

# Androgen receptor inactivation contributes to antitumor efficacy of 17 $\alpha$ -hydroxylase/17,20-lyase inhibitor 3 $\beta$ -hydroxy-17-(1*H*-benzimidazole-1-yl)androsta-5,16-diene in prostate cancer

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## Abstract

We previously reported that our novel compound 3 $\beta$ -hydroxy-17-(1*H*-benzimidazole-1-yl)androsta-5,16-diene (VN/124-1) is a potent 17 $\alpha$ -hydroxylase/17,20-lyase (CYP17) inhibitor/antiandrogen and strongly inhibits the formation and proliferation of human prostate cancer LAPC4 tumor xenografts in severe combined immunodeficient mice. In this study, we report that VN/124-1 and other novel CYP17 inhibitors also cause down-regulation of androgen receptor (AR) protein expression *in vitro* and *in vivo*. This mechanism of action seems to contribute to their antitumor efficacy. We compared the *in vivo* antitumor efficacy of VN/124-1 with that of castration and a clinically used antiandrogen, Casodex, and show that VN/124-1 is more potent than castration in the LAPC4 xenograft model. Treatment with VN/124-1 (0.13 mmol/kg twice daily) was also very effective in preventing the formation of LAPC4 tumors (6.94 versus 2410.28 mm<sup>3</sup> in control group). VN/124-1 (0.13 mmol/kg twice daily) and

VN/124-1 (0.13 mmol/kg twice daily) + castration induced regression of LAPC4 tumor xenografts by 26.55% and 60.67%, respectively. Treatments with Casodex (0.13 mmol/kg twice daily) or castration caused significant tumor suppression compared with control. Furthermore, treatment with VN/124-1 caused marked down-regulation of AR protein expression, in contrast to treatments with Casodex or castration that caused significant up-regulation of AR protein expression. The results suggest that VN/124-1 acts by several mechanisms (CYP17 inhibition, competitive inhibition, and down-regulation of the AR). These actions contribute to inhibition of the formation of LAPC4 tumors and cause regression of growth of established tumors. VN/124-1 is more efficacious than castration in the LAPC4 xenograft model, suggesting that the compound has potential for the treatment of prostate cancer. [Mol Cancer Ther 2008;7(8):2348–57]

## Introduction

Prostate cancer is the most prevalent cancer in men and the second leading cause of death in American men, resulting in 186,320 new cases and 28,660 deaths per year from this disease (1). Androgens play an important role in the development, growth, and progression of prostate cancer (2). The testes produce most of the circulating testosterone, whereas ~10% is synthesized by the adrenal glands. Testosterone is further converted in the prostate to the more potent androgen dihydrotestosterone by the enzyme 5 $\alpha$ -reductase (3). Most prostate cancers are initially dependent on androgens for their growth, and orchidectomy (either surgical or medical with a GnRH agonist) remains the standard of treatment. Although orchidectomy reduces androgen production by the testes, androgen synthesis in the adrenal glands is unaffected. Thus, orchidectomy combined with antiandrogens to block the action of adrenal androgens can be more effective and prolong survival of prostate cancer patients (4).

The mechanisms through which androgen-dependent prostate cancer tumors survive and proliferate under androgen deprivation therapy are not completely understood. However, it has been found that the androgen receptor (AR) is consistently expressed and active in multiple xenograft models of hormone refractory disease (5). Amplified expression and increased sensitivity of AR in recurrent prostate cancer may be due to its increased stability, altered growth factor signaling, and mutations that broaden ligand specificity (6–9). Additionally, reduction of AR expression in androgen-sensitive and androgen-refractory models through the use of shRNA or chemical means has resulted in marked growth

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VN/124-1 has been licensed to Tokai Pharmaceutical, Inc. (Boston, MA) for further development for the treatment of prostate cancer.

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suppression of prostate cancer cells (10–13). Further support for the role of AR and androgens in prostate cancer is the recent report of increased expression of genes of androgen-converting enzymes and persistence of androgen-regulated genes in androgen-independent prostate cancer (14–16). These observations suggest that therapies that inhibit production of androgens and target multiple points in the AR signaling cascade could offer a more effective approach for prolonging remission of prostate cancer.

In the testes and adrenal glands, the last step in the biosynthesis of testosterone involves two key sequential reactions that are catalyzed by a single enzyme, the cytochrome P450 monooxygenase 17 $\alpha$ -hydroxylase/17,20-lyase (CYP17; ref. 17). Ketoconazole, an antifungal agent and nonspecific CYP450 inhibitor used with careful scheduling (18), can produce prolonged responses in otherwise hormone-refractory prostate cancer patients. Furthermore, ketoconazole was found to retain activity in advanced prostate cancer patients with progression despite flutamide withdrawal (19). Although ketoconazole remains one of the most effective second line hormonal therapies for prostate cancer, its use is limited due to liver toxicity and other side effects. However, its antitumor efficacy suggests that more potent and selective inhibitors of CYP17 could provide useful agents for treating this disease (20).

We and others have reported a number of novel inhibitors of CYP17, and some have been shown to be strong inhibitors of testosterone production in rodent models (20–23). Jarman and colleagues recently described the effects of their steroidal CYP17 inhibitor, abiraterone, in patients with prostate cancer (24, 25). Some of our most effective CYP17 inhibitors possess additional activities, such as inhibition of 5 $\alpha$ -reductase and/or are antiandrogens with potent antitumor efficacy (26–29).

In addition to being among the strongest CYP17 inhibitors known to date, the novel steroidal compounds VN/85-1, VN/87-1, and VN/108-1 were shown to reduce dihydrotestosterone-stimulated LNCaP cell proliferation and to displace methyltrienolone (R1881), a synthetic androgen, from the mutated T877A AR at 5  $\mu$ mol/L concentration (26). 3 $\beta$ -Hydroxy-17-(1*H*-benzimidazole-1-yl)androsta-5,16-diene (VN/124-1; Fig. 1) was found to be effective *in vitro* as well as in the LAPC4 xenograft model in male severe combined immunodeficient (SCID) mice (28). In addition to inhibition of CYP17, VN/124-1 exhibited potent AR antagonism in binding studies and LNCaP luciferase transcription assays, as well as marked tumor growth suppression in LAPC4 xenografts (28). In this report, we show that VN/124-1 and other novel CYP17 inhibitors cause down-regulation of AR protein expression *in vitro* and *in vivo*. This mechanism of action seems to contribute to their antitumor efficacy. We also compared the *in vivo* antitumor efficacy of VN/124-1 with that of castration and show that VN/124-1 is more potent than castration in human prostate cancer xenograft models.

## Materials and Methods

Casodex (bicalutamide) was provided by Dr. E. Anderson from AstraZeneca (Alderley Park, Macclesfield, United

Kingdom). The compounds VN/124-1, VN/125-1, VN/85-1, VN/87-1, and VN/108-1 were synthesized in our laboratory as described previously (26, 28). AR antibody (SC-7305) was obtained from Santa Cruz Biotechnology, Inc. Tritiated methyltrienolone ([<sup>3</sup>H]R1881) was obtained from Perkin-Elmer LAS.

### Cell Culture

LAPC4 cells were grown in IMEM supplemented with 15% fetal bovine serum, 1% penicillin/streptomycin (P/S) solution, and 10 nmol/L dihydrotestosterone. LNCaP cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. PC3-AR cells were grown in the same media supplemented with 750  $\mu$ g/mL G4181 for continued selection of the AR vector.

### DNA Constructs and Transfections

The Probasin luciferase reporter construct ARR2-Luc was generated by insertion of the minimal probasin promoter ARR2 into the polyclonal linker region of PGL3-enhancer vector (Promega) as described previously (30). The pRL-null vector (Promega) was used as the internal control. PC3 cells stably transfected with the human wild-type AR (designated PC3-AR) and the T575A human AR mutation vector were kindly provided by Dr. Marco Marcelli (Baylor College of Medicine, Houston, TX; ref. 31). All transfections were carried out with LipofectAMINE 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

### Competitive Binding Assays

To determine if the CYP17 inhibitors interact with the AR, competitive binding assays were done as described previously (28). The ability of the test compounds (1 nmol/L–10  $\mu$ mol/L) to displace [<sup>3</sup>H]R1881 (5.0 nmol/L) from the AR was determined in LAPC4 cells (wild-type AR), PC3 cells transfected with wild-type AR (PC3-AR), LNCaP cells that express an endogenous AR with a mutation in the ligand binding domain (T877A), and PC3 cells transfected with an AR containing a mutation in the DNA binding domain (T575A).

### Transcriptional Activation—Luciferase Assay

LAPC4 and LNCaP cells were transferred to steroid-free medium 3 d before the start of the experiment and plated at  $1 \times 10^5$  per well in steroid-free medium. The cells were dual transfected with ARR2-Luc and the *Renilla* luciferase reporter vector pRL-null as described in "DNA Constructs and Transfections." After a 24-h incubation period at 37°C, the cells were incubated in fresh phenol red-free RPMI 1640 containing 5% charcoal-stripped fetal bovine serum and treated with 10 nmol/L dihydrotestosterone, ethanol vehicle, and/or the selected compounds in triplicate. After an 18-h treatment period, the cells were washed twice with ice-cold Dulbecco's PBS and assayed using the Dual Luciferase kit (Promega) according to the manufacturer's protocol. Cells were lysed with 100  $\mu$ L of luciferase lysing buffer, collected in a microcentrifuge tube, and pelleted by centrifugation. Supernatants (20  $\mu$ L aliquots) were transferred to corresponding wells of opaque 96-well multiwell plates. Luciferase Assay Reagent was added to each well,

and the light produced during the luciferase reaction was measured in a Victor 1420 scanning multiwell spectrophotometer (Wallac, Inc.). After measurement, Stop and Glo reagent (Promega) was added to quench the firefly luciferase signal and initiate the *Renilla* luciferase luminescence. *Renilla* luciferase luminescence was also measured in the Victor 1420. The results are presented as the fold induction (i.e., the relative luciferase activity of the treated cells divided by that of the control) normalized to that of the *Renilla*.

#### AR Down-regulation and Degradation

To determine the ability of the test compounds to modulate AR protein levels, LAPC4 and LNCaP cells were treated with concentrations ranging from 1 to 15  $\mu\text{mol/L}$  for 24 h. Cells were collected and lysates prepared. Equal amounts of total protein were analyzed for AR expression levels by Western blot analysis. Equal amounts of total protein (50–100  $\mu\text{g}$ ) were subjected to SDS-PAGE (60 V, 3 h) and transferred (90 V, 1 h) onto nitrocellulose membranes (Hybond ECL, Amersham). Immunodetections were done with mouse monoclonal antibody against human AR (Santa Cruz Biotechnology). Immunoreactive bands were visualized with enhanced chemiluminescence detection reagents (Amersham Corp.) according to the manufacturer's instructions and quantitated by densitometry using ImageQuant 5.0 software.

For degradation studies, LNCaP cells were grown in serum-free medium for 3 d and treated with 10  $\mu\text{mol/L}$  cycloheximide alone, 15  $\mu\text{mol/L}$  VN/124-1 alone, or 10  $\mu\text{mol/L}$  cycloheximide + 15  $\mu\text{mol/L}$  VN/124-1 for 0, 2, 6, 12, and 24 h. Cells were collected by centrifugation and the cell pellet was resuspended in chilled lysis buffer [0.1 mol/L Tris-HCl, 0.5% Triton X-100, protease inhibitors (Complete, Boehringer)] and sonicated for 20 s. The homogenates were transferred to Eppendorf tubes, incubated on ice for 30 min, and then spun at 14,000 rpm for 20 min. The supernatants were stored at  $-70^\circ\text{C}$ . Western blots were done as above. Protein concentrations were determined with a Bio-Rad kit.

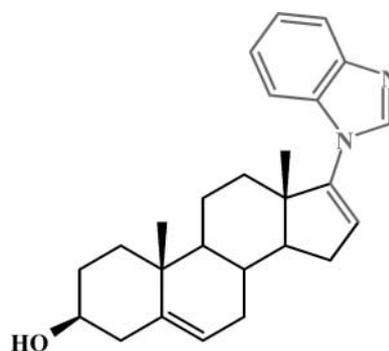
#### Cell Proliferation Assay

To determine the effect of steroids and novel compounds on cell proliferation, each cell type was transferred into steroid-free medium 3 d before the start of the experiments (steroid-free medium consisted of phenol red-free RPMI supplemented with 5% dextran-coated, charcoal-treated serum and 1% penicillin/streptomycin solution). Growth studies were then done by plating cells ( $1.5 \times 10^4$  per well) in 24-well multiwell dishes (Corning, Inc.). After a 24-h attachment period, the medium was aspirated and replaced with steroid-free medium containing vehicle or the indicated concentrations of androgens and novel compounds (1 nmol/L–10  $\mu\text{mol/L}$ ). Control wells were treated with vehicle (ethanol). Casodex (bicalutamide) was used as a reference drug for comparison to a known antiandrogen. The medium was changed every 3 d and the numbers of viable cells were compared by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT; LNCaP) assay on the 7th day.

For the MTT procedure, following incubation of cells for the above-mentioned time, 0.5 mg/mL MTT was added to each well and incubated at  $37^\circ\text{C}$  for 3 h. Following incubation, the medium was aspirated completely, with care taken not to disturb the formazan crystals. DMSO (500  $\mu\text{L}$ ) was used to solubilize these crystals. After slight shaking, the plates were read at 540 nm with a Victor 1420 scanning multiwell spectrophotometer. All results represent the average of a minimum of three wells. Additional control consisted of medium alone with no cells. XTT was done essentially as MTT, with the deletion of the solubilization step, and was preferred for the LNCaP cells that adhere poorly to the plates. A water-soluble formazan was obtained using XTT; the plates were read at 450 nm with the spectrophotometer.

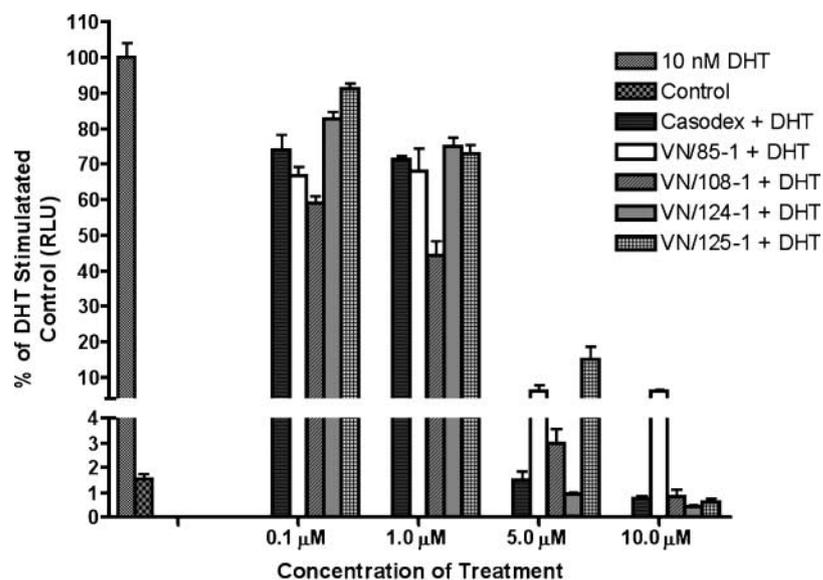
#### *In vivo* Antitumor Studies (LAPC4 Prostate Cancer Xenografts)

All animal studies were done according to the guidelines and approval of the Animal Care Committee of the University Of Maryland School Of Medicine, Baltimore. Male SCID mice, 4 to 6 wk of age, purchased from the National Cancer Institute, were housed in a pathogen-free environment under controlled conditions of light and humidity and allowed free access to food and water. Tumors were developed from LAPC4 cells inoculated s.c. into each mouse as previously described (28). LAPC4 cells were grown in IMEM with 15% fetal bovine serum plus 1% P/S solution and 10 nmol/L dihydrotestosterone until 80% confluent. Cells were scraped into Dulbecco's PBS, collected by centrifugation, and resuspended in Matrigel (10 mg/mL) at  $3 \times 10^7/\text{mL}$ . Mice were injected s.c. with 100  $\mu\text{L}$  of the cell suspension at one site on each flank. Twice per week, the mice were weighed and tumors were measured with calipers for the duration of experiment. Tumor volumes were calculated by the following formula:  $4/3\pi \times r_1^2 \times r_2$  ( $r_1 < r_2$ ). Mice in the tumor formation prevention group ( $n = 5$ ) were injected with VN/124-1 (0.13 mmol/kg twice daily) in the vehicle (0.3% saline hydroxypropyl cellulose) from the day of inoculation for the duration of the experiments. The rest of the mice were monitored until tumors reached  $\sim 500 \text{ mm}^3$ ,  $\sim 9$  wk after cell inoculation. Mice were assigned to five groups (five mice per group) for treatment so that there was no



**Figure 1.** Chemical structure of VN/124-1.

**Figure 2.** Effect of compounds on dihydrotestosterone-stimulated transcription. LAPC4 cells were transfected with the ARR2 reporter construct + the *Renilla* luciferase reporting vector pRL-null and treated with novel compounds for 18 h in the presence of 10 nmol/L dihydrotestosterone (DHT). Control, baseline activity without androgen stimulation. Androgen-stimulated luciferase activity (luminescence) was measured in a Victor 1420 plate reader. The results are presented as the fold induction (i.e., the relative luciferase activity of the treated cells divided by that of the control) normalized to that of the *Renilla*.



statistically significant difference in tumor volumes among the groups at the beginning of the treatment. The five groups were control, castration, Casodex (0.13 mmol/kg twice daily), VN/124-1 (0.13 mmol/kg twice daily), and VN/124-1 (0.13 mmol/kg twice daily) + castration. Compounds (suspensions in 0.3% hydroxypropyl cellulose) were given s.c. twice daily, 9 a.m. and 5 p.m. Mice were castrated under methoxyfluorane anesthesia. Control and castrated mice were treated with vehicle (hydroxypropyl cellulose) only. At the end of the treatment period, the animals were sacrificed under fluorothane anesthesia; tumors were excised, weighed, and stored at  $-80^{\circ}\text{C}$ . The liver and kidneys were also harvested and examined for any abnormalities. Animals were also monitored for general health status and signs of possible toxicity due to treatment.

#### Tumor Analysis

The protein extracts of the tumors from the above experiment were prepared by homogenizing the tissue in ice-cold Dulbecco's PBS containing protease inhibitors. Western blots were done as described above.

#### Statistical Analysis

The total tumor volume of each group was compared using the log scale. Because of incomplete values, we used the quasi *t* test developed by Tan et al. (32) for comparison between two groups. We compared total tumor volume from day 63 to day 93, the total tumor volume from day 76 to day 93 (the treatment effect after 2 wk), and at day 93. All computations were done using S-PLUS. The treatment groups were compared with one another at 0.05 level of significance.

## Results

### Competitive Binding to Wild-Type and Mutant ARs

LNcaP cells expressed a single class of high-affinity binding sites with  $K_d = 0.5$  nmol/L, with maximum

number of binding sites determined as  $1.18 \times 10^5$  per cell. LAPC4 cells had a similar  $K_d$  of 0.4 nmol/L with a maximum number of binding sites of  $6.1 \times 10^4$  per cell. Once the saturation concentration (5 nmol/L) was determined, evaluation of the compounds previously tested at 5  $\mu\text{mol/L}$  in LNcaP cells (VN/85-1, VN/87-1, and VN/108-1; ref. 33) was conducted over a full concentration range in both cell types. Casodex, an antiandrogen currently used as prostate cancer therapy, was included as a reference drug (Table 1). Abiraterone, a CYP17 inhibitor currently in clinical trials, was also tested.

VN/85-1, VN/87-1, VN/124-1, and VN/125-1 had the highest affinity for the wild-type AR. In contrast, abiraterone did not bind to the AR. There was no significant difference found between the wild-type AR in transfected PC3-AR cells and the wild-type AR expressed endogenously in LAPC4 cells (Table 1). VN/85-1, VN/124-1, and VN/125-1 had slightly greater ability to displace [ $^3\text{H}$ ]R1881 from the wild-type receptor than from the T877A mutant in LNcaP cells. The T877A AR contains a mutation in the ligand binding domain (34), which could account for the observed difference in binding. In contrast, VN/108-1 and VN/87-1 showed nearly identical affinities for both receptor types. PC3-T575-AR cells express an AR with a mutation in the DNA binding domain (31). Unlike the T877A AR, there was no apparent difference observed in the ability of VN/85-1, VN/124-1, or VN/125-1 to displace [ $^3\text{H}$ ]R1881 from the T575A AR when compared with wild-type. Therefore, additional compounds were not tested for AR affinity for this mutation.

### AR Antagonism

The compounds that showed strong binding affinity for the receptor were evaluated for antagonistic properties by the luciferase assay in LAPC4 and LNcaP cells transfected with the ARR2-Luc vector. These experiments were carried out against both receptor types because there are reports of some wild-type AR antagonists, such as flutamide, functioning as T877A agonists (35, 36). In both cell types,

VN/124-1, VN/125-1, and VN/108-1 inhibited dihydrotestosterone-induced transcriptional activation with similar potency as Casodex. Casodex, VN/85-1, VN/124-1, and VN/125-1 at 10  $\mu\text{mol/L}$  concentration were all able to reduce wild-type AR- and T877A AR-mediated transcriptional activation by 90% to 99% (Fig. 2). In LNCaP cells, VN/87-1 was the least effective of the compounds tested. When LNCaP-CYP17 cells were exposed to inhibitors in steroid-free media, only VN/87-1 activated luciferase transcription, indicating that it is a partial agonist of the T877A AR, similar to flutamide. This compound was therefore excluded from further studies. None of the other compounds displayed agonistic properties in either cell line.

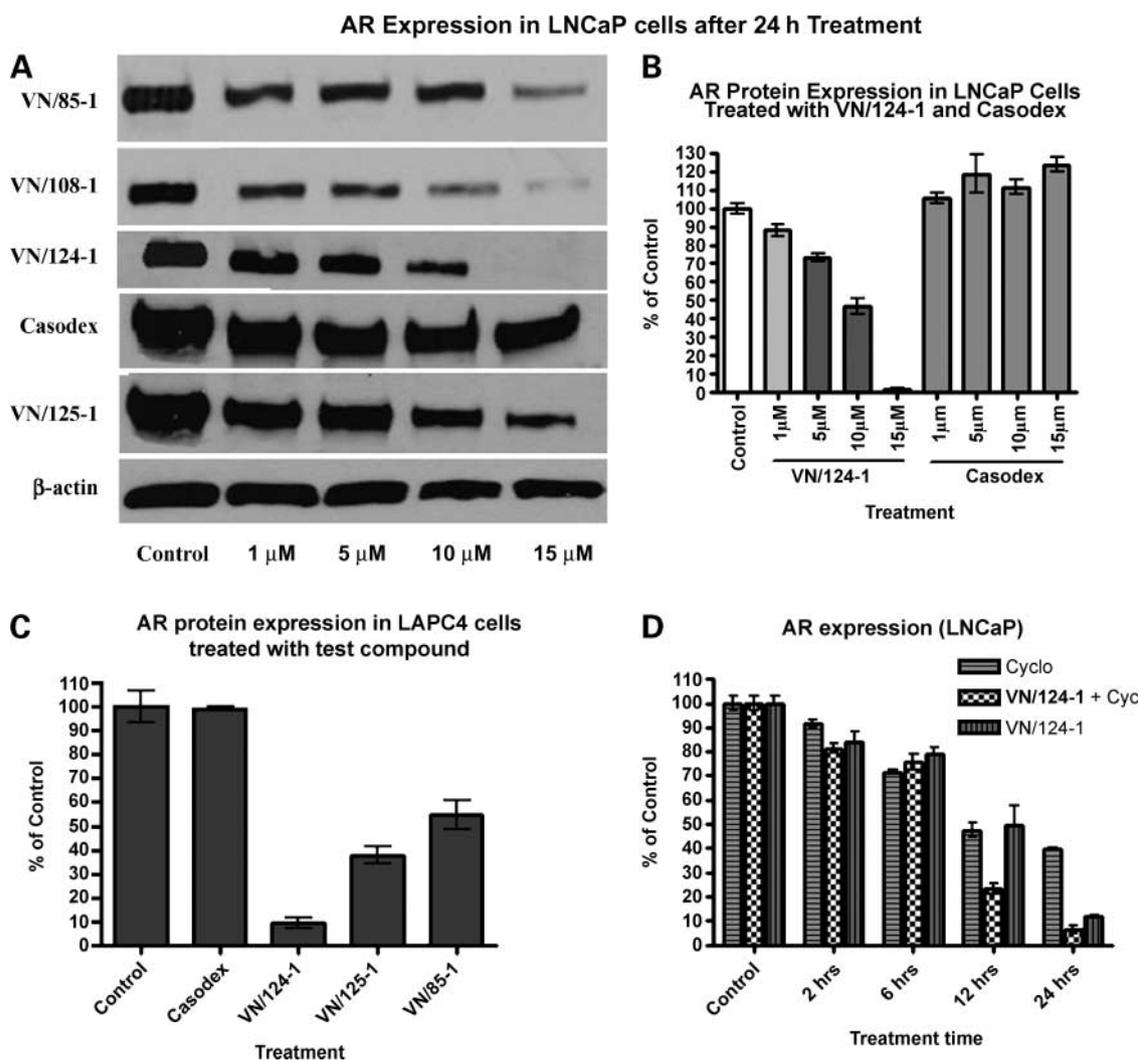
#### AR Down-regulation

The compounds showed a dramatic down-regulation of wild-type and also mutated AR receptor. In LNCaP cells, nearly all of the test compounds induced a dose-dependent

(1, 5, 10, and 15  $\mu\text{mol/L}$ ) decrease in AR levels, whereas no change in total AR level was observed with Casodex at these concentrations (Fig. 3A and B). VN/124-1 reduced expression by 50% at 10  $\mu\text{mol/L}$  and displayed nearly complete suppression at 15  $\mu\text{mol/L}$ . VN/125-1, VN/85-1, and VN/108-1 were able to reduce AR protein expression by 65%, 70%, and 90%, respectively, at a concentration of 15  $\mu\text{mol/L}$ . In LAPC4 cells, VN/124-1 reduced expression by 89% at 15  $\mu\text{mol/L}$  concentration. At the same concentration, VN/85-1 and VN/125-1 reduced expression by 50% and 66%, respectively (Fig. 3C).

#### AR Degradation

Although VN/124-1 was able to down-regulate the AR protein expression in a dose-dependent manner, it was still unclear whether the down-regulation was a result of decreased protein synthesis or increased degradation/AR destabilization. To determine protein degradation, *de novo*



**Figure 3.** Western blot analysis of AR expression *in vitro*. Cells were treated with test compounds for 24 h at the indicated concentrations (1–15  $\mu\text{mol/L}$ ). Cell extracts were prepared and probed with anti-AR and anti- $\beta$ -actin antibodies. **A**, AR expression in LNCaP cells after 24-h treatment with the indicated compounds. **B** and **C**, densitometry quantification of AR expression in LNCaP and LAPC4 cells after treatment. **D**, densitometry quantification of AR expression in LAPC4 cells after treatment with 15  $\mu\text{mol/L}$  of the indicated compounds.

**Table 1. Competitive inhibition of [<sup>3</sup>H]R1881 binding (LAPC4, LNCaP, and PC3-ART575A cells)**

Compound	Wild-type (IC <sub>50</sub> nmol/L; LAPC4/PC3-AR)	T877A (IC <sub>50</sub> nmol/L; LNCaP)	T575A (IC <sub>50</sub> nmol/L; PC3-ART575A)
VN/85-1	341	1,290	473
VN/87-1	319	422	NT
VN/108-1	868	831	1,210
VN/124-1	405	845	454
VN/125-1	248	1,240	383
Abiraterone	—	—	NT
Casodex	4,300	971	NT
Flutamide	10,985	11,600	NT

NOTE: The ability of the test compounds to displace [<sup>3</sup>H]R1881 (5.0 nmol/L) from the AR was determined in LAPC4 cells (wild-type AR), PC3 cells transfected with wild-type AR, LNCaP cells that express an endogenous AR with a mutation in the ligand binding domain (T877A), and PC3 cells transfected with an AR containing a mutation in the DNA binding domain (T575A). Cells were plated ( $2 \times 10^5$ – $3 \times 10^5$ ) in 24-well multiwell dishes in steroid-free medium and allowed to attach. The following day, the medium was replaced with serum-free, steroid-free RPMI supplemented with 0.1% bovine serum albumin and containing [<sup>3</sup>H]R1881 (5 nmol/L), test compounds (1 nmol/L–10 μmol/L), and 1 μmol/L triamcinolone acetonide to saturate progesterone and glucocorticoid receptors. Following a 2-h incubation period at 37°C, cells were washed twice with ice-cold Dulbecco's PBS and solubilized in Dulbecco's PBS containing 0.5% SDS and 20% glycerol. Extracts were removed and cell-associated radioactivity was counted in a scintillation counter. The data were analyzed by nonlinear regression using GraphPad Prism software (GraphPad Software, Inc.). NT, not tested; —, <20% inhibition at 10 μmol/L.

protein synthesis was inhibited using cyclohexamide and protein expression was measured at various time points.

Cycloheximide treatment alone reduced AR levels in a time-dependent fashion, with 50% reduction observed at 12 hours and >60% at 24 hours posttreatment. VN/124-1 treatment did not alter AR degradation rate for the first 6 hours; however, a rapid decline in AR level occurred between 6 and 12 hours posttreatment, resulting in 50% less receptor than expressed at 6 hours, and 75% less than control. The observed difference between 12 and 24 hours followed a similar pattern in both cycloheximide and VN/124-1 groups, with only an additional decline of ~10% for each (Fig. 3D). These results suggest that VN/124-1 increases the degradation rate of the AR.

#### Inhibition of Cell Proliferation

The ability of the compounds to inhibit proliferation with and without dihydrotestosterone stimulation in LAPC4 and LNCaP cells was examined. In contrast to LNCaP cells, LAPC4 cells did not exhibit strong stimulation in response to dihydrotestosterone. This is in agreement with reports by other investigators (37). As such, there was minimal difference between inhibition of dihydrotestosterone-stimulated versus nonstimulated LAPC4 cells for all test compounds, with IC<sub>50</sub>s ranging from 1 to 7 μmol/L (Table 2). VN/85-1 and VN/108-1 were able to reduce cell proliferation in a consistent dose-dependent manner, with potency equal to or greater than Casodex. VN/124-1 and VN/125-1 were also highly effective, with IC<sub>50</sub>s of 3.2 and 1.0 μmol/L, as previously reported (28). The time course to maximal effectiveness was similar among all test compounds, with onset of cell death being visually apparent no earlier than 48 to 72 hours posttreatment.

Previous results with VN/85-1, VN/87-1, and VN/108-1 have shown significant inhibition of LNCaP cell proliferation. All three compounds inhibited proliferation by 40% to 60% and inhibited dihydrotestosterone-stimulated proliferation at concentrations up to 5 μmol/L (33). Further evaluation of these compounds in dihydrotestosterone stimulated LNCaP cells, over a broader concentration

range of (0.01–100 μmol/L), indicated IC<sub>50</sub> values of 1.8, 4.6, and 3.7 μmol/L for VN/108-1, VN/85-1, and VN/87-1 respectively. To our knowledge, LNCaP cells do not express CYP17, or express very minimal amounts, because CYP17 activity is undetectable in LNCaP cells by our acetic acid releasing assay system. Therefore, LNCaP viability assays do not completely represent the extent of potential effectiveness of our novel compounds because under physiologic conditions, there would be the added effect of decreased androgen production. The fact that our compounds were equally effective against both cell lines indicates increased clinical potential because some antiandrogens such as flutamide have agonistic properties for the mutant AR as occurs in LNCaP cells.

#### VN/124-1 Causes Growth Inhibition in LAPC4 Xenograft Model

We determined the effects of VN/124-1 on prevention of LAPC4 tumor xenograft formation and also the effect of

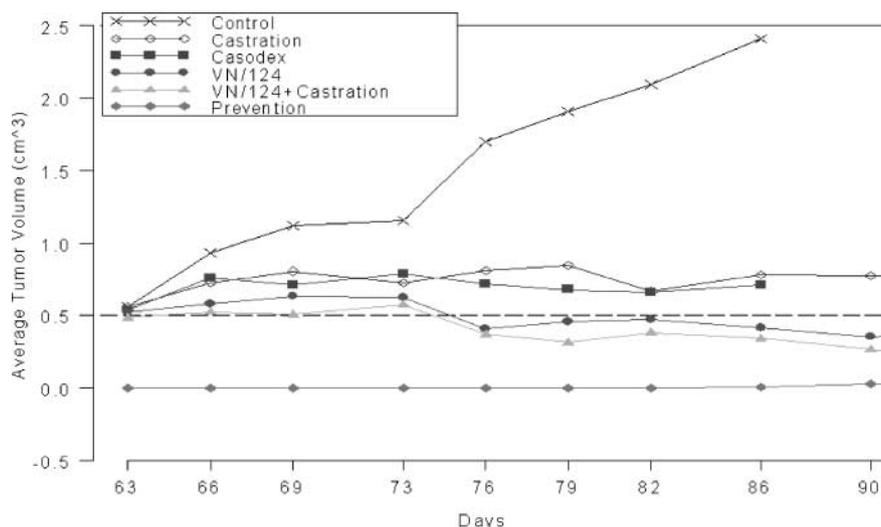
**Table 2. Effect of novel compounds on cell proliferation**

Compound	LNCaP IC <sub>50</sub> (μmol/L)		LAPC4 IC <sub>50</sub> (μmol/L)	
	+	–	+	–
DHT				
VN/85-1	3.7	1.9	4.2	3.4
VN/87-1	4.8	NT	NT	NT
VN/108-1	1.8	1.6	7	5
VN/124-1	6	2.6	3.2	4
VN/125-1	1.8	2.2	1.0	3
Casodex	8.6	4.6	10	9
Flutamide	*	*	NT	NT

NOTE: LNCaP cells were seeded at 15,000 per well in 24-well multiwell plates, and LAPC4 cells were seeded at 15,000 per well. Cells were treated with the indicated concentration of compound in steroid-free medium with or without 1 nmol/L dihydrotestosterone (LNCaP) or 10 nmol/L dihydrotestosterone (LAPC4) and allowed to grow for 7 d. The number of viable cells was compared by MTT assay (LAPC4) or XTT assay (LNCaP) on the 7th day. NT, not tested.

Abbreviation: DHT, dihydrotestosterone.

\*Stimulated proliferation.



**Figure 4.** Effects of VN/124-1, Casodex, and castration on the prevention and growth of LAPC4 human prostate xenografts in male SCID mice. Mice bearing LAPC4 human prostate tumors were grouped and treatment was started 63 d after cell inoculation except for the "prevention" group. In this group, treatment was begun with VN-124-1 on the day of cell inoculation. Treatments with both Casodex and VN/124-1 were given at a dosage of 0.13 mmol/kg twice daily. Control mice (vehicle-treated mice were sacrificed after 86 d because of large tumors and mice treated with Casodex were sacrificed due to insufficient drug). Tumors of all treated groups were significantly different from control, and the prevention group was also significantly different from all treated groups. \*, VN/124-1 alone and VN/124-1 plus castration were significantly different from castration and Casodex using multivariate analysis.

VN/124-1, VN/124-1 + castration, castration, or Casodex on tumor growth *in vivo*. LAPC4 cells were injected s.c. into SCID mice and one group of mice ( $n = 5$ ; tumor prevention group) was treated with VN/124-1 (0.13 mmol/kg twice daily) for 93 days starting the day after inoculation with LAPC4 cells. Approximately 9 weeks after inoculation, tumors had formed in the other mice ( $\sim 300 \text{ mm}^3$ ), and these animals were assigned to five treatment groups: control (vehicle), castration, Casodex (0.13 mmol/kg twice daily), VN/124-1 (0.13 mmol/kg twice daily), and castration plus VN/124-1 (0.13 mmol/kg twice daily). It should be noted that experiments with the control and Casodex groups were terminated on day 86 because of large tumors and drug shortage, respectively (Fig. 4). Treatment with VN/124-1 was very effective in preventing the formation of LAPC4 tumors (6.94 versus 2410.28  $\text{mm}^3$  in control group on day 86;  $P < 0.001$ ). Total tumor volume in the control mice increased by 4.3-fold over 3 weeks of treatment when the mice were sacrificed because of the large tumors.

The tumors in the prevention group were significantly smaller than the tumors in control group and in all treatment groups ( $P < 0.0001$ ). Compared with control groups with respect to the total tumor volume from day 76 to day 93 (effect after 2 weeks of treatment), all treatments were significantly effective after 2 weeks except for treatment with Casodex ( $P = 0.075$ ). There were no significant differences between treatment groups overall. Comparing the total tumor volumes at day 93, there were significant differences among all groups but there were no significant differences between castration and VN/124-1 groups ( $P = 0.33$ ) and between VN/124-1 and VN/124-1 + castration groups ( $P = 0.059$ ). However, we observed that in the castration and VN/124-1 groups, there was significant variation in tumor volumes due to reduced growth of tumors on either the left or right flanks of the mice. This resulted in large variations in the total tumor volumes for each group. However, when we compared the two groups (i.e., castration versus VN/124-1) based on multivariate statistical analysis (allowing for differences in left tumor

and right tumor) using  $F$  test, there were significant differences between the tumor volumes of castration and VN/124-1 groups from day 76 to day 93 ( $P = 0.031$ ) and at day 93 ( $P = 0.047$ ). Furthermore, examination of the changes in average tumor volumes in all groups clearly shows the effects of the various treatments on tumor growth.

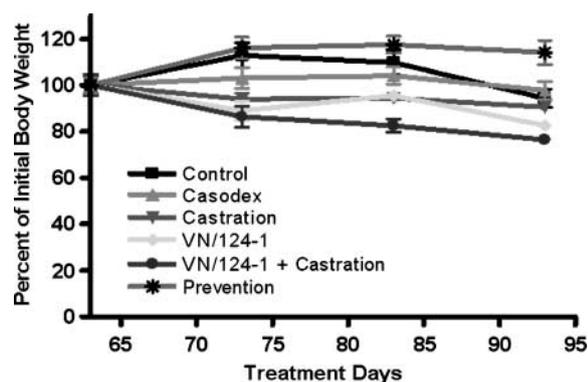
No significant changes in animal body weights were observed in all treatment groups (Fig. 5), suggesting that the treatments did not produce general toxicity in the mice.

#### Effects of Treatments on AR Expression *In vivo*

The striking difference in AR down-regulation between VN/124-1 and the other compounds, some of which had similar or better *in vitro* antiproliferation, antiandrogen, and lyase inhibition profiles, correlated with the increased activity of VN/124-1 in reducing LAPC4 tumor xenograft growth. Therefore, VN/124-1-treated LAPC4 tumor xenografts were analyzed for AR expression to determine if VN/124-1 maintained its potent down-regulation properties *in vivo* (Fig. 6). Analysis of tumors revealed that treatments with VN/124-1 or VN/124-1 + castration caused marked reduction in AR protein of 10- and 5-fold, respectively. In contrast, treatment with bicalutamide or castration caused significant AR protein up-regulation of 2.3- and 2.8-fold, respectively. Treatment with VN/124-1 in the tumor formation prevention study group caused a slight up-regulation (1.3-fold) of AR protein expression.

## Discussion

We have previously reported that some of our CYP17 inhibitors act as antiandrogens against the LNCaP AR (15, 27, 38). Because the test compounds are structurally similar to dihydrotestosterone, the most potent natural ligand for the AR, it seemed likely that the new compounds would also interact with the AR. The affinity of the compounds to the AR was assessed in competitive binding studies carried out using the synthetic ligand methyltrienolone with the wild-type and two mutant forms of the AR. To determine if



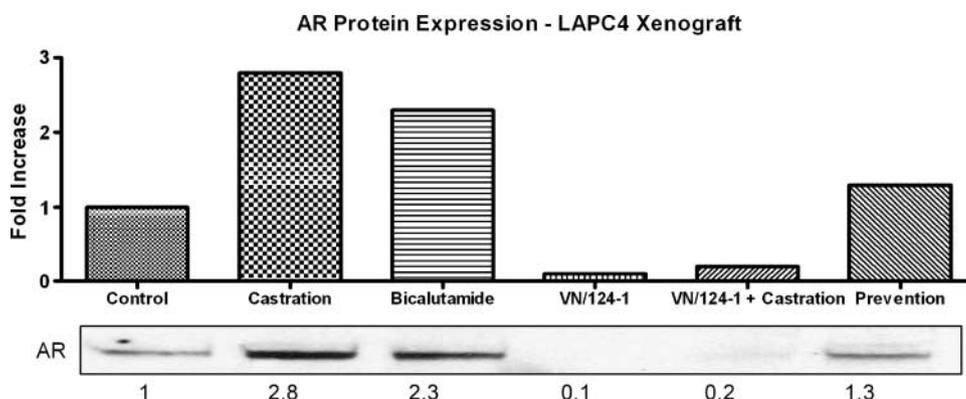
**Figure 5.** Percent change in mouse body weight over treatment duration in LAPC4 human prostate cancer xenografts in male SCID mice.

the compounds are agonists or antagonists, luciferase assays were done in the presence and absence of dihydrotestosterone. The binding affinities of the compounds were strongest for the wild-type AR, with  $IC_{50}$ s ranging from 248 nmol/L (VN/125-1). Although the strongest affinity was 10-fold weaker than dihydrotestosterone (22 nmol/L) in the same system, it is still significantly stronger than the clinically used antiandrogen Casodex (4.3  $\mu$ mol/L). Abiraterone, a CYP17 inhibitor now in clinical trials (17), did not bind to the AR. In LNCaP cells (T877A mutation), VN/85-1, VN/124-1, and VN/125-1 had lower affinities, whereas VN/108-1, VN/87-1 and flutamide had affinities equivalent to those for the wild-type AR. Casodex displayed a much stronger affinity for the T877A AR (971 nmol/L), which was approximately the same as VN/124-1 (845 nmol/L) and VN/108-1 (831 nmol/L). Conversely, all of the compounds tested against the T575A mutant, which has a mutation in the DNA binding domain (34), displayed affinities equivalent to the wild-type AR. Because the T575A mutation is within the DNA binding domain (31), it is expected that the binding affinity would remain unchanged. The difference between wild-type AR and T877A AR is not unexpected because the mutation in the latter confers broadened ligand specificity and obviously could have some effect on binding properties.

Mutations in the AR ligand binding domain have been shown to alter the affinity of ligands and antiandrogens. Bohl et al. (39) reported a 2-fold higher affinity of Casodex for W741L, a LBD AR mutant, as compared with the wild-type AR. This is similar to our results with Casodex and the T877A mutant. Interestingly, a few of our steroidal compounds exhibited a reduced affinity for the mutant T877A AR, in contrast to the increased affinity of Casodex.

Recent evidence indicates that in the majority of prostate cancer cases, even in chemotherapy-resistant disease, the AR is still expressed and required for growth (40–43). It has also been shown that the AR can be activated by cofactors and other mechanisms independent of androgen levels (44–46). In addition, it has been shown that overexpression of AR in a castration-resistant xenograft model is consistent with observations in human clinical specimens, and overexpression of AR promotes the transition from a hormone-dependent xenograft to a castration-resistant xenograft (5, 47). These observations suggest that directly targeting the AR and reducing AR levels to below a critical threshold may be a more effective approach to treatment than current antiandrogens.

In contrast to Casodex, the antiandrogen currently used clinically, our novel compounds VN/85-1, VN/108-1, VN/125-1, and VN/124-1 were all able to greatly reduce AR levels. This effect was observed in both LAPC4 and LNCaP cells, with overall AR levels decreased by  $\geq 60\%$ . In both cell lines, VN/124-1 was significantly more potent than the other compounds, with nearly complete reduction of AR expression at 15  $\mu$ mol/L in LNCaP cells and 89% in LAPC4 cells. Analysis of LAPC4 tumor samples from xenografts revealed that VN/124-1 also reduced AR *in vivo*, with a marked decrease in AR as compared with castration and control tumors. Results of analyzing tumor samples from our former study of VN/124-1 in xenografts (28) that were not previously reported showed 50% reduction in AR, expression of VN/124-1 treated (0.15 mmol/kg twice daily) versus control tumors, confirming this mechanism of action of VN/124-1 *in vivo* (data not shown). Although VN/85-1 and VN/125-1 had similar or better characteristics than VN/124-1 in terms of inhibiting the CYP17 and reducing androgen-modulated transcription, they were much less effective *in vivo*. However, these results could be explained



**Figure 6.** Western immunoblotting analysis of whole-cell lysates from LAPC4 tumors following various treatments.

in part by the greater effect of VN/124-1 on reducing AR levels *in vitro* and *in vivo*. Additional *in vitro* evidence supports this view, as reduction of AR expression produced a more pronounced effect on AR-induced transcription and cell growth than androgen deprivation in two androgen-insensitive prostate cancer cell lines, LNCaP-C42B4 and CWR22Rv1 (10).

The mechanism of AR down-regulation could occur through increased degradation or reduced protein synthesis. For our lead compound VN/124-1, AR degradation patterns were examined to determine whether AR stability was being affected. Destabilization of the AR has been shown in steroid-depleted conditions, with half-life reduced from ~6 to 3 hours (48). By using cycloheximide to inhibit new protein synthesis and measuring the rate of degradation, it was possible to determine if VN/124-1 caused additional degradation beyond that normally observed under androgen deprivation. There was a reduction of 50% in AR levels in the VN/124-1 treatment group versus control cells 6 hours posttreatment. AR levels continued to decline over 24 hours, with an additional 10% reduction over control evident at 12 and 24 hours posttreatment. These data indicate that VN/124-1 down-regulation of the AR level is at least partly due to increased AR degradation. However, it should be noted that androgens have been shown to increase AR synthesis as well (48). Therefore, the possibility of an additional effect on modulating the rate of AR expression cannot be ruled out. Consequently, we are currently investigating the effects of VN/124-1 on AR mRNA expression. In addition, the mechanism by which degradation occurs is still unknown. AR degradation has been shown to proceed through two proteolytic pathways. One relies on proteasomal degradation and occurs both in the absence (ligand-independent) and in the presence (ligand-dependent) of the hormone (reviewed in ref. 49). The second engages phosphatase and tensin homologue and caspase-3 activity (50). Interestingly, VN/124-1 is able to reduce AR levels in the presence and absence of androgens. Therefore, as long as the AR is functional, VN/124-1 may inhibit prostate cancer cell growth via AR down-regulation regardless of androgen-dependent or castration-resistant status.

In the LAPC4 xenograft, VN/124-1 is a more potent agent in reducing tumor growth than other compounds (VN/85, VN/87, and VN/108) and is more effective than castration and Casodex. VN/124-1 plus castration was also significantly better than castration alone or Casodex. VN/124-1 was most effective at preventing the formation of LAPC4 tumor xenografts, suggesting its potential as a chemopreventive agent. We show that unlike treatment with Casodex or castration, which caused significant AR protein up-regulation, treatment with VN/124-1 markedly reduced AR protein levels both *in vivo* and *in vitro*. This additional property may account for the superiority of VN/124-1 *in vivo* compared with other more potent CYP17 inhibitors such as VN/85-1.

In summary, we have determined that VN/124-1 possesses several anticancer properties that target the AR.

These include (a) CYP17 inhibition to block the synthesis of androgens from all sources thus reducing the AR ligand, (b) direct AR antagonism, and (c) AR down-regulation. The combinations of these three important activities in a single entity (VN/124-1) have potential utility in the treatment of prostate cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

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# Molecular Cancer Therapeutics

## Androgen receptor inactivation contributes to antitumor efficacy of 17 $\alpha$ -hydroxylase/17,20-lyase inhibitor 3 $\beta$ -hydroxy-17-(1*H*-benzimidazole-1-yl)androsta-5,16-diene in prostate cancer

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