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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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 (Eley 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).
Hydrophobic statins (simvastatin, lovastatin, and fluvas- 
tatin) have been shown to inhibit cancer growth. In cell-based 
experiments in in vitro and experimental animal models, these 
statins have displayed inhibitory effects on many cancers, in- 
cluding head and neck, prostate, lung, breast, colon, pancreas, 
skin (melanoma), renal cell, bladder, liver, and multiple my- 
eloma (Jakobisiak and Golab, 2010). 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. An initial case-controlled study showed that the 
use of statins is associated with a 50% reduction in the risk of 
prostate cancer (Shannon et al., 2005), a finding supported by 
another study on atorvastatin and prostate cancer clinical out- 
comes (Moyad et al., 2005). However, a study performed on 
statin use and incidence of prostate cancer in a Finnish popu- 
lation did not show any significant correlation between them 
(Haukka et al., 2010). In contrast, another study performed in a 
Finnish population showed a decreased overall relative risk of 
prostate cancer and reduced serum prostate-specific antigen 
(PSA) levels among current statin users with proportional 
changes corresponding to the amount and duration of use (Mur- 
tola et al., 2010). In addition, a number of reports published 
recently demonstrate that statin use is associated with de- 
creased chances of undergoing prostate biopsy and receiving a 
Gleason score of 7 or higher (Breau et al., 2010; Katz et al., 
2010). Reduction in serum PSA and total testosterone levels 
among statin users compared with those not using statins has 
also been reported by other groups (Mondul et al., 2010). A very 
recent study focused on characterizing the association between 
statin use and PSA recurrence after prostatectomy demon- 
strated a dose-dependent reduction in the risk of biochemical 
recurrence (Hamilton et al., 2010). Although controversial, to- 
gether, the findings of these studies suggest that long-term 
statin use can prevent or delay the onset of prostate cancer 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 the risk of biochemical 
recurrence of prostate cancer after radical prostatectomy, sim- 
vastatin was used by most of the subjects (171 of 236) involved 
in the study (Hamilton et al., 2010). In the current study, we 
focused on studying the effects of simvastatin on prostate can- 
cer cell functions in vitro as well as growth of prostate cancer 
xenograft in nude mice in vivo and characterizing the major 
molecular mechanisms regulating the process. Our findings 
indicate 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. 
Even more noteworthy, our results indicate that prostate can- 
cer cells stably expressing constitutively active Akt (myrAkt) 
were resistant to simvastatin-mediated inhibition of prostate 
cancer cell functions. We conclude that simvastatin can be de- 
veloped 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 simva- 
statin therapy.

Materials and Methods

Cell Lines, Reagents, and Antibodies. Human PC3 and 
LNCaP (lymph node carcinoma of the prostate) cell lines were 
obtained from the American Type Culture Collection (Manassas, VA). 
All cell lines were maintained in DMEM (HyClone Laboratories, 
Logan, UT) with 10% fetal bovine serum, 100 units/ml penicillin, and 
100 μg/ml streptomycin in a 5% CO2 atmosphere at 37°C. DiIC12 
fluorescence dye was purchased from BD Biosciences (San Jose, CA). 
Primary antibodies such as anti-Akt, anti-phospho-Akt (S473), 
and anti-phospho-GSK3α/β were purchased from Cell Signaling Technology 
(Danvers, MA). Primary antibodies against β-actin were purchased 
from Sigma-Aldrich (St. Louis, MO), and anti-PSA antibody was 
purchased from Pierce Biotechnology Inc. (Rockford, IL). Anti-mouse 
and anti-rabbit horsedarsh peroxidase-conjugated secondary anti- 
body were obtained from Bio-Rad Laboratories (Hercules, CA). 
Docetaxel and simvastatin were purchased from Sigma-Aldrich. 
Simvastatin was activated in the lab according to the manufacturer’s 
instructions.

Migration Assay. PC3 cells were grown to confluence, and 
a scratch was made in the monolayer, followed by treatment (control 
buffer and 25, 50, and 100 μM simvastatin). Scratch recovery was 
determined at 16 and 24 h. Microscopic pictures were analyzed using 
ImageJ software (http://rsbweb.nih.gov/ij/), and the percentage re- 
coverage was calculated using the equation 100 × (1 – T0/Tf), where 
T0 is the area at the endpoint and Tf is the area at the time 0. The data 
are presented as mean ± S.D.

Invasion Assay. The invasion of PC3 cell lines was measured 
using a 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 DiIC12 fluorescence dye and 
seeded onto the upper chamber of a 96-well Transwell plate (Cor- 
ing Life Sciences, Lowell, MA) at a density of 104 cells per well in 
400 μl of medium. DMEM containing 10% FBS was then added to 
the lower chamber. After 24 h, the cells were treated with control 
buffer or 25 and 100 μM simvastatin in DMEM. The fluorescence 
from the stained cells was measured after 12 and 24 h on an 
ELISA plate reader at 549/565 nm (ex/em). The data are presented 
as mean ± S.D.

Cell Doubling Time Assessment. In each experiment, cell dou- 
bbling time was determined according to direct cell count and 
in consideration of logarithmic growth of cancer cells (http://www. 
doubling-time.com). For a direct cell count, approximately 100 cells 
per well were seeded in 400 μl of medium on a 48-well plate in 
triplicates. After 24 h, medium was replaced, and cells were 
collected. The cells were treated with control buffer or 25 and 100 μM 
simvastatin in DMEM. At 24 h, cell counts were repeated. The cell 
doubling time was calculated as mean ± S.D.

Trypan Blue Viability Assessment. In the trypan blue method, 
cells were grown to confluence in DMEM with 10% FBS. The cells 
were treated with 25 and 100 μM simvastatin in DMEM. After 24 h, 
cells were collected and resuspended in PBS with 0.4% trypan blue 
solution. Total cells and trypan blue-stained (i.e., nonviable) cells 
were counted, and the percentage of nonviable cells was calculated.

Apoptosis Assessment. Cytoplasmic histone-associated DNA 
fragments were quantified by using the Cell Death Detection 
ELISAPLUS kit (Roche Applied Science, Indianapolis, IN) according 
to the manufacturer’s protocol. In brief, PC3 cell lines were seeded in 
96-well plates at a density of 104 cells per well. After 24 h, the cells 
were incubated in DMEM containing 25 and 100 μM simvastatin for 
16 h. Control cells were treated with 0.1% DMSO (vehicle control). 
Cells were lysed and centrifuged at 2000 g for 10 min, and the collected 
supernatant was subjected to ELISA. The absorbance was measured 
at 405 nm (reference wavelength, 492 nm). The data are presented as 
mean ± S.D.

Cell Proliferation Assay. The effect of simvastatin on the pro- 
liferation of PC3 cell lines was determined using the nonradioactive 
BrdU-based cell proliferation assay (Roche Applied Science) accord-
ing to the manufacturer’s protocol. In brief, PC3 cells were seeded in 96-well plates at a density of $5 \times 10^4$ cells per well. After 24 h, the cells were incubated in DMEM containing 25 and 100 $\mu$M simvastatin for 16 h. 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 690 nm on an ELISA plate reader. The data are presented as mean $\pm$ S.D.

**Colony Formation Assay.** Colony formation assay was performed using the standard protocol (Bradley et al., 1985). In this approach, PC3 cells were cultured on six-well plates until the monolayer was reached. The wells were treated with DMEM containing 25 and 100 $\mu$M simvastatin. Control cells were grown in DMEM. Five days after treatment, each of the wells was counted for the number of colonies, and simvastatin-treated wells were compared with the vehicle-treated control. Plates were fixed using 2% paraformaldehyde, briefly stained with crystal violet, and counted visually or by using ImageJ software. The data are presented as mean $\pm$ S.D.

**Western Analysis.** PC3 and LNCaP cell lines were cultured to reach a monolayer in DMEM in six-well plates. The wells were treated with DMEM containing 25 and 100 $\mu$M simvastatin. Control cells were grown in DMEM alone. Whole-cell lysates were prepared using lysis buffer [50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM Na$_3$VO$_4$, and 1x complete protease inhibitors (Roche Applied Science)]. Tissue obtained from mice was snap-frozen with liquid nitrogen and pulverized with a mortar and pestle. Tissue lysates were prepared using a lysis buffer. The protein concentration was measured by the DL protein assay (Bio-Rad Laboratories). Western analyses were performed using the standard Laemmli’s method as done previously (Somanath et al., 2007).

**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 resuspended 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). Mice were divided into two groups. The groups were subjected to intraperitoneal injections of simvastatin at a dose of 2 mg/kg body weight every 12 h (or 24 h in a second set of experiments) for 2 weeks. The respective controls were injected intraperitoneally with 0.9% saline every 12 or 24 h. Tumor sizes were measured on days 7 and 11. Mice were sacrificed on day 11, and tumors were dissected and weighed.

**Statistical Analysis.** Mean activities were calculated from three to five independent experiments done at least in triplicates. A 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.** Because 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 would have any effect on the phosphorylation and activity of Akt. Our results indicate that treatment with simvastatin had a robust effect on the inhibition of Akt phosphorylation in LNCaP and PC3 cells. Effects were seen starting at 25 $\mu$M, and maximum inhibition was observed when 75 $\mu$M simvastatin was used (Fig. 1A). A time course study of the effects of simvastatin on LNCaP provided further insights on the dose and time dependency of the Akt inhibition (Fig. 1B). In PC3 cells, the inhibition of Akt was observed at 25 and 100 $\mu$M simvastatin, with a peak inhibition at 72 h (Fig. 1C). These results suggest that simvastatin has a potent inhibitory effect on Akt phosphorylation in prostate cancer cells, which is dose and time dependent.

![Fig. 1. Simvastatin treatment inhibits Akt pathway in human prostate cancer cells. A, a dose-dependent (10, 25, 50, 75, and 100 $\mu$M) study on the effects of simvastatin (16 h) on phosphorylation of Akt and its downstream substrates GSK3$\alpha$ and GSK3$\beta$ in LNCaP cells. Densitometry of the corresponding bands normalized to $\beta$-actin is shown in the graph. B, time course effect of 25 $\mu$M simvastatin (4 and 16 h) on Akt and GSK3 phosphorylation in LNCaP cells. Corresponding densitometry values, normalized by $\beta$-actin, are shown in the graphs. C, time course effect of 25 $\mu$M simvastatin (4, 8, 16, and 24 h) on Akt phosphorylation in PC3 cells. Corresponding densitometry values normalized to $\beta$-actin are shown in the graph.](image)
cells indicated that although 100 μM simvastatin inhibited phosphorylation of Akt at 4 h, a maximum reduction in Akt phosphorylation by 25 μM simvastatin was observed at 16 h (Fig. 1B). Similar effects of simvastatin were observed in metastatic human PC3 prostate cancer cell lines (Fig. 1C). To determine whether the reduction in phosphorylated Akt levels in PC3 and LNCaP cells had any effect on Akt activity, we determined the 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.** Because 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 indicate that treatment with 25 μM simvastatin in PC3 cells maintained in serum-containing medium significantly impaired their ability to migrate (Fig. 2, A and B) as analyzed at 16 h (p < 0.04 for 25 μM and p < 0.05 for 100 μM) and 24 h (p < 0.0002 for 25 μM and p < 0.001 for 100 μM) after treatment (~2- and 3-fold decrease, respectively). At 24 h, treatment with 100 μM simvastatin almost completely inhibited (~90% inhibition compared with control) PC3 cell migration. Likewise, treatment of PC3 cells with 25 μM simvastatin significantly inhibited invasion in response to EGF (p < 0.005 for 12 h and p < 0.01 for 24 h) and 10% FBS (p < 0.03 for 12 h and p < 0.0002 for 24 h) (Fig. 2C). At 12 and 24 h after treatment with 25 μM simvastatin, we observed ~12 and ~15% inhibition, respectively, in EGF-stimulated PC3 cell invasion. Treatment with 100 μM simvastatin further enhanced the inhibition of PC3 cell invasion up to 26% compared with the EGF-treated control (p < 0.01). The effects of simvastatin on EGF-stimulated prostate cancer cell invasion were significantly higher compared with cells that were maintained in 10% FBS containing medium (Fig. 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 with control). Inhibition of PC3 cell invasion was slightly higher upon treatment with 100 μM simvastatin compared with the control (3.5–7.5%) (p < 0.005 for 12 h and p < 0.003 for 24 h). Overall, our data indicate 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. Metastatic PC3 cells normally have a doubling time of 10 to 14 h. Our study showed that treatment with 25 μM simvastatin resulted in a 6- to 8-fold increase in the doubling time for PC3 cells (p < 0.0003) (Fig. 3A). This effect was even greater when cells were treated with 100 μM simvastatin, reaching well above 300 h (~20-fold) (p < 0.001) (Fig. 3A). Data from the proliferation assay revealed that treatment with simvastatin resulted in significant inhibition of PC3 cell proliferation by 25 to 35% for 25 μM (p < 0.0001) and 100 μM (p < 0.00001) simvastatin, respectively (Fig. 3B). The effect of 25 μM simvastatin on proliferation was similar to the effects of a low-dose treatment with 10 nM docetaxel (Taxotere; sanofi-aventis, Bridgewater, NJ) (p < 0.0001), a currently used chemotherapy drug for the management of prostate cancer in patients (Fig. 3B). Thus, our data indicate that simvastatin significantly inhibits prostate cancer cell proliferation in vitro.

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**Fig. 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 PBS (control) and simvastatin (25 and 100 μM). A, scratch recovery as determined 16 h after simvastatin treatment. B, scratch recovery as determined 24 h after simvastatin treatment. C, invasion assay data after treatment of EGF-stimulated PC3 cells with 25 and 100 μM simvastatin for 12 and 24 h. D, invasion assay data after treatment of serum-stimulated PC3 cells with 25 and 100 μM simvastatin for 12 and 24 h. Bar graph shows the percentage inhibition of invasion in simvastatin-treated PC3 cells normalized to saline control.
Agents that can induce apoptosis in cancer cells have been an excellent choice for cancer treatment. Our study indicated that treatment with 25 μM simvastatin increased cell death by ~30% over a 12-h period in PC3 cells (p < 0.0001) (Fig. 4A). This effect was enhanced further by 100 μM simvastatin, which exhibited more than a 2-fold increase in cell death (p < 0.0001). Likewise, treatment of PC3 cells with 25 and 100 μM simvastatin resulted in 1.5-fold (p < 0.0005) and 1.75-fold (p < 0.0005) increases in apoptosis, respectively (Fig. 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 are left for an additional 5 days. Treatment with 25 μM simvastatin significantly inhibited (~25%) colony formation by PC3 cells (p < 0.04) (Fig. 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 with the effects of low-dose docetaxel (10 nM), which inhibited colony formation by PC3 cells by ~60% (p < 0.01) (Fig. 5B). To investigate whether inhibition of colony formation by simvastatin was mediated through Akt inhibition, we next determined whether prostate cancer cells expressing constitutively active Akt (myrAkt) could 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 adenovirus infections) showed that cells expressing myrAkt exhibit a significantly higher number of colonies compared with cells expressing GFP (p < 0.03) (Fig. 5C). As we hypothesized, our data indicate 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 nontreated PC3 cells expressing ad-myrAkt.
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 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 a tumor xenograft study in nude mice. In an initial study, PC3 cells were administered in nude mice and were treated with simvastatin (2 mg/kg b.wt. every day) administered intraperitoneally as performed previously (Shinozaki et al., 2010). 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 \( (p < 0.04) \) (Fig. 6A). However, no significant changes in tumor weight on day 14 \( (p < 0.9) \) between the control and simvastatin-treated mice (Fig. 6B) were observed. Next, we determined whether 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 with the saline control. Mice treated with simvastatin did not exhibit any differences or changes in tumor size between days 7 and 11 \( (p < 0.2) \) (Fig. 6, C and D) or between days 11 and 14 (data not shown).

Although the effect of simvastatin on the growth of tumor xenografts was not significant compared with saline-administered controls, a trend toward reduced growth of tumor xenografts in simvastatin-treated mice compared with 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 (2 mg/kg b.wt. every 12 h). These changes showed significant differences in prostate tumor growth between saline- and simvastatin-administered mice (Fig. 6). Overall tumor weight determined on day 14 after tumor injection showed reduced growth of prostate tumor xenograft in simvastatin-administered mice compared with the saline control \( (p < 0.03) \) (Fig. 6E). A significant reduction in tumor size was also observed in simvastatin-treated mice compared with the saline control \( (p < 0.03) \) (Fig. 6F). Next, we sought to analyze the percentage change in tumor growth between days 7 and 11 after tumor injection. Our data indicate that simvastatin inhibited growth of tumors from day 7 to day 11 compared with its original size on day 7 \( (p < 0.02) \) (Figs. 6, F and G, and 7A).

To determine whether the effect of simvastatin on the growth of PC3 tumor xenograft involves the inhibition of Akt and/or changes in the expression levels of PSA, we prepared tumor lysates and subjected them to Western analyses using...
antibodies specific for phospho-Akt and PSA. Our data indicate 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) (Fig. 7, B and C). Overall, our studies on the effects of simvastatin on a tumor xenograft in male nude mice demonstrate that simvastatin inhibits prostate tumor growth in vivo involving the inhibition of Akt activity and a reduction in PSA expression.

**Discussion**

Although the results are controversial, many recent analyses of patient samples conducted by different groups have revealed the potential benefits of statins in the management of prostate cancer (Jakobisiak and Golab, 2010). 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. Intraperitoneal administration of simvastatin in nude mice bearing PC3 tumor xenografts exhibited a significant reduction in tumor size and weight associated with a reduction in PSA expression compared with saline-administered controls. Apart from this, we also observed a significant reduction in the rate of tumor growth (from day 7 to day 11) in simvastatin-treated mice compared with control. In sum, our data clearly demonstrate 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 their potential benefits in cancer therapy. First, statins inhibit the 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 (Liao and Laufs, 2005). Second, statins are known to inhibit proliferation of smooth muscle cells in the vasculature, leading to primary and secondary prevention of cardiovascular events (Porter et al., 2002). In addition, they are known to induce apoptosis in smooth muscle cells (Porter et al., 2002) and many cancer cell types (Jakobisiak and Golab, 2010). 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 en-
Molecular mechanisms regulating statin-mediated regulation of prostate cancer by statins. Akt (protein kinase B), a serine-threonine kinase, is central to multiple prosurvival and antiapoptotic cellular pathways (Somanath et al., 2006). Akt has been shown to be one of the most frequently activated signaling molecules in cancers (Engelman, 2009), and activation of the PI3 kinase-Akt pathway as a result of phosphatase and tensin homolog deficiency is a very common cause of prostate cancer (Li et al., 1997; Blanco-Aparicio et al., 2007). Enhanced apoptosis in response to cholesterol-lowering drugs in prostate epithelial cells as well as in breast cancer and human epidermoid carcinoma cell lines was reported to be due to the inhibition of prosurvival kinase Akt, reduced expression of antiapoptotic molecule Bcl-xL, and activation of proapoptotic caspase-3-dependent pathway (Li et al., 2006). Reconstituting rafts by the addition of cholesterol restored Akt activity, resulting in the inhibition of apoptosis. A recent study performed in a different cancer type supports these findings and reports that simvastatin induces apoptosis and inhibits Akt phosphorylation and Bcl-xL expression in breast cancer cells via inhibition of nuclear factor-κB, derepression of phosphatase and tensin homolog, and subsequent inhibition of PI3 kinase (Ghosh-Choudhury et al., 2010). Statins, in general, have also shown to inhibit Akt-mammalian target of rapamycin signaling in p53-deficient hepatocellular carcinoma (Roudier et al., 2006). 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 the G1 phase via inhibition of cyclin E/cdk2 kinase (Sivaprasad et al., 2006), possibly via inhibition of Akt (Murtola et al., 2008). Our study indicated that simvastatin inhibits Akt activity in LNCaP and PC3 cells in a dose- and time-dependent manner. However, until now, a causal relationship between decreased Akt activity and reduced tumor growth by any statins in any type of cancer has not been established. Our finding that PC3 cells expressing myrAkt (constitutively active Akt) are resistant to the effects of simvastatin on colony formation demonstrates a causative relationship between the inhibition of Akt activity and impaired prostate cancer cell function. Furthermore, tumor xenografts collected from nude mice treated with simvastatin exhibited a significant reduction in phosphorylated Akt levels associated with its reduced Akt activity and impaired prostate cancer cell function. Differences in phosphorylated Akt levels associated with its reduced Akt activity and impaired prostate cancer cell function. Differences in phosphorylated Akt levels associated with its reduced Akt activity and impaired prostate cancer cell function.

A number of recent meta-analyses from medical databases
and epidemiological studies indicate the effect of statins in reducing serum PSA levels (Hamilton et al., 2010; Murtola et al., 2010). Among them, a very recent survey shows that decreases in PSA levels are correlative in subjects who are undergoing statin treatment and might influence the risk assessment for prostate cancer (Chang et al., 2010). Another recent study indicates that statins have the ability to reduce the expression of PSA mRNA via inhibition of androgen receptor (AR) protein expression in hormone-responsive LNCaP cells (Yokomizo et al., 2010). 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. Furthermore, our observations extend this information and demonstrate that the 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 with 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 used 20 to 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 in a cellular level (Habib et al., 2007), and it appears to be the dose necessary to inhibit isoprenylation of the proteins in cultured cells (Finder et al., 1997; Grosser et al., 2004). However, we have also shown that at doses very close to therapeutic concentrations (less than 10 times), simvastatin inhibits the growth of PC3 tumor xenograft in vivo. Moreover, it should be noted that the simvastatin effects that we studied on prostate cancer cells are 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 prostate epithelial cells and LNCaP and VCaP (vascular cancer cells with simvastatin significantly inhibits 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.

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Authorship Contributions

Participated in research design: Kochuparambil, Al-Husein, and Somanath.

Conducted experiments: Kochuparambil, Al-Husein, Goc, Soliman, and Somanath.

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References


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