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Interaction of Atorvastatin and CX3CR1/Fractalkine in Androgen-Dependent Prostate Cancer Cells: Effect on PI3K Pathway

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Abstract

Prostate cancer (PC) management lacks any molecular targeted therapies. Statins have shown anticancer effects against PC in both in vitro and in vivo studies. CX3CR1 is a receptor expressed by many cancer cell types, including PC. In this research, we studied the effect of interaction of atorvastatin and CX3CR1/fractalkine on androgen-dependent PC cell line 22Rv1 compared to non-tumorigenic prostatic cells, RWPE-1. Experimental settings were based on investigating the effect of atorvastatin on viability of 22Rv1 and RWPE-1 in the presence and absence of fractalkine. Neutralizing antibody specific for fractalkine was used to neutralize the activity of the receptor in these experiments. MTT viability assay, adhesion assay, and Western blot analysis for expression levels of CX3CR1, phosphorylated Akt and GSK-3 were applied. Results showed that fractalkine partially reversed the 50% inhibition of proliferation mediated by atorvastatin on proliferation and adhesion of 22Rv1 cancer cells. Similar effects were not noticed with normal RWPE-1 cells. Western blotting showed a dose-dependent reduction in the expression of CX3CR1, levels of p-Akt and p-GSK-3 in the presence of fractalkine. This effect was further enhanced when the effect of fractalkine was neutralized using specific antibody. Findings from this study revealed that deprivation of fractalkine is a potential mechanism to enhance anti-proliferative effects of atorvastatin on androgen-dependent prostate cancer cells with no effect on normal cells.

Keywords:Prostate Cancer, Fractalkine, Fractalkine receptor, Atorvastatin, Akt, glycogen synthase kinase-3.

1. Introduction

Prostate cancer (PC) is a major public health problem worldwide and is the second leading cause of cancer deaths among males in the United States (Siegel et al., 2019). PC is characterized by an uncontrolled growth of cancerous prostate cells in male reproductive system. Treatment of PC is highly dependent on stage of the disease. Options available for treatment include watchful waiting, surgery, radiation therapy, androgen deprivation therapy and chemotherapy. Furthermore, immunotherapies have been developed, such as the tumor vaccine sipuleucel-T, and pembrolizumab and were recently incorporated into cancer treatment guidelines (NCCN, 2017). To date, no molecular targeted therapies have been developed for PC.

Chemokines are cytokines that have a wide range of physiological activities such as the ability to stimulate the migration of cells (chemotaxis) and angiogenesis (Luster, 1998). Chemokines and their receptors are extensively expressed in tumors, with different cancer types having different chemokine/receptor expression profiles (Balkwill, 2003). Many studies have illustrated the effect of chemokine-receptor signaling on tumorigenesis process by promoting tumor growth and progression, angiogenesis, metastasis and modifying the tumor microenvironment to serve the tumor cells' growth (Chow and Luster, 2014). CX3C chemokine receptor 1 (CX3CR1) is a seven-transmembrane G protein-coupled receptor and is considered the only human receptor that binds fractalkine (FKN) or CX3CL1 (Bachelerie *et al*., 2013) . CX3CR1 is expressed in several immune cells, such as natural killer cells (Imai et al., 1997), monocytes/macrophages (Segerer et al., 2002), B and T lymphocytes (Wu, 2014), and microglial cells (Nishiyori et al., 1998). In addition, expression of CX3CR1 has been shown in neurons (Meucci et al., 2000) and many tumors including gliomas, breast, ovarian, and prostate cancers (Ferretti et al., 2014). CX3CR1 has a unique and sole ligand, that is FKN (Macor, 2012). FKN is found in two forms: anchored FKN that functions as an adhesion molecule (Bazan et al., 1997) and soluble FKN which functions as a chemoattractant molecule (Garton et al., 2001). FKN was shown to be overexpressed in PC spinal-metastatic sites compared to primary tumor sites, thus promoting spinal metastasis (Liu et al., 2018). The main source of FKN in spinal tissues is differentiated osteoblasts (Shulby et al., 2004) which drive chemotaxis and homing of PC cells to bone tissue. Normal epithelial prostatic cells and cancerous ones express

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* **Abbreviations**:PC: Prostate cancer; FKN: fractalkine; GSK-3: glycogen synthase kinase-3; ATCC: American Type Culture Collection; DMSO: Dimethyl sulfoxide; ANOVA: analysis of variance; FNAb: fractalkine neutralizing antibody, EDTA: Ethylenediaminetetraacetic acid. PBS: Phosphate buffer saline, HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A.

CX3CR1 on their surfaces. The expression, however, reduces as cells become androgen-independent (Xiao et al., 2012), indicating a greater role for CX3CR1 in androgen-dependent PC. Stimulation of CX3CR1 in PC cells by FKN will activate downstream singling pathways, including MAPK, PI3K, and PLC pathways. No known chemicals have the ability to downregulate CX3CR1 expression, and one group used small molecule inhibitors to study the effects of CX3CR1 inhibition (Stout et al., 2018).

Statins are lipid-lowering agents which act by inhibiting HMG-CoA reductase enzyme. Statins are used in practice for management of hypercholesterolemia. Anticancer effects of statins are mediated directly by lowering cholesterol levels and affecting growth factor receptor signaling (Gbelcova et al., 2017), or by indirect "pleiotropic" effects (Ciofu, 2012). Statins affect PC cells by lowering cholesterol levels in lipid rafts and impairing signal transduction pathways involved in cell survival (Zhuang et al., 2005). Pleiotropic effects of statins include anti-proliferative, anti-angiogenic, and pro-apoptotic effects (Kavalipati et al., 2015). Statins affect multiple signaling pathways, including PI3K/Akt and GSK pathways. Atorvastatin is considered one of the most effective statins with a preferable safety profile. A meta-analysis by Bansal et. al. showed that statins use was associated with a reduction in the risk of developing PC (Bansal et al., 2012). In addition, statins showed promising results as therapeutic agents for PC management, such as radio sensitization and direct killing effect on PC cells (He et al., 2012). Statin users were shown to have favorable clinical presentation after brachytherapy in localized PC patients (Moyad et al., 2005), reduced levels of prostate-specific antigen (PSA) (Khosropanah et al., 2011), reducing risk of PC recurrence after radical prostatectomy (Hamilton et al., 2010), and reduced mortality (Yu et al., 2013). Atorvastatin decreased FKN/CX3CR1 expression in peripheral blood mononuclear cells in coronary artery disease patients (Damås et al., 2005). It also reduced CX3CR1 levels in peripheral monocytes in patients with Crohn's disease (Grip et al., 2008). It is unknown whether atorvastatin affects the expression of CX3CR1 in PC cells and the impact of such effect is still largely unknown.

In this study, we investigated the effect of atorvastatin on androgen-dependent PC cells, focusing on FKN/CX3CR1 axis. The effect was evaluated via studying cell proliferation, cell adhesion and detecting the expression of CX3CR1 and downstream targets of PI3K pathway, namely Akt and GSK-3α/β to demonstrate the molecular mechanism of such an effect.

2. Materials and Methods

2.1. Cell Culture

Cell lines were purchased from the American Type Culture Collection (ATCC). Two cell lines were used: androgen-dependent PC cells, 22Rv1 (ATCC® CRL-2505™), and normal prostatic epithelial cells, RWPE-1 (ATCC® CRL-11609™). The 22Rv1 cells were cultured in RPMI high glucose media (Euroclone, Cat# ECB9006L) which contained 1% penicillin/streptomycin (Euroclone, Cat# ECB3001D) and 10% fetal bovine serum (Biowest, Cat# S1600). RWPE-1 cells where cultured in Keratinocyte Serum-Free Medium (K-SFM, Gibco, Cat# 10724-011) after the addition of K-SFM supplements kit (Cat#. 37000-015, providing 0.05 mg/mL bovine pituitary extract and 5 ng/mL recombinant epidermal growth factor). Cells were incubated at 37°C and 5% CO_2. Media replacement was performed every two days.

2.2. Drug Preparation

Atorvastatin calcium salt trihydrate (Sigma-Aldrich, PZ0001) was dissolved in Dimethyl sulfoxide (DMSO, Santa Cruz Biotechnology, SC-358801) to prepare stock solutions of 10 mM. The drug was aliquoted and stored at -20°C.

2.3. Study Design

Both cell lines were cultured with and without Recombinant Human Full Length CX3CL1/Fractalkine (R&D Systems, Cat# 365-FR-025/CF) with different concentrations of atorvastatin (1, 2, 5, 10 and 20 μM) vs. vehicle-control and incubated for 48 hrs. Then, effects of therapies were studied using the following assays:

2.4. MTT Cell Proliferation Assay

A total of 1×10^5 cells/200 μl of each cell line was seeded into a 96-well plate for 24 hrs. The media was withdrawn after that and 200 μl of variable concentrations of atorvastatin (1, 2, 5, 10 and 20 μM) vs. vehicle-control were added in triplicates. Then, plates were incubated for 48 hrs and 5 μg/mL FKN neutralizing antibody (FNAb) (R&D System, Cat# MAB3652) was added for 30 minutes, according to previously published work (Shulby *et al.*, 2004). Afterwards, 10 μl of a freshly prepared MTT solution (prepared by dissolving 0.5 mg of MTT powder in 1 mL of used culture media) was added to each well. After 4 hrs of incubation at 37°C, media was removed and 200 μl of DMSO was added to each well to dissolve formed formazan crystals. The plate was kept at room temperature in the dark and absorbance was read at 570 nm using microplate reader (EZ Read 400 microplate reader, Biochrom, UK). The same procedure was done in quadruplicates for each concentration while adding FKN 50 ng/mL for 24 hrs, according to previously published work (Guo *et al.*, 2012) for both cell lines.

2.5. Adhesion Assay

A 24-well plate was coated with a monolayer of 50 nM of FKN according to previously published data (Fujita et al., 2012). Coating was performed by diluting FKN with phosphate-buffered saline (PBS, Euroclone, Cat# ECB4053L) and then adding it to the plate and allowing it to dry overnight under UV-light for sterilization. Both cell lines were plated on a different 24-well plates and cultured until 70% confluent, then treated with atorvastatin (1, 2, 5, 10 and 20 μM) vs. vehicle-control for 48 hrs, each concentration in quadruplicates. Afterwards, cells were treated with 5 μg/mL of FNAb for 30 min. Next, cells were washed with PBS and were then harvested by adding trypsin-EDTA (Euroclone, Cat# ECB3052D) to each well for 5 minutes. Cells where then collected, rewashed with PBS and then were seeded on the coated plates and incubated at 37°C, 5% CO2. After 1 hour, cells that didn't attached were washed off, remaining cells were fixed with 2% paraformaldehyde for 5-10 min. Crystal violet stain (prepared by dissolving 2 g crystal violet in 20 mL of 95% ethyl alcohol then adding them to 0.8 g ammonium oxalate

monohydrate that was dissolved in 80 mL deionized water) was applied. The stain was added to each well and incubated at RT for 30 min, subsequently distilled H2O was used to wash off excess stain. Cells were counted under inverted light microscope. The same procedure was repeated for both cell lines without adding FNAb.

2.6. Western Blotting

A total of 1×10^4 cells of each cell line were seeded in a 6-well plate until 70% confluence. Atorvastatin (1, 2, 5, 10 and 20 µM) vs vehicle-control was added in duplicates for each concentration for 48 hrs. FKN 50 nM was added to each cell line for 15 minutes. Media was then removedremoved, and cells were washed with ice-cold PBS. A total of 500 µL of ice-cold lysis buffer (RIPA buffer 10x (Abcam, Cat# ab156034) diluted to 1x with PBS, with the addition of Pierce Protease and Phosphatase Inhibitor Mini Tablets (ThermoFisher Scientific, Cat# A32959), 1 tablet per 10 ml of lysis buffer) was added to each well for 5 minutes. Cells were then scraped and collected in an eppendorf tube. After 30 minutes of incubation on ice, lysates were cleared by centrifugation, and protein concentration was determined by DC™protein assay (Bio-Rad, Cat# 500-0116). Samples were mixed with 5x laemmli buffer. Equal amounts of protein were loaded into 4–20% Mini-PROTEAN® TGX™ polyacrylamide gel (Bio-Rad, Cat# 4561094DC). Afterwards, proteins were electroblotted on UltraCruz® Nitrocellulose Pure Transfer Membranes (Santa Cruz Biotechnology, Cat# sc-3724) for 1 hour at 250 mAmp constant current. Protein bands were first detected on the membrane using Ponceau S solution (1g of Ponceau S (Cat# ab146313) + 50 mL acetic acid, completed to 1 L with distilled H2O). Blots were incubated for 1 hour with 1.25% bovine serum albumin (Sigma Aldrich, Cat# A9418) in PBS then washed three times with PBS containing 1% Tween (PBST) and incubated overnight at 4°C with a 1:1000 Phospho-Akt (pSer473) antibody (Cell Signaling, Cat# 9271). This was followed by washing membrane and probing with 1:5000 secondary HRP conjugated anti-rabbit IgG antibody (Cell Signaling, Cat#7074s). Blots were re-probed with 1:1000 β-actin primary antibody (Abcam, Cat# 8227) followed by 1:5000 secondary HRP conjugated anti-rabbit IgG antibody (Cell Signaling, Cat#7074s). Same procedure was repeated using 1:1000 CX3CR1 (Abcam, Cat# ab8021), 1:1000 Phospho-GSK-3α/β (Ser21/9) antibody (cell signaling, Cat# 9331), and they were re-propped using 1:1000 GAPDH (Abcam, Cat# ab9485). For the second group, the same procedure was repeated after treating cells with (5µg/mL) of FNAb for 30 minutes (R&D System, Cat# MAB3652). Membranes were developed using Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare, Europe GmbH, Cat# RPN2235) and were imaged using C-Digit® Blot Scanner (LI-COR, Lincoln, NE). Bands were quantified using the provided Image Studio™ Software version 5.0 for the C-DiGit Blot Scanner.

2.7. Statistical Analysis

All data was presented as mean ± SEM. To determine significant differences between treatment and control groups, we used the one-way analysis of variance (ANOVA) and conducted post-hoc multiple comparison analysis. The significance was set at a P value of less than 0.05.

3. Results

In this study, we evaluated the effect of atorvastatin treatment on androgen-dependent prostate cancer cell lines in the presence or absence of FKN. The latter effect was achieved by the addition of FNAb. We used normal prostate epithelial cell lines as control for comparison.

3.1. Effects of Atorvastatin/FKN Interaction on Proliferation

Figure 1 shows the effect of atorvastatin treatment on proliferation of 22Rv1 and RWPE-1 cells. In the presence of FKN, atorvastatin treatment had no effect on viability of RWPE-1 cells; however, it significantly suppressed viability of 22Rv1 cells at a concentration of 20 µM. When FKN was blocked by FNAb, atorvastatin-induced growth inhibition was enhanced in both cell lines. Cell proliferation was significantly reduced at 20 µM and 10 µM of atorvastatin treatment in RWPE-1 and 22Rv1 cells, respectively (Figure 1).

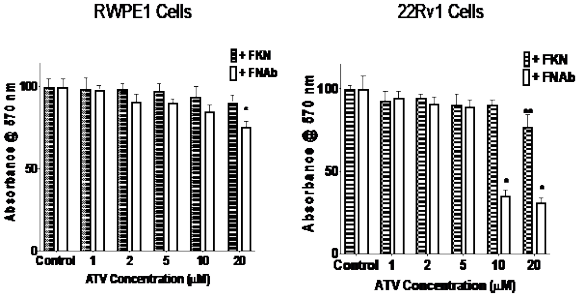

Figure 1. The effect of interaction of atorvastatin and FKN on proliferation of 22Rv1 PC cells compared to RWPE-1 non-tumorigenic cells. FKN: fractalkine; FNAb: fractalkine neutralizing antibody. (*, P < 0.05; **, P < 0.005)

3.2. Effects of Atorvastatin /FKN Interaction on Adhesion

Adhesion of cells to FKN-coated surfaces is shown in Figure 2. Atorvastatin treatment did not affect adhesion of RWPE-1 cells irrespective of the availability of FKN. On the other hand, atorvastatin induced a dose-dependent inhibition of 22Rv1 cell adhesion in the presence of FKN but not to a significant level compared to control treatment. However, neutralizing the effect of FKN by FNAb enhanced atorvastatin inhibition of 22Rv1 cell adhesion which was significant at 10 µM concentration of atorvastatin (Figure 2).

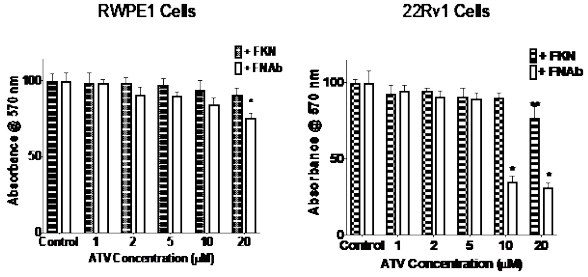

Figure 2. The effect of interaction of atorvastatin and FKN on adhesion of 22Rv1 PC cells compared to RWPE-1 non-tumorigenic cells to FKN coating material. FKN: fractalkine; FNAb: fractalkine neutralizing antibody. (**, P < 0.005; ***, P < 0.0005;)

3.3. Effects of Atorvastatin/FKN Interaction on CX3CR1 expression and downstream signaling pathways

To further understand the ATV-FKN-CX3CR1 interaction, we investigated the molecular effects of atorvastatin treatment on both cell lines in the presence of FKN as shown in Figure 3. Atorvastatin reduced expression of CX3CR1 in both 22Rv1 and RWPE-1 cells in a dose-dependent manner. In the absence of atorvastatin treatment, the presence of FKN with or without its neutralizing antibody did not alter expression of CX3CR1 compared to vehicle-treated control groups in both cell lines (Figure 3, left panel). In concordance to findings from viability studies, atorvastatin treatment significantly reduced expression of CX3CR1 at concentrations of 10 and 20 μM in 22Rv1 PC cells, in which neutralizing FNK enhanced the effects of atorvastatin (Figure 3, left panel). Atorvastatin reduced levels of phosphorylated (active) Akt and GSK-3 in both cell lines with greater suppression of expression in 22Rv1 cells (Figure 3, middle and right panels). Similarly, atorvastatin's effect on active Akt and GSK-3 levels was further enhanced when FKN was blocked using FNAb, starting at 10 μM concentration of atorvastatin in 22Rv1 cells.

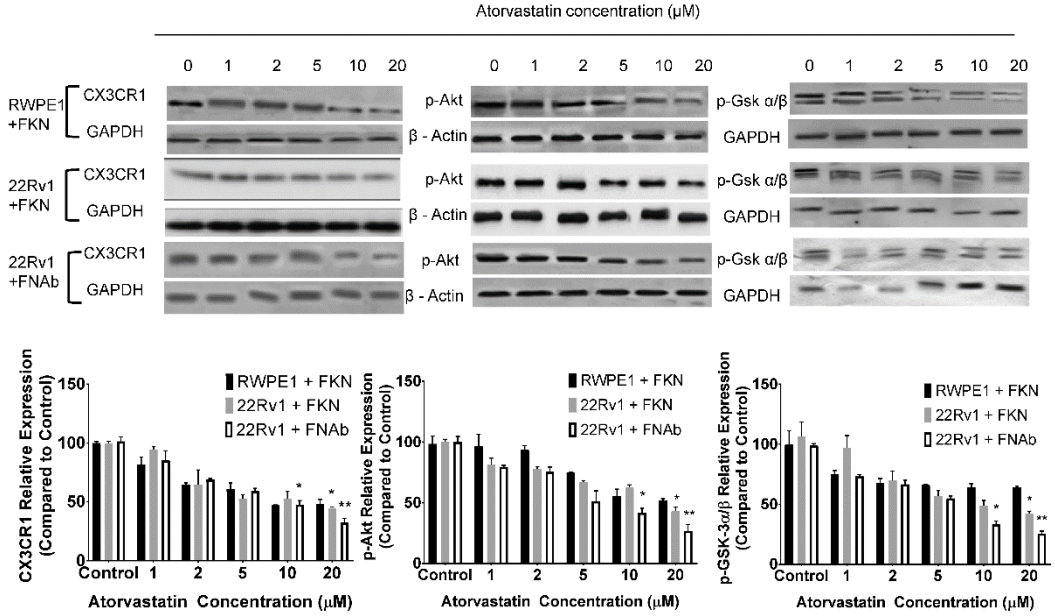

Figure 3. The effect of interaction of atorvastatin and FNK on expression of CX3CR1, p-Akt and p-GSK-3α/β in 22Rv1 PC cells and RWPE-1 non-tumorigenic cells. Top panel represent Western blot bands for the indicated proteins and the bottom panel indicates densitometric analysis for the expression of each protein compared to the respective vehicle-treated control group. FKN: fractalkine; FNAb: fractalkine neutralizing antibody. (*, $P < 0.05$; **, $P < 0.005$)

4. Discussion

In this study, we have shown the ability of atorvastatin to reduce proliferation and adhesion of 22Rv1 cells in the presence of FKN. These effects of atorvastatin were further enhanced with the use of FNAb to neutralize FNK. On the other hand, we can view those results as that atorvastatin induced reduction of proliferation and adhesion of 22Rv1 cells in the presence of FNAb, and that effect was partially reversed in the presence of FKN. This could suggest the future use of FNAb in combination with other anticancer agents to enhance their effects. Growth and adhesion inhibition induced by atorvastatin was more substantial in 22Rv1 cells compared to RWPE-1 cells. This finding supports a greater selectivity for atorvastatin effects on cancer cells compared to non-cancerous ones further reflecting the potential margin of safety for using atorvastatin. In previous work, we have studied the effect of simvastatin on proliferation of LNCaP cells and it was much higher than what we found using atorvastatin (a more potent statin) (Kochuparambil et al., 2011). Some recent work shows a higher expression profile of multiple lipids in LNCaP cells compared to 22Rv1 cells (Sorvina et al., 2018). This might explain the reduced sensitivity of 22Rv1 cells to statins, compared to LNCaP cells.

Cell adhesion is essential for growth and survival of cancer cells, as well as cell-to-cell communication (Lodish et al., 2000). Furthermore, cancer cell adhesion is an important step that mediates metastatsis to distant sites (Chen, 2012). Progression to metastasis in PC reduces 5-year survival down to 30% (American Cancer Society, 2018). Homing molecules are very important in metastasis of cancer cells in general. Those molecules include integrins, chemokines, as well as selectins. We have previously studied and found an association between integrin αvβ3 located on PC-3 cells and its adhesion potential to ICAM-1 in the presence of simvastatin (Al-Husein et al., 2013). Our current study showed reduced adhesion of 22Rv1 cells in response to atorvastatin treatment. This effect was shown in the presence of FNAb while there was no significant effect in the presence of FKN or in normal epithelial prostatic cell, RWPE-1. FKN and its receptor have been shown to enhance the migration of PC cells toward osteoblasts and to promote their adhesion (Shulby et al., 2004). Thus, impairing the interaction between FKN and its receptor could be a promising strategy to prevent progression and metastatsis of PC cells to bone, a common site for disease metastasis.

To the best of our knowledge, this is first study to show the ability of atorvastatin to reduce the expression of CX3CR1 in 22Rv1 cells in a dose-dependent fashion. This effect was enhanced by blocking the effect of FKN by FNAb. This could mean a reduction of both CX3CR1-mediated adhesion as well as CX3CR1-mediated signaling. The latter effect could have multiple implications as it means blockade of both adhesion-mediated signaling facilitated by anchored FKN, or FKN-mediated signaling as a soluble mediator. In addition, downregulation of CX3CR1 prevents the migration of PC cells towards osteoblast cells which bind to FKN on their surfaces (Mantovani, 1999). Collectively, this could be of great importance in management of not only PC, but also lung and kidney cancers, especially in the prevention of spinal metastasis (Liu et al., 2017). Multiple studies have also demonstrated the effect of atorvastatin on the expression of CX3CR1 in patients with coronary artery disease (Kureishi et al., 2000, Gotto, 2009, Nawrocki et al., 1995). Our results are in agreement with earlier studies revealing atorvastatin suppression for of CX3CR1 expression.

Signaling through the PI3K pathway results in activation of downstream Akt and GSK-3 molecules which are involved in survival and apoptotic cellular processes (Shulby et al., 2004). PI3K/Akt/GSK pathway is a major player involved in many tumor types. Simultaneously, FKN/CX3CR1 axis activation results in activation of PI3K pathway (Mantovani, 1999). Akt is has been found to be amplified in many tumorigenic processes and its presence is related to more aggressive tumors (Mitsiades et al., 2004). GSK-3 is also a downstream target in PI3K pathway and its levels are elevated in PC as it is highly involved in cell proliferation, apoptosis, and migration (Rinnab et al., 2008). Atorvastatin treatment of 22Rv1 cells reduced the level of phosphorylated/active Akt (S473), that is known to be associated with effects on cell proliferation (Gao et al., 2003), survival (Harashima et al., 2012){Gao, 2003 #55}, invasion (Shukla et al., 2007) and angiogenesis (Jiang and Liu, 2008). Similarly, levels of phosphorylated/inactive GSK-3α/β (S9/21) were decreased in response to atorvastatin in 22Rv1 cells. This effect on phosphorylation was further enhanced by FNAb addition. The inhibition of phosphorylation of GSK-3 is linked to inhibition of adhesion as well effects on transcription factors related to cell cycle progression and proliferation (El Touny and Banerjee, 2007). In agreement with our work, effect on cell cycle activation was also noticed to be mediated by FKN through effects on cyclin E and CDK2 CKD 2 and was reversed by FNAb (Tang et al., 2015). We provide additional information on the inhibitory effect of atorvastatin on the phosphorylation of Akt and GSK-3α/β in PC cells, which comes in harmony with other studies which investigated the impact of using simvastatin on LNCAP cells (Kochuparambil et al., 2011). Collectively, results show that combined use of atorvastatin and FKN further enhanced the inhibition of PI3K pathway, a major altered pathway during tumorigenesis, ultimately mediating anti-proliferative and anti-apoptotic properties (D'Haese et al., 2010).

The choice of atorvastatin in our study seems appealing as it is considered now as one of the most effective statins because of its significant reduction not only to LDL levels but also to other lipids such as triglycerides (Expert Panel on Detection and Treatment of High Blood Cholesterol in, 2001). One study has shown that a single daily dose of 10 mg of atorvastatin in 22 subjects can achieve a median concentration 10 μM, which was within the range of concentrations used in this study (Reddy et al., 2011). A 10 mg dose of atorvastatin is considered the minimum dose used to treat hypercholesterolemia (Medscape, 2019).

5. Conclusions

Atorvastatin induces anti-proliferative effect that is enhanced by blocking the effect of FKN by FNAb in PC. Moreover, atorvastatin inhibits cell adhesion of PC cells and subsequently could potentially prevent metastasis. Both effects suggest an anticancer and anti-metastatic effect of the combination of atorvastatin and FKN inhibition. Additionally, it decreases the active phosphorylated levels of Akt and GSK-3α/β. On the other hand, our study found minimal negative impact of this dual inhibition on normal prostatic epithelial cells. Figure 4 is an illustration which summarizes the findings of this study.

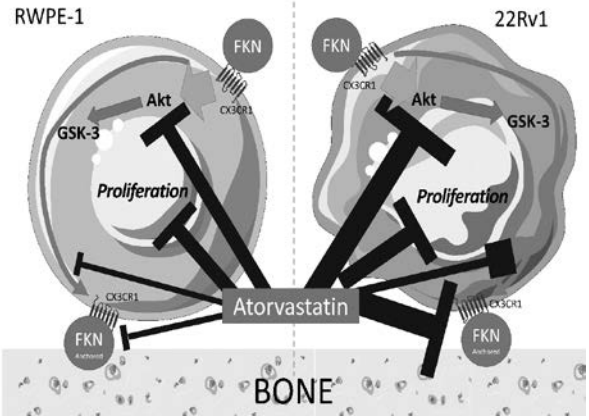

Figure 4. Schematic representation of findings of this study. Thickness of inhibition of arrow relatively expresses extent of inhibition.

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