Abstract. The mevalonate pathway plays an important role in cancer biology and has been targeted with farnesyl transferase inhibitors, although their efficacy is limited due to significant adverse effects. Statins and bisphosphonates inhibit the mevalonate pathway at different steps, thus having negative effects at various levels on cancer cells. A combination of these drugs may result in an amplified cytotoxic effect and allow for use of significantly lower doses of the drugs involved. Statins inhibit the mevalonate pathway at 3-hydroxy-3-methylglutaryl coenzyme A reductase and bisphosphonates at farnesyl pyrophosphate synthase. Our results show that low-dose combinations of simvastatin and alendronate have a synergistic cytotoxic effect on androgen-independent prostate cancer PC-3 cells, but not on androgen-dependent LNCaP or DU 145 prostate cancer cells. These two drugs cause a sequential blockade of the mevalonate pathway and significantly affect survival and apoptotic pathways by down-regulating phospho-AKT and activating c-JUN and ERK.

Prostate cancer is a leading cause of mortality and morbidity both in the United States and around the world. Based on global data from 2012 it was responsible for 1.1 million new cases and 307,000 deaths and it is the second most common cancer in men worldwide (1). Based on most recent Surveillance, Epidemiology, and End Results Program SEER data, it is estimated that approximately 15.0% of men will be diagnosed with prostate cancer at some point during their lifetime (2). The five-year relative survival for both localized (confined to organ) and regional (disease to regional lymph nodes) prostate cancer is 100.0%, but drops to 27.8% for metastatic disease (2). Thus, exists a continued need for newer treatment approaches in order to better-control metastatic prostate cancer and improve survival. In locally advanced (intermediate- and high-risk groups based on prostate-specific antigen (PSA) level, Gleason score and tumor stage), metastatic and especially castration-resistant prostate cancer (CRPC), benefits derived from available treatments are few and short lasting.

The first line of treatment for metastatic disease is androgen deprivation therapy (ADT). Since bone metastasis is very common in prostate cancer and ADT also causes significant loss of bone mineral density, intravenous bisphosphonate is a quintessential part of treatment to reduce skeletal-related events (3). Eventually, patients lose response to ADT and develop biochemical or clinical progression of metastatic disease. Understanding the molecular mechanisms of failure of ADT has been essential to recent advances in the field of treatment of CRPC. Among these, the persistence of androgen in CRPC tissue, increased expression of genes involved in conversion of testosterone to dihydrotestosterone, the ability of cells to convert cholesterol to testosterone and

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the presence of mutations on androgen receptors, are being studied extensively. Abiraterone, a CYP 17 inhibitor approved for treatment of patients who progress following docetaxel, has shown a survival benefit (4). Further knowledge of tumor-associated antigens, such as prostatic acid phosphatase, prostate-specific membrane antigen and PSA have made possible the use of immunotherapy (sipuleucel-T) in the treatment of CRPC.

In this study, we describe the differential response of prostate cancer cells to the simultaneous targeting of HMG Co-A reductase and the farnesyl transferase pathways. It has been shown that prenylated proteins from these pathways play a pivotal role in RAS, HER2 and EGFR-related signal transduction to mediate tumor proliferation and anti-apoptotic activities (5, 6). Herein we present, the synergistic effect of simvastatin and alendronate, a bisphosphonate, on the down-regulation of cell proliferation and survival of PC3 prostate cancer cells. These two agents are known to affect cholesterol metabolism and biosynthesis by inhibiting the mevalonate pathway. We recently demonstrated the role of simvastatin in down-regulating tumor proliferation in prostate cancer cell lines (7) and bisphosphonates are already important therapeutic components in prostate cancer treatment. This finding holds promise for further studies to assess the role of such therapeutic approaches to treat prostate cancer or to use in conjunction with ADT to overcome androgen resistance in CRPC.

Materials and Methods

Agents. Simvastatin and alendronate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A stock solution of alendronate was prepared in dH2O and further dilutions were made in complete cell medium. A stock solution of simvastatin was prepared in 80% ethanol and 1 N NaOH, then activated by placing in 55°C water-bath for one hour before making further dilutions in complete medium.

Cell lines and culture conditions. PC-3, DU 145 and LNCaP prostate cancer cells and RWPE-1 non-tumorigenic prostate cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell lines PC-3 and LNCaP were grown in RPMI-1640 medium supplemented with 10% serum and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). DU 145 cells were grown in minimum essential medium MEM supplemented with 10% serum and penicillin/streptomycin (Invitrogen) RWPE-1 were grown in keratinocyte serum-free medium supplied with two additives: bovine pituitary extract and human recombinant epidermal growth factor. Medium was supplemented with 10% serum and penicillin/streptomycin (Invitrogen). For 3-[4,5-ethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and luminescent cell viability assays, PC-3, DU 145 and LNCaP cells were seeded at a density of 5,000/well and RWPE-1 cells at 20,000/well in 96-well plates, grown overnight and treated the next day. For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay experiments, the cells were seeded on 12-mm round coverslips from Fisher Scientific, (Hampton, NH, USA) or 6-mm Transwell-Clear™ filters from Corning Costar (Corning, NY, USA) at high density (~5×104 cells/cm2) to obtain confluency in 2-3 days. For immunoblotting analyses the cells were seeded on six well plates (Corning) and processed as described below.

MTT assay. The cells were treated with medium, simvastatin at concentrations of 1, 2, 4, 5 μM; alendronate at concentrations of 1, 2, 5, 10, 20, 40 μM; or a combination of the two drugs at the same concentrations. The medium was removed and dosages were repeated every 24 hours for 72 h. Following 72 h of treatment, MTT was added at 50 μg/well for 3 h (Invitrogen). Formazan products were solubilized with acidified sodium dodecyl sulfate (SDS) overnight and optical density was measured at 570 nm.

Cell-viability assay. The cells were treated as described above. The medium was removed and dosages were repeated every 24 h for 72 h. Following 72 h of treatment, the viability assay was conducted according to manufacturer’s instructions. Briefly, the plates were allowed to come to room temperature before adding 100 μl/well of CellTitre Glo® reagent from Promega (Madison, WI, USA). The plates were mixed and allowed to stand for 10 min in order to allow the luciferase reaction take place. This reaction requires ATP, an indicator of cellular metabolic activity. The luminescence signal is proportional to the amount of ATP present and was measured by luminometer.

SDS-PAGE and immunoblotting. Whole cell lysates were prepared as previously described(8). Briefly, SDS-PAGE was run by loading 35 μg of protein in each lane and blotted onto nitrocellulose or polyvinylidene fluoride (PVDF) membranes (Schleicher & Schuell Bioscience, Inc., Keene, NH, USA). The signal of primary monoclonal or polyclonal antibodies was detected using secondary affinity-purified goat anti-mouse or anti-rabbit immunoglobulins coupled to peroxidase and a chemiluminescent system (Pierce, Rockford, IL, USA) and exposed on X-ray film (Kodak, Rochester, NY, USA). The intensity of the bands was estimated by digitizing the image (Image J, from the National Institutes of Health, Bethesda, MD, USA) from x-ray film. After subtracting the background, all band intensities were compared against a control.

TUNEL assay. Cells were seeded at 75% confluence and treated with either vehicle, simvastatin, alendronate or a combination of both, at 2 μM. Following 24 h of treatment, the cells were processed according to the manufacturer’s instructions (Roche Applied Science, Mannheim, Germany). Briefly, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% sodium citrate and 0.1% Triton X. DNA fragmentation was determined by TUNEL. Fluorescent images were obtained using an EVOS fluorescence microscope (AMG, Bothell, WA, USA).

Statistical analyses. Preliminary analyses on all cell lines were conducted by calculating the IC50 value as the drug concentration necessary to inhibit 50% growth compared to untreated controls. Isobologram analysis was conducted using Compusyn software (CompoSyn, Inc., Paramus, NJ, USA). In order to confirm whether the effect of alendronate and simvastatin was additive or synergistic, further isobologram analysis was performed as described before (9) This was utilized as visual assessment of the interaction of the drugs with independent statistical analysis from calculations using total dose.
in a fixed-ratio combination and with calculated additive total dose for the same effect. Isobologram analyses were carried out using a graph of equally effective dose pairs (isoboles) for a single effect level. Specifically, with a given effect level of the combination, such as 50% of the maximum, doses of alendronate and simvastatin (each alone) were calculated to give this effect and were plotted as axial points on a Cartesian plot. For non-linear dose relationships, a surface of additivity was calculated and the different types of interaction were determined (synergy, additive effects, antagonism). Cell death was determined by MTT assay, after combined treatment. Envelopes of additivity were calculated with data from independently performed experiments. Data points below the curves show a synergistic effect, data points between the curves demonstrate an additive effect and those above the curves a sub-additive response to combined treatment. Initially, dose-response relationships for alendronate and simvastatin were assessed for different concentrations, plotted and fitted to a linear-quadratic and a logistic equation.

Results

Simvastatin greatly inhibits PC3 prostate cancer cell growth and metabolic activity compared to alendronate. Based on MTT assays, cytotoxic effects of simvastatin (Figure 1A) on PC3 human prostate cancer cells were more pronounced than those of alendronate (Figure 1B). Simvastatin exerted a dose-dependent inhibition of prostate cancer cell growth, while alendronate had a lesser effect. The same was observed when ATP cellular levels were measured (Figure 1C and D). Neither simvastatin nor alendronate have a cytotoxic effect on LNCaP prostate cancer cells. Androgen-dependent LNCaP cells displayed no significant cytotoxic effect after treatment with simvastatin or alendronate as compared to controls (Figures 2A and B). MTT assays were conducted at 72 h after dosing the cells every 24 h with the drugs at the concentrations described above.

Alendronate but not simvastatin inhibit DU 145 prostate cancer and RWPE-1 epithelial prostate cell growth. Alendronate had a significant cytotoxic effect on RWPE-1 epithelial prostate cells (Figure 2E) and a lesser effect on DU 145 prostate cancer cells (Figure 2C), with sustained cytotoxicity between 20 and 40 μM. Simvastatin had no significant effect on DU 145 (Figure 2D) or RWPE-1 (Figure 2F) cells as shown via MTT assays.

Certain low-dose combinations of simvastatin and alendronate produce synergistic inhibition of cell growth of androgen-independent PC3 prostate cancer cells. Combinations of simvastatin between 1 and 5 μM and alendronate between 1 and 40 μM were tested for their effect on survival of androgen-independent PC3 prostate cancer cells (Table I). Isobolograms derived from these data with points below the curve are indicative of a synergistic effect and are shown highlighted in Table I and in Figure 3 for combinations of simvastatin/alendronate at micromolar concentrations of 1/2 (Figure 3A), 5/1 (Figure 3B), 2/2 (Figure 3C), 2/10 (Figure 3D), 1/20 (Figure 3E) and 4/1 (Figure 3F). Select drug combinations that demonstrated an additive effect are shown in
Figure 4, as evidenced by data points located between the curves. These combinations of simvastatin/alendronate at micromolar concentrations were 1/40 (Figure 4A), 5/10 (Figure 4B), 5/40 (Figure 4C) and 4/20 (Figure 4D).

Synergistic combinations of simvastatin and alendronate target survival and proliferative pathways in PC3 cells. In order to determine the mechanism responsible for the synergistic effects on PC3 cells observed using the low-dose combinations of simvastatin and alendronate shown in Figure 2, proliferative, survival and apoptotic signaling pathways were examined. PC3 cells were dosed with either 2 μM of alendronate or simvastatin, or their combination at 2 μM each and the levels of activated AKT, ERK and c-JUN were analyzed. The phosphorylation of AKT at serine 473 was unaffected by alendronate. In contrast, simvastatin inhibited this activation by 36.05% (Figure 5A), an effect further enhanced to 46.01% by use of the combination of the two
ERK activation was increased approximately five-fold by alendronate as compared to the control. Simvastatin up-regulation of pERK, albeit impressive, was not as high at approximately 2.7-fold. Following the same trend, an increase of approximately 3.5-fold was observed after treatment with the combination of the two drugs (Figure 5A and C). p-c-JUN levels were unaffected by treatment with alendronate. In contrast, PC3 cells treated with simvastatin displayed an approximately two-fold increase in activated c-JUN, a level which was sustained after treatment with the combination of alendronate and simvastatin (Figures 5A and D).

PC3 cells treated with synergistic combinations of simvastatin and alendronate display an increased number of apoptotic cells. PC3 prostate cancer cells treated with synergistic combinations of simvastatin and alendronate displayed significant DNA fragmentation (green channel) compared to cells treated with each of the drugs alone (Figure 6). Confirmation of fragmented DNA localized to the cells nuclei was confirmed by staining with DAPI (blue channel).

**Discussion**

The mevalonate pathway is an important target in cancer biology and has been targeted with inhibitors of farnesyl transferase, as well as of geranylgeranyl transferase. However, the efficacies of inhibitors of this kind have been limited due to significant observed toxicities (10). Isoprenoids are the intermediate products of this pathway and involve covalent additions of either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid onto G-protein.

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**Figure 3.** Synergistic inhibition of prostate cancer PC3 cells viability by low-dose combinations of simvastatin and alendronate. Synergistic drug combinations of simvastatin and alendronate on prostate cancer PC-3 cells are shown in isobolograms derived from MTT data using total dose in a fixed-ratio combination and with calculated additive total dose for the same effect on PC-3 prostate cancer cells. Examples of these synergistic combinations are: A: 1 μM simvastatin and 2 μM alendronate; B: 5 μM simvastatin and 1 μM alendronate; C: 2 μM simvastatin and 2 μM alendronate; D: 2 μM simvastatin and 10 μM alendronate; E: 1 μM simvastatin and 20 μM alendronate; F: 4 μM simvastatin and 1 μM alendronate. Values shown are obtained from at least seven independent experiments.

**Figure 4.** Certain low-dose combinations of simvastatin and alendronate have an additive effect on the inhibition of PC-3 prostate cancer cell viability. Drug combinations of simvastatin and alendronate with an additive effect on prostate cancer PC-3 cells are shown in isobolograms derived from MTT data using total dose in a fixed-ratio combination and with calculated additive total dose for the same effect on PC-3 prostate cancer cells. Examples of these combinations are: A: 1 μM simvastatin and 40 μM alendronate; B: 5 μM simvastatin and 10 μM alendronate; C: 5 μM simvastatin and 40 μM alendronate; D: 4 μM simvastatin and 20 μM alendronate.

**Table 1.** Synergistic effect of various combinations of simvastatin and alendronate at micromolar concentrations (highlighted). These data were derived from calculations from at least seven independent experiments analyzed by MTT.

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subunits and nuclear laminins, thus helping facilitate protein interactions in addition to termination of activity of small GTPases (11). These proteins have important biological roles, including transmembrane signal transduction (RAS), cytoskeletal reorganization (RHO), gene expression (RAS), microtubule organization and nucleocytoplasmic transport. Farnesyl is the first isoprenoid produced in the mevalonate pathway (12) and inhibition of RAS farnesylation has proven effective in inhibiting RAS-dependent cell proliferation in vitro and in vivo (13). The development of farnesyl transferase inhibitors stemmed from the discovery of mutations of the RAS oncogene found in nearly 30% of human malignancies (13, 14).

Both alendronate and simvastatin inhibit enzymes in the mevalonate pathway. Alendronate is a farnesyl pyrophosphate synthase inhibitor and simvastatin is a HMG CoA reductase inhibitor. The combination of both drugs effectively inhibits the mevalonate cascade, as well as the generation of downstream isoprenoids and geranylgeranylation of proteins (15). By combining the use of common biological targets of the mevalonate pathway, we observed a synergistic response in the growth of the malignant androgen-independent PC3 prostate cancer cells, but not of androgen-dependent LNCaP or DU 145 prostate cancer cells.

Besides these sources of disruption of oncogenic proliferative pathways, it is important to note that many of the effects of statins currently under intense study are not solely related to their effect on the mevalonate cascade (16). Among these, immunomodulation (17-19), proteasome inhibition (20) and disruption of pathways associated with inflammation (21) and angiogenesis (22) are strong candidates in validating anti-neoplastic properties of statins (23, 24). Thus, the sequential inhibition of the mevalonate pathway by simvastatin and alendronate may not solely account for the observed synergistic effect on cellular cytotoxicity. Indeed, the combination of simvastatin and alendronate significantly down-regulated AKT phosphorylated on serine 473, a recognized survival marker. The inhibition of AKT was accompanied by the concomitant up-regulation of the activated forms of ERK and c-JUN, favoring the onset of apoptosis.

Figure 5. Analysis of survival/apoptotic signaling pathways in PC3 prostate cancer cells treated with simvastatin, alendronate, or a combination of both drugs. A: Analysis of the activated forms of AKT, ERK and c-JUN after treatment with of PC3 cells with 2 μM simvastatin, or 2 μM alendronate, or a combination of both for 12 h. The cells were lysed and the sodium dodecyl sulfate SDS eluates of treated cells were separated by polyacrylamide gel electrophoresis PAGE and an SDS extract of cells exposed to vehicle alone was run as a control (first lane). The immunoblots were performed with antibodies against phospho-AKT, phospho ERK and phospho c-JUN. The membranes were reprobed for actin as a loading control. The results presented are representative of three independent assays. B-D: Graphs showing the quantification of the ratios of p-AKT/actin, pERK/actin and p-c-JUN/actin; bands were normalized to the value of the untreated cells. The values are averages±standard deviations from three independent experiments (*p<0.05).
Previous studies in PC3 cells have demonstrated that the activation of ERK and c-JUN regulate the transcriptional activity of activator protein 1 and subsequent cell death (25). It has also been reported that these activations accompanied by the inhibition of pAKT effectively induce apoptosis (26).

Alendronate stimulates osteogenic differentiation via the activation of ERK in bone marrow stromal cells (27). ERK activation is indeed observed in prostate cancer cells when treated with alendronate, as shown in Figure 5. However, this effect is significantly diminished when treated in combination with low doses of simvastatin, thus eliminating the antagonistic effect of this kinase and effectively initiating apoptosis, as shown in Figure 6. Simvastatin activates c-JUN, which regulates the expression of several stress-responsive genes. This activation increases the stability of the molecule, thereby mediating apoptosis (28-30). The level of activation of c-JUN is maintained when the cells are treated with the combination of simvastatin and alendronate. Thus, these

Figure 6. TUNEL assay. Apoptotic effect of alendronate (2 μM), simvastatin (2 μM), or a combination of both drugs as determined by TUNEL assay (green channel). 4',6-diamidino-2-phenylindole DAPI (blue channel) was used to locate the nuclei of the cells. Control panels were PC-3 prostate cancer cells treated with vehicle only. TUNEL assay was conducted 24 h after treatment.
activations are likely to support the apoptotic cascade initiated by the inhibition of the mevalonate and phosphoinositide 3-kinase PI3 kinase pathways. These results are in line with previous observations of the inhibitory effect of alendronate on growth (31, 32) and migration (33) of prostate cancer cells, as well as the antitumor effect of simvastatin via AKT, ERK and c-JUN (34, 35). Previous studies have also demonstrated the apoptotic effect of simvastatin on prostate (36), breast (37) and esophageal (38) carcinoma cells. In the study, we showed that low-dose present combinations of simvastatin with alendronate may have significant synergistic anti-carcinogenic potential against androgen-independent prostate cancer and may have the potential to serve as a safer and better tolerated alternative than previously tried drugs. In vivo studies need to be undertaken to explore further these effects and establish the safety and efficacy of the drug combinations studied in this model.

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References


