

Combination of Lipitor and Celebrex Inhibits Prostate Cancer VCaP Cells *In Vitro* and *In Vivo*

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Abstract. *Background/Aim:* Lipitor is a cholesterol-lowering drug and Celebrex is a Cyclooxygenase-2 inhibitor. We investigated the effects of Lipitor and Celebrex on human prostate cancer VCaP cells cultured in vitro and grown as orthotopic xenograft tumors in SCID mice. *Materials and Methods:* Apoptosis was measured by morphological assessment and caspase-3 assay. Nuclear factor-kappa B (NF- κ B) activation was determined by luciferase reporter assay. B-cell lymphoma-2 (Bcl2) was measured by western blotting and immunohistochemistry. Orthotopic prostate tumors were monitored by the IVIS imaging system. *Results:* the combination of Lipitor and Celebrex had stronger effects on the growth and apoptosis of VCaP cells than did either drug alone. The combination more potently inhibited activation of NF κ B and expression of Bcl2 than either drug alone. The growth of orthotopic VCaP prostate tumors was strongly inhibited by treatment with the drug combination. *Conclusion:* Administration of Lipitor and Celebrex in combination may be an effective strategy for inhibiting the growth of prostate cancer.

Prostate cancer represents one of the most frequently diagnosed malignancies in men in the United States (1). Although anti-androgen and chemotherapy options are

available for patients with advanced prostate cancer, these treatment options are only temporarily effective (2-4). In addition, anti-androgen and chemotherapy are considerably toxic to the patients. Therefore, novel and less toxic approaches for delaying the progression of prostate cancer to androgen independence would be of great benefit for patients.

Epidemiological studies showed that use of statin drugs is associated with a reduced risk of advanced prostate cancer (5-8). Recent clinical studies found that statin use was associated with a reduction in the risk of biochemical recurrence in patients with prostate cancer (9) and a decreased risk of cancer mortality (10). Statin drugs including Lipitor (atorvastatin) were found to inhibit proliferation and induce apoptosis of prostate cancer cells (11-12). Celebrex (celecoxib) is a selective Cyclooxygenase 2 (COX2) inhibitor. Many studies indicate that Celebrex has activity in prostate cancer (13-16). Studies from our laboratory showed that Lipitor and Celebrex in combination synergistically inhibited the growth and induced apoptosis of cultured 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 SCID mice more effectively than either agent alone (17, 18). Additional studies showed that the combination of Lipitor and Celebrex had a stronger effect on decreasing the levels of interleukin-6 (IL6) in cultured prostate cancer cells and in xenograft prostate tumors (19). We also found that survivin, a downstream effector of IL6/signal transducer and activator of transcription 3 (STAT3) signaling pathway, was decreased in cultured prostate cancer cells and in xenograft tumors when treated with Lipitor and Celebrex (19). Since nuclear factor-kappa B (NF κ B) is known to activate IL6 expression (20), it is of interest to determine the effect of Lipitor and Celebrex on activation of NF κ B and its downstream gene expression in

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prostate cancer cells. In the present study, we determined the effect of Lipitor and Celebrex alone and in combination on activation of NF κ B and on expression of its downstream gene B cell lymphoma 2 (*Bcl2*). We found that the combination of Lipitor and Celebrex had a more potent inhibitory effect on NF κ B activation and Bcl2 expression than either drug alone. This drug combination also strongly inhibited the growth of orthotopic VCaP prostate tumors and decreased the level of Bcl2 in the tumors.

Materials and Methods

Cells and reagents. VCaP cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Lipitor and Celebrex were provided by the National Cancer Institute's Repository. Matrigel was obtained from BD Biosciences (Bedford, MA, USA). RPMI-1640 tissue culture medium, penicillin-streptomycin, L-glutamine and fetal bovine serum (FBS) were from Gibco (Grand Island, NY, USA). VCaP cells were maintained in RPMI-1640 culture medium containing 10% FBS that was supplemented with penicillin (100 units/ml)-streptomycin (100 μ g/ml) and L-glutamine (300 μ g/ml). Cultured cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and were passaged twice a week. Proliferating VCaP cells at about 70% confluence were used for the animal experiment.

Determination of the number of viable cells. The number of viable cells after each treatment was determined using a hemacytometer under a light microscope (Nikon Optiphot; Nikon Co., Tokyo, Japan). Cell viability was determined by the trypan blue exclusion assay, which was performed by mixing 80 μ l of cell suspension and 20 μ l of 0.4% trypan blue solution for 2 min. Blue cells were counted as dead cells and the cells that did not absorb dye were counted as live cells.

Assessment of apoptotic cells by morphology and by activation of caspase-3. Apoptosis was determined by morphological assessment in cells stained with propidium iodide (21, 22). Briefly, 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 propidium iodide staining [1 μ g/ml in phosphate buffered-saline (PBS)] and analyzed using a fluorescence microscope (Nikon Eclipse TE200, Nikon Co., Tokyo, Japan). Apoptotic cells were identified by classical morphological features including nuclear condensation, cell shrinkage, and formation of apoptotic bodies (21, 22).

Caspase-3 activation was measured using an EnzoLyte AMC Caspase-3 Assay Fluorimetric kit (AnaSpec, Fremont, CA, USA) following the manufacturer instructions (23). Briefly, 1 \times 10⁵ cells were plated in triplicate in a flat-bottomed 96-well plate. After treatment with Lipitor/Celebrex, caspase-3 substrate was added to each well. Plates were incubated for 30 min at room temperature. Fluorescence intensity was measured in a Tecan Infinite M200 plate reader (Tecan US Inc., Durham NC, USA).

NF κ B-dependent reporter gene expression assay. NF κ B transcriptional activity was measured by the NF κ B-luciferase reporter gene expression assay. An NF κ B luciferase construct was stably transfected into VCaP cells and a single stable clone, VCaP/N

(24), was used in the present study. In brief, VCaP/N cells were treated with Lipitor or Celebrex alone and in combination for 24 h, and the NF κ B-luciferase activity was measured using the luciferase assay kit from Promega (Madison, WI, USA). After treatment, the cells were washed with ice-cold PBS and harvested in 1 \times reporter lysis buffer. After centrifugation, 10 μ l 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 the percentage of luciferase activity in the control cells, which were treated with dimethyl sulfoxide (DMSO) solvent. The protein level was determined by Bio-Rad protein assay kits (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Western blotting. After treatment, the cells were washed with ice-cold PBS and lysed with lysis buffer (10 mmol/l Tris-HCl (pH 7.4), 50 mmol/l sodium chloride, 30 mmol/l sodium pyrophosphate, 50 mmol/l sodium fluoride, 100 μ mol/l sodium orthovanadate, 2 mmol/l iodoacetic acid, 5 mmol/l ZnCl₂, 1 mmol/l phenylmethylsulfonyl fluoride, and 0.5% Triton X-100). The lysates were centrifuged at 12,000 \times g at 4°C for 25 min, and protein concentrations in the supernatant fractions were determined with a Bio-Rad protein assay kit according to the manufacturer's instructions. Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 110 min on 4%-12% gradient gels. Separated proteins were transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). After blocking nonspecific binding sites with blocking buffer, the membrane was incubated overnight at 4°C with primary antibody to Bcl-2 (05-729, Millipore Co, Billerica, MA, USA). β -Actin was used as a loading control. Following removal of the primary antibody, the membrane was washed three times with TBS (PBS containing 0.05% tween 20) buffer at room temperature and then incubated with fluorochrome-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz CA, USA). The membrane was then washed with TBS three times. Final detection was carried out with an Odyssey infrared imaging system (Li-Cor Co., Lincoln, NE, USA).

Orthotopic xenograft VCaP tumors in SCID mice. Male SCID mice were obtained from Taconic Farms Inc. (Germantown, NY, USA). The animals were housed in sterile filter-capped microisolator cages and were provided with sterilized 5010 rodent diet and water. VCaP cells were transfected with a luciferase-expressing vector using the Lipofectamine™ 2000 (LF2000; Invitrogen Life Technology, San Diego, CA, USA). A single clone of VCaP cells that stably expressed luciferase (VCaP-luc) was obtained and used in the present study. VCaP-luc cells (1 \times 10⁶ cells/0.05 ml/mouse) suspended in 50% Matrigel (Collaborative Research, Bedford, MA, USA) in RPMI-1640 medium were injected into the prostatic capsule of SCID mice. Four weeks after the injection, mice were examined by the IVIS imaging system to monitor the growth of orthotopic prostate tumors. Mice with orthotopic tumors were fed AIN76A diet (9 mice) or AIN76A diet containing 0.02% Lipitor and 0.05% Celebrex (10 mice) for 28 days. Growth of the orthotopic prostate tumors was determined by the IVIS imaging system. The animal experiment was carried-out under an Institutional Animal Care and Use Committee-approved protocol (RU 02-001).

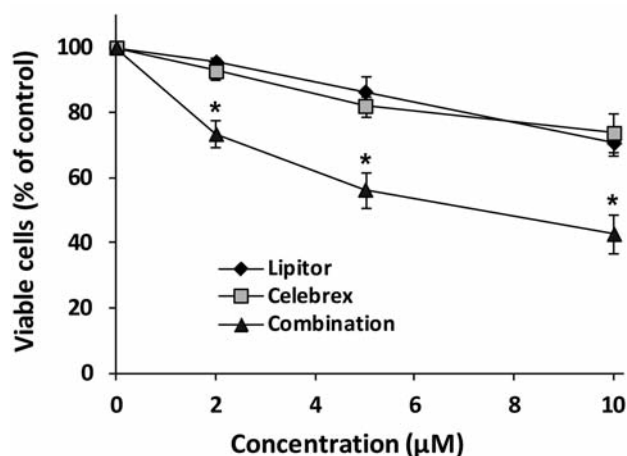


Figure 1. Effect of Lipitor and Celebrex alone and in combination on the growth of VCaP prostate cancer cells. VCaP cells were seeded at a density of 0.2×10^5 cells/ml in 35-mm tissue culture dishes (2 ml/dish) and incubated for 24 h. The cells were then treated with different concentrations of Lipitor or Celebrex alone and in combination for 96 h. The number of viable cells was determined by the trypan blue assay and expressed as a percentage that of solvent-treated control. Each value is the mean \pm S.E. from three separate experiments. Differences for the number of viable cells between a combination group and a single agent-treated group were analyzed by ANOVA with the Tukey-Kramer multiple comparison test. * $p < 0.001$ as compared to Lipitor or Celebrex alone.

Statistical analyses. Analysis of variance (ANOVA) method with the Tukey-Kramer test was used for the comparison of growth inhibition and apoptosis. Student *t*-test was used to determine the difference of tumor size between the control group and the treated group.

Results

Effects of Lipitor and Celebrex alone or in combination on the growth and apoptosis of human prostate cancer VCaP cells. The effects of different concentrations of Lipitor and Celebrex on the growth and death of VCaP cells were determined using the trypan blue assay. VCaP cells were treated with different concentrations of Lipitor (2–10 μ M) or Celebrex (2–10 μ M) for 96 h. We found that treatment of VCaP cells with Lipitor or Celebrex inhibited cell growth and caused cell death in a concentration-dependent manner. Treatment with Lipitor (2 μ M) or Celebrex (2 μ M) alone had little effect on the growth and death of VCaP cells, while their combination caused a 27% decrease in the number of viable cells, as compared to the control (Figure 1). Treatment with Lipitor (5 μ M) or Celebrex (5 μ M) alone caused a 14% and 18% decrease in the number of viable cells, respectively, and a combination of Lipitor and Celebrex (both at 5 μ M) caused a 43% decrease in viable cells (Figure 1). At a higher concentration (10 μ M), Lipitor and Celebrex in combination also caused a stronger decrease in cell viability than either agent alone (Figure 1).

Table I. Effect of Lipitor and Celebrex on apoptosis of VCaP cells. VCaP cells were seeded at a density of 0.2×10^5 cells/ml and incubated for 24 h. The cells were then treated with Lipitor (10 μ M) or Celebrex (10 μ M) alone or in combination for 96 h. Apoptosis was determined by morphological assessment and caspase-3 assay. Each value is the mean \pm S.E. from three experiments. Differences for the number of apoptotic cells and caspase-3 activation between a combination group and a single agent-treated group were analyzed by ANOVA with the Tukey-Kramer multiple comparison test.

Treatment	Apoptotic cells (%)	Caspase-3 activity
Control	2.3 \pm 0.3	1.0
Lipitor	15.2 \pm 2.6	6.7 \pm 1.2
Celecoxib	12.7 \pm 3.6	6.4 \pm 2.1
Combination	37.8 \pm 6.1*	18.1 \pm 3.4*

* $p < 0.001$ as compared to Lipitor or Celebrex alone.

Morphological assessment and caspase-3 assay were used to determine the effect of Lipitor and Celebrex on stimulating apoptosis in VCaP cells. As shown in Table I, treatment of VCaP cells with Lipitor or Celebrex alone resulted in 15% and 13% apoptotic cells, respectively. A combination of Lipitor and Celebrex increased the proportion of apoptotic cells to 38%. This drug combination also caused a stronger increase in caspase-3 activation than either drug alone (Table I).

Effects of Lipitor and Celebrex on NF- κ B transcriptional activity. An NF- κ B-luciferase reporter gene expression assay was used to investigate the effect of Lipitor and Celebrex on activation of NF- κ B. In the experiments, VCaP/N cells were treated with Lipitor (10 μ M) and Celebrex (10 μ M) alone or in combination for 24 h. Treatment of VCaP/N cells with Lipitor (10 μ M) or Celebrex (10 μ M) alone caused a modest decrease in luciferase activity, and the combination of Lipitor and Celebrex (both at 10 μ M) had a stronger effect than either agent alone (Figure 2).

Effects of Lipitor and Celebrex on the level of Bcl2. The level of Bcl2 in VCaP cells was determined by western blot analysis using an antibody against Bcl2. In the experiments, VCaP cells were treated with Lipitor (10 μ M) or Celebrex (10 μ M) alone or in combination for 24 h and analyzed by western blotting. Treatment of VCaP cells with Lipitor (10 μ M) or Celebrex (10 μ M) alone resulted in little or no change in the level of Bcl2, and the combination of Lipitor and Celebrex (both at 10 μ M) caused a stronger decrease in the level of Bcl2 than either agent alone (Figure 3). The extent of protein loading was determined by blotting for β -actin.

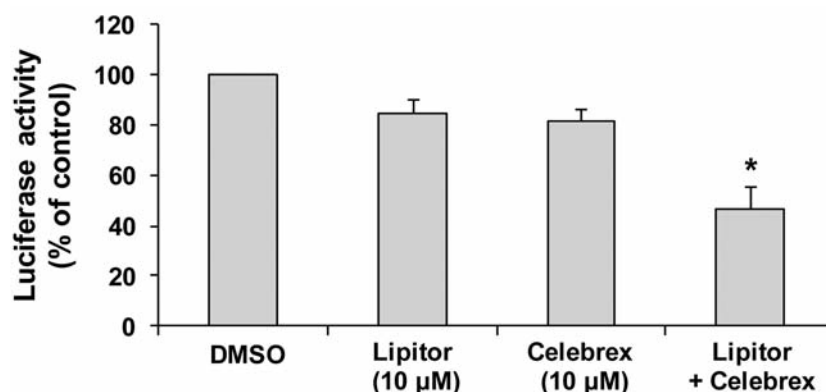


Figure 2. Inhibitory effect of Lipitor and Celebrex alone or in combination on NFκB activation in VCaP prostate cancer cells. VCaP/N cells were seeded at a density of 0.2×10^5 cells/ml of medium in 12-well plates and incubated for 24 h. The cells were then treated with Lipitor (10 μM) alone or in combination with Celebrex (10 μM) for 24 h. The NFκB transcriptional activity was measured by a luciferase activity assay. Differences in the NFκB transcriptional activity between the combination group and single agent-treated group were analyzed by ANOVA with the Tukey-Kramer multiple comparison test. * $p < 0.001$ as compared to Lipitor or Celebrex alone.

Effect of Lipitor and Celebrex on orthotopic VCaP xenograft tumors. In this experiment, mice with established orthotopic VCaP tumors, as determined by the IVIS imaging system, were fed AIN76A diet or AIN76A diet containing 0.02% Lipitor and 0.05% Celebrex for 28 days. Growth of the orthotopic VCaP tumors was monitored by the IVIS imaging system. We found that treatment of the mice with Lipitor combined with Celebrex significantly inhibited the growth of orthotopic VCaP tumors (Figure 4). Representative IVIS images of a mouse in the control group before and after treatment, and a mouse in the Lipitor and Celebrex-treated group before and after treatment are shown in Figure 4. Expression of Bcl2 in the tumors was determined by immunohistochemistry. We found that treatment of mice with Lipitor and Celebrex reduced the levels of Bcl2 immunostaining in the tumors. Representative micrographs of Bcl2 immunohistochemical staining in a VCaP tumor from the control group (Figure 4E) and a VCaP tumor from the Lipitor and Celebrex-treated group (Figure 4F) are shown.

Discussion

In the present study, we found that Lipitor and Celebrex in combination had a stronger inhibitory effect than either drug alone on the growth of cultured VCaP prostate cancer cells. This drug combination also strongly inhibited the transcriptional activity of NF-κB in the cells. In addition, Lipitor in combination with Celebrex more potently reduced the level of Bcl2 expression in VCaP cells than either drug used individually. NFκB is an important cellular regulator of growth and apoptosis in a variety of cells including prostate cancer cells (25-27). NFκB signaling has been shown to play

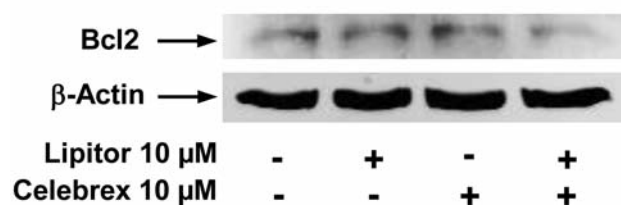


Figure 3. Effect of Lipitor and Celebrex on the level of B cell lymphoma 2 (Bcl2) in VCaP prostate cancer cells. VCaP cells were seeded at a density of 1×10^5 cells/ml of medium and incubated for 24 h. The cells were then treated with Lipitor (10 μM) or Celebrex (10 μM) alone and in combination for 48 h. Bcl2 was determined by western blotting using an antibody to Bcl2 (05-729, Millipore Co, Billerica, MA, USA).

an important role in prostate cancer growth, angiogenesis, tumorigenesis and metastatic progression (28-31). Inhibition of NFκB was shown to increase cell transmembrane receptor Fas-mediated (32) and 12-O-tetradecanoylphorbol-13-acetate-induced (33) apoptosis of PC-3 cells, and to reverse the resistance of prostate cancer cells to docetaxel (34). Inhibition of NFκB signaling may be an effective strategy for enhancing anticancer treatment in patients with prostate cancer.

In the animal experiment, we found that oral administration of 0.02% Lipitor and 0.05% Celebrex in the AIN76A diet to male SCID mice for four weeks resulted in a strong inhibition in the growth of VCaP orthotopic tumors. Our previous study showed that oral administration of 0.02% Lipitor in AIN76A diet to male SCID mice for two weeks resulted in a serum concentration of 6.1 ng/ml (35). This serum drug concentration is comparable to the level of

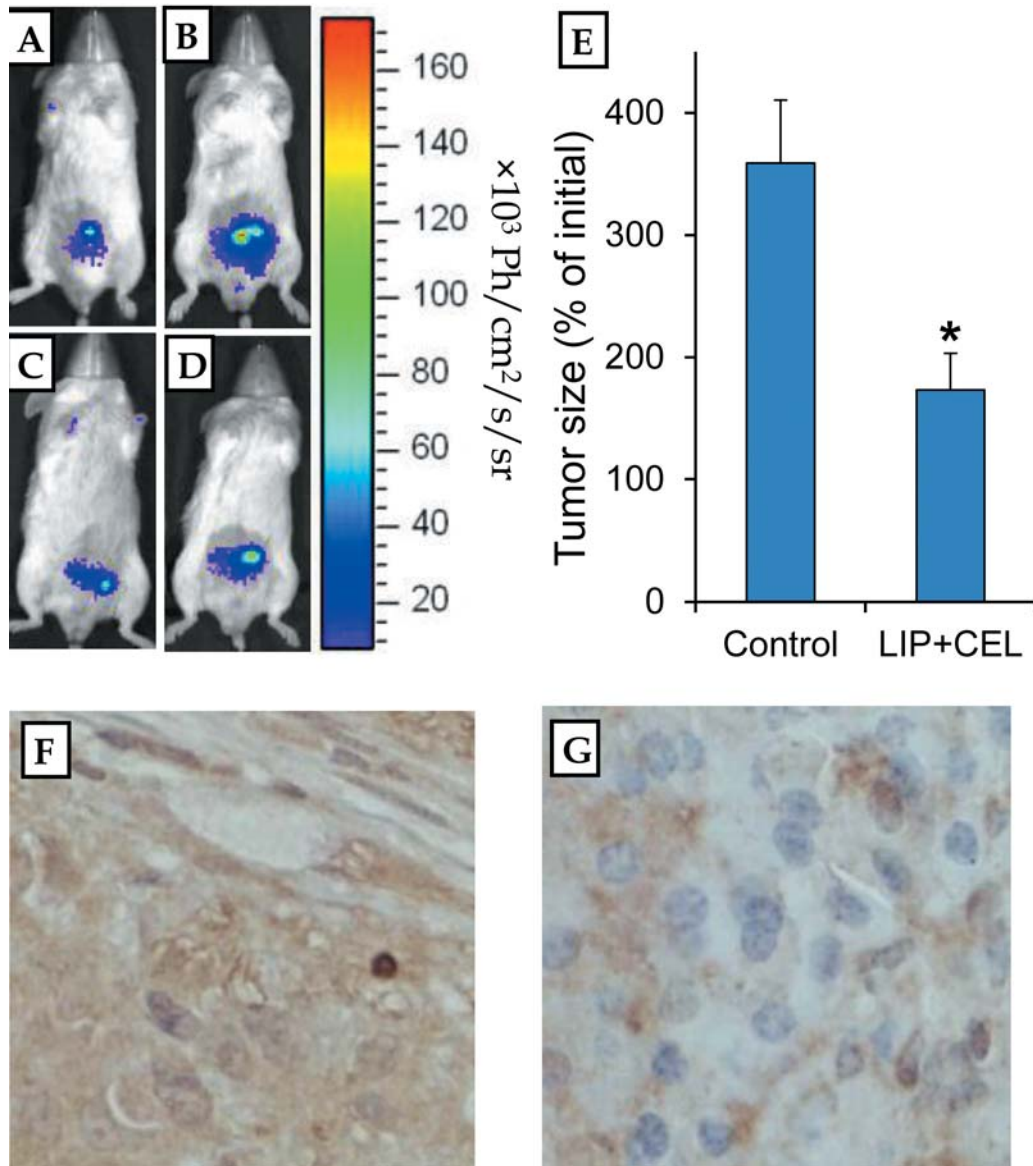


Figure 4. Effect of dietary Lipitor and Celebrex on the growth of orthotopic VCaP prostate cancer tumors in SCID mice and expression of B-cell lymphoma-2 (Bcl2) in the tumors. SCID mice were injected orthotopically with VCaP-luc cells (1×10^6 cells/mouse). After four weeks, mice with established VCaP tumors were fed AIN76A diet (9 mice) or AIN76A diet containing 0.02% Lipitor and 0.05% Celebrex (10 mice) for 28 days. Tumor size in each mouse was determined once a week using the IVIS system. Representative images are shown. A and B: A mouse of the control group before and after treatment; C and D: a mouse of the group treated with Lipitor and Celebrex before and after treatment. E: Percentage of initial tumor size in the control group and the group treated with Lipitor (LIP) and Celebrex (CEL). Each value represents the mean \pm S.E. Differences in the tumor size between the control group and the treatment group were analyzed by Student t-test. * $p < 0.001$. F: A representative micrograph of immunohistochemistry in a tumor from the control group shows brown staining of Bcl2. G: A representative micrograph of immunohistochemistry in a tumor from the Lipitor and Celebrex-treated group shows reduced brown staining of Bcl2.

~7 ng/ml reported in humans who had an oral administration of 20 mg Lipitor (36). After oral administration of Lipitor (20 mg) once a day for 14 days to humans, the peak plasma level was 15 ng/ml (37). It was also reported that oral administration of Celebrex (200 mg) to humans resulted in

a peak plasma level of 600-1300 ng/ml (38). Our previous study showed that oral administration of Celebrex (0.05% in the AIN76A diet) for two weeks in male SCID mice resulted in a plasma level of 1,090 ng/ml (35). Taken together, our results indicate that the serum levels of Lipitor

and Celebrex associated with their inhibitory effect on the growth of VCaP prostate tumors in SCID mice are achievable in humans.

In summary, the results of the present study demonstrate that a combination of Lipitor and Celebrex has a potent inhibitory effect on the growth of cultured prostate cancer VCaP cells than either drug alone. Inhibition of proliferation and stimulation of apoptosis in VCaP cells are associated with a decrease in the transcriptional activity of NF- κ B, and with a decrease in the expression of Bcl2, a downstream gene of the NF κ B signaling pathway. *In vivo* study demonstrates that oral administration of Lipitor with Celebrex strongly inhibits the growth of VCaP orthotopic tumors and the expression of Bcl2. Our results suggest that Lipitor in combination with Celebrex may be an effective regimen for inhibiting prostate cancer.

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