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The activity of the androgen receptor variant AR-V7 is regulated by FOXO1 in a PTEN-PI3K-AKT-dependent way

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Abstract

Background—The androgen receptor (AR) AR-V7 splice isoform is a constitutively active outlaw transcription factor. Transition of prostate cancer to the castration-resistant phenotype correlates with AR-V7 accumulation, suggesting that prostate cancer progression in patients refractory to conventional therapy is due to the activity of this AR isoform. The mechanism of AR-V7 constitutive activation is not known.

Methods—We analyzed potential signaling pathways associated with AR-V7 constitutive activation in PTEN (–) PC-3 and LNCaP cells. We used transient and stable transfection, reporter gene assay, RNAi technology together with a number of kinase inhibitors to determine if AR-V7 activation is linked to a kinase-dependent signaling pathway.

Results—In these cell lines, AR-V7 transcriptional activity was inhibited by LY294002, Wortmanin, and AKT inhibitor II. Analysis of the contributing mechanisms demonstrated the involvement of the Phosphatidylinositol 3-kinase (PI3K)-AKT-FOXO1 signaling pathway, and a significant reduction of AR-V7 constitutive activity under conditions of PTEN reactivation.

Conclusions—Our study identifies a pathway regulating AR-V7 constitutive activity and potential therapeutic targets for the treatment of castration resistant prostate cancer.

Keywords

Castration resistant prostate cancer; AR-V7; PI3K

INTRODUCTION

Castration-resistant prostate cancer (CRPC) represents the terminal phase of prostate cancer (PC). CRPC is marked by androgen deprivation therapy (ADT) failure and results in more

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DECLARATION OF INTEREST

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than 30,000 deaths per year among American men [1]. Although CRPC patients have been androgen-depleted by chemical or surgical castration, their androgen receptor (AR) signaling remains active and appears necessary to drive tumor growth [2, 3]. Many mechanisms have been identified to explain AR activation under conditions of androgen depletion {reviewed in [4]}, including selection of constitutively active AR isoforms such as the C-terminally truncated and constitutively active androgen receptor variants (ARVs) [5-7], or other AR isoforms containing various combinations of exon 4–8 deletions, which are also constitutively active [8, 9]. ARVs are derived from the alternative splicing of cryptic exons [6], and the most studied members of this group is AR-V7 [6, 7], which is constitutively active. AR-V7 expression increases in response to the most current therapies developed for CRPC. For instance, CRPC xenografts treated with the recently approved CYP17A1 inhibitor, Abiraterone, display a 3-fold increase in AR-V7 expression [10]. At the mRNA level AR-V7 is expressed in 24% of metastatic lesions in patients with CRPC [8], while at the protein level it is overexpressed in CRPC compared with tissue obtained from benign prostate or hormone-naïve prostate cancer [7]. Importantly, AR-V7 knockdown in hormone refractory PC cells inhibits growth in both cell culture and xenograft models [7].

The mechanism whereby AR-V7 sustains constitutive activity is extremely important to understand the basic biology of CRPC and devise novel drugs to successfully treat this disease. Wild-type AR reactivation during castration-resistant prostate cancer occurs through ligand-dependent and -independent mechanisms [4]. Among the proposed modalities of ligand-independent activation, phosphorylation is frequently postulated. Here we discuss experiments performed with prostate cancer cell lines to support the hypothesis that AR-V7 activation depends on a kinase pathway, and show the involvement of FOXO1 in a PTEN/ PI3K/AKT-dependent manner.

MATERIAL AND METHODS

Bone marrow biopsies were obtained from CRPC patients after obtaining approval from the IRB of Baylor College of Medicine, functioning according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research issued by the Royal College of Physicians of London. All patients had failed ADT. RNA extracted from bone marrow biopsies was reverse-transcribed, AR-V7 and AR-V1 expression was detected by rtPCR in each specimen (not shown). pEGFP-AR-V7 and pEGFP-AR-V1 plasmids were generated. Additional information on Material and Methods is provided in the “Supplemental Material”.

Reagents, Antibodies and Plasmids

AG490 was purchased from Selleck Chemicals; AKT inhibitor II, LY294002 and Wortmannin from EMD Chemicals; and GSK650394 from Tocris Bioscience. Antibodies against AKT, p-AKT (s473), FOXO1, and p-FOXO1 (T24) were purchased from Cell Signaling Technology. Beta-actin Ab was from Sigma-Aldrich Co, while the Anti-HA Rat monoclonal antibody (clone 3F10) was from Roche. Plasmids containing wt and mutant AKT, PTEN and FOXO1 were purchased from Addgene.

Relative Luciferase Assays

For all transfections, pools of PC-3-GFP-AR-V7 or LNCaP cells were starved in charcoal stripped and dialyzed media for a minimum of 18 hours and were transfected with the AR-responsive ARR₂PB-luciferase reporter [4] and the constitutively active pRENILLA-Luc (Promega), hereafter referred to as the dual luciferase reporters, and the plasmid of interest. After a 5 hour transfection, cells were re-plated then treated with the various drugs for 18 hours. Then, firefly and renilla luciferase activity were measured (Dual Luciferase Assay

kit; Promega). Results are reported as relative luciferase activity -firefly reporter activity divided by renilla reporter activity -to control for cell count. Each experiment was performed in triplicate and repeated a minimum of three times.

FOXO1 Immunolabeling and Image Analysis

Cells were grown on coverslips and treated with drugs for 1 hour. After treatment, cells were washed in PBS buffer and fixed for 30 min on ice in 4% formaldehyde. After fixation, auto-fluorescence was quenched using a 0.1 M NH₄Cl solution for 10 minutes. Next, cells were permeabilized for 30 min with 0.5% Triton-X, washed in PBS, then incubated for 30 minutes in 5% non-fat milk in Tris buffered Saline (TBS)-Tween (Blotto) and overnight in Blotto containing anti-FOXO1 rabbit monoclonal antibody (Cell Signaling). After labeling the primary antibody with an anti-rabbit IgG Alexa488 secondary, cells were fixed and prepared for imaging. After aspiration and washes, DAPI staining was performed with a 1 mg/mL DAPI in PBS solution. Coverslips were mounted with Slow-Fade Gold and imaged with a Nikon EZ C-1 Microscope. Images were analyzed using custom algorithms developed with CyteSeer (Vala Sciences).

siFOXO1 Assay

FOXO1 siRNA was purchased from Ambion. PC-3-GFP-AR-V7 were transfected with the dual luciferase reporters. After 5 hours, pools of cells were transfected with either control siRNA or FOXO1 siRNA using siPORT™ NeoFX™ (Ambion). After 48 hours, cells were treated with PI3K inhibitors (LY294002 or Wortmannin). After 24 additional hours luciferase activity was measured, and Western analysis confirming FOXO1 silencing was performed on parallel aliquots of cell lysates.

AR-V7 High Content Analysis (HCA)

PC-3 cells stably expressing GFP-AR-V7 and transiently transfected with an ARR₂PB-mCherry-NLS transcriptional reporter construct were treated with both traditional AR ligands [Dihydrotestosterone (2 nM) and Bicalutamide (10 μM)] and PI3K kinase inhibitors [LY294002 (30 μM) and Wortmannin (250 nM)] for 24 hours. AR-V7 HCA was performed as previously described [11, 12].

RESULTS

To confirm the constitutively active nature of AR-V7, experiments were performed in which the dual luciferase reporters were cotransfected in PC-3 cells alone or with GFP-AR-V7, GFP-AR-V1 or GFP-wtAR and treated with vehicle or 2 nM DHT. Fig. 1A shows constitutive induction of luciferase activity in cells transfected with GFP-AR-V7. In contrast, GFP-wtAR elicited luciferase activity only in the presence of ligand, while, in agreement with previous reports [9], GFP-AR-V1 remained inactive under all experimental conditions. Reporter-only transfected PC-3 cells did not produce luciferase activity under any experimental condition. Based on this validation, it was concluded that transient transfection of AR-V7 in PC-3 represents a suitable model to investigate the mechanisms causing the constitutive activation of AR-V7, hence, a stable PC-3-GFP-AR-V7 was made and used in the experiments discussed below.

Modulation of AR-V7 constitutive activity by PI3K-AKT signaling in PC-3-AR-V7 cells

Based on previous investigations elucidating the mechanism of wtAR reactivation under conditions of androgen deprivation [13, 14], we hypothesized that AR-V7 requires a kinase pathway to become transcriptionally active. To prove this point, various kinase inhibitors were used in PC-3-GFP-AR-V7 cells transiently transfected with the dual luciferase

reporters. Parallel experiments were done for MTT and immunoblot analysis. All tested inhibitors resulted in decreased MTT activity relative to vehicle (Fig 1B), but only inhibitors of PI3K-AKT signaling decreased AR-V7-dependent relative luciferase activity in parallel. Apoptosis was excluded as a cause of decreased cell count by the PARP cleavage immunoblot assay. The drugs which successfully inhibited AR-V7-dependent relative luciferase activity were LY294002, Wortmannin, and AKT inhibitor II [15], all of which decreased AKT phosphorylation (Fig 1B). Inhibition of Janus kinase 2 (JAK2) by AG490 or serine/threonine-protein kinase (SGK) by GSK650394 [16] had no effect on AR-V7-dependent relative luciferase activity. AR-V7 expression level was uniform across treatments. These data suggested that differences in luciferase activity were due to a specific effect of the kinase inhibitors, and not to differences in cell number, viability, or changes in AR protein stability. Both AKT [17] and SGK [18] are downstream effectors of PI3K signaling, however only AKT inactivation affected AR-V7 activity. Based on this, we concluded that AKT is the PI3K-regulated kinase involved in AR-V7 activity. To further substantiate the role of AKT in this signaling pathway, PC-3-AR-V7 cells were transfected with HA-tagged cDNAs encoding wild type or dominant negative (K179M) AKT expression constructs. AR-V7 activity was inhibited by LY294002 and Wortmannin by the usual 50% (Fig. 2 lanes 1, 2, 3, 4) in cells transfected with wild-type AKT. In contrast, dominant negative AKT (K179M) decreased AR-V7 activity under control conditions, and abolished the inhibitory effect of LY294002 and Wortmannin (Fig. 2 lanes lanes 5, 6, 7, 8). Concomitant immunoblot analysis demonstrated that AR-V7 levels were not affected, and that transfection with HA-tagged AKT constructs had successfully occurred with similar expression across all experimental conditions.

Single cell analysis of AR-V7 under conditions of PI3K inhibition

AR high content analysis (HCA) [11, 19] was used to determine if the observed transcriptional inhibition by LY294002 and Wortmannin could be linked to altered levels of AR-V7 protein levels and/or localization. HCA allows us to simultaneously determine total AR-V7 levels, distribution between the nucleus and cytoplasm, the degree of subnuclear hyperspeckling, and, if present, total reporter accumulation at individual cell resolution. The analysis was performed using the PC3-GFP-AR-V7 cell line transiently transfected with a ARR₂PB-mCherry reporter construct. After transfection, cells were treated with the indicated compounds for 24 hours (Fig. 3A and B), fixed, stained with DAPI (a DNA dye) and subsequently imaged and analyzed with previously developed image analysis algorithms. AR-V7 did not show a response to saturating concentrations of the AR agonist DHT or antiandrogen Bicalutamide for any of the responses that were measured (Fig. 3A). This result was not surprising since both DHT and Bicalutamide target the AR ligand-binding domain, which is not present in AR-V7. Treatment with LY294002 showed a 48% reduction in transcriptional activity. This reduction in transcriptional activity was associated with a 42% reduction in nuclear hyperspeckling, indicating a reduced level of subnuclear organization of the receptor that, according to previous studies, is linked to DNA binding, protein-interactions and transcriptional activity [11, 12, 19]. These changes were not associated with statistically significant changes in total AR-V7, nuclear AR-V7, cytoplasmic AR-V7, or overall percentage of AR-V7 protein in the nucleus. Similar results were observed with Wortmannin with a significant 39% decrease in reporter accumulation, 44% reduction in nuclear hyperspeckling, and no significant changes in the other parameters measured. Next, as a control we used this analysis on a small panel of additional kinase inhibitors. Collectively, p38 MAPK (SB203580), ERK (PD98059) or mTOR (rapamycin) inhibitors did not show any antagonistic activity on AR-V7 activation (or altered protein levels/localization) (data not shown). This information, together with the inability of AG490 and GSK650394 to affect AR-V7 activity shown in Fig. 1A, suggests that the suppression of AR-V7 activation by Wortmannin and LY294002 is a pathway-specific event affecting the

biology of AR-V7. No differences in markers of cell death were observed in cells treated with LY294002 and Wortmannin compared with the other kinase inhibitors, indicating that the decreased reporter activity was due to a specific effect on the signaling pathway activating AR-V7.

FOXO1 inhibits AR-V7 activity

Based on the results described, an analysis was initiated to identify the molecule downstream of AKT responsible for AR-V7 inhibition. AKT phosphorylates FOXO1 on three residues, Thr24, Ser256, and Ser319. This phosphorylation step leads to negative FOXO1 regulation by causing its nuclear exclusion, which results in inhibition of FOXO1 transcriptional output [20]. When unphosphorylated, nuclear FOXO1 functions also as a wild-type AR corepressor [21, 22]. In contrast, when phosphorylated, such as under the influence of AKT, nuclear exclusion of FOXO1 leaves wild type AR free to exert its maximal transcriptional activity [21-23]. Based on this paradigm, we hypothesized that FOXO1 is the downstream effector of the PI3K-AKT pathway that modulates AR-V7 transcriptional activity. Initially it was demonstrated that under control conditions active AKT (i.e. phosphorylated at Thr308 and Ser473) and inactive FOXO1 (i.e. phosphorylated at Thr24, Ser256, and Ser319) coexisted in PC-3-AR-V7 cells (Fig. 1B, lane 1), and that FOXO1 was equally represented in the nucleus and cytoplasm (Fig. 4A and B). In contrast, after PI3K and AKT inhibition with Wortmannin and LY294002, inactive AKT (Fig. 1 Lanes 3 and 4) and active FOXO1 (Fig. 1 lanes 3 and 4) coexisted, and nuclear staining increased by 22% (Fig. 4A and B). To conclusively demonstrate that FOXO1 regulates AR-V7 activity, two sets of experiments were done. In the first, PC-3-AR-V7 cells were transfected with FOXO1 or non-targeting siRNAs and, after 48 hours, with the dual luciferase reporters. With FOXO1 silenced, neither LY294002 nor Wortmannin decreased AR-V7 activity (Fig. 4C lanes 1, 2, 3). In contrast, the usual inhibition was observed with non-targeting siRNA (Fig. 4C lanes 4, 5, 6). In the second experiment PC-3-AR-V7 cells were transfected with wt FOXO1 or pcDNA3 Flag FKHR AAA, a non-phosphorylatable and constitutively nuclear form of FOXO1[24], and the dual luciferase reporters. In the presence of wild type FOXO1, both LY294002 and Wortmannin inhibited AR-V7 transcriptional activity (Fig. 5, lanes 3, 4). When pcDNA3 Flag FKHR AAA was transfected, AR-V7 activity decreased under control conditions with no further inhibition by LY-294002 and Wortmannin (Fig. 5, lanes 7, 8), or changes in the stability of the AR-V7 protein (Fig. 5). Thus, when FKHR AAA was expressed, AR-V7 activity was lower and insensitive to PI3K inhibition. These results lead to the conclusion that FOXO1 is the factor mediating inhibition of AR-V7 transcriptional activity by LY294002 and Wortmannin.

LY294002 and Wortmannin effects on AR-V7 transcriptional activity in LNCaP cells

To verify that the observations described in AR (-) PC-3 cells are not cell line specific, we chose to perform similar experiments in AR (+) LNCaP cells. LNCaP transfected with the dual luciferase reporters demonstrated detectable DHT-induced luciferase activity originating from the endogenous AR (Fig. 6A lanes 1 and 2). A robust amount of constitutive luciferase activity was detected under control conditions in LNCaP cells transfected with AR-V7 and the dual luciferase reporters (Fig. 6A lane 3), reflecting the presence of constitutive active AR-V7. As expected, addition of DHT further induced reporter activity, reflecting the activation of the endogenous full length AR (Fig. 6A lane 4). Addition of LY294002 and Wortmannin to LNCaP cells transfected with AR-V7 and the dual luciferase reporters clearly decreased relative luciferase activity (Fig. 6B lanes 2 and 3). Because simultaneous immunoblot analysis demonstrated unchanged expression of AR-V7 during this experiment, it was concluded that AR-V7 activity depends on a PI3K-AKT signaling circuit also in LNCaP cells (Fig. 6 B). We performed additional experiments to determine if inhibition of AR-V7 activity is FOXO1-dependent also in LNCaP cells. Cells

were transfected with AR-V7, the dual luciferase reporters, and wtFOXO1 or FKHR AAA. The experiment showed that luciferase activity was decreased by FKHR AAA under baseline conditions, and neither LY294002 nor Wortmannin demonstrated any further inhibitory effect (Fig. 7, lanes 5, 6, 7, 8). Addition of DHT induced luciferase activity, but to a lower degree compared to cells transfected with wild type FOXO (Fig. 7, lanes 2 and 6), indicating that FKHR AAA also inhibited wild-type AR transcriptional activity as previously described [21, 22]. This experiment showed that PI3K inhibition decreases AR-V7 activity in LNCaP cells via a FOXO1-dependent mechanism.

AR-V7 activity following PTEN manipulation

The tumor suppressor PTEN was originally identified as a negative regulator of the PI3K signaling pathway, therefore PI3K signaling is expected to be active in PTEN (–) PC-3 and LNCaP cells, and resumption of PTEN activity is expected to resume control of PI3K and to modulate AR-V7 constitutive transcriptional activity in these cell lines. This hypothesis was confirmed by transfecting wt PTEN constructs, together with AR-V7 (only in LNCaP) and the dual luciferase reporters into both LNCaP and PC-3-GFP-AR-V7 cells. This experiment resulted in a decrease of AR-V7 activity and a loss of efficacy of LY294002 and Wortmannin (Fig. 8A and B), with AR-V7 expression remaining the same.

DISCUSSION

Earlier observations [25–28] that lab-generated ligand binding domain (LBD)-truncated AR and GR (glucocorticoid receptor) are transcriptionally active in the absence of agonist have found clinical relevance in CRPC with the discovery of the AR-Vs and exon-skipping variants. Because AR variants potentially provide a unifying mechanism to explain the pathogenesis of CRPC, elucidation of their mechanism of action is priority in order to identify novel therapies. AKT is a serine-threonine kinase that stands at the crossroads of several signaling pathways responsible for cellular proliferation, apoptosis, transcription and migration; AKT activation is PTEN-dependent, and many CRPC samples and PC cell lines contain a functionally inactive tumor suppressor PTEN [29, 30]. In addition, it is known that the PTEN status of PC cell lines affects activation of wtAR signaling through AKT, with most papers [31–33] but not all [34] suggesting that wtAR is activated by this protein kinase. Using PTEN (–) PC-3-AR-V7 cells we found that PI3K, but not JAK2 or SGK inactivation was associated with inhibition of AR-V7 transcriptional activity. Further, AR-HCA also demonstrated that other drugs, such as p38 MAPK or mTOR inhibitors did not modulate AR-V7 activity. These observations support the specific involvement of the PTEN-PI3K-AKT-FOXO1 signaling pathway on AR-V7 transcription. PI3K inhibition did not change AR-V7 expression levels in our experiments. It has been reported, however, that PTEN knockout mice have reduced prostate AR protein levels [35], an effect which is partially rescued by PI3K inhibition. We may not have seen a similar effect because in our transient transfection experiments AR-V7 expression was driven by a CMV promoter. Whether the ~50% activity that remains after the inhibition of the PI3K pathway results from the involvement of other kinase pathways, the overexpression of AR coactivators or the inactivation of AR corepressors is currently being investigated.

We studied AR activity at the single cell level with automated microscopy and high content analysis (HCA) [11, 19] to better elucidate the intracellular mechanism causing AR-V7 transcriptional inhibition under conditions of PI3K inactivation. AR HCA confirmed that Wortmannin and LY294002 block AR-V7-mediated transcriptional activity. This was associated with a parallel decrease in GFP nuclear pixel intensity variance, a parameter that reflects AR dynamic interactions with the nuclear chromatin that correlates to transcription [11]. FOXO1 inhibits wtAR activity by interfering with the androgen-induced interaction of the N- and C-termini of the AR and the recruitment of p160 coactivators to its N terminus

and to the androgen response elements of natural AR target genes [23]. By analogy, it is possible that the observed decrease in GFP nuclear pixel intensity variance is due to increased intranuclear accumulation of FOXO1 occurring under conditions of PI3K inhibition (Fig. 4A), and to FOXO1 interfering with the recruitment of p160 coactivators to the N terminus of AR-V7 and to the androgen response elements of natural AR-V7 target genes. Experiments are ongoing to understand in detail the mechanism used by FOXO1 to inhibit AR-V7 transcription. Because initial experiments of immunoprecipitation have not shown a direct interaction between the two (data not shown), the ability of FOXO1 to disrupt the capacity of AR-V7 to transcribe target genes and to recruit coactivators occurs without a direct interaction. This is not necessarily in contrast with the mechanism used by wtAR described in the literature [21-23, 36], because in these papers FOXO1 and AR have only been found to interact in biochemical settings (e.g. Co-IP of overexpressed fusion proteins, GST pulldowns, or mammalian two-hybrid with AR fragments). To demonstrate that our findings were not cell line-dependent, or dependent on experimental conditions in which AR-V7 is expressed in the absence of a full length AR, these experiments and conclusions were duplicated using LNCaP cells. Thus, the PTEN-PI3K-AKT-FOXO1 pathway is responsible for AR-V7 activation in two distinct PC cell lines by mechanism shown in Fig. 9. A considerable literature links PTEN, PI3K and AKT activities to CRPC [37-40], and PI3K or AKT inhibition is an important emerging topic in cancer therapeutics [41]. Mutations or changes in the expression of PTEN-PI3K-AKT leading to the inactivation of PTEN and activation of PI3K and AKT are frequently associated with human cancer, including prostate cancer. PTEN has been the subject of many studies and its expression is decreased in 85% of prostate cancers compared to normal tissues in the same specimen [42], while loss of heterozygosity at the *PTEN* locus is present in up to 60% of prostate cancers studied [43-45]. Clinical correlations have identified a connection between lack of PTEN and prostate cancer recurrence [46] or metastatic disease to the lymph nodes [47], while simultaneous absence of PTEN and increased expression of p-AKT is correlated with PSA-recurrence [46]. Thus, the relationship described in this paper between the oncogenic activation of PTEN-PI3K-AKT and the constitutive transcriptional activation of AR-V7 represents a new mechanism for transition of prostate cancer to the CRPC phenotype. Wortmannin and LY294002 are well known first-generation PI3K inhibitors and both agents are known to have little selectivity toward the various PI3K isoforms. When dispensed in vivo, this lack of selectivity by Wortmannin and LY294004 has been associated with toxicity [48], therefore the use of second or third generation PI3K inhibitor [49] is desirable. AKT inhibition in alternative or addition to PI3K blockage is a complementary approach. Interestingly, clinical trials for CRPC have already been carried out with AKT antagonists, such as Perifosine [50] and Celecoxib [51]. The results of these trials have been only minimally successful, however therapies involving inhibitors of this pathway remain of significant interest, particularly if used in particular settings, for instance AR-V7 overexpression, or as part of a combinatorial approach, including conventional chemotherapy and hormone therapy with established (i.e. Casodex) or novel (i.e. Abiraterone) compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

A. Luciferase activity in PC-3 cells transfected with the dual luciferase reporters and with no expression plasmid (PC-3) or expression plasmids encoding GFP-wtAR, GFP-AR-V7 or GFP-AR-V1, and treated with vehicle or DHT (2nM). Data are expressed as relative luciferase activity $\pm$ SEM. B. PC-3-AR-V7 cells were transiently transfected with the dual luciferase reporters and treated with GSK (10 μ M), LY294002 (30 μ M), Wortmannin (250 nM), AKTII (6.25 μ M), AG490 (50 μ M). Parallel experiments were used to measure luciferase and MTT activities, and for immunoblot analysis. White bars express relative luciferase activity, black bars express MTT activity. Data are normalized to activity detected in vehicle treated cells. Data are expressed as mean (a minimum of 3 experiments) $\pm$ SEM. *** denotes $P < 0.001$ compared to vehicle-treated cells. C, D, E, F, G and H:

Microphotographs of cells after 18 hours of treatment with vehicle (C), LY294002 (D), Wortmannin (E), AKT II (F), GSK (G) and AG490 (H), demonstrating no changes in cellular shape at the concentration of drug used compared to vehicle.

Fig. 2.

PC-3-AR-V7 cells were transiently transfected with the dual luciferase reporters and wt(HA)-AKT or dominant negative K179M (HA)-AKT. Cells were treated with vehicle, LY294002(30 μ M) and Wortmannin(300nM). Relative luciferase activity $\pm$ SEM is plotted normalized to activity detected in vehicle treated cells transfected with wtAKT. *** denotes $p < 0.001$ compared to vehicle treated wt(HA)-AKT-transfected cells. NS denotes lack of statistically significant difference with vehicle treated K179M (HA)-AKT-transfected cells.

Fig. 3.

A. Image based HCA analysis of AR-V7 response. PC-3 cells stably expressing GFP tagged AR-V7 and transiently transfected with a probasin mCherry-NLS transcriptional reporter construct were treated with both AR ligands [DHT (10 nM) and Bicalutamide (10 μ M)] and PI3K kinase inhibitors [LY294002 (30 μ M) and Wortmannin (250 nM)] for 24 hours. The measures for each cell were normalized to the mean value from the DMSO treated control samples. Each bar represents the mean value from 4 replicate samples $\pm$ SEM. * represents $p < 0.01$, ** represents $p < 0.001$ compared to DMSO control. B. Representative Images from high content analysis. Cell images representing the nearest to the population mean for the five measures in each indicated treatment group are shown. The selected cell is marked (*).

Fig. 4.

A. Immunocytochemistry of FOXO1 in PC-3 cells transfected with AR-V7 and the dual luciferase reporters. Cells were treated with vehicle, DHT (2 nM), LY294002 (30 μM), and Wortmannin (250 nM), and labeled with DAPI and an anti-FOXO rabbit polyclonal antibody B. FOXO1 nuclear translocation of cells treated with vehicle, DHT (2 nM), LY294002 (30 μM), and Wortmannin (250 nM); *** denotes $p < 0.001$ compared to vehicle treated cell. C. PC-3 cells were transfected with GFP-AR-V7 and the dual luciferase reporters, then were transfected with either control siRNA (con-siFOXO1) or FOXO1 siRNA, then treated with LY294002 (30 μM), Wortmannin (250 nM) or vehicle. Relative luciferase activity $\pm$ SEM is plotted. ** denotes $p < 0.01$ compared with vehicle treated cells transfected with con-siFOXO1. A parallel experiment was used to verify FOXO1 silencing by immunoblot analysis (lanes 1,2 and 3).

Fig. 5.

PC-3-AR-V7 cells were transfected with wtFOXO1 or FKHR AAA, and the dual luciferase reporters. Cells were treated with vehicle, DHT, LY294002 (30 μ M) or Wortmannin (250 nM), and assays done for for luciferase and immunoblot analysis. Bars $\pm$ SEM represent relative luciferase activity. *** indicates $p < 0.001$ compared to vehicle treated cells. NS denotes lack of statistically significant difference compared to vehicle treated FOXO1 AAA transfected cells. Immunoblot analysis demonstrated that treatment with PI3K inhibitors abolished AKT and FOXO1 phosphorylation (lanes 3, 4, 7 and 8), while AR-V7 levels remained unchanged across experimental conditions.

Fig. 6.

A. LNCaP cells were transfected with the dual luciferase reporters (left) or the dual luciferase reporters and GFP-AR-V7 (right). Cells were treated with vehicle or DHT (2 nM). Bars $\pm$ SEM represent relative luciferase activity, normalized to LNCaP-AR-V7 cells treated with vehicle. **** denotes $p < 0.0001$ compared to vehicle treated LNCaP cells. ** denotes $p < 0.01$ compared to vehicle treated LNCaP-AR-V7 cells. B. LNCaP cells were transfected with the dual luciferase reporters and GFP-AR-V7 and treated with DHT (2 nM), LY294002 (30 μ M) or Wortmannin (250 nM). Assays were done for luciferase activity and immunoblot analysis. Bars $\pm$ SEM represent relative luciferase activity normalized to vehicle treated LNCaP-GFP-AR-V7. *** denotes $P < 0.001$ compared to vehicle-treated LNCaP-AR-V7 cells.

Fig. 7. LNCaP cells were transfected with GFP-AR-V7, wtFOXO1 (left) or FKHR AAA (right), and the dual luciferase reporters. Cells were treated with vehicle, DHT (2 nM), LY294002 (30 μ M) or Wortmannin (250 nM) and assays were done for luciferase and immunoblot analysis. Bars represent relative luciferase activity $\pm$ SEM normalized to LNCaP-GFP-AR-V7 transfected with wtFOXO1 and treated with vehicle. *** denotes $p < 0.001$ compared to vehicle-treated wtFOXO1-transfected cells. NS denotes lack of statistical significance compared to FKHR AAA-transfected cells.

Fig. 8.

A: PC-3-GFP-AR-V7 cells were transfected with an empty plasmid (pBabe-control) (left) or wtPTEN pBabe-PTEN (right) the dual luciferase reporters. Cells were treated with vehicle, DHT (2 nM), LY294002 (30 μ M) or Wortmannin (250 nM). Assays were done for luciferase and immunoblot analysis. Bars represent relative luciferase activity $\pm$ SEM normalized to vehicle treated pBabe-control. *** denotes $p < 0.001$ compared to vehicle-treated PC-3-GFP-AR-V7 cells transfected with pBabe-control. NS denotes lack of significant difference compared to vehicle-treated PC-3-GFP-AR-V7 cells transfected with pBabe-PTEN. B: LNCaP cells were transfected with GFP-AR-V7, an empty plasmid (pBabe-control) (left), pBabe wtPTEN (right), and the dual luciferase reporters. Cells were treated with vehicle, DHT (2 nM), LY294002 (30 μ M) or Wortmannin (250 nM). Assays were done for luciferase and immunoblot analysis. Bars represent relative luciferase activity $\pm$ SEM normalized to vehicle treated pBabe-control. *** denoted $p < 0.001$ compared to LNCaP-GFP-AR-V7 cells transfected with pBabe-control, NS denotes lack of significant difference compared to vehicle-treated LNCaP-GFP-AR-V7 cells transfected with pBabe-PTEN.

Fig. 9.

A: Under native conditions of PTEN inactivation in PC-3 and LNCaP cells, constitutive activation of PI3K activity is associated with phosphorylation/activation of AKT. pAKT phosphorylates/inactivates FOXO1 in the cytoplasm of the target cells. Under these conditions AR-V7 is transcriptionally active. B: Under conditions of PI3K inhibition with LY290004 or Wortmannin, AKT is inactive and does not phosphorylate FOXO1. FOXO1 is mostly intranuclear and interferes with the transcriptional activity of AR-V7. C: Under conditions of PTEN reactivation PI3K is inhibited, and this is associated with inactivation of the downstream pathway, which leads to a situation similar to B.