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Original Article

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 casapase-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.

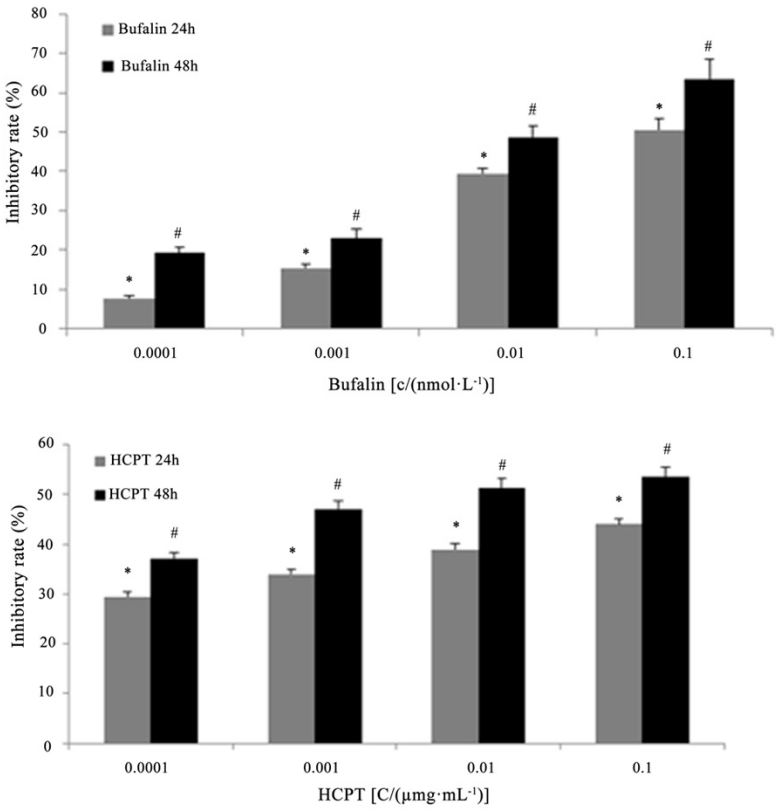

Figure 1. Growth inhibition rates of prostate cancer Du145 cells after administration of indicated bufalin or HCPT concentrations for 24 h or 48 h, *P < 0.05 significant difference between indicated concentration bufalin or HCPT and 0.0001 nmol/L bufalin or HCPT for 24 h, #P < 0.05 significant difference between indicated concentration bufalin or HCPT and 0.0001 nmol/L bufalin or HCPT for 48 h.

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° and 5% CO_2 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 × 10^4/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 Kaiji 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)/absorbance value in control group] × 100%.

The Jin's formula was employed to determine the effect of drug combination: q = 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 × 10^5/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

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

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

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 (%)

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

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 (%)

Bu+HCPT (nmol/L+μg/mL)	Bu 0	Bu 0.01	Bu 0.001	Bu 0.0001	*P*
HCPT 0	0	36.08 ± 3.89	28.07 ± 2.47	11.58 ± 0.62	
HCPT 0.01	44.82 ± 5.14	76.89 ± 1.81	73.94 ± 2.84	68.39 ± 2.23	< 0.0001
HCPT 0.001	27.15 ± 3.45	73.51 ± 0.60	70.46 ± 1.75	66.49 ± 0.56	< 0.0001
HCPT 0.0001	16.27 ± 0.58	68.40 ± 2.51	65.33 ± 2.58	63.98 ± 1.73	< 0.0001
P	< 0.0001	< 0.0001	< 0.0001	< 0.0001	

groups, two sample mean was compared with group t test; comparison of rates among the different groups was performed using analysis of variance. P < 0.05 was considered to be statistically significant.

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**).

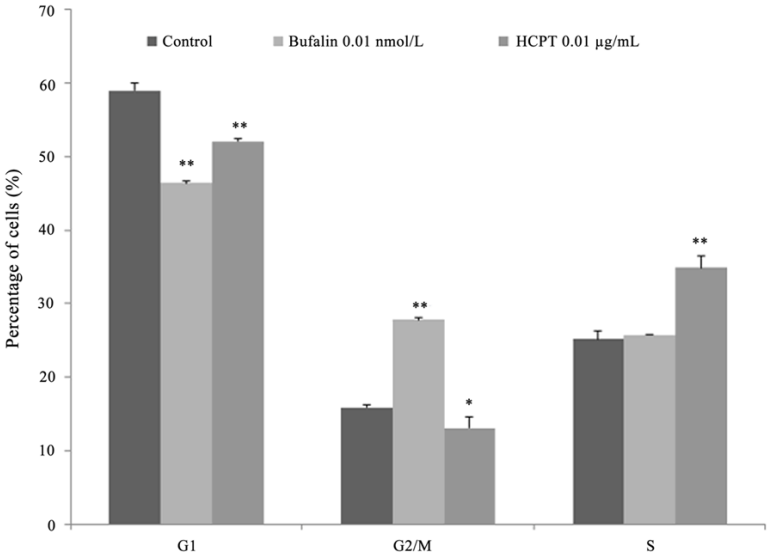

Figure 2. Flow cytometer analyses of Du145 prostate cancer cell cycle changes after treatment with 0.01 nmol/L bufalin or 0.01 μgr/ml HCPT for 24 hours. Compared to control: *P < 0.05 **P < 0.01.

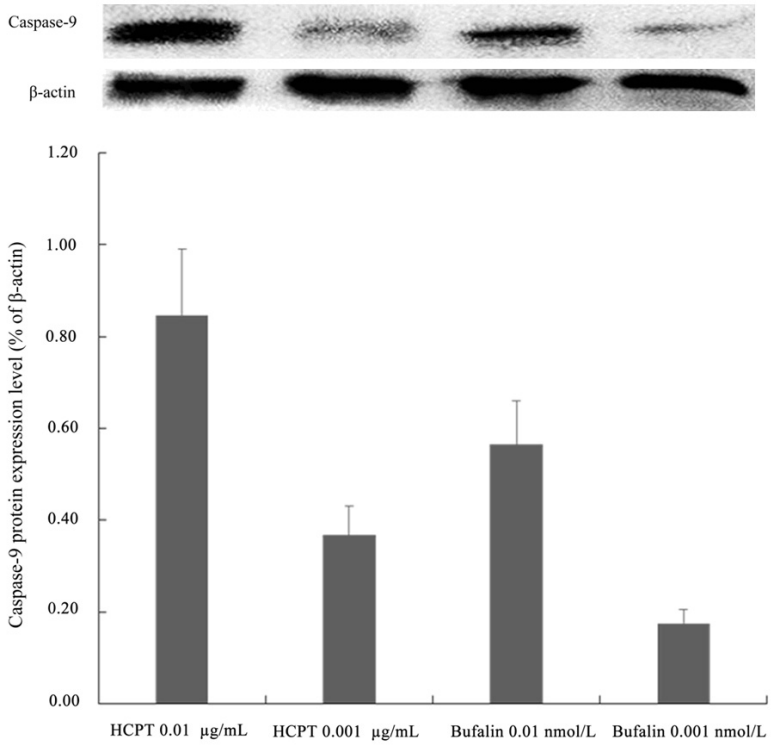

Figure 3. The protein expression levels of Caspase 9 in Du145 cells after single application with indicated bufalin or HCPT doses for 24 hours.

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

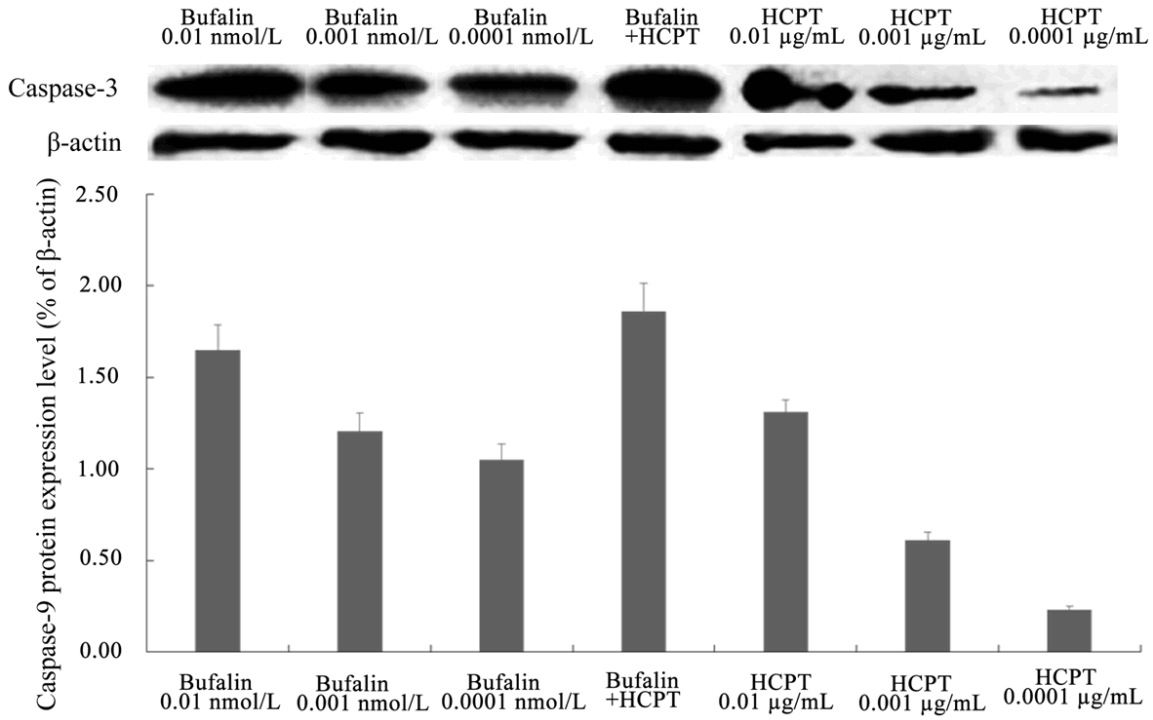

Figure 4. The protein expression Caspase 3 in Du145 cells after single or combined use of indicated bufalin and HCPT concentrations for 24 h.

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.

Disclosure of conflict of interest

None.

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References

[1] Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase. Lyon: International Agency for Research on Cancer (IARC); 2014.

[2] Du LB, Li HZ, Wang XH, Zhu C, Liu QM, Li QL, Li XQ, Shen YZ, Zhang XP, Ying JW, Yu CD and Mao WM. Analysis of cancer incidence in Zhejiang cancer registry in China during 2000 to 2009. Asian Pac J Cancer Prev 2014; 15: 5839-5843.

[3] Yanqun N, Zhangqun Y and Guang S. Chinese urology disease diagnosis and treatment guidelines version 1. Beijing: People's Medical Publishing Press; 2011.

[4] Denis LJ and Griffiths K. Endocrine treatment in prostate cancer. Semin Surg Oncol 2000; 18: 52-74.

[5] Halabi S, Small EJ, Kantoff PW, Kattan MW, Kaplan EB, Dawson NA, Levine EG, Blumenstein BA and Vogelzang NJ. Prognostic model for predicting survival in men with hormone-refractory metastatic prostate cancer. J Clin Oncol 2003; 21: 1232-1237.

[6] McGowan MH, Russell P, Carper DA and Lichtstein D. Na+, K+-ATPase inhibitors down-regulate gene expression of the intracellular signaling protein 14-3-3 in rat lens. J Pharmacol Exp Ther 1999; 289: 1559-1563.

[7] Kawazoe N, Watabe M, Masuda Y, Nakajo S and Nakaya K. Tiam1 is involved in the regulation of bufalin-induced apoptosis in human leukemia cells. Oncogene 1999; 18: 2413-2421.

[8] Hashimoto S, Jing Y, Kawazoe N, Masuda Y, Nakajo S, Yoshida T, Kuroiwa Y and Nakaya K. Bufalin reduces the level of topoisomerase II in human leukemia cells and affects the cytotoxicity of anticancer drugs. Leuk Res 1997; 21: 875-883.

[9] Liu L and Bian K. [Advance in studies on molecular mechanisms of cisplatin resistance and intervention with traditional Chinese medicines]. Zhongguo Zhong Yao Za Zhi 2014; 39: 3216-3220.

[10] Yin PH, Liu X, Qiu YY, Cai JF, Qin JM, Zhu HR and Li Q. Anti-tumor activity and apoptosis-regulation mechanisms of bufalin in various cancers: new hope for cancer patients. Asian Pac J Cancer Prev 2012; 13: 5339-5343.

[11] Hu Q, Liang B, Sun Y, Guo XL, Bao YJ, Xie DH, Zhou M, Duan YR, Yin PH and Peng ZH. Preparation of bufalin-loaded pluronic polyetherimide nanoparticles, cellular uptake, distribution, and effect on colorectal cancer. Int J Nanomedicine 2014; 9: 4035-4041.

[12] Liu Z, Zhu G, Getzenberg RH and Veltri RW. The upregulation of PI3K/Akt and MAP kinase pathways is associated with resistance of microtubule-targeting drugs in prostate cancer. J Cell Biochem 2015; 116: 1341-1349.

[13] Wu Y, Zeng FQ, Wang YB and Wang L. [Hydroxycamptothecin promotes the apoptosis of prostate cancer cell line PC-3]. Zhonghua Nan Ke Xue 2007; 13: 890-894.

[14] Nasu K, Nishida M, Ueda T, Takai N, Bing S, Narahara H and Miyakawa I. Bufalin induces apoptosis and the G0/G1 cell cycle arrest of endometriotic stromal cells: a promising agent for the treatment of endometriosis. Mol Hum Reprod 2005; 11: 817-823.

[15] Takai N, Ueda T, Nishida M, Nasu K and Narahara H. Bufalin induces growth inhibition, cell cycle arrest and apoptosis in human endometrial and ovarian cancer cells. Int J Mol Med 2008; 21: 637-643.

[16] Yu CH, Kan SF, Pu HF, Jea Chien E and Wang PS. Apoptotic signaling in bufalin- and cinobufagin-treated androgen-dependent and -independent human prostate cancer cells. Cancer Sci 2008; 99: 2467-2476.

[17] Pastor N and Cortes F. Bufalin influences the repair of X-ray-induced DNA breaks in Chinese hamster cells. DNA Repair (Amst) 2003; 2: 1353-1360.

[18] Meng G, Wang W, Chai K, Yang S, Li F and Jiang K. Combination treatment with triptolide and hydroxycamptothecin synergistically enhances apoptosis in A549 lung adenocarcinoma cells through PP2A-regulated ERK, p38 MAPKs and Akt signaling pathways. Int J Oncol 2015; 46: 1007-1017.

[19] Qingchuan Z, Jiaqi S and Yongxian Z. Perfusion of HCPT combined with Pirarubisin could prevent postoperative recurrence of superficial bladder cancer. Journal of Bengbu Medical College 2006; 31: 249-250.

[20] Yibin L, Weijian F and Gang K. Effect of sequential use of HCPT with Adriamycin in different period on portability tumors in mice. Journal of Hebei Medical University 2002; 23: 150-152.

[21] Zhu Z, Li E, Liu Y, Gao Y, Sun H, Ma G, Wang Z, Liu X, Wang Q, Qu X, Liu Y and Yu Y. Inhibition

of Jak-STAT3 pathway enhances bufalin-induced apoptosis in colon cancer SW620 cells. World J Surg Oncol 2012; 10: 228.

[22] Ding DW, Zhang YH, Huang XE, An Q and Zhang X. Bufalin induces mitochondrial pathway-mediated apoptosis in lung adenocarcinoma cells. Asian Pac J Cancer Prev 2014; 15: 10495-10500.

[23] Wu SH, Hsiao YT, Chen JC, Lin JH, Hsu SC, Hsia TC, Yang ST, Hsu WH and Chung JG. Bufalin alters gene expressions associated DNA damage, cell cycle, and apoptosis in human lung cancer NCI-H460 cells in vitro. Molecules 2014; 19: 6047-6057.

[24] Hsu CM, Tsai Y, Wan L and Tsai FJ. Bufalin induces G2/M phase arrest and triggers autophagy via the TNF, JNK, BECN-1 and ATG8 pathway in human hepatoma cells. Int J Oncol 2013; 43: 338-348.

[25] Galvez-Peralta M, Dai NT, Loegering DA, Flatten KS, Safgren SL, Wagner JM, Ames MM, Karnitz LM and Kaufmann SH. Overcoming S-phase checkpoint-mediated resistance: sequence-dependent synergy of gemcitabine and 7-ethyl-10-hydroxycamptothecin (SN-38) in human carcinoma cell lines. Mol Pharmacol 2008; 74: 724-735.