Cytotoxic effects of compounds from *Iris tectorum* on human cancer cell lines

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**A R T I C L E   I N F O**

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**A B S T R A C T**

In the course of searching for novel cytotoxic compounds which can be used in chemotherapy, several Traditional Chinese Medicines (TCM) have been screened by bioassay-guided fractionation and isolation. An extract of rhizomes of *Iris tectorum* Maxim., a TCM used to treat cancer, exhibited highest potency and led to the isolation of two flavonoids, 7-O-methylaromadendrin and tectorigenin, and four iridal-type triterpenes, iritectols A and B, isoiridogermanal and iridobelamal A. The cytotoxicities of the isolated compounds against four human cancer cell lines were evaluated by the SRB assay. Iritectol B, isoiridogermanal and iridobelamal A showed similar cytotoxicity with IC50 around 11 μM and 23 μM against MCF-7 and C32 cell lines, respectively. Cell cycle-specific inhibition and apoptosis induced by the isolated compounds were determined using flow cytometry with two sets of co-labelling systems: annexin V-FITC/propidium iodide and fluorescein diacetate/propidium iodide. Iritectol B demonstrated dose-dependent apoptotic effect against COR-L23 cells with an apoptotic rate of 33% at 100 μM. Tectorigenin (an analogue of genistin) showed cell cycle specific inhibition and arrested cells at G2/M phase up to 400 μM, but did not demonstrate apoptotic effect against COR-L23 cells up to 1 mM. The overall activities of isolated compounds observed in the present study support the traditional use of *Iris tectorum* Maxim. in the treatment of cancer.

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1. Introduction

A survey of plants used in ethnomedicine against cancer has revealed more than 3350 plant species (Graham et al., 2000). Experimental agents derived from natural products offer a means of evaluation of new chemical classes of anticancer agents as well as novel and potentially relevant mechanisms of action (da Rocha et al., 2001). It has been found that most cancer chemotherapy drugs exert cytotoxic effects on malignant cells by inducing apoptosis (Kauffman and Earnshaw, 2000), and one essential strategy for cancer therapy is to target the lesions that suppress apoptosis in the tumour cells (Evan and Vousden, 2001).

*Iris tectorum* Maxim. (Iridaceae) is a perennial herb native to China. Its Chinese botanical name is “尋常香” (Yuan Wei). It is also known as Japanese Roof Iris in some literature, because it was first observed growing on roofs by a Russian botanist, Carl Maximowicz (1827–1891) (Klingaman, 2005). The rhizome of *Iris tectorum* was introduced as a medicine in the first Chinese monograph on herbal medicines, “神农本草经” (Shen Nong Ben Cao Jing) (also known as “The Divine Farmer’s Herb-Root Classic”), which was completed about 200 AD (Tan and Wen, 2001). It was used as a bitter medicine to treat disorders described as “癒瘍屬” (Zheng Jia Jie Ju). These syndromes are similar to modern descriptions of tumours. It has also been commonly used as an anti-helminthic in China (Song et al., 2001). According to the latest edition of the Chinese Pharmacopoeia (Anonymous, 2005), *Iris tectorum* is referred to as “川射干” (Chuan She Gan), and is used to treat sore throat, to disperse phlegm, and for heat-clearing and detoxifying. It has also been used to treat abdominal distension and hepatic cirrhosis (Song et al., 2001) in China. In Japan it is used as an emetic and a laxative (Seki et al., 1994a). A field study and investigation prior to the present study revealed that the rhizome of *Iris tectorum* has been used as traditional folk medicine for the treatment of cancer in Tongren, a small town in Guizhou province, South China (Fang, 2007).

Some isoflavonoids (Wu and Xu, 1992; Morita et al., 1972), quinones (Seki et al., 1994b) and iridal-type triterpenoids (Krick et al., 1983; Ito et al., 1999) have been isolated from Iridaceous plants. Some iridal-type triterpenoids, such as 16-O-acetylisoridigermanal, belachanal and spiroiridal exhibited considerable ichthyotoxic activity with TL50 (median tolerance limit after 24 h) <3.5 μg/ml (Ito et al., 1999). As part of a search for bioactive compounds in *Iris tectorum* Maxim., two flavonoids 7-O-methylaromadendrin (1) and tectorigenin (2), and four iridal-type triterpenoids: iritectols A (3) and B (4), iridobelamal A (5) and isoiridogermanal (6) were isolated from the most cytotoxic fractions (Fang, 2007). The stereochemistry of the two novel triterpenoids iritectols A (3) and B (4) was discussed previously (Fang et al., 2007); the stereochemistry of iritectol A (3) could not be determined. In the present report, the cytotoxicity of those
MeOH 86 (5.6 mg) and sub-fractions (IT1–IT6). 12.2 mg 7-
extract of flavonoids and triterpenoids against four human cancer cell lines was not tested. Essential oil extracted by hexane could not be dissolved in aqueous medium, and was not tested.

flavonoids and triterpenoids against four human cancer cell lines was compared with two standard natural compounds genistein (7) and luteolin (8). Cell cycle-specific cytotoxicity and apoptotic activity of 7-O-methylaromadendrin (1) and iritectol B (4) are reported for the first time.

2. Materials and methods

2.1. Separation and purification

Iris tectorum Maxim. (Iridaceae) used in the present study was authenticated by Christine Leon at, and the voucher specimen “TCMK 598” is deposited in, the Chinese Medicinal Plants Authentication and Conservation Centre, Royal Botanic Gardens, Kew. For preliminary screening, 10 g of dried Iris tectorum was ground into a fine powder and extracted successively with hexane, chloroform (CHCl3), methanol (MeOH) and water (1:10 w/v) using a Soxhlet extractor for 24 h. The water solution was freeze-dried and all organic solutions were concentrated to dryness under reduced pressure, and then redissolved in DMSO at 20 mg/ml for the bioassays.

The preliminary cytotoxicity assay indicated that the CHCl3 extract of Iris tectorum (Table 1) showed high activity. Therefore, a large scale fractionation and isolation for the active compounds from Iris tectorum was carried out. Dried, ground rhizomes of Iris tectorum Maxim. (2 kg) were extracted by supercritical fluid extraction (with liquid CO2) in Shenzhen Neptunus Bioengineering Co. Ltd., China. In this step the most volatile were removed. The dry residue was exhaustively extracted into CHCl3:MeOH (1:1) and MeOH successively, and that produced six fractions IT1–IT6.

<table>
<thead>
<tr>
<th>Exacts</th>
<th>COR-L23</th>
<th>C32</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>100 ± 1.5</td>
<td>100 ± 0.8</td>
<td>100 ± 1.4</td>
</tr>
<tr>
<td>CHCl3</td>
<td>7 ± 0.7</td>
<td>27 ± 1.5</td>
<td>16 ± 0.5</td>
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<tr>
<td>MeOH</td>
<td>86 ± 0.8</td>
<td>103 ± 1.1</td>
<td>92 ± 0.6</td>
</tr>
<tr>
<td>H2O</td>
<td>101 ± 1.3</td>
<td>109 ± 0.1</td>
<td>97 ± 1.1</td>
</tr>
</tbody>
</table>

Table 1 Results of preliminary cytotoxicity assay for solvent extracts of Iris tectorum, showing percentage of remaining cells after exposure to 100 μg/ml of each extract for 48 h (n = 3, mean ± S.E.M.)

Fraction IT4 (17 g) exhibited the highest cytotoxicity in the in vitro assay. This active fraction was purified by using semi-preparative RP-HPLC (Discovery® HS C18 250 mm × 10 mm, flow rate 4 ml/min) under the following gradient: MeCN–H2O (3.7: 9.1 over 50 min) which allowed isolation of compounds 3 (11.2 mg), 4 (13.1 mg), 5 (5.6 mg) and 6 (26.8 mg).

2.2. Cell culture

Four human cancer cell lines: COR-L23 (human lung large cell carcinoma), C32 (human amelanotic melanoma) and MCF-7 (human breast adenocarcinoma) tested in present experiments were obtained from the European Collection of Cell Cultures (ECACC). HepG2 (hepatocellular carcinoma) was obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in appropriate medium (RPMI-1640, DMEM or EMEM, recommended by the supplier), and supplemented with 10% foetal bovine serum (FBS, Sigma–Aldrich, F9665), 1% penicillin (104 U/ml)/streptomycin (10 mg/ml) solution (Sigma–Aldrich, P0781) and 1% L-glutamine (200 mM, Sigma–Aldrich, G7513). At about 70–80% confluence, cells were trypsinised with trypsin/EDTA solution (Sigma–Aldrich, T4049) to form a cell suspension and counted using a haemocytometer before inoculation. Cells were plated in 96-well (Costar 3359) or 6-well plates (Costar 3516) at appropriate density which ensured that the cells were in exponential growth and left to attach for 24 h in a humidified atmosphere of 5% CO2 in air, at 37 °C prior to addition of extract or compound.

The adherent cells need to attach and spread out on the substrate before proliferation begins (Fresnhey, 2000). A seeding density of 5 × 104 cells per well in a 6-well plate proved to be appropriate in the present study, since the apoptotic rate in the controls was typically lower than 3% in most experiments. In order to spread the cells evenly in 6-well plates and avoid clump formation in the centre of each well in the first 30 min of preincubation, the plates were gently rotary shaken during the initial 15 min in the incubator. The supernatant with a few dead cells was removed from each well by careful aspiration at the end of preincubation, and then compound solutions prepared with fresh growth medium were added into each well to treat cells for another 48 h. These steps were essential for making sure that all cells were viable before treatment.

2.3. Cytotoxic assay

For determination of the dose response, cells were seeded at a density of 5 × 10^5 cells per well in 96-well plates (Costar 3359). After 24 h preincubation, solutions of the compounds were added to produce a series of concentrations, and then the plates were incubated for another 48 h. As the endpoint measurement, the sulphorhabdomine B (SRB) (Sigma–Aldrich, S1402) protein staining assay (modified by Houghton et al., 2007) was employed for measurement of in vitro cytotoxicity (Monks et al., 1991; Skehan, 1999). The cytotoxicities of the tested compounds were calculated as IC50, which provides the drug concentration causing a 50% reduction in the net increase of protein in the cells over vehicle control during drug incubation (Monks et al., 1991). Data were calculated by GraphPad Prism version 4.00 for Windows.

2.4. Detection of apoptosis

Flow cytometry (FCM) is the technique of choice for the quantitative measurement of apoptosis. The selected methods for apoptotic investigation are dual staining systems, which are based on changes in membrane permeability that occur during apoptosis. By using propidium iodide (PI) (Sigma–Aldrich, P4170) in combination with other markers, such as fluorescein diacetate (FDA) (Fluka, 31545) or fluorescein isothiocyanate-labelled annexin V (annexin V–FITC) (Sigma–Aldrich, 556420), apoptotic, viable and dead cells can be readily distinguished (Saha et al., 2003). COR-L23 cells were used to test the induction of apoptosis by the isolated compounds and genistein (7). Cells were seeded at 5 × 10^4 cells per well in 6-well plates (Costar, 3516). After 24 h preincubation, cells were exposed to compounds for another 48 h. At the end of 48 h treatment, supernatants were collected for each well and centrifuged at 1500 rpm to collect detached cells, which may contain a considerable number of apoptotic cells. Then the adherent cells were detached using 0.05%/0.04% trypsin/EDTA solution and collected by centrifugation at 1500 rpm following the methods of Sasaki et al. (1987), Ormerod et al. (1993) and Schmid et al. (1994). FDA and PI co-labeling is able to distinguish apoptotic cells (FDA+/PI−) from viable cells (FDA−/PI−) and dead cells (FDA−/PI+) by quadr-
Table 2

<table>
<thead>
<tr>
<th>Compounds</th>
<th>COR-L23</th>
<th>C32</th>
<th>HepG2</th>
<th>MCF-7</th>
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<tr>
<td>1</td>
<td>33 ± 0.5</td>
<td>33 ± 3.3</td>
<td>25 ± 1.7</td>
<td>21 ± 1.4</td>
</tr>
<tr>
<td>2</td>
<td>189 ± 0.6</td>
<td>207 ± 13.7</td>
<td>149 ± 2.5</td>
<td>105 ± 2.0</td>
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<tr>
<td>3</td>
<td>35 ± 0.9</td>
<td>40 ± 2.3</td>
<td>29 ± 2.1</td>
<td>18 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>19 ± 0.4</td>
<td>25 ± 2.0</td>
<td>20 ± 0.6</td>
<td>11 ± 1.4</td>
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<tr>
<td>5</td>
<td>14 ± 0.3</td>
<td>23 ± 1.0</td>
<td>18 ± 0.3</td>
<td>11 ± 1.2</td>
</tr>
<tr>
<td>6</td>
<td>16 ± 1.8</td>
<td>24 ± 0.8</td>
<td>22 ± 2.0</td>
<td>11 ± 2.1</td>
</tr>
<tr>
<td>7</td>
<td>63 ± 9.0</td>
<td>68 ± 2.2</td>
<td>52 ± 4.0</td>
<td>43 ± 0.5</td>
</tr>
<tr>
<td>8</td>
<td>11 ± 0.7</td>
<td>25 ± 0.9</td>
<td>9 ± 1.2</td>
<td>18 ± 1.1</td>
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</table>

Table 3

<table>
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<tr>
<th>Assays</th>
<th>1</th>
<th>2</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>3.1 ± 0.29</td>
<td>1.6 ± 0.21</td>
<td>2.6 ± 0.43</td>
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<tr>
<td>25 µM</td>
<td>5.1 ± 0.94</td>
<td>5.0 ± 0.67</td>
<td>7.2 ± 0.70</td>
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<tr>
<td>50 µM</td>
<td>6.7 ± 0.83</td>
<td>6.1 ± 0.68</td>
<td>6.5 ± 0.52</td>
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<tr>
<td>75 µM</td>
<td>10.0 ± 1.04</td>
<td>9.0 ± 1.58</td>
<td>9.8 ± 0.86</td>
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<tr>
<td>100 µM</td>
<td>13.4 ± 1.35</td>
<td>8.3 ± 1.71</td>
<td>9.8 ± 0.86</td>
</tr>
<tr>
<td>125 µM</td>
<td>18.8 ± 1.52</td>
<td>6.1 ± 1.26</td>
<td>5.16 ± 2.45</td>
</tr>
<tr>
<td>Ctrl</td>
<td>3.6 ± 0.13</td>
<td>2.4 ± 0.56</td>
<td>2.9 ± 0.97</td>
</tr>
<tr>
<td>25 µM</td>
<td>5.2 ± 1.68</td>
<td>7.5 ± 0.54</td>
<td>3.9 ± 1.57</td>
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<tr>
<td>50 µM</td>
<td>8.5 ± 2.14</td>
<td>7.1 ± 0.90</td>
<td>5.2 ± 1.67</td>
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<tr>
<td>100 µM</td>
<td>11.5 ± 3.05</td>
<td>7.6 ± 0.75</td>
<td>8.8 ± 2.71</td>
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<tr>
<td>1000 µM</td>
<td>8.0 ± 0.52</td>
<td>8.0 ± 0.52</td>
<td>13.3 ± 3.06</td>
</tr>
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</table>

Fig. 1. Percentage of COR-L23 cells in three different conditions after exposure to various concentrations of genistein (7) for 48 h. Cells stained with FDA/PI (A) and ann/PI (B) (n = 3, mean ± S.E.M.). (A) Living cells (●), dead cells (□) apoptotic cells.

3. Results

3.1. Cytotoxicity

Compounds 1–6 together with two flavonoids, genistein (7) and luteolin (8) (as positive controls), showed dose-dependent inhibition of proliferation against the four cell lines. Their cytotoxicities are shown as IC50 in Table 2. Luteolin (8) (a well-known cytotoxic flavonoid) was used as a positive control. In the present study 8 was found to be the most cytotoxic compound with IC50 of 11 µM and 9.4 µM against COR-L23 and HepG2, respectively. Broadly, the order of cytotoxicity for the four tested flavonoids was luteolin (8) > 7-O-methylaromadendrin (1) > genistein (7) > tectorigenin (2). The iridal-type triterpenes have similar levels of cytotoxicity as...
those of 1, 7 and 8. Iriectol A (3) was less cytotoxic than the other triterpene. 4, 5 and 6 showed similar cytotoxicity (IC\textsubscript{50}s around 24 \mu M and 11 \mu M against C32 and MCF-7, respectively (Table 2)).

3.2. Apoptosis

As shown in Table 3, the results of the FDA/PI and ann/PI assays are consistent with each other for the same compound. The positive control, genistein (7), induced dose-dependent apoptosis at concentrations from 100 \mu M to 400 \mu M in two sets of co-labelling apoptosis assays. As seen in Fig. 1, apoptotic cells (LR) increased from 7\% to 52\% in FDA/PI assay. Generally, the percentage of dead cells, which also increased with the concentration of 7, could be attributed to both apoptosis as well as necrosis because genistein (7) can induce necrosis at high concentrations (Baxa et al., 2005). 1 and 4 also showed dose-dependent apoptosis against COR-L23 cells (Table 3, Figs. 2 and 3). The apoptotic rates increased significantly (p < 0.01; one-way ANOVA with Tukey's multiple comparison post-test, GraphPad Prism version 4.0 for Windows) with concentration of the compounds. In contrast, 2 is the least cytotoxic flavonoid tested in the present study, and no dose-dependent apoptosis was observed up to 1 mM. It demonstrated a constant

Fig. 2. Percentage of COR-L23 cells in three different conditions after exposure to various concentrations of iritectol B (4) for 48 h. Cells stained with FDA/PI (A) and ann/PI (B) (n = 3, mean \pm S.E.M.). (□) Living cells, (■) dead cells and (■) apoptotic cells.
Fig. 3. Contour plots of annexin/PI-stained COR-L23 cells in three conditions in quadrant analysis: they are living cells (lower left; LL), dead cells (UL and UR) and apoptotic cells (LR). (A) Control; (B) exposed to 100 μM iritecitol B (4) for 48 h.

apoptotic rate about 8% from 200 μM to 1000 μM in both assays (Table 3).

3.3. Cell cycle analysis

Cell cycle analysis confirmed that the positive control genistein (7), as expected, significantly (p < 0.001) arrested COR-L23 cells at G2/M phase up to 200 μM (Fig. 4). After exposure to 7-O-methylaromadendrin (1), COR-L23 cells were significantly (p < 0.001) arrested in S phase at 60 μM and 100 μM, but no significant (p > 0.05) accumulation was observed in the G2/M phase (Fig. 5). On the other hand, the percentage of cells in G2/M phase gradually increased with the dose of tectorigenin (2) from 50 μM to 400 μM. The accumulation of cells in G2/M phase at 400 μM of 2 was significant (p < 0.001) (Fig. 6). The results with iritecitol B (4) showed that cells accumulated in the sub-G1 phase and simultaneously reduced in all the other phases as the dose of 4 increased. This means that cells were killed at all stages of cell cycle.

4. Discussion and conclusions

4.1. Cytotoxicity

Of the six isolated compounds, 2 is relatively non-cytotoxic with IC50s higher than 100 μM against various cancer cell lines. All the other compounds showed moderate cytotoxicity. 7 and 2 are both isoflavonoids with low molecular weight (270 and 300, respectively)—they differ from each other only by the presence of one extra methoxyl group in 2. However, the cytotoxicity of 7 is around 200% higher than that of 2 against various cancer cell lines. Therefore, the 6-methoxyl group on the A-ring is important to reduce the cytotoxicity in this series. 8 showed the highest cytotoxicity among those four flavonoids.

7-O-Methylaromadendrin (1) has been isolated from several plants, including Populus alba, Artemisia dracunculus, Eucalyptus maculate and Eupatorium species (Stoessl et al., 1971; Herz et al., 1972; Balza and Towers, 1984; Abdel-Sattar et al., 2000). It was reported to have acute anti-inflammatory activity in vivo, taken as a rationale for the use of the plant in skin disease (Manez et al., 1999). However, little anticancer activity has been reported. The present work is the first report of its isolation from a member of the Iridaceae and evaluation of its subsequent cytotoxicity.
4.2. Apoptosis

2 has been reported to induce differentiation and apoptotic changes in DNA fragmentation analysis of human promyelocytic leukemia HL-60 cells in vitro (Lee et al., 2001). In the present study, however, no dose-dependent apoptosis induced by 2 was observed with COR-L23 cells up to 1 mM in two apoptosis assays (Table 3). This inconsistent result may be due to the different cell lines, exposure time and methods used. Nevertheless, the present result indirectly supports the conclusion of a previous report, in which 2 was demonstrated to protect HepG2 cells against the cytotoxicity induced by tert-butyl hydroperoxide (Lee et al., 2005). This hepatoprotective effect was suggested to originate from inhibition of apoptosis. Moreover, these findings lend support to the traditional use of the original plant, because Iris tectorum has been used to treat patients with hepatic cirrhosis (Song et al., 2001). As a Traditional Chinese Medicine, besides its antioxidant activity (Jung et al., 2004), it has also been reported that Iris tectorum Maxim. The consistent results of 2 inhibited angiogenesis both in vitro and in vivo (Jung et al., 2003). It also significantly rectified the aberrant expression of several essential gene products involved in prostate cancer (Lee et al., 2001). It was reported to down-regulate prostate-derived Ets transcription factor (PDEF), prostate-specific antigen (PSA) and insulin-like growth factor-1 (IGF-1) receptor mRNA expression in vitro (Thelen et al., 2005).

4.3. Cell cycle-specific inhibition

Although it has previously been reported that genistein (7) induced cell cycle progression arrest at S and G2/M phases in human head and neck squamous cell carcinoma cells (Alhasan et al., 1999), no significant accumulation of COR-L23 cells in the S phase was observed over a range of concentrations from 20 μM to 200 μM in the present work. Similar to the cell cycle-specific activity of 7 (Fig. 4), 2 inhibited cell proliferation at G2/M phase (Fig. 6).

4.4. Conclusions

The present study provides evidence for cytotoxicity and induction of apoptosis by Iris tectorum Maxim. The consistent results of the two apoptosis assays suggested that 7-O-methylaromadendrin (1) and iriectol B (4) can induce apoptosis of COR-L23 in vitro. 1 and 2 also demonstrated S phase- and G2/M phase-specific inhibition against COR-L23 cells, respectively. However, 4 induced apoptosis of COR-L23 cells with cell cycle non-specific activity. The overall activities of isolated compounds observed in the present study support the traditional use of Iris tectorum Maxim. in anticancer treatment.

Acknowledgements

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