Prolonging hormone sensitivity in prostate cancer xenografts through dual inhibition of AR and mTOR

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BACKGROUND: To determine the mechanisms associated with loss of androgen dependency and disease progression in prostate cancer (PCa), we investigated the relationship between the androgen receptor (AR) and mTOR pathways and the impact of inhibiting both pathways in androgen-dependent and castration-resistant PCa models. 

EXPERIMENTAL DESIGN: Androgen-dependent (LNCaP) and castration-resistant PCa (HP-LNCaP) cells were grown as tumours in SCID mice. Once tumours reached 500 mm³, animals were grouped and injected subcutaneous with vehicle, our novel anti-androgen/androgen synthesis inhibitor, VN/124-1, bicalutamide, and everolimus. Tumour volumes were measured biweekly. The PSA and protein analyses were performed after completion of the treatment.

RESULTS: The addition of everolimus to bicalutamide treatment of resistant tumours significantly reduced tumour growth rates and tumour volumes. Anti-androgen treatment also increased protein expression of multiple signal transduction pathways earlier than vehicle-treated control xenografts. VN/124-1 plus everolimus acted in concert to reduce tumour growth rates in our castration-resistant xenograft model.

CONCLUSION: This study suggests that dual inhibition of AR and mTOR in castration-resistant xenograft models can restore sensitivity of tumours to anti-androgen therapy. Furthermore, after bicalutamide failure, dual inhibition with VN/124-1 and everolimus was the most effective treatment.

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The progression to castration-resistant prostate cancer (CRPC) requires the ability to overcome two major roadblocks, the initial survival of prostate cancer (PCa) cells during the acute onset of androgen deprivation and the ability to proliferate in a castrate-independent manner. During castrate-resistant progression, PCa cells adapt to alternative cellular signalling to survive in an androgen-depleted environment.

The lack of successful treatments for CRPC is, in part, a reflection of the poor understanding of the mechanisms and pathways that lead to increased cell survival after androgen ablation. Several mechanisms and pathways have been proposed to explain castrate resistance. The two main mechanisms of progression are those that involve the androgen receptor (AR) and those that bypass the AR. However, these pathways are not mutually exclusive, and cross-talk between the AR and signal transduction pathways (STPs) in CRPC has been demonstrated (Baron et al., 2004; Cinar et al., 2005; Culig et al., 2005; Ghosh et al., 2005; Edwards and Bartlett, 2005a, b).

We and others have shown that PCa cells can become hypersensitive to androgens as a means of circumventing androgen blockade (Gregory et al., 2001a, b). Hypersensitivity is believed to be caused by an increase in AR expression, which is a result of AR gene amplification (Visakorpi et al., 1995; Linja et al., 2001; Edwards et al., 2003; Chen et al., 2004). Koivisto et al. (1997) originally showed that AR amplification contributed directly to the failure of androgen deprivation by allowing cells to grow in low concentrations of androgen, resulting in hypersensitivity (Gregory et al., 2001a, b). Numerous studies have shown that despite the elimination of testicular androgens, the level of T and DHT in recurrent PCa tissue is unchanged relative to levels in benign tissue (Mohler et al., 2004; Titus et al., 2005). Intraprostatic androgen concentrations are therefore likely to be more precise indicators of the tumour environment than serum hormone levels (Habib et al., 1976; Heracek et al., 2007). Furthermore, it has been shown that DHT levels in prostatic tissue of castrated patients are sufficient to activate the AR and stimulate expression of androgen-regulated genes (Culig et al., 1999; Mohler et al., 2004; Mostaghel et al., 2007).

The exact mechanisms of steroids in promoting proliferation in CRPC remain unclear. However, there is increasing evidence to suggest that compensatory signalling mechanisms and cross-talk occur between growth factor receptor pathways and AR in...
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androgen-dependent and CRPC cell lines. Recent investigations have elucidated multiple mechanisms for regulation of AR by STPs and vice versa (Cinar et al, 2005; Yang et al, 2005). Several mechanisms of cross-talk have been reported between the AR and P13K/Akt/mTOR signalling pathway. Indeed, we have previously reported that the P13K/Akt/mTOR pathway is upregulated in LNCaP and HP-LNCaP cells (Schayowitz et al, 2008). Although the specific therapeutic impact is unknown, synergy between AR antagonists and P13K/Akt/mTOR inhibitors further suggests a functional relationship between the two pathways (Lin et al, 1999, 2001, 2004; Manin et al, 2002; Sun et al, 2003; Festuccia et al, 2005; Yang et al, 2005; Schayowitz et al, 2008).

From our previous in vitro findings (Schayowitz et al, 2008), we concluded that growth factor receptor pathways, specifically P13K/Akt/mTOR, are upregulated as LNCaP androgen-dependent cells adapt to become castrate resistant. We found that blocking both pathways with AR plus mTOR inhibitors was more effective that either inhibitor alone in reducing proliferation (Schayowitz et al, 2008). In this report, we have investigated whether tumour growth of apparent castrate-resistant tumours could be effectively inhibited by combining an mTOR inhibitor (everolimus, RAD-001, of apparent castrate-resistant tumours could be effectively inhibited by combining an mTOR inhibitor (everolimus, RAD-001, a rapamycin analogue inhibitor that blocks the downstream signalling cascade in vitro and in vivo (Boulay et al, 2004; Vignot et al, 2005)) with our novel compound VN/124-1. VN/124-1 is a potent inhibitor of CYP17 and reduces androgen synthesis. It also binds to the AR and causes its degradation. VN/124-1 is at least as effective as current anti-androgens in androgen-dependent PCa cell lines and significantly more effective in CRPC cells. Combined with inhibitors of STPs, this compound may be an effective strategy for treatment of CRPC (Schayowitz et al, 2008).

MATERIALS AND METHODS

Materials

RPMI, T-medium, penicillin/streptomycin solution (10 000 IU each), 0.25% trypsin-EDTA (1 nmol l−1) solution, and Dulbecco’s phosphate-buffered saline were obtained from Invitrogen (Carlsbad, CA, USA). Regular serum and charcoal-stripped fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). The C4-2B cells were kindly supplied by Dr Yun Qiu (University of Maryland, Baltimore, MD, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Tween 20 were obtained from Sigma Chemicals Company (St. Louis, MO, USA). The ECL chemiluminescence kit and Hybrid-ECL nitrocellulose membranes were purchased from Amersham Biosciences (Piscataway, NJ, USA). Antibodies against p-HER2 was purchased from Upstate (Temecula, CA, USA) and AR by one of us, Dr Vincent CO Njar (Handratta et al, 2002) solution (Horoszewicz et al, 1983). The CRPC (HP-LNCaP) cells were created through regular passaging of the LNCaP cell line for more than 1 year, as previously described and characterised (Schayowitz et al, 2008). Low-passage LNCaP cells (LNCaP) were designated as under passage 25, whereas high-passage cells (HP-LNCaP) were over passage 75. Both lines were maintained in identical conditions. The CRPC in vivo-derived cell line from LNCaP cells (C4-2B) were maintained in T-Medium with 10% FBS and 1% penicillin/streptomycin (Thalmann et al, 1994, 2000).

Cell growth studies

MTT proliferation assays were performed to examine the effect of R1881, a synthetic, non-metabolised androgen, on cell proliferation in LNCaP and HP-LNCaP cell lines. The IC50 values for inhibition were calculated via non-linear regression. All growth studies involving anti-androgen were performed in the presence of 0.1 nM R1881.

Western blotting

The protein extracts from tumours were prepared by pelleting homogenised tissue suspended in ice-cold DPBS and lysed with cell lysis buffer containing protease inhibitors, Tris-CL, and Triton X-100. Equal amounts (50 µg) of protein from each sample were separated on a denaturing polyacrylamide gel, transferred to nitrocellulose membrane, and western blotting was carried as previously described (Schayowitz et al, 2008). The densitometric values are corrected for loading control shown in the bottom panel of the figures. All western blots were performed at least two times; blots shown are representative of all results.

PSA ELISA

Tissue lysates and serum were loaded in the total PSA ELISA Kit (DSLabs, Webster, TX, USA) in triplicates to determine PSA concentrations. The assay standards, controls, and samples were incubated in 96-well plates following the manufacturer’s instructions. Each original experimental sample was assayed in triplicate. The PSA values (ng ml−1) were determined using log–log curve fit to the standard curve.

Tumour growth in male SCID mice

All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland School of Medicine. Male SCID mice 4–6 weeks of age were obtained from the National Cancer Institute-Frederick Cancer Research Center (Frederick, MD, USA). The mice were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum. The mice were inoculated with LNCaP or HP-LNCaP cells. These in vivo studies were performed using two PCa cell lines. Androgen-dependent cells, designated as LNCaP cells, and androgen-independent cells, denoted as HP-LNCaP cells (Schayowitz et al, 2008), were inoculated into male SCID mice and formed tumours. The mice were then treated with a combination of inhibitors to block the AR and mTOR activation. The cells were grown in regular growth conditions (RPMI, 10% FBS and 1% penicillin/streptomycin). Subconfluent cells were scrapped into DPBS, collected by centrifugation, and resuspended in Matrigel (10 mg ml−1) at 2.0 × 10⁶ cells per ml. Each mouse received a subcutaneous (s.c.) inoculation at one site per flank with 100 µl of cell suspension. Tumour formation varied between 4 and 8 weeks by cell line, although on tumours reaching 500 mm³, mice were grouped in 5 or 10 with equal tumour volumes. Tumour size was measured twice weekly with calipers, and tumour volume was calculated by the formula 4/3πr₁² × r₂, where r₁ is the smaller
radius. Treatments varied between 3 and 7 weeks by experiment. All mice were killed by decapitation under anaesthesia, and the trunk blood was collected for PSA analysis. Tumours were also excised, cleaned, weighed, and stored in −80°C for additional analysis.

Statistics

Data on tumour volume and weight were analysed separately. Growth curve models were used to summarise the pattern of response over treatment duration. The tumour growth rate (the slope of regression line) was compared between pre-specified treatment groups. The mean tumour volume was modelled as a combination of fixed effects shared by all subjects and random effects unique to a particular animal. We used random effects for both intercept and slope at the animal level. The proportions of tumours that shrank below a threshold during treatment were compared using Fisher’s exact test. The treatment groups were compared with one another at 0.05 level of statistical significance. All reported P-values were two sided. Mixed-effects models were used to compare average tumour volume on pre-specified time points. The Tukey–Kramer method was used to adjust for multiple comparisons.

RESULTS

In vitro effects of androgens on LNCaP and HP-LNCaP

As we previously reported, the proliferation of HP-LNCaP cells was significantly greater than that of LNCaP cells in the presence and absence of steroids (Schayowitz et al., 2008). The LNCaP cells demonstrated a biphasic response to the synthetic androgen, R1881 (Figure 1). Growth was stimulated by ~50% at 10⁻¹⁰ M in LP-LNCaP cells and inhibited by 50% at 10⁻⁹ M in HP-LNCaP cells. The HP-LNCaP cell proliferation was stimulated in a biphasic manner, although the dose response curves were shifted 3-log to the left (Figure 1). Thus, HP-LNCaP cells were stimulated by 50% at 10⁻¹³ M. Proliferation was inhibited at concentrations above 10⁻¹⁰ M. These results suggest that HP-LNCaP cells are hypersensitive to androgens and can respond to very low levels of androgens, thus contributing to their apparent androgen independence. These conclusions are supported by our previous observation that AR levels were increased in HP-LNCaP cells compared with LNCaP cells (Schayowitz et al., 2008). However, the absolute values differ between the different assay systems used in the different studies.

The effects of an anti-androgens and mTOR inhibition on growth of LNCaP tumours

The effect of treatment with AR and mTOR inhibitors was compared with the addition of an mTOR inhibitor to bicalutamide therapy, as tumours progressed on the anti-androgens. A total of 40 male SCID mice were inoculated with LNCaP cells and monitored weekly for tumour formation. When tumours reached 500 mm³, mice were then grouped so that tumour volumes were not significantly different (P = 0.66) and treated with bicalutamide (100 mg kg⁻¹ q.d. s.c.) or everolimus (2.5 mg kg⁻¹ every 2 weeks p.o.). When tumours had doubled in volume (tumour progression) on day 7, animals in the bicalutamide group were subgrouped (n = 10) by equal tumour volume and continued on anti-androgen only or administered everolimus (2.5 mg kg⁻¹ every 2 weeks p.o.) in addition to bicalutamide (100 mg kg⁻¹ q.d. s.c.).

As seen in Figure 2, tumour growth was controlled in the group administered bicalutamide plus everolimus, whereas tumours continued to grow during treatment with bicalutamide and everolimus alone. Inhibition of tumour growth in the combination treatment group was maintained for over 50 days.

Animals in the control and bicalutamide group were monitored for over 26 days, the everolimus group for over 33 days, and mice treated with bicalutamide plus everolimus were studied for 65 days. Linear mixed-effects models were used to analyse the data. Random effects for intercept and slope at the tissue level nested within a mouse were used to fit the model. Data on tumour volume were log transformed to satisfy the assumptions of the linear mixed-effects models. There were no differences in average tumour volume on day 0 across treatment groups; P = 0.66. The average tumour volume on day 7 was not different between mice that stayed on bicalutamide and the group administered everolimus in combination.

Figure 1 The effect of androgen on LNCaP and HP-LNCaP cell lines. Cell lines were treated with increasing concentrations of R1881 and assayed on day 8 using the MTT assay as described in Materials and Methods. Cell stimulation is expressed as percent control compared with vehicle-treated control (mean ± s.e.).
addition to bicalutamide; \( P = 0.59 \). By day 26, the growth rates between bicalutamide plus everolimus and control (\( P < 0.0001 \)) or bicalutamide (\( P < 0.0001 \)) were significantly reduced. Animals treated with everolimus alone, compared with everolimus added to bicalutamide, also had statistically different growth rates (\( P = 0.04 \)).

The results suggest that in the LNCaP xenograft model, the addition of everolimus to bicalutamide treatment of resistant tumours was more effective than either drug alone, and restored the sensitivity of tumours to anti-androgen therapy.

### The effects of anti-androgens on the expression of proteins in STPs in HP-LNCaP tumours

We sought to determine the molecular impact of the anti-androgen treatment over time in a CRPC model. For this investigation, we analysed tumours collected at the same time points. A total of 30 male SCID mice were inoculated with HP-LNCaP cells. Measurable tumours formed in \( \sim 4 \) weeks after inoculation. Mice were then grouped (\( n = 15 \)) and were treated with vehicle (control) or bicalutamide (100 mg kg\(^{-1}\) q.d. s.c.), and tumours were measured weekly (Figure 3A). Mice (\( n = 5 \)) with equal tumour volumes were isolated at three different time points (2, 3, and 4 weeks) and killed. Tumours were homogenised and prepared for protein analysis as described in Materials and Methods.

Tumours of mice treated with anti-androgens were minimally responsive as expected, as these mice were inoculated with androgen-resistant cells (Figure 3A). There was an increase in expression of proteins in the signalling pathways over time in control and treated groups. By 4–6 weeks, all proteins measured in the signalling pathways were increased. Additionally, bicalutamide treatment induced increased expression of p-Akt, p-P70S6K, and p-PS6 faster than in control mice. Changes in expression of p-HER2, p-MAPK, and p-mTOR at 4 weeks (data not shown) were about the same in control and bicalutamide-treated tumours. Serum was also collected for PSA analysis. As in tissue, serum PSA levels increased after 4 and 6 weeks compared with levels at 2 weeks in control mice. However, PSA levels in the serum, but not tissue levels, were greatly reduced at all time periods in the bicalutamide-treated group compared with controls (Figure 3C). These results suggest that serum PSA level did not reflect PSA levels in the tumour tissue or the growth of tumour.

### The effect of anti-androgens and mTOR inhibitor on HP-LNCaP xenografts

The rationale for this in vivo investigation was based on our previously described in vitro findings, which suggested that, unlike bicalutamide, VN/124-1 is effective in hormone-dependent and CRPC models (Schayowitz et al, 2008). VN124-1 may also be more effective in blocking tumour growth in the CRPC model, in combination with mTOR inhibition (Schayowitz et al, 2008).

A total of 30 male SCID mice were inoculated with HP-LNCaP cells. Approximately 4 weeks later, on tumour formation, mice were divided into six groups (\( n = 5 \)) and treatment was initiated. The six groups included control, VN/124-1, bicalutamide, everolimus, bicalutamide plus everolimus, and VN/124-1 plus everolimus.

Control tumours grew rapidly and mice were killed 27 days after the start of the treatment (Figure 4A). VN/124-1 (50 mg kg\(^{-1}\) b.i.d. s.c.) was effective for 15 days, demonstrating a slower trend in tumour growth compared with control. However, after day 15, tumours had increased growth rates and mice were killed at day 36. Everolimus (2.5 mg kg\(^{-1}\) every 2 weeks p.o.) was effective in reducing tumour volume for the first 30 days of the experiment, after that tumour growth was rapid and exponential. Dual inhibition of bicalutamide plus everolimus was more effective than either bicalutamide or everolimus alone at day 35 (Figure 4A). However, comparable with all other treatment groups, once treatment failed, tumour growth was rapid. The bicalutamide plus everolimus treatment group had a tumour volume over 300% at
resistant to the bicalutamide plus everolimus therapy, they remained sensitive to VN/124-1 plus everolimus. Tumour volumes were < 750 mm$^3$ after 43 days at termination of the experiment (Figure 4A). All treatments were well tolerated and there were no changes in body weight of the treated animals over the duration of the experiment.

For statistical analysis, animals in the control group were monitored over 29 days. In the VN/124-1 and bicalutamide groups, tumours were measured for 37 days. The three groups receiving everolimus administration were studied for 43 days. The tumour growth rate (slope) was compared between the controls and each of the five treatment groups. The growth curves were estimated over 29 days, but there were no differences found in growth rate among these groups. However, pairwise comparisons of mean tumour volume on days 29 and 43 demonstrated a statistically significant difference between VN/124-1 plus everolimus and everolimus ($P < 0.003$) and also bicalutamide ($P < 0.0001$). Therefore, the addition of VN/124-1 to everolimus therapy proved to be superior to everolimus therapy alone in the HP-LNCaP model. There was no increase in tumour growth after day 25 in the VN/124-1 plus everolimus-treated group (Figure 4A).

Serum and tumour PSA levels were also analysed. As expected, PSA concentrations in serum were significantly lower than in the tumour tissue. Mean PSA in serum was 8.36 ng ml$^{-1}$ and 454.4 ng ml$^{-1}$ in tissue from the control mice. Serum PSA after VN/124-1 treatment was 4.8 ng ml$^{-1}$ compared with bicalutamide and everolimus values of 7.4 ng ml$^{-1}$ and 6.7 ng ml$^{-1}$, respectively. The mean serum PSA levels from the VN/124-1 plus everolimus treatment group were undetectable. However, the mean PSA level in tissue (214.1 ng ml$^{-1}$), in the combination of VN/124-1 plus everolimus group, was also statistically significantly lower than all other treatment groups.

On termination of treatment, mice were killed, tumours were removed, and lysed in preparation for Western immunoblotting. Compared with control mice, treatment with bicalutamide increased expression of p-HER2 (1.4-fold), p-MAPK (3-fold), p-mTOR (2.2-fold), and p-P70S6K (4-fold) (Figure 4B). Interestingly, bicalutamide reduced IGFR1 expression significantly, compared with control and VN/124-1. VN/124-1 tumours demonstrated increase in growth factor signalling with an increase in expression of IGFR1 (6.9-fold), p-HER2 (1.9-fold), p-MAPK (3-fold), p-mTOR (1.6-fold), p-P70S6K (2.6-fold), and p-P65 (1.2-fold) (Figure 4B). The AR protein expression was increased in these groups, consistent with increased sensitivity to androgens. Everolimus treatment resulted in a 90% decrease in p-mTOR. However, there were increases in both up- and downstream proteins, including IGFR1, p-HER2, p-MAPK, p-P70S6K, and p-P65. The combination therapy of bicalutamide plus everolimus reduced IGFR1 (100%), p-Akt (25%), p-mTOR (25%), and p-P70S6K (25%), but increased expression of p-HER2 (120%), p-MAPK (300%), p-P65 (200%), and AR (80%) (Figure 4B). Tumour growth was well controlled by VN/124-1 plus everolimus, and this combination resulted in reduced expression of most proteins. Thus, dual inhibition resulted in a decrease of p-HER2 (120%), p-Akt (40%), p-mTOR (97%), p-P70S6K (100%), and p-P65 (100%) downstream of mTOR, as well as AR (100%) (Figure 4B). The IGFR1 was slightly increased compared with control, but lower than the high level due to VN/124-1 treatment alone. The p-MAPK was increased by all treatments compared with control. The increase in p-MAPK due to anti-androgen therapy is consistent with in vitro findings (Schayowitz et al., 2008).

**DISCUSSION**

Lack of effective treatment of CRPC represents a significant problem in current management of PCa. Despite initial responses to hormonal therapy, nearly all advanced PCa patients eventually
fail treatment, as tumours become resistant. This investigation was
designed to understand the molecular mechanisms that enable
androgen-dependent PCa cells to progress to CRPC. We also sought
to determine whether blocking the resistance pathway would restore
sensitivity to standard anti-androgens and/or to our novel agent
VN/124-1. Improved understanding of the interaction between the
AR and growth factor signalling could yield a better strategy for
treating disease progression, which may delay, re-sensitise, or prevent
CRPC. The data suggest that increased sensitivity to androgens and
a compensatory cross-talk mechanism between the AR and mTOR
could have a role in the progression to androgen independence. In
the two models used here, resistance to androgen deprivation was
developed in vitro in HP-LNCaP cells and in vivo in the LNCaP
tumours with anti-androgen treatment. As shown in Figure 2, LNCaP
tumours were resistant to bicalutamide after 7 days. However, when
everolimus was added to the anti-androgen therapy at 7 days, tumour
growth was effectively inhibited. Thus, the addition of everolimus to
bicatulamid treatment of resistant tumours significantly decreased
growth rate and tumour volume compared with single-agent
bicalutamide (P = 0.0001) or everolimus (P = 0.04). Additionally, in
the HP-LNCaP xenograft model (Figure 4A), dual inhibition from the
beginning with everolimus (5 mg kg⁻¹ q.d. s.c.) plus VN/124-1
(100 mg kg⁻¹ q.d. s.c.) significantly (P = 0.03) reduced tumour
volumes and growth rates compared with single-agent everolimus.
Furthermore, growth trends indicate that VN/124-1 plus everolimus
was more effective than bicalutamide plus everolimus. Although PSA
levels in tumour tissue were higher than in serum, nevertheless, PSA
levels in serum and tissues were reduced only by VN/124-1 plus
everolimus, consistent with tumour growth measurements, and
indicate the greater anti-tumour efficacy of VN/124-1 than bicatulam-
idine. It is of note that, although serum levels were reduced to
undetectable levels, tissue levels were still measurable, consistent
with incomplete tumour suppression.

VN/124-1 inhibits androgen synthesis, as well as binds to the AR
and causes its downregulation (Vasaitis et al, 2008). Inhibition of
androgen synthesis, resulting in AR instability in the presence of
everolimus, may have contributed to the greater efficacy of the
combination of everolimus and VN/124-1 over everolimus and
bicatulamid in the intact mice used here. Nevertheless, VN/124-1
alone did not decrease AR levels in the HP-LNCaP tumours.
However, these tumours were collected at autopsy and were clearly
resistant, very large, and actively growing on treatment with
VN/124-1. This is consistent with the AR still being present in the
HP-LNCaP-resistant tumours. In tumours treated with VN/124-1
in combination with everolimus, AR was markedly reduced and
tumour growth inhibited.

In vivo findings also demonstrate that inhibition of AR via
bicatulamid or VN/124-1 increases the expression and activation
of several growth factor signalling proteins. Compensatory
cross-talk is evident by changes in proteins in AR, the MAPK, and
P13K/Akt/mTOR pathways, Thus, inhibition of mTOR increased
AR protein expression in vitro as well as in vivo (Schayowitz et al,
2008). Furthermore, inhibition of AR resulted in increased
expression of IGF1R, p-HER2, and p-mTOR in vivo. We found that
serum PSA levels, but not tissue levels, were suppressed by
bicalutamide and VN/124-1, although tumour growth was not
inhibited. This suggests that tissue PSA may reflect tumour growth
more accurately, and that only a portion of the PSA produced
enters circulation. It is also possible that AR-regulated PSA is still
responsive to anti-androgens, whereas tumour growth is indepen-
dent of androgens and is responsive to other signalling pathways
such as mTOR.

Our results indicate that blockade of AR with anti-androgens
and of mTOR with everolimus improved sensitivity to these agents.
Although others (Kaarbo et al, 2010) have found that the PI3K
pathway is increased in PCa, there was little effect of the anti-
androgens or everolimus on PI3K expression. However, the
combination of VN/124-1 and everolimus significantly reduced
AR, indicating cross-talk between these signalling pathways.
Although the inhibition of AR and signal transduction proteins
with bicalutamide plus everolimus is effective, treatment with
VN/124-1 in combination with everolimus was superior in main-
taining tumour growth suppression in the HP-LNCaP xenograft.
This is consistent with previous findings that bicalutamide is less effective
than VN/124-1 as single-agent therapy, (Schayowitz et al, 2008).

In summary, our studies show that interaction between the AR
and mTOR pathways appears to be involved in tumour progres-
sion in the xenograft models. There is an increase in signal
transduction protein expression as tumours progress on anti-
androgen therapy and in control tumours that are actively
growing. Bicalutamide and everolimus treatment reduced tumour
growth to some extent in comparison with that of control tumours
in the resistant models. However, by combining mTOR inhibition
with an anti-androgen to block both the pathways from the
beginning, tumour growth was statistically significantly reduced
compared with the effect of these drugs alone in the LNCaP
and HP-LNCaP xenografts. Thus, there may be value in using an mTOR
inhibitor in combination with an AR downregulator such as
VN/124-1. VN/124-1 in combination with an mTOR inhibitor may
reduce AR levels and thereby delay transition to growth factor
receptor signalling and hormone resistance. Although everolimus
in combination with bicalutamide has not been found to be
effective in PCa patients (Bucke et al, 2010), our findings suggest
that the combination of everolimus with VN/124-1 may be effective
in resistant disease, based on the more complete inhibition of AR
and mTOR signalling. Improved understanding of the interaction
between the AR and alternate signalling pathways could yield a
better strategy for treating tumour progression that may delay,
re-sensitise, or prevent CRPC.

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