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Inhibition of Hedgehog and Androgen receptor signaling pathways produced synergistic suppression of castration-resistant prostate cancer progression

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Abstract

Metastatic prostate cancer (PCa) is initially treated with androgen ablation therapy, which causes regression of androgen-dependent tumors. However, these tumors eventually relapse resulting in recurrent castration-resistant prostate cancer (CRPC). Currently, there is no effective therapy for CRPC and the molecular mechanisms that lead to the development of CRPC are not well understood. Here, we evaluated the hypothesis that combined inhibition of Hedgehog (Hh) and Androgen receptor (AR) signaling will synergistically attenuate the growth of CRPC *in vitro* and *in vivo*. Androgen deprivation induced full-length AR protein levels in CRPC cells, but decreased its nuclear localization and transcriptional activity. On the other hand, androgen deprivation also increased a truncated form of AR (lacking ligand binding domain) that possessed transcriptional activity in CRPC cells. Androgen deprivation also promoted the expression of Hedgehog signaling components in castration-resistant prostate cancer cells, xenograft tumors, and the prostate glands of castrated mice. Importantly, while inhibition of either Hh or AR signaling alone was only moderately effective in blocking CRPC cell growth, combination of an Hh pathway inhibitor and a non-competitive AR inhibitor synergistically suppressed the growth of CRPC cells *in vitro* and *in vivo*. Finally, non-competitive inhibition of AR, but not competitive inhibition, was effective at limiting the activity of truncated AR leading to the inhibition of CRPC.

Implication Statement—Combined therapy using Hh inhibitors and a non-competitive AR inhibitor may limit CRPC growth.

Keywords

Castration-resistant prostate cancer (CRPC); Hedgehog; Androgen receptor; Smo inhibitor; Pyrrinium pamoate

Introduction

Prostate cancer (PCa) is the second most common cause of death from cancer in men of all ages and is the leading cancer diagnosed, approximately 1 in 6 American men will be diagnosed with prostate cancer during his lifetime (1). Estimated new cases and deaths from

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Conflict of interest:

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prostate cancer in the United States for 2012 include 241,740 new cases and 28,710 deaths (National Cancer Institute Facts and Figure, 2012, www.cancer.gov/cancertopic/types/prostate). Initial treatment for PCa involves androgen ablation therapy which causes regression of androgen-dependent tumors, however patients eventually fail this therapy and die of recurrent castration-resistant prostate cancer (CRPC) (2). Yet, the molecular mechanisms that promote the development of CRPC are not fully understood. Currently there is no effective therapy for CRPC and the median survival after the development of CRPC is 20–24 months (3;4). It is known that prostate tumors can select for genetic and epigenetic changes that confer growth advantage like any other cancer cells (5). Similar processes are believed to be responsible for the development of CRPC (6;7). For example, prostate tumors that recur after androgen ablation therapy have been shown to have amplified AR gene, resulting in increased AR expression (8;9) and hypersensitivity to low levels of circulating androgens. Certain growth factors such as epidermal growth factor (EGF) have been shown to activate AR in the absence of androgen (10). Overexpression of anti-apoptotic protein Bcl-2 is seen in CRPC (11). All of the above mechanisms lead to the development of CRPC under androgen depleted condition.

AR protein is organized into an N-terminal domain that comprises of the transactivation function, DNA binding domain (DBD) and a hinge region connected to the C-terminal domain or ligand binding domain (LBD) (12). AR is a member of a larger family of nuclear receptors that is activated by the binding of androgenic hormones testosterone or dihydrotestosterone (13;14). AR upon ligand binding in the cytosol translocates to the nucleus and acts as a DNA binding transcription factor where it interacts with other factors upon binding to DNA and controls the transcription of androgen-regulated genes, thereby stimulating development and maintenance of male sexual phenotype (13;15–17). Prostate cancer is androgen dependent (18). Because AR activity is dependent on ligand binding, its activity can be blocked by competitive inhibitors such as bicalutamide. On the other hand, Dehm and Tindall demonstrated the expression of truncated AR (lacking LBD) in CRPC cells that were insensitive to bicalutamide treatment (19). Hence, a non-competitive inhibitor of AR is required to inhibit truncated AR activity in CRPC cells. Here we report the use of pyvinium pamoate, which was shown to be a non-competitive inhibition of AR activity (20), to inhibit AR activity in CRPC cells.

Recent studies have demonstrated that hedgehog (Hh) signaling and other developmentally important signaling pathways are upregulated in cancer stem-like cells leading to their proliferation, invasion, metastasis and development of resistance to drug-induced apoptosis (21;22). Two studies showed that Hh signaling activity is increased in CRPC cells as compared to androgen dependent prostate cancer (ADPC) cells (23;24) suggesting that Hh signaling might contribute to the development and maintenance of CRPC. Mammalian Hh ligands are secreted lipid-modified proteins including sonic (Shh), Indian (Ihh), and Desert (Dhh) hedgehog. Shh is most ubiquitously expressed in mammals (25). They bind their cell surface receptor called Patched. Internalization of Patched after ligand binding leads to derepression and activation of Smoothened (Smo) signaling mediator, which induces activation and nuclear translocation of the Gli family members of transcription factors (21). Many of Gli target gene products are known to promote cell proliferation, self-renewal, cell survival, and epithelial-mesenchymal transition, which are traits of embryonic and adult stem cells as well as carcinoma cells. Aberrant Hh signaling in various carcinomas including prostate cancer have led to the development of several Smo inhibitors, which are currently in clinical trials with some promising outcome (26;27).

Given the important roles played by truncated AR and Hh signaling pathway in CRPC, in the current study, we further characterized their expression and function in various *in vitro* and *in vivo* models and tested our hypothesis that the combination of inhibition of both Hh

and AR pathway may generate a synergistic blockade of CRPC progression. Our results show that a combined inhibition of Hh and AR signaling pathways using Smo inhibitors and a non-competitive AR inhibitor may be a novel effective therapeutic strategy to inhibit CRPC development and progression.

Materials and Methods

Chemicals

Smo inhibitor LDE225 was provided by Novartis through a material transfer agreement, Other Smo inhibitors Cyclopamine (LC Laboratories, MA) and GDC0449 (LC Laboratories, MA) were purchased. The non-competitive inhibitor of AR (pyrvinium pamoate) and the competitive inhibitor of AR (bicalutamide [Casodex]) were purchased from Sigma, MO.

Cell Cultures

LNCaP and 22Rv-1 cells were originally from the American Type Culture Collection (ATCC, VA). The culture was maintained in McCoy's 5A medium supplemented with pyruvate, L-serine, L-arginine, L-glutamine, 100X nonessential amino acids for MEM, MEM amino acids without L-glutamine, MEM vitamins, penicillin, streptomycin, gentamycin, sodium bicarbonate and 10% fetal bovine serum (FBS). Androgen independent LNCaP and 22Rv-1 cells, which were called LNCaP AI and 22Rv-1 AI, were cultured in the above mentioned McCoy's 5A medium with 10% charcoal-stripped FBS instead of regular 10% FBS. Benign prostatic hyperplasia (BPH) epithelial cell line BPH-1 was obtained from ATCC and maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin and streptomycin.

Generation of GFP- and luciferase-expressing cell lines

22Rv-1 AI cells were stably transfected with the enhanced green fluorescent protein (GFP) in the retroviral vector, pLXSN (Clontech Laboratories, Inc. CA), to aid in the identification of these cells in animal tissues with green fluorescence imaging. For bioluminescence imaging, the cells were transduced with pLV411G effLuc-flag-IRES-hrGFP (Luc-GFP), a lentiviral expression vector, kindly provided by Dr. Brian Rabinovich at MD Anderson Cancer Center.

Transfections

For transient transfection assays, 5×10^5 cells/well were seeded into six-well plates, incubated for 24h, then transfected with 1 μ g luciferase reporter plasmid (PSA-Luc or Gli-Luc) and 0.2 μ g of -galactosidase expression plasmid using Lipofectamine 2000 (Invitrogen, NY) in a serum-free medium following the manufacturer's protocol. For stable transfection assay, Human AR cDNA was cloned in the pSDM101 lentiviral vector containing a GFP expression cassette. The empty pSDM101 and AR-pSDM101 vectors were transfected into HEK293 packaging cells, which helps in the packaging of the virus. Viral supernatant were harvested for infection into target cells. Almost all the cells expressed GFP indicating stable transfection of cells.

Animal experiments

Four to five-week-old male athymic nude mice (Harlan Sprague-Dawley, Inc. IN) were used for this study. Animals were maintained under the care and supervision of the Laboratory Animal Research facility at the University of Texas Health Science Center, San Antonio, Texas. The animal protocol was approved and monitored by the Institutional Animal Care and Use Committee.

Orthotopic and Subcutaneous injections

Briefly, 22Rv-1 AI/Luc-GFP cells were harvested from subconfluent, exponentially growing cultures. The cells were inoculated into the dorso-lateral prostate or dorsal lower back of anesthetized male nude mice for orthotopic or subcutaneous injections respectively, with a 27-gauge needle attached to a 1-ml syringe. Each mouse was injected with 1×10^5 cells in 0.01 ml of PBS for orthotopic injection or 0.05 ml of RPMI medium containing cells mixed with 0.05 ml of Matrigel for subcutaneous injections. Development of orthotopic tumors was monitored at regular intervals with whole body bioluminescence imaging for the detection of Luc-expressing tumor cells as described below. For subcutaneous tumors, the tumor sizes were measured with a caliper in two dimensions. Tumor volumes (V) were calculated with the equation $V = (L \times W^2) \times 0.5$, where L is length and W is width.

Bioluminescence imaging analysis

Bioluminescence imaging analysis was used to monitor orthotopic tumor growth in mice for ranking and grouping the mice according to their tumor burden for different drug treatments. Mice were anesthetized and D-luciferin (Xenogen, Alameda, CA) was injected i.p at 75 mg/kg in PBS. Xenogen IVIS-Spectrum Imaging system was used to acquire bioluminescence images at 10 min after injection. Acquisition time was set at 60 seconds at the beginning and reduced later on in accordance with signal strength to avoid saturation. Analysis was performed using LivingImage software (Xenogen) by measurement of photon flux (measured in photons/s/cm²/steradian) with a region of interest (ROI) drawn around the bioluminescence signal to be measured. Orthotopic tumor burden was based on photon flux in the ROI around the major bioluminescence signal in the prostate.

Cell proliferation assay

Cells were plated in a 96-well plate at 1,000 cells/well in triplicates. After treatment with LDE225, GDC0449, Cyclopamine, Casodex, and PP individually or in combination for 5 days, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, MO) solution (2mg/ml) was added and incubated for 2 hours in a tissue incubator. The blue colored formazon product was dissolved in DMSO and quantified at 595 nm wavelength on a Biotek plate reader.

Western Blot Analysis

Cells were harvested and lysed in Laemmli buffer with a cocktail of protease inhibitors. The total protein concentrations were quantified by the BCA protein assay (Thermo Scientific, Rockford, IL). An equal amount of total protein was resolved by SDS-PAGE, transferred to a nitrocellulose membrane under constant voltage and blocked with 5% non-fat dried milk in TBST (10mM Tris pH 7.5, 150mM NaCl and 0.05% Tween-20) followed by washing with TBST. Primary antibodies and secondary antibodies were diluted in TBST and applied with a washing step in between. Proteins were detected using the Amersham ECL Western blotting detection kit (GE Healthcare, Piscataway, NJ, USA). Antibody to AR was from Santa Cruz, CA, to Actin from Sigma, MO and to GAPDH from CalBioChem, MA.

Soft Agar Colony Formation Assay

Cells in 1 ml of 0.4% low melting agarose (Invitrogen, NY) with culture medium were plated at 3,000 cells/well on top of existing 0.8% agarose in 6-well plates. The wells were covered with 1 ml of culture medium containing various treatments and incubated at 37 °C in a 5% CO₂ incubator for the indicated number of days. Visualized colonies with diameter >30µm were counted after staining with *p*-iodonitrotetrazolium violet (Sigma, MO) overnight.

Real-time PCR

Real-time PCR was performed using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) in the ABI 7900HT (Foster City, CA) according to the manufacturer's instruction. The relative transcript level for each transcript was calculated according to the equation $2^{-\Delta\Delta C_t}$, where X is the target gene and N is β -actin.

Immunocytochemical Assay of Cell Lines

LNCaP AD and AI cell lines, grown as monolayers in eight-well chamber slides (Nunc International, Rochester, NY), were fixed by incubation for 20 min at RT with 2% formaldehyde in PHEM buffer (PIPES, HEPES, EGTA, MgSO₄ pH 7.0). Fixed cells were permeabilized in 0.1% Triton-X 100 in PHEM buffer for 1h. Cells were then incubated for 1 h at RT with 1% BSA in PHEM buffer to block nonspecific binding sites. After blocking, cells were incubated with Rabbit α -AR (Androgen receptor) primary antibody (Santa Cruz, CA) overnight at 4°C. Following primary antibody incubation, the cells were washed in 1X PHEM buffer and incubated with anti-rabbit secondary antibody conjugated with Alexafluor 568 fluorochrome for 1 hr at room temperature. Control incubations included a primary antibody alone and secondary antibody alone. The slides were mounted with vectashield mounting media (Vector, CA) and viewed under an Olympus Fluoview FV1000 confocal fluorescence microscope.

Small interfering RNA (siRNA)

LNCaP AD cells were transfected with AR siRNA using Lipofectamine 2000 (Invitrogen, NY). AR was silenced using siRNA pool SMARTpool, siAR #1 purchased from Dharmacon (Catalogue number M003400-00, Waltham, MA).

In vitro luciferase assay

Cells were seeded in triplicates in a 12-well plate at a density of 1.8×10^5 cells/well. When cultures were about 80% confluent, they were co-transfected with 0.2 μ g of β -galactosidase expression plasmid and 1 μ g of PSA or Gli responsive promoter-luciferase construct using Lipofectamine 2000 (Invitrogen, NY) in a serum-free medium following the manufacturer's protocol. After 5 h, the medium was replaced with the serum containing medium. After overnight incubation, the cells were lysed in a buffer (100 mM K₂HPO₄, 1 mM DTT, and 1% Triton X-100) and the luciferase activity in the cell lysate was measured as previously described (28). Luciferase activity was normalized for transfection efficiency with β -galactosidase activity.

Statistical analysis

All statistical analyses were performed using the two-tailed Student's *t*-tests or One-way analysis of variance followed by Tukey-Kramer post-hoc test. Only values with a *P*-value < 0.05 was considered as statistically significant. Error bars represent mean $\pm$ SEM.

Results

Androgen deprivation increases androgen receptor expression but not androgen receptor activity

To determine the role of androgen receptor (AR) in prostate cancer (PCa) progression under androgen deprivation condition, we assessed the levels of AR expression under *in vitro* and *in vivo* conditions in androgen dependent and androgen independent condition. Human PCa cell lines LNCaP and 22Rv-1 were cultured in McCoy's 5A medium containing 10% regular FBS and designated as androgen-dependent (AD) as the medium contains about 0.1 nM testosterone from FBS. The two cell lines were adapted to grow in McCoy's 5A medium

containing 10% charcoal-stripped FBS for more than 6 months and designated as androgen-independent (AI) as the medium contains less than 3.5 pM testosterone. RNA isolation from AD and AI cells followed by quantitative PCR analysis showed an increase in AR expression in AI cells relative to AD cells (Fig. 1A). Also there was a relative increase in AR protein level in AI cells compared to AD cells as assessed by Western blotting (Fig. 1B). To further confirm these results in an *in vivo* model, we generated CWR22 human prostate cancer xenograft tumors subcutaneously in athymic male nude mice that were implanted with slow-releasing testosterone pellet for 90 days. After the tumors reached an approximate 800 mm³, the mice were castrated and the testosterone pellet removed to provide an androgen independent condition for the xenograft tumors. Mice 2 and 5 were euthanized and tumors isolated after their tumors reached 800 mm³ in the AD condition, whereas mice 12 and 14 were castrated and testosterone pellet removed after their tumors reached 800 mm³ and these mice were maintained in the AI condition for 14 days before euthanization and isolation of their tumors. RNA isolated from mice 2, 5, 12 and 14 showed a relative increase in AR expression in tumors from mice 12 and 14 (AI condition) compared to tumors from mice 2 and 5 (AD condition) (Fig. 1C). Also there was a relative increase in the level of AR expression in mouse prostate gland after castration compared to mouse prostate gland before castration (Fig. 1D). We also found that the expression of AR was relatively increased in PCa tissues from patients treated with hormone deprivation therapy as compared to PCa tissues from patients without hormone deprivation therapy through an analysis of gene microarray data in Oncomine database from a published study (29) (Fig. 1E).

We next investigated whether relatively increased AR expression in AI condition compared to AD condition correlates with its activity. Surprisingly, we found a relative decrease in AR activity under AI condition in comparison to AD condition (Fig 2). We assessed for the AR activity by a PSA promoter luciferase reporter assay, in which an AR-responsive promoter sequence of *PSA* gene with a firefly luciferase (PSA-Luc) reporter plasmid was co-transfected with a β -gal expression plasmid into LNCaP and 22Rv-1 (AD and AI) cells. The relative Luc unit (RLU) is the Luc activity normalized by β -gal activity in the same cell lysate. There was a relative decrease in PSA expression in AI cells as compared to AD cells (Fig. 2A). Also the relative PSA expression was lower in AI cells in comparison to AD cells as assessed by quantitative real time PCR (Fig. 2B). To confirm these results in an *in vivo* model, we used the previously described CWR22 xenograft mouse model and found that upon castration the PSA mRNA level was relatively lower in comparison to non-castrated or androgen dependent tumors (Fig. 2C). Furthermore, immunocytochemical staining showed a more uniform nuclear localization of AR in AD cells than in AI cells (Fig. 2D). Consistently, immunohistochemical staining also showed that castration led to an increased cytoplasmic localization of AR castrated mouse prostate tissue when compared to the prostate gland tissue of non-castrated mice (Fig. 2E). These data clearly demonstrate that although AR expression is increased under AI condition, the activity of AR is relatively lowered in the AI condition in both *in vitro* and *in vivo* studies due to its reduced nuclear localization. Our results demonstrating a decrease in AR activity with increase in AR levels are consistent with a recently published report (30), in which active AR was shown to negatively regulate AR expression.

Increased Truncated AR (AR Δ LBD) expression in AI cells

Earlier reports have demonstrated the presence of COOH-terminally truncated AR protein isoform lacking the AR ligand binding domain (LBD) and denoted as AR 3LBD in 22Rv-1 prostate cancer cells (31). Later studies demonstrated the presence of AR Δ LBD in benign and cancerous prostate tissue samples by Western blotting (32). Together, these earlier observations suggest the presence of AR isoform lacking LBD and could be responsible for hormone therapy resistance. Here we confirmed that the level of 75–80kDa AR Δ LBD

expression was higher in LNCaP and 22Rv-1 AI cells as compared to their respective AD cells by Western blotting (Fig. 3A). To determine whether the AR LBD is functional in AI cells, we treated 22Rv-1 AD and AI cells with non-competitive AR antagonist, pyrrvinium pamoate (PP), which does not interact with AR LBD (20) or a competitive AR antagonist, Casodex, which competes with androgen for interacting with AR LBD (33) and assessed for the transcriptional activity of AR. 22Rv-1 AI cells showed a decrease in AR activity as assessed by MMTV-Luc assay when treated with PP but were insensitive to Casodex treatment (Fig. 3B). However both PP and Casodex inhibited AR activity in 22Rv-1 AD cells (Fig. 3B). Furthermore, we also utilized MDA-Kb2 cell, a breast cancer cell line stably transfected with MMTV-Luc reporter, for stable transfection with AR LBD using a lentiviral vector. We confirmed the expression of AR LBD in stably transfected MDA-Kb2 cells by Western blotting (Fig. 3C). Interestingly, overexpression of AR LBD increased level of the endogenous full length AR, which was also observed in AR LBD-transfected LNCaP AD cells (Fig. S1) indicating that overexpression of AR LBD somehow increased the stability of the full length AR. To determine the effect of PP and casodex on the transcriptional activity of AR LBD in MDA-Kb2 cells, we treated the control and AR LBD-expressing MDA-Kb2 cells with PP or casodex. Our results indicated that PP was much more effective than Casodex in inhibiting the MMTV-driven luciferase activity in AR LBD-expressing cell than in the control cell (Fig. 3D). Together, these results suggest that PP inhibits the activity of both full length AR and AR LBD whereas Casodex was effective only on full length AR and did not inhibit the AR LBD activity in 22Rv-1 AI cells and the transfected AR LBD in MDA-Kb2 cells. Our results highlight the importance of AR LBD in the development of resistance to the treatment with competitive AR antagonists.

Activation of Hedgehog (Hh) pathway in CRPC cells and prostate of castrated mice

Recent studies have demonstrated that Hh signaling activity is significantly increased in CRPC cells compared to ADPC cells (23;24). Here we assessed for the expression of Hh signaling components both *in vitro* and *in vivo* and also quantified the Hh activity in AD versus AI cells (Fig. 4). Quantitative RT-PCR assays showed a relative increase in Hh pathway components, Gli2, Smo and Shh in LNCaP and 22Rv-1 AI cells when compared to their AD cells (Fig. 4A). We assessed for Gli transcriptional activity using a Gli luciferase assay in which a Gli-responsive promoter luciferase (Gli-Luc) reporter plasmid was co-transfected with a -gal expression plasmid into LNCaP and 22Rv-1 AD and AI cells. The normalized luciferase activity measured in all four cell lines showed a relative increase in Gli activity in AI cells as compared to AD cells (Fig. 4B) suggesting an increased Hh signaling activity in AI cells in comparison to AD cells. To confirm these *in vitro* results in an *in vivo* system, we assessed the expression of Hh pathway components, Patched (Ptch), Gli2, and Smo using quantitative PCR from RNA isolated from CWR22 xenograft tumors in castrated and non-castrated mice. Our results showed an increase in Ptch, Gli2 and Smo expression in castrated CWR22 xenograft tumors compared to non-castrated tumors (Fig. 4C). We also observed a relative increase in the expression of Hh pathway components, Ptch, Gli1 and Gli2 mRNA isolated from mouse prostate after castration as compared to prostate from non-castrated mice (Fig. 4D). Furthermore, the expression of Hh pathway components was increased in PCa tissues from patients treated with hormone deprivation therapy through an analysis of gene microarray data in Oncomine from a recent study (29) (Fig. 4E). Together our results demonstrate that Hh pathway components and signaling activity are increased upon androgen deprivation in both *in vitro* and *in vivo* conditions suggesting that in addition to truncation mutation of AR, Hh pathway activation may be another mechanism supporting PCa survival and growth under androgen deprivation condition.

Hh signaling is negatively regulated by androgen dependent AR activity

The fact that Hh signaling activity was up-regulated under androgen deprivation condition indicated that Hh signaling activity is suppressed by AR activity. This was demonstrated in a published study by Sirab et al (34). We took a different approach and showed that overexpression of AR in the untransformed AR negative prostate epithelial cell line BPH-1 (Fig. 5A) resulted in a decrease in expression of Hh pathway components, Gli1, Gli2 and Smo (Fig. 5B). On the other hand, AR siRNA mediated silencing of AR in LNCaP AD cells (Fig. 5C) increased the expression of Hh pathway components, Gli1, Gli2 and Smo (Fig. 5D) and Gli transcriptional activity as assessed by Gli-responsive promoter luciferase assay (Fig. 5E). Together, these results suggest that androgen mediated AR activity suppresses Hh signaling.

Combined inhibition of Hh and AR signaling was synergistic in inhibiting the growth of PCa cells *in vitro*

Our results suggest that both Hh and AR signaling are involved in the maintenance of CRPC cell viability and proliferation. Because increased truncated AR expression in AI condition facilitates AR signaling in AI condition, we hypothesized that inhibition of both Hh and AR pathways may synergistically inhibit the growth of CRPC cells. We used Smo inhibitors Cyclopamine and LDE225 in combination with the AR inhibitor PP in a time-course study to assess the inhibition of growth of LNCaP AI cells (Fig. 6A). Our results showed that inhibiting with the Smo inhibitor or AR inhibitor alone was only marginally effective or almost had no inhibitory effects, but the combined inhibition was statistically significant in inhibiting the growth of LNCaP AI cells. Another Smo inhibitor GDC0449 was tested alone and in combination with PP on LNCaP AI cells. Similar results were seen with the combination being very effective in inhibiting the AI cell growth compared to individual treatments (Fig. 6B). To confirm the inhibitory effects on AI cells, we also tested a different AI cell line, 22Rv-1 cells with LDE225 and PP (Fig. 6C). Similar result with a combination being more effective in inhibiting AI cell proliferation was observed. We also tested LDE225 and PP individually and in combination for their effect on anchorage-independent growth of LNCaP AI cells in soft agar and found that LDE225 and PP individually inhibited the anchorage-independent growth of AI cells moderately, whereas the combination was significantly more effective (Fig. 6D). However, we did not see the synergistic inhibition of AI cell growth with Casodex + Cyclopamine and Casodex + LDE225 (Fig. S2), suggesting a significant role of the truncated AR in supporting the growth of the AI cells. We used 5–10 μ M of LDE225 and cyclopamine in our MTT assays because this concentration range was found to dose-dependently inhibit Gli transcription activity in the AI cells as shown in the Fig. S3 for LDE225 and Fig. S4 for cyclopamine, yet they showed marginal effect on cell growth (Fig. 6). Similarly, PP concentration of 50 nM or below was used in our MTT assays as it could inhibit AR transcriptional activity in AI cells (Fig. S5), but with limited growth inhibitory activity as shown in Fig. 6. The choice of these drug concentrations led to the demonstration of the synergistic growth inhibition in the AI cells when they were combined.

Combined inhibition of Hh and AR signaling was effective in inhibiting CRPC tumor growth in mice

To determine if a combinatorial approach can further enhance the efficacy of inhibiting CRPC-mediated prostate tumor progression *in vivo*, 22Rv-1 AI cells were orthotopically inoculated into athymic male nude mice. Upon detection of tumors with whole mouse bioluminescence imaging, they were castrated and divided into 4 groups (8 mice in each group) with similar tumor burden and treated everyday for 24 days with placebo, cyclopamine at 5 mg/kg/day, PP at 0.2 mg/kg/day, or cyclopamine+PP through intraperitoneal injection. After 24 days of treatment, the mice were euthanized and prostate isolated for measuring tumor weight from each group. Interestingly, the tumor incidence

was reduced from 87.5% in the control group to 50% in the cyclopamine group, 55.6% in the PP group, and 33.3% in the combination group. Furthermore, prostate tumors isolated from mice treated with Cyclopamine+PP showed a statistically significant decrease in tumor weight in comparison to the control group (Fig. 7A). To accurately track the growth of tumors as a function of cyclopamine and/or PP treatment, we also inoculated the 22Rv-1 AI cells subcutaneously so that we could measure the tumor volume with a caliper at a regular interval. Subcutaneous tumors started to arise in 2 weeks. The mice were ranked according to their tumor burden and divided into four groups with matched tumor burden. All the mice were castrated following regrouping. Four days post castration, vehicle, cyclopamine at 5 mg/kg/day, PP 0.2 at mg/kg/day, or Cyclopamine + PP was given daily through intraperitoneal injection for 11 days and the tumor volume was measured intermittently for each group (Fig. 7B). Here we present the tumor volume data in 2 ways, Figures 7B and 7C. Figure 7B shows tumor growth curve in each mouse with two tumors. We found that the treatment, especially the combination treatment, was effective in inhibiting tumor growth when the tumor volume was equal to or less than 50 mm³ at the start of the treatment. As such, Figure 7C was prepared to show the growth of the tumors in two mice with the lowest tumor burden at the start of the treatment in each group to illustrate the efficacy of each treatment. It is clear that PP alone showed no inhibitory effect while its combination with cyclopamine basically stopped tumor growth. These *in vivo* results are consistent with our *in vitro* data (Fig. 6) showing the combined inhibition of Hh and AR signaling using cyclopamine and PP was more effective in inhibiting the growth of AI prostate cancer cells than each of the agent alone.

Discussion

Prostate cancer at its early stage is dependent on androgen for growth and survival. Despite initial response rates of 80–90% of early stage tumor regression after androgen ablation therapy, nearly all men develop androgen-independent prostate cancer after 18–36 months (35). Majority of prostate cancer related deaths are due to the development of CRPC and metastasis to various organs. The molecular mechanisms that lead to the development of CRPC are not fully understood and currently there is no effective therapy for it. Although they are refractory to hormone deprivation, CRPC cells are known to express AR and rely on AR activity for survival and proliferation (36). AR activity is maintained under hormone deprivation condition via various mechanisms in CRPC, which include amplification of the AR gene (37;38), missense mutation in AR gene at T877A making it responsive to anti-androgens (39), and ligand-independent activation of AR by various growth factors such as insulin-like growth factor-1 (IGF-1) (10). In our studies, we observed an increase of AR level in CRPC cells and prostate gland of castrated mice when compared to ADPC cells or non-castrated mice. Interestingly, we observed a decreased transcriptional activity of AR in CRPC cells, presumably due to low androgen in the environment and cytoplasmic localization of inactive AR. Our result that a relative increase in AR level in CRPC cells compared to ADPC cells is consistent with a previous finding that increased androgens represses AR expression (30). On the other hand, we observed an increased expression of truncated AR proteins (AR LBD) in both LNCaP and 22Rv-1 AI cells. Previously the AR LBD proteins were shown to be present due to increased proteolytic deletion of the LBD under AI condition in LNCaP cells (40), whereas they are due to insertion mutation of AR gene and differential RNA splicing of AR transcript in 22Rv-1 cells (41). Previous studies have demonstrated increased expression levels of AR LBD and its ability to confer resistance to anti-androgens in androgen refractory PCa cells (42). As expected, AR LBD was found to be insensitive to bicalutamide (Casodex), a competitive AR inhibitor, in our study. However, its transcriptional activity was found to be inhibited by pyrvinium pamoate (PP), a non-competitive AR inhibitor, in CRPC cells suggesting that it is functionally active and may contribute to the development of CRPC as previously suggested (42).

Another mechanism that contributes to the development of CRPC was the activation of Hh signaling pathway upon hormone deprivation. Previous studies have shown that Hh signaling pathway is activated during prostate tumorigenesis and progression (43;44). More recent studies showed that androgen deprivation upregulated the expression of Hedgehog signaling components and consequently Hh signaling activity in prostate cancer cell lines and circulating prostate tumor cells (23;24). Our study not only confirmed the published findings in the prostate cancer cells, but also demonstrated upregulation of Hh pathway components *in vivo* in the human CWR22 prostate cancer xenograft tumors upon surgical androgen deprivation. Our study also confirmed a previous report demonstrating increased Hh components in the prostate gland of castrated mice (45). Consistent with these findings, genetic approaches with ectopic expression or knockdown of AR with siRNA also showed that AR level is inversely related with Hh pathway components and Gli transcriptional activity suggesting a direct suppression of Hh signaling activity by AR. As a result, androgen deprivation led to an enhanced autocrine Hh signaling activity, which can be dose-dependently inhibited by the treatment with Smo inhibitor, LDE225 in our study (Supplementary Fig. 3), or genetic knockdown of Smo (46).

These findings suggest that the increased Hh signaling and AR LBD expression might contribute to the development and maintenance of CRPC. Thus, to inhibit the development and proliferation of CRPC cells, we used Hh and AR inhibitors independently and in combination both under *in vitro* and *in vivo* conditions. We demonstrated that the non-competitive AR inhibitor PP was effective in inhibiting the AR activity in 22Rv-1 AI cells whereas the competitive AR inhibitor casodex was ineffective even though Casodex showed inhibition of AR activity similar to PP in androgen dependent 22Rv-1 cells. Hence a non-competitive AR inhibitor is effective in inhibiting AR activity under AI condition. Significantly, while various Smo inhibitors showed little or no effect on the growth of CRPC cells in micromolar concentrations, they produced synergistic inhibition of CRPC cell growth in combination with suboptimal concentrations of PP. Furthermore, the combination of PP with cyclopamine was found more effective than either agent alone in inhibiting the growth of CRPC tumors *in vivo*.

In summary, our study demonstrated activation of Hh signaling in both *in vitro* and *in vivo* models upon hormone ablation. It also indicated for the first time that the growth of CRPC cells and early stage androgen independent prostate tumors could be effectively inhibited by a Smo inhibitor in combination with a non-competitive AR inhibitor. This concept remains to be validated in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Androgen deprivation increases AR expression both *in vitro* and *in vivo*

A. Androgen independent (AI) LNCaP and 22Rv-1 cells show higher expression of AR mRNA relative to Androgen dependent (AD) cells. **B.** LNCaP and 22Rv-1 AI cells show higher expression of AR protein levels relative to their respective AD cells. **C.** Castrated mice (mouse 12 and 14) show an increase in AR expression in the CWR22 xenografts relative to those of the non-castrated mice (mouse 2 and 5). **D.** AR mRNA levels was relatively increased in wild-type mouse prostate after castration. Each bar is presented as mean $\pm$ SEM from triplicate measurements. *Indicates statistically significant difference between the two groups with a student's *t*-test at $P < 0.05$, *** $P < 0.0001$. The bars bearing different letters are significantly different ($p < 0.05$) by ANOVA. **E.** Hormone deprivation therapy significantly increased mRNA levels of AR. The data were derived from patient samples without or with hormone deprivation therapy (data provided by Dr. Taylor, prostate oncomine database [citation 29]). The bars and box represent 10–90 or 25–75 percentile of the data, respectively, from each group.

Figure 2. Androgen deprivation decreases activity and nuclear localization of AR

A. AD cells show an increase in AR activity relative to AI cells as assessed by an AR-responsive PSA promoter-luciferase (PSA-Luc) reporter plasmid co-transfected with a β -gal plasmid into the AD and AI cells, the relative Luc unit (RLU) is the Luc activity normalized by β -gal activity in the same cell lysate. **B.** AD cells show an increase in PSA mRNA expression relative to AI cells. **C.** Castrated mice (mouse 1 and 9) show a decrease in PSA mRNA expression level relative to non-castrated mice (mouse 2 and 5). Each bar is presented as mean $\pm$ SEM from triplicate measurements, and bars bearing different letters are significantly different ($p < 0.05$) by ANOVA. **D.** DAPI (Blue) stains the nucleus, α -AR (Red - alexafluor 568 fluorescent dye) stains AR protein. Immunocytochemical staining for AR shows an increased AR localization in the cytosol of AI cells compared to AD cells. **E.** Immunohistochemistry staining for AR shows a decreased AR localization in the nucleus of wild-type mouse prostate cells after castration (arrows point to the nucleus of prostate epithelial cells).

Figure 3. PP inhibits full length and truncated AR activity

A. Truncated AR (AR LBD) expression is higher in LNCaP and 22Rv-1 AI cells as compared to AD cells as assessed by western blotting. **B.** PP inhibited endogenous AR activity in both 22Rv-1 AD and AI cells, Casodex inhibited AR activity only in AD cells. **C.** AR LBD was stably transfected and overexpressed in MDA-Kb2 cells (Human breast cancer cell line). **D.** MDA-Kb2 cells were stably transfected with the androgen responsive MMTV promoter luciferase gene, and these cells report the activity of androgen receptor. Over-expression of AR LBD in these MDA-Kb2 cells increased luciferase activity as compared to the control cells. PP treatment showed significant inhibition of the over-expressed AR LBD in these MDA-Kb2 cells whereas casodex was less effective in inhibiting the over-expressed AR LBD activity. Each bar is presented as mean $\pm$ SEM from triplicate measurements. *Indicates statistically significant difference between the two groups with a student's *t*-test at $P < 0.05$, *** $P < 0.0001$, ns represents non-significant.

Figure 4. Androgen deprivation increases Hedgehog signaling components both *in vitro* and *in vivo*

A. AI cells show an increased mRNA expression of Hedgehog signaling components relative to AD cells. **B.** A Gli-responsive promoter-luciferase (Gli-Luc) reporter plasmid was co-transfected with a β -gal expression plasmid into the depicted cells, the relative Luc unit (RLU) is the Luc activity normalized by β -gal activity in the same cell lysate. AI cells exhibit an increased Hh signaling activity compared to AD cells. **C.** Castrated mice (mouse 12 and 14) show an increased mRNA levels for Hedgehog components relative to non-castrated mice (mouse 2 and 5). **D.** Increased mRNA expression of Hh components are observed in wild type mice prostate after castration. All data are mean $\pm$ SEM from three independent measurements. Bars bearing different letters are significantly different ($p < 0.05$) by ANOVA. **E.** Hormone deprivation therapy significantly increased mRNA levels of Shh, Smo, and Gli1. The data were derived from 153 and 20 patient samples without or with hormone deprivation therapy (including neoadjuvant hormone therapy) respectively (data provided by Taylor and co-workers (Citation 29 to Oncomine). The bars and box represent

10–90 or 25–75 percentile of the data, respectively, from each group. The mean of each data set is shown as “+”. The P values were obtained with two-tailed Student’s *t*-tests. The wider distribution of the data of the hormone therapy group is likely due to the small sample size and the variation of hormone therapy duration, which was not provided in the report.

Figure 5. AR negatively regulates Hh signaling

A. Overexpression of AR in BPH1 cells (AR null cells) assessed by Western blot. **B.** AR overexpression downregulates Hh signaling components Gli1, Gli2 and Smo in BPH1 cells. **C.** siRNA mediated knockdown of endogenous AR in LNCaP AD cells assessed by Western blot. **D.** AR knockdown LNCaP AD cells shows increased expression of Hh signaling components Gli1, Gli2 and Smo. **E.** AR knockdown LNCaP AD cells shows increased Gli-Luciferase activity. Each bar is presented as mean $\pm$ SEM from triplicate measurements.

Figure 6. Combined inhibition of Hh and AR signaling is synergistic in inhibiting the growth of PCa cells *in vitro*

A. Cyclopamine (10μM) or LDE225 (5μM) [Hh inhibitors] in combination with 20nM Pyvinium Pamoate (PP) (AR inhibitor) synergistically inhibits AI prostate cancer cell proliferation effectively as compared to individual treatments. **B.** GDC0449 (Hh inhibitor) and PP in combination is effective in preventing AI prostate cancer cell proliferation. **C.** LDE225 (Hh inhibitor) and PP synergistically inhibit the growth of 22Rv-1 AI cells. **D.** LDE225 (5μM) and PP (5nM) in combination effectively inhibited anchorage independent growth of LNCaP AI cells. Data is presented as mean of colony number from 6 high power fields in 3 wells for each group. All data are mean±SEM from three independent measurements, bars bearing different letters are significantly different ($p<0.05$) by ANOVA.

Figure 7. Combined inhibition of Hh and AR signaling is effective in inhibiting CRPC tumor growth in mice

A. 22Rv-1 AI cells were orthotopically inoculated into castrated athymic male nude mice and the mice were treated with cyclopamine at 5 mg/kg/day, PP at 0.2 mg/kg/day, or cyclopamine+PP through intraperitoneal injection for 24 days. Cyclopamine (Hh inhibitor) and PP (AR inhibitor) in combination was effective in inhibiting orthotopically inoculated 22Rv-1 AI (Prostate cancer cell) cell proliferation in mouse prostate as compared to individual treatments. **B.** 22Rv-1 AI prostate cancer cells were inoculated subcutaneously on the right lower back of male mice and the mice were treated with cyclopamine and PP at a dose of 5mg/kg/day and 0.2 mg/kg/day respectively for 11 days. Caliper measurements of tumors showed a steady increase in tumor volume in control and individual treatment groups, however the cyclopamine and PP combined treatment did not show a gradual increase in the tumor volume in 2 male mice (note: 2 male mice in cyclopamine and PP treatment group did not respond to the drugs as the initial tumor volume before treatment start date was already too big and hence were not responsive to the treatments). **C.** Each bar is presented as mean $\pm$ SEM from 4 subcutaneous tumors from two mice bearing the smallest tumors from each group at the beginning of the treatment. The combination treatment group did not show much increase in tumor volume in a time-course treatment with the Smo and AR inhibitors.