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Research Article

Anti-Androgen Abiraterone Acetate Improves the Therapeutic Efficacy of Statins on Castration-Resistant Prostate Cancer Cells

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Abstract

The treatment of castration-resistant (CR) prostate cancer (PCa) is limited. A sub-population of CR PCa tumors can synthesize androgens for intracrine androgen receptor (AR) activation, thus targeting androgen biosynthesis could be an effective therapeutic option for these patients. We determined that androgen biosynthesis inhibitors simvastatin, atorvastatin, and ketoconazole directly inhibit growth, migration, and colony formation of LNCaP C-81 cells, which exhibit *de novo* androgen biosynthesis, with simvastatin being the most effective. Importantly, in combination treatments, statins specifically enhanced growth suppression with added effects by anti-androgen abiraterone acetate on the CR PCa cells. Thus, statins can be used in conjunction with abiraterone acetate to enhance anti-androgen therapy for CR PCa.

Keywords: Anti-Androgens; Combination Treatments; Castration-Resistant Prostate Cancer; Prostate Cancer; Statins

Abbreviations:

AA	:	Abiraterone Acetate	CR	:	Castration-Resistant
Ab	:	Antibody	Cas	:	Casodex
AI	:	Androgen-Independent	DHEAS	:	Dehydroepiandrosterone Sulfate
ADT	:	Androgen Deprivation Therapy	DHT	:	α-Dihydrotestosterone
AR	:	Androgen Receptor	DMSO	:	Dimethyl Sulfoxide
AS	:	Androgen-Sensitive	ECL	:	Enhanced Chemiluminescence
ATCC	:	American Type Culture Collection	HMG CoA	:	3-hydroxy-3-methylglutaryl coenzyme A
			HMGCR	:	HMG CoA reductase
			EDTA	:	Ethylenediaminetetraacetic Acid
			Enz	:	Enzalutamide

FBS	:	Fetal Bovine Serum
NSAIDS	:	Nonsteroidal Anti-Inflammatory Drugs
PCa	:	Prostate Cancer
PCNA	:	Proliferating Cell Nuclear Antigen
PSA	:	Prostate-Specific Antigen
SR	:	Steroid-Reduced
TBS	:	Tris-Buffered Saline

Introduction

Prostate cancer (PCa) is the most common malignancy and the third leading cause of cancer-related death in men in the United States [1]. Metastatic PCa initially responds to androgen deprivation therapy (ADT); however, such treatment is not curative, often resulting in relapse and progression to the castration-resistant (CR) stage. Despite rapid advances in the treatments of many other advanced stage cancers, the efficacy of chemotherapy toward CR PCa is still limited. The search for novel strategies and regimens to treat CR PCa continues and remains a significant challenge.

While PCa cells can progress to the CR phenotype under ADT, those cells still require a functional androgen receptor (AR) for survival, and thus inhibition of AR remains the most common strategy in treating advanced PCa [2]. There are multiple mechanisms by which CR PCa cells can survive in androgen-deprived environments, for example, through elevated levels of AR protein and/or mutations in the AR gene, including constitutively active AR spliced variants [3-5].

It is also possible that CR PCa cells obtain an intracrine mechanism to synthesize endogenous androgens for AR activation, despite an anorchid serum concentration of androgens. Results of analyses on CR PCa tumors reveal that over 50% CR PCa tumors express enzymes that are essential for androgen biosynthesis [6] and thus exhibit intracrine regulation of AR activation. Interestingly, LNCaP C-81 PCa cells exhibit many biochemical properties of clinical CR PCa, such as being AR-positive, androgen-independent (AI), having androgen-responsive proliferation, secreting prostate-specific antigen (PSA) under steroid-reduced (SR) conditions, and developing xenograft tumors in female mice with low levels of circulating androgens. Further, AI LNCaP C-81 cells, but not its counterpart androgen-sensitive (AS) LNCaP C-33 cells, express all functional enzymes necessary for endogenous testosterone biosynthesis which results in intracrine regulation by hyper-activated AR [7]. Similarly, VCaP-AI PCa cell lines have been derived from AS counterparts, which also express AR and proliferate under SR conditions (Figure S1), and mimic the progression of PCa from AS to the AI phenotype. Thus, LNCaP C-81 and VCaP-AI cells are useful cell models of CR PCa that demonstrate clinical PCa progression.

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis pathway. These cholesterol-lowering drugs are among the most commonly prescribed medications in the United States for treatment of hypercholesterolemia or prevention of cardiovascular disease [8]. Moreover, cholesterol is an important metabolite involved in many cellular functions, including production of steroid hormones, cell membrane maintenance, and lipid raft formation. Observational case-control and cohort studies on statin drugs show a corresponding decreased incidence of many cancers, including PCa [9-15]. Furthermore, many reports have cited an association between the statin usage and a decreased risk of advanced stage PCa development [9,10,12,16-19] and/or an increased survival rate [16,20-22]. On the contrary, some studies show no benefits relating to cancer development or survival with statin usage [23-25]. There are also several PCa cell biology studies that have shown reduced PCa cell growth upon inhibition of cholesterol synthesis via statins [26] or a combination of statins and nonsteroidal anti-inflammatory drugs (NSAIDs) [27]. Additionally, Harshman et al. determined that statins reduce the uptake of an androgen precursor dehydroepiandrosterone sulfate (DHEAS) as a mechanism for the prolonged progression seen in PCa patients on statins [28]. However, the cell lines utilized in several of these reports may not be representative of the major CR PCa because of their AR expression, androgen independence, and, especially, intracrine regulation. Therefore, the clinical relevance of the data generated with those cell lines remains limited. Ketoconazole is another commonly prescribed drug primarily utilized as an antifungal agent via the inhibition of P450 cholesterol side chain cleavage enzyme involved in ergosterol biosynthesis. This enzyme is also utilized to synthesize the steroid precursor pregnenolone from cholesterol. A phase II study has demonstrated that ketoconazole treatment prolonged cancer progression in CR PCa patients [29].

Upon development of CR PCa, patients have few therapeutic options. Secondary hormonal therapies, such as anti-androgens, are often utilized as PCa tumors still largely rely on androgens for survival [30]. There are two classes of anti-androgens, which include androgen biosynthesis inhibitors, such as abiraterone acetate [31], and androgen receptor blockers, such as casodex and enzalutamide [32]. Both classes of anti-androgens lead to inhibition of the AR signaling pathway, thus inhibiting PCa growth and providing a survival advantage in both post-docetaxel and chemotherapy-naïve patients [31,32]. However, anti-androgens are not a solution to CR PCa [33]. Thus, effective therapies toward CR PCa are immediately needed.

In this study, we investigated whether statins and anti-androgens could directly inhibit CR PCa cell growth, utilizing the LNCaP C-81 cell line for its ability of endogenous testosterone biosynthesis and intracrine regulation, as seen in clinical CR

PCa [7,34]. To determine the efficacy of these compounds under ADT conditions, these compounds were analyzed in SR medium. We examined the effects of statins and ketoconazole upon PCa tumorigenicity, including cell migration and colony formation. We further examined combinational therapy of statins and anti-androgens. Interestingly, our results revealed that statins specifically enhance anti-androgen abiraterone acetate (AA) treatment of CR PCa, thus providing evidence that this combination can benefit CR PCa patients.

Materials and Methods

Materials

Fetal bovine serum (FBS), gentamicin, L-glutamine, DMEM medium, and RPMI 1640 medium were obtained from Invitrogen (Carlsbad, CA, USA). Charcoal/dextran-treated FBS was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). Molecular biology-grade agarose was procured from Fisher Biotech (Fair Lawn, NJ, USA). Anti-β-actin antibody (Ab), 5α-Dihydrotestosterone (DHT), SIGMAFAST protease inhibitor, simvastatin, and ketoconazole were purchased from Sigma (St Louis, MO, USA). Atorvastatin was obtained from LC Laboratories (Woburn, MA, USA). Anti-PCNA (#G261, 1:3000), anti-BAX (#G241, 1:1000), anti-AR (#C1411, 1:3000), anti-BclXL (#F111, 1:1000), anti-Survivin (#C271, 1:1000), horseradish peroxidase-conjugated anti-mouse (#C2011, 1:5000), anti-rabbit (#D2910, 1:5000), and anti-goat (#J0608, 1:5000) IgG Abs were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-AKT (pSer473) (#GA160, 1:1000), anti-AKT (#C1411, 1:2000), anti-Caspase 3 (#9665S, 1:1000), and anti-PARP (#9532S, 1:1000) Abs were purchased from Cell Signaling Technology (Beverly, MA, USA). Enzalutamide was purchased from Medchem Express (Monmouth Junction, NJ, USA). Casodex was obtained from Astra Zeneca (Macclesfield, Cheshire, England). Abiraterone Acetate from was obtained from Janssen (Beerse, Belgium).

Cell Culture

Human LNCaP and VCaP PCa cell lines were originally purchased from the American Type Culture Collection (ATCC) and maintained according to ATCC guidelines (Rockville, MD, USA). LNCaP cells were routinely maintained in regular steroid-containing medium, i.e., phenol red-positive RPMI 1640 medium supplemented with 5% FBS (v/v), 2 mM glutamine, and 50 μg/ml gentamicin [35,36]. VCaP cells were maintained in DMEM medium containing 15% FBS, 2 mM glutamine, 10 nM DHT and 50 μg/ml gentamicin. LNCaP cells at passage 33 or below were labeled as LNCaP C-33 cells, while LNCaP cells at passage 81 or above were labeled as LNCaP C-81 cells [35,36]. LNCaP C-33 cells are AS PCa cells, while LNCaP C-81 cells exhibit biochemical properties similar to CR PCa, including AI proliferation, PSA secretion in SR conditions, and importantly, they obtained intracrine regulation by synthesizing endogenous testosterone from cholesterol with activated AR [7]. Similarly, upon passage, VCaP cells with passage number higher than 90 obtained the AI phenotype including rapid cell proliferation in steroid-reduced media (Figure S1).

For all experiments, LNCaP C-33, LNCaP C-81, or VCaP-AI cells were seeded in regular steroid-containing medium and allowed to attach for 72 h. For experiments in SR conditions, the attached cells were then maintained in SR medium, i.e., phenol red-free RPMI 1640 medium containing 5% charcoal/dextran-treated FBS (cFBS) (v/v), 2 mM glutamine, gentamicin (50 μg/ml). To mimic the environment of ADT, 1 nM DHT was supplemented [37,38]. Cells were maintained for 48 h in SR medium before being exposed to the inhibitors in SR conditions.

Cell Growth Determination

LNCaP C-81 cells were plated at 2 x 10^4 cells per well in 6-well plates in regular steroid-containing medium for 72 h. LNCaP C-33 cells were plated at 3 x 10^4 cells per well in 6-well plates in regular steroid-containing medium for 72 h. For experiments in regular steroid-containing conditions, attached cells were treated with fresh regular steroid-containing medium containing the respective compounds. For experiments in SR conditions, attached cells were adapted to SR medium for 48 h before treatment. Cells were treated with increasing concentrations of simvastatin, atorvastatin, or ketoconazole for 72 h in SR medium. Control cells were treated with dimethyl sulfoxide (DMSO) alone. The cell number was counted by a cell counter cellometer™ Auto T4 (Nexcelom Bioscience, USA) using trypan blue exclusion dye.

Clonogenic Assay

LNCaP C-81 cells were plated in 6-well plates at 3,000 cells per well in regular steroid-containing medium for 72 h. The cells were treated with 10 μM of simvastatin, atorvastatin, or ketoconazole in fresh regular steroid-containing medium for 9 days, with a change of fresh medium every 3 days. Control cells were treated with DMSO alone. On day 12, cells were washed with HEPES-buffered saline and attached cells were stained with 0.2% crystal violet containing 50% methanol.

Transwell Migration Assay

LNCaP C-81 cell migration was analyzed using Boyden chamber transwell assay. Cells were plated in the upper chamber of the 24-well insert at 5 x 10^4 cells per insert. Regular steroid-containing medium supplemented with 10 μM of the simvastatin, atorvastatin, or ketoconazole was added to both the upper and lower chambers of the insert. Control cells were treated with DMSO alone. After 24 h, cells were stained with 0.2% crystal violet containing 50% methanol and cells remaining in the upper chamber were removed via cotton swab. Migrated cells in the

lower part of the chamber were counted at 40x magnification.

Combination Treatments with Antiandrogens

LNCaP C-81 and VCaP-AI cells were plated in 6-well plates at 2 x10^4 and 1.5 x 10^5 cells, respectively, per well in regular steroid-containing conditions for 72 h, then conditioned to SR medium for 48 h. Cells were then treated with 10 μM each of simvastatin, Casodex (Cas), AA, or enzalutamide (Enz), or a combination of the simvastatin and an anti-androgen for 72 h in SR conditions. Additionally, LNCaP C-81 cells were treated with 10 μM simvastatin in the presence or absence of 1 μM, 5 μM, or 10 μM abiraterone acetate, as well as 5 μM simvastatin with and without 10 μM abiraterone acetate. Control cells were treated with DMSO alone. Cells were harvested, and cell number was measured by a cellometerTM Auto T4 using trypan blue exclusion dye.

Immunoblotting

Cells were washed with HEPES-buffered saline, pH 7.0, harvested by scraping, and lysed in ice-cold NP-40 lysis buffer containing SIGMAFAST protease inhibitor and phosphatase inhibitors ethylenediaminetetraacetic acid (EDTA) and NaF. An aliquot of the total cell lysate was electrophoresed on SDS-polyacrylamide gels. After transfer to a nitrocellulose membrane, the membranes were blocked with 5% non-fat milk in tris-buffered saline (TBS) containing 0.1% Tween-20 for 60 m at room temperature. Membranes were incubated with the corresponding primary antibody at 4ºC overnight. Membranes were rinsed with TBS and incubated with the proper secondary antibody for 60 m at room temperature. Proteins were detected using enhanced chemiluminescence (ECL) reagent kit. β-actin was used as a loading control. The intensity of the hybridization band was semi-quantified by Image-J.

Statistical Analysis

Each set of experiments was conducted in duplicate or triplicate and repeated at least 3 times, which is labeled as 2x3 or 3x3, respectively, and the mean and standard error values were calculated. The significance of difference (p-value) was calculated using independent t-test in Microsoft Excel for comparing each experimental result with the control and a p-value less than 0.05 was considered as significant.

Results

Effects of Steroid Biosynthesis Inhibitors on PCa Cell Growth

To examine the effects of steroid biosynthesis inhibitors on PCa cell growth, LNCaP C-33 and LNCaP C-81 cells were treated with simvastatin, atorvastatin, and ketoconazole in a range of 0-20 μM for 72 h under SR conditions [35,39]. Cell proliferation was determined by cell number counting as well as western blot analysis of Proliferating Cellular Nuclear Antigen (PCNA), a marker of cell proliferation and growth. After 72 h of treatment, all three compounds inhibited cell proliferation (Figure 1A and 1C) and reduced PCNA protein levels (Figure 1B and 1D), corresponding with increased concentrations of the inhibitor. LNCaP C-33 cell proliferation was only significantly inhibited upon 20 μM treatment by all three compounds (Figure 1A). Similarly, the reduction of PCNA protein levels correlated with LNCaP C-33 cell growth reduction for all three treatments (Figure 1B). In comparison, while all compounds showed the most pronounced effect at 20 μM on LNCaP C-81 cells, growth suppression was seen at 10 μM concentrations. At 20 μM, simvastatin exhibited 70% growth inhibition, followed by ketoconazole at 65%, then atorvastatin at 60% (Figure 1C). Further analyses revealed IC50 values of simvastatin, atorvastatin, and ketoconazole to C-81 cells are 10.56 μM, 16.21 μM, and 12.76 μM, respectively. The decreased levels of PCNA in general followed the increased concentrations of simvastatin and mirrored the trypan blue dye-exclusion results, but not in atorvastatin- or ketoconazole-treated cells (Figure 1D).

Figure 1.

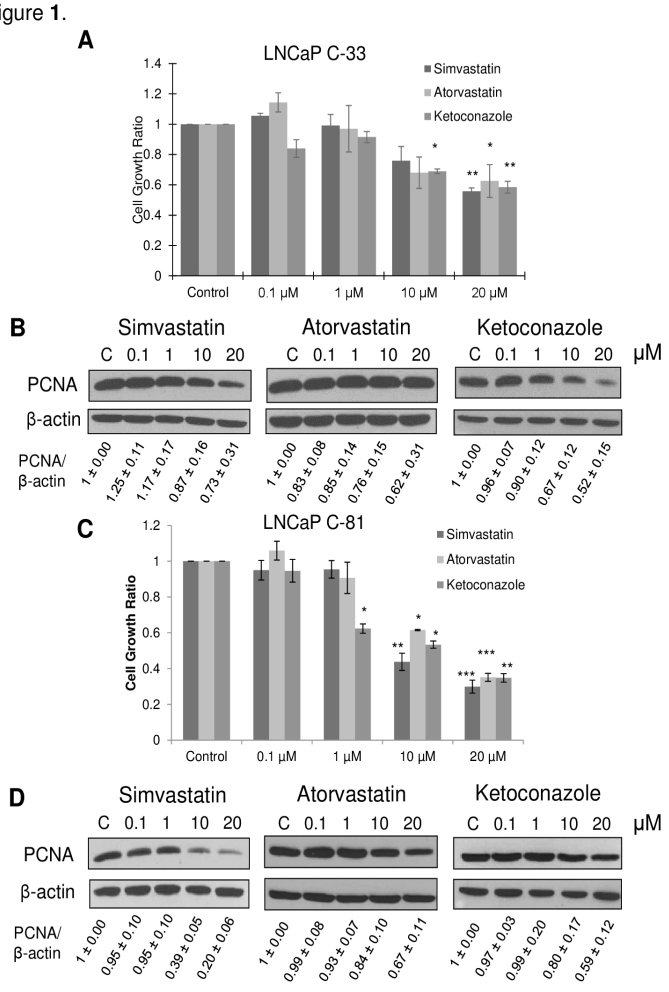

Figure 1(A-D): Effects of Statins on LNCaP C-81 PCa Cell Growth in SR Conditions. LNCaP C-33 **(A)** and LNCaP C-81 **(C)** cells were plated in regular steroid-containing medium at 3 x 10^4 and 2x10^4 cells per well, respectively, in 6-well plates. After 72 h, cells were conditioned to SR medium for 48 h, and treated with simvastatin, atorvastatin, and ketoconazole ranged from 0-20 μM concentrations in SR medium for 72 h. Control cells were treated with DMSO alone. Attached cells were harvested via trypsinization and living, shiny cells were counted with trypan blue dye. After counting, cells were lysed, and total cell lysate was analyzed for PCNA (B and D). β-actin was used as a loading control. Protein levels (**B** and **D**) were quantified with ImageJ software. Results presented are mean ± SE. n=3x3. $^{*}p<0.05$; $^{**}p<0.005$; $^{***}p<0.0005$.

Effects of Steroid Biosynthesis Inhibitors on PCa Tumorigenicity

We further determined whether statins or ketoconazole could reduce LNCaP C-81 cell tumorigenicity via Boyden chamber trans well and clonogenic assays. 10 μM treatments of the three compounds inhibited cell migration with simvastatin being the most potent at 75% inhibition, followed by atorvastatin and ketoconazole at 65% and 35% inhibition, respectively (Figure 2A). In comparison, in the clonogenic assay in which cells were grown on a plastic surface, ketoconazole exhibited about 80% inhibition of colony formation, then simvastatin at 75% inhibition, followed by a 50% reduction by atorvastatin (Figure 2B). Taken together, while these compounds had differential effects, at 10 μM concentrations, simvastatin exhibits the most potent inhibitory effects on LNCaP C-81 cell tumorigenicity in migration and colony formation.

Figure **2.**

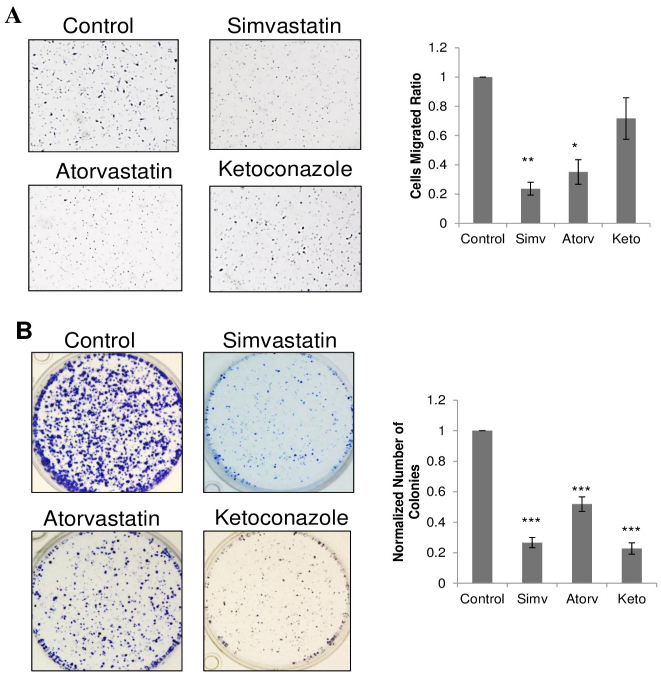

Figure 2(A-B): Effects of Steroid Biosynthesis Inhibitors on LNCaP C-81 Cell Tumorigenicity**. (A)** Cell migration via transwell assay. LNCaP C-81 cells were plated in the upper chamber of inserts at 5x10^4 cells per well in regular steroid-containing medium. Both upper and lower chambers contained medium with 10 μM of simvastatin, atorvastatin, or ketoconazole. Control cells were treated with DMSO alone. After 24 h, cells in the lower chamber were stained, while cells remaining in the upper chamber were removed via cotton swab. Images shown are at 40x magnification. Results presented are mean ± SE. n=2x3. **(B)** Clonogenic assay. LNCaP C-81 cells were plated in 6-well plates at 3000 cells per well in regular steroid-containing medium for 72 h. Cells were then treated with 10 μM of simvastatin, atorvastatin, or ketoconazole for 9 days, with fresh medium containing the specified treatment every 3 days. Control cells were treated with DMSO alone. On day 12, cells were stained, and the number of colonies was counted. Representative images are at 40x magnification. Results presented are mean ± SE. n=3x3. $^{*}p<0.05$; $^{**}p<0.005$; $^{***}p<0.0005$.

Effects of Statins on Cell Growth in Combination with Anti-Androgens

We analyzed whether simvastatin has an impact on the efficacy of anti-androgens on PCa cell growth inhibition in SR conditions. LNCaP C-81 cells were treated with 10 μM simvastatin in the presence or absence of 10 μM of each of anti-androgens: casodex, abiraterone acetate, and enzalutamide. Figure 3A showed that simvastatin alone had a 55% growth suppression, while the various anti-androgens alone inhibited cell growth by 20-30%. Unexpectedly, the combination of simvastatin and abiraterone acetate had an enhanced cell growth inhibition of about 90% decrease. Nevertheless, simvastatin and enzalutamide combination resulted in a 65% decrease in cell proliferation, and simvastatin and casodex had only a 50% decrease. The data illustrates that while simvastatin alone can reduce PCa cell growth in SR conditions; a combination of simvastatin with abiraterone acetate enhances the added growth-inhibitory effect.

To further investigate the combination treatment of simvastatin and abiraterone acetate, dose-response assays were performed. 10 μM simvastatin treatment was combined with 1 μM, 5 μM, or 10 μM abiraterone acetate (Figure 3B); conversely, 5 μM simvastatin was combined with 10 μM abiraterone acetate (Figure 3C). In Figure 5B, simvastatin alone reduces cell proliferation by about 55%. 1 μM, 5 μM, and 10 μM abiraterone acetate alone decreased cell proliferation by about 0%, 10%, and 25%, respectively. In combination, simvastatin and 1 μM abiraterone acetate inhibited cell growth by 65%, while the combination of simvastatin and 5 μM abiraterone acetate resulted in a 75% reduction in cell proliferation. The combination treatment of 10 μM simvastatin and 10 μM abiraterone acetate decreased LNCaP C-81 cell proliferation by over 85%. In parallel, Figure 3C showed that 5 μM simvastatin treatment inhibited cell growth by 45%, 10 μM abiraterone acetate reduced cell proliferation by about 30%, and the combination of these compounds resulted in an 80%

decrease in cell growth. Taken together, the data clearly show that simvastatin effectively enhances the anti-proliferative effects of abiraterone acetate on LNCaP C-81 cells.

Figure **3**.

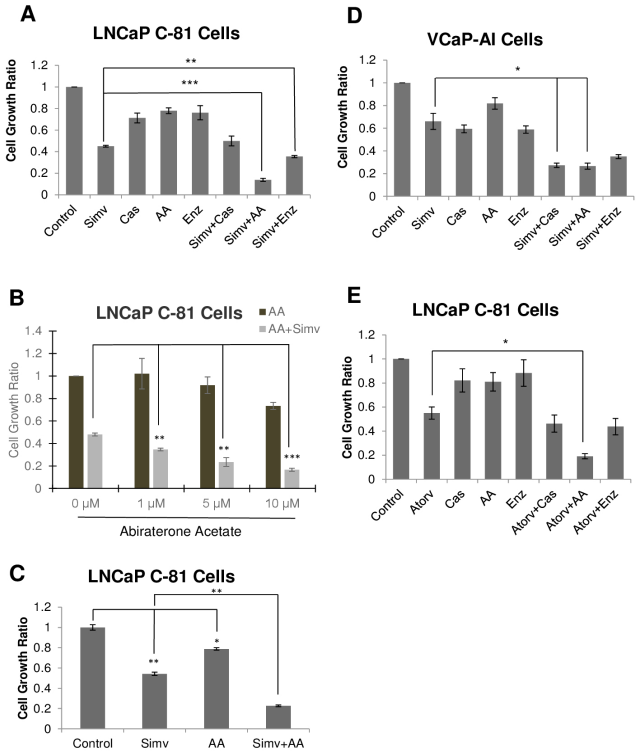

Figure 3(A-E): Effects of Combination Treatments of Simvastatin with Casodex, Abiraterone Acetate, and Enzalutamide. Combination treatments of statins and anti-androgens on LNCaP C-81 and VCaP-AI cells. LNCaP C-81 cells were plated in 6-well plates at $2x10^4$ cells per well in regular steroid-containing RPMI 1640 medium, while VCaP-AI cells were plated in regular steroid-containing DMEM medium at $2x10^5$ cells per well. After 72 h, cells were conditioned to SR medium for 48 h before treatment in SR conditions with 10 µM of statin compounds and/or 10 µM of anti-androgens: Casodex (Cas), Abiraterone Acetate (AA), or Enzalutamide (Enz) for 72 h. Control cells were treated with DMSO alone. Cells were harvested via trypsinization and counted using trypan blue exclusion dye. **(A)** Simvastatin combination treatments on LNCaP C-81 cells. **(B)** LNCaP C-81 cells were treated with 10 µM simvastatin and 1 µM, 5 µM, or 10 µM abiraterone acetate. **(C)** LNCaP C-81 cells were treated with 5 µM simvastatin and 10 µM abiraterone acetate. **(D)** Simvastatin combination treatment on VCaP-AI cells. **(E)** Atorvastatin combination treatment on LNCaP C-81 cells. Results presented are mean ± SE. n=3x3. $^{*}p<0.05$; $^{**}p<0.005$; $^{***}p<0.0005$.

For potential clinical applications, we investigated whether simvastatin can indeed enhance anti-androgen effects in another CR PCa cell line. We established VCaP-AI cells that express functional AR and obtain androgen-independent cell proliferation (Figure S1). Figure 3D demonstrated that simvastatin alone had only about 35% inhibition on VCaP-AI cells. In the context of anti-androgens, casodex, and enzalutamide resulted in 40% inhibition, and abiraterone acetate only reduced cell proliferation by 15%. In combination, simvastatin exhibited an added effect with casodex and abiraterone acetate on VCaP-AI cells, both resulting in about 75% reduction in cell proliferation, while the combination with enzalutamide inhibited growth by about 65%. Thus, simvastatin has a consistent added effect with abiraterone acetate on the inhibition of VCaP-AI cell growth in addition to LNCaP C-81 cells.

We further examined whether atorvastatin can similarly enhance anti-androgens effects on C-81 cell growth inhibition. As shown in Figure 3E, 10 µM atorvastatin treatment alone resulted in about 50% cell growth inhibition, while all three anti-androgens impeded cell growth by about 15-20%. The combination of atorvastatin and abiraterone acetate was most effective with over 80% growth inhibition, while atorvastatin combinations with Casodex or enzalutamide resulted in 55% growth suppression. In summary, both atorvastatin and simvastatin can enhance the efficacy of growth suppression by abiraterone acetate on CR PCa cells.

Effects of Simvastatin and Abiraterone Acetate Combination Treatment on LNCaP C-81 and VCaP-AI Cell Signaling under SR Conditions

To elucidate the inhibitory mechanism by combination treatment of simvastatin and abiraterone acetate, we analyzed the impacts of these compounds on survival and apoptotic proteins, both alone and in combination. The band density was semi-quantified utilizing ImageJ software (Figure 4B and 4D). As shown in Figure 4A and 4B, simvastatin alone treatment resulted in a decrease of AR protein, and abiraterone acetate alone treatment only had a slight reduction of AR protein. Unexpectedly, the combination treatment resulted in no detectable AR protein. Similar results were seen with PCNA in which simvastatin treatment alone resulted in a reduction of protein levels, abiraterone acetate alone had little impact, and the combination treatment resulted in dramatic decrease of PCNA. There was a reduction of phosphorylated and total AKT protein upon simvastatin treatment alone, while neither the protein nor its phosphorylated form was detectable upon combination treatment. Unexpectedly, abiraterone acetate treatment led to an increase in both phosphorylated and total AKT protein levels. Survivin, a downstream target of AKT, had a similar trend in which there was an increase in protein levels upon abiraterone treatment, while the combination treatment greatly reduced Survivin protein. Simvastatin alone did not appear to influence the expression of Survivin.

We also analyzed the impact of the combination treatments

on the levels of pro-apoptotic proteins. As shown in Figure 4A, abiraterone acetate and combination treatments led to a reduction of BAX protein levels, whereas simvastatin treatment had no effect on BAX level. Anti-apoptotic protein Bcl_{XL} was only reduced under simvastatin treatment, while abiraterone acetate treatment doubled the level of Bcl_{XL} and combination treatment left the protein levels unchanged. Caspase 3 levels slightly increased with simvastatin alone, and the low level of cleaved Caspase 3 was only detectable upon combination treatment with prolonged exposure. PARP levels dramatically decreased under simvastatin and combination treatment, while cleaved PARP was only present under combination treatment.

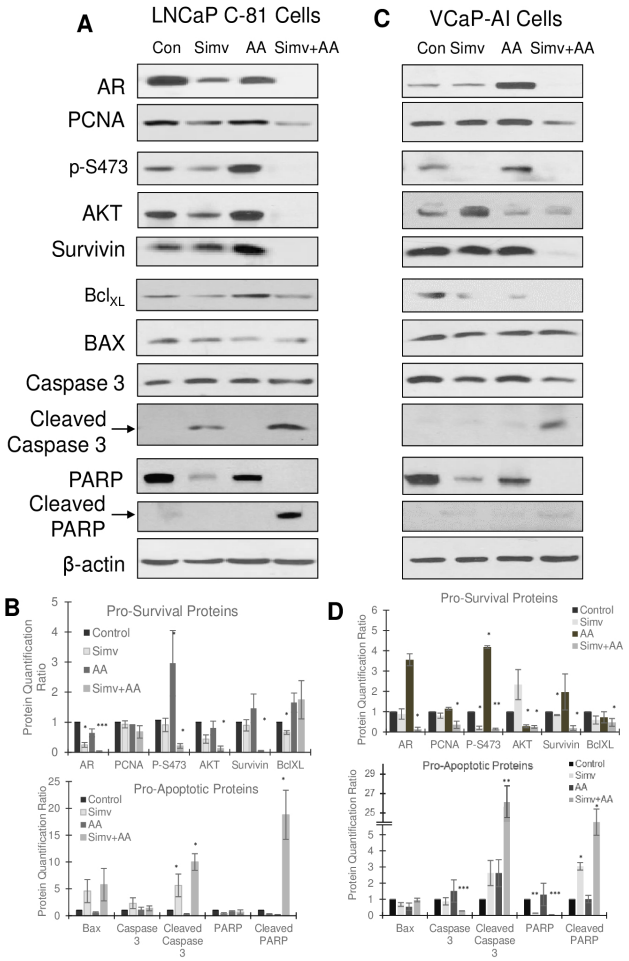

Figure 4(A-D): Western Blot Analysis of Simvastatin and Abiraterone Acetate Combination Treated LNCaP C-81 Cells. LNCaP C-81 cells **(A)** were plated in T75 flasks at 1.5 x 10^4 cells per flask in regular steroid-containing medium for 72 h, and then steroid starved for 48 h. VCaP-AI cells **(C)** were plated at 5 x 10^4 cells per T75 in regular steroid-containing DMEM medium for 72 h, then adjusted to SR conditions for 48 h. Cells were treated with 10 µM of simvastatin, abiraterone acetate, or both simvastatin and abiraterone acetate for 72 h under SR conditions. Control cells were treated with DMSO alone. Cells were harvested via scrapping and lysed. Total cell lysates were analyzed for phosphorylated AKT by site-specific Ser473 phospho-antibodies as well as total AR, PCNA, AKT, Survivin, Bcl_{XL}, BAX, PARP, Caspase 3 protein levels. β-actin protein level was used as a loading control. Protein density was determined using ImageJ Software **(B** and **D).** Results presented are mean ± SE. n=3. $^{*}p<0.05$; $^{**}p<0.005$; $^{***}p<0.0005$.

VCAP-AI cells had a similar signaling pattern to LNCaP C-81 cells upon combination treatment (Figure 4C and 4D). Unexpectedly, abiraterone acetate alone treatment led to an increase in AR and PCNA levels, while the combination of these treatments led to a large reduction in these two proteins. Simvastatin treatment alone had very little impact on the levels of pro-survival proteins, except for phosphorylated AKT. Phosphorylated AKT was undetectable upon simvastatin and combination treatments, while abiraterone acetate increased phosphorylated AKT levels. Total AKT levels were increased upon simvastatin treatment, while there was a reduction in protein levels upon abiraterone acetate and combination treatments. Pro-apoptotic protein BAX was unaffected by all three treatments in VCaP-AI cells, while Bcl_{XL} was reduced in response to all treatments with the largest reduction in combination-treated cells. Caspase 3 and PARP levels were decreased in simvastatin and combination-treated cells. Cleaved PARP was increased upon simvastatin and combination treatments, while cleaved Caspase 3 was increased upon combination treatment Figure 5.

Figure **5**.

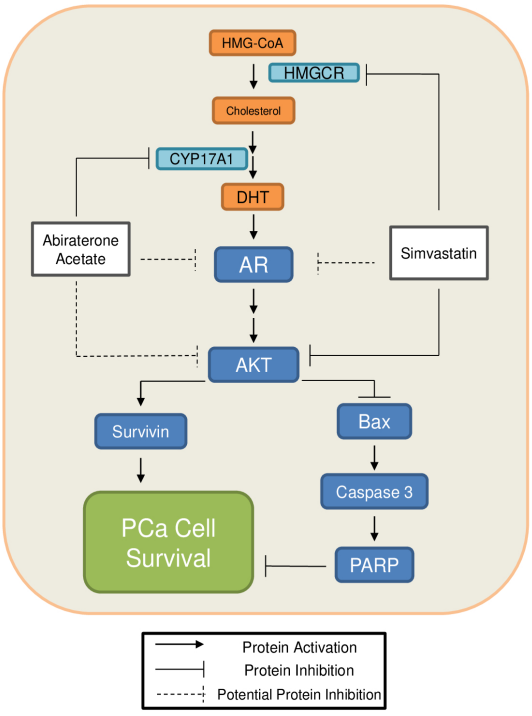

Figure 5: Proposed Mechanism of Action of Simvastatin and Abiraterone Acetate. Simvastatin inhibits HMG-CoA reductase (HMGCR), the rate-limiting enzyme of cholesterol biosynthesis. Simvastatin has been demonstrated to reduce AKT activity upon reduction of cellular cholesterol levels. AR levels are reduced in the presence of simvastatin; thus, simvastatin can potentially inhibit AR activity. Inhibition of these proteins results in the inhibition of PCa survival and growth via reduced Survivin protein levels, as well as the cleavage of Caspase 3 and PARP via Bax. Abiraterone acetate inhibits Cytochrome P450 side chain cleavage enzyme (CYP17A1) to reduce cellular DHT levels. This has the potential to result in the inhibition of AR and AKT activity, leading to inhibition of PCa growth and survival.

In summary, upon simvastatin treatment, the levels of pro-survival proteins in general decrease and pro-apoptotic proteins increase, which is greatly enhanced by the combination treatment with abiraterone acetate. Unexpectedly, in abiraterone acetate alone-treated AI cells, the levels of several pro-survival proteins increase.

Discussion

CR PCa remains a lethal disease; therefore, new effective treatment options are immediately needed. Inhibition of cholesterol biosynthesis, which reduces levels of the steroid hormone precursor, is one potential therapeutic strategy for this disease. Therefore, we investigated whether statins and/or ketoconazole, both steroid biosynthesis inhibitors, have a direct effect on CR PCa cell proliferation using the clinically relevant LNCaP C-81 cell model.

Significantly, LNCaP C-81 cells express all necessary enzymes for testosterone biosynthesis and can produce testosterone from cholesterol [7]. To the best of our knowledge, this is the first study to examine the direct effects of statins and ketoconazole on clinic-relevant AR-positive, AI PCa cells that are documented to obtain the intracrine regulation on growth and tumorigenicity. Additionally, this is the first report on the added effects of combination treatment of statins with anti-androgen abiraterone acetate.

Our results show that when growth inhibition of AS LNCaP C-33 and AI LNCaP C-81 cells by statin and ketoconazole treatments are compared under SR conditions, it is more pronounced in LNCaP C-81 cells. Because LNCaP C-33 cells do not exhibit the intracrine activity nor proliferate well in SR conditions, we determined the effects of statins on C-33 cells in regular steroid-containing medium. Importantly, a low level of growth inhibition was seen in SR conditions (data not shown). Similarly, low levels of growth inhibition were also obtained with noncancerous prostate epithelial RWPE-1 cells in regular growth conditions containing androgenic activities, as these cells also do not possess intracrine regulation (Figure S2). Thus, the inhibition of intracrine regulation is one of the mechanisms by which simvastatin effectively obstructs AI PCa cell growth.

Among the compounds analyzed, simvastatin is slightly more effective than atorvastatin and ketoconazole at suppressing LNCaP C-81 cell growth (Figure 1). Reduction of cellular cholesterol levels negatively affects cell membrane integrity [39] and lipid raft formation [40]; the statin compounds were expected to be more effective than ketoconazole, which only reduces DHT levels. Unexpectedly, only simvastatin effectively inhibited both LNCaP C-81 cell migration and colony formation, whereas atorvastatin only impeded migration and ketoconazole inhibited colony formation (Figure 2). Furthermore, simvastatin treatment is not rescued by DHT or regular FBS-containing medium, only cholesterol can rescue the effects to a limited extent (Figure S3), suggesting there are additional mechanisms involved by which simvastatin inhibits PCa growth rather than the reduction of cholesterol levels alone.

One of the general concerns over the effects of statins and the prevention of advanced cancer progression is the high dose of stains commonly used in cell culture, which are not physiologically relevant. In clinics, these compounds are often used for extended periods of time prior to PCa development to prevent high cholesterol; whereas in *in vitro* cell culture experiments, the cells were only exposed to them for a few days in culture. It is for this reason that higher concentrations of drugs were used to carry out these experiments than what is seen in patient serum during clinical trials. Supportively, *in vivo* mouse models of LNCaP xenografts have shown that prostate tumors were resistant to progression over an 8-week treatment period with clinic-relevant doses of simvastatin. Thus, this resulted in a longer survival time, providing evidence that the cell culture results translate to long-term administration of simvastatin treatment [41].

Importantly, our results can provide clarification on the inconsistent benefits of statins as a therapeutic option in combination with ADT for the treatment of PCa. There are many reports investigating the impact of statins on the risk of PCa development, progression, and survival rates, as well as in combination treatments, all with inconclusive results. It is clear that those inconsistencies may stem from a lack of analysis on the specific statins used or what types of ADT therapy the patients received [9,10-12,14-25]. Our results clearly show that the specific combination of statins and abiraterone acetate consistently exhibits an enhanced growth suppression with added effects on both AR-positive, AI LNCaP C-81 and/or VCaP-AI cells, both clinically relevant cell lines (Figure 3). Our data thus supports the statistical analyses in which CR PCa patients using statins and receiving abiraterone treatments on improved drug efficiency [42] and a better survival rate [43]. The added effect is not seen in casodex or enzalutamide combination treatments with statins. In addition, western blot analyses revealed that the anti-proliferative effects of simvastatin on LNCaP C-81 and VCaP-AI cells are enhanced by abiraterone acetate, whereas abiraterone acetate alone can

upregulate several pro-survival proteins (Figure 4). Our results are thus consistent with recent clinical reports demonstrating the benefits of the combination of statins and abiraterone acetate. Our data together with clinical observations provide an important guideline of experimental design and data analysis. Further, our data also have an important impact on the future of CR PCa treatment.

In summary, this study provides important information on the potential treatment options for PCa, especially with the widespread use of statins in the treatment of coronary artery disease and hypercholesterolemia, and also the high incidence of PCa in males in western countries. Nevertheless, it is important to clarify whether statins can provide a protective role in reducing PCa incidence.

Conflict of Interest:

The authors declare no conflict of interest.

Acknowledgements:

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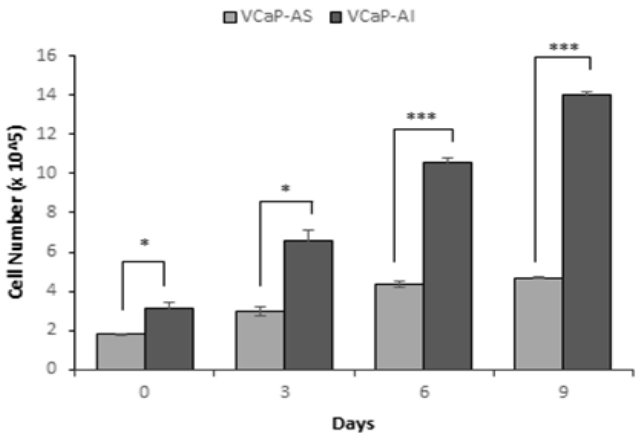

Figure S1: VCaP-AS vs. VCaP-AI Growth in SR Conditions. VCaP-AS and VCaP-AI cells were plated in regular steroid-containing medium at 1 x 105 cells per well in 6-well plates. After 72 h, cells were conditioned to SR medium for 48 h. Cells were harvested every 3 days via trypsin and counted using trypan blue exclusion dye. Results presented are mean ± SE. n=3x3.

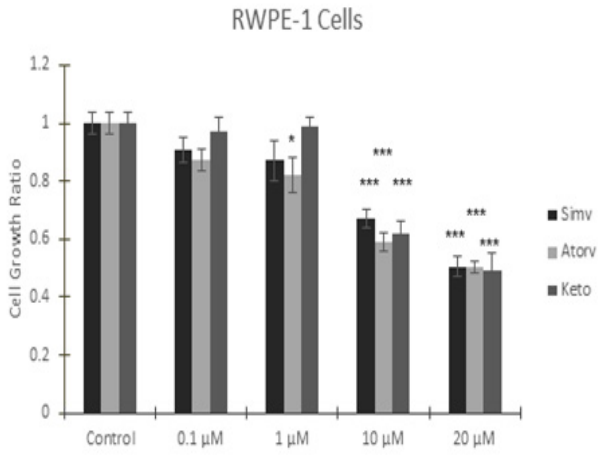

Figure S2: Effects of Statins on Normal Prostate Epithelial RWPE-1 Growth in SR Conditions. RWPE-1 cells were plated in regular steroid-containing medium at 1.5 x 105 cells per well in 6-well plates. After 72 h, cells were conditioned to SR medium for 48 h, and treated with simvastatin, atorvastatin, and ketoconazole ranged from 0-20 µM concentrations in SR medium for 72 h. Control cells were treated with DMSO alone. Attached cells were harvested via trypsinization and living, shiny cells were counted with trypan blue dye. Results presented are mean ± SE. n=3x3.

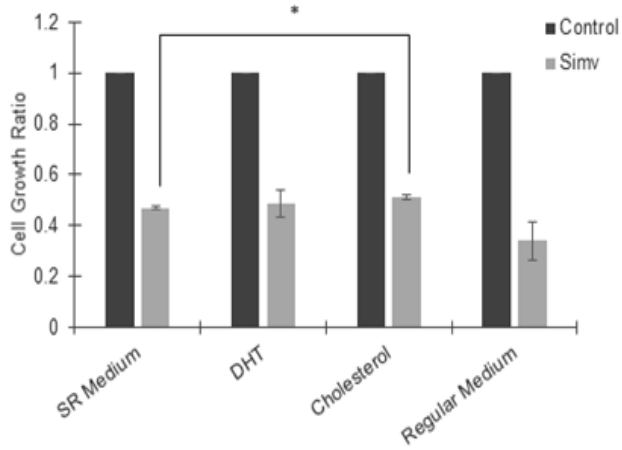

Figure S3: Rescue of Simvastatin Growth Inhibition with DHT, Cholesterol, and Regular Steroid-containing Medium. Trypan blue assay of LNCaP C-81 cells treated with simvastatin. LNCaP C-81 cells were plated at 2 x 104 cells per well in 6-well plates in regular steroid-containing medium for 72 h. Cells were conditioned to SR medium for 48 h, then treated with 10 µM simvastatin in SR medium, SR medium supplemented with 10 nM DHT or 500 nM cholesterol, or in regular steroid-containing medium for 72 h. Cells were harvested via trypsin and counted using trypan blue exclusion dye. Results presented are mean ± SE. n=3x3.

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