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Novel coumarone-derived (S,E)-4-(4-fluorobenzylidene)-3-phenylchroman-3-ol inhibits muscle-invasive bladder cancer cells by repressing the S and G2 cell cycle phases

Xin-Yi Han[1#], A-Dan Li[1#], Fa-Ying Zhou[1], Chao Li[1], Guo-Du Liu[2*], Yong Xia[1, 3*]

[1]College of Basic Medicine, Jining Medical University, Jining 272067, China. [2]College of Chemistry and Chemical Engineering, Inner Mongolia University (South Campus), Hohhot 010030, China. [3]Institute of Precision Medicine, Jining Medical University, Jining 272067, China.

[#]Xin-Yi Han and A-Dan Li are the co-first authors of this paper.

[*]**Corresponding to:** Guo-Du Liu, Inner Mongolia Key Laboratory of Fine Organic Synthesis, College of Chemistry and Chemical Engineering, Inner Mongolia University (South Campus), 24 Zhaojun Road, Yuquan District, Hohhot 010030, China. E-mail: guoduliu@imu.edu.cn. Yong Xia, Institute of Precision Medicine, Jining Medical University, No.133 Hehua Road, Taibaihu District, Jining 272067, China. E-mail: xiayong@mail.jnmc.edu.cn.

Author contributions

Yong Xia, and Guodu Liu contributed to the conception and designed the work; Xinyi Han, Adan Li, Faying Zhou and Chao Li performed the experiments; Xinyi Han, Adan Li and Yong Xia analyzed and interpreted the data for the work; Chao Li and Guodu Liu did the investigation work; Xinyi Han and Adan Li drafted the manuscript; Yong Xia revised the manuscript critically; and all the authors approved the final version to be published.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

MIBC, muscle-invasive bladder cancer; FPO, (S,E)-4-(4-fluorobenzylidene)-3-phenylchroman-3-ol; EdU, 5-ethynyl-2’-deoxyuridine; NMIBC, non-muscle-invasive bladder cancer; CCK-8, cell counting kit-8; MPO, (S,E)-4-(4-methylbenzylidene)-3-phenylchroman-3-ol; IPO, (S,E)-4-(4-isocyanobenzylidene)-3-phenylchroman-3-ol; PTCO, (S,E)-3-phenyl-4-(4-(trifluoromethoxy)benzylidene)chroman-3-ol; RPMI, Roswell Park Memorial Institute; PBS, phosphate-buffered saline; 5-FU, 5-fluorouracil.

Citation

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Abstract

Background: This study aimed to select compounds with unique inhibitory effects on muscle-invasive bladder cancer (MIBC) from coumarone derivatives with similar parent nuclear structures and to reveal their tumor-suppressive effects using various approaches. **Methods:** Bladder cancer cell lines SW780 and T24, as well as human normal bladder epithelial cell line SV-HUC-1 were selected as the study model, and these urinary system cells were co-incubated with various concentrations of (S,E)-4-(4-methylbenzylidene)-3-phenylchroman-3-ol, (S,E)-4-(4-isocyanobenzylidene)-3-phenylchroman-3-ol, (S,E)-4-(4-fluorobenzylidene)-3-phenylchroman-3-ol (FPO), and (S,E)-3-phenyl-4-(4-(trifluoromethoxy)benzylidene)chroman-3-ol. Cell activity was detected using cell counting kit-8. FPO showed the strongest inhibitory effect on MIBC cells; therefore, it was selected for further experiments. We monitored the FPO-induced T24 cell morphological changes with an inverted microscope. The FPO-inhibited migration of T24 cells was examined using a cell scratch assay. We detected the clonogenic ability of T24 cells through a clone formation test and evaluated their proliferative ability using a 5-ethynyl-2’ -deoxyuridine fluorescence staining kit. The inhibitory effect of FPO against the cell cycle was monitored using flow cytometry, and its suppressive effect on the DNA replication ability of T24 cells was detected using double fluorescence staining (Ki67 and phalloidin). **Results:** Among the four candidate coumarone derivatives, FPO showed the most significant inhibitory effect on MIBC cells and was less toxic to normal urothelial cells. FPO inhibited T24 cell growth in time- and dose-dependent manners (the half-inhibitory concentration is 8 μM). FPO significantly repressed the proliferation, migration, and clonogenic ability of bladder cancer T24 cells. Cell mobility was significantly inhibited by FPO: 30 μM FPO almost completely repressed migration occurred at after 24 h treatment. Moreover, FPO significantly suppressed the clonogenicity of bladder cancer cells in a dose-dependent manner. Mechanistically, FPO targeted the cell cycle, arresting the S and G2 phases on bladder cancer T24 cells. **Conclusion:** We discovered a novel anticancer chemical, FPO, and proposed a potential mechanism, through which it suppresses MIBC T24 cells by repressing the cell cycle in the S and G2 phases. This study contributes to the development of novel anticancer drugs for MIBC.

Keywords: muscle-invasive bladder cancer; cell proliferation; cell cycle; DNA replication; coumarone derivate

Introduction

Bladder cancer ranks the top 10 most common cancers worldwide, and its incidence ranks first among genitourinary malignancies. Statistics show approximately 550,000 new medical records for bladder cancer in 2020, showing an increasing trend each year [1]. Bladder cancer is a malignant tumor that occurs in the bladder mucosa, divided into two main subtypes: non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). The MIBC mortality rate is significantly higher than that of NMIBC [2]. MIBC often develops distant metastases, such as liver and lung metastases, leading to organ failure, and is the major reason of death in patients with bladder cancer [3]. Currently, treatment strategies for bladder cancer include surgical resection, chemotherapy, radiation therapy, minimally invasive treatment techniques, and bio-immunotherapy [4]. However, many patients still have a high rate of postoperative recurrence, and chemotherapy drugs after preoperative surgery cannot completely control bladder cancer [5]. Therefore, it is of great significance for exploring efficient and novel drugs. In the current study, our aim is to discover new drugs candidate for MIBC.

Recently, small molecule compounds have been widely used in anticancer therapy. The good druggability and pharmacokinetic properties of small molecule drugs make them highly advantageous for drug research and development [6]. Small molecule drugs have also become a research hotspot in bladder cancer treatment, and various drugs, such as cisplatin and gemcitabine, have been developed [7]. So we focused on explore novel small compounds which would contribute to developing new anticancer therapeutic medication.

Some researchers have found that compounds with coumarone as their parent structure are cytotoxic to cancer cells [8]. However, coumarone derivatives are not commonly employed as anticancer medication. In our research, we explored the anti-tumor activities of four novel coumarone derivatives with the same parent nuclear structure. We used the cell counting kit-8 (CCK-8) kit to compare the anti-tumor activities of four compounds on bladder cancer cells and found that (S,E)-4-(4-fluorobenzylidene)-3-phenylchroman-3-ol (FPO) significantly inhibited the activity of T24 cells. We focused on FPO and T24 cells using clonogenic, cell scratch, flow cytometry, 5-ethynyl-2' -deoxyuridine (EdU) staining, and Ki67 cell immunofluorescence assays, which revealed that FPO could inhibit the proliferation, migration, and clonogenicity of T24 cells. We found that FPO mainly suppressed MIBC by repressing the cell cycle in the S and G2 phases.

Since FPO showed the strongest anticancer activity of MIBC among the four candidates, we attempted to analyze the structure-activity relationship of these compounds. FPO was different from the other three compounds. The major difference was the fluorine atom which directly connected to the benzene ring of FPO. Fluorine atoms have different chemical properties from other halogens, such as the strongest electronegativity, a unique electronic structure, and smaller atomic radii. These chemical characteristics provide fluorous drugs with many advantages, such as higher biological activity, stability, and lower susceptibility to drug resistance [9, 10]. Therefore, introducing fluorine atoms into organic compounds has become an important approach to develop new antineoplastic drugs. The FDA approved 69 antineoplastic drugs between 2015 and 2020, and fluorous drugs accounted for 36.2%; however, few of these drugs targeted bladder cancer treatment [11].

FPO effectively inhibited the growth of MIBC cells with low toxicity. FPO could be a new anticancer drug candidate for MIBC treatment, because it represses the cell cycle in the S and G2 phases. These findings provide an opportunity to develop new drugs for the MIBC therapy.

Materials and methods

Reagents

The following anticancer candidates used in this study were provided by College of Chemistry and Chemical Engineering, Inner Mongolia University: (S,E)-4-(4-methylbenzylidene)-3-phenylchroman-3-ol (MPO), (S,E)-4-(4-isocyanobenzylidene)-3-phenylchroman-3-ol (IPO), FPO, and (S,E)-3-phenyl-4-(4-(trifluoromethoxy)benzylidene) chroman-3-ol (PTCO).

Cell culture

Human bladder cancer SW780 and T24 cells were obtained from Procell Life Science & Technology (Wuhan, China). Human normal urothelial cells SV-HUC-1 were purchased from BeNa Culture Collection (Beijing, China). Roswell Park Memorial Institute (RPMI)-1640 medium (Biological Industries, Kibbutz Beit Haemek, Israel) was employed to culture T24 cells; and DMEM medium (Biological Industries, Kibbutz Beit Haemek, Israel) was used to culture SW780 and SV-HUC-1 cells. Both RPMI-1640 and DMEM was supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin/streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel). The three kinds of cells suspensions were placed in a CO_2-incubator at 37 ˚C. The status and density of the cells were observed daily using a microscope (Nikon, Tokyo, Japan).

Cell viability assay

Human normal urothelial SV-HUC-1 cells and bladder cancer SW780 and T24 cells were cultured in cell culture plates (96-well) and cultured for 24 h. Various concentrations of MPO, IPO, FPO, and PTCO were co-incubated with the above cells for 24 h (three replicate wells for each concentration). After discarding the culture medium, CCK-8 proliferation assay kit (C0037, Beyotime, Shanghai, China) was employed, and the absorbance at 450 nm was examined and recorded with a microplate reader (Cytation5, Biotek, Washington, USA). Based on the absorbance, the cell viabilities and half-inhibitory concentration (IC_{50}) values were calculated.

Cell morphology observation

T24 cells were seeded at the initial cell confluence of 25% in 6-cm cell culture plates. After cells attaching to the plate bottom, they were co-incubated with a series of concentrations of FPO. During incubation, the cell morphology was preserved by taking pictures with a microscope.

Cell migration experiments

Cell migration inserts (80469, IBIDI, Grafelfing, Germany) were inserted into 12-well plates, and T24 cells were added into blank space of the inserts. After cell adhesion was complete, the inserts were removed. After plates were washed by phosphate-buffered saline (PBS), the scratches were photographed by microscope. The cells were treated with different FPO for 24 h, 48 h, and 72 h. During FPO-treatment period, the scratches were photographed and stored under a microscope every 24 h. Quantitative analysis of cell scratches was performed using ImageJ software (V1.8.0.112).

Cell clone formation assay

T24 cells were seeded at approximately 1000 cells/well in a 12-well plate and cultured for 24 h to form small clonal colonies after adding different concentrations of FPO. The cell culture medium-RPMI and FPO was renewed every 3 days. When large monoclonal cell clusters appeared in the control group, the culture was stopped. And the colonies were fixed with 4% paraformaldehyde for 20 min, and stained with crystal violet staining kit and photographed in PBS for storage. The crystal violet kit was purchased from KeyGEN Biotech Co., Ltd. (Jiangsu, China).

Cell cycle detection

Different concentrations (0–50 μM) of FPO was co-incubated with T24 cells for 48 h. After treated with EDTA-free trypsin, the T24 cells were collected in 1.5 mL EP tubes. Then 70% cold ethanol in PBS was used to fix the harvested cells. The fixed cells were washed with PBS, and

incubated in PI/RNase staining solution (Beyotime, Shanghai, China) for 20 min in the dark. The cell cycle in each phase was detected using a flow cytometry (ST579, Beyotime, Shanghai, China).

EdU cell proliferation assay

The effect of FPO on DNA replication activity on T24 cells was examined with cell proliferation detection kit (C0078L; Beyotime, Shanghai, China). After the T24 cells were co-incubated with different FPO for 48 h, EdU was added into cell culture wells, and incubated in a CO_2-incubator for 30 min. After incubation, the cells were fixed with paraformaldehyde, and permeabilized with 0.3% Triton X-100. The click reaction of EdU kit was performed according to manufacturer's instruction. H33342 staining solution (1:1000, C1022, Beyotime, Shanghai, China) was used for staining after washing and photographed under a fluorescence microscope for storage.

Ki67 immunofluorescence staining

Ki67 was used to test the anti-proliferation effect of FPO on T24 cells. Different concentrations of FPO treated T24 cells were washed with PBS and fixed with paraformaldehyde, followed by permeabilization with 0.3% Triton X-100. Blocking using blocking solution for 30 min (at room temperature) was followed by addition of primary antibody rabbit-anti-Ki67 (1:300, ab15580, Cambridge, UK), matrix (1% bovine serum albumin (Solarbio, Beijing, China) in PBS) overnight at 4 °C; after washing, secondary antibodies (SA00013-2, 488-conjugated Goat Anti-Rabbit IgG, Proteintech, Wuhan, China) were added. After 2 h incubation, the cells washed 2–3 times with PBS, and phalloidin was added to stain for 30–60 min. DAPI (C1002, Beyotime, Shanghai, China) was employed to counterstain the nuclei. The fluorescence photographs of Ki67 and DAPI double staining were observed and recorded with a fluorescence microscope.

Statistical analysis

Means ± standard deviation was shown in each figure. The significance of differences between two groups was evaluated using a Student's t-test, whereas significance of differences between more than two groups was evaluated using one-way analysis of variance. When the *P* value was less than 0.05, it was considered that there was a statistical difference among the groups. We performed the statistical analysis with Statistical Package for the Social Sciences software (version 17.0; IBM, Armonk, New York, USA).

Results

Inhibitory effects of MPO, IPO, FPO, and PTCO on bladder cancer cells

Figure 1A shows the structural formulas of MPO, IPO, FPO, and PTCO. The four compounds concentration-dependently inhibited the activity of T24, SW480, and SV-HUC-1 cells (Figure 1B–1D). The IC_{50} of FPO was 8 μM, which was significantly lower than those of the other coumarone derivates (Table 1). Among all thecoumarone derivates in this study, FPO had the strongest inhibitory effect on MIBC cell line T24 (Figure 1E). Moreover, among the three cell lines, MIBC cell line T24 showed greatest sensitivity to FPO (Figure 1F). These indicated FPO could be a potential drug candidate for MIBC.

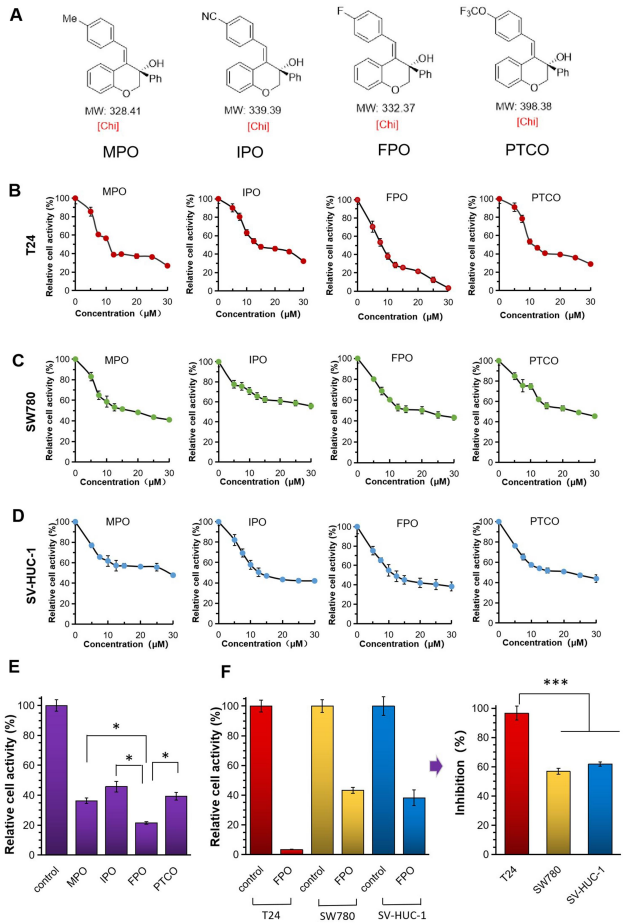

Figure 1 The effects of MPO, IPO, FPO, and PTCO on the three cell lines: T24, SW780, and SV-HUC-1. (A) The structures of the four compounds: MPO, IPO, FPO, and PTCO. The cellular activity of (B) T24, (C) SW780, and (D) SV-HUC-1 cells treated with the four compounds was determined using a CCK-8 kit. (E) The effect of the four compounds with same concentration (20 μM) on T24 cell viability ($^{*}P < 0.05$). (F) The differences in cell viability between the three cell lines treated with 30 μM FPO ($F = 52.463$, $^{***}P < 0.001$). MPO, (S,E)-4-(4-methylbenzylidene)-3-phenylchroman-3-ol; IPO, (S,E)-4-(4-isocyanobenzylidene)-3-phenylchroman-3-ol; FPO, (S,E)-4-(4-fluorobenzylidene)-3-phenylchroman-3-ol; PTCO, (S,E)-3-phenyl-4-(4-(trifluoromethoxy)benzylidene)chroman-3-ol; CCK-8, cell counting kit-8.

Effect of FPO on T24 cell morphology

In the control group, the cells grew well, with a complete structure, tight adherence, high density, and round shape. As FPO concentration increasing, the cell density gradually decreased, the death suspension phenomenon occurred, the ability to adhere to the wall weakened, and the cells were deformed (Figure 2A). As shown in Figure 2A, The yellow arrows indicate abnormal cell elongation, and the red arrows indicate that cells lose their ability to adhere to walls, or even enter cell death.

Effect of FPO on T24 cell migration ability

In the control group, the T24 cells grew well and migrated to the center of the scratch area. With the increase in time, the scratch gradually closed, nearly to complete closure after 72 h. Cells in the treated group also migrated to the center, but with the increase in FPO concentration, the scratches gradually widened, and the closure rate decreased significantly (Figure 2B). Longer treatment time potentiated the FPO inhibition of T24 cell migration (Figure 2C). After 72 h of treatment, as the concentration of FPO increased, the scratch width gradually increased, and the cell migration ability weakened (Figure 2D).

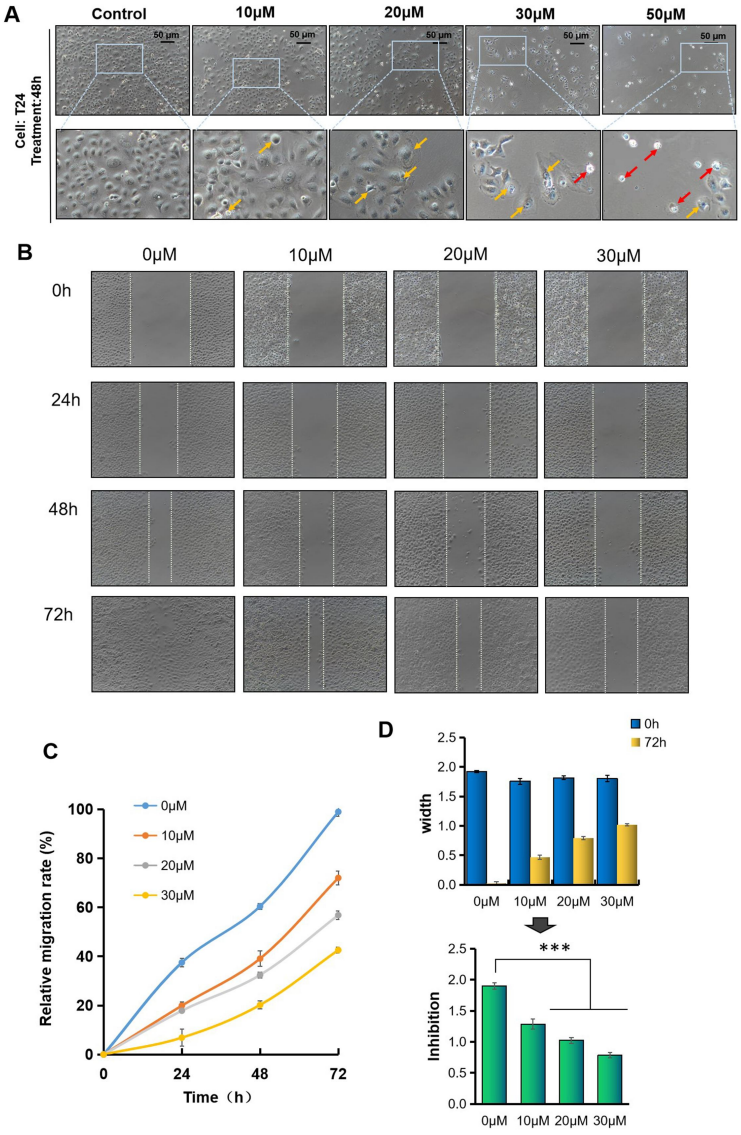

Figure 2 The inhibitory effect of FPO on T24 morphology and migration capacity. (A) Morphological display of T24 cells after treatment with various concentrations of FPO. (B) Cell scratch experiment and the effect of different FPO concentrations on T24 cell migration ability. (C) FPO affects the migration rate of T24 cells in a dose-dependent and time-dependent manner. (D) The differences in scratch widths of T24 cells at 0 h and 72 h with different concentrations of FPO ($F = 200.866$, $^{***}P < 0.001$). FPO, (S,E)-4-(4-fluorobenzylidene)-3-phenylchroman-3-ol.

Table 1 The list of IC_{50}

IC_{50} (μM)	T24	SW780	SV-HUC-1
MPO	23.0	28.0	28.4
IPO	25.0	26.2	12.9
FPO	8.0	16.3	14.0
PTCO	20.0	16.1	21.1

IC_{50}, half-inhibitory concentration; MPO, (S,E)-4-(4-methylbenzylidene)-3-phenylchroman-3-ol; IPO, (S,E)-4-(4-isocyanobenzylidene)-3-phenylchroman-3-ol; FPO, (S,E)-4-(4-fluorobenzylidene)-3-phenylchroman-3-ol; PTCO, (S,E)-3-phenyl-4-(4-(trifluoromethoxy) benzylidene)chroman-3-ol.

Effect of FPO on the clonogenic capacity of T24 cells

T24 cells in the control group formed larger cell clusters, some of which were connected into pieces. With increasing FPO concentration, the cell mass and cell density gradually decreased. When the FPO concentration was 30 μM, the cell colony could not form (Figure 3A). This indicated that FPO dose-dependently suppressed the clonal capability of T24 cells (Figure 3B).

FPO inhibition of the proliferative capacity of T24 cells

EdU kit is a measurement method that reflects cells proliferative capacity through DNA replication rate. The proportion of EdU positive cells in H33342 positive cells is also used to reflect the proliferative capacity of cells. T24 cells in the control group showed strong red and blue fluorescence, a high ratio of red and blue fluorescence, high cell density, strong DNA synthesis, and strong proliferative ability. As the FPO concentration increased, the ratio of EdU/H33342 decreased, and the number of H33342 positive cells also reduced, indicating FPO dose-dependently repressed DNA replication rate (Figure 3C, 3D).

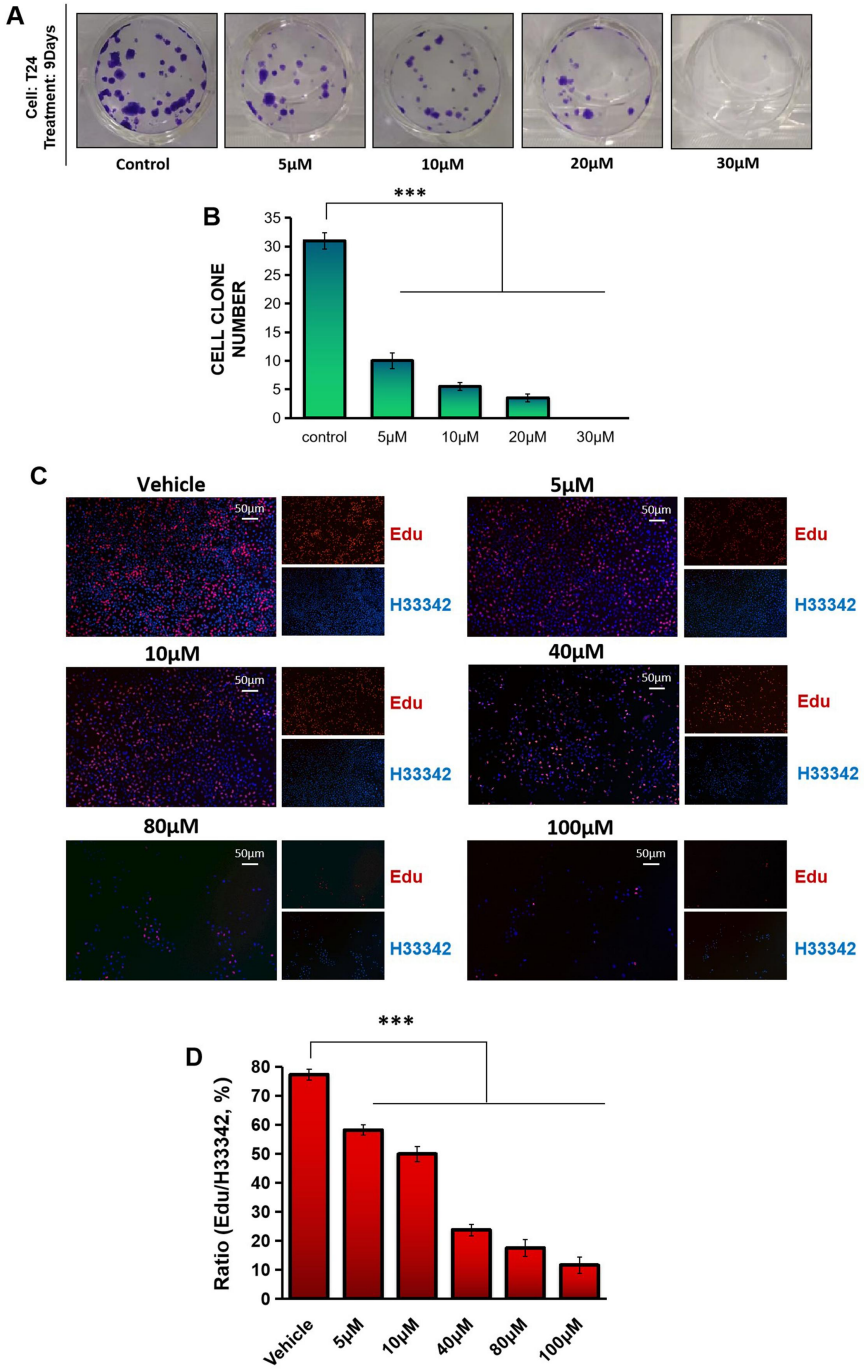

Figure 3 The effect of FPO on the clone-forming and proliferative abilities of T24 cells. (A) T24 cells treated with different doses of FPO for 9 days. Crystal violet-stained colonies were taken photos in bright field with an microscope. (B) The number of T24 cell communities at different concentrations. (C) The proliferation rate of T24 cells was monitored using an EdU cell proliferation test; EdU-positive cells were shown as red fluorescence labels, and H33342-positive cells were shown as blue fluorescence labels. (D) The red-blue fluorescence ratio of T24 cells at different concentrations ($F = 365.258$, $^{***}P < 0.001$). EdU, 5-ethynyl-2’ -deoxyuridine; FPO, (S,E)-4-(4-fluorobenzylidene)-3-phenylchroman-3-ol.

FPO inhibited both the S and G2 phases of the T24 cell cycle

Flow cytometry was employed to test the inhibitory activities of FPO on the cell cycle of T24 bladder cancer cells. As shown in Figure 4A and 4B, after 48 h treatment with FPO, the proportion of S and G2 phase cells gradually decreased with increasing FPO concentration, indicating that FPO inhibited T24 cell proliferation by blocking the S and G2 phases of T24 cells.

Verification of the inhibitory effect of FPO on bladder cancer T24 cells with Ki67 staining

Due to Ki67 was a proliferation marker, we tested Ki67 with immunofluorescence staining. 0–100 μM FPO was used to test the inhibitory effect against cell cycle of bladder cancer cells. As the concentration of FPO increasing, the Ki67 positive ratio in T24 cells reduced gradually, suggesting FPO dose-dependently suppressed cell growth via downregulation of Ki67 (Figure 5).

Discussion

Currently, the treatment strategies for bladder cancer mainly include traditional surgical resection, chemotherapy, radiotherapy, minimally invasive treatment techniques, and biological immunotherapy [4]. Radical cystectomy is the mainstream methods for treating MIBC; however, more than 50% of patients happen distant metastasis within two years, leading to poor prognosis [5]. Therefore, to improve MIBC prognosis and therapeutic strategy is of great significance. Nowadays, some guidelines suggest that patients receive cisplatin-based neoadjuvant chemotherapy before radical cystectomy; however, many patients still exhibit high postoperative recurrence rates, and preoperative and postoperative chemotherapeutic drugs cannot completely control bladder cancer. Therefore, drugs that can effectively inhibit bladder cancer cell proliferation are urgently required.

Malignant cell proliferation is an important feature of tumors, and many drugs inhibit excessive cell proliferation. For example, ERK-IN-6 is an effective anti-proliferative agent for esophageal squamous cell carcinoma: its IC_{50} for KYSE-30, KYSE-150, and HET-1A cells is 16.8, 10.07, and 13 μM, respectively [12]. In this study, we used a CCK-8 kit, clone formation assay, and immunofluorescence staining for Ki67 to confirm that FPO effectively inhibited tumor cell proliferation, with an IC_{50} of 8 μM.

Compared with NMIBC, one of the main threats of MIBC is metastasis. Migration is an important process in distal metastasis, showing the importance of inhibiting migration. AMPK activator 2 is a fluorine-containing proguanil derivatives, which can repress the migration of bladder cancer cell lines (UMUC3 and T24) [13]. The results of the cell migration assay showed that FPO effectively inhibited MIBC cell migration.

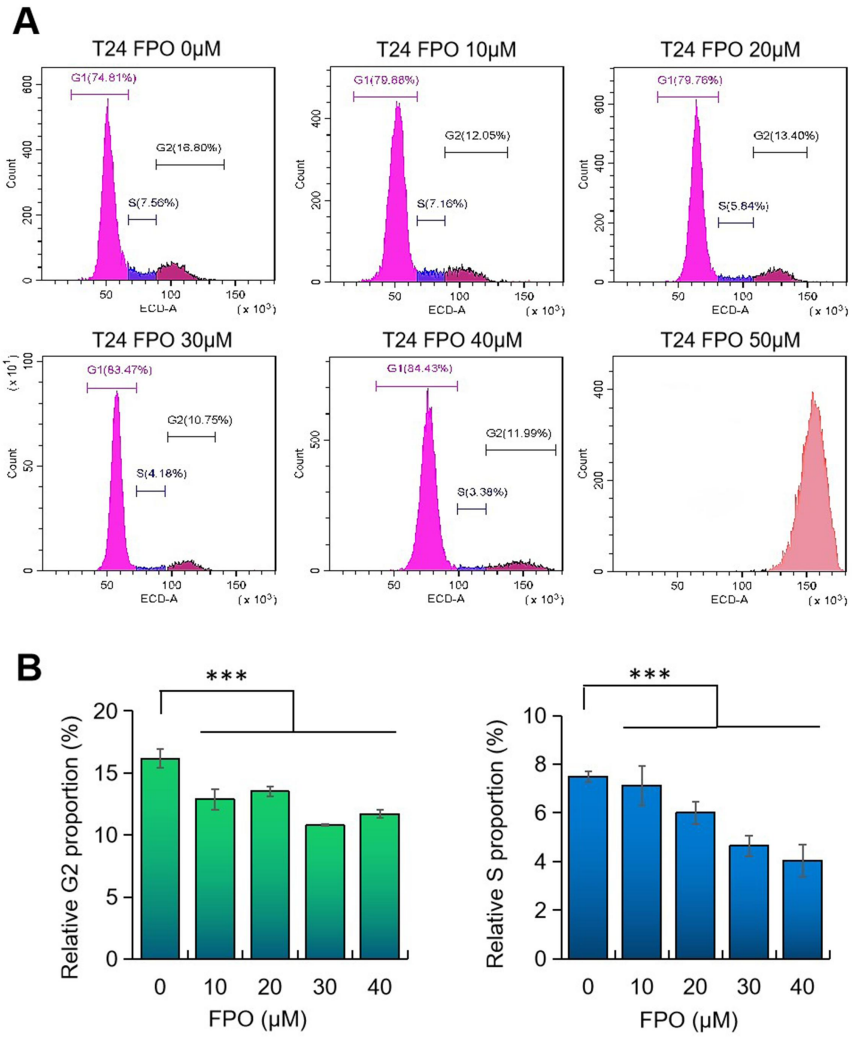

Figure 4 Inhibitory effect of FPO on the cell cycle. (A) The cell cycle data obtained from flow cytometry. The pink field represents G1-phase cells, the blue field represents S-phase cells, and the brown field represents G2-phase cells. (B) The proportion of G2- and S-phase T24 cells at different concentrations (G2-phase: $F = 41.460$, $^{***}P < 0.001$; S-phase: $F = 22.241$, $^{***}P < 0.001$). FPO, (S,E)-4-(4-fluorobenzylidene)-3-phenylchroman-3-ol.

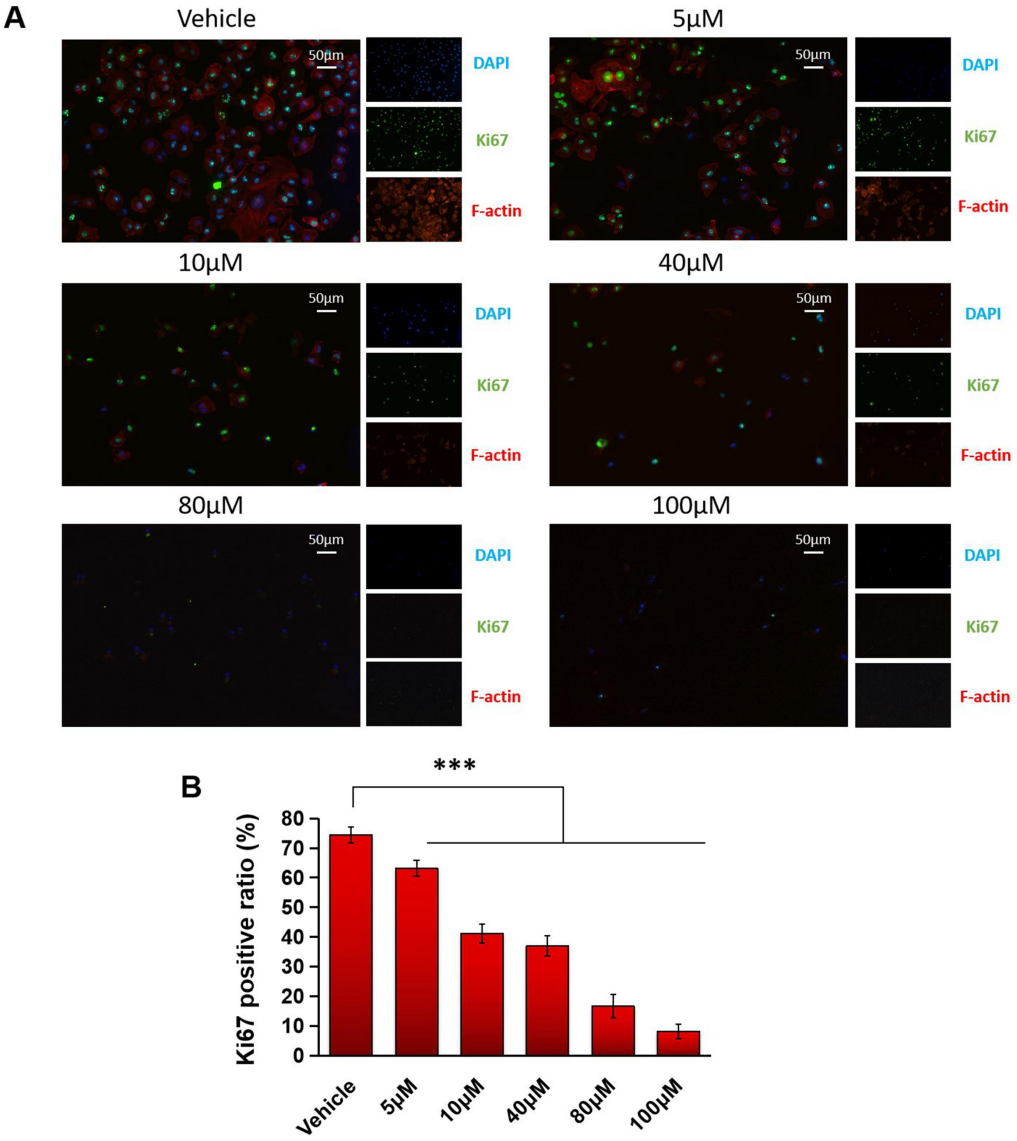

Figure 5 Inhibitory effect of FPO on T24 cell proliferation ability. (A) Detecting T24 cell proliferation using Ki67. (B) The ratio of Ki67-positive cells (green/blue fluorescence-positive cells) at different concentrations of FPO ($F = 210.245$, $^{***}P < 0.001$).

An abnormal cell cycle is characteristic of malignancy, and targeting the cell cycle is an important anticancer strategy. Many drugs exert anticancer effects by targeting the cell cycle. For example, cis,trans-germacrone and DRB18 can arrest cancer cells in the G1/S phase [14, 15]. In our study, EdU staining and flow cytometry confirmed that FPO significantly inhibited the cell cycle, especially in the S phase, indicating that FPO provides a new strategy for bladder cancer treatment.

In the present study, we explored the inhibitory effects of four coumarone derivatives with the same parent nuclear structure on bladder cancer cells. First, we examined the effects of coumarone derivatives with three different substituents on the viability of different urothial cells using the CCK-8 kit. The substituents of MPO, IPO, and FPO are hydrogen, a cyanogroup, and fluorine, respectively. Notably, FPO exhibited the most significant inhibition of cell viability in bladder tumor cells, indicating that fluorine atoms may be the main reason for its tumor-suppressing effect. Fluorinated compounds have been used in clinical practice as anticancer drugs, such as 5-fluorouracil (5-FU). Fluorine replaces hydrogen at the C-5 position of uracil in 5-FU, one of the first chemotherapeutic agents to exhibit anticancer activity [16]. Several studies have indicated that 5-FU treatment time- and dose-dependently inhibits the proliferation of colorectal cancer. Furthermore, the type of colorectal cancer cell has a considerable effect on cell growth inhibition induced by 5-FU [16]. We found that FPO exerted inhibitory effects on both MIBC and NMIBC, especially on the MIBC cell line T24. This indicates that FPO has potential as a novel anticancer drug for MIBC.

We also compared PTCO with FPO and found that an increase in the fluorine content in the substituents was not proportional to the tumor-suppressing effect. Therefore, the different inhibitory effects of coumarone derivatives containing fluorine in their substituents will be our future research direction and will provide new ideas for the exploration of new drugs to treat bladder cancer.

Conclusively, in the current study FPO showed the strongest anticancer activity of MIBC among the four candidates. FPO significantly inhibited MIBC cells proliferation and suppressed cell migration and clonality. EdU cell proliferation and Ki67 immunofluorescence staining showed that FPO effectively inhibited the proliferation of MIBC cells. FPO inhibited cell growth by inhibiting cell proliferation in the S and G2 phases. Notably, the different tumor-suppressing effects of the four coumarone derivatives with the same parent nuclear structure in this study were most likely due to their different substituents. This difference provides a direction for further research in the future.

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