MOLECULAR MECHANISMS REGULATING SIMVASTATIN-MEDIATED INHIBITION OF PROSTATE CANCER CELLULAR FUNCTIONS *IN VITRO* AND TUMOR GROWTH *IN VIVO*

By

Belal Abdelaziz Abdeljalil Al-Husein

Under the Direction of SOMANATH SHENOY

ABSTRACT

Prostate cancer is a leading killer in the United States. Lack of understanding of mechanisms of disease proliferation, progression and metastasis is a major hindrance in the proper management of this disease. Statins are drugs with known pleiotropy that could justify their introduction into various clinical conditions. Many clinical studies showed beneficial effects of statins in reducing prostate cancer progression and, to a lesser extent, development. In this research, the effect of simvastatin, a potent lipophilic statin, and the mechanism of its effect on prostate cancer cells is studied *in vitro* and *in vivo*. Results show promising effects of simvastatin on human prostate cancer proliferation, colony formation and migration, in both androgen-dependent and –independent disease. Effects were tested *in vivo* and showed a reduction of tumor size in simvastatin treated nude mice compared to controls. Mechanism of effect of simvastatin on cancer cell micrometastasis was also studied using ECIS, gene arrays,

adhesion assays immunocytochemistry and western blotting and showed its effect on integrin αvβ3 activation and consequently adhesion, as well as impairment of tumor secreted factor effect, in addition to stabilization of endothelial barrier. In this research we also studied the effect of combination on proliferation and migration *in vitro* as well as on tumor growth in vivo and showed promising effect with a highly significant reduction of proliferation, migration and tumor growth compared to controls. In conclusion, our pre-clinical research shows beneficial effect of simvastatin alone or in combination with docetaxel in the management of prostate cancer and prevention of micrometastasis.

INDEX WORDS: STATINS, SIMVASTATIN, PROSTATE CANCER, MICROMETASTASIS, DOCETAXEL, COMBINATION.

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Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2013

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ACKNOWLEDGEMENTS

I would like to thank my mentor and supervisor, Dr. Somanath Shenoy, for his continuous support and motivation and for continuing to believe in my abilities even when I lost faith in myself.

Also, I would like to thank my supervising committee, Dr. Susan Fagan, Dr. David DeRemer, Dr. Brian Cummings and Dr. Shelly Hooks for their continues support and the important feedback and fruitful comments they provided on my project research.

I would also like to thank my supporting university, Jordan University of Science and Technology, for giving me the chance to fulfill my dream and pursue my graduate study at the university of Georgia.

I would like to thank Dr. Anna Goc, who always has my back all the time, not only in lab work and being my mentor in learning so many techniques, but also for being a close friend I enjoyed chatting with and discussing my problems.

I also would like to thank Dr. Samith Kochuparambil and my best friend Sahar Soliman for their help during my initial studies and for the support they provided, especially during off hours. I would like to thank mom and dad for always keeping me in their prayers and their support during all of my days of study. I would like to thank my close friends, Alhusban, Al-Azayzih, Roshini and Sherif for being there when I needed their support.

Finally, I would like to thank the CET program for all the help and support.

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INTRODUCTION AND REVIEW OF THE RELEVANT LITERATURE AND RATIONALE

Human prostate gland:

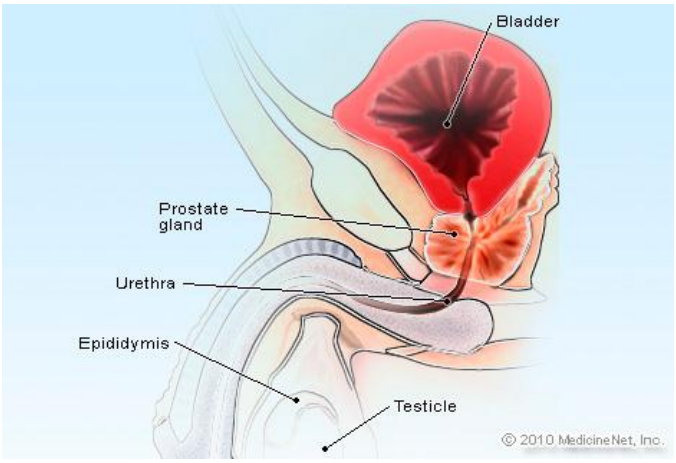


Figure 1.1: The Prostate Gland

The prostate gland is a part of the male reproductive system that contributes various secreted proteins to the seminal plasma and is responsible for modifying the semen conditions for the proper survival and function of sperm (3). Prostate secreted factors prepare the sperm for fertilization by augmenting the process of sperm 'capacitation' and 'acrosome reaction' (5). The gland is located outside the bladder and below the seminal vesicle surrounding the point where the urethra joins the seminal vesicles. Pathologically, the prostate is involved in two main conditions: benign prostatic hyperplasia (BPH) and prostate cancer. BPH involves hyperplasia rather than hypertrophy of prostate tissue in response to the continuous stimulation of the prostate gland by circulating androgens. Management of BPH is based on reduction of androgen stimulation by 5α-reductase inhibitors or symptomatic relief through relaxation of urethra's smooth muscles (by α_{1A} antagonists). Various observational studies have shown that BPH is not a risk factor for prostate cancer development (6).

Prostate cancer-epidemiology

Prostate cancer is the second leading cause of death among other cancers and is the most common cancer in men in the United States (7). Approximately 241,740 people are diagnosed with prostate cancer every year and it causes 28,170 deaths. According to the Center for Disease Control and Prevention (CDC), Georgia is one of the states with a high incidence of prostate cancer (8). Successful management of patients with cancer depends on early diagnosis. According to American Cancer Society (ACS), patients diagnosed with early local or regional disease (stages I, II and III according to TNM staging) have a 100% 5-year disease-free survival rate. The percent drops to 29% for distant (stage IV) disease.

Etiology of prostate cancer:

Prostate cancer has many reported risk factors. One major risk factor that also affects disease prognosis is an age of 65 or more. African Americans seem to have higher risk of disease development compared to other populations. Many genetic mutations are associated with prostate cancer, which include: androgen receptor (AR), BRCA1, BRCA2, PTEN, Src family kinases (9-11), E-Cadherin (12), p53, Akt (13) and many others (14). PTEN mutations correlate more with metastatic disease. Dietary factors are also associated with this disease (such as lycopenes, vitamin A and derivatives (retinol and carotenoids), high fat diet and vitamin D) (15). High fat diet causes increased production of insulin-like growth factor-1 (IGF-1), which enhances proliferation and inhibits apoptosis of cancer cells (16).

Pathophysiology of prostate cancer:

Prostate cancer mostly (95%) involves epithelial cells of the prostate gland (adenocarcinoma). The growth and proliferation of prostate cancer cells depends on the two major sources of androgens in the body, the testes and the adrenal cortex (particularly zona reticularis). Androgens are converted to the active form, dihydrotestosterone (DHT) by the enzyme 5α-reductase. DHT binds and activates androgen receptor (AR) that is then autophosphorylated and dimerizes with another phosphorylated DHT-AR complex. The dimer localizes to the nucleus where it acts as a transcription factor and stimulates growth and survival of prostate cells as well as prostate cancer cells.

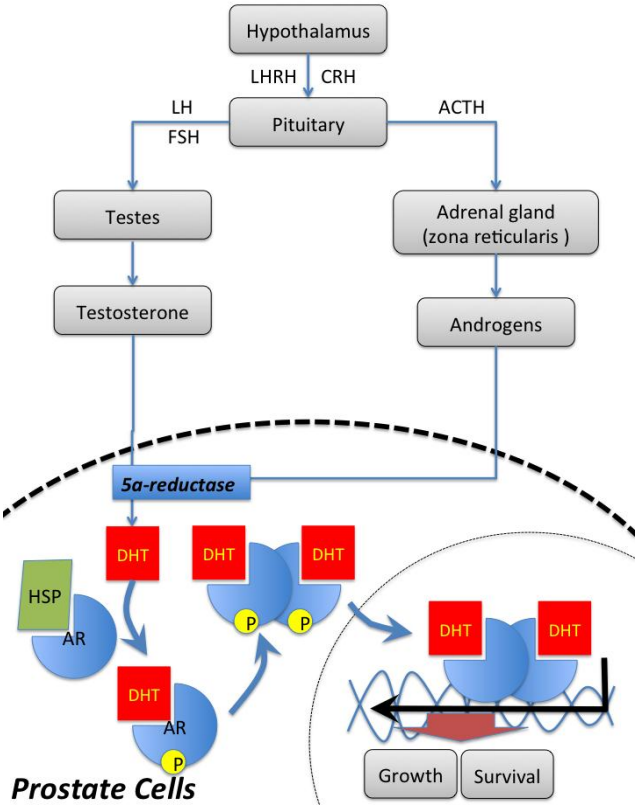


Figure 1.2: Schematic of the hormonal regulation of prostate cells [adapted from (3)]

Prostate cancer starts with an androgen-deprivation responsive phenotype. During this phase, prostate cancer tissue regresses in response to various modalities that reduce circulating androgens in the body. After exposure to anti-androgen therapy/surgical therapy, the tumor shifts to an androgen-deprivation insensitive phenotype (4). At this stage, other modalities, such as chemotherapy and immunotherapy, are required. Docetaxel-based therapy is the treatment of choice in androgen-independent disease. As the disease progresses further, more aggressive, combination-based therapy is

required. At late stages, the disease will metastasize to other tissues, such as bone, lungs, pleura, adrenals, and other tissues (17), leading to death of patients due to complications in those organs.

Molecular pathways commonly deregulated in prostate cancer

Cell survival pathways, namely PI3K/PTEN/Akt (Deocampo, N.D., Huang, H. and Tindall, D.J. (2003) The role of PTEN in the progression and survival of prostate cancer. Minerva Endocrinol. 28, 145–53.), are the main pathways that are deregulated in prostate cancer. Early disease is highly dependent upon the androgen survival pathway. After development of androgen independence, cells no longer need circulating androgen to utilize androgen survival pathway and/or stimulate their own growth, proliferation and survival through other mechanisms (4). Figure 1.3 shows the mechanism involved in androgen resistance.

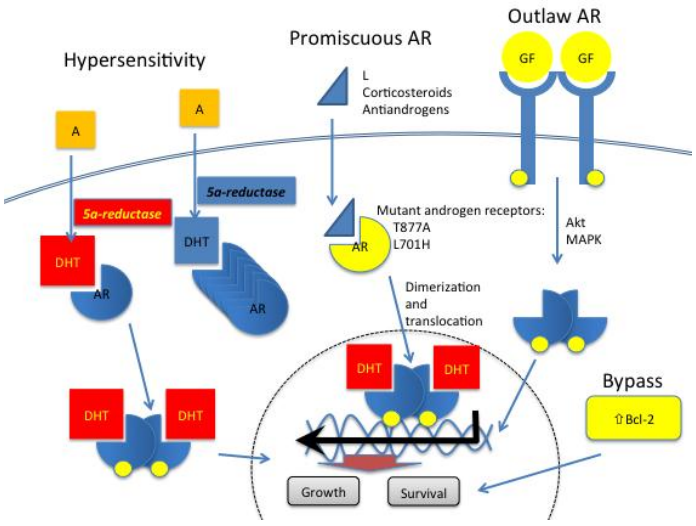


Figure 1.3: Mechanisms involved in androgen resistance, adapted from (4).

Androgen hypersensitivity mechanism involves the requirement of androgen receptor to less-than usual levels of androgens to achieve its response. This phenomenon could be due to hypersensitivity of the receptor itself, its amplification or increased activation of testosterone due to conversion to dihydrotestosterone (DHT) by the overactive 5α-reductase enzyme. Promiscuous AR involves the capability of other circulating hormones, such as corticosteroids (in case of double T877A mutation or L701H

Table 1.1: Mechanisms involved in androgen-independence in prostate cancer [reproduced from Feldman and Feldman 2001]		
Pathway	Ligand dependent?	AR dependent?
Hypersensitive AR	Yes	Yes
Promiscuous AR	Yes (atypical ligands)	Yes
Outlaw AR	No	Yes
Bypass AR	No	No
Linker cells	No	No

mutation (18)) to activate the androgen receptor. In this situation, the receptor could even be stimulated with anti-androgens (in case of T887A mutations (19)), drugs that are used for the management of early disease. AR involves the activation of the androgen receptor system without the need for an androgen or even a ligand binding. This situation could be due to over-activation of Akt or MAPK pathways. Overactive Akt and/or MAPK could phosphorylate the androgen receptor, leading to its dimerization, nuclear localization and transcription activation. Bypass AR may be the truest form of androgen independent disease, as prostate cancer progression does not require androgens or their receptors. In this mechanism, other survival pathways are activated, such as overexpression of anti-apoptotic proteins, Bcl-2 being the most common; overexpression of oncogenes; or non-functional tumor suppressor gene mutations. PI3K/Akt/PTEN pathway is a major survival pathway in prostate cancer as indicated by the fact that PTEN (a tumor suppressor protein) mutation is one of the common etiologies (20) and is more involved in metastatic disease. Table 1.1 summarizes the mechanisms involved in androgen independence according to dependence on ligand and/or AR.

Akt or protein kinase B (a serine-threonine kinase) is a major oncogene regulating growth and proliferation of prostate cancer cells. Therefore, its manipulation could be a key to the management of prostate cancer. However, Akt inhibition is associated with

various side effects attributed to the non-specificity of inhibitors to tumor cells, as Akt signaling which is crucial for growth and survival of normal tissue as well as cancer tissues. We previously showed an association between Ras/Raf/MAPK/ERK and PI3K/Akt pathways in the proliferation and colony formation of prostate cancer (21). Although angiogenesis is an important mediator of prostate cancer proliferation and metastasis, HIF-1α seems not to be influenced and doesn't correlate with incidence of prostate cancer.

Prostate cancer- signs, symptoms and screening procedures

Symptoms of prostate cancer vary according to stage of disease. In early stages of the disease, the tumor is localized and starts compressing the urethra. Symptoms include urinary incontinence/frequency. As the disease progresses it metastasizes to bones with symptoms including bone pain and spinal cord compression. Non-specific symptoms, such as weight loss and anemia, develop during late and terminal stages of disease.

Due to the high incidence of prostate cancer and its increased risk after the age of 50, ACS recommends performing two screening tests by 50: Prostate Specific antigen (PSA) and digital rectal examination. PSA is a marker expressed on the surface of prostate cancer cells. PSA also it can be detected in the circulation and, although non-specific, it could be useful for the early diagnosis of prostate cancer. Levels of PSA in blood correlate proportionally to the size of tumor tissue, making it a good test for the evaluation of the extent of the disease. Moreover, PSA can be used as a prognostic marker. Very low levels can indicate response to treatment. In addition, elevated PSA levels after disease remission could indicate recurrence. Although used extensively as a

screening, diagnostic and even prognostic tool, PSA is losing favor in evaluating prostate cancer. Lately (22), the US Preventive Services Task Force issued guidelines that recommend against using PSA to screen for prostate cancer due to the lack of specificity and sensitivity. According to the report, PSA gives a false warning in 80% of cases.

Current therapeutic approaches for prostate cancer:

Prostate cancer treatment strategies depend on the proper risk classification of the disease (23, 24). Many factors are considered, such as: age, Gleason score, clinical stage and PSA levels. Ages of 50 or 60 are cutting-edge ages, and more aggressive treatment considered for older patients as disease risk increases. Gleason score classifies prostate cancer tissue according to the degree of differentiation, with cells resembling normal tissue getting lower scores, and more unrecognizable and undifferentiated cells getting higher score. Usually, two samples are acquired from prostatic tissue, evaluated and histologically scored independently. A combined score is then reported (3). Clinical stage is the classification of cancer according to the TNM staging system (T: tumor size, N: lymph node involvement, M: metastasis). The NCCN adds expected patient survival or life expectancy to the previous four factors for further sub-classification of the disease management modalities. Assessment of life expectancy can be determined using normograms available online (www.ssa.gov/OACT/STATS/table4c6.html) (25). Management varies according to disease stage. Local disease with relatively low risk (according to previous 4 or 5 factors) is usually monitored through PSA levels. Advanced disease requires more aggressive approaches, including surgical, radiation, and hormonal therapies. Surgical

options include removal of prostatic tissue (prostatectomy) or removal of sources of androgen hormones synthesis and release. Androgens might be released from testes (resection of testes is referred to as orchiectomy), or adrenals (resection of adrenals is referred to as adrenalectomy). Indirectly, the pituitary gland can release luteinizing hormone (LH) and stimulate the synthesis and release of androgens (resection of pituitary gland is referred to hypophysectomy). Hormonal options include 1st and 2nd line therapies. First line therapies are inhibitors of LH or FSH release or direct antagonists of androgens at their receptors. Second-line therapies include estrogens, progesterones, diethylstilbestrol (DES), and androgen synthesis inhibitors. Table 2 summarizes modalities used for the management of hormone-dependent prostate cancer.

Table 1.2: Modalities used for the management of hormone-dependent prostate cancer

Modality	Example
Surgery	Prostatectomy Orchiectomy Adrenalectomy Hypophysectomy
Inhibitors of LH or FSH release	Leuprolide Goserelin
Antiandogens	Flutamide Bicalutamide Nilutamide
Androgen synthesis inhibitors	Aminoglutethimide Ketoconazole Abiraterone

LH: luteinizing hormone, FSH: follicle stimulating hormone

Usually, surgical, radiation and 1st line hormonal therapies are considered initially. Failure of such therapies requires 2nd line therapies. After exhausting these options, the cancer is referred to as castration resistant disease and more aggressive chemotherapies are required.

Chemotherapies for the management of prostate cancer

Prostate cancer has an innate resistance to chemotherapy during the initial phases of the disease and only responds to hormonal therapy (26). Later, during the phase of hormonal independence, cancer cells develop resistance to hormonal therapy and become more chemotherapy-sensitive. Docetaxel (DXL)-based regimens are the gold-standard therapy for chemotherapy treatment of prostate cancer. Other chemothearapies used include cabazitaxel, estramustine, mitoxantrone, vinblastine, satraplatin, cyclophosphamide, and vinorelbine (27). Cabazitaxel, a taxane, is increasingly used in the management of prostate cancer. Compared to the old gold-standard of prostate cancer management, mitoxantrone/prednisolone, cabazitaxel showed a significant increase in overall survival, progression free survival, and both PSA and radiologic objective response rate, with lower but not significant incidence of side effects (28). Later on, after exposure to multiple chemotherapy cycles, the disease develops more resistant forms to all treatment modalities. Although combination chemotherapy is a strategy aimed at reducing the incidence of drug side effects and the development of drug resistance. Eastham (29) has previously studied rates of recurrence in prostate cancer patients. A 56% recurrence rate was documented with a 39% incidence of local progression and 42% of metastasis.

Anti-angiogenic therapy for prostate cancer

Although being deregulated and could be one of the etiologies of prostate cancer, the efficacy of anti-angiogenic therapy for prostate cancer therapy is not clear. A search in clinicaltrials.gov, a service provided by the National Institute of Health, for reporting results of clinical trials, revealed 23 clinical trials about bevacizumab (an anti-VEGF,

antiangiogenic monocloncal antibody) in prostate cancer, 6 completed, 5 do not show results, and one shows incomplete results with no statistical analyses. A similar search in pubmed (bevacizumab + Clinical Trial as a filter for search) revealed 7 published clinical studies using bevacizumab. Three of them considered PSA as a measure of patient improvement making them less important (30-32), one reported cases of osteonecrosis of the jaws (ONJ) associated with antiangiogenic therapy (33), and three reported non-promising effects. Picus *et al,* showed no improvement of progression free survival (PFS) and complicated regimen with no additional benefit when bevacizumab was combined with docetaxel and estramustine (34). Vuky *et al,* didn't report disease-improvement endpoints and only showed no additional side effects with other therapies (35). Kelly *et al,* showed no additional improvement of overall survival (OS) although increased progression-free survival (PFS) and objective response (OR) was observed (36).

Prostate cancer: Growth, invasion, micrometastasis and metastasis:

Enhanced cell survival and proliferation is key to tumor growth as well as metastasis to various tissues; most commonly to the bone. Prostate cancer metastasis is a highly complex process. Understanding its molecular events and identifying its mediators are important steps for finding the proper therapy. For cancer cells to metastasize, they undergo a complex dynamic process that involves the interaction of cancer cells with components of the extracellular matrix, basement membrane, and endothelial cells. This miinteraction is mediated by adhesion molecules located on cancer cells and endothelial cells. Cancer cells interact via multiple molecules (such as integrins, proteoglycans, tetraspanins) with various components of the extracellular matrix (ECM).

On the other hand, cancer cells interact with endothelial cells cancer cell integrins and selectin ligands with cell adhesion molecules (CAMs), and selectins on the endothelial surface, respectively. Briefly, cancer cells detach from the core tissue and travel across the ECM through interaction with its components. When they reach blood vessels, cancer cells interact with endothelial components, disrupt the endothelial-barrier, and enter the circulation. At distant sites of metastasis, cells extravasate again by interaction with endothelial cells and disrupting the normal endothelial barrier. At this phase, cancer cells have achieved ***micrometastasis*** but are clinically undetectable in distant tissue. Once in distant tissues, cells will reside in metastatic sites, maintain their survival, proliferate to form distant ***metastatic*** cancer core. Many factors influence this process, such as tumor cells ability to secrete substances and/or their adhesion molecules expression profile which affect the interaction with ECM and endothelial cells. On the other side, endothelial cells also could respond differently to cancer cells through their adhesion molecules, which, combined with their effect on the barrier function status (adheren junctions and tight junctions), can collectively influence the ability of cancer cells to proliferate and metastasize.

Role of vascular normalization in prostate cancer

Within cancer tissue, the angiogenic blood vessels tend to be immature, leaky and have abnormal blood flow. This gives rise to two regions within cancer tissue: highly perfused areas and areas with low blood flow. Less perfused areas tend have less delivery of blood (hypoxic) as well as low levels of chemotherapeutic agents. Hypoxia leads to more stimulation of HIF1α system and more VEGF production and thus more angiogenesis, while less chemotherapy delivery gives rise to chemotherapy resistance.

Thus, the administration of agents that stabilize those immature vessels would be of benefit. This approach is termed vascular normalization. Normalization is achieved through the regulation of vascular maturation leading to a reduction in tortuosity and irregularity, as well as increased perivascular cells (PVCs) recruitment. Many modalities have been shown to induce vascular normalization in prostate cancer tissue, such as farnesyl transferase inhibitors (FTIs) (37), and androgen ablation therapy (38).

Bottlenecks facing prostate cancer management:

Metastasis is the ultimate fate of prostate cancer, especially in advanced disease. Although management of early disease shows promising results with high survival rates, this percent drops sharply with metastasis, as the 5-year disease free survival of patients with metastatic disease drops to 29% percent. Obviously, treatment strategies for late, metastatic and androgen-independent prostate cancer are lacking, given that low survival rate. For this reason, focus on the development of treatment strategies is needed for this stage. Targeting early disease and preventing progression to advanced disease is one strategy. Micrometastasis is a pre-requisite for the spread of disease and understanding this process and manipulating mediating mechanisms could be beneficial. In addition, exploring specific mechanisms for the proliferation and survival of advanced prostate cancer tumors would be valuable. Management of cancer depends on combination therapies. This approach depends on the use of the proper combinations with fewer potential for side effects and different mechanisms of action to provide proper synergism.

Statins and therapeutic applications

Statins are the second most commonly prescribed medications in 2011, with 94.1 million prescriptions written for Zocor® (simvastatin). Also, the number one best selling drug in the United States is Lipitor® (atorvastatin), with sales of $7.2 billion (39). Figure 1.4 shows the structure of the most commonly prescribed statins.

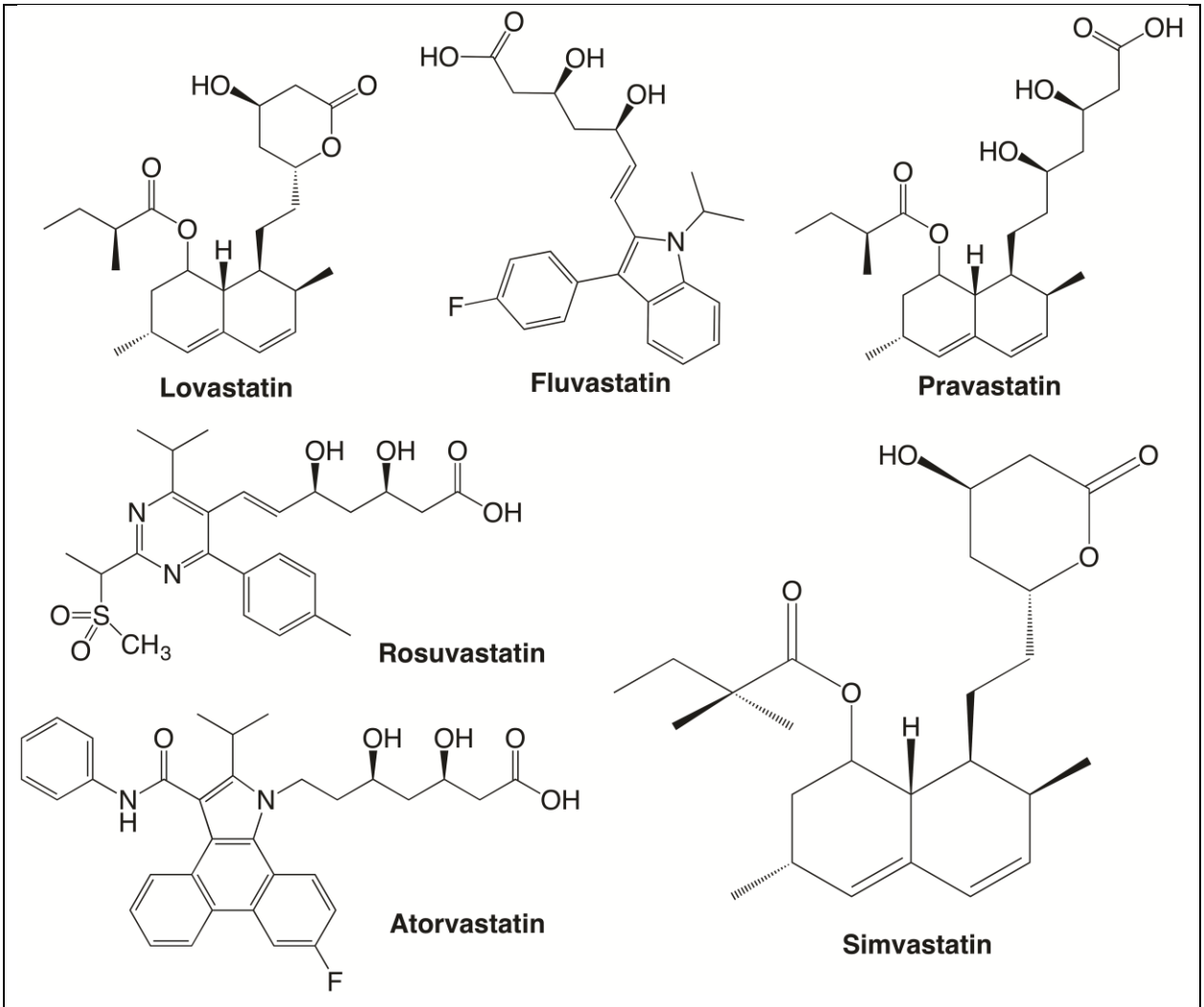


Figure 1.4: Structures of statins (Drawn using ChemDraw 13.0)

Statins are lipid-lowering agents used in cases of hypercholesterolemia and severe cases of combined hypertriglyceridemia/hypercholesterolemia. They exert their effects through inhibition of HMG-CoA reductase, the rate-limiting step in cholesterol

biosynthesis. Structurally, statins can be classified into two major groups: lipophilic (atorvastatin, lovastatin, simvastatin, fluvastatin) and hydrophilic (pravastatin and rosuvastatin) agents (40). Considering activity, Lovastatin and simvastatin are structurally inactive, and their lactone ring undergoes metabolism in liver to become the active compounds drugs (Goodman and Gilman). Among all statins, atorvastatin and simvastatin are considered the most potent (3). Statins show pleiotropic effects that can be explained partly but not totally by their mechanism of action. In clinical practice, considering their use for cardiovascular conditions, statins show beneficial effects on endothelial cells including protection, atherosclerotic plaque stabilization, as well as their anti-inflammatory, anti-oxidant and anticoagulant effects (Goodman and Gilman).

Statins and pleiotropic effects of cells

Statins could affect survival of cells through inhibition of cholesterol synthesis, a basic component of lipid rafts that control epidermal growth factor receptor (EGFR) signaling (41). Some of the intermediates of cholesterol synthesis (geranyl, farnesyl, and geranylgeranyl pyrophosphates) contribute to the process of lipid modification of Ras (an oncogene) and Rho family of small GTPase proteins (Rho, Rac, cdc42) that lead to their activation. Statins have been demonstrated to induce eNOS overexpression (42) as well as phosphorylation and activation (43). Inhibition of eNOS expression has also been reported (44). Through their antioxidant effect, statins reduced the expression of VEGF in NSCLC cells (45). Insulin-like growth factor-1 receptor (IGF-1R) expression was also proven to be controlled and inhibited by statins (46). Statins also inhibit NF-κB activation (47, 48), warranting their benefit in hematologic malignancies, especially multiple myeloma. Statins have also been reported to both activate (49) and inhibit (50)

PI3K/Akt system. Effects of statins vary according to cell type and dose used. Higher doses have inhibitory effects against cell growth proliferation and survival, while lower doses seem to have growth-promoting effects. Overall, statins have been shown to inhibit cancer cell growth yet has no effect on normal cells (51).

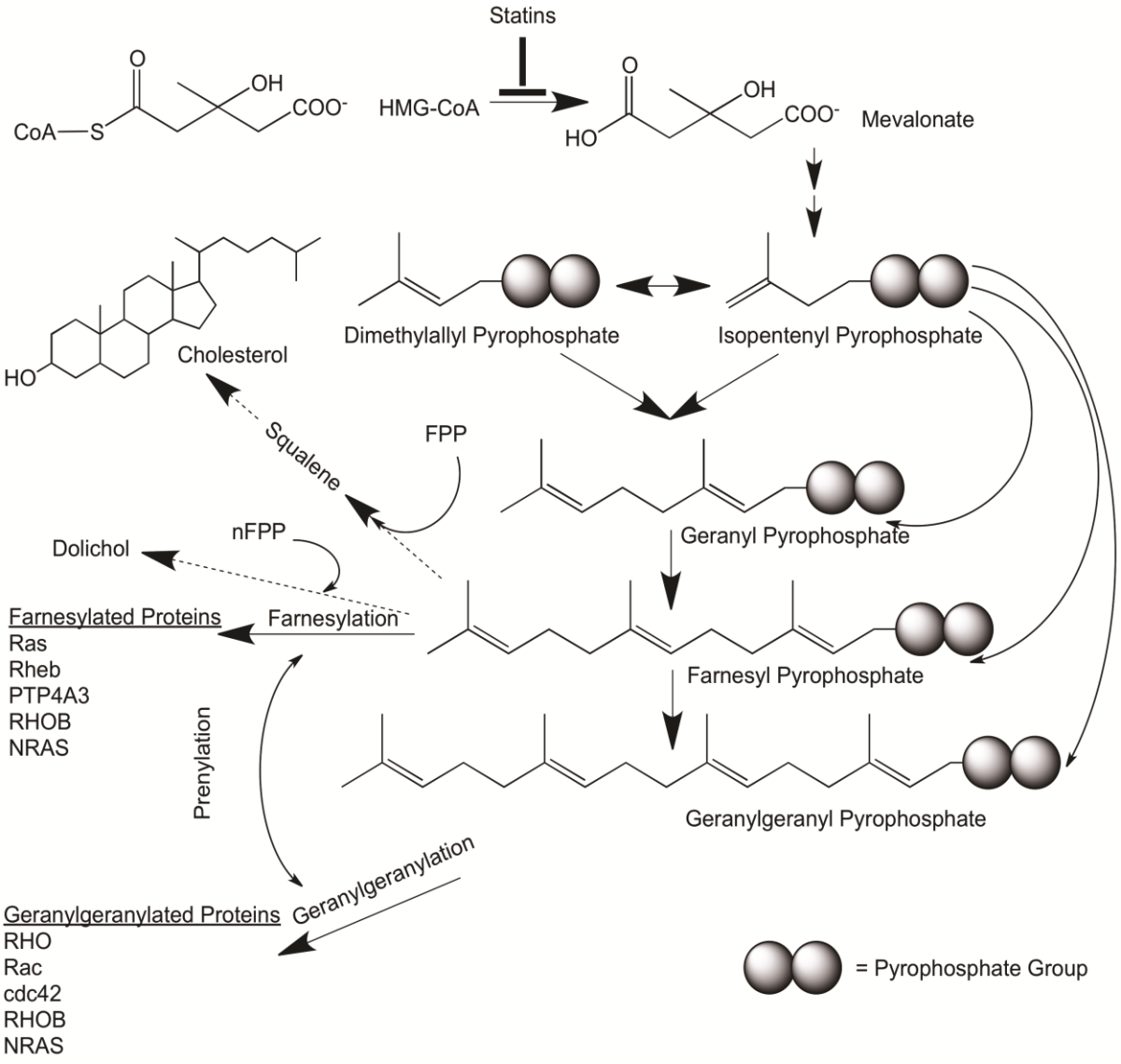


Figure 1.5: A schematic of cholesterol synthesis pathway and some of its intermediates that could explain statins pleiotropy (Adapted from (1) and (2). Drawn using ChemDraw 13.0)

Statins and cancer clinical trials:

A meta-analysis of studies considering statins use and association with cancer or influence of statins use after initial prostate cancer was conducted by Papadopoulos et. al. and showed some interesting results (52). Statins were either associated with protection against cancer, or reduced cancer progression. When those findings were corrected for the PSA testing, statins were associated with prevention of prostate cancer, even reduction cancer progression after surgical therapy/radiation therapy was observed. PSA correction was necessary due to the fact that statins-prescribed patients tend to be more closely followed up and thus had their PSA tested regularly as well as the fact that statins has the ability to reduce PSA levels. Later studies show statins to be beneficial in prostate cancer. Katz *et al,* (53) have shown a significant reduction of recurrence of prostate cancer in statin users after radical prostatectomy and radiation therapy. Gutt, (54) showed a significant improvement of relapse-free survival among statin users. In Hamilton *et al,* study (55), statin users had lower clinical stage at diagnosis, lower Gleason scores and PSA levels, significantly lower PSA recurrence rate, that was dose-dependent (lower with higher doses) with a significant improvement in survival. Breau (56) showed similar results to Hamilton's study, statins users had less incidence of prostate cancer, and even in prostate cancer diagnosed patients, statin users had less severe disease (less Gleason score). Ritch (57) also showed similar results to Hamilton's study, following radical prostatectomy. Statins users had a lower 5-year biochemical relapse-free survival compared with non-users. Moreover, Marcella (58), showed that both unadjusted and adjusted odds ratio of mortality of prostate cancer patients was significantly lower for statins users compared to non-

users, and was more significant in highly potent statins. Although not completely conclusive, evidences supporting the use of such drugs in cancer therapy for an already established disease diagnosis is appealing.

Gaps in knowledge

Statins show a promising effect on cancer management but less is known about the exact mechanism of their action. Some clinical trials showed contradicting results regarding statins effect, whether pro- or anti-cancer. But the main body of evidence supports an anti-cancer effect of statins, especially after PSA correction. An exact mechanism of action for the effect of statins on cancer cells could not be predicted. In addition, the proper dose and frequency of administration for the management of cancer could be different from the regimen used for cardiovascular conditions. Reviewing literature showed that simvastatin in clinical trials is used in 2 dosing levels, standard (20-80 mg/day) or high (15 mg/kg/day) doses. The high dose would be 1050 mg/day for a 70 kg individual, which is 13x the highest range of lower dose. In multiple myeloma, Schmidmaier et. al. (59) showed the addition of simvastatin of 80 mg/day starting 2 days before therapy and continuing 2 days after therapy, with bortezomib or bendamustine showed an increase in drug response evident by a reduction of M protein. This study has some limitations, as it didn't include a control group. Sondergaard et. al. (60) showed that a high dose of simvastatin was associated with a negative effect on bone osteoclasts in MM. In a study by van der Spek et. al. (61), high dose of simvastatin addition to vincristine, doxorubicin, and dexamethasone (VAD) regimen in MM showed a partial response for such a treatment and study had to be terminated. On the other hand in extensive disease of small cell lung cancer (62), a low

dose of simvastatin (40 mg/day) addition to irinotecan and cisplatin didn't show any benecficial effect, although showing more effect of the combination in ever-smokers compared to non-smokers.

Objectives

In this research, the effect of a lipophilic statin, simvastatin, on prostate cancer will be tested. Both *in vitro* and *in vivo* approach will be used. The in vitro part will give an idea about the proper dose that can be translated into an *in vivo* dose in mice. The proper dose and frequency of administration will be tested in the *in vivo*, xenograft model. We will also characterize the mechanisms by which simvastatin exerts its effect on prostate cancer *in vitro* and confirm it *in vivo*. The mechanism by which simvastatin mediates its effects on prostate cancer cells through apoptosis, as well as its potential to impair migration of cancer cells will be studied *in vitro* and confirmed *in vivo*.

Central Hypothesis

The central Hypothesis is that statins, due to their differential effects on normal vs. malignant cells, have the potential to be used as anti-cancer agents. While their anti-tumor cell effects can be cancer preventive, their ability to activate endothelial cells provide the benefit of using statins for the normalization of tumor vasculature prior to chemotherapy, a strategy widely applied in anti-angiogenic therapy.

This will be achieved by studying the following specific aims:

Specific Aim 1: *Determine the anti-cancer efficacy of simvastatin on prostate cancer cells and identify the molecular mechanisms regulating simvastatin-mediated effects on prostate cancer cellular functions in vitro and tumor growth in vivo.*

Specific Aim 2: *Determine the molecular mechanisms regulating simvastatin-mediated inhibition of prostate cancer micrometastasis.*

Significance of the study: Drug repurposing is gaining interest in pharmacotherapeutic interventions. Simvastatin is one of the common drugs that gained such interest, based on the fact that it elicits pleiotropic effects in multiple cell types. Repurposing of such a generic drug for management of prostate cancer will have many beneficial effects include reduced costs of therapy and a predictable profile of side effects. The only concern in this modality is whether higher doses of simvastatin used could increase the incidence of side effects.

Translational impact:

The use of already FDA-approved drugs like statins with established parameters of pharmacokinetics, pharmacodynamics, and side effects profile will have a high translational impact. Repurposing of those drugs for the management of cancer while managing other conditions treated with statins will be an interesting treatment strategy.

CHAPTER 2:

ANTI-CANCER EFFICACY OF SIMVASTATIN ON PROSTATE CANCER CELLS AND TUMOR XENOGRAFTS IS ASSOCIATED WITH INHIBITION OF AKT AND REDUCED PSA EXPRESSION

Belal Al-Husein (co-author), Samith T. Kochuparambil**,** Anna Goc, Sahar Soliman and Payaningal R. Somanath

2011, *Journal of Experimental Therapeutics*, 336, 496.

Samith T Kochuparambil and Belal Al-Husein contributed equally to this manuscript. This research was funded by the University of Georgia Research Foundation to PRS.

List of non-standard abbrevations: BrDU*, 5-Bromo-2-deoxyuridine*; FBS, Fetal bovine serum; DMSO, Dimethyl sulfoxide; PI3K, PI3 Kinase; PAGE, Polyacrylamide gel electrophoresis; TRAMP, Transgenic adenocarcinoma of the mouse prostate; LNCaP, Lymph node carcinoma of the prostate; RTK, Receptor tyrosine kinase; GSK, Glycogen synthase kinase; DMEM, *Dulbecco's* modified eagle medium; ELISA, Enzyme-Linked immunosorbent assay; ACAT, Acyl-CoA cholesterol acyltransferase; EGF, Epidermal growth factor; PSA, Prostate specific antigen, SREBP-2, Sterol-responsive Element-binding Protein; PTEN, Phosphatase and tensin homolog; VCaP, Vertebral cancer of the prostate; myrAkt, Myristoylated Akt.

Abstract

Prostate cancer is the second leading cause for cancer-associated death among men in the United States. More recently, there has been a renewed interest in the potential therapeutic benefits of statins for cancer. Simvastatin, a widely used generic drug for preventing cardiovascular events, is well known for its effects on cellular proliferation and inflammation, two key processes that also determine the rate of tumor growth. While a growing body of evidence suggests that statins have the potential to reduce the risk of many cancers, there are discrepancies over the pro- and anti-cancer effects of statins on cancers. In the current study, we sought to investigate the effects of simvastatin on the Akt pathway in prostate cancer cells with respect to the regulation of various cell functions *in vitro* and tumor growth *in vivo*. Time- and dose-effects of simvastatin on LNCaP (androgen-dependent) and PC3 (androgen-independent) cells indicated that treatment with as low as 25 μM simvastatin was sufficient to inhibit serum-stimulated Akt activity. Akin to this, treatment with simvastatin significantly inhibited serum-induced cell migration, invasion, colony formation and proliferation. Simvastatin-mediated effects on colony formation was rescued by adenovirus-mediated expression of constitutively active Akt (myristoylated Akt) in PC3 cell lines. A PC3 xenograft model performed in nude mice exhibited reduced tumor growth with simvastatin treatment associated with decreased Akt activity and reduced PSA levels. Our findings demonstrate the therapeutic benefits of simvastatin for prostate cancer and suggest a link between simvastatin, regulation of Akt activity and PSA expression in prostate tumors.

Introduction

Statins [3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase inhibitors], the second most prescribed drugs following analgesics, are also considered to be among the safest drugs. Despite the long-term nature of the treatments, use of statins have been shown to cause elevation in liver enzymes, myalgias and rhabdomyolysis (which are rare (63)), but the benefit/risk ratio could justify its use,, particularly in the management of cancer. A recent meta-analysis performed using the information retrieved from QResearch database indicated that use of statins is not associated with risk for diseases such as Parkinson's disease, rheumatoid arthritis, venous thromboembolism, dementia, osteoporosis or cancers of the gastric, colon, lung, melanoma, renal, breast or prostate (64). However, moderate increases in the risk for liver or kidney dysfunction, myopathy and cataract were associated with statin use. In humans, reports on the effects of statins on cancer have yielded varied results ranging from increased risk, to no net effect, to decreased risk of cancer (65). Many believe that these differences could be either due to variations in the doses used for the treatment of many cardiovascular conditions (66) or due to the hydrophobic nature of some, but not all statins (67). A number of pre-clinical studies have implicated that statins can modulate the efficacy of many anti-tumor therapeutic modalities (65).

Hydrophobic statins (simvastatin, lovastatin and fluvastatin) have been shown to inhibit cancer growth. In cell based experiments *in vitro* and in experimental animal models, these statins have displayed inhibitory effects on many cancers, including head and neck, prostate, lung, breast, colon, pancreas, skin (melanoma), renal cell, bladder, liver and multiple myeloma (65). Information from patient-based studies on the effects of

statins on prostate cancer has only started to trickle down, and the reports have been highly contradictory. Initial case-controlled study showed that use of statins is associated with 50% reduction in the risk of prostate cancer (68), which was supported by another study on atorvastatin and prostate cancer clinical outcome (69). However, a study performed in Finnish population on statin use and incidence of prostate cancer did not show any significant correlation between them (70). In contrast, another study performed in Finnish population showed decreased overall relative risk of prostate cancer and reduced serum PSA levels among current statin users with proportional changes corresponding to the amount and duration of use (71). Also, a number of reports published in the recent months demonstrate that statin use is associated with decreased chances of undergoing prostate biopsy and receiving a Gleason score of 7 or greater (56, 72). Reduction in serum-PSA and total testosterone levels among statin users compared to non-statin users has also been reported by other groups (73). A very recent study focused on characterizing the association between statin use and PSA recurrence after prostectomy demonstrated a dose dependent reduction in the risk of biochemical recurrence (55). Although controversial, together these studies suggest that long-term statin use can prevent or delay prostate cancer onset in men.

Simvastatin, a generic drug, is the most widely used statin for the prevention and treatment of cardiovascular events. In a recent study that established a strong correlation of statin use with decreased serum-PSA levels and risk of biochemical recurrence of prostate cancer after radical prostectomy, simvastatin was used by most of the subjects (171 out of 236) involved in the study (55). In the current study, we focused on studying the effects of simvastatin on prostate cancer cell functions *in vitro*,

growth and prostate cancer xenograft in nude mice *in vivo* and characterizing the major molecular mechanisms regulating the process. Our findings indicated that simvastatin has direct effects on prostate cancer cells in the regulation of multiple cellular functions such as cell migration, invasion, proliferation, cell survival/apoptosis and colony formation *in vitro* as well as growth of prostate tumor xenograft *in vivo*. Simvastatin treatment inhibited Akt activity in prostate cancer cells in a dose- and time-dependent manner. More importantly, our results indicated that prostate cancer cells stably expressing constitutively active Akt (myr-Akt) were resistant to simvastatin-mediated inhibition of prostate cancer cell functions. We conclude that simvastatin can be developed as a potential therapeutic agent for the management of prostate cancer. In addition, changes in Akt phosphorylation, in addition to reduced serum-PSA levels, can be an important surrogate marker to determine the patient response to simvastatin therapy.

Methods

Cell lines, reagents, and antibodies: Human PC3 and LNCaP cell lines were obtained from ATCC (Manassas, VA). All cell lines were maintained in DMEM (HyClone) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. $DiIC_{12}$ fluorescence dye was purchased from BD Biosciences (San Jose, CA). Primary antibodies such as: anti-Akt, anti-phospho-Akt^{S473} and anti-phospho-$GSK3^{S9/21}$ were purchased from Cell Signaling (Boston, MA). Primary antibodies against β-actin were purchased from Sigma (St Louis, MO) and anti-PSA antibody was purchased from Pierce Biotechnology Inc. (Rockford, IL). Anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from BioRad (Hercules,

CA). Docetaxel and simvastatin were purchased from Sigma (St Louis, MO). Simvastatin was activated in the lab using the manufacturer's instructions.

Migration assay: PC3 cells were grown to confluence, and a scratch was made in the monolayer followed by treatment with simvastatin (control buffer, 25, 50 and 100 µM). Scratch recovery was determined at 16h and 24h. Microscopic pictures were analyzed using Image J software and recovery was calculated using the equation: $[100X\ (1-T_f/T_0)]$ %, where T_f is the area at the end-point and T_0 is the area at the time zero. The data are presented as mean ± SD.

Invasion assay: The invasion of PC3 cell lines was measured using BD BioCoat Tumor Invasion Assay Kit (BD Biosciences) coated with BD Matrigel Matrix according to the manufacturer's protocol. PC3 cells were labeled with BD $DiIC_{12}$ fluorescence dye and seeded onto the upper chamber of a 96-well Transwell plate at a density of $1X10^4$ cells per well in 400 µL medium. DMEM containing 10% FBS was then added to the lower chamber. After 24h, the cells were treated with control buffer, 25 and 100 µM of simvastatin in DMEM medium. The fluorescence from the stained cells was measured after 12h and 24h on an ELISA plate reader at 549/565 nm (Ex/Em). The data are presented as mean ± SD.

Cell doubling time assessment: In each experiment, cell doubling time was determined according to direct cell count and in consideration of logarithmic growth of cancer cells (www.doubling-time.com). For direct cell count, approximately 100 cells per well were seeded in 400 µL medium on a 48-well plate, in quadruplicates. After 24h, medium was replaced and cells were counted. The cells were treated with control

buffer, 25 µM and 100 µM of simvastatin in DMEM. At 24h, cell counts were repeated. The cell doubling time was calculated as the mean ± SD.

Trypan blue viability assessment: In the trypan blue method, cells were grown to confluence in DMEM medium with 10% FBS. The cells were treated with simvastatin 25 µM and 100 µM in DMEM. After 24h, cells were collected and re-suspended in PBS with 0.4% Trypan blue solution. Total cells and Trypan blue stained (i.e., non-viable) cells were counted and percentage of non-viable cells was calculated.

Apoptosis assessment: Cytoplasmic histone-associated DNA fragments were quantified by using the Cell Death Detection ELISA^{PLUS} Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Briefly, PC3 cell lines were seeded in 96-well plates at a density of $1X10^4$ cells/well. After 24h, the cells were incubated in DMEM containing 25 and 100 µM simvastatin for 16h. Control cells were treated with 0.1% DMSO (vehicle control). Cells were lysed, centrifuged (200g for 10min) and the collected supernatant was subjected to ELISA. The absorbance was measured at 405nm (reference wavelength at 492nm). The data are presented as mean ± SD.

Cell proliferation assay: The effect of simvastatin on proliferation of PC3 cell lines was determined using the nonradioactive BrDU–based cell proliferation assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Briefly, PC3 cells were seeded in 96-well plates at a density of $5X10^3$ cells per well. After 24h, the cells were incubated in DMEM containing 25 and 100 µM simvastatin for 16h. Control cells were treated with 0.1% DMSO (vehicle control). Control cells were treated with 0.1% DMSO (vehicle control). After treatment, the cells were subjected to a 5-bromo-2-

deoxyuridine assay using the BrDU Labeling and Detection Kit III (Roche Applied Science), according to the manufacturer's protocol. BrDU incorporation into the DNA was determined by measuring the absorbance at both 450 and 690nm on an ELISA plate reader. The data are presented as mean ± SD

Colony formation assay: Colony formation assay was performed using standard protocol (74). In this approach, PC3 cells were cultured on 6-well plates till monolayer was reached. The wells were treated with DMEM containing 25 and 100 µM simvastatin. Control cells were grown in DMEM media. At 5 days post treatment, each of the wells was counted for the number of colonies and simvastatin-treated wells were compared to the vehicle treated control. Plates were fixed using 2% paraformaldehyde, briefly stained with crystal violet and counted visually or using Image J software. The data are presented as mean ± SD.

Western analysis: Cells/Tissue PC3 and LNCaP cell lines were cultured to reach a monolayer in DMEM in 6 well plates. The wells were treated with DMEM containing 25 µM and 100 µM simvastatin. Control cells were grown in DMEM alone. Whole cell lysates were prepared using lysis buffer [50 mM Tris-HCl (pH=7.4), 1 % TritonX-100, 150mM NaCl, 1mM EDTA, 2mM Na_3VO_4, and 1X Complete protease inhibitors (Roche Applied Science, Indianapolis, IN)]. Tissue obtained from mice was snap frozen with liquid nitrogen. Tissue was pulverized with mortar and piston. Tissue lysates were prepared using lysis buffer. The protein concentration was measured by the D_L protein assay (Bio-Rad, Hercules, CA). Western analyses were performed using standard Laemmli's method as done previously (75).

***In vivo* nude mouse tumor xenograft model:** All animal procedures listed in the manuscript were performed as per the protocol approved by the IACUC at the Charlie Norwood VA medical Center, Augusta (protocol # 09-07-011 dated July 10, 2009). PC3 cells were grown to confluence in 250cc flasks. Cells were re-suspended in PBS to a concentration of 1×10^6/ml. 1ml of cell suspension was injected subcutaneously (SC) in 6-8 weeks old nude mice (Athymic nude mice, Harlan Laboratories, Indianapolis, IN). Mice were divided into two groups. The groups were subjected to intraperitoneal (IP) injections of simvastatin at the dose of 2mg/kg body weight/every 12h (or 24h in a second set of experiments) for 2 weeks. The respective controls were injected IP with 0.9% saline every 12 or 24h. Tumor sizes were measured on day 7 and day 11 respectively. Mice were sacrificed on day 11 and tumors were dissected and weighed.

Statistical Analysis: Mean activities were calculated from 3-5 independent experiments done at least in triplicates. The Student's two-tailed t test was used to determine significant differences between treatment and control values.

Results

Simvastatin treatment inhibits phosphorylation and activity of Akt in prostate cancer cells in a dose- and time-dependent manner

Since Akt is central to many signaling pathways and is a known mediator of many functions of cancer cells, we sought to determine whether treatment with simvastatin will have any effect on phosphorylation and activity of Akt. Our results indicate that treatment with simvastatin had a robust effect on inhibition of Akt phosphorylation in LNCaP and PC3 cells. Effects were seen from 25 µM and maximum inhibition was observed when 75 µM simvastatin was used (Figure 2.1A). A time-course study of

simvastatin effects on LNCaP cells indicated that while 100 µM simvastatin inhibited phosphorylation of Akt in 4h, a maximum reduction in Akt phosphorylation by 25 µM simvastatin was observed at 16h (Figure 2.1B). Similar effects of simvastatin were observed in metastatic human PC3 prostate cancer cell lines (Figure 2.1C). In order to determine whether reduction in phosphorylated Akt levels in PC3 and LNCaP cells had any effect on its activity, we determined levels of phosphorylated GSK3, a well-known substrate of Akt. Our analyses indicated that, similar to its effects on Akt phosphorylation, simvastatin inhibited phosphorylation of GSK3 in PC3 and LNCaP cells in a time- and dose-dependent manner. Together, our results indicate that simvastatin inhibits Akt activity in prostate cancer cells.

Simvastatin inhibits migration and invasion of PC3 cells

Since simvastatin treatment inhibited Akt activity in prostate cancer cells, we determined whether simvastatin has any effect on prostate cancer cell migration and invasion. Our data indicated that treatment with 25 µM simvastatin on PC3 cells maintained in serum containing medium significantly impaired their ability to migrate (2.2A and 2.2B) as analyzed at 16h ($p<0.04$ for 25 µM and $p<0.05$ for 100 µM) and 24h ($p<0.0002$ for 25 µM and $p<0.001$ for 100 µM) post treatment (~2 and 3 fold decrease). At 24h, treatment with 100 µM simvastatin almost completely inhibited (~90% inhibition compared to control) PC3 cell migration. Similarly, treatment of PC3 cells with 25 µM simvastatin significantly inhibited invasion in response to EGF ($p<0.005$ for 12h and $p<0.01$ for 24h) and 10% FBS ($p<0.03$ for 12h and $p<0.0002$ for 24h) (Figure 2.2C). At 12h and 24h post treatment with 25 µM simvastatin, we observed ~12% and ~15% inhibition in EGF-stimulated PC3 cell invasion, respectively. Treatment with 100 µM simvastatin further

enhanced the inhibition of PC3 cell invasion up to 26% compared to the EGF-treated control (p<0.01). Effects of simvastatin on EGF-stimulated prostate cancer cell invasion were significantly higher compared to cells that were maintained in 10% FBS containing medium (Figure 2.2D). In the presence of 10% FBS, 25 µM simvastatin treatment resulted only in a modest inhibition of PC3 cell invasion (1.5-3% inhibition, compared to control). Inhibition of PC3 cell invasion was slightly higher upon treatment with 100 µM simvastatin, compared to the control (3.5-7.5%) (p<0.005 for 12 h; p<0.003 for 24 h). Overall, our data indicates that simvastatin treatment significantly inhibits PC3 cell migration and invasion.

Simvastatin inhibits proliferation and induces apoptosis of PC3 cells

We next determined whether simvastatin treatment has any effect on prostate cancer cell proliferation. Normally, metastatic PC3 cells have a doubling time of 10-14 hours. Our study showed that treatment with 25 µM simvastatin resulted in a 6-8 fold increase in doubling time for PC3 cells (p<0.0003) (Figure 2.3A). This effect was even greater when cells were treated with 100 µM simvastatin reaching well above 300h (~20 fold) (p<0.001) (Figure 2.3A). Data from the proliferation assay revealed that treatment with simvastatin resulted in significant inhibition of PC3 cell proliferation by 25-35% for 25 µM (p<0.0001) and 100 µM (p<0.00001) simvastatin, respectively (Figure 2.3B). The effect of 25 µM simvastatin on proliferation was similar to the effects of a low dose treatment with Docetaxel/Taxotere (10 nM) (p<0.0001), a currently used chemotherapy drug for the management of prostate cancer in patients (Figure 2.3B). Thus, our data indicates that simvastatin significantly inhibits prostate cancer cell proliferation *in vitro*.

Agents that can induce apoptosis in cancer cells have been an excellent choice for cancer treatment. Our study indicated, using viability assay, that treatment with 25 µM simvastatin increased cell death by ~30% over a 12h period in PC3 cells (p<0.0001) (Figure 2.4A). This effect was further enhanced by 100 µM simvastatin, which exhibited more than 2 fold increase in cell death (p<0.0001). Similarly, treatment of PC3 cells with 25 and 100 µM simvastatin resulted in 1.5 fold (p<0.00005) and 1.75 fold (p<0.00005) increases in apoptosis, respectively (Figure 2.4B). Our studies demonstrate that simvastatin induces apoptosis and cell death in prostate cancer cells.

Simvastatin-inhibited colony formation by PC3 cells can be partially rescued by adenovirus-mediated expression of constitutively active Akt

An important feature of the tumor cells is that they are resistant to contact inhibition and form colonies or foci. We determined whether inhibition of Akt activity by simvastatin has any effect on colony formation by prostate cancer cell lines. Our experiments show that PC3 cell lines develop colonies once they are allowed to form a monolayer and left for additional 5 days. Treatment with 25 µM simvastatin significantly inhibited (~25%) colony formation by PC3 cells (p<0.04) (Figure 2.5A), suggesting that simvastatin inhibits prostate cancer foci formation, possibly via Akt inhibition. Unlike the effects of simvastatin on proliferation, its effects on colony formation were lower compared to the effects of low dose Docetaxel (10 nM), which inhibited colony formation by PC3 cells by ~60% (p<0.01) (Figure 2.5B). In order to investigate whether inhibition of colony formation by simvastatin was mediated through Akt inhibition, we next determined if prostate cancer cells expressing constitutively active Akt (myrAkt) can resist inhibition of colony formation by simvastatin. Our initial studies comparing PC3 cells expressing

GFP (control) with those expressing myrAkt (both the transfections were performed via adeno-virus infections) showed that cells expressing myrAkt exhibit a significantly higher number of colonies compared to cells expressing GFP (p<0.03) (Figure 2.5C). As we hypothesized, our data indicated that PC3 cells expressing myrAkt were partially resistant to simvastatin-mediated inhibition of colony formation by PC3 cells. There was no significant difference between simvastatin-treated and non-treated PC3 cells expressing ad-myrAkt (p<0.5) (Figure 2.5D). In sum, these results demonstrate that treatment with simvastatin inhibits colony formation by PC3 cells and that the Akt pathway is one of the major pathways modulated by simvastatin in prostate cancer cells.

Simvastatin inhibited growth of PC3 tumor xenograft in male nude mice is associated with an inhibition of Akt activity and a reduced expression of prostate specific antigen (PSA)

Inhibition of colony formation by simvastatin provided the essential message that treatment with simvastatin may be an effective strategy to either prevent or manage prostate cancer *in vivo*. To test this, we performed tumor xenograft study in nude mice. In an initial study, PC3 cells were administered in nude mice and were treated with simvastatin (2mg/kg body wt/day), administered intra-peritoneally as performed previously (76). Analyses of tumor size on a daily basis for 14 days and the tumor weight on day 14 after tumor cell injections were made. Data did not show a significant difference in tumor size on any day except day 14 (p<0.04) (Figure 2.6A). However, no significant changes in tumor weight on day 14 (p<0.9) between control and simvastatin-treated mice (Figure 2.6B) were observed. Next, we also determined if simvastatin

would affect the growth rate of prostate tumors once they have already grown. Hence, tumor sizes measured on days 7 and 11 were used to determine the change in tumor growth in simvastatin-treated mice compared to saline control. Mice treated with simvastatin did not exhibit any differences on changes in tumor size between days 7 and 11 ($p<0.2$) (Figure 2.6C and 2.6D) or between days 11 and 14 (data not shown).

Although the effect of simvastatin on the growth of tumor xenografts was not significant compared to saline administered controls, a trend towards reduced growth of tumor xenografts in simvastatin-treated mice compared to control mice was apparent. Hence, in the next step, we modified the protocol to study the effects of simvastatin in mice based on its dose and frequency of administration. The time of simvastatin administration was increased to twice a day (2mg/kg body wt/12h). These changes showed significant differences in prostate tumor growth between saline and simvastatin administered mice (Figure 2.6). Overall tumor weight determined on day 14 post-tumor injection showed reduced growth of prostate tumor xenograft in simvastatin administered mice compared to saline control ($p<0.03$) (Figure 2.6E). A significant reduction in tumor size was also observed in simvastatin-treated mice compared to saline control ($p<0.03$) (Figure 2.6F). Next, we sought to analyze the percentage change in tumor growth between day 7 and day 11 post-tumor injection. Our data indicated that simvastatin inhibited growth of tumors from day 7 to day 11 when compared to its original size on day 7 ($p<0.02$) (Figures 2.6F, 2.6G and 2.7A).

In order to determine whether the effect of simvastatin on the growth of PC3 tumor xenograft involves inhibition of Akt and/or changes in the expression levels of prostate specific antigen (PSA), we prepared tumor lysates and subjected for western analyses

using antibodies specific for phospho-Akt and PSA. Our data indicated that simvastatin treatment in PC3 cells resulted in a significant reduction in phospho-Akt (~70% reduction) and PSA levels (~95% reduction) (p<0.0001 and p<0.002, respectively) (Figure 2.7B and 2.7C). Overall, our studies on the effects of simvastatin on tumor xenograft in male nude mice demonstrates that simvastatin inhibit prostate tumor growth *in vivo* involving inhibition of Akt activity and a reduction in PSA expression.

Discussion

Although controversial, many recent analyses of patient samples conducted by different groups have revealed the potential benefits of statins in the management of prostate cancer (65). In the current study, we report the potential benefits of simvastatin in the management of prostate cancer. In LNCaP and PC3 prostate cancer cell lines, simvastatin exhibited a dose- and time-dependent inhibition of Akt activity. Simvastatin treatment resulted in significant inhibition of cell migration, invasion, survival, doubling time, proliferation and colony formation as well as enhanced apoptosis in PC3 cells. The effect of simvastatin on colony formation was partially rescued in PC3 cells stably expressing constitutively active Akt. Intra-peritoneal administration of simvastatin in nude mice bearing PC3 tumor xenografts exhibited significant reduction in tumor size and weight associated with a reduction in PSA expression, compared to saline administered controls. Apart from this, we also observed significant reduction in the rate of tumor growth (from day 7 to day 11) in simvastatin-treated mice, compared to control. In sum, our data clearly demonstrates the ability of simvastatin to inhibit pro-tumorigenic functions of prostate cancer cells, to induce apoptosis and to inhibit tumor growth *in vivo*.

A number of characteristic effects of statins on cells provide the necessary clues for its potential benefits in cancer therapy. First, statins inhibit synthesis of mevalonate, which is necessary for the synthesis of isoprenoid compounds. Isoprenoid compounds are the precursors of cholesterol, lichol and ubiquinone and are the substrates for post-translational modifications of many proteins (77). Second, statins are known to inhibit proliferation of smooth muscle cells in the vasculature leading to primary and secondary prevention of cardiovascular events (78). In addition, they are known to induce apoptosis in smooth muscle cells (78) and many cancer cell types (65). These properties of statins can be very promising for their prospective use in inhibiting proliferation and survival of cancer cells. The dose at which statins enhance Akt activation and survival in endothelial cells (79, 80) is the same dose that inhibits Akt activity, cell proliferation and induces apoptosis in malignant smooth muscle cells in atherosclerotic lesions (81) and cancer cells. This property of statins will be extremely important in avoiding side effects when statins are used for cancer therapy. An earlier study performed in hormone-responsive LNCaP cells showed that lovastatin specifically activated caspase-7 via enhanced expression of caspase-7 mRNA (82), which was prevented by pre-treatment with mevalonate. Our results further support the existing hypothesis that statin can be developed into a potential therapeutic drug for the long-term management of prostate cancer without inflicting any major side-effects.

Molecular mechanisms regulating statin-mediated responses in cancer cells have been a recent focus of investigation. Cholesterol lowering effects of statins are believed to be a very important factor in the regulation of prostate cancer cell functions. Androgens are known to mediate cholesterol metabolism in LNCaP cells involving Acyl-CoA cholesterol

acyltransferase (ACAT) facilitating tumor progression (83). Previous studies have shown that prostate cancer cells lack sterol mediated feedback regulation of sterol regulatory element binding protein 2 (SREBP-2) in LNCaP and PC3 cells, a transcription factor regulating cholesterol homeostasis (84). Samples collected from prostate cancer patients have revealed accumulation of cholesterol (85). Cholesterol rich lipid rafts have been implicated in tumor progression and metastasis (86). Cholesterol depleting agents are known to induce apoptosis via decreased production of cholesterol-rich lipid rafts in normal prostatic epithelium, human epidermoid carcinoma (A431) and breast cancer (MCF-7 and MDA-MB-231) cell lines (87). At the same time, products of the mevalonate pathway also include dolichol, ubiquinol and isoprenoids such as farnesol and geranylgeraniol, which serve as lipid anchoring units for a number of signaling molecules such as small GTPases, Ras and Rho. These are known to mediate oncogenic transformations (88) and might account for the non-cholesterol-mediated regulation of prostate cancer by statins.

Akt (protein kinase B), a serine-threonine kinase, is central to multiple pro-survival and anti-apoptotic cellular pathways (89). Akt is the most frequently activated signaling molecule in cancers (90), and activation of the PI3 kinase-Akt pathway due to PTEN deficiency is a very common cause of prostate cancer (91, 92). Enhanced apoptosis in response to cholesterol lowering drugs on prostatic epithelial cells as well as breast cancer and human epidermoid carcinoma cell lines was reported to be due to inhibition of pro-survival kinase Akt, reduced expression of anti-apoptotic molecule Bcl-xL and activation of pro-apoptotic caspase-3 dependent pathway (87). Reconstituting rafts by the addition of cholesterol restored Akt activity resulting in inhibition of apoptosis (87). A

very recent study performed in a different cancer type supports these findings and reports that simvastatin induces apoptosis, inhibits Akt phosphorylation and Bcl-xL expression in breast cancer cells via inhibition of NFκB, de-repression of PTEN and subsequent inhibition of PI3 kinase (93). Statins, in general, can inhibit Akt-mTOR signaling in p53-deficient hepatocellular carcinoma (94). A previous study performed on PC3 and LNCaP cell lines shows that simvastatin, fluvastatin and lovastatin have profound effects on inducing a cell cycle arrest at G1 phase via inhibition of cyclin E/cdk2 kinase (95), possibly via inhibition of Akt (67). Our study indicated that simvastatin inhibits Akt activity in LNCaP and PC3 cells in a dose- and time-dependent manner. However, until today, a causal relationship between decreased Akt activity and reduced tumor growth by any statins in any type of cancer is not established. Our finding that PC3 cells expressing myrAkt (constitutively active) is resistant to the effects of simvastatin on colony formation demonstrates a causative relationship between inhibition of Akt activity and impaired prostate cancer cell function. Furthermore, tumor xenografts collected from nude mice treated with simvastatin exhibited significant reduction in phosphorylated Akt levels associated with its reduced tumor size and weight, compared to saline treated mice. Together, our results indicate that there is a causal relationship between Akt inhibition and inhibition of tumor growth by simvastatin.

A number of recent meta-analyses from medical databases and epidemiological studies indicate the effect of statins in reducing serum PSA levels (55, 71). Among them, a very recent survey show that decreases in PSA levels are correlative in subjects who are on statin treatment and might influence the risk assessment for prostate cancer (96). Another recent study indicates that statins have the ability to reduce expression of PSA

mRNA via inhibition of androgen receptor (AR) protein expression in hormone responsive LNCaP cells (97). Our results demonstrate that the effect of statins on PSA expression is not just correlative, but is a true reflection of the ability of statins to inhibit prostate cancer growth. Further, our observations extend this information and demonstrate that effects of statins are not limited to the hormone responsive stage of prostate cancer. Metastatic and hormone-insensitive PC3 cell tumor xenografts also exhibited reduced expression of PSA levels upon simvastatin treatment compared to saline treated controls, demonstrating the potential benefits of simvastatin in the management of prostate cancer. However, an important concern in our study is the dose at which simvastatin is found to exert effects on prostate cancer cells. A proper conversion of the therapeutic dose to the working concentration at a cellular level is not well-defined for statins in the existing literature. In our study, we utilized 20-50 times the prescribed therapeutic dose of simvastatin. This concentration of simvastatin has also been shown by others to be the right dose to work at a cellular level (98), and appears to be the dose necessary to inhibit isoprenylation of the proteins in cultured cells (99, 100). However, we have also shown that at doses very close to therapeutic concentrations (less than 10 times), simvastatin inhibits growth of PC3 tumor xenograft *in vivo*. Moreover, it should be noted that simvastatin effects that we studied on prostate cancer cells is on a short-term basis. At very low doses, close to therapeutic concentrations, simvastatin has been shown to enhance the inhibitory effects of acetylsalicylic acid and rosiglitazone on proliferation of normal prostatic epithelial cells and LNCaP and VCaP prostate cancer cells (101). In summary, we show that treatment of prostate cancer cells with simvastatin significantly inhibit Akt activity, prostate cancer

cell functions *in vitro* and tumor growth *in vivo* associated with a significant reduction in PSA expression. Our results suggest that long-term simvastatin medication may have beneficial effects in the management of prostate cancer.

Legends for Figures:

Figure 2.1: Simvastatin treatment inhibits Akt pathway in human prostate cancer cells: A) A dose-dependent (10, 25, 50, 75 and 100 µM) study on the effects of simvastatin (16h) on phosphorylation of Akt and its downstream substrate GSK-3 α and β in LNCaP cells. Densitometry of the corresponding bands normalized to β-actin is shown below. **B)** Time-course effect of 25 µM simvastatin (4 and 16h) on Akt and GSK-3 phosphorylation in LNCaP cells. **C)** Time-course effect of 25 µM simvastatin (4, 8, 16 and 24h) on Akt phosphorylation in PC3 cells. Corresponding densitometry values normalized to β-actin are shown below.

Figure 2.2: Simvastatin significantly inhibits PC3 cell migration and invasion: A) and B) PC3 cells were grown to confluence, and a scratch was made in the monolayer followed by treatment with control PBS and simvastatin (25 and 100 µM). **A)** Scratch recovery as determined at 16h post simvastatin treatment. **B)** Scratch recovery as determined at 24h post simvastatin treatment. **C)** Invasion assay data after treatment of EGF-stimulated PC3 cells with 25 and 100 µM simvastatin for 12 and 24h. **D)** Invasion assay data after treatment of serum-stimulated PC3 cells with 25 and 100 µM simvastatin for 12 and 24h. Bar graph shows the percentage inhibition of invasion in simvastatin-treated PC3 cells normalized to saline control.

Figure 2.3: Simvastatin inhibits PC3 cell proliferation: A) Actively growing PC3 cells were plated in 96-well plates at a density of $1x10^4$ cells/well in triplicates. After 24h incubation in a CO_2 incubator at $37^{\circ}C$, cells were treated with 25 and 100 µM simvastatin for 16h. Cell counts were performed at 0 and 24h time points and doubling time was calculated. **B)** Actively growing PC3 cells were plated in 96-well plates at a

density of $1x10^4$cells/well in triplicates. After 24h incubation in a CO_2 incubator at 37^OC, cells were treated with the indicated concentrations of 25 and 100 µM Simvastatin or 10 nM docetaxel for 16h. In Control cell DMSO was used. Cell proliferation was determined by the BrDU exclusion assay. Bar graph shows the percentage inhibition of proliferation in simvastatin-treated PC3 cells normalized to saline control.

Figure 2.4: Simvastatin induces apoptosis in PC3 cells: A) Cell viability was measured using trypan blue exclusion method. PC3 and LNCaP cells were grown to confluence and treated with simvastatin (25, 50 and 100 µM) for 24h. Cells then were collected, re-suspended in PBS with 0.4% Trypan blue solution. Total cells and Trypan Blue stained cells were counted separately and percentage of non viable cells were calculated. **B)** PC3 cells were treated with 25 and 100 µM simvastatin for 16h and subjected for apoptosis assay. Bar graph shows the fold increase in apoptosis in simvastatin-treated PC3 cells compared to saline control.

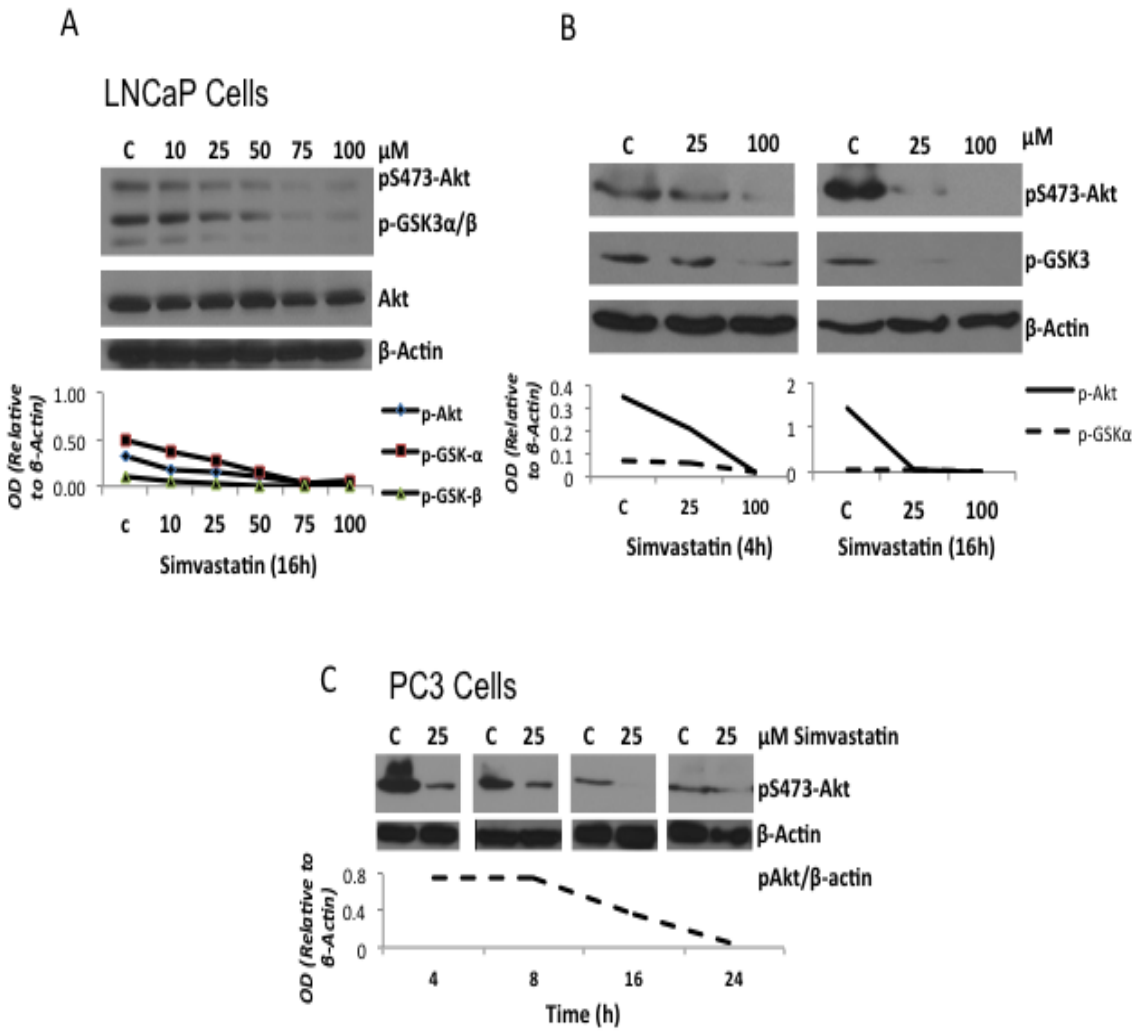
Figure 2.5: Simvastatin inhibited colony formation by PC3 cells is rescued by expression with constitutively active Akt (myrAkt): A) Cells were allowed to form a monolayer and were subjected to treatment with saline or DMSO (controls for simvastatin and docetaxel, respectively), 25 µM simvastatin or 10 nM docetaxel. On day 5, cells were fixed, stained and counted for colonies. Bar graph showing reduced number of colonies compared to control with simvastatin treatment. **B)** Bar graph showing reduced number of colonies compared to control with docetaxel treatment. **C)** Bar graph showing PC3 cells stably expressing myrAkt (constitutively active) develop significantly higher number of colonies compared to cells stably expressing GFP

(control). **D)** Bar graph showing PC3 cells stably expressing myrAkt are resistant to inhibition of colony formation by simvastatin.

Figure 2.6: Simvastatin inhibits growth of PC3 tumor xenograft in nude mice: A) and E), Bar graph showing the effect of simvastatin administered every 24h and 12h, respectively, on the weight of 2 week old tumor xenografts. **B) and F),** Bar graph showing the effect of simvastatin administered every 24h and 12h, respectively, on the size (mm^2) of 2 week old tumor xenografts. **C) and G),** Bar graph showing the effect of simvastatin administered every 24h and 12h, respectively, on the changes in tumor size (mm^2), compared to control (saline), between day 7 and day 11 tumor sizes. **D) and H),** Bar graph showing the effect of simvastatin administered every 24h and 12h, respectively, on the percentage changes in tumor size (mm^2), compared to control (saline), between day 7 and day 11 tumor sizes.

Figure 2.7: Simvastatin effects on PC3 tumor growth is associated with an inhibition of Akt activity and reduced expression of PSA: A) Pictures showing tumor xenografts isolated from nude mice treated with saline (control) and simvastatin on day 14. **B)** Western blot picture and bar graph of densitometry analyses for the phospho-Akt levels in tumor xenograft lysates collected from nude mice treated with saline (control) and simvastatin. **C)** Western blot picture and bar graph of densitometry analyses for the prostate specific antigen (PSA) levels in tumor xenograft lysates collected from nude mice treated with saline (control) and simvastatin.

Figure 2.1


A
LNCaP Cells
C 10 25 50 75 100 µM
pS473-Akt
p-GSK3α/β
Akt
β-Actin
OD (Relative to β-Actin)
1.00
0.50
0.00
p-Akt
p-GSK-α
p-GSK-β
c 10 25 50 75 100
Simvastatin (16h)
B
C 25 100 C 25 100 µM
pS473-Akt
p-GSK3
β-Actin
OD (Relative to β-Actin)
0.4
0.3
0.2
0.1
0
2
1
0
p-Akt
p-GSKα
C 25 100
Simvastatin (4h)
C 25 100
Simvastatin (16h)
C
PC3 Cells
C 25 C 25 C 25 C 25 µM Simvastatin
pS473-Akt
β-Actin
pAkt/β-actin
OD (Relative to β-Actin)
0.8
0.4
0
4
8
16
24
Time (h)

a

Figure 2.2

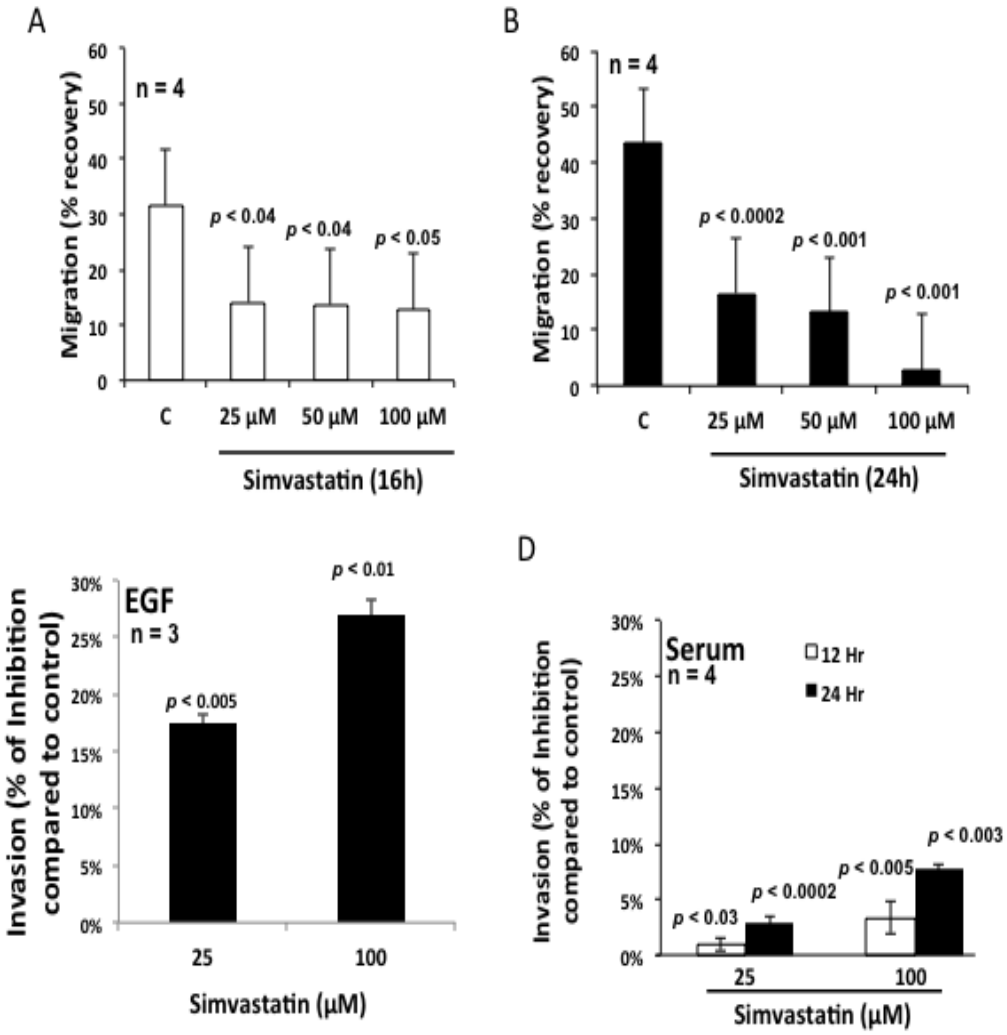

A
n = 4
Migration (% recovery)
p < 0.04
p < 0.04
p < 0.05
C
25 µM
50 µM
100 µM
Simvastatin (16h)
B
n = 4
Migration (% recovery)
p < 0.0002
p < 0.001
p < 0.001
C
25 µM
50 µM
100 µM
Simvastatin (24h)
EGF
n = 3
Invasion (% of Inhibition compared to control)
p < 0.005
p < 0.01
25
100
Simvastatin (µM)
D
Serum
n = 4
12 Hr
24 Hr
Invasion (% of Inhibition compared to control)
p < 0.03
p < 0.0002
p < 0.005
p < 0.003
25
100
Simvastatin (µM)

Figure 2.3

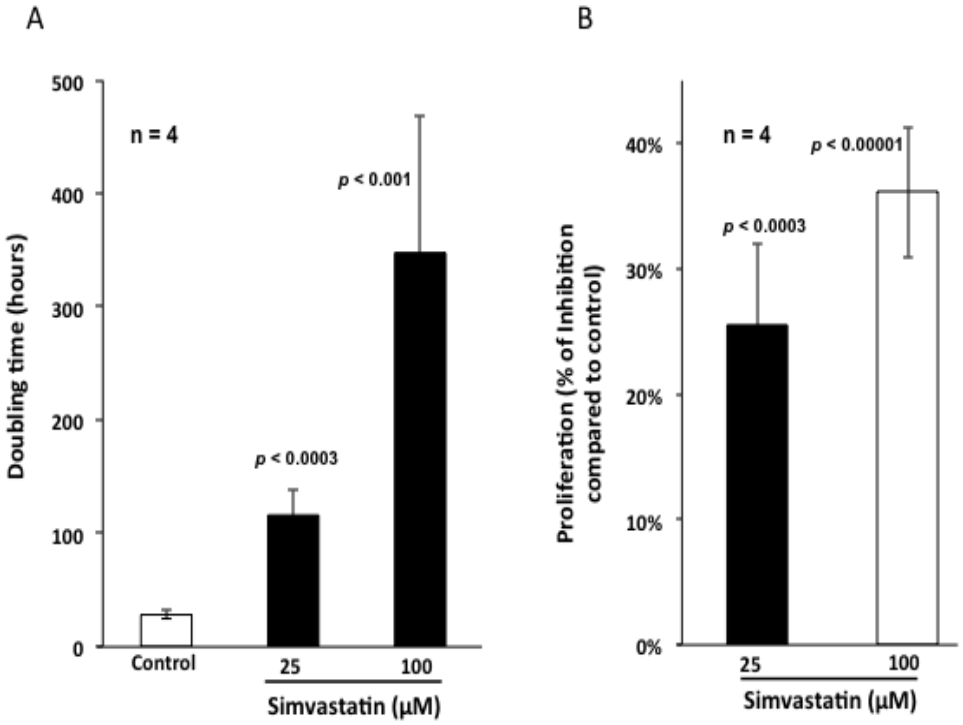

A
B
n = 4
p < 0.001
p < 0.0003
Doubling time (hours)
500
400
300
200
100
0
Control
25
100
Simvastatin (μM)
n = 4
p < 0.00001
p < 0.0003
Proliferation (% of Inhibition compared to control)
40%
30%
20%
10%
0%
25
100
Simvastatin (μM)

Figure 2.4

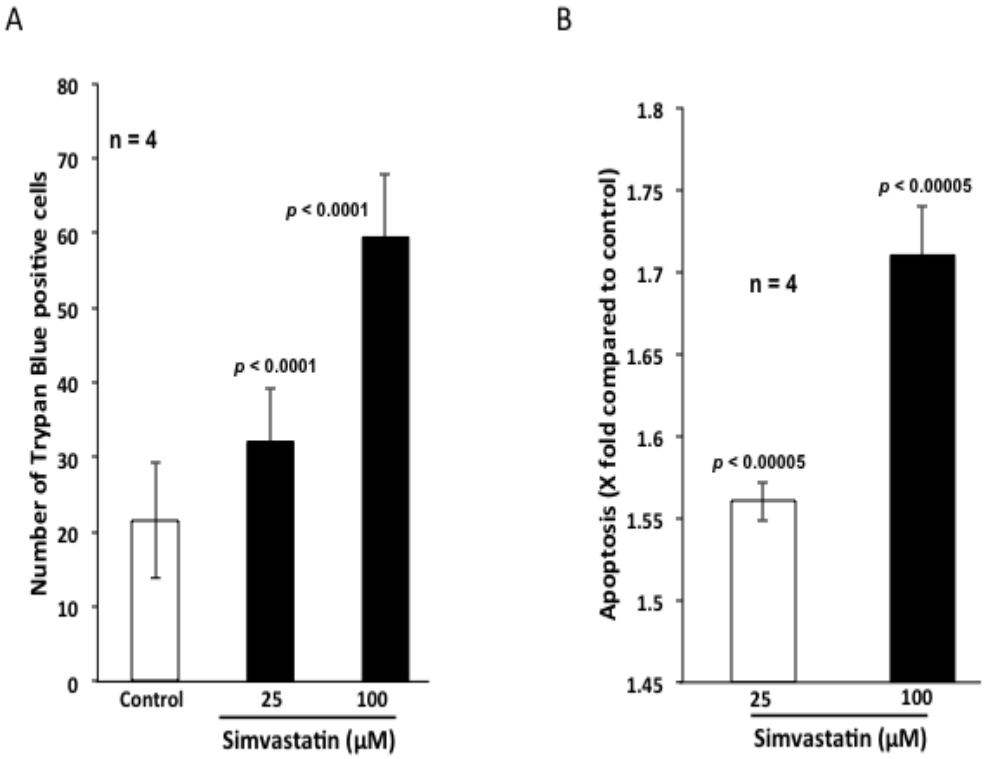

A
B
n = 4
Number of Trypan Blue positive cells
p < 0.0001
p < 0.0001
Control
25
100
Simvastatin (μM)
Apoptosis (X fold compared to control)
p < 0.00005
p < 0.00005
n = 4
25
100
Simvastatin (μM)

Figure 2.5

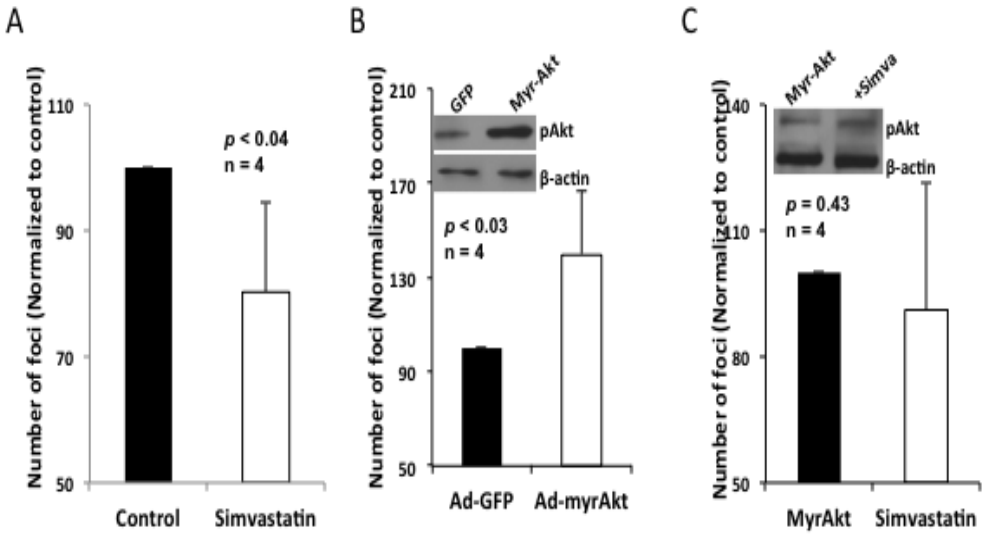
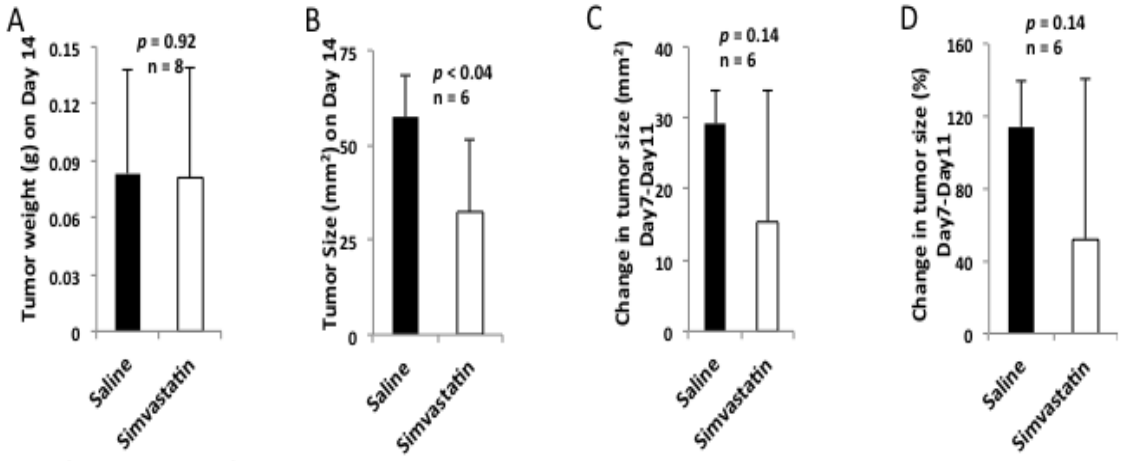

A
Number of foci (Normalized to control)
110
90
70
50
p < 0.04
n = 4
Control
Simvastatin
B
Number of foci (Normalized to control)
210
170
130
90
50
GFP
Myr-Akt
pAkt
β-actin
p < 0.03
n = 4
Ad-GFP
Ad-myrAkt
C
Number of foci (Normalized to control)
140
110
80
50
Myr-Akt
+Simva
pAkt
β-actin
p = 0.43
n = 4
MyrAkt
Simvastatin

Figure 2.6

(i) 2mg/kg body weight/day



(ii) 2mg/kg body weight/12h

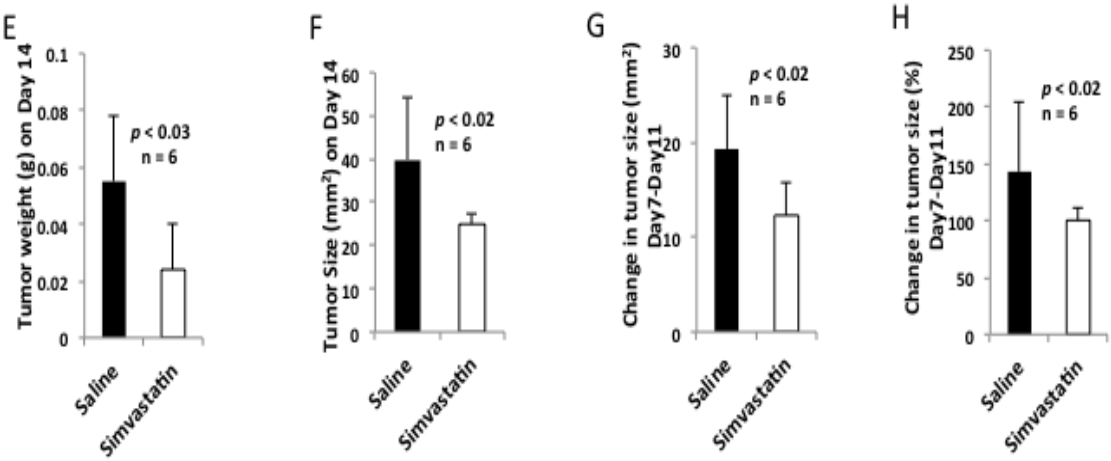
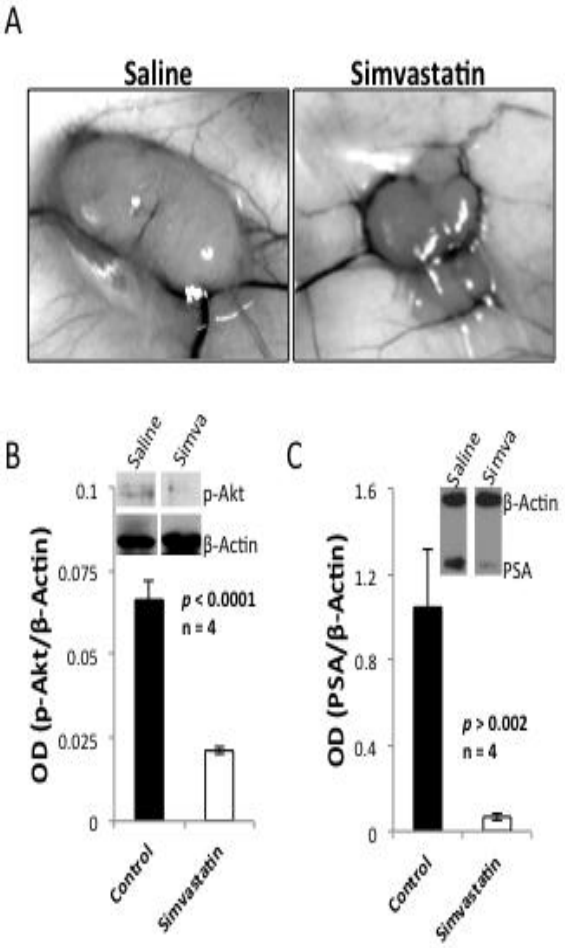


Figure 2.7


A
Saline
Simvastatin
B
Saline
Simva
p-Akt
β-Actin
OD (p-Akt/β-Actin)
0.1
0.075
0.05
0.025
0
p < 0.0001
n = 4
Control
Simvastatin
C
Saline
Simva
β-Actin
PSA
OD (PSA/β-Actin)
1.6
1.2
0.8
0.4
0
p > 0.002
n = 4
Control
Simvastatin

CHAPTER 3:

SIMULTANEOUS MODULATION OF THE INTRINSIC AND EXTRINSIC PATHWAYS BY SIMVASTATIN IN MEDIATING PROSTATE CANCER CELL APOPTOSIS

Abstract

Background: Recent studies suggest the potential benefits of statins as anti-cancer agents. Mechanisms by which statins induce apoptosis in cancer cells are not clear. We previously showed that simvastatin inhibit prostate cancer cell functions and tumor growth. Molecular mechanisms by which simvastatin induce apoptosis in prostate cancer cells is not completely understood.

Methods: Effect of simvastatin on PC3 cell apoptosis was compared with docetaxel using apoptosis, TUNEL and trypan blue viability assays. Protein expression of major candidates of the intrinsic pathway downstream of simvastatin-mediated Akt inactivation was analyzed. Gene arrays and western analysis of PC3 cells and tumor lysates were performed to identify the candidate genes mediating extrinsic apoptosis pathway by simvastatin.

Results: Data indicated that simvastatin inhibited intrinsic cell survival pathway in PC3 cells by enhancing phosphorylation of Bad, reducing the protein expression of Bcl-2, Bcl-xL and cleaved caspases 9/3. Over-expression of PC3 cells with Bcl-2 or DN-caspase 9 did not rescue the simvastatin-induced apoptosis. Simvastatin treatment resulted in increased mRNA and protein expression of molecules such as TNF, Fas-L,

Traf1 and cleaved caspase 8, major mediators of extrinsic apoptosis pathway and reduced protein levels of pro-survival genes Lhx4 and Nme5.

Conclusions: Our study provides the first report that simvastatin simultaneously modulates intrinsic and extrinsic pathways in the regulation of prostate cancer cell apoptosis *in vitro* and *in vivo*, and render reasonable optimism that statins could become an attractive anti-cancer agent.

Background

Statins, the cholesterol lowering drugs, are some of the most commonly prescribed medications. Recently, attention has focused on the development of statins as therapeutic agents for the treatment of solid and hematological cancers (65). Statins elicit pleiotropic effects on various cell types and differentially modulate cellular functions such as cell migration, proliferation, cell survival and apoptosis in normal and malignant cells (77). Lipophilicity, dose and duration of the treatment as well as cell type are all determining factors on the specific effect of a statin on the outcome of a cell function. According to the American Cancer Society, prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in American men. Many recent clinical studies have indicated that use of statins is associated with >50% reduction in prostate cancer deaths (55, 102). Our previous study showed that simvastatin, a lipophilic statin inhibited multiple prostate cancer cell functions *in vitro* such as migration, proliferation, cell survival and colony formation as well as tumor growth in a nude mouse xenograft *in vivo*, mainly via inhibition of Akt pathway (103). However, exact molecular mechanisms by which statins modulate each of the prostate cancer cell function are not clear.

One of the factors that determine the efficacy of a cancer drug is its ability to inhibit cancer cell survival and induce apoptosis. Meantime, a major concern over the use of anti-cancer drugs for therapy is the side-effects that they can inflict on normal cells. For a very long time, scientists are on the search of anti-cancer agents that specifically target tumor cells with no or minimum effects on normal cells. A very recent study indicates that simvastatin, at doses that we had previously shown to induce apoptosis in

prostate cancer cells (103), does not compromise cell survival in normal airway epithelial and fibroblast cells, while inducing apoptosis in breast, hepatocellular and lung carcinoma cells (104). Although this study provides the necessary assurance that simvastatin may be a potential drug for specifically targeting cancer cells for therapy, the molecular mechanisms by which simvastatin induces apoptosis in cancer cells remains to be determined.

Bcl-2-mediated, mitochondria associated cell survival pathway (intrinsic pathway) is one of the major pathways that are targeted for inducing apoptosis in cancer cells. In addition to this, another major pathway that promotes apoptosis in cancer cells is the death receptor-mediated pathway (extrinsic pathway) (105). Tumor necrosis factor (TNF), TNF-related apoptosis inducing ligand (TRAIL), Fas-ligand (Fas-L), TNF-related factor-1 and 2 (Traf1/2) etc. are some of the key molecules that belong to the extrinsic pathway or death receptor signaling that are known to be de-regulated in cancers (106, 107). While inhibition of Bcl-2-mediated intrinsic pathway leads to the release of cytochrome c from the mitochondria to the cytosol, resulting in the activation of caspases 9 and 3, death receptor-mediated extrinsic pathway involves caspases 10 and 8 in inducing apoptosis (105). A pre-requisite for the latter is the formation of a death-inducing signaling complex (DISC) between Fas-assciated death domain (FADD) and pro-caspase 8 (108). Resulting cleavage of pro-caspase 8 to active cleaved caspase 8 leads to the activation of downstream caspases such as caspase 3 (109).

Until recently, docetaxel-based chemotherapy is the only available treatment option for the androgen-insensitive prostate cancer patients and is shown to modestly improve survival (110), marking the first real advance after the identification of therapeutic

castration by Charles Huggins in 1941 (111). Docetaxel (Taxotere®) acts via suppression of microtubule assembly and disassembly, microtubule bundling and inhibition of Bcl-2, leading to apoptosis (112). However, use of docetaxel is associated with a number of serious side-effects due to yet unknown reasons (113, 114). According to many reports doses of statins, even 50 times higher than the prescribed doses for the treatment of cardiovascular diseases, did not inflict any serious side-effects or toxicity to liver and kidney in men (115-117). In the current study, we investigated the various mechanisms by which simvastatin induce apoptosis in prostate cancer cells as compared to the known effects of docetaxel treatment. Our study indicates that simvastatin induces apoptosis in prostate cancer cells in *vitro* and prostate tumor xenograft *in vivo* by simultaneously modulating intrinsic and extrinsic apoptotic pathways. These results suggest that simvastatin can be developed as an important drug for the treatment of prostate cancer either alone or in combination with reduced doses of chemotherapeutic drugs such as docetaxel to improve the efficacy and reduce the side-effects.

Methods

Cell lines, reagents, and antibodies

Human PC3 and LNCaP cell lines were obtained from ATCC (Manassas, VA) and maintained in DMEM High Glucose (HyClone) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin in 5% CO_2 humidified atmosphere at 37°C. Primary antibodies against pBad, Bcl-2, Bcl-xL, Bim, cleaved caspase 3, cleaved caspase 9, cleaved caspase 8, cytocrocme c, Fas-L, survivin and Traf1 were purchased from Cell Signaling (Boston, MA). Primary antibodies anti-Nme5 was obtained from

Abcam (Cambridge, MA/ San Francisco, CA), anti-Trp53inp1 was from R&D (Minneapolis, MN) and anti-β-actin was from Sigma (St Louis, MO). Anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from BioRad (Hercules, CA). Docetaxel and simvastatin were purchased from Sigma (St Louis, MO). Simvastatin was activated in the laboratory using the manufacturer's instructions.

Apoptosis assay

Cytoplasmic histone-associated DNA fragments were quantified by using the Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Briefly, PC3 cells were plated in 96-well plate at a density of either 10^4 cells/well. After 24h, the cells were treated with 25 μM simvastatin and/or 10 nM docetaxel for 16h in DMEM containing 10% FBS. Control cells received 0.1% DMSO (vehicle control). Cells were lysed and centrifuged at 200*g* for 10 min, and the collected supernatant was subjected to ELISA. The absorbance was measured at 405 nm (reference wavelength, 492 nm).

***In vivo* nude mouse tumor xenograft model**

All animal procedures listed in this article were performed as per the protocol approved by the Institutional Animal Care and Use Committee at the Charlie Norwood Veterans Affairs Medical Center, Augusta, GA (protocol 09-07-011, dated July 10, 2009). PC3 cells were grown to confluence in 250-ml flasks. Cells were re-suspended in PBS to a concentration of 10^6/ml. Cell suspension (1 ml) was injected subcutaneously in 6- to 8-week-old nude mice (athymic nude mice; Harlan, Indianapolis, IN). The mice were subjected to intraperitoneal injections of simvastatin at a dose of 2 mg/kg body weight

every 12h for 2 weeks. The respective controls were injected intraperitoneally with 0.9% saline every 12h. Mice were sacrificed on day 14, and tumors were dissected, weighed, and snap frozen using dry ice for further processing to use on western or qRT-PCR.

Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) assay

The TUNEL assay for *in situ* detection of apoptosis was performed by using the ApopTag® Fluorescein In Situ Apoptosis detection kit (Millipore, MA) according to the manufacturer's instructions. Cells were plated in 24-well flat bottom plates at a density of 1 x 10^5 cells/well and treated with 25 μM simvastatin, 10nM docetaxel or a combination of both for 24h. Following treatments, cells were fixed in 2% paraformaldehyde at 4 °C for 30 min. Fixed cells were then permeabilized in 0.1% Triton X-100 and labeled with fluorescein 12-dUTP using terminal deoxynucleotidyl transferase. Nuclei were counterstained with DAPI. Frozen nude mouse prostate tumor (PC3) xenograft sections were also processed accordingly. Cells/tissue sections were analyzed for apoptotic cells with localized green fluorescence using an inverted fluorescence microscope (Zeiss Axiovert100M, Carl Zeiss, Germany)

qReal-Time PCR arrays

PC3 cells were grown until reaching 75 % of confluence in 6-well plates and subjected to RNA isolation, followed cDNA synthesis and qPCR quantification. Briefly, cells were lysed and RNA was isolated according to manufacturer's protocol using RNAese Mini Plus Kit (Qiagen, Valencia, CA). Next, 25 μl of cDNA was produced by RT^2 First Strand Kit (SABioscience, Frederick, MD), mixed with qPCR SyberGreen master mix and loaded into Human Apoptosis RT^2 Profiler PCR Array plate (SABiosciences,

Frederick, MD). Reading was completed in Eppendorf Mastercycler realplex 2 instrument.

Western blot analysis

PC3 cells were cultured in 6-well plates to reach a monolayer. At that point, the cells were treated with 25 μM simvastatin and/or 10 nM docetaxel in DMEM supplemented with 10 % FBS. Control cells received 0.1 % of DMSO. Whole cell lysates were prepared using lysis buffer [50 mM Tris-HCl (pH=7.4), 1 % TritonX-100, 150mM NaCl, 1mM EDTA, 2mM Na_3VO_4 , and 1X Complete protease inhibitors (Roche Applied Science, Indianapolis, IN)]. Tumors isolated from mice with C53BL/6 background treated with 2mg/kg simvastatin for 11 days, were first snap frozen in liquid nitrogen and then pulverized with mortar and piston. Next, tissues lysates were prepared using lysis buffer. The protein concentration was measured by the DL protein assay (Bio-Rad, Hercules, CA). 60 μg/μl of protein was subjected to western blot analysis according to standard Laemmli's method.

Statistical Analysis

Mean activities were calculated from 3-5 independent experiments done at least in triplicates. The Student's two-tailed t test was used to determine significant differences between treatment and control values.

Results

Simvastatin induces cell death and apoptosis in prostate cancer cells

Since simvastatin inhibited activity of the cell survival kinase Akt (103), we studied whether treatment with simvastatin will compromise cell survival and induce apoptosis in prostate cancer (PC3) cells. We performed an apoptosis assay using a method that

measures the cytoplasmic histone-associated DNA fragments. Our data showed that both simvastatin and docetaxel significantly induced apoptosis in PC3 cells ($p<0.001$ and $p<0.05$, respectively) (Figure 3.1A). However, although a trend was noted, the combined effect of simvastatin and docetaxel on the apoptosis of PC3 cells was not observed. In order to further confirm our data, we performed TUNEL assay to assess DNA fragmentation as a late event in the process of apoptosis in PC3 cells. Our TUNEL staining data further confirmed that while simvastatin and docetaxel independently induced apoptosis in PC3 cells ($p<0.001$ and $p<0.05$, respectively), a combination of these drugs exhibited a modest increase in apoptosis compared to each of these drugs alone (Figure 3.1B).

Simvastatin inhibits Bcl-2-mediated intrinsic pathway in prostate cancer cells

Akt is known to modulate Bcl-2-mediated cell survival pathway via phosphorylation of Bcl-2-associated death promoter (Bad). We determined whether simvastatin treatment inhibited Bcl-2-mediated cell survival pathway in prostate cancer cells. Our data indicated that treatment with simvastatin significantly impaired phosphorylation of Bad ($p<0.05$), decreased protein expression of Bcl-2 and Bcl-xL ($p<0.01$ and $p<0.05$, respectively) as well as increased protein levels of BimL/BimS ($p<0.01$), cleaved caspase 9 and cleaved caspase 3 ($p<0.001$) (Figures 3.2). These effects were similar to the treatment of prostate cancer cells with docetaxel. Eventhough a synergistic effect on the protein expression of Bcl-2 and Bcl-xL was seen in prostate cancer cells with combined treatment of simvastatin and docetaxel, a net significant additive effect on the final products of intrinsic pathway such as cleaved caspase 3 and cleaved caspase 9

was not observed. Together, our results indicate that inhibition of Bcl-2-dependent intrinsic pathway is involved in the simvastatin-mediated effects of PC3 cells.

Simvastatin induces apoptosis in prostate tumor xenografts via inhibition of intrinsic cell survival pathway

We next determined whether simvastatin treatment has any effect on prostate tumor cell survival *in vivo*. In order to do this, frozen sections of PC3 tumor xenografts from athymic nude mice were subjected to TUNEL assay. Our data indicated that treatment with simvastatin in nude mice (2mg/kg body weight/12 hours, intra-peritoneally) significantly enhanced apoptosis in tumors compared to saline treated controls by >2-fold ($p<0.05$) (Figure 3.3A and B). Western analysis of the tumor lysates indicated that, similar to prostate cancer cells *in vitro*, treatment with simvastatin significantly impaired phosphorylation of Bad ($p<0.01$), decreased protein levels of Bcl-2 and Bcl-xL ($p<0.01$ and $p<0.001$, respectively), increased release of cytochrome C from the mitochondria to cytosol ($p<0.05$) as well as increased protein expressions of BimL/BimS, cleaved caspase 9 and cleaved caspase 3 ($p<0.05$), compared to saline treated controls (Figures 3.4).

Simvastatin modulates expression of genes involved in the death receptor-mediated apoptotic pathway in prostate cancer cells

Since over-expression of PC3 cells with Bcl-2 and/or DN-caspase 9 did not rescue from simvastatin-induced apoptosis, we hypothesized that pathways other than intrinsic cell survival pathway may also be inhibited by simvastatin. To study this, we performed Real-Time qPCR-based gene arrays specific for genes involved in the regulation of cell survival and apoptosis. From our gene array analysis, we identified several candidate

genes that are likely involved in the simvastatin-induced apoptosis in PC3 cells (Table 3.1). Some of the candidate genes whose expressions were significantly modulated by statin in PC3 cells included Bcl-2, Fas-L, Lhx4, Nme5, Traf1 and Trp53inp1 (p<0.001), many of them involved in the extrinsic death receptor-mediated apoptosis pathway (Figure 3.5).

Simvastatin, but not docetaxel is involved in the activation of Fas-L mediated extrinsic pathway in prostate cancer cells and tumor xenografts

To investigate whether these genes were regulated by simvastatin in prostate cancer cells at the protein level, we performed western analysis of PC3 cells treated with either saline control or simvastatin. Our data showed that treatment with simvastatin while significantly increased protein expression of pro-apoptotic Fas-L (p<0.05), it inhibited expression of pro-survival protein Nme5 (p<0.01) (Figure 3.6). Although a trend towards increased protein expression of Traf1 was observed with simvastatin treatment in PC3 cells lysates, this was however not significant. In any case, treatment with docetaxel did not have any effect on the expression of proteins involved in the extrinsic pathway involving Fas/Fas-L. Interestingly, we did observe some changes in cleaved caspase 8 protein levels with both simvastatin (p<0.001) and docetaxel treatment (p<0.05), suggesting that docetaxel may also be involved in the regulation of extrinsic pathway through a Fas/Fas-L independent mechanism.

Using Western analysis of the tumor lysates, we next determined whether simvastatin has effect on extrinsic pathway components in PC3 tumor xenografts *in vivo*. Our data indicated that while protein levels of Fas-L and Traf1 was significantly increased in PC3 tumors treated with simvastatin, compared to saline treated controls (p<0.05 and

p<0.001, respectively), protein expression of Nme5 was significantly reduced (*p*<0.05) (Figure 3.7). Further analysis of tumor cell lysates revealed that protein expression of cleaved caspase 8, a molecule involved in the extrinsic pathway downstream of activated caspase 10 was significantly increased in tumor xenografts treated with simvastatin, compared to saline treated controls (*p*<0.01).

Discussion

Many recent studies (65), including ours (103) show that statins are beneficial as anti-cancer agents via inhibition of prostate cancer cell functions *in vivo* such as proliferation, cell survival, cell migration and colony formation etc. In this study, we have shown that treatment of prostate cancer cells with simvastatin *in vitro* and mice bearing prostate tumor xenograft *in vivo* significantly induce apoptosis in prostate cancer cells. Simvastatin-mediated effects on prostate cancer cell viability and apoptosis was superior to the effects of docetaxel, a currently approved drug for the chemotherapy of prostate cancer patients. Although a combined effect on prostate cancer cell viability was observed by treating simvastatin along with docetaxel, this effect was not observed in assays specific for apoptosis such as TUNEL and cytoplasmic histone-associated DNA fragment assays. While Bcl-2-mediated mitochondria-associated intrinsic cell survival pathway was significantly inhibited in PC3 cells and tumor xenografts by simvastatin treatment, over-expression of PC3 cells with Bcl-2 and/or dominant negative caspase 9 did not reverse the simvastatin-mediated PC3 cell apoptosis. While simvastatin treatment reduced the expression of phosphorylated-Bad, Bcl-2, Bcl-xL and survivin in PC3 cells, it resulted in increased protein expression of Bim, cleaved caspases 9 and 3, with an increased effect in the

presence of docetaxel. Modulation of Bcl-2-pathway with simvastatin was also observed in PC3 tumor lysates. Gene arrays followed by western analysis of PC3 cell and tumor lysates treated with simvastatin identified several genes involved in the extrinsic death-receptor apoptosis pathway modulated by simvastatin, but not with docetaxel, such as tumor necrosis factor (TNF), Fas-L, Traf1 and cleaved caspase 8, along with other genes such as Lhx4, Nme5 and Trp53inp1, which are novel, yet unknown regulators of cell survival and apoptosis in prostate cancer cells. Altogether, our results have demonstrated that simvastatin induces apoptosis in prostate cancer cells via simultaneous modulation of intrinsic and extrinsic pathways (Figure 3.8).

Because of its 'crossroad' role in multiple essential signaling pathways in cancer cell maintenance, and its enhanced expression and/or activation in multiple cancer cells as compared to normal, Akt kinase is being actively pursued as a novel target for cancer therapy (21, 118-120). However, since Akt is essential for many normal cell functions (75, 89, 121), cell survival in particular, targeting Akt for cancer therapy is a bottle neck due to the serious side-effects associated with it. This asks for novel therapies that can inflict a significant but selective effect on cancer cells in inhibiting pathways like Akt without affecting the normal functioning of extra-tumor tissues. Many recently published reports suggest that statins, at certain higher doses, can be a selective and very efficient drug to treat cancers without inflicting any major side-effects (115-117). We previously showed that simvastatin, at a dose ~5 times higher than the therapeutic dose prescribed for the treatment of cardiovascular diseases, significantly inhibited Akt activity in PC3 tumor cells and prostate tumor xenograft growth *in vivo* (103). Another recent report indicated that at similar doses, simvastatin induced apoptosis in breast

cancer cells, but not in normal airway epithelial cells or fibroblasts (104). Thus, the ability of simvastatin to selectively inhibit Akt activity and induce apoptosis in prostate cancer cells without affecting the normal cells makes it an attractive candidate for drug re-purposing for cancer therapy.

Many of the effects of simvastatin on prostate cancer cell apoptosis can be credited to its ability to inhibit Akt activity. Akt is known to enhance the intrinsic mitochondria-associated cell survival pathway in cancer cells via increased phosphorylation of Bad and enhanced expression of Bcl-2 and Bcl-xL (105). Upon inhibition of Akt by simvastatin in PC3 cells, we saw reduced phosphorylation of Bad, decreased expression of Bcl-2 and Bcl-xL, associated with increased expression of Bim as well as cleaved caspases 9 and 3. Activated caspase 3 is expected to further cleave PARP in inducing apoptosis (105). Inhibition of Bcl-2-mediated pathway by statins has also been shown by other labs in multiple cancer types (51, 104, 122). However, our attempt to rescue the PC3 cells from apoptosis by re-constituting the Bcl-2 pathway by over-expressing PC3 cells with Bcl-2 and/or DN-caspase 9 did not reverse the simvastatin-induced apoptosis. This suggested that pathways other than intrinsic survival pathway are involved in simvastatin-induced apoptosis in prostate cancer cells.

On the other end, gene arrays as well as western analysis of cell and tumor lysates identified a number of novel candidates that are involved in the simvastatin-induced apoptosis in prostate cancer cells. One of the pro-survival proteins that were found to be less expressed in simvastatin-treated PC3 cells was survivin, which is also associated with mitochondria-associated cell survival pathway. Survivin is highly expressed in many cancer cells (123), including prostate cancer cells (124, 125). Regulation of

survivin expression in multiple experimental models has been linked to increase in Akt activity (126). In prostate cancer cells, survivin expression has been shown to be regulated by IGF-1 stimulated Akt-mTOR signaling (127), which Is impaired upon simvastatin treatment (103). A second pro-survival molecule that is significantly less expressed in simvastatin-treated PC3 cells is non-metastatic cells 5 (Nme5). Nme5, also known as the inhibitor of p53-induced apoptosis-beta (IPIA-beta) is known to confer protection from cell death by Bax and alter the cellular levels of several anti-oxidant enzymes such as Gpx5 (128). A third molecule that was significantly less expressed in PC3 cells with simvastatin treatment was Lhx4, a molecule abundantly expressed in many cancers (129, 130), but exact function is yet to be determined. Other molecules that are de-regulated with simvastatin-treatment in PC3 cells include CD70 (TNFRSF7), CD40, caspase-1, Trp53inp1 and TNFRSF10b etc. (Table 3.1).

Another mechanism by which apoptosis can be triggered in cancer cells is via signaling by death receptor members that belong to the tumor necrosis factor receptor super-family (131). Among the eight members of the death receptor family, most common are the TNF receptor 1 (TNFR1 or DR1) and Fas (CD95 or DR2) (105). Our gene array results indicated an increase in TNF and Fas-L in prostate cancer cells, which are ligands for TNFR1 and Fas, respectively, with simvastation treatment. Furthermore, increase in the expression of other molecules associated with the Fas receptor such as Traf1 and Fas (TNFRSF6)-associated via death domain (FADD) leading to activation of caspase-8 was also observed in PC3 cells and/or tumor lysates with simvastatin treatment. In order to induce apoptosis, TNF and Fas-L utilizes two different death receptor signaling complexes. Fas-L-mediated mechanism comprises the death-

inducing signaling complexes (DISCs) that are formed at the CD95 or Fas receptor between Fas-asscіated death domain (FADD) and pro-caspases 10 and 8 (108). Formation of DISC results in the activation of caspases 10 and 8, which place a central role in the transduction of death signal (108, 132). TNF induces apoptosis via a mechanism different from Fas-induced cell death involving two different signaling complexes (133). Complex-I is formed at the membrane and comprises TNF, TNFR1, receptor-interacting protein (RIP), TNFR-associated death domain (TRADD), TNFR-associated factors 1 and 2 (Traf-1/2) etc. and acts through a JNK-dependent mechanism. Complex-II, also known as traddosome, consists of FADD and caspase 8, which are absent in complex-I (109). An increase in the levels of cleaved caspase 8 in the PC3 tumor lysates from simvastation-treated mice indicate that one or both of the Fas-L and TNF-mediated death-receptor signaling pathway is involved in simvastatin-induced apoptosis in prostate cancer cells.

Conclusions

In conclusion, our results have demonstrated that treatment with simvastatin induces apoptosis in prostate cancer cells *in vitro* and tumor xenograft *in vivo* via simultaneous modulation of mitochondria-associated intrinsic pathway that comprises Bcl-2, Bcl-xL and caspases 9 and 3 as well as Fas-L and TNF-dependent extrinsic death receptor pathway involving caspase-8. Our study reinforces the rationale of selective pharmacologic inhibition of prostate cancer cell survival using statins and suggests re-purposing of lipophilic statins such as simvastatin for prostate cancer therapy in humans. Alternatively, statins may also be used in combination with other cytotoxic agents such as docetaxel to improve the drug efficacy and reduce the side-effects.

Abbreviations used: Bcl-2, B-Cell lymphoma-2; Bcl-xL, B-Cell lymphoma extra-large; DR, death receptor; DISC, death-inducing signaling complex; EDTA, ethylenediaminetetraacetic acid; FADD, Fas-assciated death domain; Lhx4, LIM homeobox protein-4; Nme5, non-metastatic cells 5; RIP, receptor-interacting protein; TNF, tumor Necrosis factor; TNFRSF, tumor Necrosis factor receptor superfamily; TRAIL, TNF-related apoptosis inducing ligand; TRADD, TNFR-associated death domain; Traf1/2, TNF-related factor 1 and 2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Table 3.1: Genes modulated by simvastatin in PC3 cells as identified by qRT-PCR arrays

GeneBank	Symbol	Description	Change (X fold)
NM_030693	Atf5	Activating transcription factor 5	2.0↓
NM_009741	Bcl2	B-cell leukemia/lymphoma 2	2.0↓
NM_009743	Bcl2l1/2	Bcl2-like 1 and 2	1.7↓
NM_013479	Bcl2l10	Bcl2-like 10	2.0↓
NM_008670	Birc1a	Baculoviral IAP repeat-containing 1a	2.4↑
NM_007464	Birc3	Baculoviral IAP repeat-containing 3	1.6↑
NM_009689	Birc5	Baculoviral IAP repeat-containing 5	3.0↑
NM_009807	Casp1	Caspase 1	2.8↑
NM_007702	Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	1.6↑
NM_010015	Dad1	Defender against cell death 1	2.5↓
NM_010175	Fadd	Fas (TNFRSF6)-associated via death domain	2.0↑
NM_010177	Fasl	Fas ligand (TNF superfamily, member 6)	1.9↑
NM_010548	Il10	Interleukin 10	1.6↑
NM_010712	Lhx4	LIM homeobox protein 4	3.3↓
NM_080637	Nme5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	1.8↓
NM_030152	Nol3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	1.6↓
NM_023258	Pycard	PYD and CARD domain containing	2.0↓
NM_013693	Tnf	Tumor necrosis factor	3.2↑
NM_020275	Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b	1.6↑
NM_011611	Cd40	CD40 antigen	3.5↑
NM_009425	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	1.6↑
NM_011617	Cd70	CD70 antigen	3.3↑
NM_009421	Traf1	Tnf receptor-associated factor 1	4.0↑
NM_021897	Trp53inp1	Transformation related protein 53 inducible nuclear protein 1	3.2↑

Figures and figure legends

Figure 3.1: Simvastatin induces cell death and apoptosis in prostate cancer cells. (A) Bar graph showing apoptosis in PC3 cells treated with control saline, simvastatin, docetaxel or a combination of simvastatin and docetaxel for 24 h as measured calorimetrically. (B) Bar graph showing quantification of TUNEL positive PC3 cells treated with control saline, simvastatin, docetaxel or a combination of simvastatin and docetaxel for 24 h. The data are presented as mean ± SD (n=4 of quadruplicate experiments).

Figure 3.2: Simvastatin inhibits Bcl-2- and Bcl-xL-mediated cell survival pathway in prostate cancer cells. Western blots showing reduced phosphorylation of Bad, reduced protein expression of Bcl-2 and Bcl-xL as well as increased protein expression of BimL/BimS, cleaved caspase 9 and cleaved caspase 3 after 24 h treatment with simvastatin, docetaxel or a combination of both, compared to saline treated control. (n=4 of quadruplicate experiments).

Figure 3.3: Simvastatin induces cell death and apoptosis in prostate tumor xenografts. (A) Pictures showing TUNEL staining of PC3 cell nude mice tumor xenogafts treated with control saline or simvastatin for 14 days. (B) Bar graph showing quantification of the TUNEL positive PC3 cells in tumor xenogafts treated with control saline or simvastatin for 14 days. The data are presented as mean ± SD (n=4 of quadruplicate experiments).

Figure 3.4: Simvastatin induces apoptosis in prostate tumor cells *in vivo* via inhibition of intrinsic cell survival pathway. (A) Western blots showing reduced phosphorylation of Bad, reduced protein expression of Bcl-2 and Bcl-xL, increased

release of cytochrome-c from the mitochondria as well as increased protein expression of BimL/BimS, cleaved caspase 9 and cleaved caspase 3 with 14 day simvastatin treatment, compared to saline control in PC3 cell nude mice tumor xenogafts (n=4 of quadruplicate experiments).

Figure 3.5: Simvastatin modulates expression of genes in PC3 cells involved in the extrinsic pathway regulating apoptosis. Bar graph showing changes in the mRNA levels of genes such as Bcl-2, Fas-L, Lhx4, Nme5, Traf1 and Trp53inp1with 24h simvastatin treatment normalized to multiple housekeeping genes. The data are presented as mean ± SD (n=4 of quadruplicate experiments).

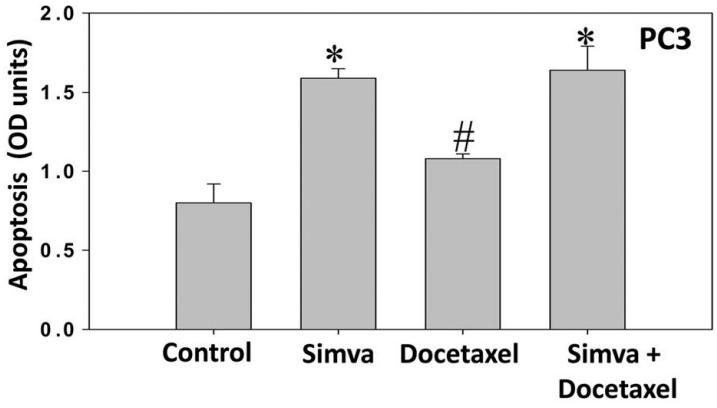
Figure 3.6: Simvastatin modulates expression of pro-apoptotic extrinsic pathway proteins in PC3 cells. Western blots showing protein expression of Fas-L, Nme5, Traf1, cleaved caspase-8 and Trp53inp1 in PC3 cells treated with simvastatin or docetaxel, compared to control saline treated cells.

Figure 3.7: Simvastatin modulates expression of Fas-L, Traf1 and cleaved caspase 8 in prostate tumor xenografts. Western blots showing protein expression of Fas-L, Nme5, Traf1, cleaved caspase 8 and Trp53inp1 in PC3 cell tumor xenografts treated with simvastatin, compared to control saline treated tumors.

Figure 3.8: Working hypothesis on the mechanisms by which simvastatin induces apoptosis in prostate cancer cells involving both intrinsic and extrinsic pathways.

Figure 3.1

A


PC3
Apoptosis (OD units)
2.0
1.5
1.0
0.5
0.0
*
#
*
Control
Simva
Docetaxel
Simva +
Docetaxel

B

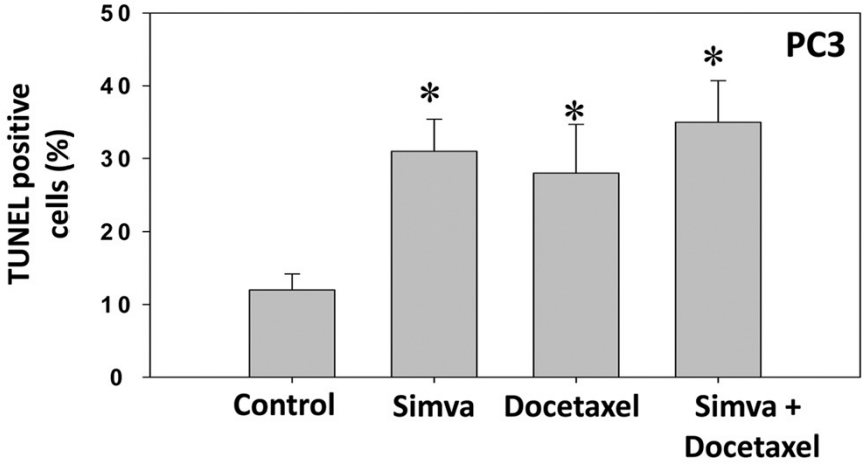

PC3
TUNEL positive
cells (%)
50
40
30
20
10
0
*
*
*
Control
Simva
Docetaxel
Simva +
Docetaxel

Figure 3.2:

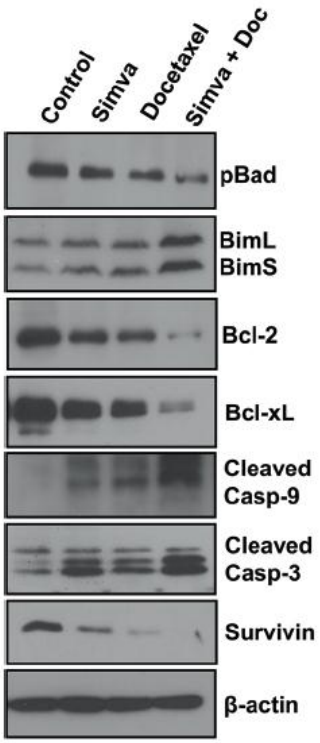

Control
Simva
Docetaxel
Simva + Doc
pBad
BimL
BimS
Bcl-2
Bcl-xL
Cleaved
Casp-9
Cleaved
Casp-3
Survivin
β-actin

Figure 3.3:

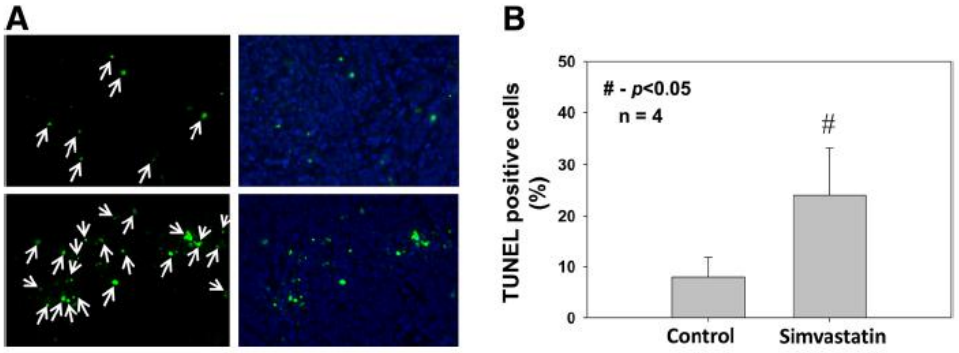

A
B
TUNEL positive cells (%)
# - p<0.05
n = 4
#
50
40
30
20
10
0
Control
Simvastatin

Figure 3.4:

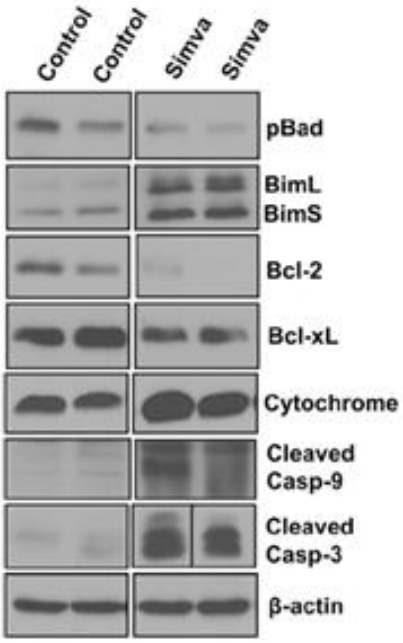

Control
Control
Simva
Simva
pBad
BimL
BimS
Bcl-2
Bcl-xL
Cytochrome
Cleaved
Casp-9
Cleaved
Casp-3
β-actin

Figure 3.5:

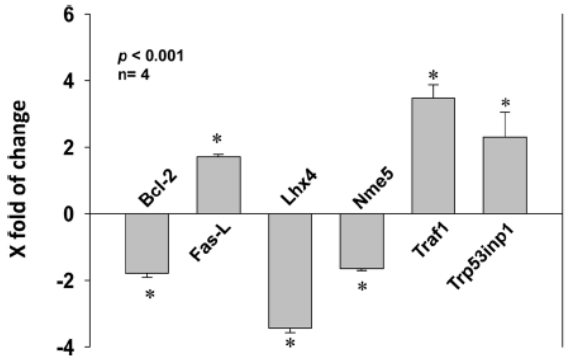

p < 0.001
n= 4
X fold of change
6
4
2
0
-2
-4
Bcl-2
Fas-L
Lhx4
Nme5
Traf1
Trp53inp1
*

Figure 3.6:

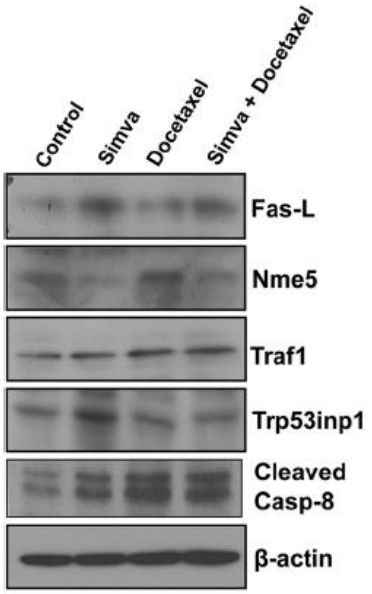

Control
Simva
Docetaxel
Simva + Docetaxel
Fas-L
Nme5
Traf1
Trp53inp1
Cleaved
Casp-8
β-actin

Figure 3.7:

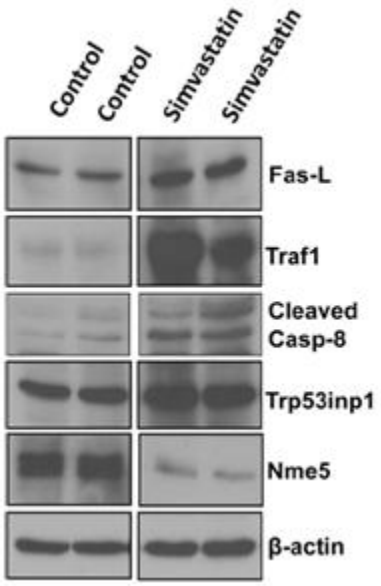
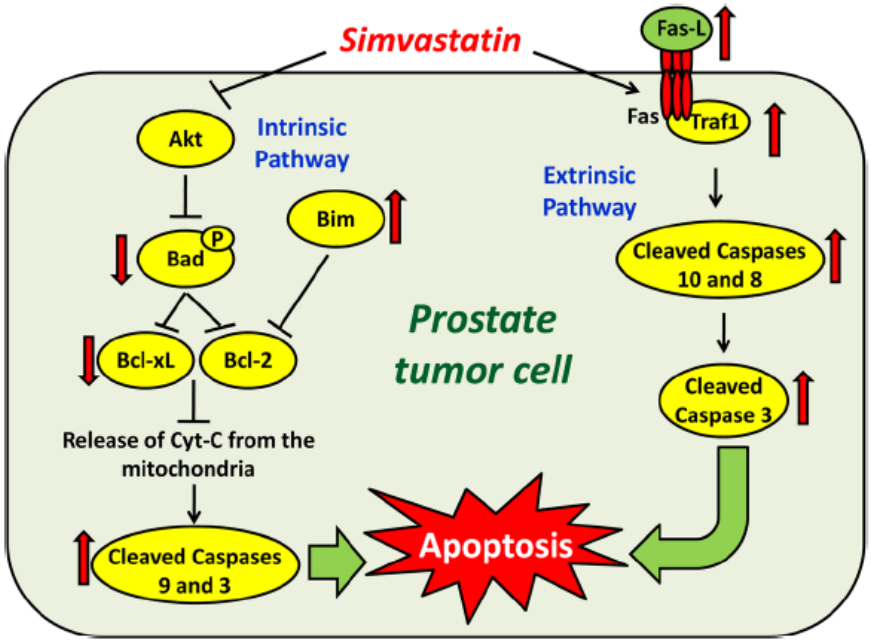

Control
Control
Simvastatin
Simvastatin
Fas-L
Traf1
Cleaved
Casp-8
Trp53inp1
Nme5
β-actin

Figure 3.8:


Simvastatin
Fas-L
Fas
Traf1
Akt
Intrinsic Pathway
Extrinsic Pathway
Bim
Bad
P
Cleaved Caspases 10 and 8
Prostate tumor cell
Bcl-xL
Bcl-2
Cleaved Caspase 3
Release of Cyt-C from the mitochondria
Cleaved Caspases 9 and 3
Apoptosis

CHAPTER 4:

SUPPRESSION OF INTERACTIONS BETWEEN PROSTATE TUMOR CELL INTEGRIN $\alpha_V\beta_3$ AND ENDOTHELIAL ICAM-1 BY SIMVASTATIN INHIBITS PROSTATE CANCER MICROMETASTASIS

Belal Al-Husein, Anna Goc and Payaningal R. Somanath

Submitted to ***Molecular Oncology on January 21st, 2013***

Abstract

Cancer micrometastasis relies on the ability of cancer cells to secrete angiogenic modulators, to interact with the vascular endothelium, and to overcome the resistance offered by the endothelial-barrier. Being an essential step prior to metastasis, blockage of micrometastasis can have potential applications in cancer therapy and metastasis prevention. Due to poorly known molecular mechanisms leading to micrometastasis, developing therapeutic strategies to target prostate cancer utilizing drugs that block micrometastasis is far from reality. Here we demonstrate the potential benefits of simvastatin in the inhibition of prostate cancer micrometastasis and reveal the novel molecular mechanisms underlying this process. First, we showed that simvastatin inhibits the ability of human PC3 prostate cancer cells for transendothelial migration in vitro. Second, our data indicated that simvastatin modulates the expression of tumor derived factors such as angiopoietins and VEGF-A at the mRNA and protein levels by the PC3 cells, thus preventing endothelial-barrier disruption. Third, simvastatin directly activates endothelial cells and enhances endothelial-barrier resistance. Apart from this, our study revealed that simvastatin-mediated effect on PC3 micrometastasis was mediated through inhibition of integrin $\alpha_v\beta_3$ activity and suppression of Interaction between prostate cancer cell integrin $\alpha_v\beta_3$ with endothelial ICAM-1.

Introduction

Although slow growing, prostate cancer is the leading cause of cancer-related death among men. Due to the limitations in prostate specific antigen (PSA) testing (22), the only available screening procedure for prostate cancer, many patients are diagnosed with prostate cancer only at an advanced stage and are left with limited treatment options. This demands additional research in developing procedures for early detection, prevention and/or the treatment of advanced stage prostate cancer.

Statins are lipid-lowering agents inhibiting HMG-CoA reductase enzyme, a key component of the cholesterol synthesis machinery (134). Apart from this, statins elicit pleiotropic effects on multiple cell types in regulating various cellular functions (77). Previous studies show that while statins are vascular protective via activation of endothelial cells (2), it inhibits proliferation of malignant cells such as hyper-active smooth muscle cells in the atherosclerotic plaques, leading to plaque stabilization and decreasing coronary artery disease-related mortality (135, 136). This clearly indicates the 'normalizing' potential of statins by activating cells at rest and inhibiting hyper-active malignant cells. This normalizing ability of statins has gathered specific interests for its potential applications in cancer for prevention and chemotherapy in combination with other drugs, thereby cancer therapy can be more effective and at the same time side-effects of chemotherapy can be minimized. In support of this view, a recent report indicates that long-term use of statins help to reduce cancer-related mortality by 15%, equivalent to the success rate of chemotherapy (137).

During the last several years, various statins have been tested for their anti-cancer efficacy on different cancer cell types *in vitro* and animal models *in vivo* (65). Despite

several controversies on the beneficial vs. adverse effects of statins on various cancers, investigations validating the use of statins for prostate cancer therapy have been highly promising (52). A recent clinical study reported a 45% reduction in the biochemical recurrence of prostate cancer after radical prostatectomy in patients taking statins (55). Statins have been reported to be safe for humans even at doses 10-50 times higher than that is prescribed for cardiovascular disease (51, 117). Previous studies from our group has demonstrated the anti-cancer efficacy of simvastatin, a highly lipophilic statin on androgen-responsive LNCaP cells and androgen-insensitive PC3 prostate cancer cell lines *in vitro* and tumor xenografts *in vivo* (103). Simvastatin also induced apoptosis in prostate cancer cells via simultaneous modulation of intrinsic cell survival and extrinsic apoptotic pathways (138). Simvastatin-induced effects on prostate cancer cells correlated with Akt inhibition, a serine-threonine kinase that has been implicated to be essential for prostate cancer progression and metastasis (21, 139, 140). Our studies have also demonstrated the pivotal role of Akt in mediating prostate cancer micrometastasis via activation of integrin $\alpha_v\beta_3$ (140), which have been reported to be elevated in prostate cancer cells (141).

The process of micrometastasis involves intravasation and extravasation of cancer cells into the blood vessels and is a pre-requisite for the metastasis of prostate cancer cells to distant tissues such as bone and lungs (142). Due to this rate-limiting nature of the micrometastasis step in cancer progression, its blockage can be developed into an effective strategy for the prevention of prostate cancer metastasis, thus providing longer window for the surgical removal of the cancer tissue. Since simvastatin inhibits Akt pathway in prostate cancer cells (103) and Akt is important for prostate cancer

micrometastasis (140) and vascular maturation (121, 143), this combined with the vascular protective role of statins lead us to hypothesize that simvastatin can be highly effective in preventing prostate cancer micrometastasis.

In the current study, we explored the effects of simvastatin on prostate cancer micrometastasis. We first demonstrated that simvastatin inhibited expression of VEGF and enhanced expression of angiopoietin-1 at the RNA and protein levels, implicating its effects on stabilizing the endothelial-barrier. Our results provide strong evidence that while simvastatin performs vascular normalization through Akt-mediated activation of endothelial cells, thus protecting the endothelial-barrier; it prevents micrometastasis of prostate cancer cells *in vitro* via suppression of interactions between prostate cancer cell integrin $\alpha_v\beta_3$ and endothelial ICAM-1. To our knowledge, we provide the first evidence demonstrating the potential application of statins in the prevention of prostate cancer and endothelial interactions in order to prevent its micrometastasis.

Materials and Methods

Cell culture

PC3 human prostate cancer cells were grown in DMEM/High glucose media supplemented with 10% FBS and 100 U/mL of penicillin-streptomycin. Human Microvascular Endothelial Cells (HMVECs) were grown in EBM-2 Basal Medium supplemented with EGM-2 MV SingleQuot Kit and Blasticidine (12.5 mg/ml) (Lonza, Fisher Scientific, Pittsburgh, PA).

Real-time PCR

PC3 human prostate cancer cells were grown in DMEM/High glucose media supplemented with 10% FBS and 100 U/mL of penicillin-streptomycin (Fisher Scientific,

Pittsburgh, PA). When reaching 90% confluence, cells were treated with activated Simvastatin 25 μM vs. control for 12 h. Cells were harvested and lysed for mRNA using RNeasy Mini Kit (Qiagen, Valecia, CA), cDNA was then produced from mRNA using RT² First Strand Kit (SA Biosciences, Valecia, CA). A total of 25 μg of cDNA was applied on each Cancer PathwayFinder PCR Array® (SA Biosciences, Valecia, CA) well, and PCR was run using an Eppendorff realplex2 equipment. Results were plugged into the associated tool available in SA Biosciences website to compare difference in expression using ΔΔC method after normalization to housekeeping genes.

Electric Cell Substrate Impedance Sensing (ECIS)

Electric Cell Substrate Impedance Sensing (ECIS) (Applied Biophysics, Troy, NY) was performed according to manufacturer's recommendations. Briefly, arrays were first washed with 10 mM glycine for 10 minutes, washed with media, stabilized using the device, and HMVECs were plated on the array wells. When reaching monolayer, arrays were plugged to ECIS device to take baseline readings and cells were treated with: simvastatin (5, 10 and 25 μM vs. control), detached PC3 cells pretreated with simvastatin vs. control, or media collected from simvastatin-treated PC3 cells. For cell detachement, cells were first treated with simvastatin (5, 10 and 25 μM vs. control), media was washed with PBS, and then sterile Ethylenediamine Tetraacetic acid (EDTA, 20 mM) containing PBS was added to cells (for 5-7 minutes, 37°C) to detach them from ECM without digesting cell surface receptors. Cells were then collected, pelleted, re-suspended in media and counted. A total of 5×10^4 cells were added to each ECIS array well. For media collection, cells were treated with Simvastatin for 3 h and then media was removed, washed with serum free medium and incubated further in serum free

medium to collect secreted factors over 12 h. Following this, media was collected, centrifuged to remove cell debris, and a total of 200 µl of the conditioned media was added to each ECIS array well. For AP7.4 (β3-integrin activation antibodies; kindly provided by Thomas Kunicki) experiments, PC3 cells were grown to a 70% confluence, treated with Simvastatin for 10 h, treated with AP7.4 (5 µg/ml) for another two hours, collected using cell dissociation buffer followed by adding to the ECIS array wells containing endothelial monolayer. Real-Time resistance/impedance were measured and recorded by the equipment automatically.

Protein precipitation and immunoblotting

A ratio of 1:4 trichloroacetic acid and the conditioned media was mixed and kept overnight in 4°C. Precipitated proteins were pelleted at 14000 rpm, washed twice with acetone and dried for 5 minutes at 95°C and then mixed with lamelli sample buffer and β-mercaptoethanol (9:1) followed by boiling for 5 minutes at 95°C. Equal volumes of samples were loaded in a 12% SDS-PAGE and subjected for Western blotting as done previously (138).

Immunocytochemistry

Endothelial cells were plated on glass cover-slips, grown to confluence, treated with simvastatin vs control. At endpoint (1 and 6 h of treatment), media was removed, cells were washed using PBS and fixed using 4% paraformaldehyde washed thrice with PBS, blocked using 5% goat serum albumin/0.3% Triton X-100 for 30 min, incubated with primary antibodies for VE-Cadherin, or β-Catenin (both from Cell Signaling, Danvers, MA) overnight at 4°C. Cells were washed three times with PBS and then incubated with secondary antibodies labeled with alexa-488 (goat anti-mouse) or alexa-597 (goat anti-

rabbit) for 1 h followed by three times washing with PBS. Cover slips were mounted on to the microscope slides using DAPI-containing vectashield (Vector laboratories, Burlingame, CA). Slides were viewed using Carl-Zeiss Fluorescent microscope (Carl Zeiss, Germany).

Cancer cell-endothelium adhesion assay

PC3 cells were infected with GFP-expressing empty vector adenovirus particles. Briefly, 10 µl of a 10^9 PFU of adenovirus particles were added to a 6-well plate. 48 h later, cells were treated with Simvastatin (25 µM) vs. control for 4 h and 16 h, followed by plating of cells on top of a monolayer of HMVECs for adhesion. After 1 h, excess cells that were not attached were washed off, fixed with 2% paraformaldehyde and viewed under fluorescent microscope. Attached cells were identified by the GFP fluorescence and quantified.

Integrin $\alpha_v\beta_3$-ligand binding assays

PC3 cells were plated in a 12-well plate and cultured until achieving monolayer by visual inspection, cells were then treated with simvastatin vs. control for 12 h. Media was removed and integrin $\alpha_v\beta_3$ specific ligand Fibrinogen-Alexa-488 (12.5 µg/ml or 100 µg/ml, respectively; Invitrogen, Calrsbad, CA) was added to the wells for 40 min. Excess Fibrinogen-Alexa-488 was washed off and fluorescence was measured at 488/519 using a BioTek multi-plate reader (Biotek, Winooski, VT).

Activation-dependent ligand, WOW-1 Fab, was used to determine the activation status of integrin $\alpha_v\beta_3$ in PC3 cells as previously described (140). A monolayer of PC3 cells was treated with simvastatin for 12 h. WOW-1 Fab (30 µg/ml) was added and incubated for additional 40 min. Wells were washed once with PBS, incubated for 1 h with

secondary goat anti-mouse antibody labeled with Alexa-488, washed three times with PBS, fixed with 2% paraformaldehyde and fluorescence was measured at 488/519 using a BioTek multi-plate reader (Biotek, Winooski, VT).

PC3 cells-ICAM-1 adhesion assay

Human soluble ICAM-1 (R&D Systems, Minneapolis, MN) at a final concentration of 12.5 µg/ml was plated on to each well of a 12 well plate and PC3 cells, pre-treated with various concentrations of simvastatin (5 and 25 µM) were added on top of the wells pre-coated with ICAM-1 at a concentration of 1 X 10^4 cells/well. After 40 Min, wells were washed three times with PBS to remove unbound cells and adhered cells were stained with crystal violet and counted.

Assessment of tumor angiogenesis in nude mice tumor xenografts

Tumor xenografts were implanted and collected as done previously (103). Tumor sections were stained for antibodies specific to laminin (Sigma, St. Louis, MO) followed by incubation with Alexa-488 labeled secondary antibodies (Invitrogen, Calrsbad, CA). Cover slips were then mounted on slides using vectashield with DAPI and imaged using Carl-Zeiss Fluorescent microscope (Carl Zeiss, Germany).

Statistical analysis

All the data are presented as mean ± SEM. To determine significant differences between treatment and control values, we used the Student's two-tailed t test. The significance was set at 0.05 levels (marked with symbols wherever data are statistically significant).

Results:

Simvastatin inhibits transendothelial migration (micrometastasis) of PC3 cells

The ability of cancer cells to disrupt the endothelial-barrier is the initial step to mediate their entry into the circulation and come out of the vasculature to metastasize to distant tissues. Hence, using the ECIS equipment, we first studied the effect of simvastatin on PC3 cells on their ability to interact with endothelial cell monolayer and micrometastasis *in vitro*. PC3 cells were treated with various doses of simvastatin (5, 10 and 25 µM) vs. control for 12 h, collected and re-suspended in DMEM before introducing them on top of a monolayer of HMVECs. Our results indicated that simvastatin inhibited micrometastasis of PC3 cells *in vitro* in a dose dependent manner (Figure 4.1A and B), as compared to vehicle treated cells. Although the effect of simvastatin on PC3 micrometastasis was not statistically significant, there was a dose-dependent trend towards the inhibition of PC3 micrometastasis with simvastatin treatment (Figure 4.1A and B). These results suggested the inhibitory effect of simvastatin on PC3 cell micrometastasis *in vitro*.

Simvastatin inhibits endothelial-barrier disruption induced by the PC3 cell conditioned medium

A major factor that drives cancer cell micrometastasis is the ability of tumor cells to secrete growth factors and cytokines that have the potential to modulate the endothelial-barrier integrity. Hence, we next attempted to study the effect of simvastatin on the secretion of tumor-derived factors, and in turn, modulation of the endothelial-barrier resistance. To do this, PC3 cells were treated with various doses of simvastatin (5, 10 and 25 µM) vs. control for 2 h, followed by replacing the medium with serum free

medium. After incubation for another 10 h, conditioned media was collected; cell debris was removed by centrifugation and plated on to ECIS array wells previously maintaining a fully confluent HMVEC monolayer to record the changes in the electrical impedance. Similar to our results on PC3 cell micrometastasis, although not significant, simvastatin exhibited a trend towards impaired endothelial-barrier disruption by the PC3 cell conditioned medium as compared to vehicle treated controls (Figure 4.1C and D). Our results once again suggested the effect of simvastatin on inhibiting secretion of PC3 tumor derived factors in the modulation of endothelial-barrier resistance.

Simvastatin inhibits expression of prostate tumor cell-derived factors

Next, we sought to identify the key growth factors and cytokines regulating simvastatin-mediated inhibition of HMVEC-barrier disruption by PC3 tumor cell-derived factors. First, using CancerPathway Finder arrays, we performed a qPCR for PC3 cells pre-treated with 25 μM simvastatin for 12 h vs. control-treated. Our results indicated that while simvastatin inhibited the mRNA expression of pro-angiogenic and/or endothelial-barrier disrupting growth factors such as VEGF-A, IGF-1 and angiopoietin-2, it resulted in the decreased expression of angiopoietin-1, a growth factor known to arrest endothelial-barrier break-down and enhance vascular maturation (Figure 4.2A). These results were corroborated with our Western analysis data, which showed that while simvastatin treatment resulted in significant decrease in the secretion of VEGF-A (2-fold) into the conditioned media by the PC3 cells, secretion of angiopoietin-1 was significantly increased (2-fold) (Figure 4.2B-D). These results demonstrated the effect of simvastatin on the secretion of various pro- and anti-angiogenic factors such as VEGF-A and angiopoietins in the modulation of endothelial-barrier resistance.

Simvastatin has no significant effect on the rate of tumor angiogenesis

Since expression of pro-angiogenic and vascular permeability modulators VEGF-A and Angiopoietin-1 in PC3 cells were altered by simvastatin treatment, we investigated the effects of simvastatin on tumor angiogenesis *in vivo* in PC3 tumor xenografts developed in athymic nude mice (103) (Figure 4.3). Interestingly, simvastatin treatment did not inflict any significant changes in the vascular area in PC3 cell tumor xenografts as evidenced by the laminin statining, demonstrating that simvastatin is more involved in the normalization of tumor vasculature than modulating tumor angiogenesis.

Simvastatin inhibits PC3 cell interactions with the endothelium

Although synthesis and secretion of endothelial-barrier modulating growth factors were altered by simvastatin, our results suggested that the simvastatin-mediated inhibition of prostate cancer cell micrometastasis is reliant on factors other than its effects on tumor cell-derived factors. Hence, in the next step, we investigated the effect of simvastatin on interactions between prostate tumor cell and the endothelial cells. To do this, GFP transfected cells (using adenovirus expressing GFP) were treated with 25 µM simvastatin for 12 h, followed by isolation by cell dissociation buffer. Cells after re-suspending in DMEM were counted and equal number of cells was plated onto a monolayer of endothelial cells. One hour following the cell addition, wells were washed with PBS and fixed using 2% paraformaldehyde. Images were taken using an inverted fluorescent microscope and the GFP expressing PC3 cells attached to the endothelial monolayer was quantified. Our study indicated that simvastatin significantly inhibited adhesion of PC3 cells to the endothelial monolayer in a time-dependent manner (Figure 4.4 A-D). While 20% decrease in PC3 cell adhesion to the endothelium was observed in

4 h post simvastatin treatment (Figure 4.4A and B), there was >60% inhibition of PC3 cell adhesion to the endothelium after 16 h (Figure 4.4C and D).

Inhibition of prostate cancer cell interactions with the endothelium is mediated through integrin $\alpha_v\beta_3$

Cell-surface integrin $\alpha_v\beta_3$ has been implicated in mediating interactions between lymphocytes and vascular endothelial cells in mediating diapedesis (144). Integrin $\alpha_v\beta_3$ is highly expressed in invasive and metastatic prostate cancer cells and our previous study has demonstrated the role of Akt-integrin $\alpha_v\beta_3$ cooperation in mediating prostate cancer cell micrometastasis (140). Therefore, we tested if simvastatin-mediated inhibitory effects on prostate cancer cell interactions with the endothelium involved integrin $\alpha_v\beta_3$. Our study utilizing Alexa 488-labelled fibrinogen, a specific ligand for integrin $\alpha_v\beta_3$ and WOW-1 Fab (30 µg/ml; kindly gifted by Sanford Shattil, Scripps Research Institute, CA) that detects only active integrin $\alpha_v\beta_3$ on the cell surface demonstrated significantly impaired interaction of fibrinogen-Alexa 488 (Figure 4.5A) and WOW-1 (Figure 4.5B) with PC3 cells pre-treated with 5 and 25 µM simvastatin for 12 h, indicating impaired affinity of integrin $\alpha_v\beta_3$ for its extracellular matrix ligands.

To confirm this further, we performed a rescue experiment utilizing AP7.4, a specific clone of antibodies that bind to the extracellular domain of the cell-surface integrin $\alpha_v\beta_3$ and induce activating conformational changes. Treatment of PC3 cells with AP7.4 enhanced their micrometastasis, compared to the control (Figure 4.5C). While simvastatin impaired the ability of PC3 cells to micrometastasize, our results indicated that pre-treatment of PC3 cells with AP7.4 (5 µg/ml) significantly rescued simvastatin-mediated inhibition of micrometastasis *in vitro* on HMVECs (Figure 4.5C and D).

Simvastatin suppresses interactions between PC3 cell integrin $\alpha_v\beta_3$ and endothelial ICAM-1

One common method by which circulating cells adhere to endothelium to mediate diapedesis is by utilizing their surface integrin $\alpha_v\beta_3$ to bind to specific ligands-cum-adhesion molecules on the endothelium such as VCAM-1 and ICAM-1 (145). Since integrin $\alpha_v\beta_3$ is also necessary for prostate cancer micrometastasis, we tested whether simvastatin treatment had any effect on interactions between PC3 cell-surface integrin $\alpha_v\beta_3$ and ICAM-1, adhesion molecules that are abundant on endothelial cell surface. Our data demonstrated that treatment with 5 and 25 μM simvastatin significantly inhibited PC3 cell interaction with ICAM-1, indicating that simvastatin suppressed interactions between PC3 cell-surface integrin $\alpha_v\beta_3$ and ICAM-1 (Figure 4.6 A and B).

Simvastatin enhances endothelial cell-barrier integrity via VE-Cadherin stabilization

To investigate this effect, we performed immunocytochemistry analysis of endothelial monolayer treated with various doses of simvastatin (5, 10 and 25 μM) for 1 h and 6 h, with antibodies specific for VE-Cadherin. As figure 4.7 shows, VE-Cadherin expression analysis of HMVECs treated with simvastatin for 6 h indicated a significantly elevated VE-Cadherin expression in HMVEC-barrier junctions with 5 μM simvastatin with modest, but significant reduction in expression VE-Cadherin with 25 μM simvastatin. These results indicated that simvastatin elicits vascular protective effects via endothelial-barrier enhancement. However, our results also provide the need for caution at the use of very high doses of simvastatin for prostate cancer treatment, as higher doses can be toxic to endothelial cells and normal vasculature.

Discussion

The process of micrometastasis involving intravasation and extravasation of cancer cells is an essential pre-requisite for the cancer cells to metastasize to distant tissues (144). Our current study demonstrates the potential benefits of simvastatin in the inhibition of prostate cancer metastasis (Figure 4.9). We first showed that simvastatin treatment inhibited transendothelial migration of highly invasive PC3 cells in an ECIS array equipment *in vitro*. Simvastatin treatment on PC3 cells also significantly reduced the effect of cancer cell conditioned medium on endothelial-barrier break-down. Our gene array experiment, followed by Western analysis identified a decrease in VEGF-A expression and increase in Angiopietin-1 expression by PC3 cells in response to simvastatin treatment. WOW-1 binding assay that measures the surface expression of activated integrin $\alpha_v\beta_3$ in PC3 cells and fibrinogen binding assay demonstrated the effect of simvastatin on significantly inhibiting the affinity of integrin $\alpha_v\beta_3$ to bind to its ligands. Co-treatment with AP7.4, integrin $\alpha_v\beta_3$ activating antibodies, rescued simvastatin-mediated inhibition of PC3 cell micrometastasis. While pre-treatment with simvastatin significantly impaired the ability of PC3 cells to bind to the endothelial cell surface, adhesion of PC3 cells to soluble ICAM-1, adhesion molecules that are abundantly expressed in endothelial cells (146) often implicated in mediating transendothelial migration of inflammatory cells (145) and cancer cells (147) was significantly blunted by treatment with simvastatin. Finally, simvastatin treatment stabilized the vascular adherens junctions and protected endoethelial-barrier by enhancing β-catenin expression. Collectively, our data demonstrates the potential therapeutic benefits of simvastatin in preventing prostate cancer micrometastasis.

Much of the effects of simvastatin on prostate cancer micrometastasis have been attributed to its ability to differentially modulate cell signaling in normal cells at rest and malignant cells. While statins have been previously shown to inhibit proliferation of vascular smooth muscle cells in atherosclerotic plaques (148) and inhibition of cell motility and migration of various cancer cells (140), including prostate cancer cells (21, 138), vascular endothelium is protected by statins via activation of Akt-eNOS pathway (42). Hence, while statins protect the vasculature, it inhibits tumor growth and may prevent micrometastasis. This ability of statins to normalize the tumor cells and the cells in the microenvironment provides the benefit of improving the efficacy of chemotherapeutic drugs when treated in combinations with statins.

Our previous results related to the effects of simvastatin on prostate cancer cells *in vitro* and tumor xenograft growth *in vivo* are completely in agreement with the general perception that statins normalize the deregulated signaling pathways in malignant cells. We showed that while simvastatin inhibited prostate cancer cell proliferation, migration and colony formation *in vitro*, it impaired the growth of tumor xenografts *in vivo* (103). In addition, simvastatin induced apoptosis in prostate cancer cells via inhibition of intrinsic cell survival as well as activation of extrinsic death-receptor pathways (138). Results from the current study on the specific effects of simvastatin on micrometastasis of prostate cancer cells *in vitro* further strengthens our overall idea of utilizing statins for prostate cancer therapy either alone (for prevention) or in combination with chemotherapeutic drugs such as Taxotere® (for therapy). Our studies suggest that dual effects of simvastatin on inhibition of interactions between the prostate cancer cells and the endothelium, and on the modulation of tumor-derived factors such as VEGF and

angiopoietins will provide a net stabilizing effect on the tumor vasculature, by reducing the vascular permeability, an important feature of tumor vasculature (149).

A very recent study has also demonstrated the effect of atorvastatin on VEGF expression by the human small non-cell lung carcinoma cell lines (45). While our study confirms this report to be true also for prostate cancer cells, we provide additional information on the inhibitory effect of simvastatin on the expression of angiopoietin-2, a signaling companion of VEGF in the modulation of tumor vascular permeability (150), and enhance the expression of angiopoietin-1, a growth factor that stabilizes the vascular endothelial-barrier junctions, thus stabilizing the endothelial-barrier and reducing vascular permeability (150). Other prominent pro-angiogeneic growth factors or signaling molecules identified in the gene array that are regulated by simvastatin in PC3 cells include TEK-Receptor tyrosine kinase, Insulin like growth factor-1 (IGF-1) and FGF Receptor-2, all of which are inhibited by simvastatin. Since IGF-1 has been implicated in the development of castration resistant prostate cancer (151), simvastatin treatment appears to be an effective treatment strategy to prevent recurrence after prostatectomy as has been shown recently (55). Furthermore, staining of endothelial-barrier junctions for βcatenin, a predominant adherens junction protein indicated enhanced endothelial-barrier protection upon treatment with simvastatin, accompanied by enhanced barrier-resistance. Interestingly, these changes in gene and protein expression of angiogenic modulators by simvastatin did not have a net effect on tumor angiogenesis *in vivo*, indicating that simvastatin provides a normalizing and stabilizing effect on the tumor endothelium. This priming effect on the tumor vasculature is a feature considered essential for anti-angiogenic therapy (152).

Impaired micrometastasis of PC3 cells pre-treated with simvastatin in an ECIS array suggested that factors other than tumor derived factors may be involved in the modulation of simvastatin-mediated prostate cancer micrometastasis. An important mechanism by which circulating blood cells and cancer cells transmigrate the endothelial-barrier is via a direct heterophilic interaction with the endothelium mediated through cell-surface integrins and cellular adhesion molecules (145, 147). Statins have previously shown to impair transendothelial migration of inflammatory cells via inhibition of their interaction with the endothelium (145). Our previous studies have shown that Akt and Rac pathways enhance prostate cancer micrometastasis involving integrin $\alpha_v\beta_3$ (138, 140), which are extracellular matrix (ECM) receptors abundantly expressed in prostate cancer cells and have been implicated to be necessary for prostate cancer metastasis (141). Since Akt and RhoGTPases are important targets of simvastatin in cancer cells (103, 153) we postulated that simvastatin-mediated inhibition of PC3 micrometastasis may involve integrin $\alpha_v\beta_3$. Our findings supported this hypothesis and demonstrated that while simvastatin treatment resulted in impaired inside-out activation of integrin $\alpha_v\beta_3$ and thus reduced their affinity for specific ligands such as fibrinogen. Furthermore, co-treatment of PC3 cells with simvastatin and AP7.4 (integrin $\alpha_v\beta_3$ activating antibodies) rescued the impaired micrometastasis, once again confirming that simvastatin has a direct effect on integrin $\alpha_v\beta_3$ on reducing its ligand affinity.

Cell-surface integrins on circulating blood cells and cancer cells often interact with cell adhesion molecules expressed on endothelial cells such as VCAMs and ICAMs (145). Among these, interaction between integrin $\alpha_v\beta_3$ and ICAM-1 is the best characterized (147). Our data indicated that pre-treatment with simvastatin significantly impaired the

ability of PC3 cells to recognize and adhere to human soluble ICAM-1, thus demonstrating the direct effect of simvastatin on inhibiting interactions of prostate cancer cells with cellular adhesion molecules. Although not tested in the current study, statins have been previously reported to reduce the expression of cellular adhesion molecules such as ICAMs and VCAMs on endothelial cell surface (146). Hence, simvastatin is expected to elicit a much more potent inhibitory effect on prostate cancer micrometastasis *in vivo* as compared to our unidirectional approach in the *in vitro* experiments. Altogether, this study identifies simvastatin as a potent inhibitor of prostate cancer micrometastasis mostly via inhibition of cancer cells adhesion to endothelial cells, inhibition of prostate cancer cell transendothelial migration and by stabilizing the endothelial-barrier and to a less extent via inhibition of tumor derived factor expression.

Conclusion

Precise role of statins in the regulation of reciprocity between prostate cancer cells and the tumor endothelium in the regulation of micrometastasis remains to be determined. Moreover, mechanisms regulating prostate cancer micrometastasis are also poorly understood. In this study, we identified the importance of interactions between prostate cancer cell-surface integrins and endothelial cell adhesion molecules and the potential benefits of simvastatin in blocking these interactions leading to the inhibition of micrometastasis. Apart from this simastatin modulated expression of various pro- and anti-angiogenic factors by the PC3 cells. These results combined with the anti-inflammatory and endothelial-barrier stabilizing effects of simvastatin suggest that statins could be re-purposed for the management of prostate cancer either alone or in combination with chemotherapeutic regimen.

Authorship contributions

Conceived and designed the experiments: BA, AG and SPR

Performed the experiments: BA and AG

Analyzed the data: BA, AG and SPR

Wrote the manuscript: BA and SPR

Conflict of interest

None

Acknowledgements

Funds were provided by the National Institutes of Health grant (R01HL103952) and American Heart Association Scientist Development Grant (0830326N) to PRS and in part by the University of Georgia Research Foundation, Wilson Pharmacy Foundation and UGA College of Pharmacy and Clinical and Administrative Pharmacy Department through intramural grants to PRS. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Legends for Figures:

Figure 4.1: Simvastatin inhibits the transendothelial migration of PC3 cells: A) and B) PC3 cells were treated with different doses of simvastatin (0, 5, 10 and 25 µM) for 12 h and introduced into ECIS array wells pre-plated with a confluent monolayer of HMVECs. Figure shows transendothelial migration of PC3 cells in response to different doses of simvastatin as measured by the reduced electrical resistance. C) and D) PC3 cells were treated with different doses of simvastatin for 12 h and the conditioned media was collected and introduced into ECIS array wells pre-plated with a confluent monolayer of HMVECs. Figure shows disruption of endothelial-barrier by the PC3 cell conditioned media in response to different doses of simvastatin. Data presented as Mean ± SEM.

Figure 4.2: Simvastatin inhibits binding of PC3 cells to the endothelial monolayer: PC3 cells expressing GFP were treated with 25 µM simvastatin for 4 h A) and B) and 12 h C) and D), and introduced into 12-well plates containing a confluent monolayer of HMVECs. One hour later, medium was removed, wells were washed with PBS and the number of GFP-positive PC3 cells were determined.

Figure 4.3: Simvastatin modulates expression of secreted angiogenic factors by the PC3 cells: A) Bar graph showing qRT-PCR array data indicating key genes modulated by simvastatin. B) Western analysis of PC3 cell conditioned medium concentrated by TCA-precipitation showing changes in the expression of VEGF-A and Ang-1. C) and D) Band-densitometry analysis of the Western blots of PC3 cell conditioned medium indicating changes in the expression of VEGF-A and Ang-1 normalized to coomassie staining.

Figure 4.4: Simvastatin does not inhibit prostate tumor angiogenesis: A) Immunofluorescence staining of PC3 tumor xenograft sections from nude mice showing laminin staining as a measure of tumor angiogenesis. B) Bar graph showing no differences in the vascular area in tumor xenografts between control (saline) and simvastatin (2 mg/kg/12 h) treated mice.

Figure 4.5: Simvastatin abolishes PC3 cell interaction with the endothelium via inhibition of PC3 cell surface integrin αvβ3 affinity for its ligands: A) Bar graph showing the effect of simvastatin on PC3 cell interactions with Alexa486-labelled fibrinogen, an integrin αvβ3 ligand. B) Bar graph showing impaired binding of WOW-1, a specific ligand for activated integrin αvβ3, by the PC3 cells treated with 25 μM simvastatin compared to control. C) and D) Figure showing significant inhibition of PC3 cell transendothelial migration with 25 μM simvastatin compared to control saline treated cells. Co-treatment with AP7.4, integrin αvβ3 activating antibodies abolished simvastatin-mediated effects.

Figure 4.6: Simvastatin inhibits interaction between PC3 cell-surface integrin αvβ3 and endothelial ICAM-1: Figure shows treatment with 5 and 25 μM doses of simvastatin significantly inhibits adhesion of PC3 cells on pre-coated soluble human ICAM-1.

Figure 4.7: Simvastatin stabilizes the endothelial-barrier in mediating normalization of the tumor vasculature: A) Fluorescent microscopic images of HMVEC monolayer stained with adherens junction protein β-catenin. B) Bar graph of the above data indicating changes in expression with 5, 10 and 25 μM doses of simvastatin.

Figure 4.8: Schematic representation of the working hypothesis.

Figure 4.1:

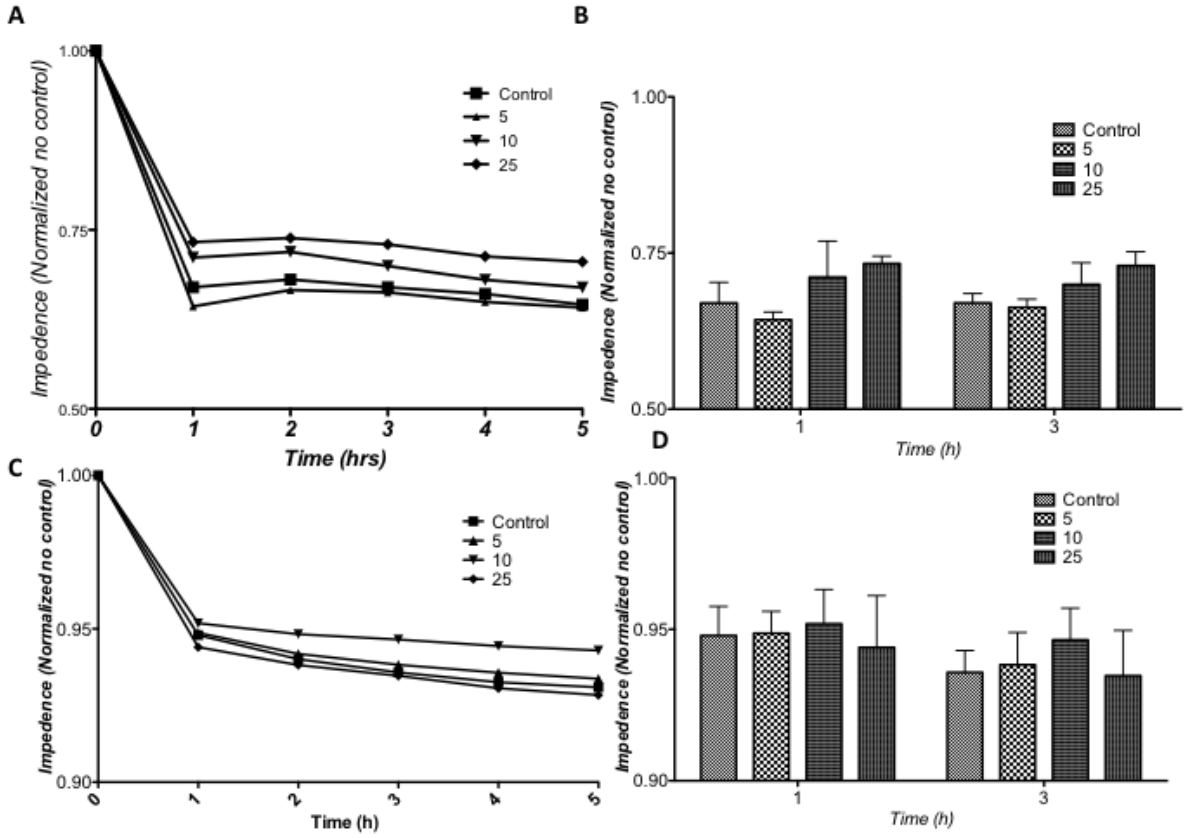

A
B
C
D
Impedence (Normalized no control)
Time (hrs)
Time (h)
Control
5
10
25
1.00
0.75
0.50
0.95
0.90
0
1
2
3
4
5

Figure 4.2:

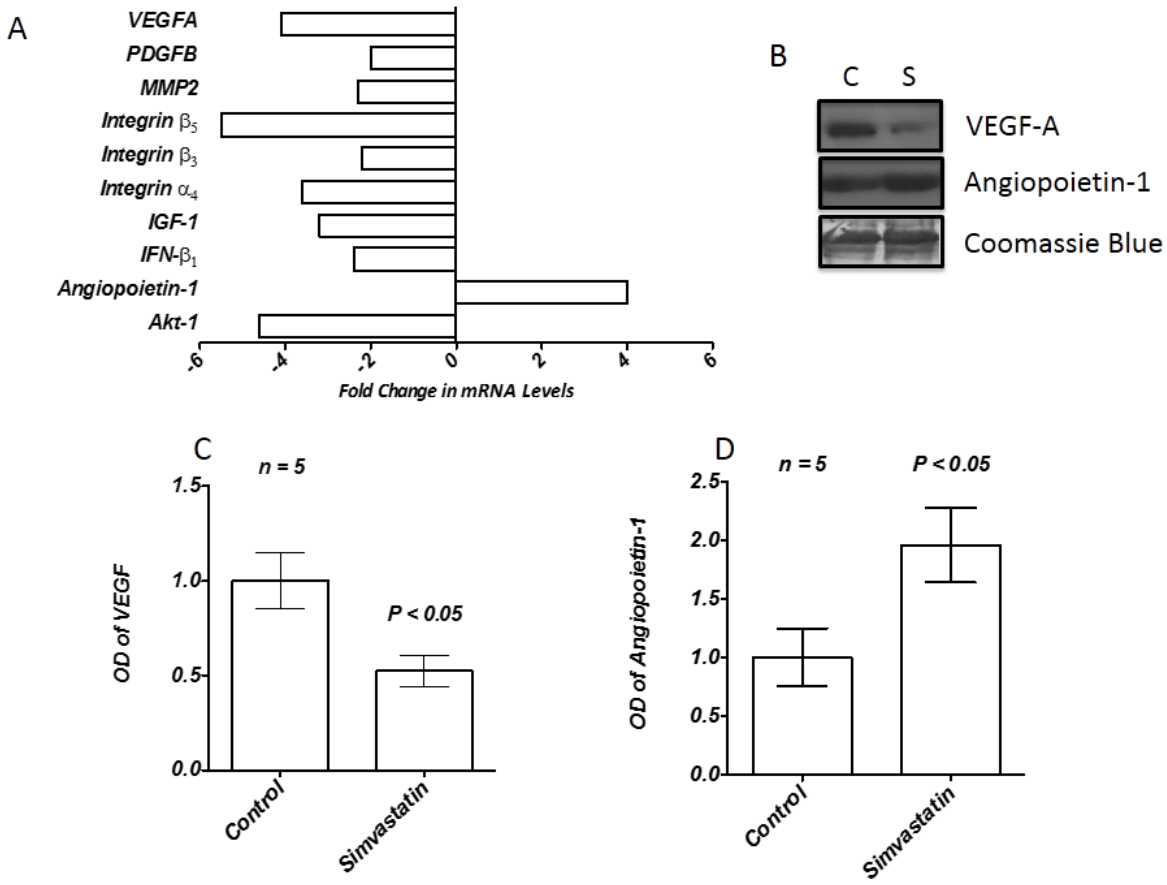

A
VEGFA
PDGFB
MMP2
Integrin β5
Integrin β3
Integrin α4
IGF-1
IFN-β1
Angiopoietin-1
Akt-1
-6
-4
-2
0
2
4
6
Fold Change in mRNA Levels
B
C
S
VEGF-A
Angiopoietin-1
Coomassie Blue
C
n = 5
P < 0.05
OD of VEGF
1.5
1.0
0.5
0.0
Control
Simvastatin
D
n = 5
P < 0.05
OD of Angiopoietin-1
2.5
2.0
1.5
1.0
0.5
0.0
Control
Simvastatin

Figure 4.3:

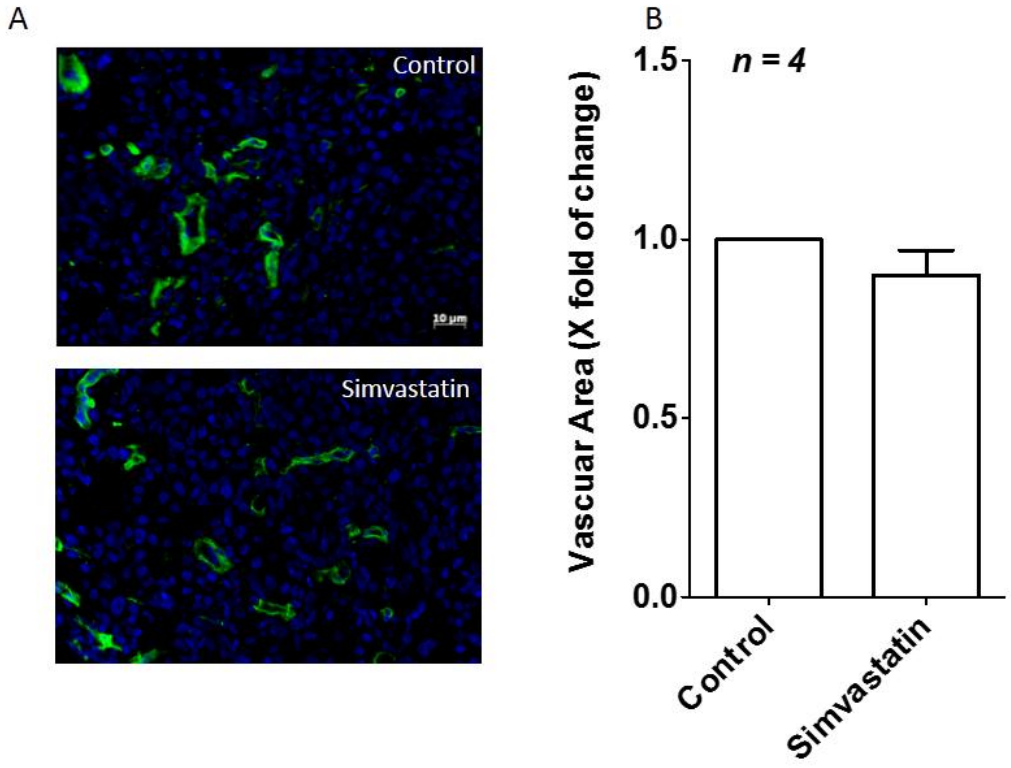

A
Control
10 µm
Simvastatin
B
n = 4
Vascuar Area (X fold of change)
1.5
1.0
0.5
0.0
Control
Simvastatin

Figure 4.4:

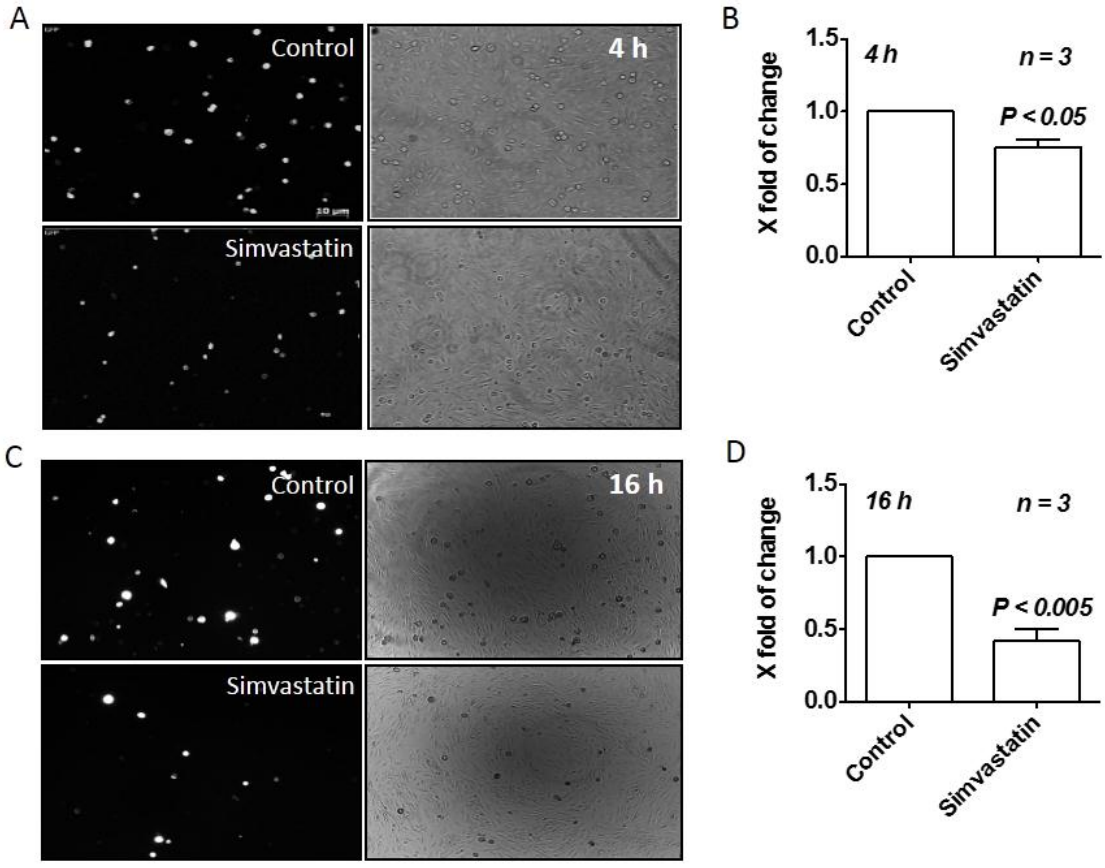

A
Control
4 h
Simvastatin
B
4 h
n = 3
P < 0.05
X fold of change
1.5
1.0
0.5
0.0
Control
Simvastatin
C
Control
16 h
Simvastatin
D
16 h
n = 3
P < 0.005
X fold of change
1.5
1.0
0.5
0.0
Control
Simvastatin

Figure 4.5:

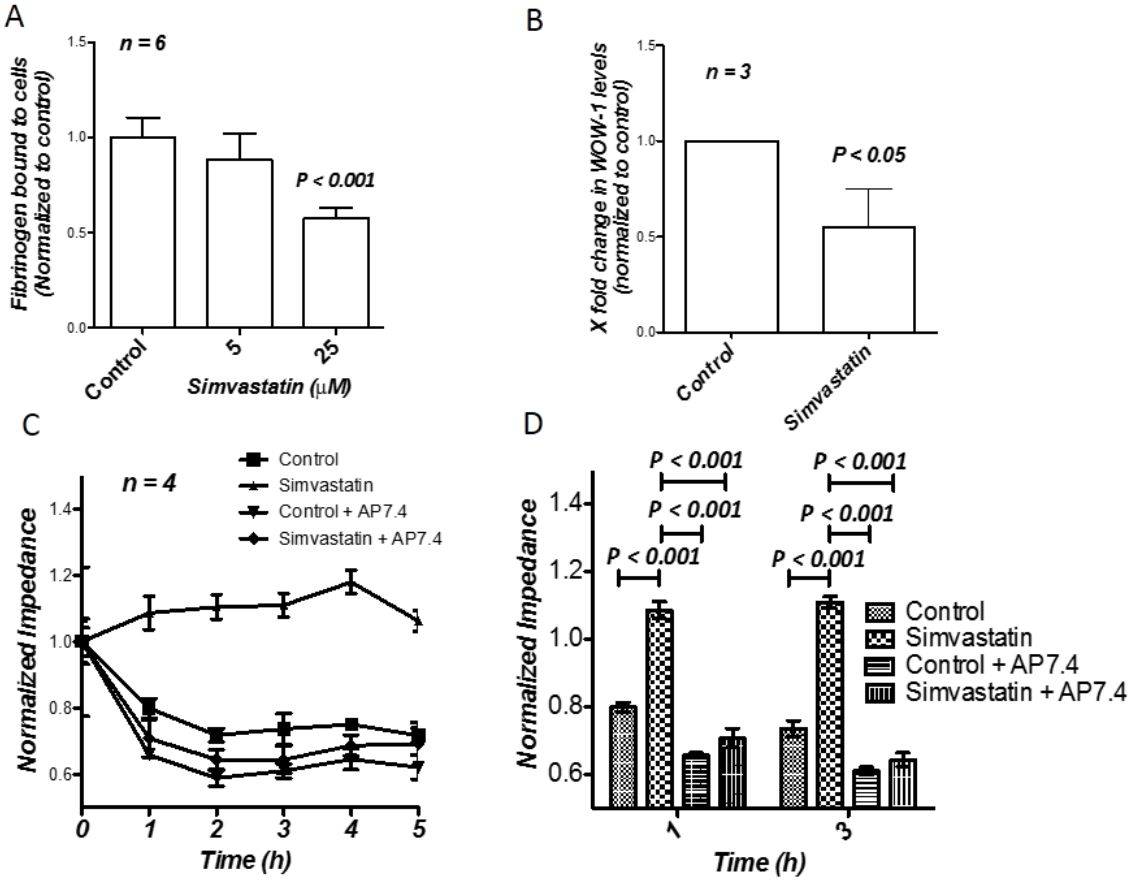

A
n = 6
Fibrinogen bound to cells (Normalized to control)
P < 0.001
Control
5
25
Simvastatin (μM)
B
n = 3
X fold change in WOW-1 levels (normalized to control)
P < 0.05
Control
Simvastatin
C
n = 4
Control
Simvastatin
Control + AP7.4
Simvastatin + AP7.4
Normalized Impedance
Time (h)
D
P < 0.001
Normalized Impedance
Control
Simvastatin
Control + AP7.4
Simvastatin + AP7.4
Time (h)

Figure 4.6:

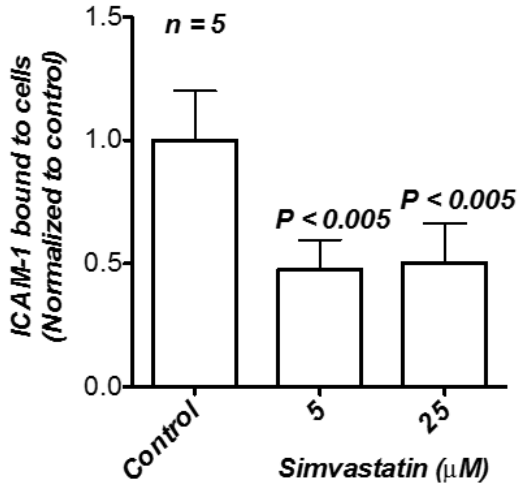

n = 5
P < 0.005
P < 0.005
ICAM-1 bound to cells
(Normalized to control)
1.5
1.0
0.5
0.0
Control
5
25
Simvastatin (μM)

Figure 4.7:

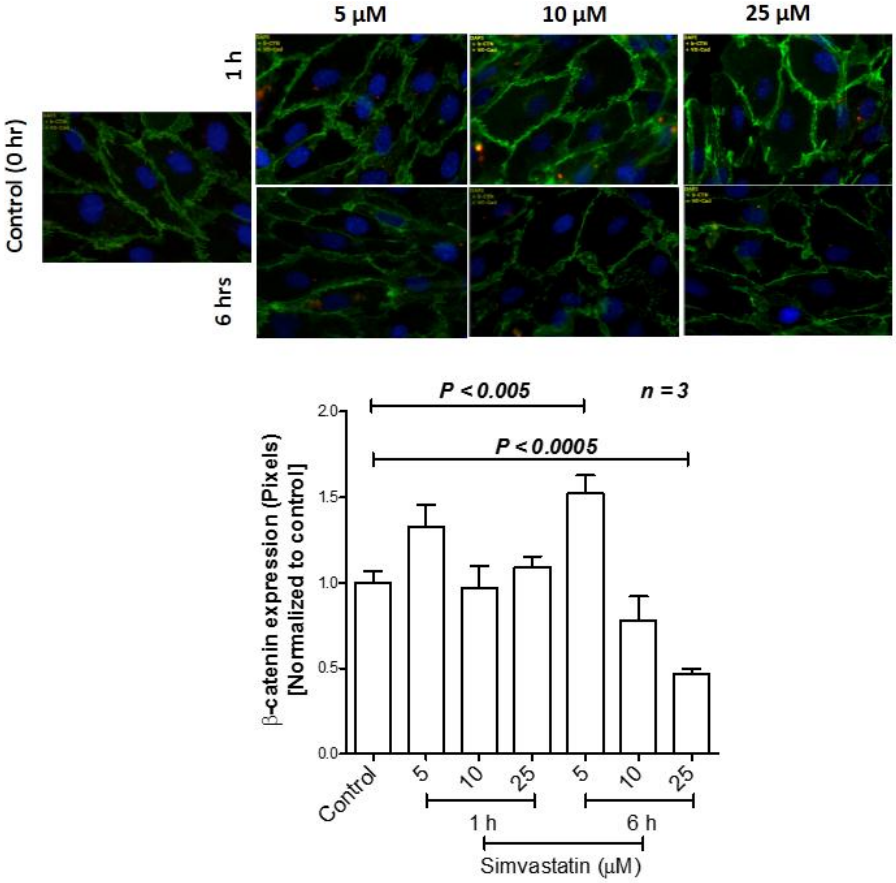
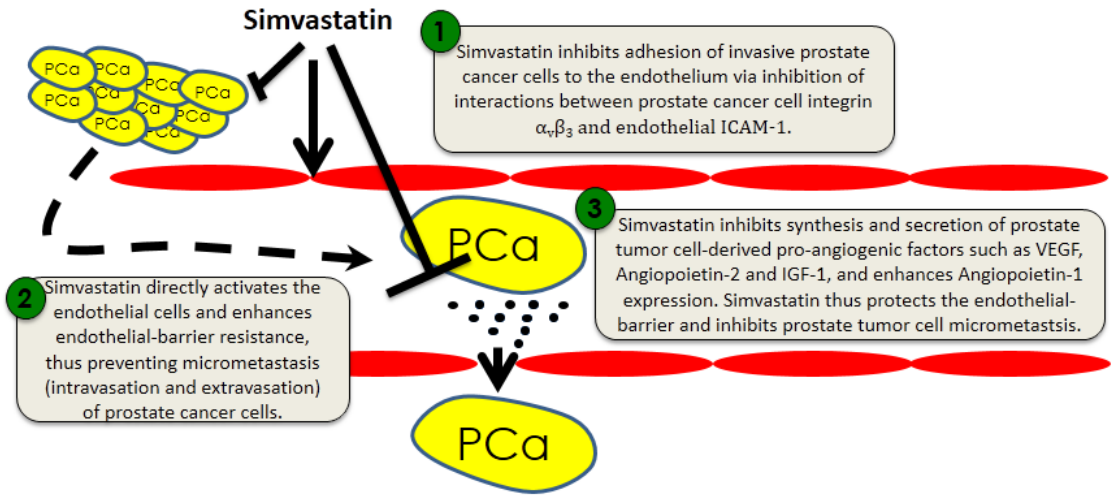

5 µM
10 µM
25 µM
1 h
6 hrs
Control (0 hr)
P < 0.005
n = 3
P < 0.0005
β-catenin expression (Pixels)
[Normalized to control]
2.0
1.5
1.0
0.5
0.0
Control
5
10
25
5
10
25
1 h
6 h
Simvastatin (µM)

Figure 4.8:


Simvastatin
1
Simvastatin inhibits adhesion of invasive prostate cancer cells to the endothelium via inhibition of interactions between prostate cancer cell integrin $\alpha_v\beta_3$ and endothelial ICAM-1.
PCa
3
Simvastatin inhibits synthesis and secretion of prostate tumor cell-derived pro-angiogenic factors such as VEGF, Angiopoietin-2 and IGF-1, and enhances Angiopoietin-1 expression. Simvastatin thus protects the endothelial-barrier and inhibits prostate tumor cell micrometastsis.
2
Simvastatin directly activates the endothelial cells and enhances endothelial-barrier resistance, thus preventing micrometastasis (intravasation and extravasation) of prostate cancer cells.
PCa

CHAPTER 5:

SYNERGISTIC EFFECT OF SIMVASTATIN IN COMBINATION WITH DOCETAXEL ON THE GROWTH OF HUMAN PROSTATE CANCER CELLS *IN VITRO* AND TUMOR XENOGRAFT GROWTH *IN VIVO*

Belal Al-Husein, Anna Goc, Katerina Katsanevas, Uvette Lou, Somanath Shenoy

To be submitted to Journal of Pharmacology and Experimental Therapeutics

Abstract:

Prostate cancer is the second leading cause of death among cancer patients in the United States. Strategies should be implemented for the management of such a disease. Combination therapy is one of the basic strategies for the proper management of cancer patients. Clinical evidence is in favor of use of statins in combination with other chemotherapies.

Introduction

Prostate cancer is the second leading cause of death in the United States and the second leading killer among all cancers (154). Progression of this disease is an inevitable step during the course of cancer but it is not observed much in prostate cancer patients, as the incidence of this cancer is higher in older people with death preceding progression to an advanced stage. When metastasis occurs, chemotherapy is the treatment of choice. One basic strategy for the pharmacological management of cancer is the use of combination therapies. This strategy reduces the doses of agents used, thus minimizing their side effects. Another rationale for the use of combination chemotherapy is the reduction of incidence of resistance and enhancement of efficacy by using agents with different mechanisms of action. Table 5.1 summarizes basic principles of combination chemotherapy.

Table 5.1: Basic principles of the use of combination chemotherapy in cancer settings (adapted from (155) and (65)).
Drugs should be active alone for use in combinations
They should have a different mechanism of action with additive or synergistic effect
They should have different dose-limiting toxicities
They should be used at their optimal dose and schedule
They should be given at consistent intervals, with minimal time of treatment-free periods.
They should have different mechanisms of resistance
They could block development or mechanism of resistance of the other agent.

Statins in conjunction with other various chemotherapies have been studied on different types of cancers (Reviewed in (65)), and have shown promising effects by increasing the efficacy and reducing chemotherapy resistance developed by cancer cells. We previously showed that simvastatin has effects similar to docetaxel on prostate cancer (PC3 cells) colony formation (103). Also, we showed an additive/synergistic effect of simvastatin when combined with docetacxel in the induction of apoptosis (138). In the

following unpublished studies, we also observed synergistic effect of simvastatin in combination with docetaxel on prostate cancer cell migration. Furthermore, we also delineate below the effects of simvastatin on prostate tumor growth *in vivo*, in combination with docetaxel.

Materials and methods

Migration assay

PC3 cells were used to perform cell migration assay as previously described (Kouchu, 2011). Briefly, PC3 cells were grown to a monolayer, and then a scratch was made in the presence of 10% fetal bovine serum, docetaxel (25 nM), simvastatin (25 μM), and the combination docetaxel and simvastatin (25 nM and 25 μM consecutively). Experiments were performed in triplicates and data was analyzed using paired student t-test.

Tumor xenografts

Animal procedures were conducted in Charlie Norwood VA medical center and were compliant with the Institutional Animal Care and Use Committee (IACUC). Athymic nude mice (designation Hsd:Athymic Nude-*Foxn1*^{nu}, purchased from Harlan Laboratories) consisting of 5 mice at 6-7 weeks of age were used for each treatment group. PC3 cancer cells where grown according to protocols previously used(103). When a proper amount of cells was achieved, cells where detached using a 10 mM EDTA solution, and a total of $2x10^6$ PC3 tumor cells were reconstituted in 100 μl of PBS and were administered subcutaneously into the flank of mice (2 xenograft injections per mouse). Mice were treated with (1) control (5% DMSO solution/0.9% NaCl), (2) docetaxel (5 mg/kg administered 3 times per cycle and cycle is repeated every 14 days), and (3)

docetaxel + simvastatin (docetaxel as treatment group 2, simvastatin: 2 mg/kg twice daily started 3 days before tumor injection). Tumors were followed for 4 weeks. Animals were sacrificed and tumors were collected and either snap-frozen (with dry ice) for homogenization and WB, or fixed in 4% paraformaldehyde for immunohistochemistry.

Results

Simvastatin enhances the inhibitory effect of docetaxel on migration of PC3 cells

Figure 5.1 shows that the effect of simvastatin on migration at 16 hrs was more pronounced than docetaxel. At 24 hours, the effect was equal and significantly showing synergistic/additive effect of the combination.

Simvastatin enhances the tumor growth inhibitory effect of docetaxel on PC3 xenografts in nude mice

Figure 5.2A shows representative pictures of tumors isolated from mice for each treatment group. One mouse died from each treatment group. Figure 5.2B represents analysis of tumor weights normalized to average weights of control tumors. The figure shows that docetaxel produced a 55% reduction in tumor size compared to controls. On the other end, a combination of both docetaxel and simvastatin produced a 96% reduction in tumor size. The one animal that died in the docetaxel arm suffered from petechial hemorrhage in this animal that could be the cause of mortality. Although both the control and the combination arms had a single mortality, they were not associated with the skin lesions.

Discussion

Results show possible beneficial effect of the combination of simvastatin and docetaxel by increasing the efficacy on prostate cancer cells. Docetaxel's most common side

effects include myelosuppression and hypersensitivity (although hypersensitivity is predicted to be mediated by vehicle used (156)). Docetaxel requires premedication with corticosteroids but not antihistamines (as it is the case with paclitaxel which requires premedication with both corticosteroids and antihistamines). Simvastatin, with its known anti-inflammatory effect, have the potential to reduce the incidence of side effects of docetaxel. Simvastatin also has a different mechanism of action compared to docetaxel. Docetaxel works as a microtubule polymerization enhancer and can arrest cells in G2/M phase of cell division. This drug combination is predicted to produce synergistic/additive effect.

Conclusion

The combination of simvastatin and docetaxel seems to be appealing for the management of prostate cancer. Both agents individually showed chemotherapeutic effect on prostate cancer cells. Further analysis of tumors acquired from this work is necessary to determine the exact effects of this combination on prostate cancer cells.

Legends for figures

Figure 5.1: Simvastatin combination with docetaxel showed synergistic/additive effect on reduction of migration of PC3 cancer cells.

Figure 5.2: Simvastatin combination with docetaxel showed synergistic effect on the inhibition of growth of tumor xenografts *in vivo*: A) Representative pictures showing a reduction in tumor size with combination therapy. B) Analysis of tumor weights from animals of the three treatment groups.

Figure 5.1

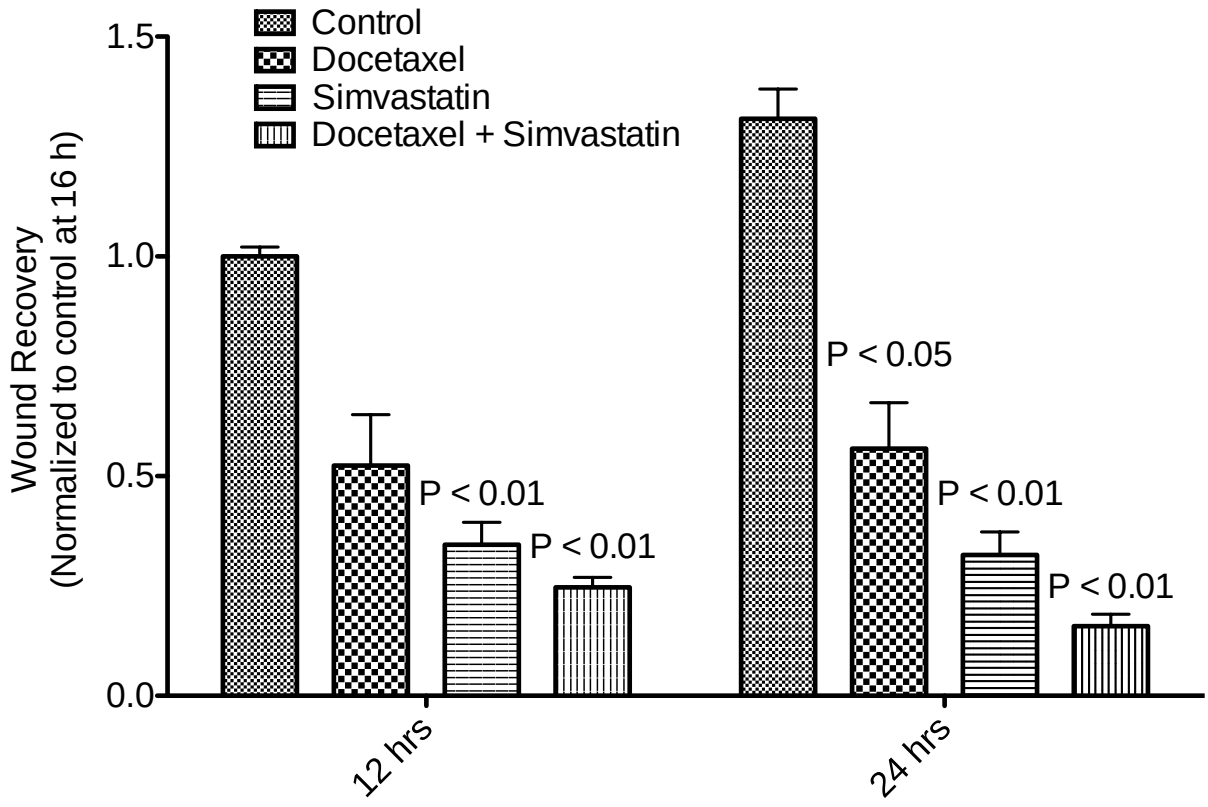
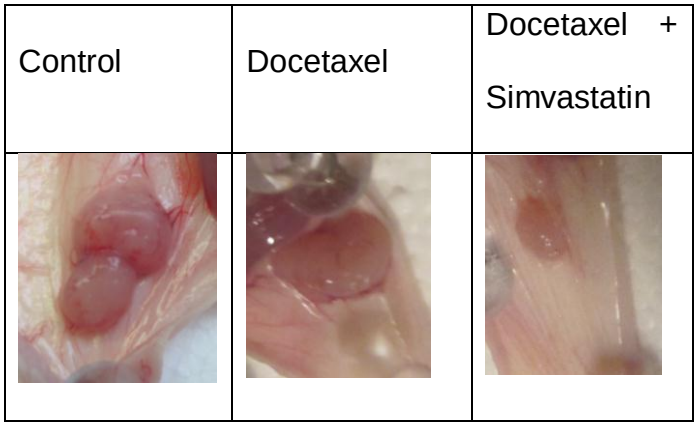
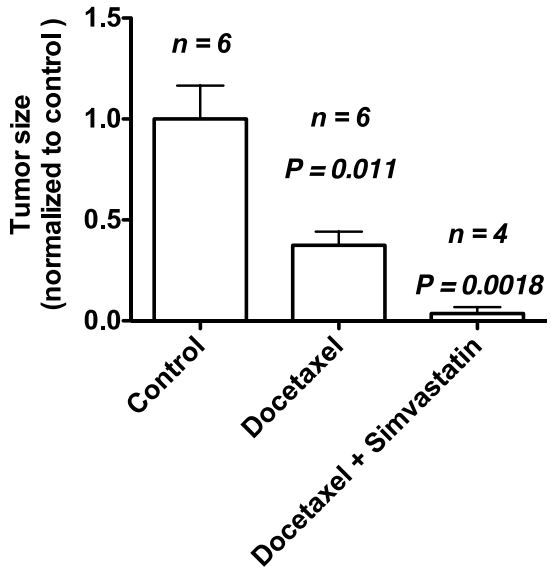

Control
Docetaxel
Simvastatin
Docetaxel + Simvastatin
Wound Recovery
(Normalized to control at 16 h)
1.5
1.0
0.5
0.0
P < 0.01
P < 0.01
P < 0.05
P < 0.01
P < 0.01
12 hrs
24 hrs

Figure 5.2

A


Control
Docetaxel
Docetaxel + Simvastatin

B


Tumor size (normalized to control)
1.5
1.0
0.5
0.0
n = 6
n = 6
P = 0.011
n = 4
P = 0.0018
Control
Docetaxel
Docetaxel + Simvastatin

CHAPTER 6:

SUMMARY, CONCLUSION AND TRANSLATIONAL IMPACT

From our literature review, the main body of evidence supports the benefit of a lipophilic and highly potent statin, simvastatin, on prostate cancer progression and to a lesser extent on its incidence and development. The main findings in our research are summarized as follows: (1) simvastatin inhibits growth of human prostate cancer cells *in vitro* and *in vivo* in a xenograft model and (2) simvastatin influences metastasis of human prostate cancer cells by inhibiting secretion of factors that interfere with their adhesion to endothelial cells while not negatively influencing endothelial cells. Simvastatin proved to be effective in the inhibition of Akt activity in both androgen-dependent and independent human prostate cancer cells *in vitro*. Akt inhibition is a major survival pathway for prostate cancer cells. *In vivo*, androgen independent growth in nude mice was inhibited by a dose of simvastatin that translates to 5 times the levels used for *de novo* cholesterol synthesis inhibition.

Next, we studied the effects of simvastatin on transendothelial migration (micrometastasis) of prostate cancer cells. Simvastatin was capable of inhibiting the expression of a few major mediators of prostate cancer transendothelial migration and tumor angiogenesis. Simvastatin also inhibited the ability of prostate cancer cells to adhere to components of endothelial cells and ECM. The effect of simvastatin on cancer cells was mediated through impaired inside-out signaling (affinity modulation) of integrin αvβ3. Simvastatin also reduced the effect of tumor cell conditioned media on endothelial

cells. Gene array analysis revealed many changes induced by simvastatin treatment on tumor cells. Simvastatin reduced mRNA levels of Akt1, IFN-β1, IGF-1, integrin α4, integrin β3, integrin β5, MMP-2, PDGF-B and VEGFA, and increased levels of angiopoietin-1.

In conclusion, our findings provide new insight supporting the use of statins, simvastatin in particular, for the management of prostate cancer. Given the combined effect of simvastatin and docetaxel on prostate cancer cells, this might support the use of simvastatin in combination with other chemotherapies for the management of cancer. Keeping in mind that cancers have an immune component and simvastatin also has anti-inflammatory effects, use of statins would have profound effects if used in the proper dose and frequency. Also, some side effects of anticancer drugs might have an inflammatory component, which can be reduced by statins. A recent study (157) showed the ability of lovastatin, a statin, to reduce hepatotoxicity induced by doxorubicin, an anthracyclin chemotherapeutic agent.

Translational impact

Drug repurposing has recently gained interest in the world of therapeutics. Drugs with pleiotropic effects, such as statins, are good candidates for drug repurposing. Simvastatin is a relatively old drug with known profile of side effects. Repurposing such a drug for use in cancer has many benefits. Simvastatin is a generic drug now and would have less cost for patients. In addition, simvastatin is a safe drug, even with the high doses being used for cancer treatment. A recent clinical trial showed that high doses of simvastatin did not have an increase in side effects compared to control.

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biologiia. 2003 Jan-Feb;37(1):97-111. PubMed PMID: 12624952. Epub 2003/03/11. Profili ekspressii proteinkinaz i fosfataz, poluchennye metodom gibridizatsii s uporiadochennymi naborami kDNK (cDNA arrays): molekuliarnyi portret raka predstatel'noi zhelezy cheloveka. rus.

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APPENDIX

Cover page of the following manuscripts:

1. P21 activated kinase-1 (Pak1) promotes prostate tumor growth and microinvasion via inhibition of TGFβ expression and enhanced MMP9 secretion. Goc A, Al-Azayzih A, Abdalla M, Al-Husein B, Kavuri S, Lee J, Moses K, Somanath PR. J Biol Chem. 2012 Dec 20. PMID: 23258534
2. Antiangiogenic therapy for cancer: an update. Al-Husein B, Abdalla M, Trepte M, Deremer DL, Somanath PR. Pharmacotherapy. 2012 Dec;32(12):1095-111. PMID: 23208836
3. Simultaneous modulation of the intrinsic and extrinsic pathways by simvastatin in mediating prostate cancer cell apoptosis. Goc A, Kochuparambil ST, Al-Husein B, Al-Azayzih A, Mohammad S, Somanath PR. BMC Cancer. 2012 Sep 14;12:409. PMID: 22974127
4. PI3 kinase integrates Akt and MAP kinase signaling pathways in the regulation of prostate cancer. Goc A, Al-Husein B, Kochuparambil ST, Liu J, Heston WW, Somanath PR. Int J Oncol. 2011 Jan;38(1):267-77. PMID: 21109949
5. Anticancer efficacy of simvastatin on prostate cancer cells and tumor xenografts is associated with inhibition of Akt and reduced prostate-specific antigen expression. Kochuparambil ST, Al-Husein B, Goc A, Soliman S, Somanath PR. J Pharmacol Exp Ther. 2011 Feb;336(2):496-505. PMID: 21059805

6. PAK1 as a therapeutic target. Kichina JV, Goc A, Al-Husein B, Somanath PR, Kandel ES. Expert Opin Ther Targets. 2010 Jul;14(7):703-25. PMID: 20507214

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 5, pp. 3025–3035, February 1, 2013

P21 Activated Kinase-1 (Pak1) Promotes Prostate Tumor Growth and Microinvasion via Inhibition of Transforming Growth Factor β Expression and Enhanced Matrix Metalloproteinase 9 Secretion*

Received for publication, October 2, 2012, and in revised form, December 20, 2012 Published, JBC Papers in Press, December 20, 2012, DOI 10.1074/jbc.M112.424770

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Background: The significance of Pak1 in prostate cancer remains unclear.
Results: Pak1 knockdown impaired prostate tumor growth via increased expression of TGFβ and reduced secretion of MMP9.
Conclusions: We demonstrated that Pak1 is a more potent mediator of prostate cancer cell migration and tumor growth than Pak6, the predominant isoform in the prostate.
Significance: A novel role of Pak1 in prostate cancer is identified.

P21-activated kinases (Paks) are major effectors downstream of the small Rho family of GTPases. Among the six isoforms, Pak1 is the most ubiquitous and the best characterized member. Previous studies have shown that inhibition of Pak6, which is predominantly present in the prostate compared with other tissues, inhibits prostate tumor growth *in vivo*. Even though Pak1 has been identified in normal prostatic epithelial cells and cancer cells, its specific role in the development of prostate cancer remains unclear. We report here that highly invasive prostate cancer cells express significantly higher levels of Pak1 protein compared with non-invasive prostate cancer cells. Furthermore, prostate tumor tissues and prostate cancer metastasized to lungs showed a higher expression of Pak1 compared with normal tissues. Interestingly, Pak6 protein expression levels did not change with the invasive/metastatic potential of the cancer cells or tumors. Although inhibition of Pak1, and not Pak6, resulted in impaired PC3 cell migration, the effects of Pak1 knockdown on transendothelial migration (microinvasion), tumor growth, and tumor angiogenesis was higher compared with Pak6 knockdown. Finally, gene array data revealed reduced expression of matrix metalloproteinase 9 with the ablation of either Pak1 or Pak6 gene expression in PC3 cells, whereas protein levels of TGFβ was elevated significantly with specific modulation of Pak1 activity or ablation of the Pak1 gene. Our observations suggest that although some level of functional redundancy exists between Pak1 and Pak6 in prostate cancer cells, targeting Pak1 is a potential option for the management of prostate tumor growth, microinvasion, and metastasis.

P21-activated kinases (Paks)[2] are a family of six serine-threonine kinases that are categorized into group I and group II Paks on the basis of their mechanism of activation (1). Group I Paks differ from their group II counterparts on their activation by small Rho GTPases such as Rac and cdc42 (2) as well as their specific involvement in inducing cytoskeletal changes and lamellipodia and filopodia formation in mammalian cells in the promotion of cell motility (1, 3). Group II Paks lack the auto inhibitory domain, acidic and Pix-binding regions, as well as the cdc42/Rac interacting binding (CRIB) domain, which are present in all group I Pak isoforms (4–6).

Among the Pak isoforms, group I Paks (Pak1 and Pak2 in particular) are the best characterized and most deregulated in cancers (1, 3). We have shown previously that Pak1 is necessary for inducing cytoskeletal changes in normal cells (7) and in reconciling the effects of the Akt and ERK pathways, two major pathways deregulated in multiple cancers, thus mediating oncogenic transformation (8). Although present in the prostate, group I Paks are the least studied in prostate cancer, probably because the prostate is known for its higher expression of group II Paks, Pak4 and Pak6 (9, 10), which are less expressed and less known for cancer incidences in other tissues. In support of this assumption, inhibition of either Pak4 or Pak6 has been reported to inhibit prostate cancer cell function *in vitro* and tumor growth *in vivo* (11, 12). Our recent study has demonstrated that activation of Rac1 driven by the dimerization of protein 14-3-3ζ induces cytoskeletal changes in prostate cancer

* This work was supported, in whole or in part, by National Institutes of Health Grant R01HL103952 (to P. R. S.). This work was also supported by the University of Georgia Research Foundation, by the Wilson Pharmacy Foundation, and by the University of Georgia College of Pharmacy through intramural grants (to P. R. S.) and by American Heart Association Scientist Development Grant 0830326N (to P. R. S.).

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[2] The abbreviations used are: Pak, p21-activated kinase; MMP, matrix metalloproteinase; ECM, extracellular matrix; ECIS, electric cell substrate impedance sensing.

Antiangiogenic Therapy for Cancer: An Update

Belal Al-Husein, M.S., Maha Abdalla, Pharm.D., Morgan Trepte, Pharm.D., David L. DeRemer, Pharm.D., and Payaningal R. Somanath, Ph.D.

The idea of antiangiogenic therapy was the brainchild of Dr. Judah Folkman in the early 1970s. He proposed that by cutting off the blood supply, cancer cells would be deprived of nutrients and, hence, treated. His efforts paid off when bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor, was approved as antiangiogenic therapy in 2004 for the treatment of colon cancer. Since then, an array of antiangiogenic inhibitors, either as monotherapy or in combination with other cytotoxic and chemotherapy drugs, have been developed, used in clinical trials, and approved for the treatment of cancer. Despite this important breakthrough, antiangiogenic therapy for cancer met with a number of hurdles on its way to becoming an option for cancer therapy. In this article, we summarize the most current information on the mechanisms of tumor angiogenesis, proangiogenic and antiangiogenic factors, potential targets and their mechanisms of action, and experimental evidences, as well as the most recent clinical trial data on antiangiogenic agents for cancer therapy.

Key Words: tumor angiogenesis, bevacizumab, antiangiogenic therapy, cancer.
(Pharmacotherapy 2012;32(12):1095–1111)

Traditional chemotherapeutic agents are limited by their narrow therapeutic index due to their lack of specificity, resulting in damage to both cancerous and normal cells; severe adverse effects; variable dosing regimens; and the development of drug resistance with subsequent disease relapse. In the 1970s, Dr. Judah Folkman observed that in the absence of neovascularization, tumors cannot grow more than 2–3 mm,[1] and this gave rise to the field of angiogenesis and laid the foundation for antiangiogenic cancer therapy.[2, 3] In 1971, Dr. Folkman proposed the concept of antiangiogenesis as a modality in cancer therapy to prevent "new vessel sprouts from penetrating into early tumor implant."[1] It was not until 2004 that the first antiangiogenic drug, bevacizumab, was approved by the United States Food and Drug Administration (FDA) for the management of advanced colon cancer.[4] In this review, we will discuss pathways regulating tumor angiogenesis, identify potential therapeutic targets for antiangiogenic cancer therapy, and provide an update on the various clinical trials evaluating this therapy.

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Dr. Somanath received support from the University of Georgia (UGA) Research Foundation, Wilson Pharmacy Foundation of the UGA College of Pharmacy, and the Department of Clinical and Administrative Pharmacy through intramural grants. Dr. Somanath also received funding from a National Institutes of Health grant (R01HL103952) and an American Heart Association Scientist Development grant (0830326N).

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Tumor Angiogenesis

Angiogenesis is the process of forming blood vessels from preexisting ones, unlike neovascularization,

Goc et al. BMC Cancer 2012, 12:409
http://www.biomedcentral.com/1471-2407/12/409

BMC Cancer

RESEARCH ARTICLE Open Access

Simultaneous modulation of the intrinsic and extrinsic pathways by simvastatin in mediating prostate cancer cell apoptosis

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Abstract

Background: Recent studies suggest the potential benefits of statins as anti-cancer agents. Mechanisms by which statins induce apoptosis in cancer cells are not clear. We previously showed that simvastatin inhibit prostate cancer cell functions and tumor growth. Molecular mechanisms by which simvastatin induce apoptosis in prostate cancer cells is not completely understood.

Methods: Effect of simvastatin on PC3 cell apoptosis was compared with docetaxel using apoptosis, TUNEL and trypan blue viability assays. Protein expression of major candidates of the intrinsic pathway downstream of simvastatin-mediated Akt inactivation was analyzed. Gene arrays and western analysis of PC3 cells and tumor lysates were performed to identify the candidate genes mediating extrinsic apoptosis pathway by simvastatin.

Results: Data indicated that simvastatin inhibited intrinsic cell survival pathway in PC3 cells by enhancing phosphorylation of Bad, reducing the protein expression of Bcl-2, Bcl-xL and cleaved caspases 9/3. Over-expression of PC3 cells with Bcl-2 or DN-caspase 9 did not rescue the simvastatin-induced apoptosis. Simvastatin treatment resulted in increased mRNA and protein expression of molecules such as TNF, Fas-L, Traf1 and cleaved caspase 8, major mediators of intrinsic apoptosis pathway and reduced protein levels of pro-survival genes Lhx4 and Nme5.

Conclusions: Our study provides the first report that simvastatin simultaneously modulates intrinsic and extrinsic pathways in the regulation of prostate cancer cell apoptosis *in vitro* and *in vivo*, and render reasonable optimism that statins could become an attractive anti-cancer agent.

Keywords: Prostate cancer, Simvastatin, Docetaxel, Apoptosis, Bcl-2, Fas-L

Background

Statins, the cholesterol lowering drugs, are some of the most commonly prescribed medications. Recently, attention has focused on the development of statins as therapeutic agents for the treatment of solid and hematological cancers [1]. Statins elicit pleiotropic effects on various cell types and differentially modulate cellular functions such as cell migration, proliferation, cell survival and apoptosis in normal and malignant cells [2]. Lipophilicity, dose and duration of the treatment as well as cell type are all determining factors on the specific effect of a statin on the outcome of a cell function. According to the American Cancer Society, prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in American men. Many recent clinical studies have indicated that use of statins is associated with >50% reduction in prostate cancer deaths [3,4]. Our previous study showed that simvastatin, a lipophilic statin inhibited multiple prostate cancer cell functions *in vitro* such as migration, proliferation, cell survival and colony formation as well as tumor growth in a nude mouse xenograft *in vivo*, mainly via inhibition of Akt pathway [5]. However, exact molecular mechanisms by which statins modulate each of the prostate cancer cell function are not clear.

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PI3 kinase integrates Akt and MAP kinase signaling pathways in the regulation of prostate cancer

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DOI: 10.3892/ijo_xxxxxxxx

Abstract. PI3 kinase (PI3K), Akt and MAP kinase (MAPK) pathways are central to many classical signaling cascades and are often de-regulated in many cancers. Due to this, inhibitors for a number of key signaling molecules in these pathways such as PI3K, Akt, mTOR, Raf and ERK are currently in clinical trials. In the current study, we investigated the effects of specific inhibition of these signaling molecules, alone or in combinations, on prostate cancer cells. Our study showed that integration of Akt-mTOR and MAPK signaling by PI3K was essential for the EGF-stimulated TRAMP cell migration, proliferation, survival and invasion as well as PC3 and LNCaP C4-2 (C4-2) colony/foci formation. Adenovirus-mediated expression of constitutively active Akt (Ad-myrAkt) in PC3 cells resulted in significant increase in number of foci. Even though PI3K inhibition significantly reduced foci formed by C4-2 cells, none of the Akt, ERK or mTOR inhibitors showed any significant inhibition. This indicated that functional redundancies and/or feed back loops between Akt-mTOR and MAPK signaling exist in prostate cancer. Further studies on cotargeting these signaling molecules revealed that combined inhibition of Akt (or mTOR) and ERK, but not Akt and mTOR, resulted in significant reduction in number of foci formed by the C4-2 cells. Overall, our study demonstrated that the effects of PI3K-mediated prostate cancer growth necessitates a synergism between the Akt and MAPK pathways and suggests cotargeting Akt (or mTOR) and MAPK as an effective method for prostate cancer therapeutic interventions.

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Abbreviations: BrDU, 5-bromo-2-deoxyuridine; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; PI3K, PI3 kinase; mTOR, mammalian target of rapamycin; PAGE, polyacrylamide gel electrophoresis; TRAMP, transgenic adenocarcinoma of the mouse prostate; ERK, extracellular regulated kinase; C4-2, LNCaP C4-2; RTK, receptor tyrosine kinase

Key words: PI3 kinase, Akt, mammalian target of rapamycin, mitogen-activated protein kinase, prostate cancer

Introduction

Age-related epithelial cancers such as prostate cancer are often caused due to somatic mutations in cell signaling molecules (1). The occurrence of androgen-insensitive prostate cancers, often due to the resistance developed by activating mutations in many signaling molecules, makes it even more difficult to treat these cancers using conventional androgen therapy (2). Research has for a long time been focused on targeting growth factors (e.g. Bevacizumab), receptor tyrosine kinases (RTKs) (e.g. Sorafenib) and integrins (e.g. Cilengitide) (3) for treating many cancers. Many of these trials demonstrated development of tumor resistance to therapy (4). Hence, attention has recently shifted to use of intra-cellular signaling molecules to target for cancer therapy.

Mutations in oncogenes such as Ras (5), Src (6), Raf (7) and myc (8). have been linked to oncogenic transformation, tumor progression and metastasis. De-regulation of one or more of these signaling pathways is always associated with tumor growth and metastasis (1). The loss of the tumor suppressor gene encoding phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is common in prostate cancer (9,10). Loss of PTEN activity, in turn, results in the activation of many signaling pathways that are deregulated during prostate cancer that include classical PI3 kinase (PI3K)-Akt (10,11) and MAP kinase (MAPK) cascades (12). Because of this significance, there is considerable interest in the importance of downstream signaling components of PTEN, mainly the PI3K/Akt/mTOR and MAPK pathways in developing therapeutics for prostate cancer.

PTEN functions as a lipid phosphatase that is responsible for the dephosphorylation of phosphatidylinositol triphosphate (PIP3), a second messenger of PI3K at the plasma membrane (13,14). In the absence of PTEN activity, intracellular PIP3 levels will be elevated due to the conversion of

0022-3565/11/3362-496–505
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 336:496–505, 2011

Vol. 336, No. 2
174870/3660086
Printed in U.S.A.

Anticancer Efficacy of Simvastatin on Prostate Cancer Cells and Tumor Xenografts Is Associated with Inhibition of Akt and Reduced Prostate-Specific Antigen Expression

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Received September 7, 2010; accepted November 5, 2010

ABSTRACT

Prostate cancer is the second-leading cause of cancer-associated death among men in the United States. There has been renewed interest in the potential therapeutic benefits of statins for cancer. Simvastatin, a widely used generic drug for preventing cardiovascular events, is well known for its effects on cellular proliferation and inflammation, two key processes that also determine the rate of tumor growth. Although a growing body of evidence suggests that statins have the potential to reduce the risk of many cancers, there are discrepancies over the pro- and anticancer effects of statins. In the current study, we sought to investigate the effects of simvastatin on the Akt pathway in prostate cancer cells with respect to the regulation of various cell functions in vitro and tumor growth in vivo. Time- and dose-dependent effects of simvastatin on LNCaP (androgen-dependent) and PC3 (androgen-independent) cells indicate that treatment with simvastatin at concentrations as low as 25 μM was sufficient to inhibit serum-stimulated Akt activity. Akin to this, treatment with simvastatin significantly inhibited serum-induced cell migration, invasion, colony formation, and proliferation. Simvastatin-mediated effects on colony formation were rescued by adenovirus-mediated expression of constitutively active Akt (myristoylated Akt) in PC3 cell lines. A PC3 xenograft model performed in nude mice exhibited reduced tumor growth with simvastatin treatment associated with decreased Akt activity and reduced prostate-specific antigen (PSA) levels. Our findings demonstrate the therapeutic benefits of simvastatin for prostate cancer and suggest a link between simvastatin, regulation of Akt activity, and PSA expression in prostate tumors.

Introduction

Statins [3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase inhibitors], the second-most prescribed drugs after analgesics, are also considered to be among the safest drugs. Despite the long-term nature of the treatments, the use of statins has not been shown to inflict any serious side effects; instead, it has been shown to yield additional benefits, particularly in the management of cancer. A recent meta-analysis performed using the information retrieved from the QResearch database indicates that the use of statins is not associated with a risk of diseases such as Parkinson's disease, rheumatoid arthritis, venous thromboembolism, dementia, osteoporosis, or cancers of the stomach, colon, lung, skin, kidney, breast, or prostate (Hippisley-Cox and Coupland, 2010). However, moderate increases in the risk of liver or kidney dysfunction, myopathy, and cataracts were associated with statin use. In humans, reports on the effects of statins on cancer have yielded varied results ranging from increased risk, to no net effect, to decreased risk of cancer (Jakobisiak and Golab, 2010). Many believe that these differences could be due to either variations in the doses used for the treatment of many cardiovascular conditions (Elewa et al., 2010) or the hydrophobic nature of some, but not all, statins (Murtola et al., 2008). A number of preclinical studies have implicated that statins can modulate the efficacy of many antitumor therapeutic modalities (Jakobisiak and Golab, 2010).

This research was funded by the University of Georgia Research Foundation (to P.R.S.).

S.T.K. and B.A.-H. contributed equally to this work.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.110.174870.

ABBREVIATIONS: PSA, prostate-specific antigen; myrAkt, myristoylated Akt; DMEM, Dulbecco's modified Eagle's medium; GSK, glycogen synthase kinase; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; BrdU, 5-bromo-2-deoxyuridine, bromodeoxyuridine; EGF, epidermal growth factor; GFP, green fluorescent protein.

Expert Opinion

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PAK1 as a therapeutic target

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Importance of the field: P21-activated kinases (PAKs) are involved in multiple signal transduction pathways in mammalian cells. PAKs, and PAK1 in particular, play a role in such disorders as cancer, mental retardation and allergy. Cell motility, survival and proliferation, the organization and function of cytoskeleton and extracellular matrix, transcription and translation are among the processes affected by PAK1.

Areas covered in this review: We discuss the mechanisms that control PAK1 activity, its involvement in physiological and pathophysiological processes, the benefits and the drawbacks of the current tools to regulate PAK1 activity, the evidence that suggests PAK1 as a therapeutic target and the likely directions of future research.

What the reader will gain: The reader will gain a better knowledge and understanding of the areas described above.

Take home message: PAK1 is a promising therapeutic target in cancer and allergen-induced disorders. Its suitability as a target in vascular, neurological and infectious diseases remains ambiguous. Further advancement of this field requires progress on such issues as the development of specific and clinically acceptable inhibitors, the choice between targeting one or multiple PAK isoforms, elucidation of the individual roles of PAK1 targets and the mechanisms that may circumvent inhibition of PAK1.

Keywords: allergy, angiogenesis, cancer, p21-activated kinases, Rho GTPases, signal transduction

Expert Opin. Ther. Targets [Early Online]

1. Introduction

In the early-1990s, Manser and co-workers made the seminal observation that specific downstream effects of a group of Rho GTPases (P21) in rat brain cytosol is mediated through a p21-activated kinase (PAK) [1]. In a gel-overlay assay, these researchers identified three proteins, of 68, 65 and 62 kDa, in a specific screen designed for identifying the binding partners of Rho GTPases. From subsequent studies, these three proteins were identified to be the members of the group I PAK family: PAK1, PAK2 and PAK3 that are activated by GTP-bound, but not GDP-bound, Rac and cdc42 [2-4]. With this discovery, a new area of research on serine–threonine kinase PAK was originated, that later was found to be important for many physiological function and as a major underlying cause of many pathological conditions. The structure, substrate specificity and functional role of group I PAKs have been evolutionarily conserved right from the protozoa to yeast and mammals [5]. PAKs are important for a variety of cellular functions such as cytoskeletal remodeling, focal adhesion assembly, cell migration [6], survival [7] and mitosis [8] as well as transcriptional regulation and protein synthesis involving ERK and NF-κB pathways [9,10]. Currently, PAKs are among the best characterized downstream effectors of the Rho family GTPases Rac and cdc42 in the regulation of lamellipodia and filopodia formation, respectively [6,11]. Furthermore, deregulation of PAK activity has been linked to a variety of cancers [12]. In addition to Rac and

10.1517/14728222.2010.492779 © 2010 Informa UK Ltd ISSN 1472-8222