Phosphodiesterase 4D Inhibitors Limit Prostate Cancer Growth Potential

Ginny L. Powers1, Kimberly D.P. Hammer1, Maribella Domenech2,3, Katsiaryna Frantskevich1, Rita L. Malinowski1, Wade Bushman4, David J. Beebe2, and Paul C. Marker1

Abstract

Phosphodiesterase 4D (PDE4D) has recently been implicated as a proliferation-promoting factor in prostate cancer and is overexpressed in human prostate carcinoma. However, the effects of PDE4D inhibition using pharmacologic inhibitors have not been examined in prostate cancer. These studies examined the effects of selective PDE4D inhibitors, NVP-ABE171 and cilomilast, as anti–prostate cancer therapies in both in vitro and in vivo models. The effects of PDE4D inhibitors on pathways that are critical in prostate cancer and/or downstream of cyclic AMP (cAMP) were examined. Both NVP-ABE171 and cilomilast decreased cell growth. In vitro, PDE4D inhibitors lead to decreased signaling of the sonic hedgehog (SHH), androgen receptor (AR), and MAPK pathways, but growth inhibition was best correlated to the SHH pathway. PDE4D inhibition also reduced proliferation of epithelial cells induced by paracrine signaling from cocultured stromal cells that had activated hedgehog signaling. In addition, PDE4D inhibitors decreased the weight of the prostate in wild-type mice. Prostate cancer xenografts grown in nude mice that were treated with cilomilast or NVP-ABE171 had decreased wet weight and increased apoptosis compared with vehicle-treated controls. These studies suggest the pharmacologic inhibition of PDE4D using small-molecule inhibitors is an effective option for prostate cancer therapy.

Implications: PDE4D inhibitors decrease the growth of prostate cancer cells in vivo and in vitro, and PDE4D inhibition has therapeutic potential in prostate cancer. Mol Cancer Res; 13(1); 149–60. ©2014 AACR.

Introduction

Prostate cancer is the second leading cause of cancer deaths among men and is the most commonly diagnosed noncutaneous male cancer (1). Although there are well-defined histologic alterations that have been identified in prostate cancer, the nature of this genetically heterogeneous disease has limited the identification of novel oncogenes that can be used as therapeutic targets. Using a transposon insertion mutagenesis screen, our laboratory previously identified phosphodiesterase 4D (PDE4D) as a novel proliferation-promoting factor for prostate cancer (2). PDE4D was expressed in prostate cancer cell lines but not primary prostate epithelial cells, and knockdown of PDE4D significantly decreased prostate cancer cell growth and migration in vitro. Furthermore, knockdown of PDE4D by shRNA significantly reduced xenograft wet weights and xenograft cell proliferation in vivo. Subsequent studies that performed whole-genome sequencing of human prostate tumors detected internal rearrangements of the PDE4D gene in approximately 20% of the tumors sequenced (3). These results are consistent with results in other cancers that have rearrangements that are predicted to produce overexpression of short PDE4D isoforms (4).

Phosphodiesterases (PDE) hydrolyze cyclic AMP (cAMP) and GMP (cGMP) and play a major role in cellular signaling. The PDE4 gene family has 4 isoforms: PDE4A, PDE4B, PDE4C, and PDE4D. In addition, the PDE4D gene has many variants attributed to alternative splicing and the use of multiple promoters (5, 6). PDE4D enzyme degrades cAMP but does not affect cGMP (5). The distribution pattern of PDE4D variants varies within both cells and tissues (7). In the prostate, PDE4D expression was observed in both the stroma and in epithelium, and its hydrolytic activity was restricted to the cytosolic compartment (8). In human prostate tumor microarrays representative of multiple stages of prostate cancer, PDE4D expression was most prominent in the epithelial cells (2). PDE4D was overexpressed in prostate adenocarcinoma samples when compared with benign prostatic hyperplasia samples in a human prostatic tissue microarray (2).

Previous studies have demonstrated the importance of cAMP signaling in prostate cancer. However, it remains unclear whether cAMP signaling promotes or inhibits prostate cancer progression. Transient increases in cAMP have been shown to be mitogenic in LNCaP prostate cancer cells by activation of extracellular signal–related kinase 1/2 (ERK1/2), whereas prolonged increases in cAMP led to growth arrest and neuroendocrine differentiation (9). In contrast, increased cAMP levels in androgen-insensitive...
PC3 cells resulted in growth inhibition (10). cAMP/protein kinase A (PKA) activity has also been shown to affect the activity of the androgen receptor (AR) leading to increased AR-responsive gene expression in androgen-independent conditions (11, 12).

In addition, cAMP/PKA activity negatively regulates the hedgehog (HH) pathway in some cell types including leukemia cells and basal cell carcinomas, and increasing cAMP signaling leads to growth arrest and death (13, 14). While the effects of PKA on the hedgehog pathway have not been investigated in prostate cancer cells, the hedgehog pathway has been shown to be an important driver of the prostate cancer phenotype in DU145 and PC3 (15). Furthermore, both DU145 and PC3 cells are growth-inhibited by PDE4D inhibitors (2). In prostate cancer, increased expression of hedgehog pathway components is correlated with high Gleason grade (grades 7 and 8) and lymph node metastasis (16). In prostate development, epithelial cells secrete sonic hedgehog which binds to the mesenchymal smoothened (Smo) receptors which is essential for the formation of prostate buds (17). While it is clear that the hedgehog pathway is a critical pathway in prostate cancer progression, it remains unclear whether paracrine, autocrine, or both mechanisms of hedgehog signaling are important for driving prostate cancer progression (18). However, on the basis of the effects of cAMP signaling, the literature suggests that alterations in PDE4D expression seen in the prostate cancer could potentially affect many signaling pathways including MAPK, AR, and hedgehog. This suggests that pharmacologic inhibition of PDE4D could affect several pathways that are linked to prostate cancer.

Phosphodiesterases have historically been active experimental targets for a wide variety of conditions including erectile dysfunction (PDE5), depression (PDE4D), asthma, chronic obstructive pulmonary disorder (COPD; PDE4D), and inflammation (PDE4D, PDE7; ref. 19). Currently, the FDA-approved PDE inhibitors include a variety of PDE inhibitors used in the treatment of erectile dysfunction and a non-subtype-selective PDE4 inhibitor, rolipram, used in combination therapy for COPD (in combination with bronchodilator; ref. 20). First-generation PDE4D inhibitors, such as rolipram, were initially developed and tested in the treatment of respiratory disorders, and second-generation PDE4D inhibitors such as NVP-ABE171 (21) and cilomilast (Arltlo, SB207499) were designed to have an improved potency and subtype selectivity compared with first-generation inhibitors (22). NVP-ABE171 was reported to have increased potency and selectivity for PDE4D compared with cilomilast, although both small-molecule inhibitors were more selective for PDE4D than other PDE family members (21). NVP-ABE171 has been tested with in vitro and in vivo models of airway inflammation and was generally effective and well tolerated in rats and mice (21). Among PDE4D-selective inhibitors, cilomilast was the most extensively tested in human clinical trials (23), but cilomilast was not FDA-approved because of a lack of efficacy in phase III clinical studies for respiratory diseases. However, these clinical trials established doses (15-mg twice daily) of cilomilast that were safe for human use, with mild, transient side effects (22).

Currently, the effects of PDE4D in cancer are not well understood and only few studies examined the role of PDE4D and its inhibitors in cancer therapy. PDE4D inhibitors have not been tested in prostate cancer models. Studies in A549 lung cancer cells demonstrated that TGFβ stimulation increased PDE4D expression and activity which promoted epithelial to mesenchymal transition, which could be attenuated with PDE4D inhibitor rolipram (24). A second study revealed that hypoxia via hypoxia-inducible factor 1α (HIF1α) regulated PDE4D in a lung cancer model and that in vivo treatment with first-generation PDE4D inhibitor, rolipram, decreased A549 xenograft weight and proliferation (25). Recently, a third study indicated that PDE4D expression increased in melanoma and endometrial carcinomas and that 26β, a novel PDE4D inhibitor, decreased in vivo growth of HGC-27 gastric carcinomas cells and A375 melanoma cells (26).

On the basis of our previous studies that showed that stable knockdown of PDE4D in cell lines reduced the growth of the prostate cancer epithelial cells and the published literature demonstrating in vivo efficacy on lung, melanoma, and gastric cancer xenografts, we hypothesized that the second-generation PDE4D inhibitors NVP-ABE171 and cilomilast would reduce the growth of prostate cancer cells. We chose these second-generation PDE4D inhibitors based on their selectivity for PDE4D as well as their previous use in other disease models with limited side effects observed in both humans and animal models. The results of the current study demonstrate that PDE4D inhibitors are effective at reducing growth of prostate cancer cells both in vitro and in vivo. In addition, PDE4D inhibition also reduced growth of benign prostate cells and modestly decreased the overall prostate weight in wild-type C57BL/6 mice.

Materials and Methods

Cell lines

Prostate cell lines BPH1 [LNCaP (ATCC) and LNCaP-C4 (ref. 27)] were cultured in 5% heat-inactivated FBS (Sigma), 100 μg/ml penicillin/streptomycin (pen/strep; Gibco), 1× HEPES (Gibco), 1× RPMI-1640 (CellGro) with 10⁻⁵ mol/L dihydrotestosterone (Sigma) or in RPMI-1640 (ATCC) 10% FBS (Omega Scientific) with 100 μg/ml pen/strep. UGSM-2 and Gli3 (18, 28) cells were cultured in 10% heat-inactivated FBS (Sigma), 100 μg/ml pen/strep (CellGro), 1× ITS (Lonza) with 10⁻⁵ mol/L dihydrotestosterone (Sigma), BPH1 and NPH1 cells were cultured in DMEM/F12 5% FBS, 0.4% bovine pituitary extract (Hammnod Cell Tech), 0.005 μg/ml EGF, 1 μg/ml ITS (Lonza), and 100 μg/ml pen/strep (29). P2 cells were cultured as previously described (30). For reduced serum experiments, 1% heat-inactivated FBS was used with the appropriate media.

Small-molecule inhibitors

PDE4D-selective inhibitors were obtained commercially (cilomilast from Selleck Chemicals and ChemPacific, NVP-ABE171 from Synthace) and dissolved in DMSO for in vitro experiments. Cyclopamine was obtained commercially (Toronto Research Chemicals or Enzo Life Sciences) and diluted in DMSO. PDE4D-selective inhibitors were diluted first in EtOH, followed by olive oil for a final concentration of 10% EtOH, 90% olive oil. PDE4D inhibitors were administered daily by oral gavage at a dose of 1 mg/kg NVP-ABE171 or 25 mg/kg cilomilast.

Cell growth and proliferation assays

Prostate cancer cell lines were plated at 2,000 cells per well in 96-well plates in the presence of PDE4D-selective inhibitors (NVP-ABE171, cilomilast) or vehicle-only (DMSO) control. Cells were grown for 72 hours with daily changes of the growth media, which contained vehicle or PDE4D inhibitor. Following the
growth period, relative cell numbers were quantified using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega).

**Western blotting**

Blots were performed as previously described (31). Primary antibodies for pERK1/2 and total ERK1/2 were obtained from Cell Signaling.

**Microculture assays**

A total of 5,000 to 7,500 cells were plated in microculture devices as previously described (32). LNCaP or LNCaP-C4 cells were cultured with either UGSM-2, UGSM-2 + exogenous sonic hedgehog (Shh, Curis), or Gli3−/− UGSM cells (UGli3−/−; ref. 33). Drugs were added on day 1 and replenished until day 5. Following the growth period, cell proliferation was quantified using an EdU assay kit (Invitrogen) or RNA was extracted using DynaBeads (Invitrogen; ref. 33). EdU assay images were obtained on an inverted Nikon Eclipse Ti using a ×4 objective. Fluorescent nuclear counts and GPF intensities were determined using ImageJ v1.38 (NIH). Percentage of EdU+ cells was obtained by dividing total EdU+ to total cell number (Hoescht nuclear stain) × 100. Cell proliferation was assessed for significant differences by Wilcoxon Mann–Whitney test. Significant differences have P < 0.05.

**qRT-PCR**

A StepOne Plus (Applied Biosystems) or an iCycler (Biorad) thermocycler was used to perform qRT-PCR. Primers used were Ptch1, Gli1 (34), AR (forward: 5′-TTTGGATGCCTCCTATCCCATC-3′ and reverse: 5′-GCAATGTACCAATGCTGC-3′), prostate-specific antigen (PSA; forward: 5′-CATTAGAAACAAAACCTGTA-3′ and reverse: 5′-ATATCGTACGCCGTTGGC-3′), and transmembrane protease, serine 2 (TMPRSS2; forward: 5′-CCATTGTCAGCATCGTTCG-3′ and reverse: 5′-GATGTTGCTTGCCGAGC-3′). Control housekeeping genes primers for 18S rRNA (Ref. 32) were used and data are presented as 2^ΔΔCt.

**Xenografts and animal experiments**

All experiments using mice were performed using protocols approved by the University of Wisconsin Institutional Animal Care and Use Committees. LNCaP-C4 cells (1 × 10⁶) were combined with Matrigel (BD Biosciences) and grafted under the kidney capsule of male nu/nu mice (Charles River). After 1 week of xenograft growth, mice were given PDE4D-selective inhibitors or vehicle-only control by gavage once daily for 6 weeks. After the treatment period, grafts were excised, weighed, and photographed. Xenografts were fixed in formalin, processed, and embedded in paraffin blocks. For experiments in wild-type mice, 6-week-old C57BL/6 mice (Charles River) were treated daily with vehicle, cilomilast, or NVP-ABE171 for 6 weeks. After the treatment, mice were sacrificed and overall body weight and prostate weight were measured. Prostates were formalin fixed, processed, and embedded in paraffin blocks.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described using antibodies for E-cadherin (Cell Signaling), smooth muscle actin (Sigma), p63 (Santa Cruz Biotechnology), Ki67 (Vector Laboratory, VP-K452), or p21 (Santa Cruz Biotechnology; sc-397; ref. 31). Slides were dewaxed, rehydrated with a graded alcohol series, antigen retrieval was performed with antigen unmasking solution (Vector Labs), and endogenous peroxidases were quenched by incubation in H₂O₂. Slides were blocked with 2.5% serum, incubated with primary antibody overnight, washed, and incubated with biotinylated secondary antibody (Vector Labs) followed by incubation with ABC reagent (Vector Labs) and color development with DAB (Vector Labs). Slides were counterstained with hematoxylin, dehydrated, and mounted. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assays were performed as specified by the manufacturer using the DeadEnd Colorimetric TUNEL Assay System (Promega) and were counterstained with hematoxylin. Slides were imaged with a 20× objective on a Leica DMLB microscope and acquired using QCapture Software (QImaging Software). Labeling indices were calculated by blinded individuals who counted the number of positive cells (Ki67, p21, TUNEL) and the total number of cells. Data are presented as the percentage of positive cells.

**Results**

To examine the effects of PDE4D inhibition on prostate cancer, LNCaP-C4 cells were treated with increasing concentrations of PDE4D inhibitors cilomilast and NVP-ABE171 (Fig. 1). After 72 hours, the effects on cell growth were assayed in a cell titers assay and demonstrated that there are dose-dependent decreases in the growth of LNCaP-C4 cells with both cilomilast (Fig. 1A) and NVP-ABE171 (Fig. 1B). Cilomilast showed decreased growth with doses of 5 μmol/L, but NVP-ABE171 was more potent and decreased growth with doses as low as 0.5 nmol/L. PDE4D inhibition by cilomilast (Fig. 1C) or NVP-ABE171 (Fig. 1D) decreased the growth of BPH1, an SV-40 immortalized benign prostate cells at doses of 5 μmol/L and 50 nmol/L, respectively. In addition, dose-dependent decreases in growth with increasing doses of NVP-ABE171 were also observed in P2 (Pten+/−) mouse prostate cancer cells (Fig. 1E). The impact of PDE4D inhibition was also tested in normal spontaneously immortalized (untreated) prostate epithelial cell lines BPH1E1 (Fig. 1F) and NHPE1 (Fig. 1G) which were unaffected by either cilomilast or NVP-ABE171 treatment. These data suggested that PDE4D inhibition has a modest effect on growth in vitro of a transformed prostate cell lines.

The main effector of cAMP is PKA which has impacts on multiple cell signaling pathways that are important in cancer (reviewed in ref. 35). Therefore, the effects of PDE4D inhibition on PKA regulated pathways were examined in LNCaP-C4 cells (Fig. 2). Effects on the MAPK pathway, which is regulated by PKA (35), were examined by measuring phosphorylation of downstream effector ERK (Fig. 2A) by Western bloting. While NVP-ABE171 decreased phosphorylated ERK (p-ERK) protein, the decrease is observed at 250 nmol/L, a dose 5-fold greater than the dose that produced growth inhibition. In addition, expression of AR, which is a primary therapeutic target of prostate cancer treatment and has previously been reported to be regulated by cAMP (36), was decreased by high doses of cilomilast and NVP-ABE171 (5–10 μmol/L), but not at the low doses of NVP-ABE171 where growth was inhibited (Fig. 2B). Similar to AR, expression of AR target gene PSA decreased...
with high doses of cilomilast and NVP-ABE171 (Fig. 2C). A second AR target gene, transmembrane protease, serine 2 (TMPRSS2), was unaffected by either of the PDE4D inhibitors (Fig. 2D).

Previous studies have demonstrated negative regulation of the hedgehog signaling pathway via PKA in other cell types (37, 38), and the role of the hedgehog pathway has been well established in prostate cancer (15). The effects of cilomilast and NVP-ABE171 treatment on the hedgehog pathway were measured using qRT-PCR to measure expression of Gli1 and Patched1 (Ptc1), genes that are upregulated in response to hedgehog pathway activation. Doses of cilomilast (5 μmol/L) and NVP-ABE171 (50 nmol/L) that resulted in growth inhibition also resulted in a statistically significant decrease in the expression of sonic hedgehog target genes Ptc1 and Gli1 (Fig. 3A and B). The potential for complimentary effects between PDE4D inhibition and hedgehog inhibitor cyclopamine was examined. LNCaP-C4 cells were treated with doses of
NVP-ABE171 (25 nmol/L) or cyclopamine (0.5 μmol/L) that did not affect growth on their own (Fig. 3C). However, in combination, suboptimal doses of NVP-ABE171 and cyclopamine were able to decrease growth compared with control or either treatment alone.

Because there are conflicting reports regarding the importance of autocrine and paracrine hedgehog signaling in prostate cancer, the connection between PDE4D and the sonic hedgehog pathway was explored further using a relevant model of stromal–epithelial signaling. LNCaP cells have been previously reported to lack autocrine hedgehog signaling capacity; however, the contributions of autocrine and paracrine hedgehog signaling in various cell lines are still controversial (34). LNCap or LNCap-C4 cells were plated in microculture devices (33) with mesenchymal cell lines UGSM-2 (wild-type mouse UGM cells), UGSM-2 cells with Shh (ligand that binds to and activates Smo receptor), or UGli3−/− cells (mesenchymal cells with constitutively active Shh signaling; ref. 18). Proliferation of LNCap or LNCap-C4 cells in coculture with the indicated mesenchymal cells treated with PDE4D inhibitors NVP-ABE171 or cilomilast were measured by the incorporation of EdU (Fig. 4A and B). Coculture using mesenchymal cells with activated hedgehog signaling, either by the addition of Shh or genetic (UGli3−/−), resulted in increased proliferation of both LNCap and LNCap-C4 cells. In addition, UGli3−/− cells are also unresponsive to common hedgehog inhibitors such as cyclopamine that target activation of the cell surface receptor Smo (33). Proliferation of LNCap and LNCap-C4 cocultured with UGSM-2 cells was unaffected by cilomilast.
and NVP-ABE171. However, both NVP-ABE171 and cilomilast significantly inhibited proliferation induced by sonic hedgehog activation by coculture with UGSM-2 + Shh and UGI13−/− stromal cells (Fig. 4A and B). RNA was isolated from the mesenchymal cells that were cocultured with the LNCaP epithelial cells to measure the effects of PDE4D inhibitors on hedgehog target genes Gli1 and Ptc1 (Fig. 4C and D). As anticipated, activation of hedgehog signaling (UGSM-2 + Shh and UGI13−/−) increased Gli1 and Ptc1 mRNA expression in the mesenchymal cells. Inhibition of PDE4D by cilomilast and NVP-ABE171 significantly decreased the expression of Gli1 and Ptc1 in UGSM-2 cells treated with Shh or in UGI13−/− cells, which suggests that PDE4D may be affecting prostate cancer growth by modulating hedgehog paracrine signaling that occurs via stromal and epithelial interactions.

Although data from the LNCaP and LNCaP-C4 indicated that PDE4D inhibition affected growth and signaling in prostate cancer cell lines, the effects of PDE4D inhibition on the normal prostate were unknown. In the course of experiments, it was also observed that the prostates of the PDE4D inhibitor–treated nude mice that were used as hosts in xenografts experiments appeared smaller (data not shown). The effects of PDE4D inhibition were examined in wild-type C57BL/6 mice. Male C57BL/6 mice were treated daily with vehicle, cilomilast, or NVP-ABE171 for 6 weeks. The prostate wet weight (Fig. 5A) and total body weight (Fig. 5B) were measured at sacrifice. NVP-ABE171–treated mice has a statistically significant reduction in prostate weight and cilomilast treatment resulted in a similar trend, but the overall body weight and wet weights of the lungs, liver, spleen, and seminal vesicles of the mice were not impacted (Fig. 5A and B and data not shown). IHC was performed on prostate sections to determine the effects of PDE4D inhibition on morphology and organization (Fig. 5C). The overall morphology and organization of the prostate appeared normal in the cilomilast and NVP-ABE171–treated prostates as assessed by hematoxylin and eosin (H&E) staining. In addition, markers for stromal (smooth muscle actin) and epithelial (E-cadherin) compartments were unchanged. While there was a trend to an
increase in the basal epithelial subtype (p63), this increase was not statistically significant. Neuroendocrine markers, neurophilin and chromogranin A, were examined because of a previous report that in an in vitro model PDE inhibitors caused a neuroendocrine differentiation (9). However, positive staining for neuroendocrine markers (as compared with a positive control) was not observed in any of the control, cilomilast, or NVP-ABE171–treated prostates (data not shown). The expression of hedgehog-activated genes, Shh, Gli1, and Ptch1, in the mouse prostate was not significantly affected by cilomilast or NVP-ABE171 treatment (data not shown). However, this was not unexpected as the level of hedgehog signaling in the adult mouse prostate is quite low compared with the human prostate or the developing prostate (39).

The effects of PDE4D inhibition were also measured in vivo using a prostate cancer xenograft model. LNCaP-C4 cells were implanted under the kidney capsule of nude mice. Host mice received daily oral doses of vehicle or PDE4D inhibitors cilomilast or NVP-ABE171 for 6 weeks. Images of the xenografts from vehicle and treated animals are shown in Fig. 6A. PDE4D inhibition with cilomilast or NVP-ABE171 resulted in a statistically significant decrease in the weight of the LNCaP-C4 xenograft (Fig. 6B). Compared with the effects in vitro where the decrease in growth compared with control were only decreased by approximately 50%, the effect of PDE4D inhibitors in vivo was robust where cilomilast and NVP-ABE171 decreased xenograft wet weight 85% and 70%, respectively. To identify a potential mechanism to explain how PDE4D inhibitors decreased the size of LNCaP-C4 xenografts, xenografts were fixed and paraffin embedded to examine proliferation (Ki67), apoptosis (TUNEL), and senescence (p21) markers by IHC (Fig. 6C and D). Labeling indices were performed to compare the number of positive cells between treatment conditions (Fig. 6D). NVP-ABE171 treatment resulted in a statistically significant decrease in Ki67–positive cells (proliferation). Both cilomilast and NVP-ABE171 treatment resulted in a significant increase in TUNEL–positive cells (apoptosis). Neither cilomilast nor NVP-ABE171 led to a change in the senescence marker p21.

Discussion

Resistance to existing prostate cancer therapies is a constant problem in prostate cancer as current treatments are not curative. Therefore, targeting novel pathways may provide alternatives to existing treatments for prostate cancer. Previous studies in our laboratory have identified PDE4D as a proliferation-promoting factor that has increased expression in prostate cancer (2). Because phosphodiesterases are good targets for small-molecule inhibitors, the effects of PDE4D inhibitors were investigated as potential therapies for prostate cancer. To date, there have been no studies published which investigate PDE4D inhibitors in prostate cancer, although others have investigated the potential for use of PDE4D inhibitors in cancer therapy in vivo using animal models of cancer of lung cancer (25) and...
**in vivo** studies have shown efficacy of PDE4D inhibitors in lung, melanoma, and leukemia (25, 26, 40). The results of these studies suggest that PDE4D inhibition has the potential to be an effective prostate cancer therapy.

These studies demonstrate the efficacy of PDE4D inhibitors in both **in vivo** and **in vitro** prostate cancer models. **In vitro** moderate inhibition of LNCaP-C4 and P2 cell growth was observed, and **in vivo** dramatic decreases in LNCaP-C4 xenografts wet weight were observed in PDE4D inhibitor-treated mice. It is unclear why the **in vivo** response to PDE4D inhibitors was so strong compared with the **in vitro** response; however, these results highlight the importance of examining the effect of potential therapeutics using multiple experimental approaches (41). While **in vitro** models of cell culture are models of growth in an isolated cell population, they provide a framework where the mechanism of action of a particular drug or pathway can be interrogated. In the current study, **in vitro** models identified effect of PDE4D inhibitors on several PKA-mediated pathways (SHH, AR, and MAPK). Because **in vivo** studies add complexity, including interaction with a relevant microenvironment (including extracellular matrix, multiple additional cell types, paracrine signaling), growth in a 3-dimensional environment, they provide valuable insights into how a drug may perform in conditions relevant to the disease being targeted. Data from coculture experiments (Fig. 4) provide additional complexity compared with **in vitro** monoculture and demonstrated that PDE4D inhibitors affected multiple cell types (epithelial and stromal) found in the prostate cancer microenvironment. These data suggest that PDE4D inhibitors may exert their effects by modulating paracrine signaling and influencing the prostate microenvironment. The **in vivo** studies with LNCaP-C4 xenografts, which are subject to the host microenvironment, demonstrated dramatic decrease in tumor size and increased apoptosis in response to PDE4D inhibitors. Ultimately, the complimentary data from **in vivo** and **in vitro** studies support the idea that overall PDE4D inhibitors may be efficacious as an anti-prostate cancer therapy.

Although PDE4D inhibition affected multiple cell types, the effects of PDE4D inhibition appear to have some selectivity for cancer and/or transformed rapidly proliferating immortalized cells. **In vitro** studies with the small-molecule inhibitors revealed that both NVP-ABE171 and cilomilast decreased growth of
the LNCaP-C4, and P2 prostate cancer cell lines as well as decreasing growth of the benign BPH1 cell line. BPH1 cells were immortalized with SV40 large T-antigen (42), which is known to inactivate multiple tumor suppressors including Rb and p53 and can have transforming effects on cells (reviewed in ref. 43) which may be responsible for the efficacy of PDE4D inhibitors on BPH1 cell growth. This is also supported by the data that demonstrated spontaneously immortalized prostate cells (NHPrE1 and BHPrE1) that express high levels of tumor suppressors p53, RB, and p21 (29) were unaffected by either cilomilast or NVP-ABE171 treatment. This is further supported by the in vivo data, where PDE4D inhibition had dramatic effects on prostate cancer xenografts but only modest effects on the wild-type prostate. In addition, in vivo the effect of the inhibitors was not apparent in many other tissues (lungs, liver, spleen, or seminal vesicles) and the overall weight of the animals was also not impacted. These
data suggest an overall selectivity for transformed cell populations both in vivo and in vitro.

In vitro mechanistic studies revealed that PDE4D inhibition affected several PKA-mediated pathways, but only high doses of NVP-ABE171 lead to a decrease in MAPK and AR signaling pathways. However, the inhibition of hedgehog signaling pathway was best correlated with growth inhibition, which was observed at doses as low as 50 nmol/L NVP-ABE171. A previous study used purified PDE4A, B, C, and D to determine the IC50 values of both NVP-ABE171 and cilomilast. NVP-ABE171 had the lowest IC50 for PDE4D (1.5 nmol/L), the next lowest was PDE4B (34 nmol/L), and the IC50 values for both PDE4A and PDE4C were greater than 600 nmol/L (21). For cilomilast, the results showed a similar trend for IC50, but with less potency where the IC50 for PDE4D and PDE4B was the lowest at 63 and 288 nmol/L, respectively. As a comparison, the prototypal PDE4 inhibitor rolipram is most potent for inhibition of PDE4A (IC50 of 3 nmol/L) followed by PDE4B (130 nmol/L) and PDE4D (240 nmol/L; ref. 44). The in vitro studies demonstrated growth inhibition by NVP-ABE171 effects on doses as low as 40 to 50 nmol/L and cilomilast doses of 5 µmol/L which have the potential to inhibit both PDE4D and PDE4B. Further experiments will be required to determine the relative contribution of each PDE4 isoform to the effects of NVP-ABE171 and cilomilast. Offentimes, inhibition in whole cells or in vivo requires a higher concentration of the drug, which is consistent with treatments used in this study. This suggests that the results observed are likely due to the inhibition of PDE4D, but the impact of PDE4B cannot be completely excluded. The role of the hedgehog pathway in well-characterized in vitro studies with prostate development, where paracrine signaling occurs from epithelial cells that secrete Shh that binds to Smo on the mesenchymal cells and ultimately promotes prostate budding and development (17). In the adult mouse prostate, hedgehog activity is diminished compared with the developing prostate, but in the human adult, prostate hedgehog signaling remains relatively high (39). Both human prostate tumors and mouse models of prostate cancer have increased hedgehog expression and activity, particularly in aggressive and androgen-independent cancers (15, 45). However, in prostate cancer, it is unclear whether the paracrine hedgehog signaling remains intact or if there is autocrine signaling that occurs. Studies examining prostate tumor histology have demonstrated that the localization of hedgehog pathway components or the level of androgens or AR signaling, as the histology of the H&E from the prostate appears normal and does not demonstrate the atrophy and loss of ducts observed in castrated mice (50). Also, the wet weight of seminal vesicles, which are highly androgen-sensitive, was unchanged by PDE4D inhibition (data not shown). Further studies are required to identify whether there are a subset of patients who are likely to benefit from these inhibitors. In addition, on the basis of the impacts of PDE4D inhibition on several key pathways in prostate cancer, hedgehog, and AR, these results also suggest future studies to examine the effects of PDE4D inhibitors in combination with AR or hedgehog inhibitors. The results of this work suggest that there may be a precedent for further preclinical exploration of PDE4D inhibitors in prostate cancer, particularly in those model systems that have elevated PDE4D levels.
References
Molecular Cancer Research

Phosphodiesterase 4D Inhibitors Limit Prostate Cancer Growth Potential


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-14-0110

Cited articles
This article cites 49 articles, 14 of which you can access for free at:
http://mcr.aacrjournals.org/content/13/1/149.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/13/1/149.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.