Cellular Physiology and Biochemistry

Cell Physiol Biochem 2017;43:1003-1011
DOI: 10.1159/000481698
Published online: October 02, 2017
Accepted: July 25, 2017

Original Paper

Fangchinoline Induces Apoptosis, Autophagy and Energetic Impairment in Bladder Cancer

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Key Words
Bladder cancer • Fangchinoline • Autophagy • Apoptosis • Proliferation

Abatract
Background/Aims: Tetrandrine and Fangchinoline (Fcn) are two natural products that are found in *Stephania tetrandra*. Tetrandrine is a known anti-bladder cancer compound, but the effects of Fcn on bladder cancer have been previously unclear. In the present study, we focused on the anti-tumor effects of Fcn on bladder cancer. ***Methods and Results:*** We treated T24 and 5637 bladder cancer cell lines with Fcn *in vitro*. We observed that Fcn inhibited the viability of bladder cancer cells in a concentration-dependent manner. The expression of PCNA, a biomarker of proliferation, was down-regulated. Fcn treatment induced both apoptosis and autophagy in bladder cancer cells, as shown by the increased cleavage of caspase-3, an up-regulated LC3-II/LC3-I ratio and the down-regulated p62 level. Blocking autophagy with 3-MA (3-Methyladenine) enhanced Fcn-induced apoptosis, indicating that Fcn-induced autophagy was adaptive. Additionally, we observed that Fcn treatment inhibited mTOR and reduced the intracellular ATP levels. The exogenous addition of methyl pyruvate (MP) to compensate metabolic substrates alleviated Fcn-induced apoptosis and autophagy. ***Conclusions:*** Our data indicated that Fcn caused an impairment in energy generation, which led to apoptosis and adaptive autophagy in bladder cancer. These results demonstrated that Fcn may be a potential candidate for use in the prevention and treatment of bladder cancer.

Introduction

Bladder cancer is a urological malignant tumor that causes a heavy burden to patients and society. For advanced patients, combined chemotherapy is needed. However, resistance to chemotherapy drugs may gradually occur and significantly reduce the effects of treatment. Therefore, the discovery of novel low-toxicity drugs is beneficial for the prevention and treatment of the disease.

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Tetrandrine (Tet), a widely investigated compound, exhibits potent anti-tumor effects in lung cancer, prostate cancer, breast cancer and bladder cancer [1-6]. In bladder cancer, Tet induces apoptosis and reverses the epithelial-mesenchymal transition [2, 6]. Tet and Fangchinoline (Fcn) are the two main bis-benzylisoquinoline alkaloids that exist in the plants of *Stephania tetrandra*. The structures of both Tet and Fcn are similar, but Fcn was reported to be associated with the inhibition of acetylcholinesterase and glutamate release in neurological diseases [7-9]. Fcn is a potent antioxidant, showing protective effects in oxidative cell injury [10]. This compound was also found to inhibit the growth of several cancers, such as lung cancer, breast cancer and prostate cancer, through proliferation inhibition and/or apoptosis [11-14] and induces autophagic cell death in hepatocellular cancer cells [15]. More recently, Fcn was found to block the well-known P-glycoprotein, indicating that Fcn may enhance the effects of chemotherapy drugs and prevents multidrug resistance [16]. However, whether Fcn can inhibit bladder cancer remains unclear. In the present study, we focused on the effects of Fcn on bladder cancer.

Materials and Methods

Chemicals

Fcn was purchased from Sigma-Aldrich (MO, USA), dissolved in DMSO to create an 80-mM stock solution and stored at -80 °C for use. Z-VAD-FMK was obtained from Enzo Life Sciences (New York, USA). The mouse anti-PCNA antibody, 3-MA (3-Methyladenine) and Necrostatin-1 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Methyl pyruvate (MP) was purchased from Acros (Geel, Belgium). The rabbit anti-LC3B antibody was purchased from Sigma-Aldrich. The rabbit anti-p62, rabbit anti-cleaved caspase-3, rabbit anti-caspase-3, rabbit anti-GAPDH, rabbit anti-p-4E-BP1, and rabbit anti-4E-BP1 primary antibodies and the goat anti-rabbit and anti-mouse HRP-tagged secondary antibodies were all purchased from Cell Signaling Technology (Boston, MA, USA).

Cell culture

Human bladder cancer T24 and 5637 cells were obtained from the Cell Resource Center of Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences). The cells were cultured in RPMI-1640 medium (Thermo Fisher) with 10% (v/v) of fetal bovine serum (FBS) containing 100 units/ml penicillin and 0.1 mg/ml streptomycin in an atmosphere of 5% CO_2 at 37 °C.

Proliferation measurement

The proliferation of the cells was measured using a Cell Counting Kit-8 (CCK-8) assay. In brief, 1 × 10^4 cells/well were seeded into a 96-well plate. After the treatment, 10 μl/well of CCK-8 reagent (Dojindo, Kumamoto, Japan) was added. The plate was then incubated at 37 °C for 2 h. The cell viability was measured as the relative optical density read at 450 nm.

Protein analysis

The total protein from each group was extracted with RIPA lysis buffer, quantified, mixed with loading buffer for Western blotting, and heated to 100 °C for 5 min. Then, 20 μg of total protein in each group was loaded onto a SDS-PAGE gel. The protein samples were separated by electrophoresis and were blotted onto a nitrocellulose membrane. The membrane was then blocked with 5% non-fat milk for 1 h at room temperature. The membrane was then soaked in the corresponding primary antibodies at 4 °C overnight. The primary antibodies were diluted as 1:1000 in a Tris-buffered saline (TBS)/Tween solution (TBST). The membrane was then washed with TBST 4 times over 20 min. After that, the membrane was incubated with the goat anti-rabbit or goat anti-mouse HRP-tagged secondary antibody (1:2000) for 1 h at room temperature. The membrane was then washed with TBST 4 times over 20 min. The bands were detected using a HRP substrate (Millipore, Bedford, MA, USA), and the band intensities were quantified using the Quantity One software V4.6.2 (Bio-Rad, CA, USA).

ATP measurement

T24 and 5637 cells were seeded in a 96-well plate and treated with 5 μM of Fcn for 24 h. The levels of ATP were detected using the ATPlite luminescence assay system (PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol. In brief, the cells were lysed, and the relative ATP levels were reflected by the luminescence intensity in each group detected using a Varioskan 96-well plate reader (Thermo Fisher, Waltham, MA, USA).

Statistical analysis

All data in this study are presented as the mean ± SD. A two-tailed Student's t-test or a one-way ANOVA was performed by using the SPSS 13.0 software (SPSS Inc., IL, USA). $P<0.05$ was considered statistically significant.

Results

Fangchinoline inhibits bladder cancer proliferation

To investigate whether Fcn exhibits anti-cancer effects in bladder cancer, T24 and 5637 cells were treated with a range of concentrations of Fcn for 24, 48 and 72 h. The cell viability was detected using a CCK-8 assay. We observed that the cell viabilities of both T24 and 5637 cells were significantly inhibited by Fcn (≥5 μM) at 24, 48 and 72 h ($P<0.01$) in a dose-dependent manner (Fig. 1A and 1B). The IC50 values of Fcn in T24 cells were 19.0 μM, (24

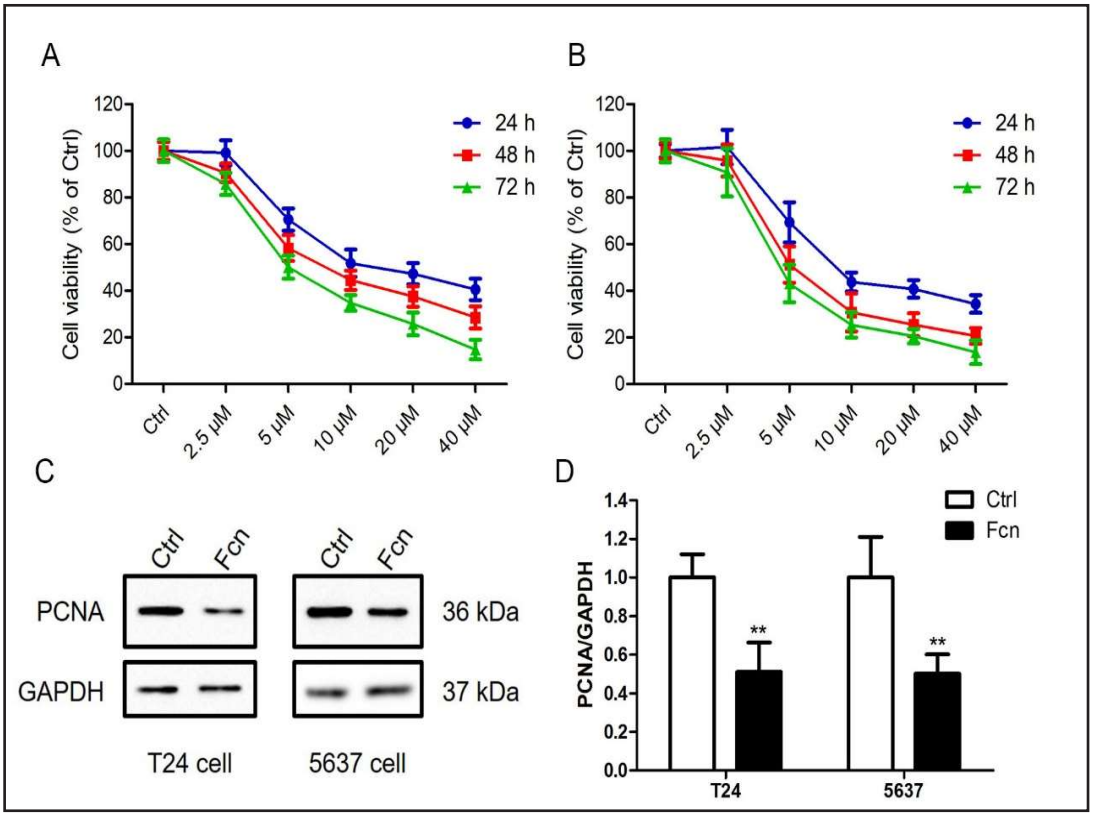

Fig. 1. Fangchinoline inhibits bladder cancer proliferation. (A, B) T24 (A) and 5637 (B) cells were treated with the indicated concentrations of Fangchinoline (Fcn) for 24, 48 and 72 h, and the cell viability was detected using a CCK-8 assay. (C, D) T24 and 5637 cells were treated with 5 μM Fcn for 24 h. The expression of PCNA was detected using Western blotting (C). The densitometric analysis for the relative expression of PCNA was determined using the PCNA/GAPDH ratio (D). The data are expressed as the mean ± SD; **$P<0.01$ compared with the control (Ctrl) group.

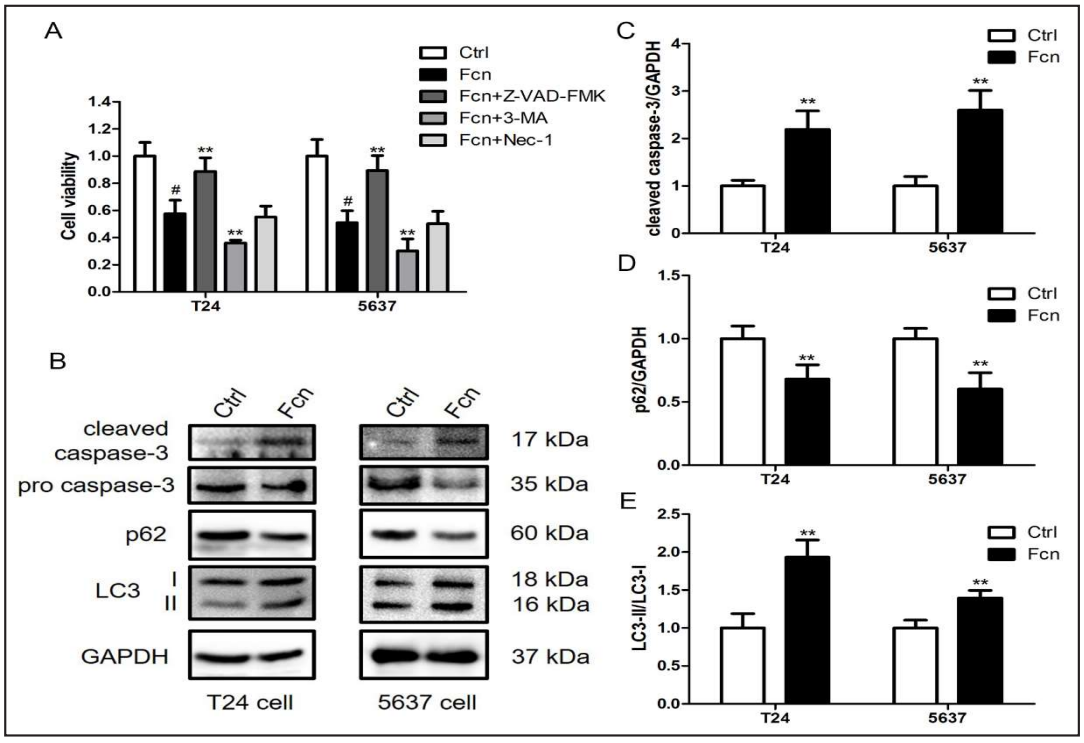

Fig. 2. Fangchinoline induces apoptosis and autophagy in bladder cancer cells. (A) T24 and 5637 cells were treated as indicated for 24 h. The cell viability was detected using a CCK-8 assay. The data are expressed as the mean ± SD; **P<0.01 compared with the Fcn group and #P<0.01 compared with the control (Ctrl) group. (B-E) T24 and 5637 cells were treated with 5 μM of Fcn for 24 h. The cleavage of caspase-3, the expression of p62 and the conversion of LC3 were detected using Western blotting (B). The densitometric analysis for the ratios of cleaved caspase-3/GAPDH (C), p62/GAPDH (D) and LC3-II/LC3-I (D) was measured. The data are expressed as the mean ± SD; **P<0.01 compared with the control (Ctrl) group.

h), 12.0 μM (48 h) and 7.57 μM (72 h), and 11.9 μM (24 h), 9.92 μM (48 h) and 7.13 μM (72 h) in 5637 cells.

We then detected the expression of proliferating cell nuclear antigen (PCNA) as a biomarker of proliferation. Consistent with the results from the CCK-8 assay, we found that the expression of PCNA was down-regulated in the two cell lines after Fcn treatment (Fig. 1C and 1D). These results indicated that Fcn was able to inhibit the proliferation of bladder cancer.

Fangchinoline induces apoptosis and autophagy in bladder cancer

To explore whether Fcn induces cell death, we treated the cells with 50 μM of Z-VAD-FMK (apoptosis inhibitor), 30 μM of Necrostatin-1 (necroptosis inhibitor) or 2 mM of 3-MA (autophagy inhibitor) in combination with Fcn. The cell viability was then detected using a CCK-8 assay. We observed that Z-VAD-FMK partially rescued cell viability under treatment of Fcn. Additionally, 3-MA synergized the inhibitory effects of Fcn, but Necrostatin-1 did not alter the viability in the presence of Fcn (Fig. 2A).

We then detected the cleavage of caspase-3, a well-known biomarker of apoptosis. We observed that treating T24 and 5637 cells with Fcn caused a significant increase in the cleavage of caspase-3 (Fig. 2B and 2C). For autophagy, we detected the conversion of LC3 and the expression of p62. We found that Fcn induced a significant increase in the LC3-II/LC3-I ratio and a decrease in p62 in both T24 and 5637 cells (Fig. 2B, 2D and 2E). These results indicated that both apoptosis and autophagy were induced by Fcn in bladder cancer cells.

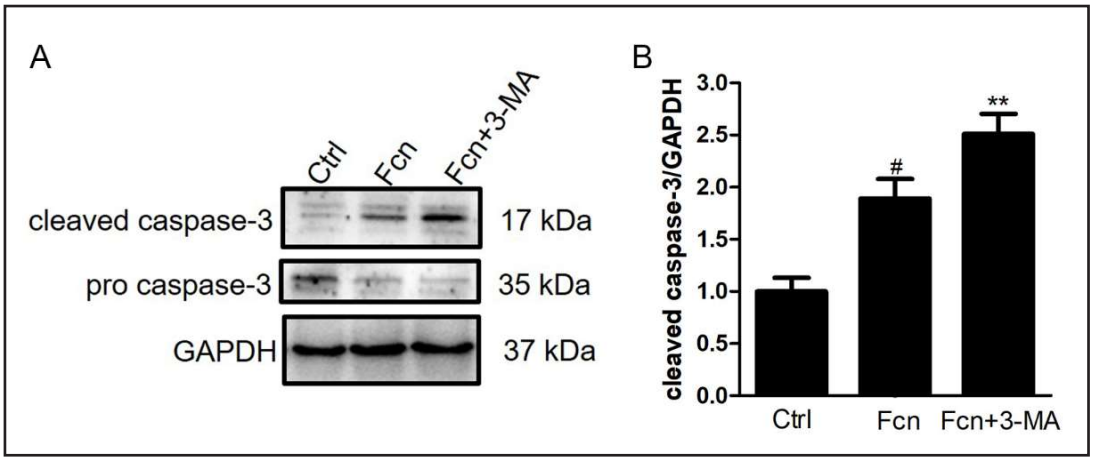

Fig. 3. Blunting autophagy enhances Fcn-induced apoptosis in bladder cancer cells. (A) T24 cells were treated with 5 μM of Fangchinoline (Fcn) in the presence or absence of 3-MA (2 mM) or vehicle (as a control) for 24 h. The cleavage of caspase-3 was detected using Western blotting. (B) The densitometric analysis for the ratios of cleaved caspase-3/GAPDH was measured. The data are expressed as the mean ± SD; **P<0.01 compared with the Fcn group and #P<0.01 compared with the control (Ctrl) group.

Inhibition of autophagy increased Fangchinoline-induced apoptosis

Autophagy is closely associated with the regulation of metabolism and cell survival control [17, 18]. Blocking autophagy with 3-MA synergized the inhibitory effects of Fcn on bladder cancer cells (Fig. 2A). Therefore, we hypothesized that the activation of autophagy might be an adaptive response induced by Fcn. To investigate the role of autophagy in Fcn-induced cell death, we treated T24 cells with Fcn in the presence or absence of 3-MA. We observed that the cleavage of caspase-3 was enhanced by 3-MA (Fig. 3). These results indicated that the activation of autophagy might be an adaptive response in Fcn-treated bladder cancer cells, whereas the blockade of autophagy enhanced Fcn-induced cell death.

Fangchinoline inhibited the mTOR pathway in bladder cancer

The mTOR pathway is closely related to autophagy regulation. To clarify the mechanism of Fcn-induced autophagy activation, we detected the phosphorylation of 4E-BP1, a direct substrate of mTOR, in bladder cancer cells. T24 cells were treated with Fcn for 6, 12 and 24 h. We observed that the phosphorylation of 4E-BP1 at the residues T37 and T46 was significantly inhibited (Fig. 4A and 4B), indicating that Fcn may induce autophagy through the inhibition of the mTOR pathway.

Fangchinoline induces energetic impairment in bladder cancer

The mTOR complexes play important roles in the regulation of nutrition and energy generation. The inhibition of mTOR induced by Fcn reflected a decrease in the metabolic substrates in bladder cancer cells. To verify whether the inhibitory effects of Fcn are associated with energy impairment, we detected the ATP levels after treatment with 5 μM of Fcn for 24 h. We observed that the relative ATP levels were significantly down-regulated in both T24 cells and 5637 cells (Fig. 4C). We then employed methyl pyruvate (MP) to compensate as a metabolic substrate for energy generation. MP could generate pyruvate acid, an important substrate for tricarboxylic acid cycle (TCA). In the presence of MP, the cell viability was increased after treatment with Fcn in both T24 and 5637 cells (Fig. 4D). Consistently, MP partially antagonized the Fcn-induced cleavage of caspase-3 in T24 cells (Fig. 4E and 4F). In addition, we observed that the addition of MP also reduced the ratio of LC3-II/LC3-I under the treatment of Fcn (Fig. 4E and 4F). These data indicated that the impairment of energy generation may contribute to Fcn-induced autophagy and apoptosis in bladder cancer.

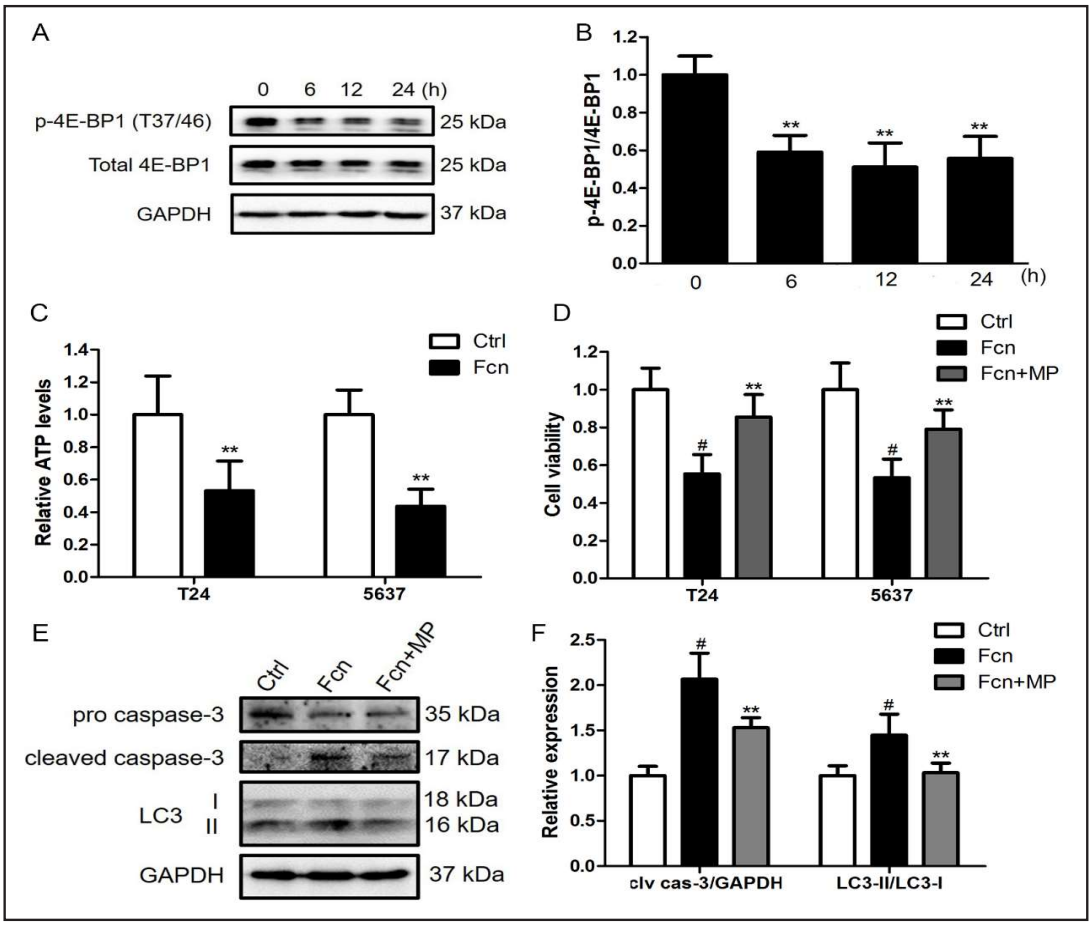

Fig. 4. Fangchinoline inhibits the mTOR pathway and induces energetic impairment in bladder cancer cells. (A, B) T24 cells were treated with Fcn (5 μM) for the indicated time. The phosphorylation levels of 4E-BP1 were detected (A), and the densitometric analysis for the p-4E-BP1/4E-BP1 ratios is shown (B) The data are expressed as the mean ± SD; **P<0.01 compared with the 0 h group. (C) T24 and 5637 cells were treated with 5 μM of Fangchinoline (Fcn) for 24 h. The relative ATP levels were measured. (D) T24 and 5637 cells were treated with 5 μM of Fcn in the presence or absence of methyl pyruvate (MP; 2 mM) or vehicle (as a control) for 24 h. The cell viability was detected using a CCK-8 assay. (E, F) T24 cells were treated as described as in (D). The cleavage of caspase-3 and the conversion of LC3 were detected using Western blotting (E). The densitometric analysis for the ratios of cleaved caspase-3/GAPDH and LC3-II/LC3-I was determined (F). The data are expressed as the mean ± SD; **P<0.01 compared with the control (Ctrl) group (C) or the Fcn group (D, F), and #P<0.01 compared with the control (Ctrl) group (D, F).

Discussion

The present study highlights the anti-tumor effects that a natural product, Fcn, exhibits against bladder cancer. This compound could inhibit proliferation and induce apoptosis. For the first time, we revealed that Fcn induced protective autophagy in bladder cancer, inhibited the mTOR pathway, and thus eventually induced energetic impairment in bladder cancer. Our data indicated that Fcn is a potent anti-tumor compound that may be a potential candidate for the prevention and treatment of bladder cancer.

The anti-tumor effects of Fcn has been reported in many other cancers, such as lung cancer, prostate cancer, glioblastoma and breast cancer [11-14, 19, 20]. This compound

antagonizes multidrug resistance-related drugs by targeting P-glycoprotein, which may improve drug resistance in chemotherapy [21]. Fcn targets the PI3K/Akt/GSK-3β pathway and induces apoptosis [13]. Fcn can also modulate cell cycle-related proteins, such as PCNA, p27 and cyclin D, and thus induces G1/S arrest in prostate cancer [20]. In the present study, we investigated the effects of Fcn on two widely used bladder cancer cell lines, T24 and 5637 cells. Notably, 5637 cells may have lower chemotherapy resistance than T24 cells [22]. We observed that Fcn inhibited proliferation and activated apoptosis in bladder cancer. Fcn treatment resulted in the cleavage of caspase-3, whereas blocking caspase activation with Z-VAD-FMK antagonized Fcn-induced cell death. These results indicated that Fcn-induced caspase-dependent apoptosis in bladder cancer. However, these effects were similar in the two cell lines.

Autophagy is an important process that regulates cell survival, metabolism and energy generation [23]. The activation of autophagy induced by stimulants may be adaptive or maladaptive [24]. It has been reported that Fcn can induce autophagic cell death in hepatocellular carcinoma cells, indicating the presence of a Fcn-induced maladaptive response in these cells [15]. This effect is associated with the p53/sestrin2/AMPK signaling pathway [15]. We observed that Fcn could also significantly activate autophagy in bladder cancer cells, as shown by the increased LC3-II/LC3-I ratios and decreased p62 levels. However, our data indicated that the activation of autophagy in bladder cancer induced by Fcn was an adaptive response, whereas the blockade of autophagy enhanced Fcn-induced cell death. The appropriate activation of autophagy always plays a protective role in response to harmful irritation, such as stress injury and energy depletion [23, 25-27]. The activation of autophagy could sequester protein aggresomes and injured organelles to limit the injury [28, 29]. The cargoes in the autophagosomes are degraded, and the products were released as substrates of metabolism [28, 29]. However, the excessive activation of autophagy may induce cell death. Our results indicated that the autophagy induced by Fcn in bladder cancer cells was protective rather than a process leading to cell death. These data indicated that Fcn-induced autophagy may play multiple roles according to the context of the cancer.

The mTOR signaling pathway is one of the key pathways that control the autophagy level [30]. Our data revealed that Fcn significantly inhibited the mTOR pathway. Additionally, mTOR also plays an important role in controlling intracellular nutrition, and it could be inhibited during the nutrition depletion [31, 32]. In the present study, we observed that Fcn caused a decrease in the ATP level, whereas the exogenous supplement of tricarboxylic acid cycle substrates partially antagonized Fcn-induced apoptosis and autophagy. On the one hand, these results support that the Fcn-induced apoptosis was due to energetic impairment, possibly through the mTOR pathway. On the other hand, the inhibition of mTOR induced by Fcn activated autophagy, which could up-regulate the degradation of intracellular components to generate metabolic substrates for the compensation of energetic depletion stress. This mechanism may explain why blocking this adaptive autophagy led to increased cell death.

Many natural products show anti-tumor effects in bladder cancer [33]. The present study indicated that Fcn, an anti-tumor compound, inhibited bladder cancer. Our data supports Fcn as a potential candidate for the prevention and treatment of bladder cancer.

Acknowledgements

We thank Dr. Yongqing Shen at the Hebei University of Chinese Medicine for the advice on our autophagy investigation.

Disclosure Statement

The authors declare no competing financial interests.

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