Mechanistic Study of Inhibitory Effects of Atorvastatin and Docetaxel in Combination on Prostate Cancer

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Abstract. Aim: To investigate the effects and mechanisms of docetaxel and atorvastatin administered individually or in combination on prostate cancer cells. Materials and Methods: Cell growth and apoptosis were determined by the trypan blue exclusion assay and morphological assessment of cells was performed with propidium iodide. NF-ĸB activity was determined by luciferase reporter gene assay and the western blot assay was used to determine the levels of Bcl-2, phospho-Akt, VEGF, and phospho-Erk1/2. Results: Results showed that following pre-treatment with cholesterol, resistance of PC-3 prostate cancer cells to docetaxel was increased. The combination of docetaxel with atorvastatin potently inhibited growth and induced apoptosis in PC-3 cells. Mechanistic studies indicated that induction of apoptosis in PC-3 cells was associated with significant decreases in the levels of Bcl-2, phospho-Akt, VEGF, phosphor-Erk1/2. Conclusion: Treatment with cholesterol decreased the sensitivity of prostate cancer cells to docetaxel. Docetaxel in combination with cholesterol-lowering drugs such as atorvastatin may be an effective strategy for inhibiting the growth of prostate cancer.

Prostate cancer (PCa) is the fourth most common cancer in both sexes collectively and the second most common cancer in men. In 2012, an estimated 1.1 million cases were diagnosed worldwide, accounting for 15% of cancers diagnosed in men and almost 70% of them (759,000) occurring in more developed regions (1). According to Cancer Statistics, PCa accounts highest for 27% of newly diagnosed cancers in 2014 (2). Consequently, it is urgent to gain a better understanding of mechanisms driving PCa in developed countries and to develop new agents (3).

PCa incidence has been linked to western diet, which includes high levels of red meat, saturated fat, and dairy products (4, 5). Epidemiological studies have associated a fat/cholesterol enriched diet with hypercholesterolemia and an increased risk of PCa by causing intra-tumoral steroidogenesis, increased inflammation, and increased proliferation. For example, preclinical models involving feeding mice a high cholesterol diet have been shown to significantly increase the volume of human PCa xenografts (6). Meanwhile, cholesterol-lowering drugs reduce the risk of advanced PCa. Therefore, cholesterol has an important role on PCa progression.

Furthermore, normal prostate epithelial cells have abnormally high cholesterol content and cholesterol accumulates in solid tumors, with cholesterol levels further increasing during progression of PCa (7-11). Older studies also demonstrated that cholesterol homeostasis breaks-down in the prostate during aging and the transition to malignant state. Since cholesterol is a precursor to androgen production, a decrease in cholesterol levels can reduce PCa risk by decreasing the levels of circulating testosterone. This reduction of cholesterol reduces the levels of interprostatic dihydrotestosterone, which is a strong ligand for the androgen receptor and a target to PCa therapy (12-14). There is a certain potential relationship between cholesterol and PCa. Cholesterol has been also shown to accumulate in lipid...
rafts and regulate the activation of the phosphatidylinositol 3-kinase/Akt pathway (14). We investigated whether alterations of cholesterol in PCa will affect treatment refractory in vitro.

Docetaxel, one of the promising chemotherapeutic treatments for carcinomatosis is a member of the taxoid drug class, which is semi-synthetically produced from the needles of the Pacific yew tree (Taxus brevifolia). As an antineoplastic agent, docetaxel has a more beneficial affect against progressive human prostate cancers than that of conventional anticancer agents (15-19). The principal chemotherapeutic targets of docetaxel are microtubules, which cause cell-cycle arrest and apoptosis by increasing tubulin polymerisation, promoting microtubule assembly, and inhibiting tubulin depolymerisation. It has been assumed that docetaxel is able to induce the phosphorylation of Bcl-XL/Bcl-2 members and thus inactivate their anti-apoptotic capacity. The down-regulation of Bcl-2 and/or the up-regulation of p53 are certainly one of the important modes of apoptosis induction by docetaxel (20, 21). High concentrations of docetaxel greatly induce the formation of extensive bundles of microtubules and inhibit cell proliferation. However, use of high doses of docetaxel induce toxic reactions. In cancer clinical trials, tolerability and toxicity are concerns, particularly since most prostate cancer patients are elderly or have other medical problems and so the use of docetaxel as a monotherapy for cancer needs improvement (22, 23).

In an initial experiment, we tested the anti-proliferative effects of docetaxel on PCa cell line PC-3 and discovered that pre-treatment with cholesterol decreased the sensitivity of docetaxel. Treatments with agents that specifically target the biochemical synthesis of cholesterol may increase the sensitivity of docetaxel. Atorvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, has been approved for cholesterol reduction and considered to be among the safest drugs (14, 24, 25). The present study was therefore designed to explore the potential synergistic effect of docetaxel in combination with atorvastatin on growth and apoptosis in human prostate cancer cells.

Materials and Methods

Cell culture and reagents. The human prostate carcinoma cell lines PC-3(AR negative) and LNCaP (AR sensitive) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). PC-3 cells are human prostatic carcinoma cell lines derived from a bone metastasis of an androgen-independent prostatic adenocarcinoma that has a greatly reduced dependence upon serum for growth when compared to that of normal prostatic epithelial cells. The cells do not respond to androgens, glucocorticoids, epidermal, or fibroblast growth factors (26). LNCaP cells are androgen-sensitive human prostate adenocarcinoma cells derived from the left supraclavicular lymph node metastasis from a 50-year-old caucasian male in 1977. The PC-3 and LNCaP cells sustained exponential growth by being treated twice a week in RPMI 1640 tissue culture medium with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), penicillin (100 U/ml)-streptomycin (100 mg/ml), and L-glutamine (300 μg/ml). Atorvastatin and Docetaxel were provided by the National Cancer Institute. Cultured cells were placed into tissue culture flasks and dishes and grown at 37°C in a humidified atmosphere of 5% CO₂. Atorvastatin and docetaxel were dissolved in DMSO, and the final concentration of DMSO in all the experiments was 0.1%.

Determination of the number of viable cells. The number of viable cells after each treatment was determined by the trypan blue exclusion assay. After single cells were dissociated, they were seeded in 35-mm dishes containing growth mediums at a final cell concentration of approximately 2.5x10⁴ cells/ml and incubated at 37°C in an atmosphere of 5% CO₂. We used a hemocytometer under a light microscope (Nikon Optiphot, Nikon, Tokyo, Japan) to complete the measurement of cell viability, which was performed by mixing 80μl of cell suspension and 20 μl of 0.4% trypan blue solution for 2min. Blue cells were marked dead and the cells that did not absorb dye were marked alive.

Assessment of apoptotic cells by morphology and by activation of caspase-3. Apoptosis was determined by morphological assessment in cells stained with propidium iodide (PI). Apoptotic cells were identified by their classical morphological features such as nuclear condensation, cell shrinkage, and formation of apoptotic bodies. Cytospin slides were prepared after each experiment and cells were fixed with acetone/methanol (1:1) for 10 min at room temperature, followed by 10 min with PI staining (1 μg/ml in PBS: Phosphate Buffered Saline), and finally analyzed using a fluorescence microscope (Nikon Eclipse TE200, Nikon). At least 200 cells were counted in each sample and the percentage of apoptotic cells was presented.

Western blotting. After treatment, the cell lysates were prepared as described earlier. Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking nonspecific binding sites with blocking buffer, the membranes were incubated overnight at 4°C with primary antibodies (#2870 for Bcl-2, #4501 for phosphor-Akt, #4376 for phosphor-Erk1/2, and #9272 for total-Akt, all from Cell Signaling Co., Beverly, MA; ab46154 for VEGF from Abcam, Cambridge, MA, USA). β-actin (#4970 from Cell Signaling Co., Beverly, MA ) was used as a loading control. Following the removal of the primary antibodies, the membranes were then washed three times with TBS (PBS containing 0.05% tween 20) buffer at room temperature and later incubated with fluorochrome-conjugated secondary antibody (Santa Cruz Biotechnology Inc., CA, USA). The membrane was then washed with TBS three times. Final detection was done with an Odyssey infrared imaging system (Li-Cor Biotechnology, Lincoln, NE, USA).

NF-kB-dependent reporter gene expression assay. NF-kB transcriptional activity was measured by the NF-kB-Luciferase reporter gene expression assay. An NF-kB luciferase construct was stably transfected into PC-3 cells and a single stable clone of PC-3 cells that were stably transfected with the NF-kB luciferase reporter gene, became the PC-3/N cell line, which was used in the present study. PC-3/N cells were treated with docetaxel and atorvastatin.
individually or in combination for 24 h, and the NF-κB-luciferase activities were measured using the luciferase assay kits from Promega (Madison WI, USA). After treatments, the cells were washed with PBS and harvested in 1 x reporter lysis buffer. After centrifugation, 10 μl aliquots of the supernatants were measured for luciferase activity by using a Luminometer from Turner Designs Instrument (Sunnyvale, CA, USA). The luciferase activity was normalized against known protein concentrations and expressed as a percentage of luciferase activity in the control cells, which were treated with DMSO solvent. The protein level was determined by Bio-Rad protein assay kits (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions.

Statistical analyses. The potential synergistic effect of docetaxel or atorvastatin was assessed by the isobole method (27), using the equation AC/AE+BC/BE=combination index (CI). Ac and Bc represent the concentration of drug A and drug B used in the combination, and Ae and Be represent the concentration of drug A and B that produced the same magnitude of effect when administered alone. If CI is <1, then the drugs act in an antagonistic or additive manner, respectively. The analysis of variance (ANOVA) model with Tukey-Kramer adjustment was used for the comparison of growth inhibition of NF-κB activity and apoptosis ratios among different treatment groups at the end of the treatment.

Results

Pre-treatment with cholesterol affects the inhibitory effect of docetaxel on cultured prostate cancer cells. To determine whether pre-treatment with cholesterol affects the inhibitory effects of docetaxel on prostate cancer cells, we treated PC-3 cells with cholesterol for 5 days, then treated with docetaxel for 72h (Figure 1A), and finally determined cell viability by the trypan blue exclusion assay. As presented in Figure 1B, treatment with cholesterol reduced the inhibitory effects of docetaxel on cultured PC-3 cells, especially under 5 μM cholesterol. Cholesterol decreased docetaxel inhibition
activity by 72.7% and 22.4% following treatment of docetaxel 2 nM and 5 nM. The results indicate that cholesterol pre-treatment may suppress the inhibitory effects of docetaxel on PC-3 prostate cancer cells.

Effects of docetaxel and atorvastatin in combination on the growth and apoptosis of human prostate cancer. The growth-inhibitory activities of docetaxel and atorvastatin alone or in combination were assessed in two prostate cancer cell lines: PC-3 (androgen-independent) and LNCaP (androgen-dependent). The effects of different concentrations of docetaxel and atorvastatin on the growth and death of PC-3 and LNCaP cells were determined by using the trypan blue exclusion assay. As shown in Figure 2, nanomole concentrations (1-5 nM) docetaxel alone and in combination with atorvastatin caused a dose-dependent inhibition of the proliferation of both cell lines, with 5 nM docetaxel combined with 5 μM atorvastatin being the most effective treatment in both cell lines. Treatments with docetaxel (2 nM) or atorvastatin (5 μM) alone had small effects on the growth and death of PC-3 and LNCaP cells. The combination caused a momentous increase in the percentage of growth inhibition. As shown in Figure 2, potent additional inhibition occurred when docetaxel was combined with atorvastatin on the two prostate cancer cell lines, particularly PC-3 cells, which increased 35.25% and 29.76% of growth inhibition, compared to that of docetaxel alone in concentration of 2 nM and 5 nM. The increased number of dead cells suggests similar potency. With concentrations of 5 nM docetaxel combined with 5 μM atorvastatin, showing the more potent increases in inhibitory effect of the combined treatment in comparison to the treatments with single agent. This increase is 29.76% in PC-3 cells and 45.54% in LNCaP cells. As shown in Figure 2, lower concentrations of docetaxel (5 nM) in combination with atorvastatin (5 μM) produced a better inhibitory effect than docetaxel at higher concentrations (10 nM).

Morphological assessments of PC-3 and LNCaP cells after their exposure to either docetaxel (1-5 nM), atorvastatin (5 μM), or a combination of both drugs were performed by staining with propidium iodide, which stains the nuclei of cells. As shown in Table I and Figure 3, after combination treatment, more PC-3 and LNCaP cells showed morphological changes, characteristic of apoptosis in contrast to that observed in cells treated with each agent alone, indicating a more potent stimulatory effect on apoptosis.
The combination index (CI) for IC_{50} were calculated as 0.48 for PC-3 cells and 0.67 for LNCaP cells, respectively. The results (Figure 2, Figure 3 and Table I) indicate that the combination of docetaxel and atorvastatin synergistically inhibits the growth of cultured prostate cancer cells and induces cell apoptosis.

Effect of docetaxel and atorvastatin on NF-κB transcriptional activity. A luciferase reporter gene expression assay was used to determine the effect of docetaxel and atorvastatin when used alone or in combination on the activation of NF-κB. In initial experiments, PC-3/N cells were treated with docetaxel (1-5 nM) and atorvastatin (5 μM) individually or in combination for 24 h, and the activity of NF-κB in prostate cancer cells was reflected by luciferase activity. As shown in Figure 4, Treatment of PC-3/N cells with docetaxel (1, 2 or 5 nM) and atorvastatin (5 μM) alone or in combination for 48 h. Apoptosis was determined by morphological assessment. Each value is the means±S.E. from 3 different experiments. Differences in the number of apoptotic cells between a combination group and a single agent-treated group were analyzed by ANOVA with the Tukey-Kramer multiple comparison test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% apoptotic cells</th>
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<tbody>
<tr>
<td></td>
<td>LNCaP</td>
</tr>
<tr>
<td>Control</td>
<td>1.83±1.52</td>
</tr>
<tr>
<td>Doc (1 nM)</td>
<td>9.66±2.51</td>
</tr>
<tr>
<td>Doc (2 nM)</td>
<td>20.00±5.29</td>
</tr>
<tr>
<td>Doc (5 nM)</td>
<td>31.83±3.78</td>
</tr>
<tr>
<td>Ator (5 μM)</td>
<td>8.17±4.04</td>
</tr>
<tr>
<td>Doc (1 nM) + Atorn(5 μM)</td>
<td>18.67±6.50*</td>
</tr>
<tr>
<td>Doc (2 nM) + Ator(5 μM)</td>
<td>30.83±6.80*</td>
</tr>
<tr>
<td>Doc (5 nM) + Ator(5 μM)</td>
<td>39.67±3.21*</td>
</tr>
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The proposed strategy is beneficial to the treatment of PCa because it inhibits proliferation and induces apoptosis in human prostate cancer cells. The observed potent inhibitory effect in vitro suggests a direction to reduce docetaxel resistance and foster a more beneficial therapy for treating PCa in future clinical trials (29).

Initial treatments for PCa are generally androgen-ablation therapy, prostatectomy, radiation therapy, and cytotoxic therapies. For patients with androgen-independent prostate cancer, chemotherapy is used in the treatment of PCa. The role of hormones and chemotherapy is also important in the treatment of PCa. In general, in vitro therapy is usually repeated after a certain period of time, which is called a cycle. The extra cycle of chemotherapy is repeated after the second cycle of chemotherapy. This cycle treatment is called a cycle of chemotherapy. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. For patients with androgen-independent prostate cancer, hormone therapy is used in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa. The role of hormones and hormone therapy is also important in the treatment of PCa.
chemotherapy (30). However, many patients are not cured by these therapy treatments and cancer re-occurs and develops from androgen-dependent to androgen-independent prostate cancer (AIPC) (31). At present, treatment of AIPC remains futile. Docetaxel is one of the most promising chemotherapeutic treatments; however, the disease response, survival percentage, and toxicity effects of this anti-prostate cancer drug are not promising (32). Evidence from several studies suggests that hypercholesterolemia is associated with increased risk of aggressive prostate cancer. Cholesterol has...
been shown to accumulate in solid prostate tumors (12, 33). Studies have shown that docetaxel exhibits chemo resistance with cholesterol stimulatory effect (Figure 1). This suggests that decreasing cholesterol levels may be a novel strategy to increase the sensitivity of AIPC to docetaxel treatment. A large number of studies have shown that the combination of different anticancer agents at lower concentrations had even more potent effects on growth inhibition and apoptosis induction than the effects produced when either agent was used alone at higher concentrations.

In the present study, we found that combination of docetaxel and atorvastatin increased growth inhibition and apoptosis induction compared to that of either drug alone on cultured PC-3 (androgen-independent) or LNCaP (androgen-dependent) prostate cancer cells. As shown in Figure 2, potent inhibition occurred when docetaxel was combined with atorvastatin on the two prostate cancer cell lines, particularly PC-3 cells, compared to that of docetaxel alone at concentrations of 2 nM and 5 nM. The increased number of dead cells suggests similar potency. Moreover, our data demonstrated that the apoptotic cell rate of combined treatment of docetaxel and atorvastatin significantly increased both in PC-3 (p<0.001) and LNCaP (p<0.05) cell lines in comparison to the apoptotic cell rates of each agent used alone. Apoptotic cells were identified by general morphological features, including nuclear condensation, DNA breakdown, plasma and nuclear membrane intense convolution, cell shrinkage, and formation of apoptotic bodies (34, 35). Docetaxel combined with atorvastatin treatment cells showed the morphological changes typical of cells undergoing apoptosis such as shrinkage, blebbing and DNA fragmentation (Figure 3).

To help determine whether the strong effects on PC-3 cells following combination treatment were mediated through apoptosis-related signaling pathways, we evaluated the transcriptional activity of NF-κB, which activates genes associated with limitless replicative potential, angiogenesis, tissue invasion, metastasis, and the suppression of apoptosis in prostate cancer cells (36, 37). Previous studies have demonstrated that constitutive deactivation of NF-κB in human prostate cancer exhibits increased levels of apoptosis (36-39). Combined treatment of PC-3/N cells with docetaxel (5 nM) and atorvastatin (5 μM) showed that NF-κB activity was significantly inhibited (Figure 4), while no striking
effect was observed with docetaxel or docetaxel used alone (p<0.001). Besides, proteins encoded by Bcl-2 family genes are important regulators of programmed cell death and apoptosis and there is evidence that links NF-κB survival pathway with the upregulation of Bcl-2 (40-42). Bcl-2 expression was remarkably down-regulated in the combined treatment of docetaxel and atorvastatin (Figure 5). Overall, data from the trypan blue exclusion assay, along with the NF-κB luciferase reporter gene expression assay and western blot, provide strong evidence that docetaxel (5 nM) combined with atorvastatin (5 μM) effectively inhibits the growth and induces apoptosis in human prostate cancer cells.

Earlier studies have shown that the regulation of Akt pathway plays a central role in growth, proliferation, motility, survival, and angiogenesis in tumor cells (43, 44), and both normal and pathological angiogenesis are regulated predominantly by the vascular endothelial growth factors (45). Akt activation and VEGF overexpression prompt PCA progression (46). Results of the present study suggest that the combination of low concentrations of docetaxel and atorvastatin apparently decreased the level of phospho-Akt and VEGF, while each agent used alone even in higher concentration had very slight effects on the level of phospho-Akt and VEGF (Figure 5). Human prostate cancer cells contain cholesterol-rich lipid rafts that mediate epidermal growth factor (EGF)-induced and constitutive signaling through the Akt serine-threonine kinase (6). When the prostate cancer cells were treated with docetaxel combined with atorvastatin, cholesterol was down-regulated, lipid rafts were disrupted, VEGF and Akt phosphorylation were inhibited and autonomous cell survival was reduced. Another signaling pathway that is associated with prostate cancer growth and progression is the mitogen activation protein kinase (MAPK). Erk1/2 belongs to a subfamily of MAPK. The level of activated Erk1/2 increased with increasing Gleason score and tumor stage. In the present study, we found that the combination of docetaxel and atorvastatin had a potent effect on decreasing the level of Erk1/2 in the cells (Figure 5). Taken together, considering the molecular level results, simultaneous inhibition of Akt and Erk pathways and downregulation of VEGF and Bcl-2 may initiate a strong inhibitory effect on proliferation and a strong stimulatory effect on apoptosis in prostate cancer cells with the combined treatment of docetaxel and atorvastatin (47, 48).

In conclusion, the present study demonstrated that docetaxel and atorvastatin in combination strongly inhibited growth and induced apoptosis in human prostate cancer cells. The effects of docetaxel and atorvastatin on growth inhibition and apoptosis in prostate cancer cells were associated with inhibition of NF-κB activation, increased caspase-3 activation, and decreased levels of Bcl-2, VEGF, phospho-Akt, and phospho-Erk1/2. The combination of docetaxel and atorvastatin may be an effective adjuvant therapy for inhibiting the growth of prostate cancer.

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