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Original Research Article

6-Methyl-1-(3-(trifluoromethyl)phenyl)-1H-pyrazolo[4,3-c]pyridin-4(5H)-one (FMPPP) exhibits anti-proliferative effect on prostate cancer via autophagy induction and mTOR/p70S6K inhibition

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Abstract

Purpose: *To investigate 6-methyl-1-(3-(trifluoromethyl)phenyl)-1H-pyrazolo[4,3-c]pyridin-4(5H)-one (FMPPP) as anti-proliferative agent against prostate cancers.*
Methods: *The FMPPP-mediated changes in cell proliferation were measured using cell counting kit-8 (CCK-8). Flow cytometry and propidium iodide staining were used for cellular DNA content determination. Proteins expression in cells was probed by western blotting assay.*
Results: *A significant ($p < 0.05$) dose-dependent suppression of DU145 and PC 3 cell proliferation was observed following FMPPP treatment. FMPPP treatment at 20 μM raised DU145 cell fraction to 75.08 ± 4.87 % in G1 phase when compared to 48.32 ± 3.44 % for control. The population of PC 3 cells in G1-phase reached to 72.78 ± 5.21 % on treatment with 20 μM FMPPP compared to 49.65 ± 4.62 % in control. The FMPPP treatment of DU145 and PC-3 cells elevated LC3-II expression and suppressed SQSTM1/p62 expression. In FMPPP-treated DU145 and PC-3 cells, p-ERK1/2 level was promoted whereas mTOR and p70S6K phosphorylation significantly decreased. Exposure to U0126 (ERK pathway inhibitor) reduced FMPPP-induced increase of LC3-II expression and promotion of p-ERK1/2 level in DU145 and PC-3 cells.*
Conclusion: *FMPPP exhibits anti-proliferative effect by increasing autophagy in prostate cancer cells. The cytotoxicity of FMPPP involves elevation of ERK1/2 phosphorylation and targeting mTOR pathway in DU145 and PC-3 cells. Therefore, FMPPP may be beneficial for the treatment of prostate cancer in patients.*

Keywords: *Prostate cancer, Phosphorylation, Drug development, Autophagy*

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INTRODUCTION

Prostate cancer is the most common type of malignant tumor detected in men and its incidence is increasing worldwide every year [1,2]. It is considered as a highly complicated type of cancer caused by changes in intrinsic as well extrinsic processes in various kinds of cells [3]. Multiple strategies like surgical removal, hormonal therapy, radiation/chemotherapies and

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very high-intensity ultrasound either alone or in combination are used to inhibit prostate tumor growth [4]. Autophagy regulates homeostasis at cellular level by eliminating dysfunctional proteins as well as organelles from cells [5]. It increases survival response and is triggered by various stresses like viral infections and nutrient starvation to cells [6]. Activation of autophagy by cellular stimuli result in programmed but non-apoptotic death of cells [7]. Moreover, in mammalian cells regulation of autophagy is associated with mammalian target of rapamycin (mTOR) pathway [8]. Another pathway known as extracellular signal-regulated kinases 1/2 (ERK1/2) also plays significant role in regulating the autophagy [9].

Pyrazolo[4,3-*c*]pyridin-4(5*H*)-ones have been demonstrated as potentially attractive heteroaromatic compounds in drug development programme [10].

The structural arrangement of hydrogen bond-donating and accepting groups in the bicyclic scaffold fulfils the demand of competitive ATP binding to kinases [11]. The pharmacological implications of pyrazolo[4,3-*c*]pyridin-4(5*H*)-ones has led to synthesis and investigation of these compounds for diverse medicinal fields. Medicinal chemistry is dependent on synthesis of chemical compounds which can interact with several enzymes to demonstrate efficient pharmacokinetic properties [11]. The bicyclic heterocyclic aromatic compounds are known for pharmacokinetic properties and are preferred in medicinal chemistry because of relatively easy synthetic methods [11]. In the present study, the anti-proliferative potential and underlying mechanism of 6-methyl-1-(3-(trifluoromethyl)phenyl)-1H-pyrazolo[4,3-c]pyridin-4(5H)-one (FMPPP; Figure 1) against prostate cancers was investigated.

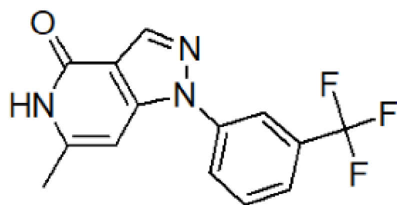

Figure 1: Structure of 6-methyl-1-(3-(trifluoromethyl)phenyl)-1H-pyrazolo[4,3-c]pyridin-4(5H)-one (FMPPP)

EXPERIMENTAL

Cell lines and culture

The cell lines, PNT2 DU145 and PC-3 were provided by American Type cell Culture Collection (Manassas, VA, USA) and maintained in DMEM. The medium was also supplemented with antibiotics such as 1 % penicillin/streptomycin (Sigma-Aldrich) and fetal bovine serum (10 %). Cells were cultured in incubator at 37 °C and under 5 % CO_2 humid atmosphere.

Cell viability assay

To measure changes in proliferation by FMPPP-treatment cell counting kit-8 (CCK-8; Inc., Kumamoto, Japan) was used. The PNT2, DU145 and PC-3 cells were distributed at 1 x 10^5 cells/well density in 96-well plates containing culture medium, 10 % FBS and antibiotics. Incubation in an incubator under 5 % CO_2 atmosphere was carried out for 12 h at 37 ˚C. The cells were treated with FMPPP at 1.5, 3.0, 6.0, 12 and 20 μM in 10 μL concentrations for 48 h. At completion, CCK-8 (10 μl) was poured into each well to incubate cells for 3 h more. Measurement of absorbance for each well was made at 457 nm in microplate reader to determine cell viability.

Cell cycle analysis

Cells treated with FMPPP at 20 μM or untreated cells were trypsinized at 48 h of incubation and then fixed in ethyl alcohol (70 %) for overnight. Then washing of cells was done in PBS two times followed by centrifugation at 230 x g for 15 min. The cells were treated for 10 min with 50 μl of RNase before staining with 50 μg/ml solution of propidium iodide (Sigma-Aldrich) at room temperature. Following 1 h of staining, cellular DNA was examined using a FACSAria-II flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blot analysis

The cells treated with FMPPP at 20 μM or untreated cells were collected at 48 h of incubation and then washed in ice-cold PBS. The cells were treated with lysis buffer [NP-40 (1 %), NaPPi (5 mM), NaCl (150 mM), Tris-HCL (20 mM; pH 7.5), Na_3VO_4 (5 mM), PMSF (1 mM) and leupeptin (10 μg/mL)] to obtain lysate. After 40 min, lysate was subjected to centrifugation for 20 min at 13,000 x g and protein level in the lysate was estimated using Bradford method. The proteins were resolved on 10-15 % SDS-PAGE and subsequently transferred to PVDF membranes. Incubation of membrane for 1.5 h was carried out at room temperature with 5 % non-fat milk to block non-specific sites. Proteins were probed by overnight incubation of membranes with primary antibodies at 4˚C. Washing with PBS/Tween-20 (0.1 %) was

followed by incubation at room temperature with horseradish peroxidase-conjugated secondary antibodies for 1 h. The blots were detected using enhanced chemiluminescence reagent (ECL) and X-ray films. The primary antibodies included against: LC3II, p-ERK1/2, SQSTM1/p62, ERK1/2, mTOR, p-mTOR, p-p70S6K and β-actin (Cell Signaling Technology, Inc., Danvers, MA, USA).

Statistical analysis

The data are expressed as mean ± standard deviations of triplicate measurements. The statistical analysis of obtained data was made using one-way analysis of variance (ANOVA) and Bonferroni's post-hoc tests for multiple comparisons. At $p < 0.05$, differences were taken as statistically significant.

RESULTS

Proliferation suppression by FMPPP in DU145 and PC-3 cells

Proliferation changes by FMPPP-treatment in PNT2, DU145 and PC-3 cells at 48 h were measured to assess its cytotoxicity (Figure 2). The cells exposed to FMPPP at 1.5, 3.0, 6.0, 12 and 20 μM doses and then subjected to Cell Counting assay. In PNT2 cells proliferation was not changed by FMPPP treatment in 1.5 to 20 μM concentration range. However, proliferation of FMPPP-treated DU145 and PC-3 cells showed significant ($p < 0.05$) dose-dependent suppression when compared to control. In FMPPP-treated DU145 cells, proliferation was suppressed to 88.26 and 19.53 %, respectively at dose of 1.5 and 20 μM doses. Proliferation of 1.5 and 20 μM FMPPP-treated PC-3 cells was reduced to 90.42 and 21.72 %, respectively at 48 h.

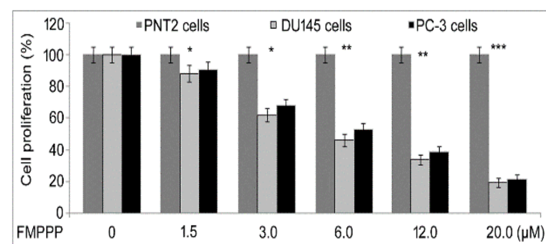

Figure 2: Effect of FMPPP on PNT2, DU145 and PC-3 cells. The FMPPP treatment at 1.5, 3.0, 6.0, 12 and 20 μM was followed by proliferation measurement of normal (PNT2) and cancer (DU145 and PC-3) cells at 48 h using Cell Counting assay. *$P < 0.0476$, **$p < 0.0196$ and ***$p < 0.0109$ vs. untreated cells

Cell cycle arrest by FMPPP in G1-phase

Distribution of DNA in FMPPP treated or untreated DU145 and PC-3 cells were examined by flow cytometry (Figure 3). In DU145 and PC-3 cells FMPPP treatment at 20 μM dose raised fraction of cells in G1 phase significantly compared to untreated cells. Treatment with FMPPP at 20 μM raised DU145 cell fraction to 75.08 ± 4.87 % in G1 phase when compared to 48.32 ± 3.44 % in untreated cells. The G1-phase PC-3 cell population increased to 72.78 ± 5.21 % on treatment with 20 μM FMPPP when compared to 49.65 ± 4.62 % in untreated cells. The DU145 cellular count in G2/M-phase reduced to 17.41 ± 2.14 % (control 28.20 ± 3.08 %) and in S-phase to 8.27 ± 213 % (control 23.30 ± 3.08 %) on treatment with 20 μM FMPPP. In case of PC-3 cellular population in G2/M-phase reduced to 18.51 ± 2.19 % (control 30.51 ± 3.15 %) and in S-phase to 8.45 ± 213 % (control 19.61 ± 3.54 %) on treatment with 20 μM FMPPP.

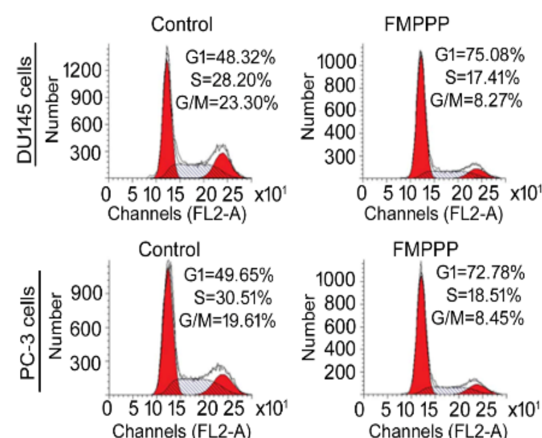

Figure 3: Effect of FMPPP on cell cycle progression. The FMPPP treatment at 20 μM dose was followed by Annexin V-FITC/PI staining of DU145 and PC-3 cells at 48 h to assess the DNA content distribution

Autophagy activation by FMPPP in DU145 and PC-3 cells

Effect of 20 μM FMPPP dose on markers of autophagy in DU145 and PC-3 cells was analyzed by western blotting (Figure 4). Treatment with FMPPP (20 μM) for 48 h markedly promoted LC3-II expression in DU145 and PC-3 cells when compared to untreated cells. The SQSTM1/p62 expression in FMPPP (20 μM) treated DU145 and PC-3 cells was significantly ($p < 0.05$) suppressed at 48 h relative to untreated cells.

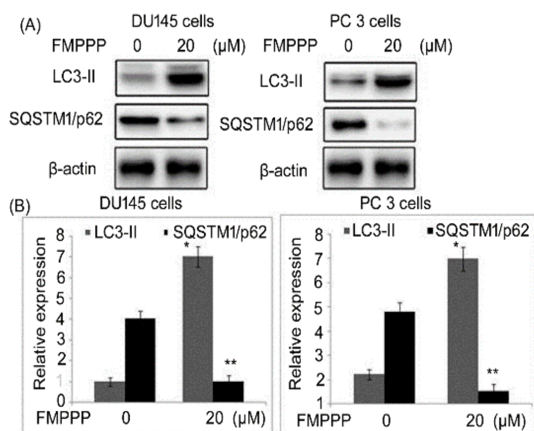

Figure 4: Effect of FMPPP on markers of autophagy. (A) The FMPPP treatment of DU145 and PC-3 cells was followed by LC3-II and SQSTM1/p62 expression assessment at 48 h using western blotting. (B) Immunoblots were quantified. *$P < 0.05$ and **$p < 0.01$ vs. untreated cells

FMPPP regulates p-ERK1/2 and mTOR signaling in DU145 and PC-3 cells

In FMPPP-treated DU145 and PC-3 cells elevated p-ERK1/2 expression was observed compared to untreated cells (Figure 5). The FMPPP treatment of DU145 and PC-3 cells down-regulated mTOR phosphorylation significantly relative to untreated cells. The p70S6K phosphorylation in DU145 and PC-3 cells was also suppressed on treatment with 20 μM dose of FMPPP.

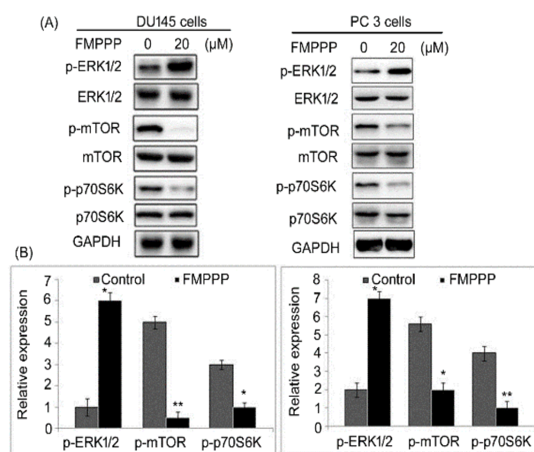

Figure 5: Effect of FMPPP on mTOR and ERK pathways. (A) The FMPPP treatment of DU145 and PC-3 cells was followed by ERK1/2, mTOR and p70S6K activation assessment at 48 h using western blotting. (B) Immunoblots were quantified. *$p < 0.0476$ and **$p < 0.0189$ vs. untreated cells

Effect of U0126 FMPPP induced p-ERK1/2 and mTOR signaling in DU145 and PC-3 cells

Exposure to U0126 (ERK pathway inhibitor) reduced FMPPP-induced increase in LC3-II expression in DU145 and PC-3 cells (Figure 6). The elevated p-ERK1/2 expression was effectively alleviated by U0126 exposure in DU145 and PC-3 cells. Moreover, U0126 exposure elevated mTOR phosphorylation and p70S6K activation in FMPPP-treated DU145 and PC-3 cells.

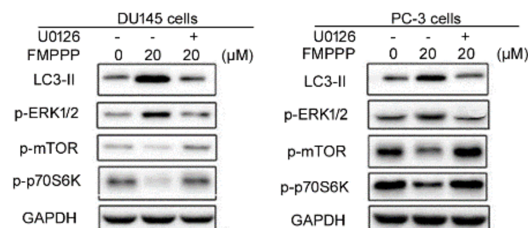

Figure 6: Effect of U0126 exposure on mTOR and ERK pathways in FMPPP treated cells. The U0126-exposed DU145 and PC-3 cells were treated with FMPPP and LC3-II expression, ERK1/2, mTOR and p70S6K activation assessment was made by western blotting

DISCUSSION

Autophagy, a process which transfers intracellular unwanted constituents to lysosomes for decomposition plays crucial role in maintaining homeostatic in cells. Studies have reported activation of autophagy via multiple factors in tumor cells on administration of anti-cancer drugs [12,13]. Cell death via non-apoptotic pathway is induced by activation of autophagy in tumor cells [14]. The main indicator of cellular autophagy is the formation of LC-3II [15]. There is increased LC3 puncta formation during the cell death induced by autophagy [15]. Besides, p62 levels are suppressed during autophagy because of its degradation along with the autophagosomal contents in autophagosomes [16]. The hallmark of autophagic flux is the breakdown of SQSTM1/p62 in various cells [17].

In the present study, cytotoxicity of FMPPP was investigated against PNT2 (normal), DU145 and PC-3 (carcinoma) cells. The FMPPP treatment decreased proliferation of DU145 and PC-3 cancer cells selectively and significantly without affecting PNT2 cells. This indicates that FMPPP exhibits cytotoxic effect on DU145 and PC-3 cancer cells. Flow cytometry of FMPPP treated cells demonstrated arrest of cell cycle in G1-phase which was evident by higher cell fraction.

For further mechanistic clarification effect of FMPPP on markers of autophagy was analyzed by western blotting. In FMPPP treated DU145 and PC-3 cells marked elevation in LC3-II expression was observed when compared to untreated cells. Moreover, SQSTM1/p62 expression in FMPPP-treated DU145 and PC-3 cells showed a marked down-regulation relative to untreated cells. These findings revealed that FMPPP treatment excessively activates autophagy in prostate cancer cells to suppress proliferation.

Nutrient starvation mediated cell autophagy is regulated by two important pathways known as mTOR and ERK1/2 pathways. Activation of ERK pathway is associated with cadmium or TNFα treatment of the cells resulting in autophagy [18]. Moreover, ERK is directly activated in cells by phosphorylated-MEK over-expression leading to autophagic death [19]. Studies demonstrated that cellular autophagy is negatively regulated following AKT/mTOR activation [20]. In the present study, FMPPP-treatment of DU145 and PC-3 cells elevated p-ERK1/2 expression when compared to untreated cells.

In FMPPP-treated DU145 and PC-3 cells down-regulation of mTOR phosphorylation was observed significantly relative to untreated cells. Moreover, p70S6K phosphorylation in DU145 and PC-3 cells was also suppressed on treatment with FMPPP. The activity of MAPK/ERK kinases such as MEK1 and MEK2 at cellular level is inhibited on treatment with U0126 and therefore is used as selective inhibitor for ERK1/2 activation [21]. In the present study U0126 exposure alleviated FMPPP-induced increase of LC3-II expression in DU145 and PC-3 cells. It elevated p-ERK1/2 expression, mTOR phosphorylation and p70S6K activation in FMPPP treated DU145 and PC-3 cells.

CONCLUSION

FMPPP exhibits anti-proliferative effect by activating autophagy in prostate cancer cells. Its cytotoxicity involves elevation of ERK1/2 phosphorylation and targeting mTOR pathway in DU145 and PC-3 cells. Therefore, FMPPP is a potential treatment strategy for prostate cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yanlin Wu - conceived and designed the study; Weichong Zhao, Lei Ning, Lihui Wang, Lei Qi, Ruihong Yang - collected and analyzed the data; Weichong Zhao, Lei Ning, Lihui Wang, Lei Qi - wrote the manuscript. Yanlin Wu - approved final version of the manuscript. All authors read and approved the manuscript for publication.

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