Bufalin combined with hydroxycamptothecin affect in vitro cell growth and apoptosis of human Du145 prostate cancer cells

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Abstract: Objectives: To investigate the effect of different bufalin doses combined with 7-ethyl-10-hydroxy-camptothecin (HCPT) on the growth and apoptosis of human Du145 prostate cancer cells in vitro and analyze related mechanisms. Methods: We investigated the inhibiting effect of single, sequential or combined use of different bufalin and HCPT doses on human prostate cancer cells Du145 via flow cytometry and cell viability assays and determined caspase 3 and 9 expression. Results: The growth rates of Du145 cells were significantly reduced by single applications of bufalin and HCPT in dose and time dependent manners. Bufalin arrested the Du145 cells in the G2/M whereas HCPT in the S phase. Combined simultaneous application of bufalin and HCPT led to antagonistic effects with reduced growth inhibitions, whereas combined sequential application led to synergistic effects with highest proliferation reduction, particularly with initial HCPT followed by bufalin exposure of the cells. The expression of caspase 3 and 9 was upregulated after single use of HCPT and bufalin and further upregulated after sequential use. Conclusions: Sequential use of bufalin after HCPT exposure led to the highest synergistic growth inhibition effects on Du145 cells, which might be attributed to the ability of the drugs to block cell cycles in both G2/M and S phases and upregulation of caspase-3 and caspase 9 apoptosis factor expressions.

Keywords: Prostate cancer, bufalin, hydroxycamptothecin, G2/M phase, caspase 3, caspase 9

Introduction
Prostate cancer is the most common non-skin malignancy in the US and the second most common cancer in men globally [1]. In recent years, the incidence of prostate cancer in China is significantly increasing [2] and ranks third among urinary tract tumors [3]. Surgery, radiotherapy, chemotherapy and endocrine therapy are the main therapeutic strategies, but the later one with moderate results [4]. Especially for hormone refractory prostate cancer patients the median survival time is only about 18 to 24 months [5]. Bufalin is a cardiotonic steroid isolated from toads of Bufo species that potently inhibits Na+, K+-ATPase activity [6] and induces apoptosis in various human tumor cell lines. It has been demonstrated, that bufalin activates the Rac1, PAK, and c-Jun NH2-terminal kinase (JNK) pathway in leukemia HL60 and U937 cells [7]. In the human leukemia cells ML1 and U937 bufalin enhanced the induction of cell death, markedly decreased topoisomerase II activity and increased the inhibitory effects of cisplatin and retinoic acid on cell growth [8]. The later effect has been used for application of bufalin as adjunct medication for cisplatin resistant tumor cell treatments [9]. Yin et al. summarized the apoptotic effects of bufalin on leukemia, prostate cancer, gastric cancer, liver cancer, and breast cancer [10]. HCPT is formed via hydrolysis of irinotecan, which is a derivate of Camptotheca acuminata, by a carboxylesterase and a potent inhibitor of DNA topoisomerase I being mainly used as a cytostatic drug for treatment of metastatic colon or rectal cancers. Previous research demonstrated that bufalin also significantly inhibited the growth and metastasis of colorectal cancer [11].

In our present study we analyzed the effect of combined in vitro bufalin and HCTP application on growth and apoptosis of human prostate cancer Du145 cells.
**Materials and methods**

**Cell lines and cell culture**

The human DU145 prostate cancer cells (Chinese academy of sciences, Shanghai) were routinely cultured in 1640 complete medium (Gibco® Thermo Fisher Scientific Inc., Rockford, IL, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Hyclone™, GE Healthcare Life Sciences, Utah, USA) at 37°C and 5% CO₂ under fully saturated humidity. Cells were maintained in the logarithmic growth phase by sub-culturing every 2 to 3 days.

**Determination of the viable cell number**

Du145 cells in the logarithmic growth phase were diluted to 5 × 10⁴/ml and seeded into 96-well plates. After 24 h 0.0001, 0.001, 0.01, 0.1 nmol/L bufalin (Sigma-Aldrich Co. LLC., St. Louis, USA) and/or 0.0001, 0.001, 0.01, 0.1 µg/ml of HCPT Shenzhen Wanle pharmaceutical co., LTD, Shenzhen, China) were added. After incubation for 24 h/48 h, 10 µl CCK-8 reagent (Cell Counting Kit-8 (CCK-8), Nanjing Kaji biological development co., LTD, Nanjing, China) was added for 2 hours. Then absorbance at 450 nm was monitored with a microplate reader (689 type) and 3 duplicate measurements were repeated for 3 times. The cell proliferation inhibition rate = [absorbance value in control group/absorbance value in experimental group] × 100%.

The Jin’s formula was employed to determine the effect of drug combination: 

\[ q = \frac{E (a+b)}{Ea+Eb-Ea×Eb}. \]

(E (a+b) = inhibition rate of the combined two drugs, Ea and Eb = inhibition rates of single drugs). q < 0.85 indicates antagonistic effect, 0.85 ≤ q ≤ 1.15 indicates additive effect, and q > 1.15 indicates synergistic effect.

**Detection of change of cell cycle by flow cytometry**

Du145 cells in the logarithmic growth phase were collected and seeded into 6 well plates in a dilution of 1 × 10⁴/ml. After the indicated treatments, we determined the cell cycle stages with a real-time detection kit (Wuhan Boster Biological Engineering Co., Ltd, Wuhan, China) and a flow cytometry instrument (FCASCalibur™ BD, Franklin Lakes, NJ, USA).

**Statistical analysis**

SPSS13.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) software was used for statistical analyses. All data were expressed as x ± sd, single factor analysis of variance was used for comparison among
Results

Both bufalin and HCPT inhibited the growth of Du 145 prostate cancer cells

The cell growth inhibition rates positively correlated with the bufalin and HCPT dosages, but at low doses (0.0001, 0.001 and 0.01) HCPT led to a higher inhibition rates than bufalin at the same dosages, whereas at the highest dosage (0.1) both drugs inhibited the growth rates to similar extends. For both drugs, 48 hours of incubation was more effective than 24 hours in all concentrations measured (Figure 1).

Effect of combined simultaneous use of bufalin and HCPT on Du 145 prostate cancer cell growth

As shown in Table 1, 48.56 ± 7.28% cell proliferation inhibition was achieved by single use of HCPT at a concentration of 0.01 μg/ml and 0.01 nmol/L bufalin led to 44.21 ± 4.36% decrease. With increasing dose of bufalin as simultaneous co-treatment, the inhibition rate by 0.01 μg/ml HCPT dropped to 24.45 ± 0.71% at 0.01 nmol/L bufalin. The q value was < 0.85 indicating an antagonistic effect (Table 1).

If the cells were exposed first for 24 hours to bufalin and then for 24 hours to HCTP, the Du145 cell growths was also significantly inhibited in dose and combination dependent manners but the extent was higher than with simultaneous application of the drugs. The q value was > 1.15 (Table 2).

Table 1. Effect of combined use of bufalin and HCPT for 24 h on prostate cancer Du145 cell growth inhibition (%)

<table>
<thead>
<tr>
<th>Bu+HCPT (nmol/L+µg/mL)</th>
<th>Bu 0.00</th>
<th>Bu 0.01</th>
<th>Bu 0.001</th>
<th>Bu 0.0001</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCPT 0.00</td>
<td>0</td>
<td>44.21 ± 4.36</td>
<td>15.32 ± 2.01</td>
<td>8.23 ± 4.04</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HCPT 0.01</td>
<td>48.56 ± 7.28</td>
<td>24.45 ± 0.71</td>
<td>35.5 ± 3.03</td>
<td>36.21 ± 3.81</td>
<td>0.0012</td>
</tr>
<tr>
<td>HCPT 0.001</td>
<td>41.67 ± 2.52</td>
<td>17.42 ± 2.82</td>
<td>30.12 ± 2.64</td>
<td>34.34 ± 2.35</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HCPT 0.0001</td>
<td>36.33 ± 4.72</td>
<td>15.84 ± 1.43</td>
<td>22.77 ± 1.66</td>
<td>26.69 ± 5.66</td>
<td>0.0012</td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Effect of sequential incubation with first bufalin for 24 h and then HCPT for 24 h on prostate cancer Du145 cell growth inhibition (%)

<table>
<thead>
<tr>
<th>Bu+HCPT (nmol/L+µg/mL)</th>
<th>Bu 0</th>
<th>Bu 0.01</th>
<th>Bu 0.001</th>
<th>Bu 0.0001</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCPT 0</td>
<td>0</td>
<td>39.63 ± 1.68</td>
<td>14.94 ± 1.32</td>
<td>6.12 ± 2.56</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HCPT 0.01</td>
<td>42.09 ± 1.36</td>
<td>73.58 ± 0.87</td>
<td>68.31 ± 2.39</td>
<td>60.99 ± 1.28</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HCPT 0.001</td>
<td>21.21 ± 3.57</td>
<td>70.50 ± 1.68</td>
<td>64.93 ± 2.29</td>
<td>57.89 ± 1.06</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HCPT 0.0001</td>
<td>18.09 ± 5.78</td>
<td>69.25 ± 1.85</td>
<td>62.16 ± 2.87</td>
<td>55.57 ± 0.26</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Effect of sequential incubation with first HCTP for 24 h and then bufalin for 24 h on prostate cancer Du145 cell growth inhibition (%)

<table>
<thead>
<tr>
<th>Bu+HCPT (nmol/L+µg/mL)</th>
<th>Bu 0</th>
<th>Bu 0.01</th>
<th>Bu 0.001</th>
<th>Bu 0.0001</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCPT 0</td>
<td>0</td>
<td>36.08 ± 3.89</td>
<td>28.07 ± 2.47</td>
<td>11.58 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>HCPT 0.01</td>
<td>44.82 ± 5.14</td>
<td>76.89 ± 1.81</td>
<td>73.94 ± 2.84</td>
<td>68.39 ± 2.23</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HCPT 0.001</td>
<td>27.15 ± 3.45</td>
<td>73.51 ± 0.60</td>
<td>70.46 ± 1.75</td>
<td>66.49 ± 0.56</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HCPT 0.0001</td>
<td>16.27 ± 5.78</td>
<td>68.40 ± 2.51</td>
<td>65.33 ± 2.58</td>
<td>63.98 ± 1.73</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>
Bufalin for prostate cancer

In case of initial incubation with HCTP for 24 hours followed by incubation with bufalin for 24 hours the growth inhibition pattern of the Du145 cells was similar to Table 2 with significant dose and combination dependent inhibitions, but the extend was slightly higher. The q value was > 1.15 (Table 3).

Cell cycle changes after treatment with bufalin or HCPT for 24 hours

Both treatments significantly reduced cells in the G1 phase (P < 0.01) of the prostate cancer Du145 cells, but they were enhanced arrested in the G2/M phase by 0.01 nmol/L bufalin, whereas 0.01 µgr HCPT led to less G2/M phase (P < 0.05) and enhanced S phase (P < 0.01) arrest (Figure 2).

Bufalin and HCPT incubation enhanced expression levels of caspase 3 and caspase 9 in prostate cancer Du145 cells.

The initiator caspase 9 was dose dependent enhanced expressed in prostate cancer Du145 cells after 24 hour incubation with solely bufalin and solely HCPT (Figure 3).

As shown in Figure 4, the effector caspase 3 expressions in Du145 cells were dose dependent elevated after solely bufalin or HCPT incubations and reached a maximum after combined sequential application of 0.01 µgr/mL HCPT and 0.01 nmol/L bufalin (Figure 4).

Discussion

Our results revealed that bufalin and HCPT could inhibit the growth of prostate cancer Du145 cells in an incubation time and dose dependent manner, which is in agreement with previous literature [12, 13]. Antitumor effects of bufalin, a component of the Chinese medicine chan’su, have also been...
Bufalin for prostate cancer

Bufalin for prostate cancer demonstrated previously for endometrial and ovarian as well as prostate cancer cells [14-16]. Bufalin has been shown to be a potent inhibitor of topoisomerase II [8, 17]. Combined simultaneous application of the topoisomerase I and II inhibitors resulted in a q value of < 0.85 indicating an antagonistic effect, which is in contrast to a previous finding, that simultaneously combined use of HCPT with the Tripterygium wilfordii extract triptolide showed synergistic effects in inhibiting proliferation of lung cancer A549 cells [18], which is probably due to the different antineoplastic effects of triptolide, but needs further evaluation.

However, sequential use of bufalin with HCPT produced significant higher inhibitory effects on growth of Du145 cells compared with single use of the drugs indicating a synergistic effect with a q value of > 1.15. Initial exposure to HCPT followed by bufalin yielded the highest cell growth inhibition rate of 76.89 ± 1.81%. Effect enhancing sequential use of HCPT with adriamycin and pirarubicin has also been reported previously for bladder cancer T24 cells and in a mouse tumor model [19, 20].

Bufalin has been shown to induce apoptosis in various cancer cells [21-23]. Also in our study, we found that HCPT as well as bufalin could increase the expressions of caspase 3 and 9 with highest levels after sequential application, which reflected also in the highest Du145 cell growth inhibition under the same condition. In accordance with previous literature, our cell cycle analyses revealed, that Du145 cells were arrested in the G2/M phase by bufalin [24] and in the S phase by HCPT [25].

Our analyses were performed by in vitro experiments and animal experiments as well as clinical trials are necessary to confirm the effectiveness and safety of bufalin and HCPT co-treatments in vivo.

In summary, our in vitro experiments revealed, that bufalin and HCPT had similar effects on human Du145 prostate cancer cells regarding growth rate inhibition and caspase 3 and 9 expression, but bufalin arrested the cells in the G2/M phase whereas HCPT in the S phase. Simultaneous application of bufalin and HCPT led to antagonistic effects with reduced human Du145 prostate cancer growth rate inhibitions compared to single usage, but sequential and particularly bufalin following HCPT application showed synergistic effects with significantly highest growth rate inhibition rates.

Figure 4. The protein expression Caspase 3 in Du145 cells after single or combined use of indicated bufalin and HCPT concentrations for 24 h.
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Disclosure of conflict of interest

None.

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References


