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Research Article

# Synergistic effect of obeticholic acid and fasting-mimicking on proliferative, migration, and survival signaling in prostate cancer

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## Abstract

The systemic and resistant nature of the androgen-independent stage of prostate cancers makes it largely incurable even after intensive multimodal therapy. Apoptosis and epithelial-mesenchymal transition (EMT) are two fundamental events that are deeply linked to carcinogenesis. Hence, it is necessary to find a new combination of several therapies targeting apoptosis and EMT without causing side effects. Several recent studies have indicated that the Farnesoid X receptor is extensively associated with human tumorigenesis. The FXR agonist obeticholic acid (INT 747) has preliminarily exhibited a tumor suppressor potential. In this present study, we assess the potential synergism of FXR activation under nutrient deprivation in prostate cancer cell lines to investigate whether FXR activation enhances starvation-induced apoptosis in PC3 cells. In this study, PC-3 treatment with INT 747 significantly repressed cell proliferation and clonogenic potential. In addition, it significantly induced apoptosis of PC-3 cells and decreased their cancerogenic potential, as evaluated by annexin v apoptosis and transwell migration assay, respectively. The decreased expression of pro-caspase 3 by western blot analysis further confirmed INT 747-induced apoptosis. Furthermore, the fasting-mimicking diet (FMD) potentiated the antiproliferative, pro-apoptotic, and antimetastatic effects of INT 747. Mechanistically, these effects were mediated through the downregulation of cyclin D1 and upregulation of PTEN. In conclusion, INT 747 alone markedly decreases, and when combined with FMD abrogates the growth and migration of PC-3 cells.

## Keywords

FXR, FMD, prostate cancer, proliferation, apoptosis, migration

## Introduction

Prostate cancer (PCa) is the most common male malignancy in many regions of the world and the second leading cause of cancer-related death among men worldwide (Kimura and Egawa 2018). Androgenic phase in PCa can be efficiently cured by chemical castration (Ebner et al. 2021). Unfortunately, the death rate of prostate cancer is caused by bone and lymph node metastases and the reversion of androgen-dependent prostate growth to androgen independent (Westhofen et al. 2021). The failure of androgen-independent therapy is due to metastasis and chemoresistance of cancer cells. Based on this, innovative therapies are needed to interfere with new signals during the establishment of metastatic CRPC.

Belonging to the super-family of metabolic nuclear receptors, the bile acid Farnesoid -X receptor (FXR) shows

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a vital role in the regulation of the homeostasis of bile acid (J. M. Lee et al. 2014). However, evidence was accumulating supporting the essential role of FXR in the formation of human tumors (Peng et al. 2019). The reduced FXR levels of mRNA and protein was reported in prostate cancer tissue and even more significant reduction was observed in CRPC (Z. Wang et al. 2018). However, FXR functions in the androgen-independent stage of prostate cancer, and the exact molecular mechanism remains unidentified.

Many previous studies show that chronic caloric restriction reduces and delays cancer incidence, and inhibits tumor progression and metastasis (Wang 2018). Fasting mimicking diet (FMD) has the same benefits as traditional fasting, such as a drop in IGF-1 levels, but with less adverse effects (Stefano et al. 2016). FMD has been shown in studies to improve the therapeutic efficacy of many anticancer drugs (De Groot et al. 2020; Di Tano et al. 2020; Salvadori et al. 2021).

Therefore, the purpose of this study is to assess the effects of obeticholic acid (INT747); a potent bile acid derived FXR agonist; on the growth, survival and metastatic growth of PC-3 cells. However, the intricate tumor heterogeneity, on the other hand, may limit the efficacy of INT 747 alone in cancer treatment (Di Ciaula et al. 2017). Consequently, this study was aimed to evaluate the potential synergy of the treatment of INT 747 and FMD to determine their potential impact on proliferation, survival, and metastasis of castration -resistant prostate cancer.

# Materials and methods

## Chemicals and their sources

Obeticholic acid (INT 747) was obtained from MedChemExpress (South Brunswick, NJ) and. Purified Guggulsterone (GS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used were of analytical grade.

## Cell culture and fasting mimicking condition:

In this study, PC-3 (human prostate cell carcinoma) was used as a model for prostate cancer that is not caused by androgen (Huang et al. 2011). PC-3 cells were purchased from (ATCC, USA) and were used according to the rules of the Code of Ethics of the World Medical Association. Cells were grown in two different types of medium and kept at 37 °C in a humidified environment containing 5% $CO_2$. The control media (Control) consisted of 2 g/L glucose RPMI 1640 medium (Euro clone, Italy) supplemented with 10% fetal bovine serum (FBS) and 0.1% DMSO (Sigma-Aldrich). While FMD was emulated using glucose-free RPMI 1640 medium (Euroclone, Italy) supplemented with glucose 0.5 g / L (Santa Cruz, CA) and 1% FBS , then cells were incubated in this medium for 48 h, as described elsewhere (Stefano et al. 2016; Di Tano et al. 2020).

## Cell viability assay:

PC-3 cell lines were -seeded and kept overnight at 37 °C in a -humidified incubator. Cells were treated with several 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 amount of live cells was estimated using the MTT assay using Promega Cell-Titer 96 Nonradioactive Cell Proliferation Assay as described (Yang et al. 2019). Cyclophosphamide was used as a positive control. The IC50 values of INT 747 against PC-3 cancer cell line were calculated using GraphPad Prism 7. The percentage of cell viability was calculated as the following equation:

$$\text{Cell viability \%} = [\text{OD530, 630 (sample)} / \text{OD530, 630 (control)}] \times 100.$$

$$\text{Cytotoxicity \%} = 100 - \text{Cell viability \%}$$

## Anchorage-Independent Colony Formation Assay (CFA)

The cells (2 ml) were grown in a 6-well plate at 500 cells per well, then kept overnight for 24 hours. After that, the cells were rinsed in PBS, and the medium was replaced with a new regular RPMI 140 or FMD medium. The PC-3 cells were then kept in incubator for 2 weeks at 37 °C and 5% CO2 with INT 747 added, where indicated. Every three days, a new media was introduced. Finally, the colonies were washed twice in PBS, fixed in PBS with 2.5 percent glutaraldehyde, stained in 20% Me-OH with 0.4 percent crystal violet solution, and then imaged.

## ROS detection by the nitrogen-blue tetrazolium assay

The NBT assay was used to assess the amount of ROS produced by the treated cells. The lowering of NBT by ROS causes a proportionate change in the medium's light absorption at 630 nm. Briefly, PC-3 cells were grown in triplicate in a 96-well plate at a density of 1104 cells/well and incubated overnight before being treated with INT 747/ and/or FMD for 48 hours. After removing the culture fluid, cells were incubated for 1 hour at 37 °C in the dark in PBS containing 1 mg / ml of NBT. The absorbance of NBT was measured at 630 nm using an ELISA plate reader after it was reduced by ROS to a dark blue insoluble form of NBT that was solubilized in a KOH 2 M and DMSO 5 M solution.

## Transwell migration assay

300,000 cells/well (300 µL/well) suspended in either normal or FMD medium were added to the upper chamber of a transparent PET membrane with a 8.0-µm pore size (SPLInsert™). 800 µL RPMI1640 supplemented with 10%FBS was added to the lower chamber. After that, cells were immediately treated with the indicated concentration of INT 747 and incubated at 37 °C to allow them to

migrate for 48 h. The cells on the top chamber of the insert were removed and migrated cells on the lower part of insert were fixed with methanol, discolored with crystal violet, and then the OD was measured at 530 nm by using a plate reader.

## Annexin V/PI apoptosis assay

Fluorescence cytometry and Annexin V-FITC Apoptosis Kit (Elabscience) were used to detect apoptotic cells. PC-3 cells were grown at a density of $1\times10^4$ cells per well in 6-well plates. When cells were 60% confluent, they were refreshed with new specified media and then exposed to INT 747 and/or FMD for 48 hours. The trypsin-free floating and adhering cells were collected and centrifuged after incubation. The cells collected were washed twice in phosphate buffer saline, then mixed in 1X binding buffer (100 µl), and kept for 20 minutes at room temperature with annexin, double staining solution V / PI. Flow cytometry (BD FACSVerseTM) was used to evaluate stained cells and the percentage of apoptotic cells was determined using, FlowJo 10.2 software (TreeStar, Ashland, OR, USA).

## Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the PC-3 cells using TRIzol (Gibco; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, then reversely transcribed into cDNA using the Easy Script One Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech Co., Ltd.). Then, the PerfectStart Green qPCR Super Mix (TransGen Biotech Co., Ltd.) was used for the amplification stage in quantitative RT-PCR., and all results were standardized to GAPDH, the housekeeping gene. All reactions were carried out in duplicate using the particular primers specified below in (Table 1).

**Table 1.** PCR primers.

| PCR primers | Forward | Reverse |
|---|---|---|
| cyclin D1 | AAACAGATCATCCGCAAACAC | GTTGGGGCTCCTCAGGTTC |
| PTEN | AAGGGACGAACTGGTGTAATG | GCCTCTGACTGGGAATAGTTAC |
| GAPDH | TGCAC CACCAACTGCTTAGC | GGCATGGA CTGTGGTCATGAG |

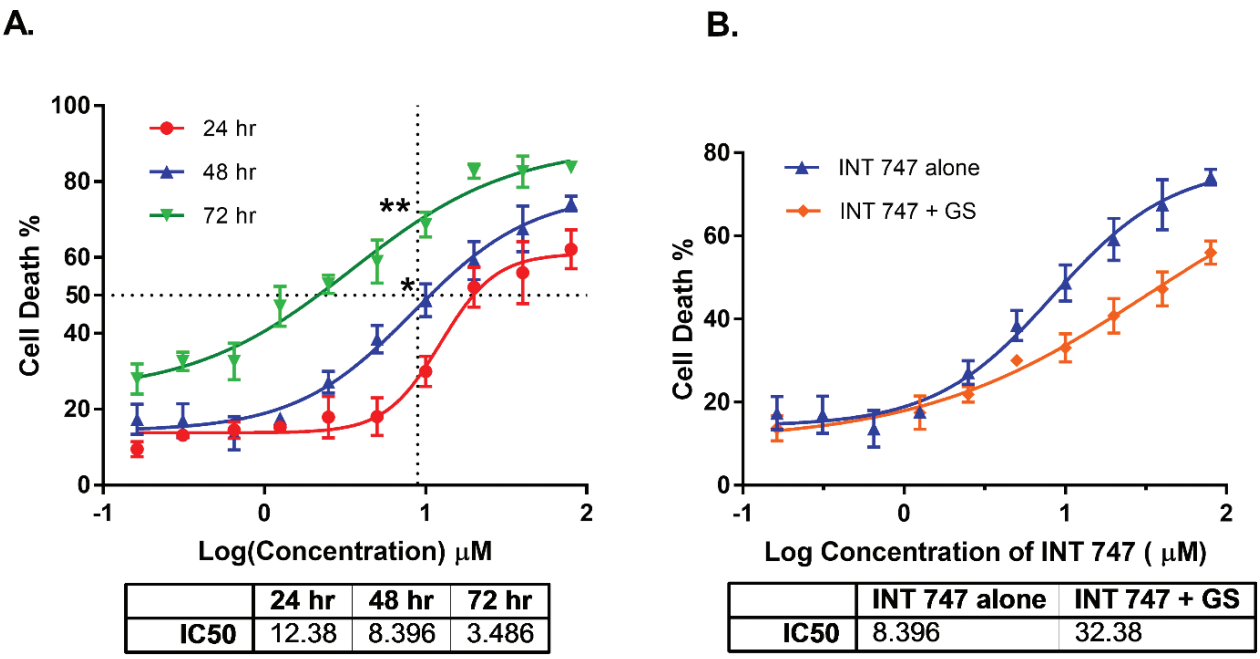
## Statistical analysis

Microsoft Excel and GraphPad Prism 7 were used to conduct statistical analyses (Graph Pad Software Inc., San Diego, CA, USA). Student's t test or one-way ANOVA was used to compare differences between means with SD, 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

## INT 747 represses the cell viability of prostate cancer cells

As shown in (Fig. 1A), PC-3 cell viability decreased significantly over time due to increased INT 747 concentrations, indicating that INT 747 may inhibit PC3 cell viability in a time-dependent and dose -dependent manner. However,




| | 24 hr | 48 hr | 72 hr |
|---|---|---|---|
| IC50 | 12.38 | 8.396 | 3.486 |

| | INT 747 alone | INT 747 + GS |
|---|---|---|
| IC50 | 8.396 | 32.38 |

**Figure 1.** Effect of INT 747 on prostate cancer cell proliferation. **(A)** Time course dose-response curves for PC-3 cells treated with INT 747 for determinations of IC50. **(B)** Dose response plots of INT 747, alone and combined with the FXR antagonist, GS, on PC-3 cell lines after 48 h exposure, as detected by MTT assay.

pretreatment with the FXR antagonist (Z) -Guggulsteron (GS) has been shown to effectively inhibit the cell cytotoxicity caused by INT 747 (Fig. 1B). GS (10 μM) caused a 4 fold increase in the IC50 of INT 747 in PC 3 cell lines at 48 h , These data showed that INT 747 induce cell cytotoxicity of prostate cancer cells specifically through FXR dependent manner.

## The starvation conditions potentiate the antiproliferative effects of INT 747 in cultured cancer cells

Next, we investigate whether FMD potentiates the anticancer effect of INT 747 against the prostate cancer cell line model. As shown in Fig. 2, the viability of prostate cancer (PC-3) after 48 and 72 h was significantly reduced to INT 747 and FMD when both were used as a single agent. Interestingly, the viability of PC-3 cells grown under a combination of FMD conditions and INT 747 treatment was strongly reduced (Fig. 2A, B). Morphology studies have also shown that the cell proliferation is significantly inhibited and cells exhibit morphological changes such as cell loss and cell separation. As shown in Fig. 2D. Furthermore, 8 μM doses of INT 747 were administered in colony formation assays in the presence and absence of FMD. The colony was smaller in size and number with the combination of INT 747 and FMD (Fig. 2E). Quantitative analysis revealed further that the number of colonies decreased considerably by incorporating FMD into the INT 747 treatment. (Fig. 1F, left panel). In general, when FMD was added to the INT 747, the average surface of a single clone was also reduced. (Fig. 1F, right panel).

## Apoptotic effects of the combination of FXR activation and FMD on PC-3 cells

To further confirm that FXR could induce apoptosis, PC-3 cells were treated with INT 747 and / or FMD, double stained with annexin V-FITC/PI then the degree of apoptosis was measured by flow cytometry The results (Fig. 3A and B) indicate that cells have undergone early and late apoptosis. The percentage of early apoptosis cells treated with INT 747 or FMD alone was 22.5% and 15.6%, respectively, compared to the control (4.13%). A similar trend has also been observed for the population of late apoptosis. The percentage of late apoptosis cells treated with either INT 747 or FMD was 21.7% and 13.3%, respectively, compared to the control (1.64%). However, the combination of INT 747 and FMD increased the population of early and late apoptotic cells by about 6 and 58 times, respectively, compared to untreated control cells (Fig. 3B). Furthermore, decreased level of pro-caspase-3 (Fig. 3D) confirmed that INT 747 could induce apoptosis in PC-3 cells.

## Effect of FXR activation and /or FMD on genes associated with cell proliferation

Because FXR has been shown to decrease cell growth, as a result, we further studied the impact of treatment on gene expression involved in cell proliferation and survival under nutrient deprivation. As shown in Fig. 4, PTEN increased substantially with INT 747 treatment, according to quantitative PCR analysis. Interestingly, and consistent with their observed effects on cell growth and proliferation, coupled FMD and FXR amplified levels of PTEN expression by about 3.2 folds as compared to control cells. Comparatively, more than two folds in response to FXR activation or FMD alone attenuated the expression of cyclin D1 at the mRNA levels. Finally, FMD cooperated significantly with INT 747 to reduce the level of cyclin D1 in PC3 (Fig. 4b).

## Involvement of ROS production in INT 747 and /or FMD-treated prostate carcinoma cells

Notably, our study showed that the combination of FMD and INT 747 strongly induced cell death in PC-3 cells line (Fig. 2), suggesting that oxidative stress may either participate in mediating this cytotoxic effect or may be a secondary event; therefore, the NBT assay was performed to determine the level of ROS in PC-3 cells. Consistently, and as shown in Fig. 5, there was increase in production of ROS (i.e. increase NBT reduction )was observed in PC-3 cells treated with INT 747 relative to the untreated control, while more statistically significant differences ($p < 0.001$) in percentage of reduction in NBT were observed in PC-3 cells incubated in FMD alone. In fact, the combination of FMD and INT 747 exacerbated ROS production in PC-3 cells compared to control cells.

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

To study the effect of FXR on the migration of prostate cancer cells, a key event in carcinogenesis, PC-3 cells were tested for transwell migration. The treatment of PC-3 cells with INT 747 remarkably reduced cellular migration by more than 4 folds ($p < 0.0001$ versus control). However, there were no significant differences in incubated PC-3 in FMD alone ($p > 0.0425$ versus untreated). Remarkably, FXR activation in cells incubated in FMD largely reduced cellular migration by more than 30 folds ($p < 0.0001$ vs. untreated), as shown in (Fig. 6).

# Discussion

In this study, we show for the first time that the FXR agonist INT 747 that is commonly administered to improve bile excretory function becomes potentiated in its activity by fasting mimicking diet (FMD) in prostate cancer. The main findings of our study indicate that in human androgen-independent prostate cell cultures: INT 747 had anti -neoplastic effects by reducing the survival and viability of PC -3 cell lines in time and dose dependent ways. A concentration of 8.4 μM of INT 747 for 48 h was successful

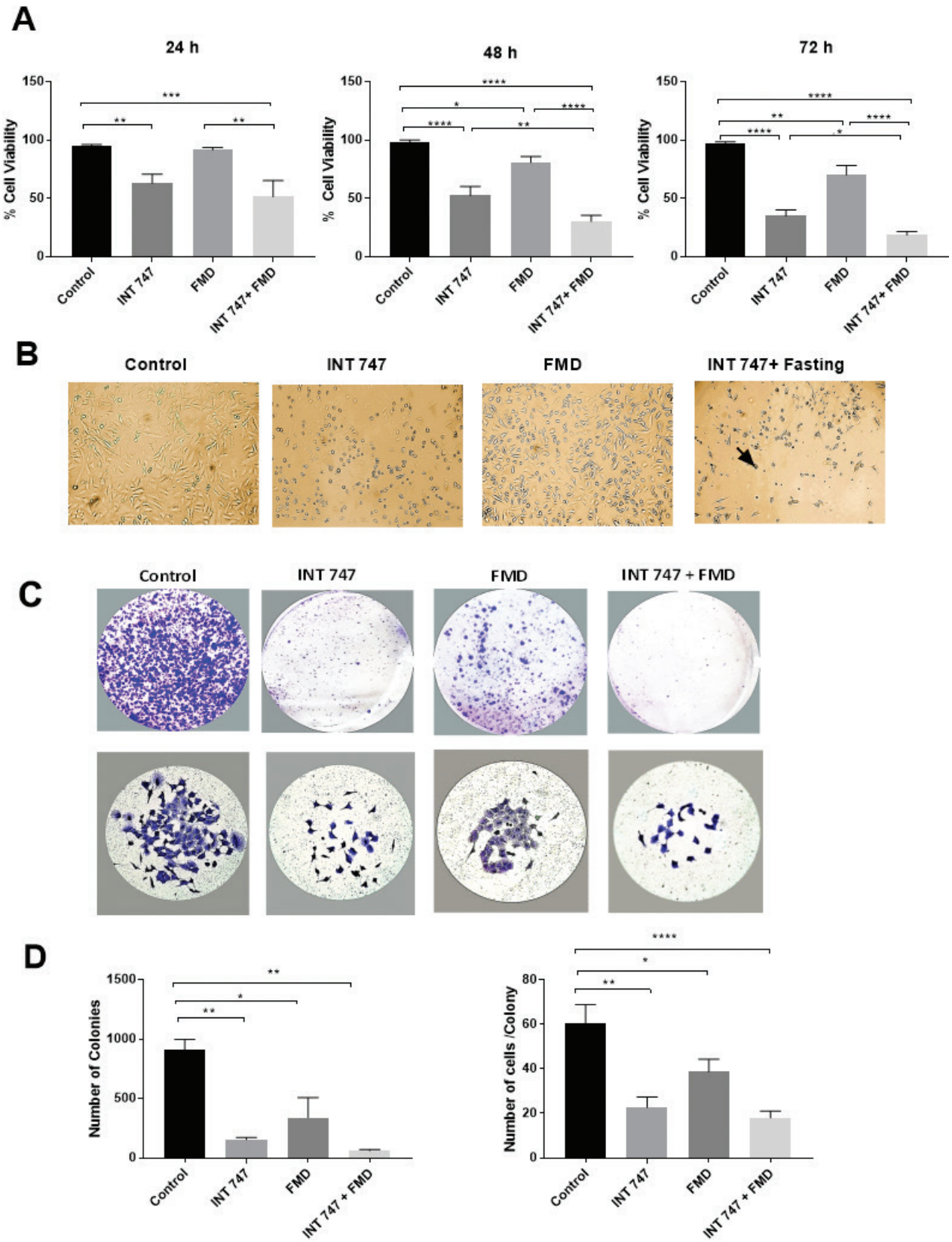



**Figure 2.** FMD improved the inhibitory effect of INT 747 on the growth of prostate cancer cells. **(A)** The cell viability assay was performed on cells growing in regular medium with DMSO (Control) or starvation medium (FMD) After 24 h, 48 h, or 72 h, 8 μM INT-747 was added when indicated. Later, viability was detected by the MTT assay. **(B)** Cell morphology of PC-3 cells after treatment with INT-747 for 48 h was imaged under an inverted microscope at 10 × to detect any changes. **(C)** and **(D)** representative images of the colony number in each group (top panel) and proliferation of single cell clones (bottom panel), stained with crystal violet.Scale bar: 80 μm.

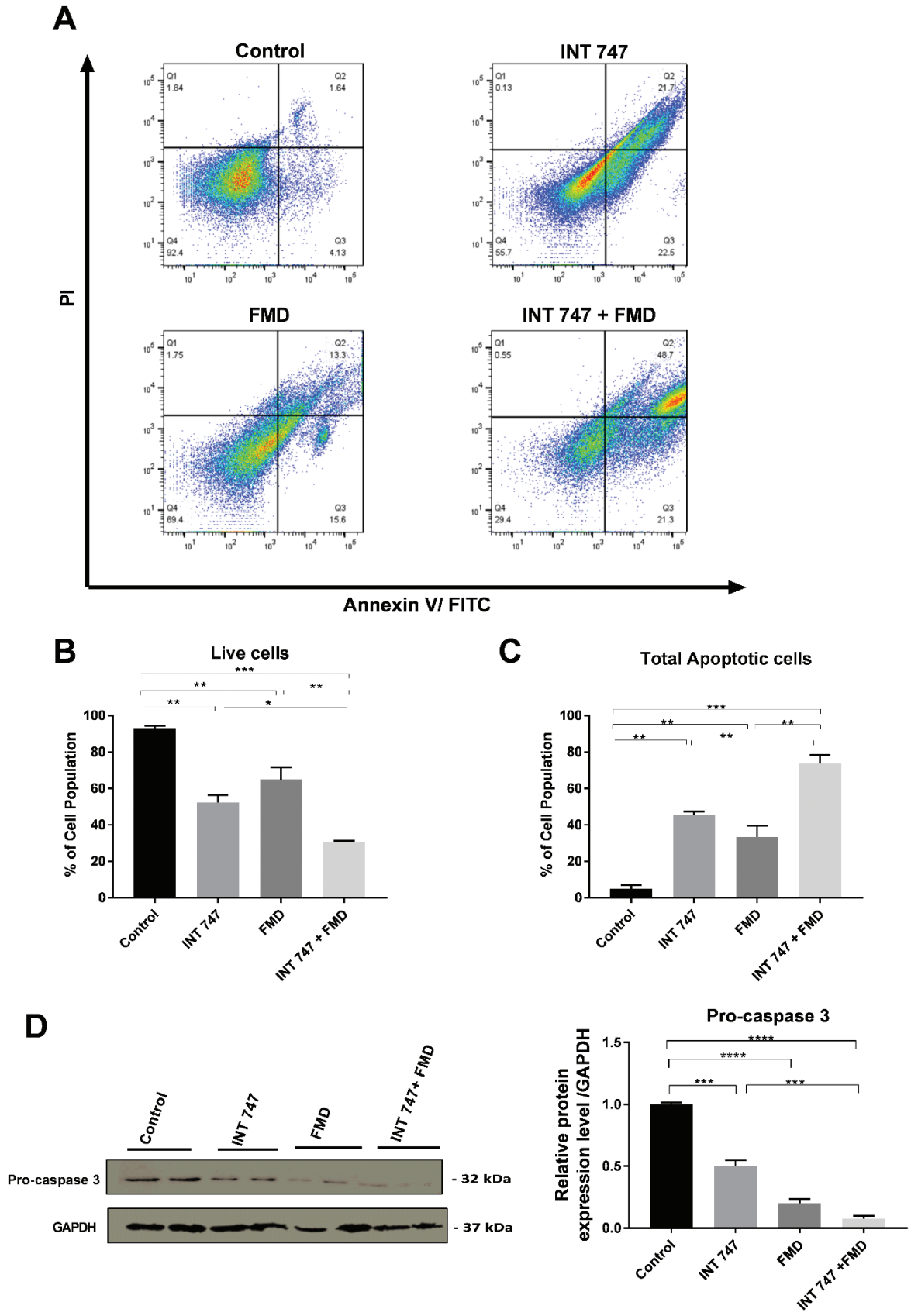



**Figure 3.** Flow cytometry analysis of PC-3 stained with annexin V and PI after 48 h of treatment with INT 747, FMD, and their combination. (**A**) Pseudo color dot plots represent responses to therapy with indicated compound(s) for PC-3 cells. Apoptosis was measured by annexin V-FITC/PI double staining and flow cytometry. **(B)** Graphical representation of live cells. **(C)** A graphical representation of total apoptotic cells (a sum of dead, early, and late stages) (D) expressions of pro-caspase 3 detected by Western blot. GAPDH was used as a loading control. FITC, fluorescein isothiocyanate; PI, propidium iodide.

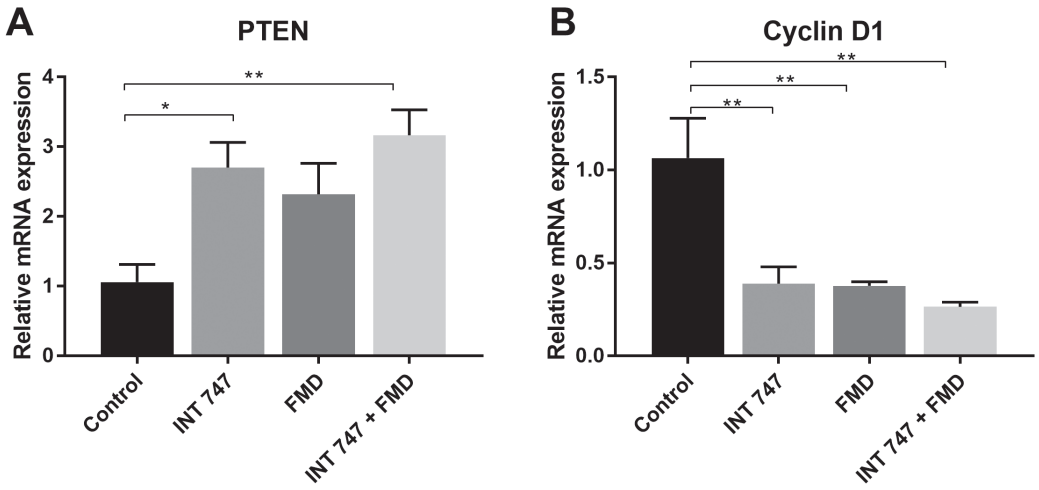



**Figure 4.** Effect of INT 747/ FMD combination on cell proliferation and metastasis gene expression in prostate cancer. mRNA expression was evaluated by real-time qPCR of cell culture in absence or presence of FMD and INT 747 at IC50 dose for 48 h . GAPDH was used as housekeeping control gene to normalize RT-PCR reaction.

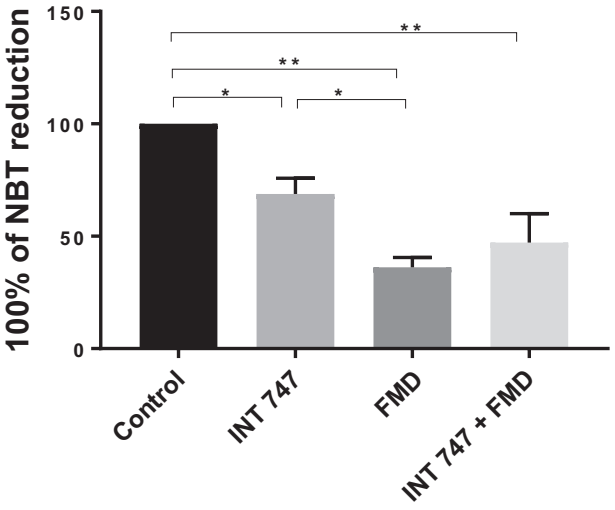



**Figure 5.** Effect of INT 747 on the production of reactive oxygen species. ROS production was measured by reduction in nitro blue tetrazolium (NBT) in the PC-3 cell line at different treatments for 48 h. NBT reduction was calculated as the percentage of the control.

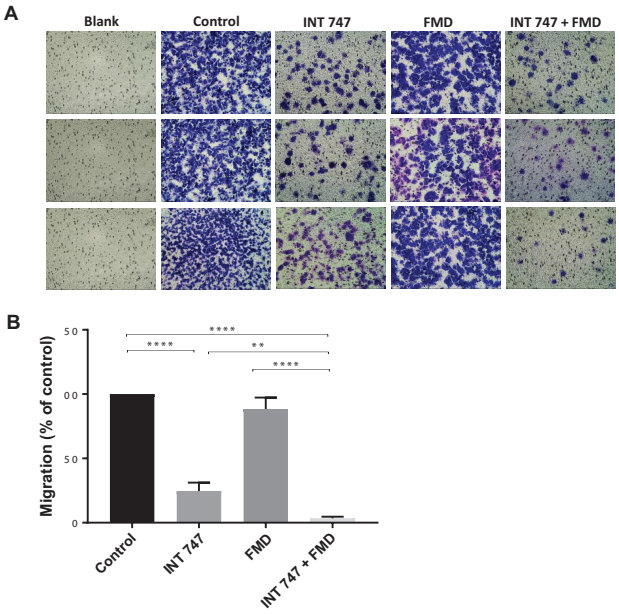



**Figure 6.** The combined inhibitory effect of FXR and FMD on the migration of PC-3 cells. **(A)** Images of migrated PC-3 cells that were treated with INT 747 (8 μM) and control solvent in the presence and absence of normal or FMD. **(B)** Image quantified the relative number of migrating cells treated with or without INT 747 cells in different medium and values were normalised against DMSO-treated cells and expressed as percentages of the control, which was assumed to be 100%.

in achieving IC50. However, guggulsterone treatment reduced the effect of FXR induced cell death. Furthermore, this study showed that INT 747 altered PC-3 cell colony formation as well. Interestingly, co-administration of INT 747 and FMD to PC-3 cells had a greater effect on cell viability and colony formation than administration of either treatment alone.

Motility is also one of the characteristics in cancer cells that is required for migration from the primary site to a secondary organ. The current study showed that in addition to suppressing the proliferation of prostate cancer cells, FXR inhibits in vitro migration. Obviously, the impairment to cell migration and the reduced rate of cell growth induced by FXR was much greater in cells incubated in FMD compared to cells incubated only with the FXR agonist. This decrease in proliferation and migration was associated with a drastic decrease in cyclin D1 gene expression. Many previous studies showed cyclin D1 is commonly overexpressed in human prostate cancers and promotes proliferation and migration by mediating progression through key check points in G 1 phase of the cell cycle and regulation of Cdk4-paxillin-Rac1 axis, respectively (Fusté et al. 2016; Cao et al. 2020; Drobnjak et al. 2000).

Next, we evaluate alterations in the apoptotic phenotype of PC-3 cells in order to understand the mechanisms involved in death. Remarkably, our study showed FXR

activation also exerted synergistic pro-apoptotic effects alone or with FMD, which is responsible for the features of morphological alterations of PC-3 cells associated with apoptosis (S. T. Lee et al. 2013). Most importantly, our data showed that INT 747 and / or FMD significantly decrease pro-caspase-3. This suggests that INT 747 and / or FMD induced a caspase-dependent apoptosis in PC-3 cells. Procaspase-3 levels decreased significantly 48 h after application of INT 747 or FMD at death-inducing concentration (8 μM in PC-3 cells). After 48 h, the level of procaspase-3 in cells treated with both INT 747 and FMD was extremely low. The decrease of procaspase-3 levels correlated with dramatically increased levels of apoptosis 48 h after treatment application. Apoptotic cell death was further confirmed in these cells by the appearance of the cell population with the hypodiploid sub-G1 DNA content (our unpublished data). The collected data are consistent with Anwar et al, who showed that expression of procaspase 3 in prostatic adenocarcinoma correlates with tumor grade (Anwar et al. 2004).

Consistent with this, the analysis of the RT-PCR genes revealed an increase in PTEN expression, which suppresses cell growth through the negative regulation of cell cycle and cell survival signaling, confirming the outcome of this study (Yan and Huang 2019; Braglia et al. 2020). In prostate cancer tissue, abnormal methylation of PTEN genes was observed, leading to inactivation of PTEN and hyperactivation of the Akt signal that recruits apoptosis (Wang et al. 2020). Therefore, in our study the upregulation of PTEN expression due to FXR activation led to activation of caspase-3 as evidenced by appearance of low procaspase-3 isoforms on immunoblotting. Consequently, the new interaction between the PC-3 cell PTEN signal and the FXR could be a new drug target for treating prostate cancer (Liu et al. 2014). Furthermore, and in agreement with our study. FXR agonist INT 747 induced cell death is confirmed via increase ROS production in PC-3 cell line.

An interesting aspect that emerged from previous studies was that FXR accumulation was found to be significantly lower, at both mRNA and protein levels, in human prostate cancer tissues compared to adjacent normal tissues (Cariello et al. 2018). Genes that are dysregulated in cancer are clinically attractive as a candidate prognostic marker and therapeutic target. The second important point is that FMD was described to cause favorable environments that can decrease the ability of prostate cancer cells to survive (Longo and Mattson 2014). Furthermore, both nutrients deprivation and FXR activation were reported previously to enhance PPAR-α expression (Pineda Torra et al. 2003; Contreras et al. 2013). As far as PPAR-α activation plays an important role in the prevention of prostate cancer, by inhibiting the AP-1-mediated proliferation and survival of cancer cells and affecting the Warburg effect (Vamecq et al. 2012; Tan et al. 2021); therefore, modulating PPAR-α activity by fasting-mimicking may play a role in the observed synergism with obeticholic acid. Based on these results, one can suggest that FXR activation under starvation condition modulate transcription of genes involved in tumorigenesis and metabolism (Han 2018; Fu et al. 2019; Mao et al. 2020).

## Conclusions

The results reported a restraining in, proliferation, survival and migration of prostate cancer cells by predisposes cells to up-regulation of the tumor suppressor PTEN and down-regulation of Cyclin D1 and procaspase-3 signaling in PC-3 cells, resulting in remarkable suppression in growth and metastasis and consequently starving cancer cells to death. Taken together, this work reports for the first time that FXR alone or in combination with FMD may be a tumor suppressor in the progression of metastatic castration prostate cancer.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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