compounds (10 μM) for 48 h were observed by Hoechst 33342 staining. As shown in Fig. 3A, compared with the control cells, the treated cells exhibited condensed nuclei. The treated cells were also stained by Annexin V-FITC and PI, and the population of apoptotic cells was analyzed by flow cytometry. As seen in Fig. 3B, the drug treatment significantly increased the proportion of apoptotic cells. These results demonstrate the ability of these two compounds to induce apoptosis in HepG2 cells.

Table 1. IC50 of Compounds 1 and 2 on Protein Tyrosine Kinases (Mean Values from Two Independent Experiments)

<table>
<thead>
<tr>
<th>Protein Tyrosine Kinase</th>
<th>Compound 1 IC50 (μM)</th>
<th>Compound 2 IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pp60c-src (3 U/ml)</td>
<td>ca. 20 μM</td>
<td>ca. 30 μM</td>
</tr>
<tr>
<td>GST-v-src protein</td>
<td>33 μM</td>
<td>41 μM</td>
</tr>
<tr>
<td>(0.1 mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPC-A-1 total protein</td>
<td>67 μM</td>
<td>71 μM</td>
</tr>
<tr>
<td>tyrosine kinase (0.5 mg/ml)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. IC50 of the Two Anthraquinones on Human Cancer Cell Lines (Mean Values from Three Independent Experiments)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Compound 1 IC50 (μM)</th>
<th>Compound 2 IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>51</td>
<td>62</td>
</tr>
<tr>
<td>SPC-A-1</td>
<td>66</td>
<td>79</td>
</tr>
<tr>
<td>BCA3P7</td>
<td>57</td>
<td>65</td>
</tr>
<tr>
<td>SW480</td>
<td>&gt;125</td>
<td>&gt;125</td>
</tr>
</tbody>
</table>

Fig. 1. Structure of Compounds 1 and 2
tial (ΔΨm), which is a crucial dysfunction resulting in mitochondrial release of apoptogenic factors and decrease of ATP generation, leading to cell death.17) To gain a better understanding of the mechanism underlying apoptosis induced by the compounds, we examined the effects of these two compounds on ΔΨm by rhodamine 123 retention. As shown in Fig. 4, the rhodamine 123 fluorescence appeared to decrease after compound treatment compared with the untreated control, indicating that the compound treatment incurred a collapse in the mitochondrial membrane potential. The result suggested that the mitochondrial pathway is involved in apoptosis induced by these two compounds.

Role of Caspase-3 in Compound-Induced Apoptosis in HepG2 Cells

Caspase-3 is a major mediator of apoptosis acting downstream of the mitochondrial signaling pathway. The dysfunction of mitochondria provoked us to measure the changes of caspase-3 activity in HepG2 cells following the compound treatment. As shown in Fig. 5, after exposure to the compound 1 and compound 2 separately, the activity of caspase-3 increased gradually from zero time point to 12 h, followed by an abrupt augment approximately to a flat stage after 12 h. Of note, compound 1 led to a more prominent change in the caspase-3 activity than compound 2.
which also showed antitumor effects,\textsuperscript{19,20} these two compounds possess a more simple structure, with only two substituents in one of the aromatic rings of the molecule. Their structures may provide clues for molecular modification of existing compounds to improve antitumor activity. We demonstrated that these two compounds can inhibit v-src, c-src and total cell lysates tyrosine protein kinase activity. Whether they can antagonize tyrosine protein kinases other than Src and act through other pathways remains uncertain. We can not exclude the possibility that these two compounds might be deleterious to normal cells as well, as a result of suppressing other signaling pathways involving tyrosine kinases, such as the insulin signaling pathway. More studies are necessary to address this question.

In conclusion, our results reveal anthraquinones derived from \textit{Hedyotis diffusa} \textsc{Willd.} are effective components of the herb when applied in cancer therapy, and highlight their molecular function. In light of this study, targeted therapy against c-src or other tyrosine protein kinase may present an important investigational strategy for cancer research.

\section*{REFERENCES}
\begin{enumerate}
\item Liao L. L., Chen C. H., Chen G. C., \textit{Taiwan Yi Xue Hui Za Zhi.}, \textbf{78}, 658–660 (1979).
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