Physalins A and B Inhibit Androgen-Independent Prostate Cancer Cell Growth through Activation of Cell Apoptosis and Downregulation of Androgen Receptor Expression

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Androgen deprivation therapy is a common treatment strategy for advanced prostate cancer. Though effective initially, the tumor often progresses to androgen independent stage in most patients eventually after a period of remission. One of the key factors of development of resistance is reflected in expression of androgen receptor (AR). In this study, we showed that two natural compounds, physalins A and B, both secosteroids from Physalis alkekengi var. franchetii, significantly inhibited the growth of two androgen-independent cell lines CWR22Rv1 and C42B, induced apoptosis via c-Jun N-terminal kinase (JNK) and/or extracellular signal-regulated kinase (ERK) activation, and decreased AR expression. In addition, physalins A and B down-regulated the expression of prostate specific antigen (PSA) in C42B cells which is a target gene of AR. Our results suggest that physalin A and B might be useful agents in preventing the growth of androgen-independent prostate cancer (AI-PCa).

Key words physalin A; physalin B; androgen-independent prostate cancer; apoptosis; androgen receptor

Prostate cancer is the second leading cause of cancer-related deaths in American males.1) When diagnosed with advanced prostate cancer, androgen depletion therapy would initially be used to treat these patients.2) The majority of patients would respond to the treatment and resulting in shrinkage of tumors. Unfortunately, relapse of tumors often occur in most of the patients, leading to androgen-independent prostate cancers (AI-PCa). Currently, there is no curative therapy for AI-PCa.3) The exact molecular mechanisms responsible for the development of AI-PCa are not understood. The available data support the significance of the expression and activity of androgen receptor (AR) in AI-PCa progression. In a relatively large number of AI-PCa patients, AR is expressed, overexpressed, mutated or amplified.4,5) AR itself may exert an important function in PCA progression and has been a promising target for treating AI-PCa.

Natural products have become more and more attractive in the treatment of cancers, therefore, we focused on natural products as a source for screening active components that target AR signaling pathway and inhibit cell growth in PCA cell lines. Our previous study showed that lignans from Campylotropishirtella (Franch.) Schindl. significantly down-regulated AR expression and also its target gene, prostate specific antigen (PSA), in an androgen dependent cell line, LNCaP, for the first time.6) The compound that showed the best activity on AR inhibition also significantly inhibited cell growth. In this study, we further investigated two natural compounds, physalins A and B, both secosteroids isolated from Physalisalkekengi var. franchetii and provided by Qiu.7) In China, the calyces of Physalisalkekengi var. franchetii (Solanaceae), were provided by Qiu. The detailed isolation and identification procedures of these two compounds were described by Qiu et al.7) They were dissolved in dimethyl sulfoxide (DMSO) to obtain the desired concentrations.

MATERIALS AND METHODS

Physalin A and B Physalins A and B, purified from Physalisalkekengi var. franchetii (Solanaceae), were provided by Qiu. The detailed isolation and identification procedures of these two compounds were described by Qiu et al.7) They were dissolved in dimethyl sulfoxide (DMSO) to obtain the desired concentrations.

Cell Lines and Cell Culture Two androgen-independent cell lines CWR22Rv1 and C42B were used in the present study. They were generous gifts from Dr. Franky Chan (the Chinese University of Hong Kong). CWR22Rv1 was maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. C42B was maintained in T medium (Invitrogen) supplemented with 5% fetal bovine serum and 100 U/ml penicillin/streptomycin.

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streptomycin. Both cell lines were cultured at 37 °C, 5% CO₂.

Cell Proliferation Assay Eight thousand cells per well were seeded in a 96-well plate. After 24 h, different concentrations of physalins were added. The cells were incubated for the designated time, and the cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay kit (Boeringher, MO, U.S.A.), according to the manufacturer’s protocol.

Colonies Formation Assay Experimental details have been described elsewhere.13) Briefly, 300 CWR22Rv1 cells were seeded per well in 6-well plates and 5 doses of physalin A or B were added. After 11—13 d incubation, the cells were fixed in 70% ethanol and stained with 10% (v/v) Giemsa (MERCK, Damstadt, Germany). Colonies that consisted of more than 50 cells were counted. Two wells were used for each dose and 2 wells treated with solvent only served as controls. Colony forming ability after drug treatment was calculated as the ratio between the number of colonies in the treated wells and the untreated controls multiplied by 100. The results represented the average of 3 independent experiments.

Western Blot Analysis Experiments were carried out as previously described.14) Briefly, 250000 cells were seeded in 6-well plates. Different concentrations of physalins were added and the cells were incubated for the designated time. The medium was removed completely, the cells were collected, and resuspended in 30—50 μl of lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonylfluoride). After centrifugation, lysates were obtained and protein concentrations were measured using the DC protein assay kit (Bio-Rad, CA, U.S.A.). Equal amounts of proteins (20 μg) were loaded into a 10% SDS polyacrylamide gel for electrophoresis and then transferred onto a polyvinylidenedifluoride membrane (Amersham, NJ, U.S.A.). The membrane was blocked with milk for 1 h and then incubated with primary antibody for 1 h at room temperature against [Caspase-3, poly(ADP-ribose) polymerase (PARP), JNK, phospho-JNK, phospho-c-Jun, ERK, phospho-ERK, P38, phospho-P38 (Cell Signaling Technology, MA, U.S.A.), AR (Santa Cruz Biotechnology, CA, U.S.A.), PSA (Dako Cytomation, Denmark), and Actin (Santa Cruz Biotechnology)]. After incubating with appropriate secondary antibody, signals were visualized by enhanced chemiluminescence (Amersham).

RESULTS AND DISCUSSION

Physalins A and B, as shown in Fig. 1, are secosteroids which possess an unusual 13, 14-seco-16,24-cyclo-steroidal ring skeleton in nature. The cell line C42B is derived from an androgen dependent prostate cancer cell line LNCaP.15) C42B expresses AR and secretes PSA, but is no longer sensitive to androgen. CWR22Rv1 is originally from a human prostatic carcinoma xenograft, CWR22R.16) CWR22Rv1 is also androgen independent, expresses AR, but does not secrete PSA. These two cell lines closely mimic clinical prostate cancer progression from androgen dependence to androgen independence. We selected C42B and CWR22Rv1 to explore the possible functions of physalins A and B in preventing AI-PCa progression.

We found that physalins A and B significantly inhibited cell growth by measuring cell viability using MTT method (Table 1). A range of concentrations of these two compounds were tested at three different time points; i.e. 24, 48 and 72 h. Table 1 lists the IC₅₀ values after physalins A and B treatment on each cell line at different time points. For physalin A, they were 53.8, 13.5, and 9.6 μM on C42B, and 81.5, 18.2 and 14.2 μM on CWR22Rv1 at 24, 48 and 72 h, respectively. For physalin B, they were 19.1, 7.0 and 2.6 μM on C42B, and 24.4, 8.8 and 3.7 μM on CWR22Rv1 at same time points as physalin A. The results clearly showed that physalin B was more potent than physalin A on both cell lines while C42B was more sensitive to these compounds than CWR22Rv1.

Since the colony forming assay is more closely correlated with antitumor activity in preclinical studies, we further performed colony forming assay on CWR22Rv1cells. As shown in Fig. 2, both compounds significantly inhibited colony formation. Lower concentrations of physalin B compared to physalin A were needed to inhibit colony formation in CWR22Rv1 cells. Though previous studies have reported physalin B showed growth inhibitory effect on a variety of cancer cell lines,9—12) there is no data available for its effects on C42B and CWR22Rv1. On the other hand, Magalhães et al.13) demonstrated that physalin inhibited sarcoma 180 tumour growth with toxic effects to the liver and kidney in vivo, but the effects are reversible.

The following investigation was conducted to determine if the growth inhibitory effects of physalins A and B were caused by activating apoptosis pathways in cancer cells. Caspase 3 has been implicated as an “effector” caspase associated with the initiation of the “death cascade” and is there-

![Physalin A](image1.png)

![Physalin B](image2.png)

Fig. 1. Structures of Physalins A and B
fore an important marker of the cell’s entry point into the apoptotic signaling pathway. During activation, caspase 3 is cleaved to yield two subunits of 17 and 12 kDa which dimerize to form an active enzyme.17) PARP is an important substrate cleaved by activated caspase 3. It also has been used as hallmark of apoptosis together with caspase 3. Therefore, we examined these two proteins and their activated forms (cleavages) after both cell lines were treated with various concentrations of physalins A and B at different time points. In Fig. 3, activation of caspase-3 was represented by a decrease in the intensity of procaspase-3 (32 kDa) protein band and the appearance of its active subunit p17 (17/19 kDa). PARP degradation was represented by a decrease intensity of the 116-kDa PARP protein band and appearance of an 85-kDa PARP cleavage product. The results clearly showed a dose and time-dependent activation of caspase-3 and PARP, suggesting the induction of apoptosis by physalin A and B on these two cell lines. Also, we found that there are more significant cleavages in C42B than in CWR22Rv1 when treated with physalin A (10 μM) and physalin B (4 μM) in the different time points from 12 to 48 h. So these results are consistent with the growth inhibitory data that C42B was more sensitive to the physalins than CWR22Rv1.

In order to explore the mechanisms involved in the apoptotic effects of physalins A and B, we examined the regulation of the mitogen-activated protein kinases (MAPKs) signaling pathways after treatment with these two compounds in C42B and CWR22Rv1 cells. MAPKs, which include the ERK1/2, JNK, and p38MAPK subgroups, are suggested to play important roles in cells proliferation and apoptosis.18) Our results (Fig. 4) showed that the phosphorylation levels of JNK and its downstream target, c-Jun were increased after treatment with physalins A and B in both cell lines. The phosphorylation level of ERK1/2 was increased in the CWR22Rv1, but not in the C42B. The phosphorylation level of p38 was not observed in either cell line. The information from literature indicates that the roles of MAPKs may differ from experiment to experiment and are strongly dependent on cell type as well as the types of agents used.19) The activation of the ERK1/2 pathway is generally considered to be as-

### Table 1. The Values of IC₅₀ for Physalin A and Physalin B on C42B and CWR22Rv1 through MTT Method

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<th>Physalin B (IC₅₀ μM)</th>
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<td>24 h</td>
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<td>C42B</td>
<td>19.1</td>
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<td>CWR22Rv1</td>
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Fig. 2. The Effects of Physalins A and B on Colony Forming Ability of CWR22Rv1. The percentage of colonies formed after the treatment of two compounds was calculated as the ratio between the number of colonies in the treated wells and the untreated controls multiplied by 100. Results were presented as the mean and standard deviation from 3 experiments. Note that colony forming ability of the treated groups was significantly lower than vector control and physalin B was more potent than physalin A.
associated with cell proliferation and survival. However, ERK1/2 activation has been shown to induce apoptosis in T-cells.\(^6\) Also, ERK activation was required for Cisplatin-induced apoptosis in Hela cells.\(^7\) P38 MAPK has been demonstrated to either induce or prevent apoptosis in different cell types\(^8,9,22,23\) while JNK is activated by many cytotoxic drugs, and its activation is generally believed to be involved in cell deaths.\(^24,25\) Our results suggested JNK activation might be involved in physalins A and B induced cell apoptosis and that ERK activation might be required in CWR22Rv1 cells, but not in C42B cells. Based on the aforementioned results, we concluded that physalins A and B had significant growth inhibitory effects on both these androgen-independent cell lines, CWR22Rv1 and C42B.

We next determined whether they have specific functional effects on AI-PCa, namely expression of AR and secretion of PSA. AR has been indicated to play an important role in the progression of AI-PCa. Once diagnosed with advanced prostate cancer, treatment strategies to block androgen-mediated cell proliferation would be initiated. However, almost in all cases the end result is the development of androgen resistant PCa. A significant body of evidence has suggested that in many AI-PCa cases, AR is highly expressed and hypersensitive to low, castrated-levels of androgens or even can be activated by non-androgens to induce tumor cell growth.\(^26\) Chen et al.\(^27\) reported that a two- to five-fold increase in AR mRNA was the only change in gene expression consistently associated with the development of resistance to anti-androgen therapy using an isogenic prostate cancer xenograft model. The expression of the AR itself might contribute to this resistance. Such findings provide insight toward the target of new therapeutics for AI-PCa. The next target of this study was to evaluate if physalins A and B would affect AR expression. The results are shown in Fig. 5. At the lowest dosages used for physalins A and B, 5 \(\mu\)M and 2.5 \(\mu\)M, respectively, both compounds significantly decreased AR expression, and a dose-dependent effect was observed at higher concentrations in both cell lines. The low molecular weight AR band (LMW AR) in CWR22Rv1 cell line is truncated AR which should be an androgen independent isoform of AR. Here we can see both LMW AR and AR were decreased in C42B, as PSA is detectable in C42B, we also tested PSA level after the treatment. PSA is a target gene of AR, and is also a functional biomarker of AR activation.\(^28\) PSA is well-accepted as a clinically marker for diagnosis and prognosis of prostate diseases. After interaction with androgen, AR is activated and the AR-androgen complex is translocated into the nucleus to regulate transcription of its target genes, like PSA. As predicted, PSA expression was significantly decreased in C42B after the treatment of physalins A and B. This further suggested that physalin A and B not only affected the expression level of AR, but also inhibited its transcriptional activity.

In summary, the results of this study provided the first evidence that physalins (A and B) could down regulate AR expression, a key factor in the development of resistance of PCa to androgen deprivation therapy and the progression of AI-PCa. Also, we showed that physalins A and B significantly inhibited C42B and CWR22Rv1 proliferation, and induced apoptosis. Previous studies have shown that down regulation of AR inhibited prostate cancer cell proliferation and induced apoptosis. Liao et al. reported that small-interfering RNA induced AR silencing leads to apoptotic cell death in prostate cancer.\(^29\) Using similar method, Haag et al. showed that down regulation of AR induced cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cell.\(^30\) We hypothesize that down regulation of AR by physalins A and B, contributes to, at least partly, their proliferation inhibitory effects and apoptosis induction of these two cell lines. We further explored the possible mechanisms of their apoptotic effects by measuring activation of the MAPK pathway. Previous studies have shown evidence of cross-talk between the MAPK pathways and AR signaling.\(^31,32\) One recent report demonstrated that expression of constitutively active MAP kinase kinase 1 (MEKK1) inhibited AR signaling and induced apoptosis in androgen receptor-positive but not in androgen receptor-negative prostate cancer cells.\(^33\) In our study, two possible mechanisms are that physalins A and B inhibit AR signaling through regulating MAPK pathway, then cause apoptosis on these two cell lines, or physalins A and B down regulate AR activity, which, then activates MAPK pathway to induce apoptosis, further experiments need to be done to determine the exact mechanism. Based on the current results, we suggested that physalins A and B should be considered for use in the prevention of AI-PCa progression and treatment for AI-PCa.

**REFERENCES**


