Inhibition of Hedgehog and Androgen Receptor Signaling Pathways Produced Synergistic Suppression of Castration-Resistant Prostate Cancer Progression

Pramod S. Gowda¹, Jianhong D. Deng¹, Sweta Mishra¹, Abhik Bandyopadhyay¹, Sitai Liang¹, Shu Lin¹, Devalingam Mahalingam², and Lu-Zhe Sun¹,²

Abstract

Metastatic prostate cancer 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 androgen receptor protein levels in CRPC cells, but decreased its nuclear localization and transcriptional activity. However, androgen deprivation also increased a truncated form of androgen receptor (lacking ligand-binding domain) that possessed transcriptional activity in CRPC cells. Androgen deprivation also promoted the expression of Hh signaling components in CRPC cells, xenograft tumors, and the prostate glands of castrated mice. Importantly, although inhibition of either Hh or androgen receptor signaling alone was only moderately effective in blocking CRPC cell growth, combination of an Hh pathway inhibitor and a noncompetitive androgen receptor inhibitor synergistically suppressed the growth of CRPC cells in vitro and in vivo. Finally, noncompetitive inhibition of androgen receptor, but not competitive inhibition, was effective at limiting the activity of truncated androgen receptor leading to the inhibition of CRPC.

Implications: Combined therapy using Hh inhibitors and a non-competitive AR inhibitor may limit CRPC growth.

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Introduction

Prostate cancer 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 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 prostate cancer 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; ref. 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 to 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 androgen receptor gene, resulting in increased androgen receptor expression (8, 9) and hypersensitivity to low levels of circulating androgens. Certain growth factors such as EGF have been shown to activate androgen receptor in the absence of androgen (10). Overexpression of antiapoptotic protein Bcl-2 is seen in CRPC (11). All of the above mechanisms lead to the development of CRPC under androgen-depleted condition.

Androgen receptor 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; ref. 12). Androgen receptor is a member of a larger family of nuclear receptors that is activated by the binding of androgenic hormones testosterone or dihydrotestosterone (13, 14). Androgen receptor 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 androgen receptor activity is dependent on ligand binding, its activity can be blocked by competitive inhibitors such as bicalutamide. However, Dehm and Tindall showed the expression of truncated androgen receptor (lacking LBD) in CRPC cells that were insensitive to bicalutamide treatment (19). Hence, a noncompetitive inhibitor of androgen receptor is required to inhibit truncated androgen receptor activity in CRPC cells. Here we report the use of pyrvinium pamoate, which was shown to be a noncompetitive inhibition of androgen receptor activity (20), to inhibit androgen receptor activity in CRPC cells.

Recent studies have showed 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 androgen receptor and Hh signaling pathway in CRPC, in this 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 androgen receptor pathway may generate a synergistic blockade of CRPC progression. Our results show that a combined inhibition of Hh and androgen receptor signaling pathways using Smo inhibitors and a noncompetitive androgen receptor 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) and GDC0449 (LC Laboratories) were purchased. The noncompetitive inhibitor of androgen receptor (pyrvinium pamoate) and the competitive inhibitor of androgen receptor [bicalutamide (casodex)] were purchased from Sigma.

Cell cultures

LNCaP and 22Rv-1 cells were originally from the American Type Culture Collection (ATCC, Manassas, VA). The culture was maintained in McCoy’s 5A medium supplemented with pyruvate, 1-serine, 1-arginine, 1-glutamine, 100× nonessential amino acids for MEM (Minimum Essential Medium purchased from Life Technologies), MEM amino acids without 1-glutamine, MEM vitamins, penicillin, streptomycin, gentamycin, sodium bicarbonate, and 10% 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 GFP in the retroviral vector, pLXSN (Clontech Laboratories Inc.), 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-IRE-S-hrGFP (Luc-GFP), a lentiviral expression vector, kindly provided by Dr. B. Rabinovich at MD Anderson Cancer Center.

Transfections

For transient transfection assays, 5 × 10⁵ cells/well were seeded into 6-well plates, incubated for 24 hours, then transfected with 1 μg luciferase reporter plasmid (PSA-Luc or Gli-Luc) and 0.2 μg of β-galactosidase expression plasmid using Lipofectamine 2000 (Invitrogen) in a serum-free medium following the manufacturer’s protocol. For stable transfection assay, human androgen receptor 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.) 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 \( 1 \times 10^5 \) cells in 0.01 mL of PBS for orthotopic injection or 0.05 mL of RPMI medium containing cells 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 later. For subcutaneous tumors, the tumor sizes were measured with a caliper in 2 dimensions. Tumor volumes (V) were calculated with the equation \( V = (L \times W^2) \times 0.5 \), where L is the length and W is the 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 i.v. luciferin (Xenogen) was injected intraperitoneally at 75 mg/kg in PBS. Xenogen IVIS-Spectrum Imaging system was used to acquire bioluminescence images at 10 minutes 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 conducted 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 pyrvinium pamoate individually or in combination for 5 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) solution (2 mg/mL) was added and incubated for 2 hours in a tissue incubator. The blue-colored formazon product was dissolved in dimethyl sulfoxide 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 bichinchoninic acid protein assay (Thermo Scientific). An equal amount of total protein was resolved by SDS-PAGE, transferred to a nitrocellulose membrane under constant voltage and blocked with 5% nonfat dried milk in TBST (Tris buffered saline-Tween 20 containing 10 mmol/L Tris pH 7.5, 150 mmol/L 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 Amer sham ECL Western blotting detection kit (GE Healthcare). Antibody to androgen receptor was from Santa Cruz, to actin from Sigma, and to GAPDH from CalBioChem.

Soft agar colony formation assay

Cells in 1 mL of 0.4% low melting agarose (Invitrogen) 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 >30 μm were counted after staining with p-iodonitrotetrazolium violet (Sigma) overnight.

Real-time PCR

Real-time PCR (RT-PCR) was conducted using Brilliant SYBR Green QPCR Master Mix (Stratagene) in the ABI 7900HT according to the manufacturer’s instruction. The relative transcript level for each transcript was calculated according to the equation \( 2^{-\Delta\Delta CT} \), where X is the target gene and N is the β-actin.

Immunocytochemical assay of cell lines

LNCaP AD and AI cell lines, grown as monolayers in 8-well chamber slides (Nunc International), were fixed by incubation for 20 minutes at room temperature with 2% formaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 7.0). Fixed cells were permeabilized in 0.1% Triton-X 100 in PHEM buffer for 1 hour. Cells were then incubated for 1 hour at RT with 1% BSA in PHEM buffer to block nonspecific binding sites. After blocking, cells were incubated with Rabbit α-AR primary antibody overnight at 4°C. Following primary antibody incubation, the cells were washed in 1× PHEM buffer and incubated with anti-rabbit secondary antibody conjugated with Alexafluor 568 fluorochrome for 1 hour 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.

siRNA

LNCaP androgen-dependent cells were transfected with androgen receptor siRNA using Lipofectamine 2000 (Invitrogen). Androgen receptor was silenced using siRNA pool SMARTpool, siAR #1 purchased from Dharmacon (Catalogue number M003400-00).

In vitro luciferase assay

Cells were seeded in triplicates in a 12-well plate at a density of \( 1.8 \times 10^5 \) cells/well. When cultures were about 80% confluent, they were cotransfected with 0.2 μg of β-galactosidase expression plasmid and 1 μg of PSA or Gli responsive promoter-luciferase construct using...
Lipofectamine 2000 (Invitrogen) in a serum-free medium following the manufacturer’s protocol. After 5 hours, the medium was replaced with the serum-containing medium. After overnight incubation, the cells were lysed in a buffer (100 mmol/L K2HPO4, 1 mmol/L 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 conducted using the 2-tailed Student 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 ± SEM.

**Results**

**Androgen deprivation increases androgen receptor expression but not androgen receptor activity**

To determine the role of androgen receptor in prostate cancer progression under androgen deprivation condition, we assessed the levels of androgen receptor expression under *in vitro* and *in vivo* conditions in androgen-dependent and androgen-independent condition. Human prostate cancer cell lines LNCaP and 22Rv-1 were cultured in McCoy’s 5A medium containing 10% regular FBS and designated as androgen-dependent as the medium contains about 0.1 nmol/L testosterone from FBS. The 2 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 as the medium contains less than 3.5 nmol/L testosterone. RNA isolation from androgen-dependent and androgen-independent cells followed by quantitative PCR analysis showed an increase in androgen receptor expression in androgen-independent cells relative to androgen-dependent cells (Fig. 1A). Also there was a relative increase in androgen receptor protein level in androgen-independent cells compared to androgen-dependent 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-dependent condition. The activity of androgen receptor is relatively lower in comparison to noncastrated or androgen-dependent tumors (Fig. 2C). Furthermore, immunocytochemical staining showed a more uniform nuclear localization of androgen receptor in androgen-dependent cells than in androgen-independent cells (Fig. 2D). Consistently, immunohistochemical staining also showed that castration led to an increased cytoplasmic localization of androgen receptor castrated mouse prostate tissue when compared to the prostate gland tissue of noncastrated mice (Fig. 2E). These data clearly show that although androgen receptor expression is increased under androgen-independent condition, the activity of androgen receptor is relatively lowered in the androgen-independent condition in both *in vitro* and *in vivo* studies due to its reduced nuclear localization. Our results showing a decrease in androgen receptor activity with increase in androgen receptor levels are consistent with a recently published report (30), in which active androgen receptor was shown to negatively regulate androgen receptor expression.

**Increased truncated androgen receptor (AR ΔLBD) expression in androgen-independent cells**

Earlier reports have showed the presence of COOH-terminally truncated androgen receptor protein isoform lacking the androgen receptor LBD and denoted as AR ΔLBD in 22Rv-1 prostate cancer cells (31). Later studies showed the presence of AR ΔLBD in benign and cancerous prostate tissue samples by Western blotting (32). Together,
these earlier observations suggest the presence of androgen receptor isoform lacking LBD and could be responsible for hormone therapy resistance. Here we confirmed that the level of 75 to 80 kDa AR LBD expression was higher in LNCaP and 22Rv-1 AI cells as compared to their respective androgen-dependent cells by Western blotting (Fig. 3A). To determine whether the AR LBD is functional in androgen-independent cells, we treated 22Rv-1 AD and androgen-independent cells with noncompetitive androgen receptor antagonist, pyrvinium pamoate, which does not interact with AR LBD (20) or a competitive androgen receptor antagonist, casodex, which competes with androgen for interacting with AR LBD (33) and assessed for the transcriptional activity of androgen receptor. 22Rv-1 AI cells showed a decrease in androgen receptor activity as assessed by MMTV-Luc assay when treated with pyrvinium pamoate but were insensitive to casodex treatment (Fig. 3B). However, both pyrvinium pamoate and casodex inhibited androgen receptor activity in 22Rv-1 AD cells (Fig. 3B). Furthermore, we also used 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 androgen receptor, which was also...
observed in AR ΔLBD-transfected LNCaP androgen-dependent cells (Supplementary Fig. S1) indicating that overexpression of AR ΔLBD somehow increased the stability of the full-length androgen receptor. To determine the effect of pyrvinium pamoate and casodex on the transcriptional activity of AR ΔLBD in MDA-Kb2 cells, we treated the control and AR ΔLBD-expressing MDA-Kb2 cells with pyrvinium pamoate or casodex. Our results indicated that pyrvinium pamoate was much more effective than casodex in inhibiting the MMTV-driven luciferase activity in AR ΔLBD-expressing cell than in the control cell (Fig. 3D). Together, these results suggest that pyrvinium pamoate inhibits the activity of both full-length androgen receptor and AR ΔLBD, whereas casodex was effective only on full-length androgen receptor and did not inhibit the AR ΔLBD activity in 22Rv-1 AI cells and the transfected AR ΔLBD in MDA-Kb2 cells. Our results highlight the importance of AR ΔLBD in the development of resistance to the treatment with competitive androgen receptor antagonists.

Activation of Hh pathway in CRPC cells and prostate of castrated mice

Recent studies have showed 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 androgen-dependent versus androgen-independent 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 androgen-dependent 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 cotransfected with a β-gal plasmid into LNCaP and 22Rv-1 AD and AI cells. The normalized luciferase activity measured in all 4 cell lines showed a relative increase in Gli activity in androgen-independent cells as compared to androgen-dependent cells (Fig. 4B), suggesting an increased Hh signaling activity in androgen-independent cells in comparison to androgen-dependent 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 noncastrated mice. Our results showed an increase in Ptch, Gli2, and Smo expression in castrated CWR22 xenograft tumors compared to noncastrated 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 noncastrated mice (Fig. 4D). Furthermore, the expression of Hh pathway components was increased in prostate cancer tissues from patients treated with hormone deprivation therapy through an analysis of gene microarray data in oncomine from a recent study (ref. 29; Fig. 4E). Together our results show 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 androgen receptor, Hh pathway activation may be another mechanism supporting prostate cancer survival and growth under androgen deprivation condition.

Hh signaling is negatively regulated by androgen-dependent androgen receptor activity

The fact that Hh signaling activity was upregulated under androgen deprivation condition indicated that Hh signaling...
activity is suppressed by androgen receptor activity. This was showed in a published study by Sirab and colleagues (34). We took a different approach and showed that overexpression of androgen receptor in the untransformed androgen receptor 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). However, androgen receptor siRNA mediated silencing of androgen receptor in LNCaP androgen-dependent 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 androgen receptor activity suppresses Hh signaling.

Combined inhibition of Hh and androgen receptor signaling was synergistic in inhibiting the growth of prostate cancer cells in vitro

Our results suggest that both Hh and androgen receptor signaling are involved in the maintenance of CRPC cell viability and proliferation. Because increased truncated androgen receptor expression in androgen-independent condition facilitates androgen receptor signaling in androgen-independent condition, we hypothesized that inhibition of both Hh and androgen receptor pathways may synergistically inhibit the growth of CRPC cells. We used Smo inhibitors cyclopamine and LDE225 in combination with the androgen receptor inhibitor pyrvinium pamoate 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 androgen receptor 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 pyrvinium pamoate on LNCaP AI cells. Similar results were seen with the combination being very effective in inhibiting the androgen-independent cell growth compared to individual treatments (Fig. 6B). To confirm the inhibitory effects on androgen-independent cells, we also tested a different androgen-independent cell line, 22Rv-1 cells with LDE225 and pyrvinium pamoate (Fig. 6C). Similar result with a combination being more effective in inhibiting androgen-independent cell proliferation was observed. We also tested LDE225 and pyrvinium pamoate individually and in combination for their effect on anchorage-

Figure 5. Androgen receptor negatively regulates Hh signaling. A, overexpression of androgen receptor in BPH1 cells (androgen receptor null cells) assessed by Western blot. B, androgen receptor overexpression downregulates Hh signaling components Gli1, Gli2, and Smo in BPH1 cells. C, siRNA-mediated knockdown of endogenous androgen receptor in LNCaP androgen-dependent cells assessed by Western blot. D, androgen receptor knockdown LNCaP androgen-dependent cells shows increased expression of Hh signaling components Gli1, Gli2, and Smo. E, androgen receptor knockdown LNCaP androgen-dependent cells show increased Gli-Luciferase activity. Each bar is presented as mean ± SEM from triplicate measurements.
independent growth of LNCaP AI cells in soft agar and found that LDE225 and pyrvinium pamoate individually inhibited the anchorage-independent growth of androgen-independent cells moderately, whereas the combination was significantly more effective (Fig. 6D). However, we did not see the synergistic inhibition of androgen-independent cell growth with casodex + cyclopamine and casodex + LDE225 (Supplementary Fig. S2), suggesting a significant role of the truncated androgen receptor in supporting the growth of the androgen-independent cells. We used 5 to 10 μmol/L of LDE225 and cyclopamine in our MTT assays because this concentration range was found to dose-dependently inhibit Gli transcription activity in the androgen-independent cells as shown in Supplementary Fig. S3 for LDE225 and Supplementary Fig. S4 for cyclopamine, yet they showed marginal effect on cell growth (Fig. 6). Similarly, pyrvinium pamoate concentration of 50 nmol/L or less was used in our MTT assays as it could inhibit androgen receptor transcriptional activity in androgen-independent cells (Supplementary 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 androgen-independent cells when they were combined.

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

To determine whether a combinatorial approach can further enhance the efficacy of inhibiting CRPC-mediated
prostate tumor progression \textit{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, pyrvinium pamoate at 0.2 mg/kg/day, or cyclopamine + pyrvinium pamoate 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

Figure 7. Combined inhibition of Hh and androgen receptor 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, pyrvinium pamoate at 0.2 mg/kg/day, or cyclopamine + pyrvinium pamoate through intraperitoneal injection for 24 days. Cyclopamine (Hh inhibitor) and pyrvinium pamoate (androgen receptor 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 pyrvinium pamoate at a dose of 5 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 pyrinium pamoate combined treatment did not show a gradual increase in the tumor volume in 2 male mice (note: 2 male mice in cyclopamine and pyrinium pamoate 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 ± SEM from 4 subcutaneous tumors from 2 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 androgen receptor inhibitors.
group, 55.6% in the pyrvinium pamoate group, and 33.3% in the combination group. Furthermore, prostate tumors isolated from mice treated with cyclopamine + pyrvinium pamoate 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 pyrvinium pamoate 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 4 groups with matched tumor burden. All the mice were castrated following regrouping. Four days post castration, cyclopamine at 5 mg/kg/day, pyrvinium pamoate 0.2 at mg/kg/day, or cyclopamine + pyrvinium pamoate 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 (Fig. 7B and C). Figure 7B shows tumor growth curve in each mouse with 2 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, Fig. 7C was prepared to show the growth of the tumors in 2 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 pyrvinium pamoate alone showed no inhibitory effect whereas 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 androgen receptor signaling using cyclopamine and pyrvinium pamoate was more effective in inhibiting the growth of androgen-independent 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% to 90% of early stage tumor regression after androgen ablation therapy, nearly all men develop androgen-independent prostate cancer after 18 to 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 androgen receptor and rely on androgen receptor activity for survival and proliferation (36). Androgen receptor activity is maintained under hormone deprivation condition via various mechanisms in CRPC, which include amplification of the androgen receptor gene (37, 38), missense mutation in androgen receptor gene at T877A making it responsive to anti-androgens (39), and ligand-independent activation of androgen receptor by various growth factors such as insulin-like growth factor-1 (10). In our studies, we observed an increase in androgen receptor level in CRPC cells and prostate gland of castrated mice when compared to ADPC cells or noncastrated mice. Interestingly, we observed a decreased transcriptional activity of androgen receptor in CRPC cells, presumably due to low androgen in the environment and cytoplasmic localization of inactive androgen receptor. Our result that a relative increase in androgen receptor level in CRPC cells compared to ADPC cells is consistent with a previous finding that increased androgens repress androgen receptor expression (30). However, we observed an increased expression of truncated androgen receptor proteins (AR ΔLBD) in both LNCaP and 22Rv-1 AI cells. Previously, the AR ΔLBD proteins were shown to be present because of increased proteolytic deletion of the LBD under androgen-independent condition in LNCaP cells (40), whereas they are due to insertion mutation of androgen receptor gene and differential RNA splicing of androgen receptor transcript in 22Rv-1 cells (41). Previous studies have shown increased expression levels of AR ΔLBD and its ability to confer resistance to anti-androgens in androgen refractory prostate cancer cells (42). As expected, AR ΔLBD was found to be insensitive to bicalutamide (casodex), a competitive androgen receptor inhibitor, in our study. However, its transcriptional activity was found to be inhibited by pyrvinium pamoate, a non-competitive androgen receptor 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 Hh 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 showed 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 showing increased Hh components in the prostate gland of castrated mice (45). Consistent with these findings, genetic approaches with ectopic expression or knockdown of androgen receptor with siRNA also showed that androgen receptor level is inversely related with Hh pathway components and Gli transcriptional activity suggesting a direct suppression of Hh signaling activity by androgen receptor. 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. S3), or genetic knockdown of Smo (46).

These findings suggest that the increased Hh signaling and AR ΔLBD expression might contribute to the development and maintenance of CRPC. Thus, to inhibit the development and proliferation of CRPC cells, we used Hh and androgen receptor inhibitors independently and in combination both under in vitro and in vivo conditions. We showed that the noncompetitive androgen receptor inhibitor pyrvinium pamoate was effective in inhibiting the androgen signaling using cyclopamine and pyrvinium pamoate was more effective in inhibiting the growth of androgen-independent prostate cancer cells than each of the agent alone.
receptor activity in 22Rv-1 AI cells; whereas, the competitive androgen receptor inhibitor casodex was ineffective even though casodex showed inhibition of androgen receptor activity similar to pyrvinium pamoate in androgen-dependent 22Rv-1 cells. Hence, a noncompetitive androgen receptor inhibitor is effective in inhibiting androgen receptor activity under androgen-independent condition. Significantly, although 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 pyrvinium pamoate. Furthermore, the combination of pyrvinium pamoate with cyclopamine was found more effective than either agent alone in inhibiting the growth of CRPC tumors in vivo.

In summary, our study showed 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 an Smo inhibitor in combination with a noncompetitive androgen receptor inhibitor. This concept remains to be validated in clinical trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
Inhibition of Hh and AR Synergistically Suppresses CRPC

Inhibition of Hedgehog and Androgen Receptor Signaling Pathways Produced Synergistic Suppression of Castration-Resistant Prostate Cancer Progression

Pramod S. Gowda, Jianhong D. Deng, Sweta Mishra, et al.


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