

Role of Fasting Mimicking Diet in Farnesoid x Receptor for Suppressing Epithelial-to-Mesenchymal Transition, Cell Cycle Progression, and Viability of Prostate Cancer Cells

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Abstract

The systemic and resistant nature of metastatic castration-resistant prostate cancers (mCRPC) renders it largely incurable even after intensive multimodal therapy. Proliferation, survival, and epithelial-mesenchymal transition (EMT) are three fundamental events that are deeply linked to carcinogenesis. Hence, it is necessary to find a new combination of several therapies, targeting those vital mechanisms without causing side effects. Significant research works have shown differential low expression of the metabolic Farnesoid X receptor (FXR) in primary and metastatic prostate cancer suggesting their importance in prostate pathogenesis. Obicholic acid (INT 747), a potent FXR agonist is widely used in primary biliary cholangitis, and Fasting mimicking Diet (FMD) both were drastically showed effects on different cancer progression. The purposes of the present study were to test the hypothesis that FXR agonist INT 747 is a novel therapeutic target for prostate cancer progression and metastasis. Besides, to assess the synergistic effects of fasting-mimicking diet with INT 747 on PC-3 cells.

In the present work, the anti-proliferative and cytotoxic effects of FXR and FMD were analyzed by the MTT, colony formation and tumor spheroidal assays. While, the cell cycle distribution was performed by using fluorescent dyes propidium iodide (PI) and then analyzed by flow cytometry, The nuclear morphology of apoptotic cells stained with crystal violet were detected by phase contrast microscopy. Besides, this study conducted a series of *in vitro* experiments to investigate most of the biological phenotype steps involved in the metastasis such as wound healing (scratch) assay and matrigel invasion assay. Finally, this work Analyzed gene and protein expression by RT-qPCR and western blotting to elucidate how INT 747 and /or FMD functions in prostate cancer cell line.

The results of the present study showed that INT 747 treatment caused apoptotic morphological changes and significantly reduced the survival of human prostate cancer cell line (PC-3) cells incubated in normal medium. Furthermore, this study showed that the combination of the INT 747 and FMD was much more harmful to cancer cells than the treatment with INT 747 or FMD alone. Moreover, our study showed that INT 747 either alone or combined with FMD robustly induced cell cycle arrest at the G1 phase. Interestingly, the combination treatment on human prostate cancer cell line (PC-3) cells not only showed several lines of evidence of apoptotic cells death, but also inhibited carcinogenic potential and metastasis capacity as evaluated by impairment of spheroid formation capacity, delayed wound healing and matrigel invasion assays. Mechanistically, FXR agonism was also capable of withdrawing the molecular variations associated with Epithelial to –mesenchymal transition, which a crucial mechanism governing cancer cell migration and invasion, as was showed by reduced vimentin (a mesenchymal marker) expression. This anti-carcinogenic effect facilitated by FXR was also complemented by decreased mRNA gene expression of Matrix metalloproteinase 9 (MMP-9), which destroy the extracellular matrix. Furthermore, FXR activation resulted in proteolytic activation of procaspase -3 protein. The present study further revealed that FMD alone could restrained growth and metastasis of androgen refractory prostate cancer cells, but when combined with FXR synergistically augmented all the anti-cancer effects of FXR against these cells. In summary, our findings suggest that activation of FXR and improving its function through FMD could be a hopeful treatment option for mCRPC.

Keywords: FXR, FMD, Epithelial-Mesenchymal Transition, Cell Cycle, Migration, Invasion.

دور النظام الغذائي المحاكي للصيام لتعزيز مفاعل مستقبلات فارنيسويد إكس الأيضي لقمع الانتقال

والانتشار وتطور دورة الخلايا في ورم السرطان البروستاتا الخبيث

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الخلاصة

سرطان البروستات المقاوم هو احد أنواع السرطان الغير قابل للشفاء الى حد كبير حتى بعد العلاج المكثف بالعقاقير المتعددة. بعد الانتقال والبقاء والانتقال الثانوي ثلاثة أحداث أساسية ترتبط ارتباطاً وثيقاً بالسرطنة. وبالتالي، من الضروري إيجاد مزيج جديد من عدة علاجات تستهدف تلك الآليات الحيوية دون التسبب في آثار جانبية. أظهرت الأعمال البحثية الهامة انخفاض التعبير التفاضلي لمستقبلات فارنيسويد إكس الأيضي في

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سرطان البروستات الأولي والثانوي مما يشير إلى أهميتها في التسبب في أمراض البروستات. يستخدم حمض الاوبتي كولك، وهو أحد منبهات القوية لمستقبلات فارنيسويد إكس الأيضي ويستعمل على نطاق واسع في التهاب الأفتية الصفراوية الأولي. إضافة إلى ذلك قد أظهرت دراسات متعددة أن النظام الغذائي المحاكى للصيام له تأثيرًا كبيرًا على تطور السرطان المختلف. في هذه الدراسة، افترضنا أن لمستقبلات فارنيسويد إكس الأيضي والنظام الغذائي المحاكى للصيام قد يمنع الانتشار والنمط الظاهري النقلي في خلايا سرطان البروستات. تم إجراء تحليلات لتكاثر ونمو الخلايا، وتوزيع دورة الخلية، ومفاسات الهجرة والغزو، وتحليل التعبير الجيني والبروتيني في هذا العمل لتوضيح كيفية وظائف حمض الاوبتي كولك و/ أو النظام الغذائي المحاكى للصيام في خط خلايا سرطان البروستات. هذه الدراسة كشفت أن حمض الاوبتي كولك أدى إلى تغيرات مورفولوجيا سببها موت الخلايا المبرمج وقل بشكل كبير من بقاء خلايا خط خلايا سرطان البروستات البشرية المحتضنة في الوسط الطبيعي. علاوة على ذلك، أظهرنا أن الجمع بين حمض الاوبتي كولك والنظام المحاكى للصيام كان أكثر ضررًا للخلايا السرطانية من العلاج مفردًا. وإضافة إلى ذلك، أظهرت دراستنا أن حمض الاوبتي كولك كان إما بمفرده أو مدمجًا سبب لتوقف دورة الخلية المستحث بقوة في المرحلة صناعة الحمض النووي. ومن المثير للاهتمام، أن العلاج المركب على خلايا خط خلايا سرطان البروستات البشرية لم يُظهر فقط عدة خطوط من الأدلة على موت الخلايا المبرمج، بل أدى أيضًا إلى تثبيط إمكانية انتشار السرطان والقدرة على الانبثاث كما تم تقييمه من خلال ضعف قدرة تكوين كرويات السرطان، وتأخر حركة الخلايا وفحوصات الانتشار الخلوي والغزو السرطاني. ميكانيكيًا، تم التوسط في الغالب في التأثيرات المضادة للورم الخبيث والمضادة للبقاء من خلال تقليل تعبير كلا من مصفوفة البروتين المعدني وبروتين الفايمنتين. علاوة على ذلك، أدى إلى تنشيط بروتين بروكاسياس. باختصار، تشير النتائج التي توصلنا إليها إلى أن تنشيط مستقبلات فارنيسويد إكس الأيضي وتحسين وظيفته من خلال مرض النظام المحاكى للصيام بانه يمكن أن يكون خيارًا علاجيًا مأمولًا لـ سرطان البروستات النقلي والمقاوم.

الكلمات المفتاحية: مستقبلات فارنيسويد إكس الأيضي، النظام المحاكى للصيام، تغيير نمط الخلية، توقف دورة الخلية

Introduction

Prostate cancer (PCa) is the most prevalent invasive cancer in men worldwide and the second-leading cause of cancer-related death among men⁽¹⁾. In 2020, there were 1,898,160 cases and 608,570 deaths globally. The androgen-dependent stage of prostate cancer may be effectively cured with androgen-deprivation therapy (ADT)⁽²⁾. However, prostate cancer mortality happens due to reversion from androgen-dependent to androgen-independent prostatic growth⁽³⁾. As a result, the prostate cancer changed to metastatic castration-resistant prostate cancer (mCRPC) associated with high mortality risk, and short survival of only 16–18 months⁽⁴⁾. Failure of therapy in the androgen-independent is due to metastasis to adjacent tissues and chemo resistance of cancer cells. Based on that, urgent research is needed to explore new efficient therapeutic agents interfering with novel signaling pathways during the treatment of mCRPC^(3, 5).

The epithelial-mesenchymal transition (EMT) has been identified as a complex molecular and cell process involved in tissue reconstruction that plays essential roles in cell invasion, migration, and chemoresistance in many cancer types including prostate cancer⁽⁶⁾. During EMT, cells undergo transformation from epithelial to mesenchymal state⁽⁴⁾. The acquired mesenchymal features are cell mobility, invasiveness, acquiring stem cell characteristics, and protective resistance to apoptosis⁽⁷⁾.

Obticholic acid (INT 747), a potent FXR agonist is widely used in primary biliary cholangitis, can inhibit different diseases disorders by downregulation or upregulation of transcription of its target genes such as SHP, OATP, SREBP-1c, PPAR α , PEPCK and glucose-6-phosphatase which play an important role in metabolism^(8, 9). Furthermore, many epidemiological studies have shown that the use of INT 747 is associated with a reduction in the incidence of different types of cancers. Most notably in cholangiocarcinoma, hepatocellular cancer and colon cancer⁽¹⁰⁻¹²⁾.

However, whether there is activity against prostate cancer remains to be shown.

Fasting mimicking diet has fewer adverse effects, but the same benefits as traditional fasting, such as a drop in insulin-like growthFactor-1 (IGF-1) levels⁽¹³⁾. FMD has been shown to improve the therapeutic efficacy of anticancer drugs.^(14, 15) Moreover, previous studies showed that chronic caloric restriction reduces and delays cancer incidence, and inhibits tumor progression and metastasis⁽¹⁶⁾.

Consequently, our aim in this study is to assess the potential synergy of the treatment of INT 747 and FMD and determination their potential impact on proliferation, survival, and metastasis of castration-resistant prostate cancer cell line. This research evaluated these potential effects *in vitro* by the cell viability assay, spheroid formation assay, morphological assay, flow cytometry assay, and migration and invasion assays in prostate cancer cells.

Materials and Methods

Chemicals and their sources

Obticholic acid (INT 747) was obtained from MedChemExpress (South Brunswick, NJ). All chemicals used were of analytical grade.

Cell culture and fasting-mimicking condition

In this study PC-3 (human adenocarcinoma prostate cancer cells) was used as a model for mCRPC that is not caused by androgen⁽¹⁷⁾. Compared to other models of prostate cancer cell lineage, PC3 cells have a high metastatic potential⁽¹⁷⁾. Human PC-3 cells purchased from the ATCC, USA. PC-3 cells were cultured in various media containing penicillin and streptomycin and kept at 37 degrees Celsius in a humidified environment containing 5% CO₂. The control media (Control) consisted of 2g/L glucose RPMI 1640 medium (Euroclone, Italy) supplemented with 10% fetal bovine serum (FBS) and DMSO (Sigma-Aldrich). Fasting was emulated by incubating cells in this medium for 48 h, as described elsewhere, using glucose-free RPMI 140 (Euroclone, Italy)

supplemented with glucose 0.5 g / L (Santa Cruz, CA) and 1% FBS. (Di Tano et al. (2020).

Cell viability assay

PC-3 cell lines were seeded in 96-well culture plates and incubated overnight at 37°C in a humidified incubator. INT-747 was purchased from Medical Chemical Express. Cells were treated with various concentrations of INT 747 for 24, 48, and 72 hours or left untreated in the control group. INT 747 (was added either alone or in combination with FMD. The number of viable cells was determined by MTT assay using Promega CellTiter 96™ Nonradioactive Cell Proliferation Assay (MTT). Absorbance was measured by using plate reader. The optical density(OD) of 530 and 630 nm. The percentage of cell viability was calculated as the following equation as described , where OD is the measured optical density at 530 and 630 nm ⁽¹⁸⁾:

$$\text{Cell viability} = \frac{OD_{530,630}(\text{sample})}{OD_{530,630}(\text{control})} \times 100.$$

Cell morphological analysis

To visualize morphological changes, PC-3 cell lines at a density of 2.0×10^5 were seeded into sterilized coverslip mounted in a 6-well plate and exposed to INT 747 treatment with or without FMD for 48 h. Subsequently, the cells were washed with cold PBS and then fixed in 1% formaldehyde in DPBS for 10 minutes at room temperature. Next, the cells were stained with a mixture of 0.5% crystal violet in 20% methanol for 30 minutes. Then, the cells were washed two times with water and visualized using a phase-contrast microscope Optika, IM-3 (Italy) at 400× total magnification.

Cell cycle analysis

PC-3 was exposed to INT 747 treatment (8 μM) with or without FMD for 48 h. Next, the cells were trypsinized and recovered by centrifuging at 1000×g for 5 min. Then, the cells were washed by DPBS, fixed in 70% ethanol at 4 °C overnight and subsequently re-suspended in 400μL buffer containing PI and RNase for 30 min. The rates of cell cycle distribution were determined by Flow cytometry (BD FACSVerser™) and the pattern of cell distribution was determined using, BD FACSDiva™ Software (USA).

In vitro assessment of cell migration and wound closure scratch assay

It is an in vitro methodology widely used to estimate the healing capacity of different drugs⁽¹⁹⁾. In the current study, 150,000 cells/well of PC-3 cells were seeded in 24-well tissue culture plates at 37°C and 5% CO₂ for 48 h. Once the cells were a nearly confluent cell monolayer, the culture medium was removed, and a wound was created using a sterile 200 μL tip and the debris was removed by washing with PBS. Next, INT 747 and/or FMD were added to corresponding wells then incubated for 48 h. The cell migration and wound closure were detected at

diverse time intervals under an inverted microscope, and a digital camera captured images. The space area caused by cell migration at different time intervals was measured with the ImageJ software. The rate of wound closure was expressed as the ratio of wound closure (The wound area at 0 hours is set to 100%) and it measured through the following equation ⁽²⁰⁾:

$$\text{Wound closure (\%)} = \frac{(\text{Wound area at 0 h} - \text{Wound area at time})}{\text{Wound area at 0 h}} \times 100$$

Matrigel invasion assay

To investigate the effects of INT 747 and/ or FMD on invasiveness' features of PC 3 cells, CytoSelect™ 24-Well Cell Invasion Assay (CBA-100-C, Cell Biolabs, CA, USA) was used. A total of 300,000 cells/well (300 μL/well) suspended in either normal or FMD medium were added to the upper chamber, and 500 μL RPMI 1640 supplemented with 10% FBS was added to the lower chamber. Cells were immediately treated with the indicated concentration of INT 747 and incubated at 37°C. Cells were allowed to invade for 48h. The medium of the insert was aspirated and non-invasive cells were removed with a cotton-tipped swab. Cells were stained with cell stain solution, rinsed in dH₂O, and dried. Invaded cells were visualized and counted from five random fields with an inverted microscope (Optika, IM-3, Italy). Then, the optical densitometry was measured at a wavelength of 530 nm using a microplate reader. The cell images were analyzed using ImageJ software (ImageJ Software, NIH, USA).

Tumor sphere formation assay

Spheroids were produced using a previously described agarose-overlay method ^(21, 22). In short, a non-adhesive agarose plate was prepared by solidifying agarose solution (0.5% agar dissolves in a complete culture medium) into 24 well cell culture plates. Next, the PC-3 cells were suspended in the indicated media (normal or FMD) at a density of 50 cells/well and treated with INT 747 where indicated. Then the cell suspension was added to the solid agarose and incubated at 37 C at 5% CO₂. After 10 days of culture, tumor-spheroids were visualized, counted, and sized using light microscopy. The results of the tumorsphere formation assay were displayed as a relative fold decrease in size of tumorspheres formed compared to control and analyzed by ImageJ software ⁽²¹⁾.

Quantitative real-time PCR (qRT-PCR)

The RNeasy Micro Kit (Qiagen) was used to extract total RNA from cells that had been treated or not treated with INT 747 for 48 hours, according to the manufacturer's recommendations. 1 g RNA was applied to each sample for cDNA synthesis using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit, as directed by the manufacturer. The PerfectStart Green qPCR

SuperMix was used for the amplification stage in quantitative RT-PCR. All reactions were carried out. The RNeasy Micro Kit (Qiagen) was used to extract total RNA from cells that had been treated or not treated with INT 747 for 48 hours, according to the manufacturer's recommendations. 1 g RNA was applied to each sample for cDNA synthesis using the EasyScript One-Step gDNA Removal and cDNA

Synthesis SuperMix kit, as directed by the manufacturer. The PerfectStart Green qPCR SuperMix was used for the amplification stage in quantitative RT-PCR. All reactions were carried out in duplicate using the particular primers specified below, and all results were standardized to GAPDH, the housekeeping gene.

Table.1 sequences of primers used in qPCR

PCR primers	Forward	Reverse
MMP9	TTGACAGCGACAAGAAGTGG	GCCATTACGTCGTCCTTAT
GAPDH	TGCAC CACCAACTGCTTAGC	GGCATGGA CTGTGGTCATGAG

Western blots

The PC-3 cells were lysed with RIPA lysis buffer, complemented by a cocktail of protease and phosphate inhibitors. (Elabscience). Next, the cell lysates (30µg protein per well) in 5× loading buffer were resolved by 12% SDS-PAGE and transferred on polyvinylidene fluoride membranes (PVDF). After blocking with 5% skimmed milk for 1 h, the membranes were incubated with primary rabbit antibodies against GAPDH (1:2000, Elabscience), Vimentin (1:1000, Elabscience), Procaspase-3 (1:1000, Elabscience) overnight at 4 °C. The primary antibodies were detected with peroxidase-conjugated goat anti-rabbit IgG (H + L) secondary antibody (1: 5000). The blots were incubated with enhanced chemiluminescence substrate (Elabscience) and the signals were quantified using a Bio-Rad western blot analysis system (ChemDoc XRS Plus, Bio-Rad, USA) with ImageLab software (Bio-Rad Laboratories).

Statistical analysis

The data are presented as means with standard deviations (S.D.). Microsoft Excel and GraphPad Prism 7 were used to conduct statistical analyses (GraphPad Software Inc., San Diego, CA, USA). The Student's t-test or one-way ANOVA was used to compare the differences between the experimental and control groups, and statistically significant differences are indicated by asterisks as

follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Results

FXR activation and FMD inhibits PC-3 viability and induce apoptotic morphological changes

The effect of FXR and FMD on the growth and proliferation of prostate cancer cells was measured. In this study, PC-3 cells were treated by INT 747(8µM) in either, normal or fasting media conditions for 48 h of incubation, then cell viability was determined by MTT assay. As shown in (Figure 1. A), INT 747 reduced the degree of cell viability in the aggressive prostate cancer cells (PC-3), as compared to the corresponding control. In PC 3 cells treated with INT 747 in normal media (NCM), the cell viability was 67.17± 0.89, compared to untreated control cells. Interestingly, the incubation of PC-3 cells with INT 747 in FMD had a more noticeable effect (about a 3-fold decrease) on cell viability (34.9±0.32). These effects are visible by inverted microscopy (Figure 1B). Although cell shape and number are influenced by the INT 747 or FMD used only, combined treatment is much more powerful. The apoptotic phenotype included rounded cells with cytoplasmic blebbing, cells not attached well, and their number was reduced as compared to PC-3 cells treated with either INT 747 or FMD alone.

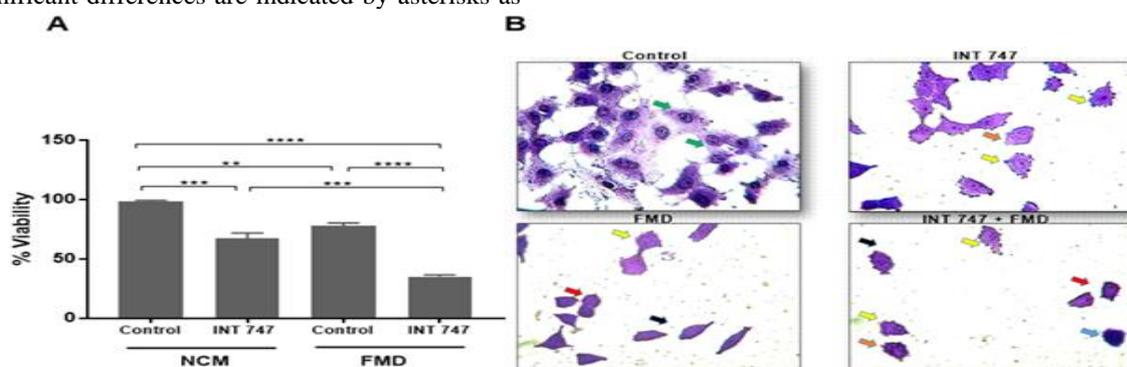


Figure 1 . Inhibition of viability by INT 747 in prostate cell carcinoma (PC-3) cells. (A) PC-3 cells were treated with 8 µM INT 747 in either normal medium (NCM) or FMD for 48 h. The cell viability was monitored by MTT assay. **(B)** Representative photomicrograph shows morphological changes of prostate cancer cells and imaged by inverted phase-contrast microscope (total magnification 400x). Arrows indicate (Green) control cell with intact nuclei, (blue) condensed nuclei, (red) cell shrinkage, (yellow) membrane blebbing (black) loss of cell-cell contact, (orange) nuclei are cracked into two or multiple apoptotic bodies.

FXR activation inhibits proliferation in prostate cancer cell lines in vitro by inducing G1 phase arrest and apoptosis

Next, this study investigated whether FXR and FMD might effect on cell cycle distribution and mediating the growth and proliferation inhibition on PC-3 cells. Cells treated with INT 747 and/or FMD were stained with PI and then analysed by flow cytometry. In the present study, as presented in Figure 2, there was a significant difference between the INT 747 incubated in normal media and untreated control in the cell cycle analysis. INT 747 in normal media caused a significant increase in

the cells in the G1 phase ($P < 0.029$), and decreased the percentage of S phase cells by 6.20 folds ($P < 0.0011$) as compared to control. Whereas in FMD alone group, cell growth was significantly arrested at the G1 phase where accumulated cells reached 75% as compared to cells incubated in control normal media ($p = 0.0004$). Remarkably, INT 747 combined with FMD has also caused a more significant increase ($P < 0.0001$) in the percentage of cells in the G1 phase in the PC-3 cell line and high increase in the sub-G1 apoptotic phase.

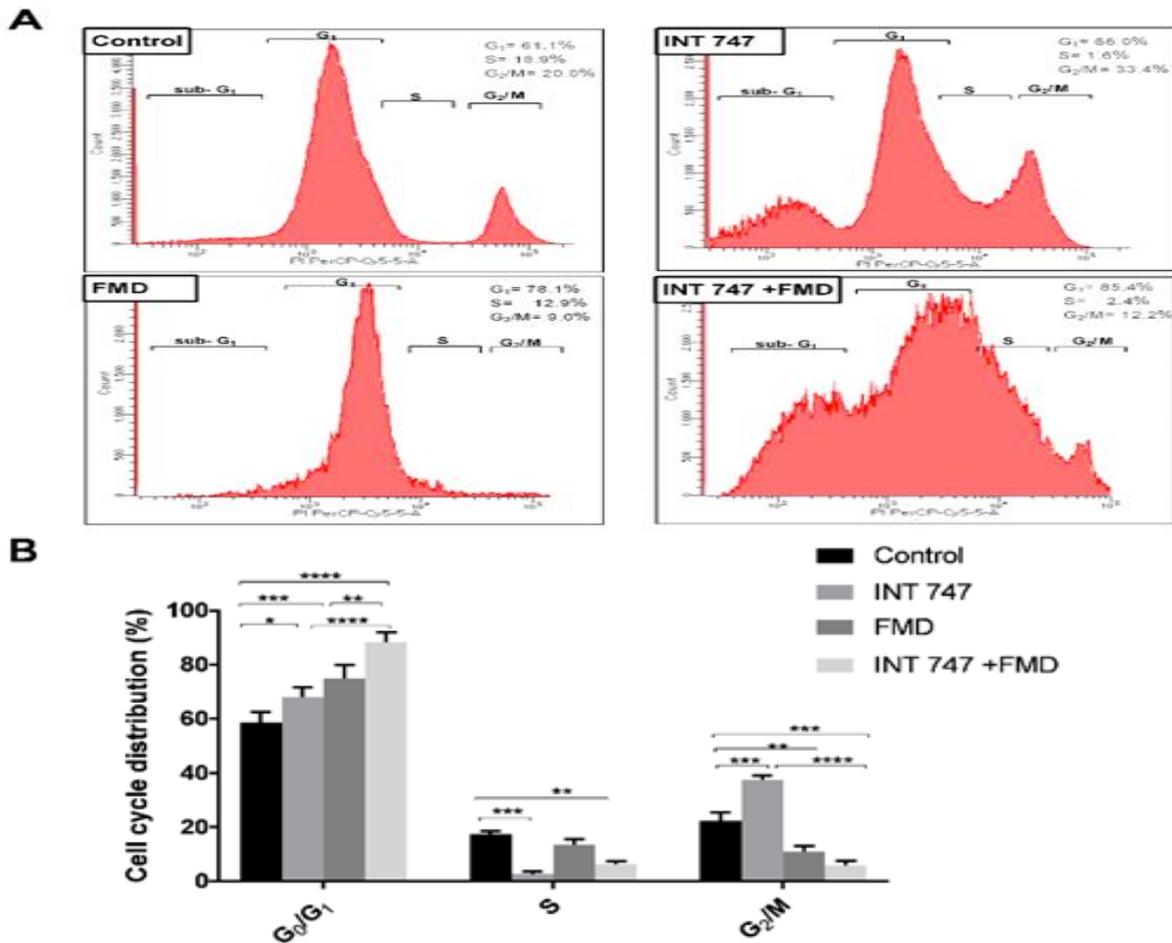


Figure 2. INT 747 induces G1 phase arrest of prostatic cancer cells. (A) Cell cycle detection of PC-3 cells following treatment with INT 747 at different media conditions. The percentages of each phase of the cell cycle were obtained by flow-cytometry analysis. Statistically significant differences between the control group and the treated groups were analyzed using GraphPad Prism 7 software and Differences between the groups were considered to be significant at * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$. (B) Quantification of sub-G1, G0/G1, S, and G2/M phase cells in cells

FXR and FMD inhibit the initiation of prostatic cancer spheroid formation

A 3-dimensional spheroidal formation assay was performed to determine whether FXR and/or FMD inhibits the initiation of cancer spheroids formation. The ability of a cell to grow and colonize in agarose indicates that the cells have an aggressive phenotype *in vitro* that contributes to its

invasiveness, metastatic, and resistance to therapy⁽²¹⁾. As shown in Figure 3, inhibition of tumorspheres by both INT 747 and FMD was markedly impaired compared to untreated cells. Specifically, the spheroid formation was almost totally impaired by INT 747 combined with FMD in comparison with the control group, ($P < 0.0001$). These results indicated that metastatic progression

of the PC-3 cells was suppressed by FXR activation and under tumor starvation.

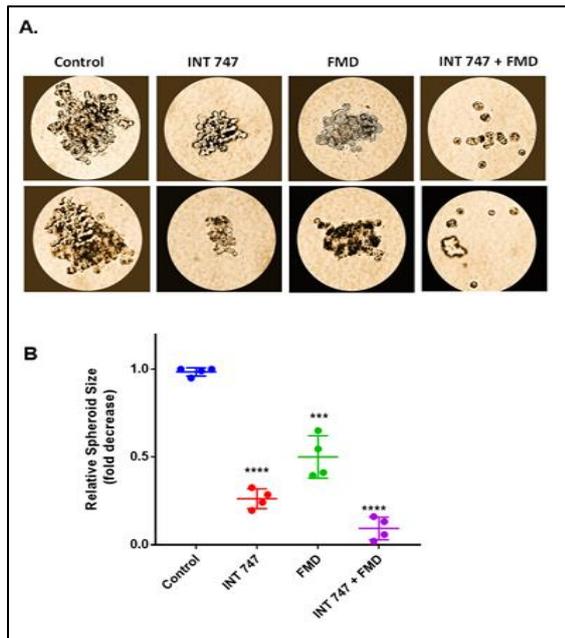


Figure 3. INT 747 treatment impedes prostate cancer cells spheroid formation. (A) PC-3 cells were grown in low adherent plates (prepared by pre-coating with 0.5% agarose in RPMI 164 media), and treated with INT 747 and/or FMD. After 10 days, the spheroids were photographed. (B) The relative Spheroid size was counted by ImageJ and performed bar diagram.

FXR activation and FMD suppresses in vitro cell migration of prostate cancer cells

The wound-healing assay was performed to investigate the effect of FXR on prostate cancer cell migration, a key event in carcinogenesis. In this study, PC-3 cells were imaged following treatment with 8 μM INT747 with or without FMD at the same marked site. The difference in wound width was measured at three-time points (0 h, 24 h, and 48 h). As shown in Figure 4, the group treated with INT 747, demonstrated significantly impaired wound healing ability after 24 and 48 hours (Fig. 4 B, P<0.001) compared with controls. The cell-free area increased by approximately two folds after 24 h compared to INT 747-free PC-3 cells. This inhibition effect was even stronger after 48 h when cells were treated with INT 747 at the same dose (Figure 4 A.B). Interestingly, INT 747 in combination with FMD highly significantly reduced cell migration of PC3 compared to INT 747 alone. Moreover, FMD alone was as effective as INT 747 treatment in inhibiting cell migration. These data indicated that FXR and /or FMD negatively regulated prostate cancer cell migration.

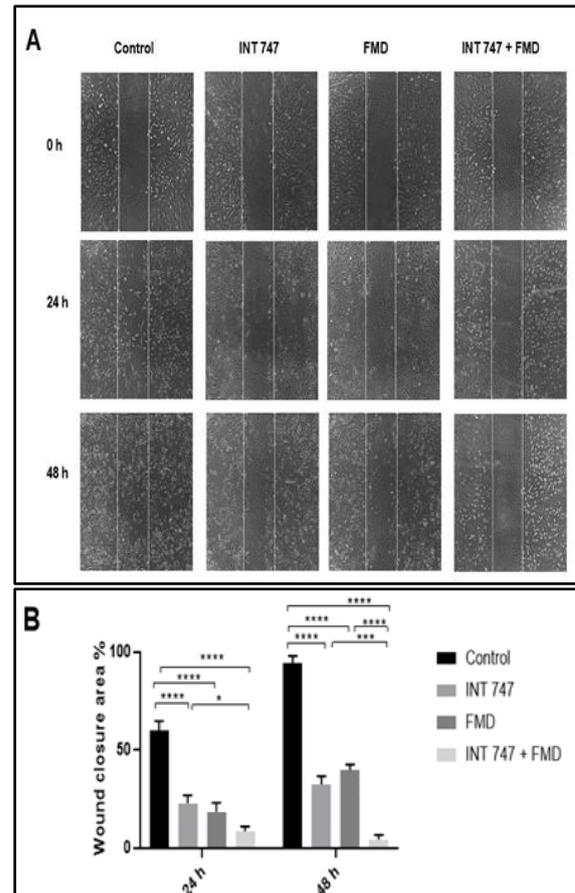


Figure 4. INT 747 inhibits prostate cancer cell migration in vitro (A) Inverted microscopy (at lower magnification lens 4 X) shows that the wound closure area of PC-3 cells control monolayers is nearly closed after 48 h. In cells treated by different conditions of the INT 747 and/or FMD, the closure of the wounded area is calculated. (B) The images were analyzed using the Image J software to evaluate the scratch area after 24, 48 h respectively. The diagram shows the average value and standard error of the three experiments in quadruples. Test Anova followed; ***p < 0.001. Scale bar in (A): 200 μm.

FXR activation and starvation synergistically inhibit prostate cancer cell invasion

An invasion assay was performed to further explore the role of FXR in the invasiveness' capacity of prostate cancer. In this study, PC-3 cells treated with INT 747 in the presence and absence of FMD for 48 hours. As shown in Figure 5, treatment of PC-3 cells with INT 747 considerably reduced cellular invasion by about more than 2.5 folds (p = 0.0045 vs. untreated PC-3 cells). Likewise, significant differences were also noted for PC-3 cells incubated in FMD alone (p > 0.0425 vs. untreated PC-3). Furthermore, FXR activation in cells incubated in FMD largely reduced cellular invasion by more than 8 folds (p = 0.0009 vs. untreated PC-3 cells). To determine the possible causes of reduced cancer cell invasion and migration

during activation of FXR, the expression of MMP9 was analyzed by RT-PCR. As shown in Figure 5, we observed that MMP9 mRNA expression was relatively very low in PC-3 cells treated with either INT 747 or FMD alone. Remarkably and consistent with our result, INT 747 combined with FMD highly decrease MMP9 expression level ($p > 0.0056$ vs. untreated PC-3).

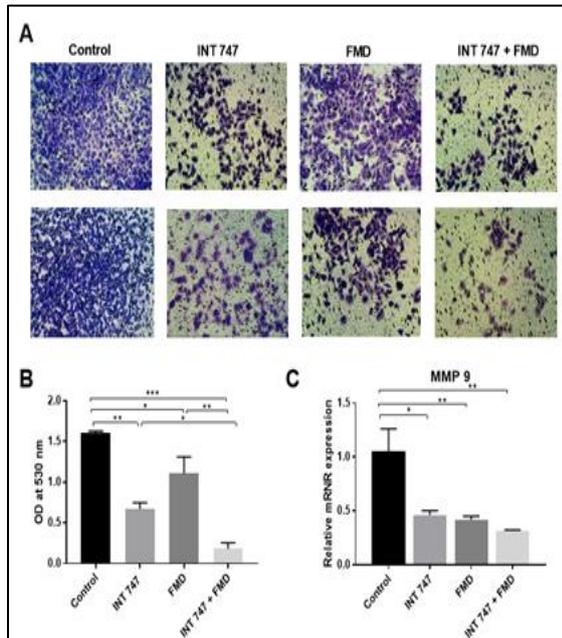


Figure 5. The combined inhibitory effect of FXR and FMD on the invasion of PC-3 cells. (A) Images of the invaded PC-3 cells that were treated with INT 747 (8 μ M) in the presence or absence of normal or FMD. (B) Quantitative analysis was performed by destaining and reading the OD at 530 nm. (C) The levels of gene expression were estimated with the relative qRT-PCR method (a fold-change of untreated control samples normalized to GAPDH). Values are presented as means \pm S.D. from three independent experiments performed in triplicates. (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ vs control). Scale bar=100 μ m.

FXR inhibits epithelial-to-mesenchymal transition and survival of PC-3 cells via vimentin suppression and procaspase-3 proteolytic activation

Epithelial-to-mesenchymal transition (EMT) and apoptosis are two of the most important processes in enabling cancer cells to have increased survival, migration, and invasive capacity (7, 23). Therefore, key biomarker molecules of these signaling pathways were evaluated. According to functional studies, the inhibitory effects of FXR and/or FMD on survival and EMT are consistent with Western Blotting analysis. Which indicated a significant decrease in the mesenchymal marker; vimentin in the PC-3 cell incubated with INT 747 (Fig.6). Remarkably, our results further showed that the protein levels of full-length procaspase in PC-3 cells significantly decreased upon INT 747

treatment, which is decreased due to proteolytic cleavage and activation to promote the apoptotic cell death. Moreover, we also found that FMD alone also significantly decreased the protein levels of vimentin and procaspase3 as compared to the PC-3 cells in the control group. Interestingly, we observed that both vimentin and procaspase-3 expression were relatively very low in PC-3 cells treated with the combination treatment as compared with control ($p > 0.008$, $p > 0.0001$ respectively).

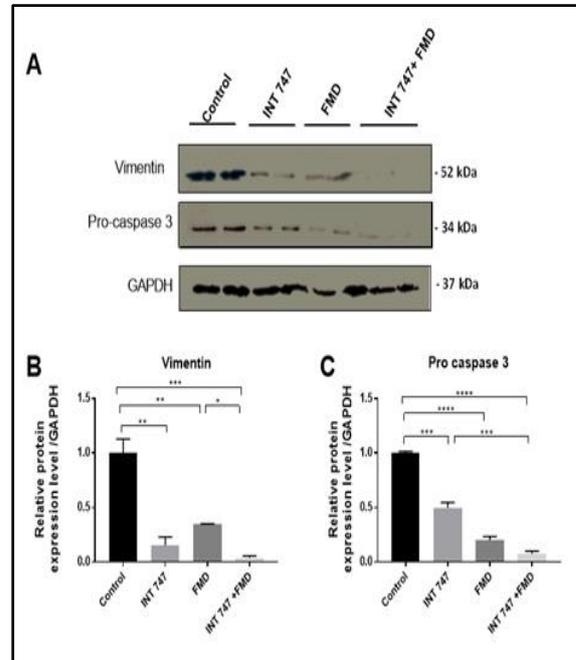


Figure 6. The effects of INT 747 and/or FMD on both EMT and apoptosis-related protein expression in PC-3 cells. (A) Cells were treated with INT 747 and/or FMD for 48h. Cell lysates were analysed by Western Blot. Here, GAPDH is detected as internal control. (B) Protein bands were quantified by ImageJ software, standardized with GAPDH levels, and expressed as normalizations of control. The significance was determined by one way Anova (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

Discussion

In many patients with metastatic castration-resistant prostate cancer (mCRPC), the hormonal therapy can be ineffective due to the high resistance of cancer cells to various anticancer treatments. Thus, innovative non-AR-dependent treatment approaches should be explored in the future. In several studies, the induction of cell cycle arrest and apoptosis have been emphasized as a crucial targets of controlling the infinite growth of cells⁽³⁾. FXR is a bile acid orphan nuclear receptor (NR), which is important in the homeostasis of bile acids, glucose metabolism and lipid metabolism^(24, 25). Recent evidence also suggests that FXR plays an important role in apoptosis and cancer.^(26, 27)the inhibitory effects of FXR agonists have been investigated in some other tumors, such as liver, colon, breast cancer^(11, 28, 29). In the present study, we further

validated the above results by assessment its inhibitory role on prostate cancer. Furthermore, a drug combination study was utilized to identify whether the fasting mimicking diet (FMD) act synergistically with INT 747 in prostate cancer cells. PC-3 cell cultures was used that recapitulate the typical features of metastatic human androgen independent adenocarcinoma, including high representation of CSC markers and EMT traits, and high cancerogenic potential in vitro and in vivo ⁽³⁰⁾.

In this study, we first showed that INT 747 had a significant inhibitory effect on the viability of PC-3 cells (Fig. 1). In particular, an 8 μ M INT 747 treatment was sufficient to inhibit PC-3 cell growth approximately half that of non-treated cells at 48 h after treatment. In the next step, the effect of FXR on the cell cycle was studied. As shown in Figure 2, flow cytometry indicated that before inducing cellular apoptosis, INT 747 also arrest cell cycle by increases the number of cells in the G1 phase and decreases the portion of those in the S1 phase cells. Cells subsequently accumulated in the sub-G1 phase of the cell cycle, suggesting that INT 747 mediated sequential cell cycle arrest and apoptosis. The morphological and cell cycle quantitative assessments in this study further suggested that INT 747 treatment-induced cell death and eventually cell cycle arrest. The cell cycle is the basis for cell proliferation ⁽³¹⁾. Our results further show that INT 747 increases the fraction of cells in the G1 phase of the PC-3 cells, illuminating that some cells are blocked at the G1 phase. Cell arrested of cell cycle progression provide an opportunity for cells to either undergo repair mechanisms or follow the apoptotic pathway ⁽³²⁾. When apoptosis is initiated, it ultimately leads to a repair failure of DNA damage; this result is catastrophic for cell proliferation ⁽³³⁾. The apoptotic cell death is also confirmed by the decreased protein levels of procaspase-3 due to proteolytic cleavage and activation. However, on using an antibody specific for procaspase-3, which detects the full-length 32-kDa form which according to the manufacturer's instructions; therefore, we could not detect the cleaved form. Caspase-3 plays an important in the execution of apoptosis and leads to the characteristic of morphology changes of the final event of apoptosis such as nuclear condensation and fragmentation ⁽³⁴⁾. In agreement with other studies, FXR agonists induced procaspase-3 proteolytic cleavage in many cancer cells ^(11, 35). These findings suggest INT 747 modulates cell cycle regulatory machinery leading to S cell cycle arrest, and it prompts the caspase-3 activation triggering apoptotic pathway. The combination of INT 747 plus FMD exerts synergistic tumor inhibition in PC-3 human prostate cancer in vitro and in vivo by cooperatively induced cell cycle arrest and cell apoptosis. These findings confirm those of earlier studies, such as, fasting has

been shown to be multi-functional on tumor progression ⁽³⁶⁻³⁸⁾.

Epithelial-mesenchymal transition (EMT) is identified to play an important role in cancer development, metastasis and drug resistance ⁽³⁹⁾. This research demonstrated that FXR and FMD activation inhibited the migratory and invasiveness capacity of PC-3 cells. Based on the cell migration photomicrographs, PC-3 cells incubated with approximately 8 μ M of INT 747 have displayed wide wound gaps between cells compared to untreated cells after 48 h. however, wider wound gaps were noted as PC-3 cells incubated in combination of both INT 747 and FMD, meanwhile, the untreated cells filled the entire wound gaps at 48 h.. Similar to the cell migration data, the combination of INT 747 and FMD produced a decrease in cellular invasion of PC-3 cell line equal to the calculated additive effects of INT 747 or FMD used alone. Accordingly, the anti-invasiveness additive effect of the combination of INT 747 and FMD was also confirmed by a synergistic loss of matrix metalloproteinase9 (MMP9) and vimentin expression in PC-3 cells. Many previous studies showed that both MMPs and Vimentin in general have been involved with the EMT and other stages of malignancy, including primary tumor growth, angiogenesis, invasion of the basement membrane and stroma ^(4, 17). Consequently, this study is consistent with earlier studies, which showed that FXR activation by INT 747 treatment impaired the invasive and migratory potential of colon cancer cells by arresting EMT through vimentin repression ⁽²⁹⁾. Another earlier study also found that INT 747 abrogated the induction of IL-6-induced EMT in an intrahepatic cholangiocarcinoma in vivo model and thereby inhibit their metastasis potential ⁽²⁸⁾. Moreover, may scientific works also showed that FMD suppressed the invasion of various cancer cells including liver and breast cancer cells ^(38, 40). Very few studies have investigated INT 747 anti-cancer effects in cancer therapy. In this context, we demonstrate that INT 747 combined with FMD has an additive impact on prostate cancer cell viability, spheroid formation and invasion, and synergistically decreases cell migration.

Conclusion

This study demonstrated for the first time the FXR ligand INT 747 that is commonly used to improve bile excretory function; becomes potentiated in their anticancer activity by Fasting mimicking diet (FMD) in prostate cancer cells. The present paper documents the anti- proliferative, anti-EMT and anti-metastatic capabilities of INT747 and/or FMD and lists the mechanisms that may cause these effects in prostate cancer cells. At cellular level, these effects are mediated mainly by suppressing Vimentin and MMP-9 expression; promote apoptosis and cell cycle arrest, which all

further supports the notion that FXR agonist could be a negative regulator of androgen refractory prostate tumorigenesis. This work also demonstrated that FMD synergistically enhanced the efficacy with which INT 747 blocks cell cycle signaling and consequently affects crucial events in tumor survival, invasion, and metastasis. These results are particularly important as they provide new possibilities for specially designed fasting-mimicking diet programs in combination with FXR agonists in oncology. It could replace much more toxic and less effective chemotherapy treatments.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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