Feselol Enhances the Cytotoxicity and DNA Damage Induced by Cisplatin in 5637 Cells

Samaneh Mollazadeh, Maryam M. Matin, Ahmad Reza Bahrami, Mehrdad Iranshahi, Morteza Behnam-Rassouli, Fatemeh B. Rassouli, and Vajiheh Neshat

Introduction

Two main histological forms of bladder cancer are transitional cell carcinoma (TCC) and squamous cell carcinoma (SCC) (Sengupta et al., 2004). TCC of the bladder is the second most common urological malignancy after prostate cancer (Pow-Sang, 2000). Although various options such as surgery and radiotherapy are available for the management of bladder cancers, these tumours are rather resistant to most chemotherapy regimes (Niell et al., 1985).

Cisplatin is a chemotherapeutic drug, which is used widely against different types of malignant tumours (Cohen and Lippard, 2001). It is believed that the cytotoxic function of cisplatin is related to inhibition of DNA replication and/or transcription mediated by the formation of platinum-DNA adducts (Zamble and Lippard, 1995). It has been shown that cisplatin-based combination therapy is the mainstay of the treatment for high-grade bladder cancers (Roth and Bajorin, 1995).

Many studies have shown that plants are a suitable source of drugs, and finding novel therapeutic compounds from medicinal plants has received considerable attention. The genus *Ferula* (Apiaceae), which is distributed throughout the Mediterranean area and Central Asia (French, 1971), is a rich source of coumarin derivatives (Bukreeva and Pimenov, 1991; Iranshahi et al., 2004, 2007, 2008; Mirjani et al., 2005; Barthomeuf et al., 2008). Sesquiterpene coumarins of the drimane type (diportlandin, conferone, mogoltacin, and feselol, Fig. 1) can increase the accumulation and effectiveness of several anticancer agents (Madureira et al., 2004; Barthomeuf et al., 2006; Neshati et al., 2009; Behnam Rassouli et al., 2009; Rassouli et al., 2011a; Mollazadeh et al., 2010).

The aim of the present study was to investigate the effects of feselol, a sesquiterpene coumarin extracted from *Ferula badrakema* (Bukreeva and Pimenov, 1991), on cisplatin cytotoxicity in 5637 cells, a subline of TCC, and the [3-(4,5-dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide (MTT) assay and the alkaline version of the comet assay were used to investigate cell survival and DNA damage, respectively.
Material and Methods

Preparation of solutions of feselol and cisplatin

Feselol was isolated from the fruits of *F. badrakema*, using silica gel chromatography, as previously described (Mollazadeh et al., 2010). In order to prepare various concentrations of feselol (8, 16, 32, 64, and 128 μg/mL), 2 mg of feselol powder were dissolved in 1 mL dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) and serially diluted in culture medium. Since feselol was dissolved in DMSO, equivalent amounts of DMSO (0.4%, 0.8%, 1.6%, 3.2%, and 6.4%, respectively) were used as control groups. To identify the half maximal inhibitory concentration (IC₅₀) of cisplatin (EBEWE Pharma, Vienna, Austria) in 5637 cells, increasing concentrations of cisplatin (10, 20, 50, 100, and 200 μg/mL) were prepared in complete culture medium just before the experiments.

Culture of 5637 cells

Human 5637 cells, which are epithelial-like adherent cells, were obtained from the Pasteur Institute (Tehran, Iran). They were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS) (Gibco), and incubated at 37 °C in a humidified atmosphere with 10% CO₂. For subculture, flasks with confluent cells were incubated with 0.25% trypsin and 1 mM EDTA (Gibco) for 3–5 min. Then fresh medium was added to the detached single cells which were transferred to new labeled flasks.

Cytotoxicity assay

In order to assess the cytotoxic effects of feselol, cisplatin, and their combination, the MTT (Sigma, Deisenhofen, Germany) assay was used. The MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme of viable cells to convert the tetrazolium bromide into purple formazan crystals (Mosmann, 1983). To do the test, 8000 cells/well were seeded in 96-well plates (Falcon, Becton-Dickinson, Mississauga, Ontario, Canada), and the total volume of each well was increased to 200 μL by adding fresh medium. The cells were allowed to grow for 24 to 48 h, and then they were treated with different concentrations of feselol and cisplatin and incubated for 3 d. After determination of the IC₅₀ values of cisplatin, cells were incubated with various combinations of feselol + cisplatin concentrations and equivalent DMSO + cisplatin solutions, for three consecutive days. To assess cell viability, 20 μL of MTT solution were added to each well and incubated for 4 h at 37 °C. After removing the MTT solution from each well, the produced formazan was dissolved in DMSO (200 μL/well), and the optical density (OD) of each well was read at 570 nm using a multiwell scanning spectrophotometer (ELISA reader; Awareness, Palm City, USA). All experiments were performed in triplicate. Percentages of living cells were calculated as follows: living cells (%) = (absorbance of treated cells per well)/(mean absorbance of control cells) · 100.

Morphological alterations

5637 cells were coincubated with different concentrations of feselol + cisplatin. Then morphological alterations such as cell density, cytoplasm granulation, and adhesiveness to the flask were observed using a light microscope 24, 48, and 72 h after drug administration.
Alkaline comet assay

To detect DNA damage caused by cisplatin, the alkaline version of the comet assay (Singh et al., 1998) was used. Briefly, untreated cells, cells treated with 32 μg/mL feselol + 1 μg/mL cisplatin, and cells treated with an equivalent amount of DMSO (1.6% DMSO) + 1 μg/mL cisplatin were trypsinized and centrifuged at 1066 x g for 10 min (Orto Alresa Digicen 20, Madrid, Spain). The resulting cell pellets were resuspended in 100 μL of 1% (w/v) low-melting point agarose (LMA; Fermentas, St. Leon-Rot, Germany), layered onto glass microscope slides precoated with 1% (w/v) normal-melting point agarose (Helicon, Moscow, Russia), and kept at 4 °C for 20 min for solidification. Then slides were recoated with 100 μL of 1% (w/v) LMA and kept for another 20 min at 4 °C. Next, slides were immersed in fresh ice-cold lysing buffer [2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 2% (v/v) Triton X-100, pH 10] and kept at 4 °C for 4 h. Slides were then washed with cold distilled water, placed in an electrophoresis chamber filled with fresh cold alkaline electrophoresis buffer (1 mM EDTA, 0.3 M NaOH, pH 13) and kept at 4 °C for 30 min. Electrophoresis was carried out at 25 V, 300 mA, and 4 °C for 20 min under highly alkaline conditions, which allowed the damaged DNA to migrate away from the nucleus. Then, slides were washed with ice-cold neutralizing buffer (0.4 M Tris-HCl, pH 7.5), dried with 96% ethanol, stained with ethidium bromide (20 μg/mL), and visualized under a fluorescent microscope (Olympus, Tokyo, Japan) attached to a CCD camera. For each slide 50 cells were analysed, and the average of the comet tail moment was determined by Cometscore version 1.5 software. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. The DNA damage was expressed as: DNA in tail (%) = [tail DNA/(head DNA + tail DNA)] · 100.

Statistical analyses

The significance level was ascertained by one way ANOVA, followed by Tukey multiple comparison test. A P-value of <0.05 in the Tukey test was considered significant. Results were expressed as means ± SD. Statistical procedures were performed using SPSS, JMP4, and MSTATC softwares.

Results

5637 cells were exposed to different concentrations of feselol for 24, 48, and 72 h. Since feselol was dissolved in DMSO, which is a toxic compound, equivalent amounts of DMSO were used as controls. The MTT assay revealed that feselol on its own did not have any significant toxic effects on these cells (data not shown).

To determine the IC₅₀ values of cisplatin in 5637 cells, they were treated with various concentrations of cisplatin for three consecutive days, and the viability of cells was then measured by the MTT assay. The IC₅₀ values of cisplatin in these cells were 12 μg/mL after 24 h and 8 μg/mL after 48 and 72 h, respectively (Fig. 2).

To test the effects of feselol on cisplatin cytotoxicity, 5637 cells were treated with 15 different combinations of feselol (8, 16, 32, 64, and 128 μg/mL) and cisplatin (1, 5, and 10 μg/mL), i.e. close to and below its IC₅₀ values, for three consecutive days. Equivalent volumes of DMSO in combination with cisplatin were also used as control groups. Comparing the effects of feselol + cisplatin with DMSO + cisplatin on the cells indicated that cell viability was significantly decreased in feselol + cisplatin combinations (Table I). Final statistical analyses (LSD test) showed that, 24 h after treatment, the concentration of 32 μg/mL feselol increased the cytotoxicity of 1 μg/mL cisplatin by 37% (Fig. 3).

The effects of feselol on cisplatin cytotoxicity were also confirmed by morphological observations. 5637 cells treated with 30 different concentrations of feselol + cisplatin and DMSO +
Table I. Comparison of cell survival percentage in 90 treated groups; combination of 8, 16, 32, 64, and 128 μg/mL feselol with 1, 5, and 10 μg/mL cisplatin and their DMSO equivalents (mean ± SD).

<table>
<thead>
<tr>
<th>Treated group</th>
<th>Cisplatin [μg/mL]</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>0.4% DMSO</td>
<td>1</td>
<td>105 ± 0.11</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>90 ± 0.01</td>
</tr>
<tr>
<td>8 μg/mL</td>
<td>1</td>
<td>86 ± 0.04*</td>
</tr>
<tr>
<td>Feselol</td>
<td>5</td>
<td>86 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>86 ± 0.01</td>
</tr>
<tr>
<td>0.8% DMSO</td>
<td>1</td>
<td>89 ± 0.07</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>77 ± 0.02</td>
</tr>
<tr>
<td>16 μg/mL</td>
<td>1</td>
<td>65 ± 0.01*</td>
</tr>
<tr>
<td>Feselol</td>
<td>5</td>
<td>63 ± 0.01*</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>61 ± 0.02*</td>
</tr>
<tr>
<td>1.6% DMSO</td>
<td>1</td>
<td>94 ± 0.11</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>71 ± 0.05</td>
</tr>
<tr>
<td>32 μg/mL</td>
<td>1</td>
<td>57 ± 0.01*</td>
</tr>
<tr>
<td>Feselol</td>
<td>5</td>
<td>64 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>62 ± 0.02</td>
</tr>
<tr>
<td>3.2% DMSO</td>
<td>1</td>
<td>86 ± 0.03</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>92 ± 0.14</td>
</tr>
<tr>
<td>64 μg/mL</td>
<td>1</td>
<td>64 ± 0.02*</td>
</tr>
<tr>
<td>Feselol</td>
<td>5</td>
<td>60 ± 0.03*</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>58 ± 0.03*</td>
</tr>
<tr>
<td>6.4% DMSO</td>
<td>1</td>
<td>84 ± 0.05</td>
</tr>
<tr>
<td>10</td>
<td>88 ± 0.08</td>
<td>92 ± 0.02</td>
</tr>
<tr>
<td>128 μg/mL</td>
<td>1</td>
<td>56 ± 0.02*</td>
</tr>
<tr>
<td>Feselol</td>
<td>5</td>
<td>60 ± 0.01*</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>60 ± 0.01*</td>
</tr>
</tbody>
</table>

The results of the Tukey test compared the effects of all combinations of feselol and cisplatin concentrations with their DMSO analogues. * P < 0.05 shows significant difference between test and control groups.

cisplatin were monitored during 72 h after drug administrations. The most obvious changes were observed 24 h after the coincubation of 5637 cells with the combinations of 32 and 64 μg/mL feselol with 1 and 10 μg/mL cisplatin, respectively. The cells became rounded and deformed with granulated cytoplasm, and the cell numbers were significantly decreased in comparison with controls (Figs. 4A–C).

In order to determine the mechanism involved in the cytotoxic effects of feselol + cisplatin, a comet assay was performed. In comparison with untreated and control cells, the combination of
32 μg/mL feselol with 1 μg/mL cisplatin significantly \((P < 0.05)\) increased DNA damage by 31% (Figs. 4D–F and Fig. 5).

**Discussion**

The main obstacle in cancer chemotherapy is resistance to anticancer chemotherapeutic agents, which is caused by decreased drug accumulation, altered intracellular drug distribution, increased detoxification, and DNA repair (Germann, 1996; Borst and Schinkel, 1997). Cisplatin is typically responsible for cellular toxicity and induction of apoptosis because of its strand-breaking and cross-linking potency (Konstantakou et al., 2009). Although cisplatin-based combination is the mainstay treatment of bladder cancer, its efficacy is limited due to inherent or acquired drug resistance (Roth and Bajorin, 1995; Clifford et al., 1996; Borst et al., 2000). In the present study, the effects of feselol, a sesquiterpene coumarin from fruits of *F. badrakema*, on cisplatin cytotoxicity was investigated. The MTT assay indicated that feselol increased the cisplatin toxicity by 37%. Moreover, comet photomicrographs revealed that most of the nuclei in untreated cells were undam-

![Fig. 4. Photomicrographs and comet images of 5637 cells after 24 h, (A, D) without any treatment, (B, E) treated with 1.6% DMSO + 1 μg/mL cisplatin, and (C, F) treated with 32 μg/mL feselol + 1 μg/mL cisplatin.](image)

![Fig. 5. DNA lesion of untreated 5637 cells in comparison with cells treated with 1.6% DMSO + 1 μg/mL cisplatin and cells treated with 32 μg/mL feselol + 1 μg/mL cisplatin. Results are means ± SD. * Indicates significant \((P < 0.05)\) difference to control cells; • indicates significant \((P < 0.001)\) difference to untreated cells.](image)
aged, while in cells treated with DMSO + cisplatin, DNA damage was significantly increased. On the other hand, addition of feselol caused more prominent lesions, which is in agreement with the results of the MTT assay and morphological observations.

One of the mechanisms involved in cisplatin resistance is overexpression of the multidrug resistance-related protein 2 (MRP2), which facilitates the cisplatin efflux (Kawabe et al., 1999). Moreover, expression of the MRP gene affects cellular accumulation of some chemotherapeutic drugs such as doxorubicin, epirubicin, and vinblastine which are used in the treatment of advanced bladder TCC (Clifford et al., 1996). Therefore, using agents that revert drug resistance could be a potential approach to enhance the sensitivity of bladder TCCs to chemotherapy (Wu et al., 2006).

It has been shown that sesquiterpenes from the Celastraceae and Euphorbiaceae families could act as reversal agents (Spivey et al., 2002; Madureira et al., 2004). The probable mechanism of feselol action could be its interaction with and inhibition of the function of the MRP2. Furthermore, we have previously shown that, besides increasing the cytotoxic effects of anticancer agents (Neshati et al., 2009; Behnam Rassouli et al., 2009; Rassouli et al., 2011a; Mollazadeh et al., 2010), terpenoid derivatives from Ferula species show cytotoxic effects in vitro (Rassouli et al., 2011b). However, more research is needed to test whether feselol has the same effects on other drug-resistant cells and also to analyse its mode of action.

In conclusion, since inherent or acquired overexpression of drug efflux pumps is a major cause of poor responses to chemotherapy, the potency of sesquiterpene coumarins, as blockers of MRP-mediated drug transport activity, would make these components ideal choices for future in vivo and clinical approaches.

Acknowledgements

The authors would like to thank Prof. Javad Behravan and Dr. Fatemeh Soltani for their excellent scientific and technical advice. We are also grateful to Mrs. Zeinab Neshati and Mr. Nakhaei for their great assistance. This work was partially supported by a grant from Ferdowsi University of Mashhad.


Iranshahi M., Kalategi F., Rezaee R., Shahverdi A. R., Ito C., Furukawa H., Tokuda H., and Itoigawa M. (2008), Cancer chemopreventive activity of terpe-
noid coumarins from Ferula species. Planta Med. 74, 147–150.


