Effects of low-dose bufalin combined with hydroxycamptothecin on human castration-resistant prostate cancer xenografts in nude mice

RENZE GU and QINGCHUAN ZHANG

Department of Urology, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200062, P.R. China

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Abstract. Prostate cancer is the most prevalent tumor found in men worldwide. Despite the efficiency of primary endocrine prostate cancer therapies, more efficient drugs are needed to tackle the most advanced and resistant forms of this condition. The present study investigated the antitumor effects of low-dose bufalin combined with hydroxycamptothecin on castration-resistant prostate cancer (CRPC) in mice, as well as the possible mechanisms of apoptosis induction. CRPC xenograft tumors were generated in mice and, subsequently, mice received appropriate doses of bufalin, hydroxycamptothecin or a combination of the two drugs. Tumors from each treatment group were removed, and the tumor volume, weight and inhibition rate of each group was determined. Hematoxylin and eosin staining was performed for pathological analysis and TUNEL staining was used to assess the level of apoptosis in the xenografts. Immunohistochemistry was used for the analysis of proliferating cell nuclear antigen expression and the expression of Bax, Bel-XL, p53, programmed cell death 4 (PDCD4), phosphorylated (p)-AKT and glycogen synthase kinase (GSK)-3β was determined by western blotting. Treatment with bufalin significantly (P<0.05) reduced tumor volumes compared with the negative control group, reducing tumor volumes to lower levels when combined with hydroxycamptothecin. The combination of bufalin (0.6 or 0.8 mg/kg) and hydroxycamptothecin significantly (P<0.05) induced higher levels of cell apoptosis compared with the administration of bufalin or hydroxycamptothecin alone. The combination of bufalin and hydroxycamptothecin also increased the expression of apoptosis-related proteins Bax, p53, PDCD4 and GSK-3β, and decreased the expression of Bel-XL and p-AKT compared with a single drug treatment. The present study suggested that the combination of bufalin and hydroxycamptothecin improved the inhibitory effects of both drugs on CRPC tumors in vivo, potentially via the regulation of the PI3K/AKT/GSK-3β and p53-dependent apoptosis signaling pathways.

Introduction

Prostate cancer is a high-risk malignant tumor of the urinary tract, typically diagnosed in middle-aged and elderly men (1). The worldwide incidence rate of prostate cancer accounted for 15% of the total number of malignant tumors in males in 2012 (2), displaying a significant upward trend each year (3). Prostate cancer is the third largest cause of cancer-associated death in males in the United States (4) and the second largest cause worldwide (2). At present, the treatment of prostate cancer includes surgery, radiotherapy, chemotherapy and endocrine therapy (5). However, for advanced prostate cancer, surgery is ineffective and other treatment options are not satisfactory, with the majority being associated with severe adverse reactions (5). The main therapeutic strategy for prostate cancer is endocrine therapy, however, after a median of 18-20 months, the majority of patients will eventually develop androgen resistance, with a median survival of 12 months (6,7). Therefore, the treatment of castration-resistant prostate cancer (CRPC) has become a focus in urology research and the identification of novel treatment options is required (8).

Bufalin is a steroidal terpene compound, and is one of the active ingredients in traditional Chinese medicine (9). Bufalin has been reported to be useful in the treatment of liver and pancreatic cancers, as well as other tumors (9-12). Previous studies have reported that bufalin can significantly inhibit the proliferation of PC3 CRPC cells in vitro (13,14). However, the therapeutic dose is close to the toxic dose, which limits its clinical application (15,16). Bufalin is a DNA topoisomerase (TOP)II inhibitor (17), and its mechanism of action is related to the inhibition of tumor proliferation, metastasis and angiogenesis, as well as the reversal of tumor resistance (18-21). Cortés and Piñero (22) reported that DNA TOPI inhibition in ovarian cells by the TOPI inhibitor irinotecan resulted in increased levels of TOPII mRNA and protein expression, allowing DNA metabolism to continue (23,24). This phenomenon is called the...
side-channel sensitivity of TOP, and is required to maintain
the normal physiological state of the cells (25,26). In oral
cancer research, Ding et al (27) combined the TOPI inhibitor
irinotecan and the TOPII inhibitor doxorubicin to improve
treatment efficacy.

Previous studies have reported that the DNA TOPI inhibi-
tor hydroxycamptothecin and the TOPII inhibitor bufalin can
inhibit the growth of the CRPC cell line DU 145 in vitro (28,29).
These studies also suggested that simultaneous administration
of the two inhibitors was not as effective as individual drug
administration, due to antagonism, but sequential administra-
tion significantly improved the results obtained (28,29).

The present study investigated whether bufalin in combi-
nation with hydroxycamptotecin would exhibit the same effect
in vivo, as has been reported in previous in vitro studies.
Furthermore, the present study aimed to identify a dose of
low-toxicity bufalin combined with hydroxycamptothecin for
further clinical applications.

Materials and methods

Cell lines and cell culture. The human prostate cancer cell line
DU 145, purchased from Thermo Fisher Scientific, Inc., was
cultured in RPMI 1640 complete medium (Sigma-Aldrich;
Merck KGaA) containing 10% fetal bovine serum (FBS;
Sigma-Aldrich; Merck KGaA) and 1% penicillin and strep-
tomycin solution, at 37°C with 5% CO₂ and fully saturated
humidity. Cells were subcultured each for 2-3 days.

Establishment of a CRPC xenograft model in nude mice and
treatment administration. A total of 41 male BALB/c nude
mice (age, 22-25 g; 6-8 weeks), obtained from the Experimental
Animal Center of Shanghai University of Traditional Chinese
Medicine were housed under pathogen-free condition at
22±2°C with 40-60% humidity, 12-h light/dark cycles and free
access to food and water. Cell suspensions of DU 145 cells in
the logarithmic growth phase were prepared at a concentra-
tion of 1x10⁵ cells/ml, using physiological saline. To induce
tumor formation, five mice were subcutaneously injected in
the abdomen with 0.2 ml cell suspension. At three weeks
post-inoculation, when the diameters of the primary implanted
tumors had grown to 1 cm³, tumors were removed and abdomi-
inally implanted into the other 36 mice. After 8 days, drugs
were administered once every other day for 30 days. For drug
administration, the 36 tumor-bearing mice were randomly
divided into six groups, with six mice in each group. The
groups were as follows: Normal saline negative control group
(SN), hydroxycamptothecin (2 mg/kg; BioCrick) single drug
positive control group (H), bufalin (1 mg/kg, Sigma-Aldrich;
Merck KGaA) single drug positive control group (B) and
hydroxycamptocetin (2 mg/kg) sequentially combined with
0.4 mg/kg bufalin treatment group (H4B), 0.6 mg/kg bufalin
treatment group (H6B) or 0.8 mg/kg bufalin treatment group
(H8B). No adverse reactions were reported in the mice in the
combined treatment groups. The sequential co-administration
method involved administration of the corresponding dose of
bufalin 8 h after the administration of hydroxycamptothecin,
as previously described (30). The present study was approved
by the Institutional Ethics Committee of Shanghai University
of Traditional Chinese Medicine.

Morphological and histological observation of tumors. A
total of one day post-treatment, the six mice in each group
were sacrificed and the tumors were completely removed. The
tumors from each group were compared by morphological
analysis. The long diameter (a) and short diameter (b) of the
tumor were measured. Tumor parameters were calculated
using the following formulae: Tumor volume (V; mm³)=(ab²)/2;
tumor-inhibition rate (%)=[(V administration group/V nega-
tive control group)] x100; and tumor mass was determined
using an electronic balance. A section of the tumor mass was
removed, fixed in 10% formalin (pH 7.4) at room temperature
for 24 h and embedded in paraffin. The paraffin-embedded
samples were sectioned (4 µm). For pathological analysis,
sections were stained at room temperature using haemo-
xytoxin for 3 min and eosin for 30 sec (HE). Subsequently,
tissue sections were examined using the Leica DM B light
microscope (Leica Microsystems Inc.; magnification, x4, x10
and x40).

TUNEL detection. The TUNEL Assay kit-FITC (cat.
no. ab66108; Abcam) was used to detect apoptosis in
paraffin-embedded sections, according to the manufac-
turer's protocol. The staining of tumor cells in each group
was observed under a fluorescence microscope (Leica DMLB;
Leica Microsystems Inc.; magnification, x400) and necrotic
areas were avoided. TUNEL-positive cells were observed
in five randomly-selected high-power fields. The integrated
optical density (IOD) values of the images were analyzed using
Image-Pro Plus software (version 6.0; Media Cybernetics,
Inc.) to assess the extent of apoptosis in tumor cells.

Detection of proliferating cell nuclear antigen (PCNA) protein
expression by immunohistochemistry. Immunohistochemical
staining was performed to detect the expression of PCNA in
the xenograft tumors. Paraffin sections were rehydrated. The
sections were subsequently treated as follows: Microwave
antigen retrieval (700 W for 8 min; twice in 10 mM sodium
citrate; pH 6.0) was followed by incubation with 3% hydrogen
peroxide to block endogenous peroxidase and 10% goat serum
(cat. no. 5560-0007; Seracare Life Sciences, Inc.) at 4°C
for 30 min to block non-specific binding. PCNA was detected
with rabbit anti-PCNA (1:100; cat. no. ab18197; Abcam)
overnight at 4°C and horseradish peroxidase-conjugated goat
anti-rabbit immunoglobulin G secondary antibody (1:400;
cat. no. ab205718; Abcam) for 1 h at room temperature.
After that, 3,3-diaminobenzidine tetrahydrochloride (DAB;
cat. no. 30015; Biotium, Inc.) was used to detect apoptosis in
tumor cells. The tissue sections were examined using the Leica
FDC300 FX light microscope (Leica Microsystems Inc.; IL;
magnification, x400). PCNA-positive cells, displaying brown-yellow
granules in the nuclei, were observed in five randomly-selected
high-power fields. The integrated optical density (IOD) values of the images were analyzed using
Image-Pro Plus software (version 6.0; Media Cybernetics,
Inc.). For the negative control, the primary antibody was replaced by normal rabbit IgG.

Western blotting. A homogenizer was used to prepare lysates
from xenograft tumor tissues. The xenograft tumor tissues
Comparison of tumor size in different groups. After successful establishment of CRPC xenografts in mice, the mice were weighed and no statistically significant difference was observed between treatment groups (Table I; P>0.05). The mice in the treatment groups displayed no abnormal changes in body weight or behavior. The mice were sacrificed one day post-treatment and comparisons of tumor size of the xenograft tumors isolated from different groups were performed (Fig. 1A).

The volume and weight of the xenograft tumors were measured. All drug treatments significantly reduced the tumor volume compared with the SN group (P<0.05; Fig. 1B). Among the different drug treatment groups, the H6B and H8B groups were more effective at inhibiting increases in the
differences were found between the H4B group and the single drug administration groups (Fig. 3A and B).

**Differential expression of PCNA protein.** The PCNA protein is ubiquitously expressed in the nucleus, and its nuclear content is consistent with the synthesis of DNA, making PCNA an indicator of cell proliferation (31). The expression of PCNA in prostate cancer xenograft tumors was analyzed by immunohistochemistry. PCNA-positive cells were stained brown or yellow, indicating that the cells were proliferating and dividing (Fig. 4A).

The xenograft tumors of the drug-administered groups displayed significantly reduced PCNA levels compared with the SN group (P<0.05; Fig. 4A and B). The H6B and H8B groups displayed the lowest levels of PCNA expression compared with all the other treatment groups (P<0.05; Fig. 4A and B). There was no significant difference (P>0.05) between the PCNA expression in xenograft tumors from the H4B group and the single drug administration groups (H and B). The H6B group exhibited the most significant effect on PCNA expression, and there was a statistical difference compared with the single drug administration groups (P<0.05; Fig. 4B).

**Expression of apoptosis-related proteins Bax, p53, programmed cell death 4 (PDCD4) and glycogen synthase kinase (GSK)-3β.** To further explore the mechanisms of drug inhibition on tumor growth, the expression of cytoplasmic proteins in xenograft tumors was determined by western blotting. All drug treatments increased the protein expression levels of the tumor suppressor genes p53 and PDCD4, the mitochondrial apoptosis-related protein Bax and the PI3K/AKT/GSK-3β apoptosis signaling pathway-related protein GSK-3β, compared...
with the SN group (Fig. 5). Furthermore, all drug treatments decreased the protein expression levels of the mitochondrial apoptosis-related protein Bcl-XL and the PI3K/AKT/GSK-3β apoptosis signaling pathway-related protein p-AKT, compared
with the SN group (Fig. 5). Additionally, the H4B, H6B and H8B groups significantly increased the protein expression levels of Bax, p53, PDCD4 and GSK-3β, and decreased the protein expression levels of Bcl-XL and p-AKT compared with the single drug administration groups (H and B; P<0.05; Fig. 5). Among the combination treatment groups, the changes were the most prominent in the H6B group, followed by the H8B and H4B treatment groups, respectively, although there was no significant difference among the three groups.

Discussion

The use of bufalin for the inhibition of tumor cell growth has been researched extensively in precious years (32-37). Low-dose bufalin displays an inhibitory effect on the growth of prostate cancer DU 145 cells in a time and dose-dependent manner (38). Administration of a combination of a specific dose of hydroxycamptothecin with a single agent was found to be effective against cancer cells (28). Similar synergy was also reported for the combination of hydroxycamptothecin and etoposide in human colon carcinoma HT-29 cells (39).

The present study suggested that in a nude mouse CRPC xenograft model, low-dose bufalin inhibited increases in tumor volume and weight, but bufalin (0.6 and 0.8 mg/kg) combined with hydroxycamptothecin had an improved effect on tumor volume, weight and inhibition rate compared with the administration of either drug alone. Histopathological analysis of sections of the xenograft tumors indicated increased cell death with the combined administration of bufalin and hydroxycamptothecin compared with the other groups. The TUNEL assay suggested that the H6B and H8B groups promoted higher levels of tumor cell apoptosis compared with the single drug administration groups. Immunohistochemical staining indicated that the H6B and H8B groups were more effective at inhibiting the proliferation of prostate cancer cells than all other treatment groups. The proapoptotic and growth-inhibiting effects of bufalin, hydroxycamptothecin or their combination may be related to the mitochondrial, p53-related and PI3K/AKT/GSK-3β apoptotic signaling pathways.

In recent years, bufalin has been reported to exhibit proapoptotic effects in a number of tumors (40-44,41), but the effective dose (≥1.5 mg/kg) utilized in previous studies is close to the toxic dose. According to the Dictionary of Traditional Chinese Medicine, the median lethal dose (LD₅₀) of bufalin in nude mice is 2.2 mg/kg (45), and a number of previous studies have reported that 1.5 mg/kg bufalin significantly promoted the
apoptosis of transplanted tumor cells and exhibited antitumor effects in nude mice (37,46,16,37). In the present study, the dose of bufalin used in the combination treatment groups (0.4, 0.6 and 0.8 mg/kg) was much lower than the LD_{50} value. There was no significant difference in the body weight of mice in the treatment groups compared with the SN group. The effects of the three combined treatment groups were no less than those of the bufalin (1.0 mg/kg) alone positive control group. Therefore, it can be suggested that the use of low-dose bufalin combined with hydroxycamptothecin may have a significant therapeutic effect, and may not be associated with toxicity, providing rationale for the clinical use of low-dose bufalin. However, the administration of a bufalin and hydroxycamptothecin combination would need to follow a specific protocol. The present study further suggested that the simultaneous administration of bufalin and hydroxycamptothecin combination was effective in the treatment of CRPC. The use of bufalin and hydroxycamptothecin simultaneously leads to drug antagonism, but sequential administration may lead to a synergistic effect (47). Therefore, administration of hydroxycamptothecin for a certain period of time prior to the administration of bufalin may be more effective (47). The present study suggested that the sequential administration of bufalin (0.6 and 0.8 mg/kg) 8 h after the administration of hydroxycamptothecin (2 mg/kg) was more beneficial. However, the role of hydroxycamptothecin in CRPC requires further investigation.

Bel-XL and Bax belong to the Bcl-2 protein family (48). By controlling the permeability of the mitochondrial inner membrane structure, Bel-XL and Bax affect proapoptotic factors in the cytoplasm, including cytochrome C, and transmit apoptotic signals to regulate cell death (49). Bax is an important component of mitochondrial membrane ion channels (50). After receiving the apoptotic signal, Bax expression is increased, proapoptotic factors in the mitochondria, such as cytochrome C, enter the cytoplasm and the caspase protein family is activated to induce apoptosis (51-53). Bcl-XL is primarily located in the cytoplasm and can be translocated to the mitochondrial outer membrane to bind Bax and form Bcl-XL/Bax heterodimers, under the action of apoptotic signals (20,21). Subsequently, the Bcl-XL/Bax heterodimers maintain the integrity of the mitochondrial outer membrane and interfere with apoptosis induction (54,55). The sequential administration of bufalin 8 h after the administration of hydroxycamptothecin enhanced the expression of Bax and inhibited the expression of Bcl-XL, potentially promoting apoptosis. The present study suggested that the combination of hydroxycamptothecin and bufalin, at the dose of 0.6 mg/kg, was the most beneficial treatment option.

Both p53 and PDCD4 are tumor suppressor genes, which play roles in cell apoptosis and DNA damage repair (56,57). Under physiological conditions, p53 levels are low in the cell (58). When DNA damage occurs in cells, p53 accumulates in the cells and promotes the apoptosis of abnormal cells via the p53/Bax apoptosis regulatory signaling pathway to prevent excessive proliferation of abnormal cells (59,60). The present study suggested that the apoptotic effect of bufalin on tumor cells is related to the activation of p53 and an increase in PDCD4 expression, which could potentially prevent the excessive proliferation of prostate cancer cells.

The PI3K/AKT signaling pathway is important for cell membrane receptor signaling (61). AKT regulates the
proliferation of downstream proteins, including caspase 9, Bad, NF-κB and GSK-23, by phosphorylation, thereby regulating cell proliferation, differentiation, apoptosis and migration (62). GSK-3β can inhibit the expression of transcription factors, including β-catenin, Nrf2 and NFAT and activate the caspase pathway to induce apoptosis (63). The combination of bufalin and hydroxycamptothecin promoted the expression of GSK-3β and inhibited the expression of p-AKT, potentially inhibiting the growth of tumor cells. Furthermore, the combination of hydroxycamptothecin and bufalin at a dose of 0.6 mg/kg was the most effective at promoting GSK-3β expression and inhibiting p-AKT expression.

To conclude, sequential administration of bufalin and hydroxycamptothecin inhibited the growth of CRPC xenograft tumors. The dosage used for co-administration influenced the degree of drug inhibition. The administration of hydroxycamptothecin (2 mg/kg) followed by the administration of bufalin (0.6 mg/kg) 8 h later was the most effective treatment method assessed in the present study. The proapoptotic effect of bufalin and hydroxycamptothecin may occur via signaling pathways associated with mitochondrial apoptosis, P13K/AKT/GSK-3β apoptotic signaling and p53-dependent apoptosis regulation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

QZ performed the experimental work and collected and interpreted the data. RG designed the study, performed the analysis of the data, interpreted the data and drafted the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of Shanghai University of Traditional Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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