Mechanistic Study of Inhibitory Effects of Metformin and Atorvastatin in Combination on Prostate Cancer Cells in Vitro and in Vivo

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Metformin is a commonly used drug for the treatment of type II diabetes and atorvastatin is the most prescribed cholesterol-lowering statin. The present study investigated the effects and mechanisms of metformin and atorvastatin in combination on human prostate cancer cells cultured in vitro and grown as xenograft tumor in vivo. Metformin in combination with atorvastatin had stronger effects on growth inhibition and apoptosis in PC-3 cells than either drug alone. The combination also potently inhibited cell migration and the formation of tumourspheres. Metformin and atorvastatin in combination had a potent inhibitory effect on nuclear factor-kappaB (NF-κB) activity and caused strong decreases in the expression of its downstream anti-apoptotic gene Survivin. Moreover, strong decreases in the levels of phospho-Akt and phospho-extracellular signal-regulated kinase (Erk)1/2 were found in the cells treated with the combination. The in vivo study showed that treatment of severe combined immunodeficient (SCID) mice with metformin or atorvastatin alone resulted in moderate inhibition of tumor growth while the combination strongly inhibited the growth of the tumors. Results of the present study indicate the combination of metformin and atorvastatin may be an effective strategy for inhibiting the growth of prostate cancer and should be evaluated clinically.

Key words: prostate cancer; atorvastatin; metformin; apoptosis; combination

Prostate cancer is one of the common types of malignant disease in males in the world and is the second leading cause of cancer-related death in the United States. The American Cancer Society estimates for 2016 approximately 180890 new cases will be diagnosed and 26120 men will die of the disease, accounting for about 8.3% of male cancer-related deaths.1) Androgen deprivation therapy is an effective treatment for advanced prostate cancer. Unfortunately, almost all patients treated with androgen deprivation therapy will progress to castration-resistant prostate cancer (CRPC).2) Docetaxel is the most commonly prescribed first-line chemotherapy for CRPC.4) Although docetaxel provides a modest (2.4-month) increase in median overall survival, many patients with CRPC cannot tolerate this cytotoxic chemotherapy due to advanced age, medical comorbidities, or limited bone marrow reserves.5) Therefore, the identification of an effective alternative therapy with less toxicity may lead to increased survival and an improved QOL.

Metformin is a well-established agent for the treatment of type II diabetes. In addition to its efficacy in lowering glucose levels, recent reports indicate it may have antitumor effects in various cancers, including prostate cancer.6) Diabetic patients receiving metformin have been shown to have a reduced cancer incidence and a decrease in cancer-specific mortality.7) Further, epidemiologic evidence revealed a decreased incidence of prostate cancer in men taking metformin,8,9) and both animal and in vitro models demonstrate its activity in prostate cancer cell lines.10,11) Finally, a recent study suggested statin use moderates the risk of prostate cancer among type II diabetic patients using metformin.12)

Epidemiological studies have shown a reduced risk of advanced prostate cancer with the use of statins.13–16) Statin use was also found to be associated with a reduction in the risk of biochemical recurrence in patients with prostate cancer17) and a decreased risk of cancer mortality.18) Recent studies have shown statin drugs including atorvastatin (Lipitor®) inhibit proliferation and induce apoptosis of prostate cancer cells.19,20) In our previous studies, we found that atorvastatin enhanced the inhibitory effect of docetaxel on the growth of prostate cancer cells.21) We also found atorvastatin and celecoxib in combination synergistically inhibited the growth and induced apoptosis in prostate cancer cells. This combination inhibited the progression of androgen-dependent LNCaP tumors to androgen independence and the growth of androgen-independent PC-3 prostate tumors in severe combined immunodeficient (SCID) mice more effectively than either agent alone.22,23) Clinically, however, cardiac toxicity is a concern for celecoxib with long-term use increasing the risk of cardiovascular events limiting the development of the combination of atorvastatin and celecoxib in prostate cancer.24)

Based on epidemiologic evidence and the preclinical data for metformin and atorvastatin individually in prostate cancer, we assessed the effects of metformin and atorvastatin alone or in combination on cultured prostate cancer cells and in xenograft prostate tumors in SCID mice. Metformin and atorvastatin in combination exhibited potent inhibitory effect on the growth of prostate cancer cells in vitro and in vivo. The drug combination strongly stimulated apoptosis in prostate cancer cells.
MATERIALS AND METHODS

Cells and Reagents The PC-3 human prostate carcinoma cell line (ATCC, Rockville, MD, U.S.A.) were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 Ag/mL). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. Metformin and atorvastatin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Atorvastatin was dissolved in dimethyl sulfoxide (DMSO). Metformin was dissolved in sterile water.

Assay of Cell Viability PC-3 were seeded at a density of 0.25×10⁵ cells/mL in 35 mm tissue culture dishes and incubated for 24h. The cells were then treated with metformin (1–10 mM), atorvastatin (2–20 µM) or metformin (2 mM)+atorvastatin (5 µM) for 72h. Du-145 cells and LNCaP were seeded at a density of 0.25×10⁵ cells/mL in 35 mm tissue culture dishes and incubated for 24h. The cells were then treated with metformin (2 mM) or atorvastatin (5 µM) alone or in combination for 72h. Viable cells were determined by the trypan blue exclusion assay. The hemocytometer was used for the measurement of cell viability and the number of viable cells was counted under a light microscope, which was performed by mixing 80 µL of cell suspension and 20 µL of 0.4% trypan blue solution for 2 min. Dead cells were stained as blue cells and live cells did not absorb the blue dye.

Determination of Apoptosis PC-3 cells were seeded at a density of 0.25×10⁵ cells/mL in 35 mm tissue culture dishes and incubated for 24h. The cells were then treated with metformin (2 mM) or atorvastatin (5 µM) alone or in combination for 72h. After treatment, cells were stained with propidium iodide (PI) and apoptosis was measured by morphological assessment. Cytospin slides were fixed with a mixture of acetone–methanol (50 : 50) for 10 min. After fixation, the slides were stained with propidium iodide (1 µg/mL in phosphate buffered saline (PBS)) for 10 min. Analysis of apoptosis was done using a fluorescence microscope (Nikon Eclipse TE200, Nikon, Japan). Classical morphological features of apoptosis were used to identify apoptotic cells.

Cell Migration Assay The migration ability of PC-3 cells was used by a scratch wound-healing assay. PC-3 cells were seeded at a density of 1×10⁵ cells/mL in 35 mm tissue culture dishes and incubated for 24h. An artificial wound was created by mixing 80 µL of medium in 35 mm tissue culture dishes and incubated for 24h. The cells were then treated with metformin (2 mM) and/or atorvastatin (5 µM) for 24h. The cells were washed with ice-cold PBS and added in the lysis buffer. The 10 µL aliquots of the supernatants were measured for luciferase activity by using Luminometer (Sunnyvale, CA, U.S.A.). After normalization the concentrations of proteins, the luciferase activity was expressed as percent of luciferase activity compared with DMSO-treated. The Bio-Rad protein assay kits was used to measure the protein level in each sample.

Western Blot Analysis PC-3 cells were seeded at a density of 1×10⁵ cells/mL of medium in 100 mm culture dishes and incubated for 24h. The cells were then treated with atorvastatin (5 µM) and metformin (2 mM) alone and in combination for 24h. The cells were lysed with 200 µL RIPA buffer that was supplemented with 10% protease inhibitor cocktail and 10% phosphatase inhibitor cocktail. The cells were scraped, lysate was collected in a microfuge tube and passed through a 25G ¾ needle to break up cell aggregates. Homogenates were centrifuged at 13000×g at 4°C for 15 min, and supernatant was obtained to use. The protein concentrations were determined with a Bio-Rad protein assay kit. The samples (30 µg protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis at 150 V for 60 min on 12% gradient gels. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After blocking with 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS) and Tween 20 (TBST), the membrane was incubated 12 h at 4°C with primary antibodies. β-Actin was used as a control. After incubation with the primary antibodies, the membrane was washed with TBST and then incubated with secondary antibodies for 90 min. The membranes were detected using an ECL kit (Thermo, Rockford, IL, U.S.A.). The images were analysed by Bio-Rad ChemiDoc™ XRS Imager. The levels of proteins were analyzed by optical density measurement.

NF-κB Luciferase Reporter Assay An NF-κB-luciferase reporter gene expression assay was used in this experiment.25) PC-3/N cells were seeded at a density of 0.2×10⁵ cells/mL of medium in 12-well plates and incubated for 24h. The cells were then treated with metformin (2 mM) and/or atorvastatin (5 µM) for 24h. The cells were washed with ice-cold PBS and added in the lysis buffer. The 10 µL aliquots of the supernatants were measured for luciferase activity by using Luminometer (Sunnyvale, CA, U.S.A.). After normalization the concentrations of proteins, the luciferase activity was expressed as percent of luciferase activity compared with DMSO-treated. The Bio-Rad protein assay kits was used to measure the protein level in each sample.

PC-3 Xenografts Male SCID mice were provided ad libitum access to sterilized drinking water and diet. PC-3 cells were obtained and suspended in a mixture of Matrigel and RPMI 1640 medium (1 : 1). Two million cells (0.1 mL) were injected subcutaneously into the right flank. After the formation of the xenograft tumors (about 4 weeks), mice with tumors were randomly assigned into 4 groups (each group had 10 mice). Mice in group 1 were intraperitoneally (i.p.) injected with vehicle, mice in group 2 were i.p. injected with atorvastatin (5 mg/kg), mice in group 3 were i.p. injected with metformin (200 mg/kg), and mice in group 4 were i.p. injected with atorvastatin (5 mg/kg)+metformin (200 mg/kg) three times a week for 14d. The vehicle consisted of propylene glycol, polysorbate 80, benzyl alcohol, ethanol and water (40:0.5:1:10:48.5). Tumor size (length×width) and body weight were measured three times a week. After experiment, mice were sacrificed and the xenograft tumors were excised and prepared for paraffin tissue sections.26) The animal experiment was approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC).

Statistical Analyses Statistical analyses were done by using the software InStat (GraphPad Software, Inc., La Jolla,
CA, U.S.A.). The ANOVA was used for the comparison of growth inhibition, apoptosis and nuclear factor-kappaB (NF-κB) in the in vitro studies, and for comparison of tumor size and body weight in the in vivo studies. The isobole method, using the equation $\frac{A_c}{A_e}+\frac{B_c}{B_e}=\text{combination index (CI)}$ was used to analyze the potential synergistic effect. 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. The combined effect of the drugs are considered to be antagonistic or additive if CI is >1 or <1, respectively. The analysis was performed at least in triplicate.

RESULTS

Effects of Metformin and Atorvastatin Alone or in Combination on the Growth and Apoptosis of PC-3, Du-145 or LNCaP Cells  Treatment of PC-3 cells with metformin or atorvastatin inhibited cell growth in a concentration-dependent manner (Figs. 1A, B). Treatment of PC-3 cells with 1 to 10 mM metformin for 72 h resulted in a 14 to 46% decrease in the number of viable cells when compared with control cells treated with DMSO solvent (Fig. 1A). Atorvastatin (2–20 µM) caused 2 to 58% decrease in the number of viable PC-3 cells (Fig. 1B). The number of viable cells was significantly lower in cells treated with 5–20 µM atorvastatin and 1–10 mM metformin as compared with the control (Figs. 1A, B; **p<0.01, ***p<0.001). The effects of metformin and atorvastatin in combination on the growth of PC-3 cells were also determined. We found that the drug combination caused a stronger growth inhibition as compared with either agent alone (Fig. 1C). Treatment with metformin (2 mM) or atorvastatin (5 µM) alone had little effect on the growth of PC-3 cells, whereas their combination caused a 50% decrease in the number of viable cells. The number of viable cells in the combination treatment group was significantly lower (p<0.001) than that in atorvastatin- or metformin-treated groups.

We found that IC$_{50}$ was 9.1 mM for metformin and 16.6 µM for atorvastatin. The combination of 2 mM metformin with 5 µM atorvastatin caused a ca. 50% decrease in the number of viable cells. Therefore, the CI for IC$_{50}$ was calculated as 0.52 indicating that the combination of metformin and atorvastatin synergistically inhibits the growth of PC-3 cells.

In additional experiments, we determined the effects of metformin and atorvastatin in combination on LNCaP (androgen-dependent) and Du-145 (androgen-independent) prostate cancer cells. We found that treatment with metformin (2 mM) and atorvastatin (5 µM) in combination had significant inhibition on the growth of Du-145 (Fig. 1D) and LNCaP cells (Fig. 1E). Our result indicated that the drug combination inhibited both androgen-dependent (LNCaP) and androgen-independent (Du-145 and PC-3) cells.

Representative micrographs of apoptotic cells are shown in Figs. 2A–D. As shown in Fig. 2E, treatment of PC-3 cells with atorvastatin (5 µM) alone resulted in 9% apoptotic cells and treatment with metformin (2 mM) alone resulted in 15% apoptotic cells. The combination of metformin (2 mM) and atorvastatin (5 µM) resulted in 31% apoptotic cells, significantly higher than the metformin or atorvastatin alone treated group (p<0.001).

Effects of Metformin and Atorvastatin Alone or in Combination on the Migration of PC-3 Cells  Migration rates
of PC-3 cells were determined by the scratch wound-healing assay and expressed as percentages of original scratch area. Figure 3A shows representative micrographs of cells treated with metformin (2 mM) alone or in combination with atorvastatin (5 µM). As shown in Fig. 3B, the migration rate was around 55.54% for cells treated with atorvastatin (5 µM) and 48.09% in cells treated with metformin (2 mM). Treatment with a combination of 2 mM metformin and 5 µM atorvastatin resulted in 25.56% migration rate. The migration rate was significantly lower in cells treated with the combination of metformin and atorvastatin than that in the cells treated with either drug alone (p<0.001).

Effects of Metformin and Atorvastatin Alone or in Combination on the Formation of Tumorsphere in PC-3 Cells

The forming efficiency and size of tumorspheres formed in PC-3 cells treated with metformin and atorvastatin were determined by sphere culture in serum-free medium. As shown in Figs. 4A to D, the number and size of PC-3 tumorspheres markedly decreased in the combination treatment group than in metformin- and atorvastatin-treated groups. As shown in Fig. 4E, the sphere forming efficiency was 0.44% in the control group, 0.28% in cells treated with atorvastatin (5 µM) and 0.42% in cells treated with metformin (2 mM). The sphere forming efficiency was 0.04% in cells treated with the drug combination (Fig. 4E). The sphere forming efficiency was significantly lower in the combination treatment group than the metformin-treated group (p<0.001) and the atorvastatin-treated group (p<0.01). The size of tumorspheres was also decreased by a combination of metformin (2 mM) and atorvastatin (5 µM), as evidenced by significantly decreased number of large tumorspheres (100−150 µm) by treating with metformin (2 mM) and atorvastatin (5 µM).

Inhibitory Effect of Metformin and Atorvastatin on NF-κB Transcriptional Activity

Treatment of PC-3/N cells...
with atorvastatin or metformin resulted in moderate decreases in NF-κB luciferase activity (Fig. 5). Treatment of the cells with the combination of metformin and atorvastatin resulted in a much stronger decrease in NF-κB luciferase activity than either agent alone (Fig. 5). The differences for the luciferase activity between the atorvastatin-treated group and the combination-treated group ($p<0.001$), and between the metformin-treated group and the combination-treated group ($p<0.01$) were statistically significant.

**Effects of Metformin and Atorvastatin on the Levels of Survivin, Phospho-Akt and Phospho-Extracellular Signal-Regulated Kinase (Erk)1/2**

The levels of phospho-Akt and phospho-Erk1/2 were determined by the Western blotting, and the levels of these proteins were analyzed by optical density measurement and normalized for actin.

Fig. 4. Effects of Metformin and Atorvastatin on the Formation of Tumorsphere in PC-3 Cells

The cells were seeded at a density of $1\times10^3$ cells/mL in 24 well plate and incubated for 14d in the presence of metformin (2mM), atorvastatin (5μM) or metformin (2mM)+atorvastatin (5μM). At the end of the 14-d incubation, micrographs were taken to measure the number and size of tumorspheres. Each value represents the mean±S.E. from three separate experiments. Statistical analysis was performed using ANOVA (**$p<0.01$, ***$p<0.001$).
percentage of initial tumor size and body weight. (A) Percentage of initial tumor size. (B) Body weight was expressed as percentage of initial body weight.

Inhibitory effects on PC-3 tumor growth while the combination of metformin and atorvastatin (Fig. 6). Treatment of PC-3 cells with metformin had a moderate effect on decreasing the level of phospho-Erk2, and atorvastatin alone caused small to moderate decrease in the level of phospho-Erk1 and Erk2. The combination of these two drugs had a strong effect on decreasing the level of phospho-Erk1/2 (Fig. 6). The level of Survivin decreased moderately in cells treated with metformin or atorvastatin alone and a strong decrease in the level of this protein was found in the cells treated with the combination of metformin and atorvastatin (Fig. 6).

In Vivo Effect of Atorvastatin and Metformin on the Growth of PC-3 Tumors As shown in Fig. 7A, treatment of the mice with atorvastatin or metformin alone had a small inhibitory effect on the growth of the PC-3 tumors, and the combination of the two drugs more potently inhibited the growth of the tumors. The mean±standard error (S.E.) for percentage of initial tumor size was $168.5\pm12.0$ for the control group, $145.9\pm6.8$ for the atorvastatin-treated group, $149.2\pm6.8$ for the metformin-treated group and $113.1\pm6.7$ for the combination-treated group. The average tumor size in the combination-treated group was significantly smaller than in the atorvastatin- or metformin-treated group ($p<0.05$). The percent initial body weight of each animal, shown in Fig. 7B, did not significantly decrease with the combination, atorvastatin or metformin alone.

**DISCUSSION**

In our current study, we demonstrated that the inhibitory effect of metformin and atorvastatin in combination on the growth of cultured PC-3 cells was much stronger than that of either agent alone. We also showed the combination had a potent effect on induction of apoptosis in PC-3 cells. In addition, the formation of tumorspheres in non-adherent serum-free cultures was strongly inhibited by the combination of metformin and atorvastatin suggesting a suppression on the cancer stem cells was demonstrated for the first time. Our in vivo study showed that atorvastatin or metformin alone had small inhibitory effects on PC-3 tumor growth while the combination exhibited a potent inhibitory effect on the growth of PC-3 xenograft tumors in immunodeficient mice. Results of the present study, coupled with epidemiological studies, provide a strong rationale for clinically evaluating the combination of metformin and atorvastatin in prostate cancer patients.

Metformin is a commonly used biguanide drug for type II diabetes treatment. Recent studies indicate that besides its anti-diabetic effect, metformin may have anti-tumor effects on various cancers, including prostate cancer. A preliminary phase-II trial suggested metformin may slow the progression of advanced prostate cancer. Understanding the mechanisms of this activity is critical prior to clinical evaluation. It has been shown that the type 1 insulin-like growth factor receptor (IGF-IR) up regulation induced by androgen was inhibited by metformin via disrupting membrane-initiated androgen signaling. A recent study showed that metformin inhibited epithelial–mesenchymal transition in prostate cancer cells via up regulation of miR30a and down regulation of SOX4. Metformin was also shown to enhance the antiproliferative and apoptotic effects of the androgen receptor inhibitor bicalutamide in prostate cancer. In the present study, we found that metformin alone had a moderate effect on NF-κB activity. Metformin also had a weak inhibitory effect on Erk2 and Survivin. These results, together with findings from the studies described above, indicate that multiple mechanisms may be involved in the inhibitory effects of metformin on prostate cancer cells.

Statins inhibit the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase leading to decrease in the biosynthesis of cholesterol. Statin drugs are commonly used as a safe and effective treatment for hypercholesterolemia. In addition to their effect on lowering cholesterol, statin drugs were found to inhibit cell proliferation and stimulate apoptosis. Statins were shown to inhibit NF-κB, an important transcription factor for regulation of cell growth and death. The target of the statins, HMG-CoA reductase, is involved in the generation of farnesylpyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). FPP and GGPP are required for the process of Ras activation which is important for regulating the cell growth and apoptosis. Our present study showed that atorvastatin inhibited NF-κB activation and reduced the levels of phospho-Akt and phosphor-Erk, the downstream targets of the Ras pathway.
Molecular mechanisms for the combined effect of statins and metformin in prostate cancer cells are still largely unknown. Recent studies showed that metformin in combination with simvastatin induced G1-phase cell cycle arrest, and Ripk1- and Ripk3-dependent necrosis in prostate cancer cells.\textsuperscript{38,39} The combination of metformin and simvas- tatin was found to decrease the levels of phospho-Akt and phospho-AMPK\(_{1/2}\).\textsuperscript{40} The inhibitory effect and mecha- nisms of metformin in combination with atorvastatin have previously not been reported. In the present study, we found the potent effects of metformin in combination with atorvas- tatin on prostate cancer cells were associated with a strong inhibition of the activation of the transcription factor NF-\kappa B. We also found the drug combination strongly decreased the levels of phospho-Akt and phospho-Erk, indicating an inhibition on the activation of these proteins. Our results suggest the combination of atorvastatin and metformin targets multiple signaling pathways regulating prostate cancer cell growth and survival. Simultaneous inhibition of these important pathways may result in a strong growth inhibition and apoptosis in prosta- tice cancer cells.

In the in vivo study, SCID mice bearing PC-3 xenograft tumors were treated with metformin alone or in combination with atorvastatin. Metformin or atorvastatin alone only had small inhibitory effect on the growth of PC-3 xenograft tumors. Treatment of the mice with a combination of metformin and atorvastatin caused stronger inhibition than either drug used individually on the growth of PC-3 tumors. Treatment of the mice with metformin (200 mg/kg) and atorvastatin (5 mg/kg) alone or in combination did not cause body weight loss. In the end of the experiment, no abnormalities was found in major organs of the mice. These results indicate that metformin and atorvastatin were not toxic to the mice at the dose used in our experiment.

In conclusion, our study demonstrates that metformin in combination with atorvastatin had strong inhibitory effect on the growth and strong stimulatory effect on apoptosis in human prostate cancer cells. The potent combined effects of these two drugs were associated with suppression of NF-\kappa B, decreases in the levels of Survivin, phosphorylated Akt and phosphorylated Erk1/2. Moreover, our in vivo study indicates a strong combined effect of metformin and atorvastatin on in- hibiting the growth of PC-3 tumors in SCID mice. Combined metformin with atorvastatin may represent an effective approach for inhibiting prostate cancer and should be evaluated clinically.

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Conflict of Interest The authors declare no conflict of interest.

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