Effect of peucedanin and bergapten (5-MOP), furanocoumarins isolated from *Peucedanum tauricum* Bieb. (*Apiaceae*) fruits, on apoptosis induction and heat-shock protein expression in HeLa cells

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Summary

In the research effects of bergapten (5-MOP) and peucedanin, furanocoumarins isolated from fruits of *Peucedanum tauricum* Bieb. (*Apiaceae*), on apoptosis, necrosis and heat-shock proteins Hsp 27 and Hsp 72 expression in human carcinoma cells HeLa B (ECACC No 85060701) were preliminary studied. The purity of analysed compounds was confirmed by RP HPLC, EI-MS and 1D, 2D NMR. For apoptosis and necrosis detection the mixture of Hoechst 33342 and propidium iodide (PI) was used. Early apoptosis was detected using Annexin V Test. Cells were incubated with furanocoumarins at the concentrations of 1, 5, 10 and 15 μg/ml. The level of heat-shock protein expression was determined by Western blot technique. Quantitative analysis of Hsp 27 and Hsp 72 was done with the Bio-Profil Bio-1D Windows Application V99.03 program. The obtained results were analysed for significance by one-way ANOVA test. As a result of our experiments we found that peucedanin was more effective in apoptosis induction than bergapten. In HeLa cells treated with peucedanin only low necrosis was observed. The incubation with 15 μg/ml of peucedanin for 24 hours inhibited Hsp 72 expression in cells by 77.5% (± 0.05), and Hsp 27 by 74.0% (± 0.02) after five hours of treatment as compared to the control. The inhibition of Hsps expression after peucedanin treatment may be responsible for increased apoptosis induction (11.4%) detected by Annexine V test. Incubation

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with furanocoumarins has an influence on morphology of HeLa cells (many cytoplasmic protrudings and bulges of “blebbing” cytoplasm were observed).

Key words: furanocoumarins, apoptosis, heat-shock proteins, Peucedanum tauricum

INTRODUCTION

Coumarins are very common secondary metabolites widely distributed in plants [1, 2]. They have been extensively studied for their phototoxic properties [3, 4]. They are used in therapy of psoriasis, mycosis fungoides and vitiligo. Some reports indicate their effect on tumor cells [5]. The inhibition of DNA synthesis by coumarins (e.g. peucedanin) in mouse skin and Ehrlich tumour, the growth inhibitory effect on human hepatocellular carcinoma cells by arresting cell cycle at the G2/M phase by coumarins [6] and apoptosis induction activity were also observed [7]. The process of apoptosis is closely associated with carcinogenesis and many cancer cells have defective machinery for self-destruction.

It is known that tumor cells are very resistant to cell death and the reason for that can be the ability of Hsps to protect cells for apoptosis. In tumor cells accumulation of heat-shock proteins may be responsible for drug and chemotherapy resistance and poor prognosis [8, 9]. Thus, decreasing Hsps level in cancer cells would be beneficial. In this research the effects of bergapten (5-MOP) and peucedanin, furanocoumarins isolated from fruits of Peucedanum tauricum Bieb. (Apiaceae), on apoptosis, necrosis and heat-shock protein expression in human carcinoma cells were preliminary studied.

MATERIAL AND METHODS

Isolation and structure elucidation of furanocoumarins

Bergapten (5-MOP) and peucedanin used in our experiments were isolated from petroleum ether extract of P. tauricum fruits by means of LC and preparative TLC [10, 11], and identified by RP HPLC, EI-MS and 1D, 2D NMR (COSY, NOESY, HMBC and HMQC) [11].

Cell and culture conditions

Human cervix carcinoma cell line (HeLa B, ECACC No 85060701) cultured in RPMI 1640 medium supplemented with 5% FBS (fetal bovine serum) (v/v) was used in this study. Cells at a density of 1x10⁵ cells/ml were seeded on cover slides (for apoptosis detection) or in Falcon vessels (for heat-shock proteins identification) and incubated at the temperature of 37°C in humidified atmosphere with 5% CO₂. Cells were incubated with furanocoumarins at concentrations of 1, 5, 10 and 15 µg/ml for 5 and 24 hours.
Morphology

After incubation with coumarins cells were washed four times with PBS, fixed for 10 minutes in 3.7% paraformaldehyde in PBS, washed with PBS. All the processes were carried out at room temperature. Next the cells were examined under a Nikon E 800 microscope.

Apoptosis detection

The cells were stained with a mixture of Hoechst 33342 and propidium iodide (PI) for 5 minutes at 37°C [12]. Cells exhibiting blue fluorescent nuclei (intact or fragmented) were interpreted as apoptotic cells. For early apoptosis detection HeLa cells were stained with a solution of Annexin V-Cy3 and 6-carboxyfluorescein diacetate (Annexin V-Cy3 Kit, Sigma) for 10 minutes at room temperature. Cells exhibiting green (6-carboxyfluorescein diacetate) and red (Annexin V-Cy3) fluorescence were interpreted as apoptotic cells. Necrotic cells were stained only with Annexin V-Cy3. Morphological analysis was performed under a fluorescence microscope (Nikon E 800). At least 1000 cells in randomly selected microscopic fields were counted. The obtained results were analysed for significance by one-way ANOVA test.

Determination of Hsps expression

In order to determine the expression of the heat-shock proteins Hsp27 and Hsp72, HeLa cells were incubated with peucedanin (15 µg/ml) for 5 to 24 hours.

For heat-shock protein identification, Western blot analysis was used [8]. Cells were lysed in hot SDS-loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 100 mM DTT), boiled in water bath for 10 minutes, centrifuged at 10000 g for 10 min and then the supernatant was collected. The protein concentration was determined by the Bradford method [13]. Next, samples of supernatants containing exactly 80 µg of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis [14]. Proteins were then transferred onto Immobilon P membrane (Sigma). After the transfer, the membrane was blocked with 3% low fat milk in PBS for 1 hour, then incubated overnight with mouse monoclonal antibodies against Hsp27 (SPA-800, StressGen) and against Hsp72 (SPA-810, StressGen) (dilution 1:1000). The membrane was washed three times for 10 minutes with PBS containing Triton X-100 and incubated for 2 hours with a 1:30000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). The membrane was visualized with alkaline phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro-blue tetrazolium (NBT) in N,N-dimethylformamide (DMF) [15]. The experiment was repeated independently three times. Quantification of heat-shock proteins was done with the Bio-Profil Bio-1D Windows Application V.99.03 program. Significant levels were calculated using the one-way ANOVA test. P-values lower than 0.05 were considered statistically significant.
RESULTS

A five-hour incubation of HeLa cells with 5-MOP had no effect on apoptosis induction. Nor was Bergapten effective in apoptosis and necrosis induction after a 24-hour incubation (except for one case). Incubation with bergapten at the concentration of 10 μg/ml increased the percentage of necrotic cells (0.5% in the control cells, while 4.3% in treated cells: Table 1).

Table 1

<table>
<thead>
<tr>
<th>Concentration of bergapten (μg/ml)</th>
<th>5 h (%)</th>
<th>24 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hoehst 33342</td>
<td>Annexin V Test</td>
</tr>
<tr>
<td>0 (control)</td>
<td>0.20 ± 0.07</td>
<td>0.25 ± 0.07</td>
</tr>
<tr>
<td>1</td>
<td>0.40 ± 0.10</td>
<td>2.75 ± 1.2*</td>
</tr>
<tr>
<td>5</td>
<td>0.60 ± 0.14</td>
<td>0.95 ± 0.9</td>
</tr>
<tr>
<td>10</td>
<td>0.70 ± 0.20</td>
<td>1.65 ± 0.9</td>
</tr>
<tr>
<td>15</td>
<td>0.83 ± 0.05</td>
<td>0.35 ± 0.2</td>
</tr>
</tbody>
</table>

Note: n=3, *p<0.05.

In HeLa cells treated with peucedanin at the concentration of 15 μg/ml for 5 hours 8.35% apoptotic cells were observed. The percentage of apoptotic cells after a 24-hour incubation with peucedanin (15 μg/ml) increased (0.5% in the control cells against 11.4% in treated cells: Table 2). Some correlations between Hsps expression and the induction of apoptosis in HeLa cells after treatment with 15 μg/ml of peucedanin were observed. A 24-hour incubation with the drug inhibited Hsp 72 expression in cells by 77.5% (± 0.05). The level of Hsp 27 was diminished by 74.0% (± 0.02) after 5 hours of incubation as compared to the control (Fig. 1). Inhibition of Hsps expression may be responsible for apoptotic effect of peucedanin shown in test with Annexine V. Interestingly, only low necrosis in HeLa cells treated with peucedanin was observed. Incubation with furanocoumarins has an influence on morphology of HeLa cells. The control cells were spindle- or star-shaped with only few cytoplasmic protrudings of different length (Fig. 2 a, c, e). Incubation of HeLa cells with peucedanin at the concentration of 15 μg/ml caused pronounced changes of their shapes. Many cytoplasmic protrudings were observed (Fig. 2 b, d, f). Cytoplasmic bulges formed tiny microvilli or they had structures of long, thin and branching protrudings. On the surface of the cells bulges of “blebbing” cytoplasm of various shapes, mostly oval, were also present (Fig. 3 b,d,f).
Peucedanin — induction of apoptosis in HeLa cells.

<table>
<thead>
<tr>
<th>concentration of peucedanin (µg/ml)</th>
<th>time of incubation</th>
<th>5 h (% ± SD)</th>
<th>24 h (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>apoptosis</td>
<td>necrosis</td>
<td>apoptosis</td>
</tr>
<tr>
<td></td>
<td>Hoehst 33342</td>
<td>Annexin V Test</td>
<td>PI</td>
</tr>
<tr>
<td>0 (control)</td>
<td>0.20 ± 0.07</td>
<td>0.25 ± 0.07*</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>0.13 ± 0.05</td>
<td>4.65 ± 1.1*</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>0.25 ± 0.07</td>
<td>4.80 ± 0.42*</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>10</td>
<td>0.20 ± 0.01</td>
<td>3.00 ± 0.1**</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>15</td>
<td>0.60 ± 0.20</td>
<td>8.35 ± 0.6**</td>
<td>0.5 ± 0.4</td>
</tr>
</tbody>
</table>

Note: n=3; *p < 0.05; **p < 0.005; ***p < 0.001.

Figure 1. The effect of peucedanin (15 µg/ml) on Hsp 27 and Hsp 72 expression in HeLa cells (K – control).
Figure 2. The influence of incubation with peucedanin on morphology of HeLa cells; a, c, e — Control HeLa cells (mag. 400x); b, d, f — HeLa cells incubated with peucedanin at the concentration of 15 μg/ml (mag. 400x).

CONCLUSIONS
1. As a result of our experiments we have found that peucedanin is more effective in apoptosis induction than bergapten.
2. Only low necrosis in HeLa cells treated with peucedanin was observed.
3. Inhibition of Hsps expression may be responsible for the apoptotic effect of peucedanin shown in tests carried out with Annexine V.
4. The processes of apoptosis and necrosis are complex and depend on many different factors, including the incubation time and drug concentration.
5. Incubation with peucedanin has an influence on morphology of HeLa cells.
REFERENCES


Wpływ peucedany i bergaptenu, furanokumaryn izolowanych z owoców Peucedanum tauricum Bieb. (Apiaceae), na indukcję apoptozy i ekspresję białek szoku termicznego w komórkach HeLa

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Streszczenie

Podjęto próbę określania wpływu peucedaniny i bergaptenu, związków furanokumarynowych wyizolowanych z owoców gorysza krymskiego (Peucedanum tauricum Bieb.) na indukcję apoptozy i nekrozy oraz ekspresję białek szoku termicznego Hsp 27 i Hsp 72 w komórkach HeLa (ECACC No 85060701). Czystość analizowanych związków naturalnych potwierdzono metodami chromatograficznymi i spektralnymi: RP HPLC, MS, 1D i 2D NMR. Identyfikacji apoptozy i nekrozy w badanych komórkach HeLa dokonano przy użyciu mieszaniny barwiącej fluorochromów: Hoechst 33342 i jodku propidionowego (PI). Do określania wcześniejszej apoptozy wykorzystano test z aneksyną V. W badaniach stosowano różne stężenia związków kumarynowych (1, 5, 10 i 15 μg/ml) oraz różny czas inkubacji. Aby zbadać wpływ związków kumarynowych na ekspresję białek szoku termicznego, wykonano elektroforezę białek na żelu poliakryloamidowym SDS/PAGE i technikę immunoblotingu. Do ilościowej analizy ekspresji białek szoku termicznego Hsp 27 i Hsp 72 wykorzystano program Bio-Profil Bio-1D Windows Application V.99.03. Do analizy statystycznej uzyskanych wyników badań biologicznych użyto testu ANOVA.

Rezultaty badań pozwalały stwierdzić, że z obu analizowanych substancji peucedanina w większym stopniu indukuje apoptozę. Jednocześnie zaobserwowano tylko nieznaczną nekrozę komórek poddanych działaniu peucedaniny. Po inkubacji z peucedaniną (przy stężeniu 15 μg/ml) stwierdzono obniżenie ekspresji białek szoku termicznego Hsp 72 po 24-godzinnej inkubacji do 77,5% (+0,05), a Hsp 27 po pięciogodzinnej inkubacji do 74,0% (+0,02) w porównaniu z materiałem kontrolnym. Obniżenie ekspresji białek szoku może mieć związek ze wzmożonym efektem proapoptotycznym peucedaniny (w teście wcześniejszej apoptozy wzrost do 11,45%; w materiale kontrolnym — 0,5%). Ponadto w obrazie morphologicznym komórek poddanych działaniu związków kumarynowych zaobserwowano zmiany w porównaniu z materiałem kontrolnym (wydłużanie się komórek, pojawianie się wypustek cytoplasmatycznych i uwypukleń).

Słowa kluczowe: furanokumaryny, apoptoza, białka szoku termicznego, Peucedanum tauricum