



Downregulation of ZFX is associated with inhibition of prostate cancer progression by baicalein

Wenhui Zhu^{1*}, Jimeng Hu^{1*}, Mengbo Hu¹, Haowen Jiang^{1,2}

¹Department of Urology, ²Institute of Urology, Huashan Hospital, Fudan University, Shanghai 200040, China

Contributions: (I) Conception and design: W Zhu, J Hu, H Jiang; (II) Administrative support: W Zhu, M Hu, H Jiang; (III) Provision of study materials or patients: W Zhu, J Hu; (IV) Collection and assembly of data: W Zhu, J Hu, H Jiang; (V) Data analysis and interpretation: W Zhu, M Hu, H Jiang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

*These authors contributed equally to this work.

Correspondence to: Haowen Jiang. Huashan Hospital, Fudan University, 12 Urumuqi Middle Road, Shanghai 200040, China. Email: haowenjzwh@163.com.

Background: Baicalein is a bioactive flavonoid that is widely used in China. However, its effects on prostate cancer (PCa) and relative mechanisms have not been extensively studied. We intended to study the potential anti-tumor effects of baicalein and the related mechanisms on PCa.

Methods: We analyzed the inhibitory effects of baicalein on PCa by utilizing a cell viability assay and an *in vivo* tumor growth assay. Wound healing assay and transwell assay were performed to investigate cancer cell migration and invasion abilities. Then the expressions of zinc finger protein X-linked (ZFX) in PCa cells and xenograft tumor were respectively detected by western blot and immunohistochemistry assay.

Results: Baicalein significantly inhibited cell proliferation *in vitro* and tumor growth of PCa *in vivo*. Consistently, baicalein induced significantly inhibition on the migration and invasion abilities of PCa cells. In addition, ZFX expressions were downregulated by baicalein in PCa both *in vitro* and *in vivo*.

Conclusions: Our study provides evidence that baicalein plays important roles in PCa therapy probably by downregulating ZFX expression, which was demonstrated to be involved in PCa progression, and thus may be developed as a novel and efficient candidate agent for PCa therapy.

Keywords: Baicalein; prostate cancer (PCa); xenografts; zinc finger protein X-linked (ZFX); cell migration/invasion

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Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed lethal tumors around the world (1). For decades, androgen-deprivation therapy (ADT) has been the standard treatment for advanced PCa (2). However, ADT becomes ineffective no more than 2 years for most PCa progressing into castration-resistant prostate cancer (CRPC) stage, which results in high mortality due to lack of cheap and effective alternative (3,4). Therefore, the development of pharmacological interventions is vital.

Baicalein is a bioactive flavonoid extract obtained from *Scutellaria baicalensis georgii*'s dry root, which are widely

used in China. Recently, increasing evidence has suggested that baicalein has anti-tumor effect (5,6). Cellular and animal experiments have verified that baicalein could inhibit tumor cell proliferation, invasiveness and metastasis (7-11) in many cancers, including PCa (12,13). However, the anti-tumor effects and the specific molecular mechanisms in PCa is yet to be further elucidated.

It has been suggested that the inhibition of gallbladder cancer cells by baicalein is correlated with the downregulation of zinc finger protein X-linked (ZFX) (14), which is one kind of zinc-finger protein encoded by *ZFX* gene (chr Xp21.3). It has also been demonstrated that ZFX plays a vital role in the self-renewal of both embryonic

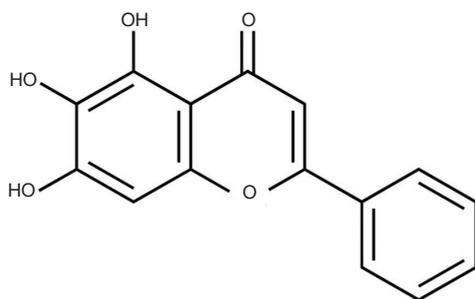


Figure 1 Molecular structure of baicalein.

and hematopoietic stem cells (15). Increasing evidence suggests that ZFX acts as an oncogene in many malignant tumors, including glioblastoma (16), hepatocellular (17) and oral squamous carcinoma (18), performing in the regulation of those genes which are essential for cell growth, differentiation and apoptosis. Our previous results also demonstrated that knockdown of ZFX inhibited cell proliferation and induced apoptosis in PC-3 and DU145 cells (19), which indicated that ZFX was critical in PCa cell proliferation and survival. Therefore, we sought to determine whether baicalein could inhibit PCa progression by downregulating ZFX expression.

Methods

Chemicals and antibodies

Baicalein was obtained from Sigma-Aldrich (St Louis, Missouri, USA), and its chemical structure is shown in *Figure 1*. Antibody against ZFX was purchased from Abcam (Cambridge, MA, UK), and antibody against β -actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Drug treatment

Baicalein [dissolved in dimethyl sulfoxide (DMSO)] was used for experiment group. The final concentration of DMSO (Sigma-Aldrich, St Louis, USA) in each group was 0.1% (v/v). Control cells were treated with vehicle (DMSO group) or blank (CTRL group).

Cell culture

PCa cells of PC-3, DU145 and LNCaP were purchased from Chinese Academy of Sciences (CAS, Shanghai, China).

PC-3 and DU145 were cultured in DMEM/F12 medium (Hyclone, USA), and LNCaP was cultured in RPMI-1640 medium (Hyclone, USA) containing 10% FBS. All media were supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin; Gibco, USA). All cells were grown at 37 °C/5% CO₂.

CCK-8 assay

Cell proliferation and viability were determined using Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay. Briefly, cells were seeded at 5×10^3 cells/well (100 μ L) in 96-well plates and cultured overnight. Then different concentrations of baicalein (12.5, 25, 50, 75, 100, 150 and 200 μ M) were added. Blank control group (CTRL) and negative control group (vehicle: DMSO) were set up. After treatment for 24, 48, and 72 h, CCK-8 (10 μ L, 10%) was added to each well for incubation at 37 °C for 4 h. Then we measured the absorbance at 450 nm with a microplate reader (Molecular Devices, CA, USA). All experiments were repeated in triplicate.

Western blot

Cells were stimulated with different concentrations of baicalein [0, 100 and 200 μ mol/L (μ M)] for 48 h, then harvested on ice and lysed in RIPA buffer (Beyotime, China) containing 1% protease inhibitors. Proteins were extracted, then separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane for western blotting analyses. The membrane was blocked in 5% non-fat milk in TBST for 2 h. Primary antibodies against ZFX (1:1,000) and β -actin (1:1,000) were incubated at 4 °C overnight. HRP-conjugated IgG were used as secondary antibodies at 1:5,000. The signals were visualized using the enhanced chemiluminescence method.

Immunohistochemistry

Antigen retrieval was conducted in 0.01 mol/L citric acid (pH 6.0). Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide. Primary antibody was diluted 1:100 and incubated overnight at 4 °C, followed by incubation with a biotinylated secondary antibody (1:200) for 1 hour. Immunohistochemical staining was conducted following the avidin-biotin peroxidase complex method with diaminobenzidine as a chromogen. Slides were

counterstained with hematoxylin, dehydrated and mounted. Brown cytoplasmic staining of stromal or tumor cells was considered positive.

Wound healing assay

Cells were seeded in 24-well plates and cultured to at least 90% of confluence. Adherent cells were scratched with a plastic 200 μ L pipette tip and then washed with phosphate-buffered saline (PBS). Baicalein (25, 50, 100 and 200 μ M) was added and cells were cultured at 37 °C/5% CO₂. Blank control group (CTRL) and negative control group (vehicle: DMSO) were included. The “wounded” areas were photographed by phase contrast microscopy at 0, 24 and 48 h.

Transwell invasion assay

The cell invasion ability was analyzed by transwell assay as previously described (20). Briefly, cells were pretreated with either 100 μ M baicalein or DMSO vehicle control for 24 h, then surviving cells were collected and incubated in transwell chamber (Corning Incorporated, Corning NY, USA) for 24 h. Afterwards, the inserts were taken out from the chamber, and cells on the upper side were carefully removed by swabbing, while cells on the bottom side were fixed, stained and measured.

Establishment and treatment of DU145 xenografts

All animal experiments were approved by the Huashan Hospital Animal Ethics Committee (KY2016-112). Male BABL/c nude mice (The Animal Center of Chinese Academy of Science, Shanghai, China) at the age of 5 weeks were subcutaneously inoculated in the right armpit of each mouse with DU145 cells (5×10^6 cells/mouse). The mice were housed in a constant room temperature with a 12/12 h light/dark cycle, and ad libitum accessed to food and water. After 2 weeks of tumor formation, mice were randomly divided into two groups (5/group). In these two groups, nude mice bearing DU145 tumor were i.p. injected with 0.1% DMSO and baicalein (30 mg/kg, once/day) for 21 days, respectively. Tumors were measured and tumor weights were detected.

Statistical analysis

The results are presented as mean \pm standard deviation (SD).

Statistical analysis was performed using *t*-test by GraphPad Prism. $P < 0.05$ is considered significantly different. Each experiment was repeated at least three times.

Results

Baicalein suppressed the proliferation ability of PCa cells

When compared with the control groups (CTRL and DMSO), baicalein significantly inhibited PC-3, DU145 and LNCaP cells proliferation at various concentrations for 24, 48 and 72 h ($P < 0.05$, *Figure 2A*). And this inhibition effect increased gradually in a time- and dose-dependent manner from 24 to 72 h and from low to high concentration.

Baicalein inhibited the migration and invasion abilities of PCa cells

To analyze the cell metastatic potential, we detected the cell migration capacity used wound healing assay. As shown in *Figure 2B*, cells in baicalein groups had weaker migration ability than those of control groups (CTRL and DMSO) for 24 and 48 h in PC-3, DU145 and LNCaP cells. And in higher concentration of baicalein groups, the scratch width was wider at 24 and 48 h. These results showed that baicalein inhibited the migration ability of PCa cells in a time- and dose-dependent manner. Transwell assays were performed to assess cell invasion ability. Consistently, the results showed that the number of cells which invaded to the lower chamber in baicalein group was lower than DMSO vehicle control group (Baicalein/DMSO: 33.7%, 20.3%, 46.2% in PC-3, DU145 and LNCaP cells, respectively). Baicalein significantly inhibited the invasion ability of cells compared with DMSO vehicle control ($P < 0.05$, *Figure 2C*).

Baicalein suppressed the tumor growth of DU145 xenografts

To test the antitumor activity of baicalein in vivo, nude mice bearing human prostate adenocarcinoma (DU145) tumors were treated with baicalein or DMSO (as control) by intraperitoneal injection for 3 weeks. As shown in *Figure 2D*, baicalein inhibited growth of human prostate adenocarcinoma (DU145) cells and decreased tumor size in appearance when compared with the control group (DMSO). Similarly, the tumor weights were significantly inhibited by baicalein when compared with DMSO (0.336 ± 0.092 vs. 0.548 ± 0.176 g, $P < 0.05$).

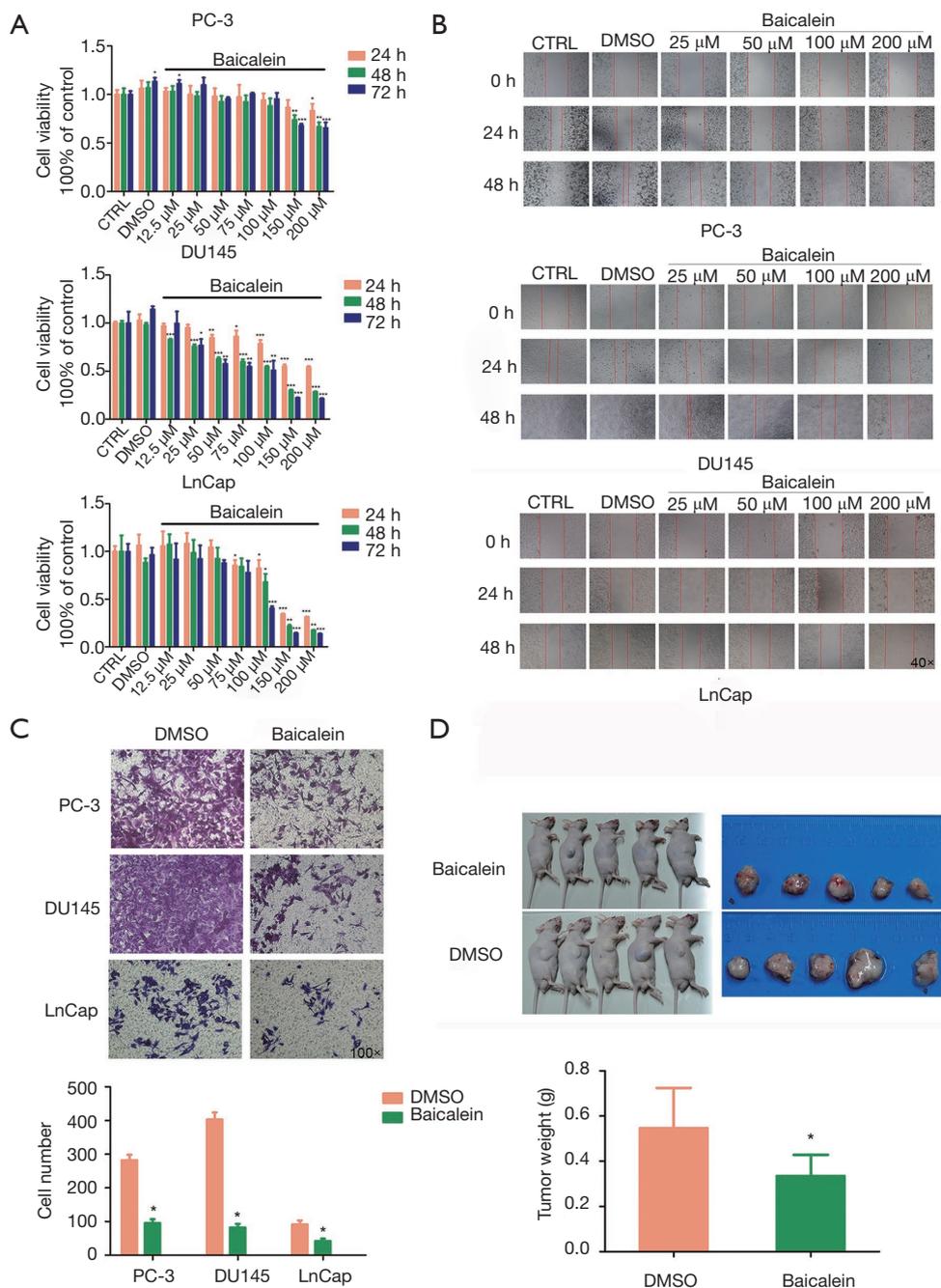


Figure 2 Baicalein inhibited the prostate cancer progression *in vitro* and *in vivo*. (A) Inhibition of cell proliferation by baicalein in a time- and dose-dependent manner. CTRL, DMSO, and 12.5–200 μM baicalein-treated cells measured using CCK-8 assay. Results were expressed as a percentage of control levels. Data were mean ± SD, n=3. *P<0.05; **P<0.01; ***P<0.001; (B) baicalein inhibited the migration ability of prostate cancer cells. Representative images of wounded CTRL, DMSO and various concentrations of baicalein for 0, 24 and 48 h, respectively (magnification, ×40); red line, border of the scratch; (C) baicalein inhibited the invasion of prostate cancer cells. Representative images of three experiments are shown for each group, respectively (magnification, ×100). Columns, mean; bars, SD. *P<0.05; (D) baicalein inhibited tumor growth in nude mice transplanted with DU145 cells. Nude mice were i.p. injected with 0.1% DMSO and baicalein (30 mg/kg, once/day) for 21 days, respectively. Images of nude mice and DU145 tumors. Columns, mean; bars, SD. *P<0.05. CTRL, cells treated with blank; DMSO, dimethyl sulfoxide; SD, standard deviation.

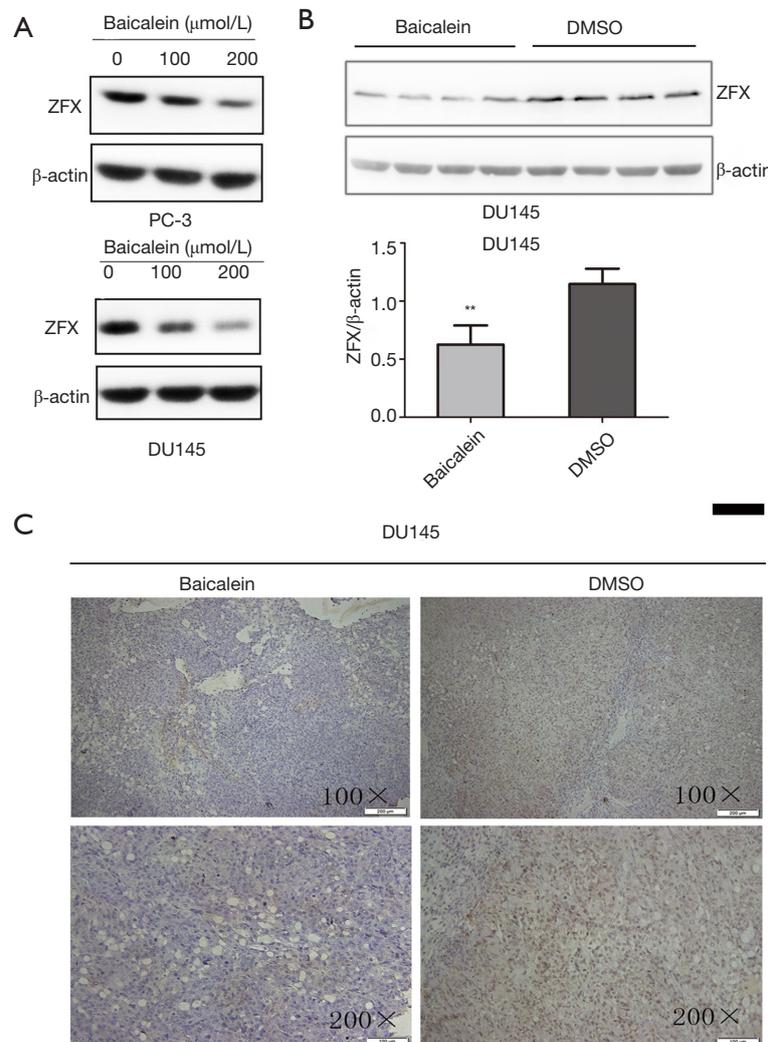


Figure 3 Baicalein inhibited the expression levels of ZFX protein in prostate cancer *in vitro* and *in vivo*. (A) Western blot analysis of ZFX *in vitro*. Cells were incubated with different concentrations (0, 100, 200 μmol/L) of baicalein for 48 h. Antibody against β-actin was used as control; (B) western blot analysis of ZFX *in vivo*. Nude mice bearing DU145 tumor were i.p. injected with 0.1% DMSO and baicalein (30 mg/kg, once/day) for 21 days, respectively. Then tumors were taken for Western blot assay (upper, Western blot; lower, the graph of ZFX/β-actin expression). ** $P < 0.01$; (C) immunohistochemistry analysis of ZFX. Nude mice bearing DU145 tumor were i.p. injected with 0.1% DMSO and baicalein (30 mg/kg, once/day) for 21 days, respectively. Then tumors were taken for immunohistochemistry assay (magnification, upper, 100×; lower, 200×). ZFX, zinc finger protein X-linked; DMSO, dimethyl sulfoxide.

Baicalein inhibited the expression of ZFX protein *in vitro* and *in vivo*

To assess whether baicalein downregulates ZFX protein expression in PCa, the levels of ZFX were detected after baicalein treatment using western blot analysis and immunohistochemistry assay. As expected, baicalein treatment induced a decrease in ZFX protein levels *in vitro*

and *in vivo*. *In vitro*, western blot analysis (Figure 3A) showed that ZFX protein levels decreased in a dose-dependent manner with the baicalein administration in PC-3 and DU145 cells. To further study the downregulation effects *in vivo*, the nude mouse transplantation tumor experiment and immunohistochemistry assay were used. Nude mice bearing human prostate adenocarcinoma (DU145) tumors were administered baicalein or DMSO vehicle control by

intraperitoneal injection for 3 weeks. Then we conducted western blot and immunohistochemistry assay to examine the expression of ZFX protein in tumors from nude mice mentioned above. As shown in *Figure 3B,C*, the levels of ZFX decreased significantly in the tumors following treatment with baicalein compared with DMSO.

Discussion

In our study, we show that baicalein has a significantly inhibitory effect both *in vitro* and *in vivo* on progression of human PCa cells. These findings are similar to prior study (13), except that they conducted their *in vivo* study on SCID mice by oral administration of baicalein. In our study, we administrated baicalein by i.p. injection because its reabsorption is not easy due to its poor permeability from the gastrointestinal tract (21). Further, we show that baicalein suppress the expression of ZFX, knockdown of which inhibited PCa cell proliferation and induced apoptosis in our previous study (19).

Patients have limited methods of treatment when progressing into metastatic PCa due to loss of sensitivity to androgen withdrawal. Formerly, PC-SPES had been one kind of prevalent herbal supplements chosen by those patients (22,23). As a mixture of 8 herbs, including baicalein as a main ingredient (24), PC-SPES quickly lowered PSA levels in patients with PCa and also improved quality of life and reduced pain (25). However, because FDA-regulated compounds such as the synthetic nonsteroidal estrogen diethylstilbestrol (DES) were included in PC-SPES (26), it was taken off the market in 2002.

Baicalein was seen as a major component of PC-SPES in inhibiting cancer growth (24). It is an important member of flavonoids family, which have been reported to have anti-allergic, anti-inflammatory and anti-microbial effects (27,28), and have been widely used in China for a long time. In addition, many studies have suggested that flavonoids possess anti-tumor ability against various cancer (29-31), the underlying mechanisms include suppression of cell proliferation, induction of apoptosis and cycle arrest (32). In addition, Gu *et al.* revealed that baicalein had a potential feature of targeting cancer stem cells of multiple myeloma (33), and Liu *et al.* showed that baicalein downregulated ZFX expression in gallbladder cancer cells (14).

In non-malignant embryonic stem cell, the transcriptional role of ZFX in the self-renewal of both embryonic and hematopoietic stem cells was described (15,34). Using a

new software program, Ouyang *et al.* identified ZFX as one of 12 transcription factors upregulated in embryonic stem cells (35). And in malignant cells, previous studies have indicated that ZFX can function as an oncoprotein in various types of cancer like gastric cancer (36), gallbladder cancer (37), hepatocellular cancer (17) and PCa (19). Therefore, suppression of ZFX would be an operative therapeutic approach to cancer treatment, including PCa.

Conclusions

We demonstrated that baicalein could significantly inhibit the progression of PCa, and downregulate the expression of ZFX *in vitro* and *in vivo*. Due to the important role of ZFX in PCa progression, we have a reasonable speculation that the inhibition is probably associated with the downregulation of ZFX, and we expected that baicalein could play significant role in PCa treatment in the future.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2017.09.11>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived. The study was approved by the Huashan Hospital Animal Ethics Committee (No. KY2016-112).

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