Novel PI3K inhibitor 5d inhibits prostate cancer cell growth in vitro and in vivo

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Abstract

**Purpose** To investigate the therapeutic effect and possible mechanism of PI3K inhibitor 5d on prostate cancer.

**Methods** The MTT cell proliferation assay was used to assess the PC-3 prostate cancer cell viability. The effect of compound 5d on cell cycle and apoptosis was detected by flow cytometry, and the expression of proteins was estimated by Western blotting. The xenograft models of DU145 and PC-3 cell lines were established to further explore the inhibitory activity of 5d on prostate cancer in vivo.

**Results** The results of MTT experiment showed that 5d could significantly inhibit the proliferation of PC-3 cells in vitro. The flow cytometry results showed that 5d could arrest PC-3 cell cycle in G0 / G1 phase and promote apoptosis. Western blot analysis showed that 5d could effectively inhibit the activation of PI3K signaling pathway. The results of in vivo experiments showed that the tumor weights in the 5d treatment group was significantly reduced compared with the control group, and western blot results show that 5d can effectively inhibit the expression of PI3K signaling pathway in both tumor tissues.

**Conclusion** PI3K inhibitor 5d can effectively inhibit the proliferation of prostate cancer in vitro and in vivo.

1. Introduction

Prostate cancer (PCa) represents the second most common cancer after lung cancer and accounts for 7% of newly diagnosed cancers in men globally (15% in developed regions) in men\(^1\),\(^2\). In the USA, there were an estimated 268,490 new cases of prostate cancer and 34,500 deaths in 2022\(^3\). Generally, for localized non-metastatic disease, suggested treatments include active surveillance and local ablation through surgical or radiotherapeutic intervention with or without antihormonal treatment\(^4\). However, once the disease metastasizes to bone and lymph nodes, there are no curative treatments. Suppression of androgen receptor (AR) signaling through Androgen deprivation therapy (ADT) is the mainstay of treatment for metastatic PCa. But the disease becomes resistant with the emergence of metastatic castration-resistant prostate cancer (mCRPC)\(^5\). Thus, new therapeutic agents are urgently required.

Molecular targeted therapy drugs can act on specific signaling pathways in the process of tumor occurrence and development. By inhibiting the expression of certain specific molecules to block the signal pathway of tumors, and then achieve anti-tumor effect, it has become one of the most effective methods to treat tumors. The PI3K/AKT/mTOR signaling pathway is one of the most important signaling pathways and plays an important role in regulating cell growth, proliferation, migration, apoptosis\(^6\). This signaling pathway is frequently activated in many cancers, including prostate cancer\(^7\). Compound 5d is a novel PI3K inhibitor synthesized by the Institute of Materia Medica, Chinese Academy of Medical Sciences\(^8\). In this study, we investigated whether 5d could inhibit prostate cancer proliferation in vivo and in vitro and focus on its potential mechanisms.
2. Materials And Methods

2.1 Drugs

Compound 5d was synthesized by the Institute of Materia Medica, Chinese Academy of Medical Sciences. PF-04691502 (hereinafter referred to as PF#), a small molecule Pan-PI3K inhibitor, was used as the positive control drug[9].

2.2 Cells and reagents

Human prostate cancer cell lines (DU145 and PC-3) were purchased from Cell Resource Center at the Institute of Medical Science, Peking Union Medical College. Both cell lines were maintained in complete RPMI 1640 medium with 10% FBS. 100 units/mL penicillin and 100 mg/mL streptomycin were also added in all the medium above. Anti-β-Actin, anti-P-AKT(S473), anti-Pan-AKT, anti-P-S6RP and anti-S6RP antibodies were purchased from Cell Signaling Biotechnology. FxCycle PI/RNase cell cycle Staining Solution was purchased from Thermo Fisher Scientific Inc. Annexin V-FITC/PI apoptosis detection kit was purchased from Beijing Kang Run Cheng Ye Biotech Co. Ltd.

2.3 Animals

Six- to eight-week-old (18–20 g) BALB/c nude male mice were obtained from the Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). All mice in this experiment were housed in a specific pathogen-free environment with free access to food and water. All the experiments involving animals were in accordance with the guidelines of the Committee on Animals of the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College.

2.4 Cell viability assay

The cell viability was detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenytetrazolium bromide (MTT) assay. The cell lines PC-3 was evaluated during this experiment. Briefly, 2×10^3/well/100 µL cells were seeded in 96-well plates and cultured in cell incubator (37 °C, 5% CO2) overnight. Then, 100 µL cell culture medium containing relevant concentrations of tested compounds or DMSO controls were added to the corresponding wells. The cells were placed in cell incubator for another 72h. After that, 20 µL MTT solution (5 mg/mL, dissolved in PBS) were added and continuously cultured in cell incubator for another 4h. The supernatants were carefully discarded, and 180 µL DMSO was added to each well to dissolve the formazan. The plates were shaken and the optical density value (OD) was determined at the wavelength of 570 nm by a microplate reader. The cells treated with 0.1% DMSO were used as control. The inhibitory rate of the compounds was calculated as: Inhibition rate (%) = (1-OD_A/OD_B) × 100%. (OD_A: Average OD value of compound treatment cells; OD_B: Average OD value of control). The value of half maximal inhibitory compound concentration (IC_{50}) was calculated by SPSS.

2.5 Cell cycle and apoptosis assay
Flow cytometry were used for detection of cell cycle distribution and apoptosis. Briefly, PC-3 cells were firstly seeded in 6-well plates and cultured overnight. The cells were incubated with PF# and 5d for 24 h, and then harvested for further detection. As for cell cycle arrest assay, the cells were firstly fixed in cold 70% ethanol at -20 °C overnight, and then stained with FxCycle propidium iodide (PI)/ RNase staining solution for 30 min. As for apoptosis assay, cells were firstly stained with Annexin V-FITC for 30 min, and then stained with PI. Both cell cycle distribution and apoptosis assay were measured by BD FACS verse flow cytometer and analyzed by Flowjo V10.

2.6 Western blot assay of PC-3 cells

The cells were firstly washed with cold PBS twice, then lysed with RIPA lysate buffer containing 1% protease inhibitor cocktail (Roche). Relevant protein samples (10 µg) were separated on 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (0.22/0.45 µM). The membranes were then blocked with TBST (5% skimmed milk added) for 1 h at room temperature, and incubated overnight with relevant primary monoclonal antibodies (1:1000 dilution) at 4°C. After incubated with secondary antibodies (1:2000 dilution) for 1 h at 37°C, the bands were detected by enhanced chemiluminescence (Tanon, Shang Hai)

2.6 DU145 and PC-3 tumor xenograft model

DU145 and PC-3 tumor tissues were separately cut and inoculated subcutaneously in the right flank of BALB/c nude mice. The mice were divided once the tumors reached to 100-300mm³, and administrated with control, PF# (10mg/kg), 5d (2.5mg/kg), 5d (5 mg/kg) and 5d (10 mg/kg) once daily for 15 days. In addition, tumor volume (TV) was measured twice a week. After drug treatment for 15 days, the mice were sacrificed and tumor weight (TW) was measured. The tumor tissues were collected for western blot assay. TV was calculated by the formula: TV = 1/2×L×W² (L: the maximum length of the tumor tissue; W: the maximum width of the tumor tissue). The tumor growth inhibition rate (TGI%) was calculated by the formula: TGI% = (1-TW_treatment/TW_vehicle) ×100%.

2.7 Western blot assay of tumor tissues

3 samples in the control group and 3 samples in the 5d (10 mg/kg qd) group were randomly selected for western blot detection. The rest of the steps are the same as 2.6.

2.8 Statistical analysis

All the statistics were performed using GraphPad Prism software 9.0. Differences of mean values between two groups were analyzed using Student’s t-test. A value of P < 0.05 was considered as statistically significance. (*p < 0.05, **p < 0.01, ***p < 0.001)

3. Results

3.1 5d inhibits the growth of PC-3 in vitro
As the MTT assay results showed, 5d can effectively inhibit cell proliferation when they act on PC-3 cells for 72h (Fig. 1). The half-inhibitory concentration (IC$_{50}$) of 5d was $5.119 \times 10^{-9}$M, while the IC50 of PF# was $1.439 \times 10^{-7}$M, which demonstrated 5d was more effective in inhibiting cell proliferation than PF#.

### 3.2 5d arrests cell cycle and induces apoptosis of PC-3 in vitro

Then we evaluated 5d’s ability to arrest cell cycle and induce apoptosis in PC-3 cells (Figs. 2 and 3). After treatment with 5d, the cell cycle was dose-dependently arrested at the G0/G1 phase. The percentage of apoptotic cells increased dose-dependently with the treatment of 5d.

### 3.3 5d inactivated the PI3K/Akt pathway in PC-3 cells

After treatment with 5d for 4 h, the phosphorylation of AKT (S473), S6RP was inhibited by 5d in a concentration-dependent manner (Fig. 4). As the result showed, the inhibition of phosphorylation by 5d at 50 nM is comparable to that of PF at 500 nM.

### 3.4 5d potently inhibits DU145 and PC-3 growth in vivo

Inhibitory effects of 5d were further evaluated by examining the growth of DU145 and PC-3 tumor xenografts in the nude mice (Fig. 5). After p.o. administration for 15 consecutive days, 5d showed great anti-tumor efficacy with a TGI value of 69.8% at 10 mg/kg in DU145 and a TGI value of 59.2% at 10mg/kg in PC-3.

### 3.4 5d inactivated the PI3K/Akt pathway in DU145 and PC-3 tumor tissues

We detected the phosphorylation of AKT (S473) and S6RP in both DU145 and PC-3 tumor tissues (Fig. 6). The results demonstrated that 5d can effectively inhibit the phosphorylation of proteins in the PI3K signaling pathway in both cell lines, thereby inhibiting the activation of this signaling pathway.

### 4. Discussion

Prostate cancer is one of the leading causes of death in male cancer patients. Advanced prostate cancer poses a greater threat to human survival\[5\]. The androgen receptor (AR) signalling pathway is critical for the growth and survival of prostate cancer cells, demonstrating that AR is a valid therapeutic target for treating prostate cancer. Accordingly, ADT remains the standard treatment for patients with metastatic disease. Current ADT includes surgical castration or medical castration using luteinizing hormone-releasing hormone (LHRH) agonists or antagonists with or without anti-androgen drugs. Besides, the next-generation AR-targeting inhibitors, such as abiraterone, have been the standard of care for patients
with CRPC\cite{10,11}. However, resistance to AR-targeted therapy remains a challenge in the treatment of prostate cancer.

The PI3K signaling pathway is one of the known factors that play a key role in the process of cell proliferation and apoptosis. Phosphoinositide 3-kinase (PI3K) can be classified into three types, which regulate different life processes respectively. The class I PI3K is composed of p85 and p110 catalytic subunits and can be classified into IA and IB. IA includes p110α, p110β and p110δ; IB is p110γ, also known as PI3Ky\cite{6}. After being activated, the class I PI3K can convert phosphatidylinositol 2 phosphate (PIP2) to phosphatidylinositol 3 phosphate (PIP3). PIP3 activates AKT/mTOR signaling pathways by performing the function of the second messenger, thereby regulating various cellular activities. AKT is a serine/threonine kinase that converts signals from various cytokines and growth factors into intracellular messages by producing phospholipids that stimulate AKT as well as downstream effector pathways. As a major PI3K effector, AKT can trigger the rapamycin-sensitive mTOR complex 1 (mTORC1) signaling pathway, while the rapamycin-insensitive mTORC2 can phosphorylate AKT on key targets\cite{12,13}. PTEN is a bispecific protein tyrosine phosphatase, and its mutation has been detected in multiple tumors\cite{14}. Previous studies have shown that PTEN negatively regulates the PI3K-AKT-mTOR cascade in prostate cancer, affecting multiple biological processes such as cancer cell apoptosis, metabolism, cell proliferation and growth\cite{15}. In the PI3K pathway, both the activation of PI3K and the loss of PTEN have the same effect on the induction of PIP3. According to sequencing analysis, approximately 15% of primary prostate cancer and 50% of CRPC harbour alterations in this pathway\cite{16}. In addition, abnormal activation of the PI3K pathway is associated with metastasis and resistance to therapy in prostate cancer patients\cite{17,18}. Thus, PI3K signaling pathway inhibitors may be useful for the treatment of prostate cancer.

Compound 5d is a novel PI3K inhibitor synthesized by the research group of Xu Heng of the Institute of Materia Medica, Chinese Academy of Medical Sciences, and has strong inhibitory activity on each subtype of PI3K. IC50 of compound 5d for class I PI3Ks (p110α, p110β, p110γ and p110δ) were 0.87, 0.70, 1.60 and 1.10 nM, respectively\cite{8}. The purpose of this experiment was to use the human prostate cancer cell lines DU145 and PC-3 to investigate the inhibitory activity of 5d in vitro and in vivo. PF-04691502, an oral small molecule ATP-competitive inhibitor of PI3K, has strong inhibitory effects on recombinant class I PI3K kinase activity, Akt phosphorylation and proliferation\cite{19}, and were used as positive control.

Firstly, the inhibitory activity of compound 5d on PC-3 cell in vitro was detected by MTT assay. The results showed that the IC50 of compound 5d was 5.119×10^{-9}M, and the IC50 of PF# was 1.439×10^{-7}M. Compound 5d was nearly 100 times lower than PF#, showing stronger inhibitory activity in vitro. Then, the effects of compound 5d on PC-3 cell cycle arrest and apoptosis were further detected by flow cytometry. The results showed that both 5d could dose-dependently arrest PC-3 cells in G0/G1 phase for 24 h, thus effectively inhibiting cell proliferation, which is more consistent with the results of MTT. Annexin V-FITC/PI double-stained cell apoptosis detection results showed that both PF# and 5d could
effectively increase the proportion of apoptotic cells. These results show that compound 5d can significantly inhibit the in vitro proliferation activity of PC-3 cells, block the cell cycle in G0/G1 phase and promote cell apoptosis. Then the inhibitory activity of 5d on the activation of PI3K/AKT signaling pathway was further investigated. The results showed that compared with the control, compound 5d can dose-dependently down-regulate the expressions of P-AKT(S473) and P-S6RP; thereby effectively inhibiting the activation of PI3K signaling pathway in PC-3 cells.

Based on the results of in vitro experiments, DU145 and PC-3 tumor xenografts were established to further explore the inhibitory effect of 5d on the proliferation of prostate cells in vivo. The results showed that after continuous administration for 15 days, compound 5d could effectively inhibit the proliferation of tumors in vivo. The tumor inhibition rates of the administration group 5d (10mg/kg) in DU145 and PC-3 reached 69.8% and 59.2%, respectively. The next step was to study the inhibitory effect of 5d on the activation of PI3K/AKT signaling pathway of tumor tissues. The results showed that compared with the control group, 5d could significantly down-regulate the expressions of P-AKT (S473) and P-S6RP; thereby inhibiting the activation of PI3K signaling pathway in both DU145 and PC-3 tumors.

5. Conclusions

In summary, we conclude that PI3K inhibitor 5d can effectively inhibit the proliferation of prostate cancer PC-3 cells in vitro and induce PC-3 cells to appear G0/G1 phase arrest, promote its apoptosis by inhibiting the PI3K signaling pathway. In vivo experiments, 5d can inhibit the proliferation of both DU145 and PC3 tumor tissues, and inhibit the activation of PI3K signaling pathway in both tumors. Our results provide evidence that 5d may be a promising medicine for prostate tumor treatment.

References


**Figures**
Figure 1

Effects of PF# and 5d on the viability of PC-3 cells lines. Cell growth was measured by MTT assay.

Figure 2

Compound 5d induced cell cycle arrest in PC-3 cells. (A) Flow cytometry analysis of cell cycle arrest by PI staining after treatment of PC-3 with 5d and PF# at relevant concentrations for 24 h. (B) Statistic analysis of the percentage of pC-3 cells in each phase after treatment with PF# and 5d for 24 h.
Figure 3

Compound 5d induced apoptosis in PC-3 cells. (A) Flow cytometry analysis of apoptosis via Annexin V-FITC/PI staining after treatment of PC-3 cells with PF# and 5d at relevant concentrations for 24 h. (B) Statistic analysis of apoptotic PC-3 cells after treatment with PF# and 5d.

Figure 4

Compound 5d inhibited the PI3K/AKT/mTOR pathway in PC-3 cells. PC-3 cells were treated with PF# (500 nM) and different concentrations of 5d for 4 h. The expression levels of AKT, S6RP and their phosphorylated forms were analyzed by western blotting. β-Actin was used as a loading control. *p < 0.05, **p < 0.01, and ***p < 0.001 vs control group.
Figure 5

Anti-tumor efficacy of 5d and PF# in the DU145 (A, B) and PC-3 (C, D) prostate cancer xenograft models. Oral administration of 5d caused a significant reduction in both tumor weight (A,C) and tumor volume (B,D) in the tumor-bearing mice. *p < 0.05, **p < 0.01, and ***p < 0.001 vs control group.

Figure 6

Compound 5d inhibited the PI3K/AKT/mTOR pathway in DU145 (A) and PC-3 (B) tumor tissues. The expression levels of AKT, S6RP and their phosphorylated forms were analyzed by western blotting. β-Actin was used as a loading control. *p < 0.05, **p < 0.01, and ***p < 0.001 vs control group.