

Synergistic effect of a novel antiandrogen, VN/124-1, and signal transduction inhibitors in prostate cancer progression to hormone independence *in vitro*

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Abstract

This study was carried out to determine the mechanisms associated with loss of androgen dependency and disease progression in prostate cancer. We investigated the role of the androgen receptor and its relationship to other signal transduction proteins. A hormone-refractory prostate cancer cell line [high-passage LNCaP (HP-LNCaP)] was established *in vitro*. Cells were treated with inhibitors of mammalian target of rapamycin and tyrosine kinase receptors. Expression of these proteins and the androgen receptor were measured by Western immunoblotting. Analysis of the model and various treatments was also assessed through proliferation assays, luciferase activation assays, binding assays, and ELISA. Our novel antiandrogen, VN/124-1, effectively inhibited proliferation of hormone-resistant prostate cancer cell lines (HP-LNCaP), which were no longer sensitive to bicalutamide and had increased expression of the androgen receptor. Treatment with everolimus or gefitinib resulted in an increase in protein expression and activation of the androgen receptor. Conversely, inhibition of the androgen receptor resulted in increased expression of IGF1R β , pHER2, pmTOR, and pAkt. The addition of bicalutamide to everolimus or gefitinib inhibited cell proliferation in HP-LNCaP cells. However, the addition of VN/124-1 has proven to be superior to bicalutamide, and the combination was synergistic ($P < 0.05$) compared with either agent alone. This

study suggests that compensatory cross-talk between the androgen receptor and various signaling pathways may account for decreased sensitivity to androgen receptor antagonists and the progression to hormone-resistant prostate cancer. Furthermore, these findings suggest that inhibition of both pathways may provide effective control in hormone-resistant prostate cancer and restore sensitivity to androgen antagonists in hormone-refractory patients. [Mol Cancer Ther 2008;7(1):121–32]

Introduction

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths in males in the United States (1). In the initial stages of prostate cancer, cell survival and proliferation are regulated through the androgen receptor. Testosterone and dihydrotestosterone play a vital role in the tumorigenesis and progression of early-stage prostate cancer. Testosterone, predominantly produced in the testes, is converted to dihydrotestosterone by 5 α -reductase, an enzyme present in the prostate. Dihydrotestosterone binds with higher affinity to the androgen receptor. In addition, type II 5 α -reductase is also found in the skin and other organs. The adrenal glands produce about 10% of available androgens. Early diagnosis of prostate cancer via detection of prostate-specific antigen (PSA) in serum followed by aggressive surgical and radiation treatment of organ-confined disease results in favorable outcomes. However, prognosis is poor once progression occurs and the androgen-independent phenotype develops.

Androgen deprivation therapy is the leading treatment for hormone-dependent prostate cancer. Antiandrogens, such as bicalutamide (Casodex), prevent HSP90 dissociation, dimerization, and translocation of the androgen receptor to the nucleus (2–4). Blockade of the androgen receptor is initially an effective method of treatment. However, nearly all patients progress to a hormone-refractory state generally in ~16 to 24 months (5, 6). Understanding the mechanisms of resistance to androgen deprivation therapy is therefore important in efforts to improve treatment of this disease.

Luteinizing hormone-releasing hormone analogues or surgical orchiectomy reduce androgen production in the testes but does not affect adrenal androgen synthesis. We have described previously a novel potent CYP-17 inhibitor, VN/124-1, which is also an androgen receptor antagonist (7–9). This lead compound is the first to show superior efficacy compared with castration in prostate cancer xenograft models (7). Here, we describe its effects in combination with inhibitors of signal transduction pathways in hormone-sensitive and hormone-refractory disease.

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Loss of sensitivity to androgen ablation therapy may have a variety of causes. This investigation focuses on the activation of signal transduction pathways. These include tyrosine kinase receptors, epidermal growth factor receptors, insulin-like growth factor receptors, mitogen-activated protein kinases, phosphatidylinositol 3-kinase, Akt, and mammalian target of rapamycin (mTOR). All have been heavily implicated in the progression of hormone-resistant prostate cancer (5, 10–16).

The epidermal growth factor and epidermal growth factor receptor antagonist gefitinib (Iressa, ZD 1839) have been investigated in several cancer settings. Although an *in vitro* investigation had shown an additive antitumor effect of blocking both tyrosine kinase pathway and androgen receptor pathway by combining gefitinib with bicalutamide (17), clinical trials did not show similar benefit. These trials involved dual epidermal growth factor receptor/HER2 tyrosine kinase inhibitor gefitinib but failed to provide additional clinical advantage over docetaxel and estramustine in prostate cancer (18, 19).

The mTOR is a 289-kDa serine/threonine kinase downstream of Akt. mTOR and other upstream proteins, such as phosphatidylinositol 3-kinase and Akt, have been implicated in the proliferation, differentiation, and malignant transformation in prostate cancer progression (20). mTOR activates ribosomal p70S6K and eukaryotic initiation factor EIF-4E and therefore translation. Everolimus (RAD-001) is a potent derivative of rapamycin, which has been shown to inhibit activation of mTOR and block the downstream signaling cascade (21, 22).

Hormone-dependent LNCaP cells have been used as a model for preclinical research for over 30 years (23). Although this model has numerous sublines, prostate cancer tumors are heterogeneous and current models do not mimic all physiologic conditions. The mechanisms of prostate cancer progression from hormone sensitivity to independence remain unclear. Originally immortalized from a hormone-sensitive metastatic lymph node, LNCaP cells gradually become more aggressive and resistant to antiandrogens through regular passage in growth medium and normal serum (24–26). In accordance with previously published literature, we have found that these cells naturally develop resistance to hormonal therapy. This model is therefore a useful tool in studying the mechanisms associated with disease progression.

High-passage LNCaP (HP-LNCaP) cells (over passage 75), used predominately in this investigation, exhibit increased activation of the phosphatidylinositol 3-kinase/Akt pathway as reported previously (27) and decreased activation of the androgen receptor compared with low-passage cells (LNCaP; over passage 25). As resistance to hormonal therapy and increased invasiveness occurs naturally through passaging in regular growth conditions, this cell line represents a good model for tumor progression. However, the model does not mimic androgen ablation therapy. Previously described androgen-independent C4-2B cells serve as another valid model for studying growth inhibition in advanced disease (28, 29).

There is increasing evidence to suggest compensatory signaling mechanisms and cross-talk occur between growth factor receptor pathways and androgen receptor in androgen-dependent and hormone-resistant prostate cancer cell lines. Recent investigations have elucidated several mechanisms for regulation of androgen receptor by signal transduction pathways and vice versa (30, 31). Our own studies are consistent with these findings and provide a rationale for dual inhibition of androgen receptor and signal transduction pathway. The enhanced efficacy of VN/124-1 and its other mechanisms of action render the compound more effective in combination with signal transduction inhibitors than bicalutamide. Thus, dual inhibition with VN/124-1 and everolimus or gefitinib has proven to be more efficacious in a hormone-resistant prostate cancer model than combination with bicalutamide. These findings highlight the importance of the interaction between the androgen receptor and growth factor receptor pathways and suggest the potential for combining therapy in hormone-resistant prostate cancer with inhibitors of these signaling pathways.

Materials and Methods

Materials

RPMI 1640, T medium, penicillin/streptomycin solution (10,000 IU each), 0.25% trypsin-EDTA (1 mmol/L) solution, and Dulbecco's PBS were obtained from Invitrogen. Regular and charcoal-stripped fetal bovine sera were obtained from Hyclone. C4-2B cells were kindly supplied by Dr. Yun Qiu (University of Maryland). DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Tween 20 were obtained from Sigma. Enhanced chemiluminescence kit and Hybond-ECL nitrocellulose membranes were purchased from Amersham Biosciences. Antibodies against pAkt (Ser⁴⁷³ and Thr³⁰⁸), pmTOR (Ser²⁴⁴⁸ and Ser²⁴⁸¹), pMAPK, IGFR, p70S6K, pS6, immobilized Akt, and β -actin were purchased from Cell Signaling Technology. pHER2 was purchased from Upstate, and PSA and androgen receptor (SC-441) were obtained from Santa Cruz Biotechnology. The total PSA ELISA kit was purchased from Diagnostic Systems Laboratories. Everolimus was provided by Dr. D. Evans (Novartis Pharmaceuticals), stored as a dry powder at 4°C, and dissolved in 100% DMSO for cell culture application. Gefitinib was supplied by Dr. A. Wakeling (AstraZeneca Pharmaceuticals), stored as a dry powder at 22°C, and dissolved in 95% ethanol for cell culture application. 3 β -Hydroxy-17-(1*H*-benzimidazol-1-yl)androst-5,16-diene (VN/124-1) was designed and synthesized by one of us (V.C.O.N.; ref. 7).

Cell Culture

Hormone-dependent prostate cancer cells derived from a metastatic lymph node of a prostate cancer patient (LNCaP) cells were routinely maintained in RPMI 1640 with 10% fetal bovine serum and 1% penicillin/streptomycin solution (23). HP-LNCaP cells were created through regular passaging of the LNCaP cell line for more than 1 year. Low-passage cells (LNCaP) were designated under passage 25, whereas high-passage cells (HP-LNCaP) were

over passage 75. Both lines were maintained in identical conditions. Hormone-independent *in vivo* derived cell line from LNCaP cells (C4-2B) was maintained in T medium with 10% fetal bovine serum and 1% penicillin/streptomycin (28, 29).

Cell Growth Studies

Growth studies were done in 24-well plates coated with poly-L-lysine (0.05 mg/mL) for 15 min and washed with Dulbecco's PBS. Cells (1×10^5 per well) were plated on day 1, washed with 10 mL Dulbecco's PBS, and treated with 1 mL regular growth medium per well containing vehicle or indicated concentration of compounds on days 2 and 5 and counted on day 8 using the MTT assay. MTT (0.5 mg/mL) was added to each well in serum-free medium and cells were incubated for 2 h. Medium was removed, the formazan dye trapped in the living cells was dissolved in DMSO, and absorbance at 560 nm was measured in a spectrophotometer. Viability results were expressed as a percentage of control \pm SE. IC₅₀ values for inhibition were calculated via nonlinear regression. All growth studies involving antiandrogens were done in the presence of 0.1 nmol/L exogenous R1881.

Western Blotting

The protein extracts from cell lysates were prepared by pelleting cells suspended in ice-cold Dulbecco's PBS and lysed with cell lysis buffer containing protease inhibitors, Tris-HCl, and Triton X-100. Equal amounts (50 μ g) of protein from each sample were separated on a denaturing polyacrylamide gel and transferred to nitrocellulose membrane. The protein-bound membranes were then incubated for 1 h at room temperature with 0.1% Tween 20 in TBS and 10% nonfat dry milk to block nonspecific antibody binding. The membranes were then incubated with respective primary antibodies as specified in manufacturer's protocol, and specific binding was visualized by using species-specific IgG followed by enhanced chemiluminescence detection and exposure to enhanced chemiluminescence X-ray film. Bands were quantitated by densitometry using Molecular Dynamics software (ImageQuant). The densitometric values are corrected for loading controls for each individual blot and expressed a fold change compared with vehicle-treated controls. A representative control blot is shown in the bottom of all panels. All Western blots were done at least thrice; blots shown are representative of all results.

Coimmunoprecipitation

Coimmunoprecipitation was done by preclearing cell lysates with protein A/G agarose beads for 30 min at 4°C followed by centrifugation of the pellet, the nonspecific binding protein A/G beads. The supernatant was incubated with immobilized Akt immunoprecipitation beads overnight in 4°C. Beads were spun and washed thrice with ice-cold Dulbecco's PBS and then prepared with sample buffer and heated at 100°C for 5 min according to Western blot procedures. IgG loading controls were also done using precleared lysates. Control samples were immunoprecipitated as stated above with protein A/G then probed for androgen receptor.

PSA ELISA

Five hundred thousand HP-LNCaP cells were plated in cell culture flasks and grown in regular growth conditions for 3 days. On day 4, the medium was changed and cells were treated with bicalutamide, VN/124-1, everolimus, gefitinib, or control vehicle. Twenty-four hours later, 20 μ L cell supernatant was removed and loaded in the Total PSA ELISA kit (DSLabs) in triplicates to determine PSA concentrations. In the assay standards, controls and samples were incubated in 96 wells following the manufacturer's instructions. Each original experimental sample was assayed in triplicate. PSA values (ng/mL) were determined using log-log curve fit to standard curve.

Luciferase Transactivation Assay

Transcriptional activation assay was carried out as described previously by Kim et al. (32) with minor modifications. The probasin luciferase reporter construct ARR2-Luc was generated by insertion of the minimal probasin promoter ARR2 (kindly provided by Dr. R. Matusik, Vanderbilt University Medical Center; ref. 33) into the polyclonal linker region of PGL3 enhancer vector (Promega). The pRL-null (Promega) was used as the internal control. Briefly, cells grown in 24-well plates coated with poly-L-lysine were transfected with ARR2-Luc in RPMI 1640 containing 10% fetal bovine serum (Hyclone). Twenty-four hours after transfection, the cells were incubated with fresh medium with or without R1881 and inhibitors for 18 h. Luciferase activities were measured in triplicates by using the dual luciferase assay system according to the manufacturer's instruction (Promega). The results are presented as the fold induction (that is, the relative luciferase activity of the treated cells divided by that of the control or percent of control).

Competitive Androgen Receptor Binding Studies

As described previously, 24-well plates were coated with poly-L-lysine to facilitate attachment. Androgen receptor binding studies were carried out as described previously by Handratta et al. (7).

Statistics

All experiments were done at least twice in replicates of three or more and the results are expressed as mean \pm SE where applicable. The effect of the treatments was compared with control cells using Student's *t* test. Comparison across multiple treatment groups was made with vehicle-treated control using one-way ANOVA (Tukey's test). All results were evaluated using GraphPad Prism 4, and *P* values less than 0.05 were considered statistically significant.

Results

Development of a Hormone-Refractory Prostate Cancer Cell Model

LNCaP cells showed a biphasic response to synthetic androgen R1881. Growth of these cells was stimulated by \sim 50% at 0.1 nmol/L R1881, whereas proliferation of HP-LNCaP and hormone-independent C4-2B cells was not stimulated by R1881 as reported by other investigators (data not shown; refs. 26, 34).

In comparison with LNCaP cells, HP-LNCaP cells grew more aggressively in regular steroidal growth conditions as shown by a 20-fold increase in cell proliferation (Fig. 1A). In the absence of steroids, HP-LNCaP cells showed a 5-fold increase in cell proliferation compared with the slow growth of LNCaP cells. As expected, C4-2B cell growth was unaffected by the absence or presence of steroids (Fig. 1A). Consistent with proliferation studies and previously published literature, androgen receptor activation is hypersensitive to R1881 in HP-LNCaP cells compared with LNCaP cells (refs. 16, 35, 36; Fig. 1B).

Several investigations have shown the involvement of growth factor pathways in the progression to and proliferation of hormone-resistant prostate cancer (12–14, 37). Therefore, we examined the expression of various growth factor receptors and downstream signaling proteins. Compared with LNCaP cells, HP-LNCaP cells expressed a 1.6-fold increase in IGFR1 β and C4-2B cells showed a 3.8-fold higher level in this growth factor receptor (Fig. 1C). Expression of activated HER2 (pHER2) was also increased 2.5-fold (HP-LNCaP) and 2-fold (C4-2B) compared with LNCaP cells (Fig. 1C). Both androgen-independent cell lines showed increased protein expression of other down-

stream signaling proteins, including pmTOR (1.8-fold; Fig. 1C). There was also a 1.3-fold increase in pp70S6K and a 1.5-fold increase in pS6 in the HP-LNCaP cell lines compared with the LNCaP cell line (Fig. 1C). HP-LNCaP and C4-2B cells also showed 1.5- and 2-fold increase, respectively, in androgen receptor protein expression (refs. 38–43; Fig. 1C). Our findings show a minor increase (1.3-fold) in activated mitogen-activated protein kinase between LNCaP and HP-LNCaP cell lines. It has been reported previously that pMAPK plays a central role in the progression of prostate cancer; however, our data did not support this conclusion. This finding highlights the importance of multiple models to investigate prostate cancer progression (15, 16, 44).

Growth Inhibition by Antiandrogens and Modulation of Protein Expression

To further show hormone sensitivity and resistance among the LNCaP, HP-LNCaP, and C4-2B cell lines, the effect of bicalutamide, the clinically used antiandrogen, and VN/124-1 on cell viability was determined. Viability of LNCaP cells was inhibited by bicalutamide at a lower concentration (IC₅₀, 2.3 μ mol/L) compared with HP-LNCaP cells (IC₅₀, 18 μ mol/L; data not shown). Bicalutamide did

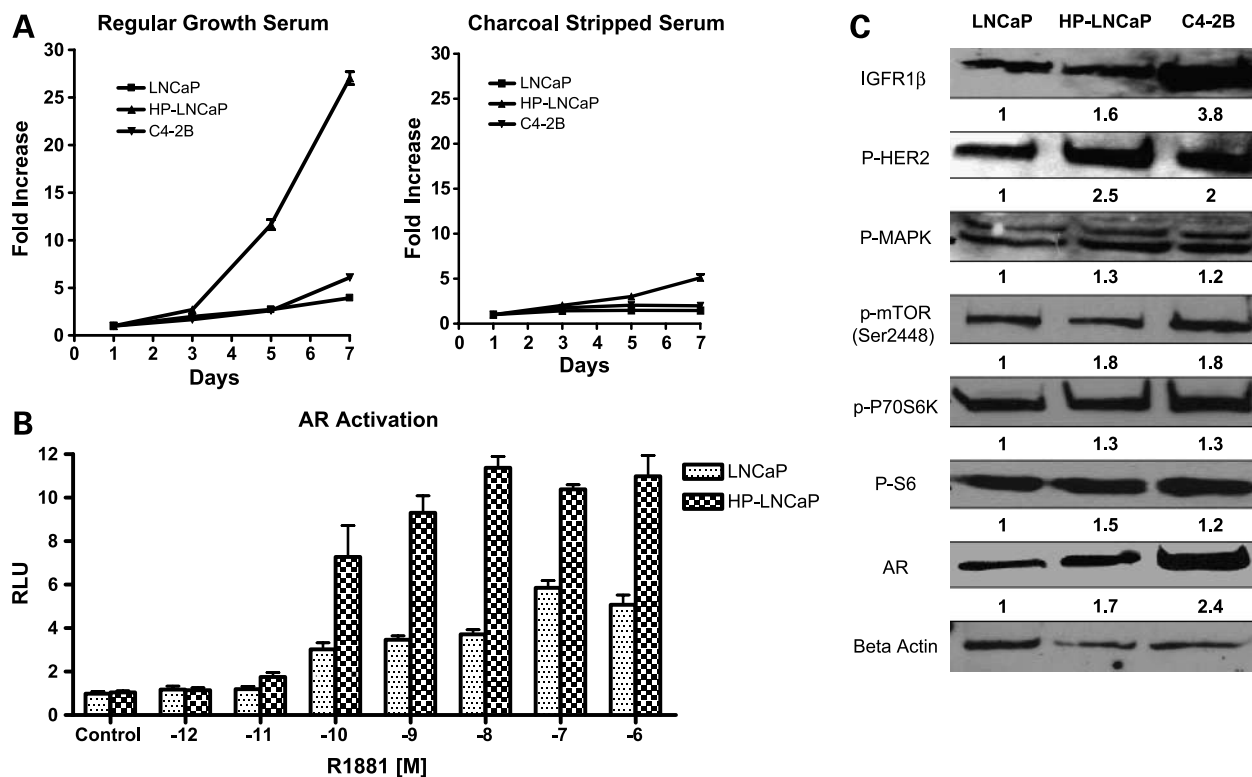


Figure 1. **A**, effect of regular growth serum and medium compared with charcoal-stripped steroid-free growth serum and medium on LNCaP, HP-LNCaP, and C4-2B cell lines. Cell lines were cultured in specified conditions and assayed at 1, 3, 5, and 7 d using the MTT assay as described in Materials and Methods. Cell viability is expressed as fold increase compared with initial absorbance at day 1. **B**, androgen receptor transcriptional activity measured by luciferase in LNCaP and HP-LNCaP cell lines treated with R1881. HP-LNCaP cells are hyperstimulated by R1881 compared with LNCaP. For the luciferase activity, cells were plated in regular growth serum and medium, transfected, and assayed as described in Materials and Methods. Transcriptional activity is expressed in relative luciferase units (RLU). **C**, Western immunoblotting analysis of whole-cell lysates from LNCaP, HP-LNCaP, and C4-2B cells cultured *in vitro* for various signaling proteins. Experimental protocol was described in Materials and Methods. Lane 1, LNCaP; lane 2, HP-LNCaP; lane 3, C4-2B.

not inhibit C4-2B cell viability beyond 40% (data not shown). In contrast, VN/124-1 effectively inhibited proliferation of LNCaP (IC₅₀, 4.9 μmol/L), HP-LNCaP (IC₅₀, 2.9 μmol/L), and C4-2B (IC₅₀, 9.7 μmol/L) cell lines (data not shown). Using a luciferase reporter assay, inhibition of androgen receptor activity was also measured. Bicalutamide inhibited androgen receptor activation in LNCaP cells (IC₅₀, 319 nmol/L), whereas there was little inhibition of androgen receptor activity detected in HP-LNCaP cells (IC₅₀, 3.57 μmol/L; Fig. 2A). VN/124-1 effectively inhibited androgen receptor activation in LNCaP cells (50%) and HP-LNCaP cells (70%) at 1 μmol/L (Fig. 2B). Although bicalutamide decreased androgen receptor activation via luciferase assay in hormone-sensitive LNCaP cells (Fig. 2A), there was no change in activation in HP-LNCaP cells. Additionally, there was no change observed in androgen receptor protein expression following treatment with 1 μmol/L bicalutamide (Fig. 2C). VN/124-1, however, decreased activation of the androgen receptor in both LNCaP cells (IC₅₀, 1 μmol/L) and HP-LNCaP cells (IC₅₀, 411 nmol/L; Fig. 2B) and down-regulated androgen receptor protein expression by 50% after 24 h of treatment (Fig. 2D).

Interestingly, bicalutamide treatment also resulted in a 6-fold increase of IGFR1β protein expression within 1 h of treatment (Fig. 2C). There were no other significant changes in protein levels following treatment with bicalutamide (Fig. 2C). VN/124-1 showed increased expression and activation of several growth factor signaling pathways, including IGFR1β (8.9-fold increase), pHER2 (1.5-fold increase), and pmTOR (Ser²⁴⁴⁸; 3.8-fold increase) 3 h following treatment (Fig. 2D). Unlike bicalutamide, VN/124-1 down-regulated androgen receptor protein expression at 24 h, providing an additional mechanism of action compared with bicalutamide potentially increasing activity (45).

Growth Inhibition of Signal Transduction Inhibitors and Modulation in Protein Expression

Based on increased expression and activation of IGFR1β, pHER2, and pmTOR, we examined the effect of inhibiting growth factor receptors and downstream signaling pathways in all cell lines. Everolimus was equally potent in inhibiting proliferation in hormone-sensitive and hormone-refractory cells lines. The IC₅₀ for LNCaP cells was 0.9 pmol/L, whereas the IC₅₀ for HP-LNCaP and C4-2B cells was 378 and 8.9 pmol/L, respectively (Fig. 3A). Everolimus decreased pmTOR (Ser²⁴⁴⁸) expression by 60% as well as decreased downstream activation of p70S6K (Fig. 3B). We also observed a decrease in protein expression of IGFR1β (50%) and pHER2 (50%) after 24 h of treatment (Fig. 3B). Additionally, androgen receptor expression increased 3.4-fold within 15 min of treatment (Fig. 3B).

Gefitinib was also effective at inhibiting growth of hormone-dependent and hormone-refractory prostate cancer cell lines but at substantially higher concentrations. LNCaP, HP-LNCaP, and C4-2B cells were all sensitive to gefitinib with IC₅₀ values of 5.5, 3, and 7.6 μmol/L, respectively (Fig. 3C). It should be noted that gefitinib was only effective at concentrations higher than 1 μmol/L. Inhibition in HP-LNCaP cells resulted in 80% decrease in

pHER2 within 15 min (Fig. 3A). A 50% decrease in pAkt (Ser⁴⁷³) as well as a 90% decrease in pmTOR at 24 h (Fig. 3D) was also noted. There was no change in IGFR1β expression. Protein analysis supports the specificity of gefitinib and inhibition of downstream pathways. Interestingly, similar to everolimus, gefitinib treatment resulted in a 1.8-fold increase in androgen receptor protein expression within 15 min.

PSA concentration measured by ELISA in the supernatant of HP-LNCaP cells was reduced with the treatment of all agents. After 24 h of treatment, 1 μmol/L bicalutamide decreased PSA to 0 ng/mL, whereas 1 μmol/L VN/124-1 reduced PSA to 0.2 ng/mL (Fig. 4). Comparable with clinical trials gefitinib reduced PSA levels to 0.5 ng/mL (refs. 18, 19; Fig. 4). Treatment of everolimus also reduced PSA levels to 1 ng/mL or 30% of untreated HP-LNCaP control cells (Fig. 3E). There was no statistical difference between any of the treatment groups, although compared with the vehicle-treated control all groups were significantly different.

Cross-talk between Androgen Receptor and Signaling Pathways

To examine further the cross-talk between various signaling pathways and androgen receptor, coimmunoprecipitation was done by preclearing cell lysates with protein A/G and immunoprecipitating with an Akt antibody followed by immunoblotting with androgen receptor antibody. As shown in Fig. 5A, there is interaction between the two pathways in both LNCaP and HP-LNCaP cell lines. However, there is a 7-fold increase in the interaction in HP-LNCaP cells compared with LNCaP cells.

Dual reporter luciferase assays were done to examine the effect of androgen receptor-regulated transcription in the presence of everolimus and gefitinib. Treatment with 0.1 pmol/L everolimus or 1 μmol/L gefitinib resulted in a significant ($P < 0.005$) increase in androgen receptor-mediated transcription compared with vehicle-treated controls of HP-LNCaP cells in the presence of either inhibitor (Fig. 5B and C). It should be noted that androgen receptor activation occurs at subtherapeutic doses of signal transduction inhibitors, as PSA, an androgen receptor reporter gene, is reduced following treatments with high concentrations of inhibitors. Competitive binding assays confirmed insignificant ($P > 0.05$) binding to the androgen receptor, confirming that the compounds did not have an agonistic effect on the androgen receptor activation (Fig. 5B and C).

Despite increased activation of the androgen receptor as a result of treatment with everolimus and gefitinib, we have shown, through coimmunoprecipitation, a modest decrease in the interaction of the androgen receptor and Akt pathway with bicalutamide or VN/124-1 (40% and 60%, respectively; Fig. 5D). Everolimus alone was also modestly effective (40%) at reducing the interaction as well (Fig. 5D). The combinations of everolimus plus bicalutamide or VN/124-1 markedly inhibited the interaction in HP-LNCaP cells. However, everolimus plus VN/124-1 has proven to be superior to the combination with bicalutamide, resulting in

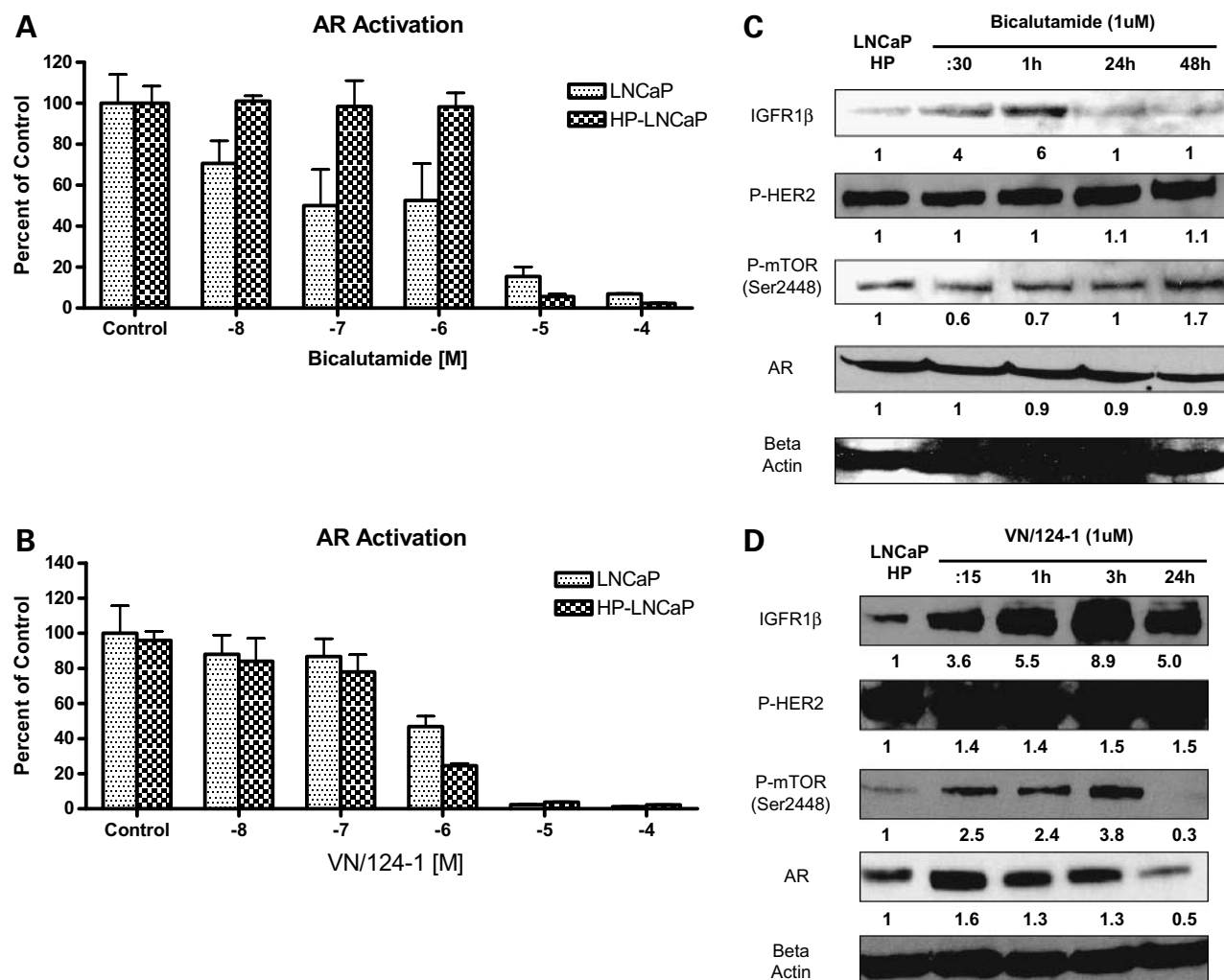


Figure 2. **A**, androgen receptor transcriptional activity measured by luciferase in LNCaP and HP-LNCaP cell lines treated with bicalutamide. LNCaP cells were sensitive to bicalutamide as androgen receptor activation was inhibited in a dose response. Androgen receptor activation in HP-LNCaP cells was only inhibited at higher concentrations of bicalutamide. **B**, androgen receptor transcriptional activity measured by luciferase in LNCaP and HP-LNCaP cell lines treated with VN/124-1. Both LNCaP and HP-LNCaP cells were sensitive to VN/124-1, as androgen receptor activation was inhibited in a dose response in both cell lines. **C**, Western immunoblotting analysis of whole-cell lysates from HP-LNCaP cells cultured *in vitro* in the presence of 1 μ mol/L bicalutamide for 0.5, 1, 24, and 48 h compared with vehicle-treated HP-LNCaP cells. Experimental protocol was described in Materials and Methods. Lane 1, HP-LNCaP; lane 2, bicalutamide, 0.5 h; lane 3, bicalutamide, 1 h; lane 4, bicalutamide, 24 h; lane 5, bicalutamide, 48 h. **D**, Western immunoblotting analysis of whole-cell lysates from HP-LNCaP cells cultured *in vitro* in the presence of 1 μ mol/L VN/124-1 for 0.25, 1, 3, and 24 h compared with vehicle-treated HP-LNCaP cells. Experimental protocol was described in Materials and Methods. Lane 1, HP-LNCaP; lane 2, VN/124-1, 0.25 h; lane 3, VN/124-1, 1 h; lane 4, VN/124-1, 3 h; lane 5, VN/124-1, 24 h.

nearly undetectable expression of androgen receptor immunoprecipitate (Fig. 5D).

Combination of Antiandrogens and Signal Transduction Inhibitors in Hormone-Sensitive and Hormone-Refractory Cell Lines

Further evidence that androgen receptor and signal transduction pathways interact was obtained from a set of experiments in which the growth factor receptor pathways were inhibited by everolimus or gefitinib in combination with bicalutamide or VN/124-1 in LNCaP, HP-LNCaP, and C4-2B cell lines.

After 1 μ mol/L VN/124-1 treatment, cell viability in LNCaP cells was $56.96 \pm 7.6\%$ compared with vehicle-

treated controls. When combined with gefitinib, cell viability decreased to $21.01 \pm 7.2\%$, a decrease that was significant compared with gefitinib alone ($P < 0.05$; Fig. 6A). Bicalutamide (1 μ mol/L) treatment resulted in $92.34 \pm 5.3\%$ cell viability in LNCaP cells as single agent. Although the addition of bicalutamide to gefitinib reduced viability to $66.01 \pm 5.12\%$, compared with control, the difference was not statistically significant when adjusted for multiple comparisons (Fig. 6A). Everolimus was also investigated in combination with bicalutamide and VN/124-1 in LNCaP cells. However, no significant differences were noted with the addition of either hormonal agent to the mTOR inhibitor. Inhibition of epidermal growth factor receptor,

mTOR, and the androgen receptor via triple combination of everolimus, gefitinib, and bicalutamide was more effective in reducing cell viability ($30.31 \pm 3.9\%$) than bicalutamide alone or bicalutamide plus gefitinib ($P < 0.05$). However, triple combination therapy involving VN/124-1 reduced cell viability to $4.37 \pm 0.76\%$ of controls. This result was

not statistically significantly different from gefitinib plus VN/124-1 or bicalutamide plus everolimus plus gefitinib (Fig. 6A).

Further evidence of increased synergism between inhibition of androgen receptor and growth factor receptor pathways was noted by protein expression resulting from

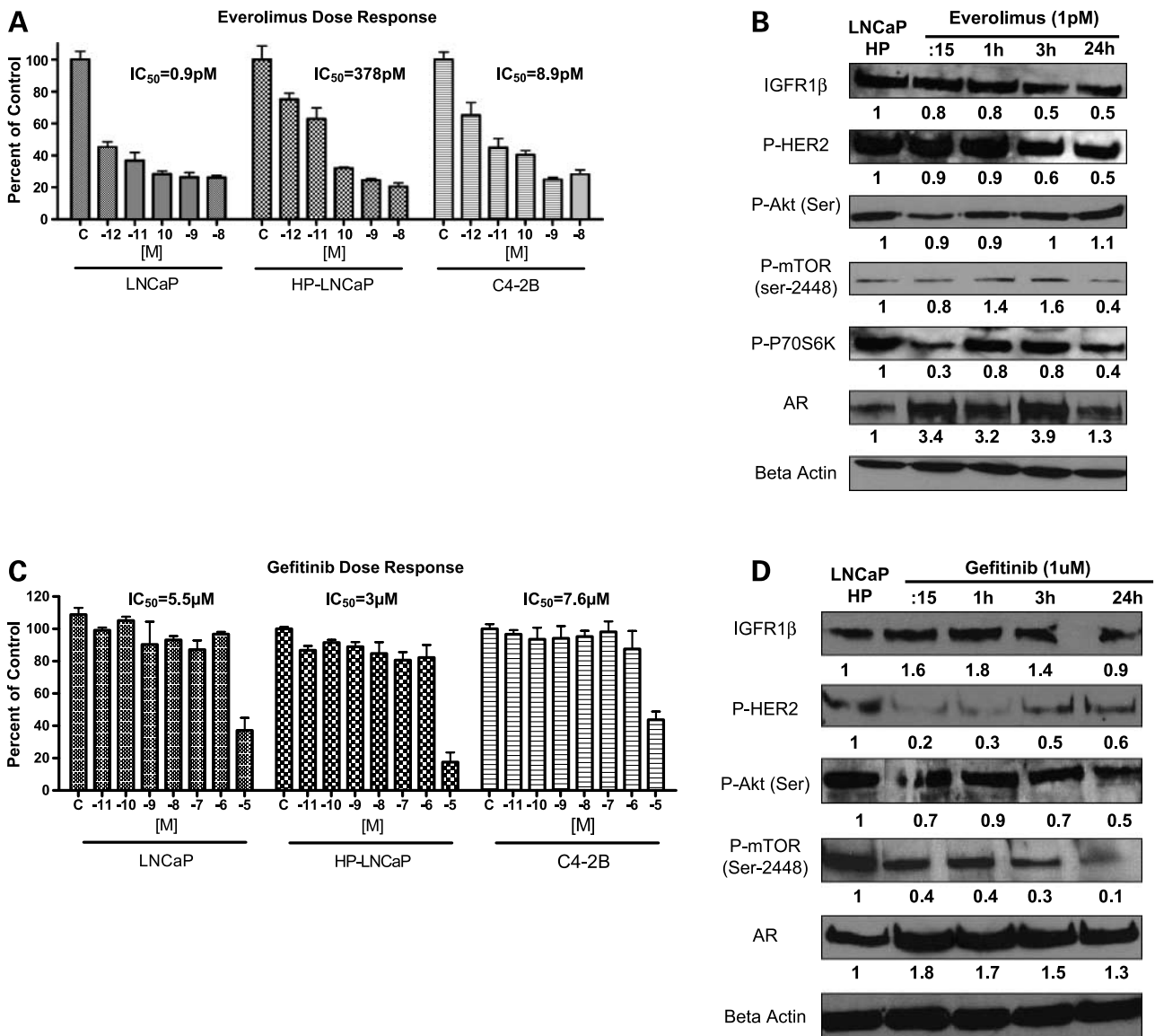
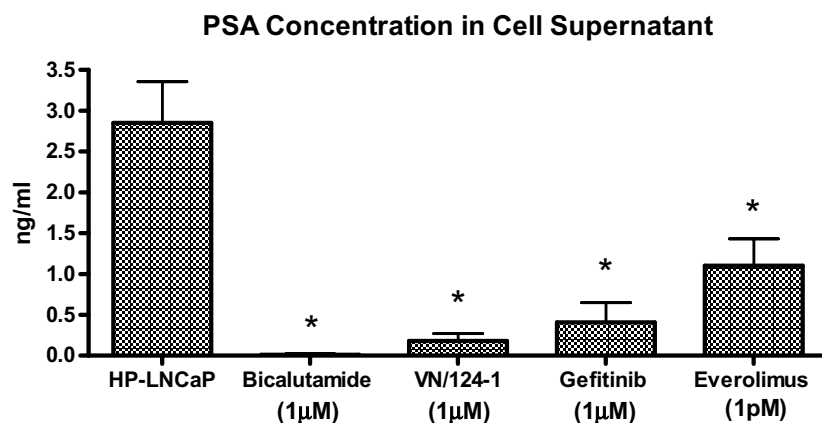


Figure 3. **A**, effect of everolimus on LNCaP, HP-LNCaP, and C4-2B prostate cancer cell lines *in vitro*. Cell lines were cultured in regular serum and medium, plated in triplicate wells, and treated with indicated concentration of everolimus for 8 days and the medium was refreshed every 3 days. Cell viability was measured on day 9 using the MTT assay as described in Materials and Methods. Cell viability is expressed as a percentage of the cells compared with control wells. **B**, Western immunoblotting analysis of whole-cell lysates from HP-LNCaP cells cultured *in vitro* in the presence of 1 pmol/L everolimus for 0.25, 1, 3, and 24 h compared with vehicle-treated HP-LNCaP cells. Experimental protocol was described in Materials and Methods. Lane 1, HP-LNCaP; lane 2, everolimus, 0.25 h; lane 3, everolimus, 1 h; lane 4, everolimus, 3 h; lane 5, everolimus, 24 h. **C**, effect of gefitinib on LNCaP, HP-LNCaP, and C4-2B prostate cancer cell lines *in vitro*. Cell lines were cultured in regular serum and medium, plated in triplicate wells, and treated with indicated concentration of gefitinib for 8 days and the medium was refreshed every 3 days. Cell viability was measured on day 9 using the MTT assay as described in Materials and Methods. Cell viability is expressed as a percentage of the cells compared with control wells. **D**, Western immunoblotting analysis of whole-cell lysates from HP-LNCaP cells cultured *in vitro* in the presence of 1 μ mol/L gefitinib for 0.25, 1, 3, and 24 h compared with vehicle-treated HP-LNCaP cells. Experimental protocol was described in Materials and Methods. Lane 1, HP-LNCaP; lane 2, gefitinib, 0.25 h; lane 3, gefitinib, 1 h; lane 4, gefitinib, 3 h; lane 5, gefitinib, 24 h.



* $p < .05$ - One Way ANOVA, pair wise comparison by Tukey Test

Figure 4. Total PSA ELISA from HP-LNCaP cell supernatant treated with various inhibitors. The experimental protocol was described in Materials and Methods.

several combination treatments in LNCaP and HP-LNCaP cell lines. LNCaP exhibited a 50% decrease in protein expression of IGFR1 β after treatment of VN/124-1 and everolimus or gefitinib. However, the addition of bicalutamide to everolimus or gefitinib was not as effective in reducing protein expression (Fig. 6B). Combination therapy with VN/124-1 and gefitinib was also effective in reducing pMAPK expression by 50% compared with vehicle-treated controls (Fig. 6B).

Similar results were observed with HP-LNCaP cells, although an even greater synergy was observed as a result of the increased cross-talk and interaction between the androgen receptor and signal transduction pathway in HP-LNCaP cells compared with LNCaP cells. Gefitinib alone minimally decreased HP-LNCaP cell viability ($98.75 \pm 5.8\%$ of controls) compared with vehicle-treated controls. Bicalutamide and VN/124-1 also showed a modest decrease in cell viability ($82.59 \pm 13.3\%$ and $76.5 \pm 10.6\%$, respectively; Fig. 6C) compared with controls. However, the addition of VN/124-1 to gefitinib significantly ($P < 0.05$) decreased cell viability in HP-LNCaP cell viability ($24.6 \pm 9.1\%$) compared with the gefitinib or VN/124-1 alone (Fig. 6C). Everolimus reduced cell viability by $74.18 \pm 9.6\%$. The addition of bicalutamide to everolimus reduced viability to $54.63 \pm 13.51\%$ of controls, although the addition of VN/124-1 resulted in a significantly greater decrease in cell viability ($47.43 \pm 12.12\%$) than bicalutamide ($P < 0.05$). Triple combination therapy of gefitinib, everolimus, and bicalutamide reduced cell viability to $21.64 \pm 3.8\%$ of controls, whereas triple combination with VN/124-1 reduced cell viability to $4.9 \pm 1\%$. However, there does not appear to be any benefit of triple therapy compared with dual therapy of gefitinib plus VN/124-1 (Fig. 6C).

Examination of protein expression after combination therapy in HP-LNCaP cells supported results of proliferation studies and the cross-talk hypothesis. HP-LNCaP cells exhibited marked decrease in multiple signaling proteins, including pAkt, pmTOR, and pMAPK, compared with LNCaP protein expression. In HP-LNCaP cells, VN/124-1 plus everolimus or gefitinib decreased protein ex-

pression of IGFR1 β (40%), pmTOR (Ser²⁴⁴⁸; 50%), pS6 (20%), pMAPK (40%), and androgen receptor (40%; Fig. 6D). Similar findings were also observed with the addition of bicalutamide to signal transduction inhibitors.

Viability of C4-2B cells was significantly decreased with VN/124-1 plus gefitinib treatment ($68.19 \pm 2.57\%$) compared with control, which was significantly different ($P < 0.05$) compared with gefitinib alone (data not shown). The addition of bicalutamide or VN/124-1 to everolimus also significantly reduced cell viability by $51.09 \pm 2.20\%$ and $54.66 \pm 3.3\%$, respectively ($P < 0.05$; data not shown). However, despite decreased cell viability, protein expression was largely unchanged (data not shown).

Discussion

Our model indicates that continual passage of LNCaP cells in regular growth serum and medium results in changes in the phenotype of hormone-dependent cells. The cell culture conditions mimic physiologic conditions in patients where tumor cells continuously proliferate. This allows HP-LNCaP cells to function as a good model for investigation of hormone-resistant prostate cancer and the progression to advanced disease. Unlike LNCaP cells, HP-LNCaP cells grow well in charcoal-stripped serum and phenol red-free medium and are exponentially more aggressive in regular serum conditions. Additionally, HP-LNCaP cells are hypersensitive to androgens compared with LNCaP cells.

Compensatory cross-talk between the androgen receptor and the mitogen-activated protein kinase, phosphatidylinositol 3-kinase, Akt, and mTOR pathways was observed, as inhibition of signal transduction pathways resulted in an increase in androgen receptor protein expression and inhibition of androgen receptor resulted in increased expression and activation of IGFR1 β , pHER2, and pmTOR. The data indicate that hormone-resistant prostate cancer cells exhibit survival mechanisms, including increased activation of signaling pathways in the presence of pharmacologic inhibitors. Additionally, the rapid increase in androgen receptor protein expression within 15 min of

mTOR inhibition suggests that androgen receptor expression may be regulated through nongenomic mechanisms. An understanding of the mechanisms of growth and survival along with the effect of androgen receptor and signal transduction pathway inhibition provides a rationale for combination treatment to inhibit androgen receptor and signal transduction pathways.

Our results indicate that dual blockade of androgen receptor with antiandrogens and signal transduction pathway with signal transduction inhibitors has the potential to restore sensitivity to hormonal therapy and delay the use of cytotoxic chemotherapy. Although the dual inhibition of signal transduction pathways with bicalutamide is effective, as both pathways are activated, treatment of VN/124-1 in combination with signal transduction inhibitors is superior and synergistic. This is a result of

two findings. First, there is a significant increase in compensatory signaling and cross-talk mechanisms found in HP-LNCaP cells. Second, bicalutamide is less effective than VN/124-1 as single-agent therapy in HP-LNCaP cells.

The synergistic action of VN/124-1 plus gefitinib or everolimus in HP-LNCaP cells can be attributed to blockade of the proposed compensatory signaling mechanisms. Inhibition of androgen receptor activation with VN/124-1 increased signal transduction pathway activation more than bicalutamide. Additionally, the increased efficacy in HP-LNCaP cells is potentially due to the down-regulation of the androgen receptor as well as other currently undetermined mechanisms of action.

First-line therapy to achieve androgen deprivation by pharmacologic or surgical castration does not inhibit all of the available testosterone in patients. It is estimated that

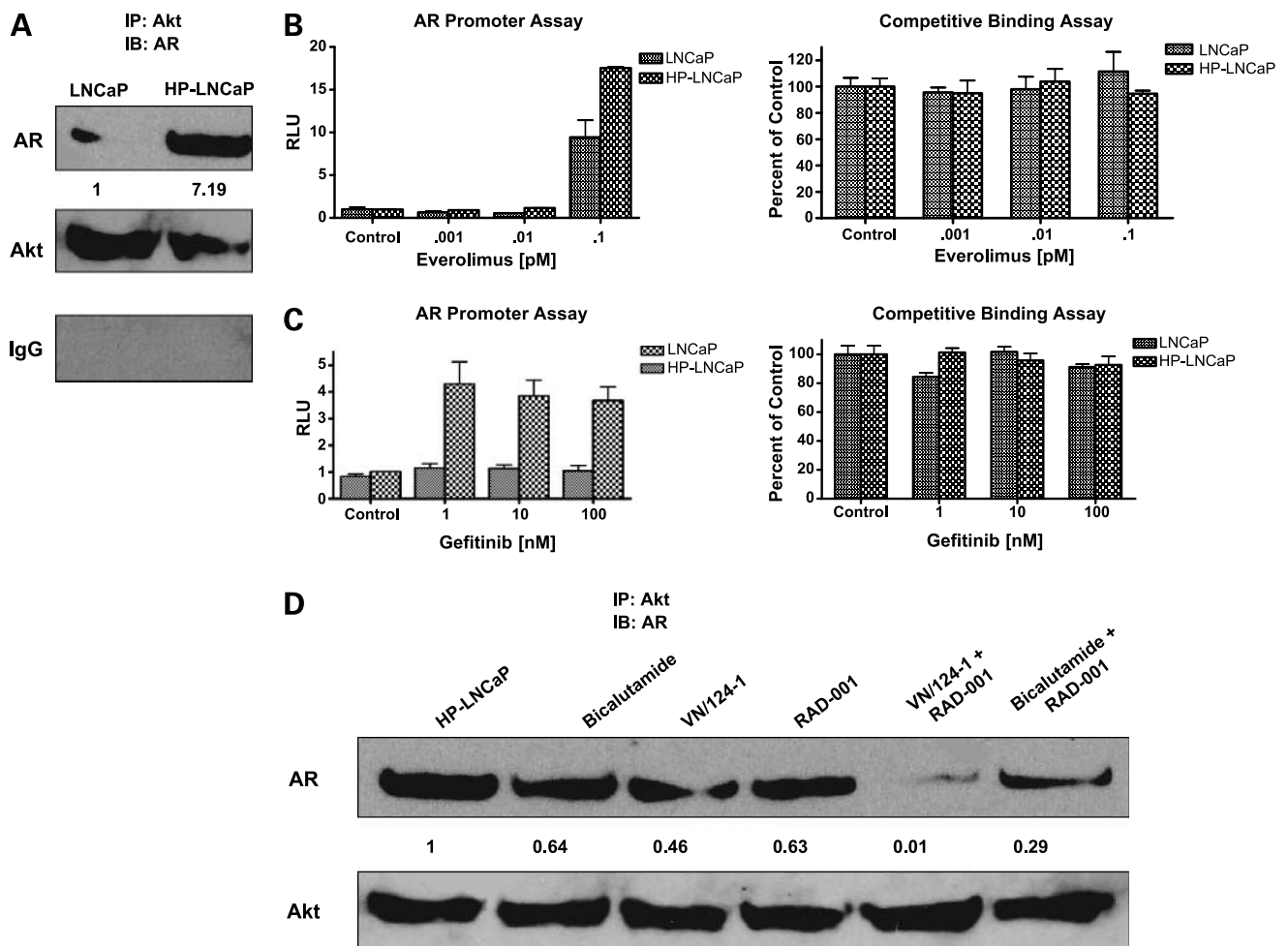


Figure 5. **A**, coimmunoprecipitation of whole-cell lysates from LNCaP and HP-LNCaP cells. The experimental protocol was described in Materials and Methods. Cell lysate (200 μ L) was subjected to immunoprecipitation with anti-Akt antibody then subjected to Western immunoblotting using androgen receptor antibody. **B**, transcriptional activity of luciferase and competitive binding assays in the presence of varying concentrations of everolimus or gefitinib. **C**, transcriptional activity is expressed in relative luciferase units in LNCaP and HP-LNCaP cell lines, whereas binding assays were expressed in percentage of control. The experimental protocol was done as described in Materials and Methods. **D**, coimmunoprecipitation of whole-cell lysates from HP-LNCaP cells treated with bicalutamide (1 μ mol/L), VN/124-1 (1 μ mol/L), everolimus (1 pmol/L), VN/124-1 (1 μ mol/L) plus everolimus (1 pmol/L), and bicalutamide (1 μ mol/L) plus everolimus (1 pmol/L) for 24 h. The experimental protocol was described in Materials and Methods. Cell lysate (200 μ L) was subjected to immunoprecipitation with anti-Akt antibody then subjected to Western immunoblotting using androgen receptor antibody.

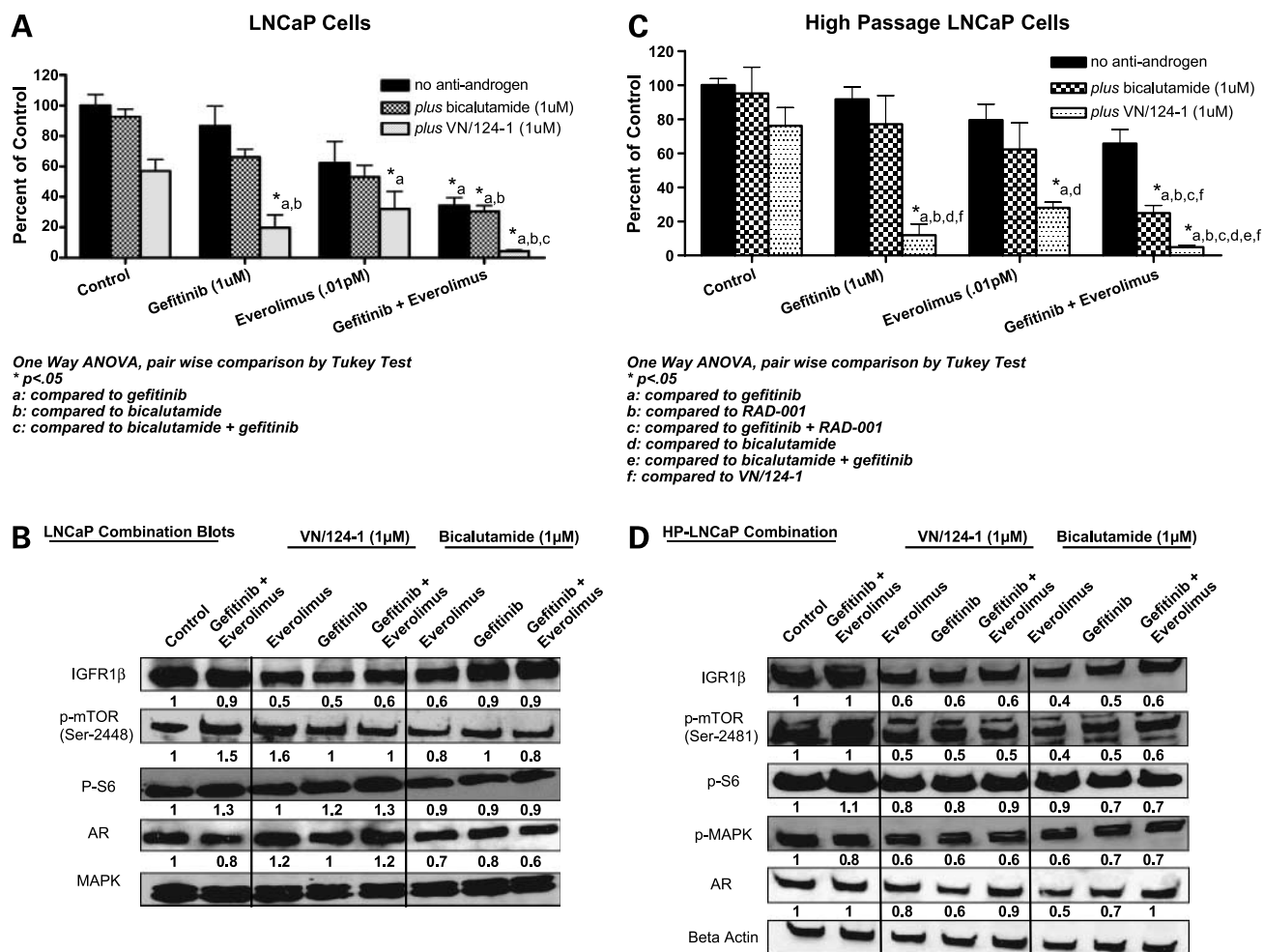


Figure 6. **A**, *in vitro* antiproliferative effect of combination antiandrogen (bicalutamide or VN/124-1) and signal transduction inhibitor (gefitinib or everolimus) in LNCaP cells. Effect of the signal transduction inhibitor alone or in combination with bicalutamide or VN/124-1. Cell viability was assayed using MTT as described in Materials and Methods. **B**, Western immunoblotting analysis of whole-cell lysates from LNCaP cells cultured *in vitro* in the presence of gefitinib (1 μ mol/L) and everolimus (1 pmol/L) alone or in combination with VN/124-1 or bicalutamide at 24 h compared with vehicle-treated LNCaP cells. Experimental protocol was described in Materials and Methods. Lane 1, HP-LNCaP; lane 2, gefitinib (1 μ mol/L) + everolimus (1 pmol/L); lane 3, everolimus (1 pmol/L) + VN/124-1 (1 μ mol/L); lane 4, gefitinib (1 μ mol/L) + VN/124-1 (1 μ mol/L); lane 5, gefitinib (1 μ mol/L) + everolimus (1 pmol/L) + VN/124-1 (1 μ mol/L); lane 6, everolimus (1 pmol/L) + bicalutamide (1 μ mol/L); lane 7, gefitinib (1 μ mol/L) + bicalutamide (1 μ mol/L); lane 8, gefitinib (1 μ mol/L) + everolimus (1 pmol/L) + bicalutamide (1 μ mol/L). **C**, *in vitro* antiproliferative effect of combination antiandrogen (bicalutamide or VN/124-1) and signal transduction inhibitor (gefitinib or everolimus) in HP-LNCaP cells. Effect of the signal transduction inhibitor alone or in combination with bicalutamide or VN/124-1. Cell viability was assayed using MTT as described in Materials and Methods. **D**, Western immunoblotting analysis of whole-cell lysates from HP-LNCaP cells cultured *in vitro* in the presence of gefitinib (1 μ mol/L) and everolimus (1 pmol/L) alone or in combination with VN/124-1 or bicalutamide at 24 h compared with vehicle-treated HP-LNCaP cells. Experimental protocol was described in Materials and Methods. Lane 1, HP-LNCaP; lane 2, gefitinib (1 μ mol/L) + everolimus (1 pmol/L); lane 3, everolimus (1 pmol/L) + VN/124-1 (1 μ mol/L); lane 4, gefitinib (1 μ mol/L) + VN/124-1 (1 μ mol/L); lane 5, gefitinib (1 μ mol/L) + everolimus (1 pmol/L) + VN/124-1 (1 μ mol/L); lane 6, everolimus (1 pmol/L) + bicalutamide (1 μ mol/L); lane 7, gefitinib (1 μ mol/L) + bicalutamide (1 μ mol/L); lane 8, gefitinib (1 μ mol/L) + everolimus (1 pmol/L) + bicalutamide (1 μ mol/L).

10% of baseline testosterone levels are a result of peripheral conversion in the adrenal glands (46). It is also postulated that the adrenal production of androgens may be partially responsible for the progression to hormone-resistant prostate cancer. It has been shown that more complete androgen blockade containing an androgen synthesis inhibitor and antiandrogen causes a statistically significant delay in disease progression despite modest clinical efficacy (47). Additionally, recent clinical findings indicate the success of other androgen synthesis inhibitors in

hormone-resistant prostate cancer patients using PSA response as a measurable endpoint (48). Our potent novel androgen synthesis inhibitor with antiandrogenic activity, VN/124-1, has potential as treatment in hormone-dependent prostate cancer and possibly hormone-resistant prostate cancer, providing further clinical benefit over pure antiandrogens (7).

This report provides additional evidence that signaling pathways, such as HER2, Akt, mitogen-activated protein kinase, and mTOR, are implicated in the progression to

hormone-resistant prostate cancer as shown by others (12–14, 49). However, our data indicate the progression to advanced disease is not a shift from the androgen receptor pathway to a signal transduction pathway but rather a balance between the two pathways. The activation of Akt, mTOR, and p70S6K and sensitivity to signal transduction inhibitors in hormone-dependent LNCaP cells suggests the importance of these pathways from the onset of the disease. Given the modest compensatory signaling observed in LNCaP cells, there may be a clinical benefit in using a signal transduction inhibitor in combination with androgen receptor down-regulators in hormone-dependent prostate cancer patients, possibly delaying transition to tyrosine kinase receptor signaling and hormone resistance.

In summary, our data suggest that our novel androgen synthesis inhibitor is more effective than current antiandrogens in hormone-dependent prostate cancer. In addition, our studies reveal that this compound has some activity in hormone-resistant prostate cancer, although the mechanism remains to be determined. Combined with inhibitors of signal transduction pathways, this compound may be an effective strategy for treatment of hormone-resistant prostate cancer.

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