Elevated LIM Kinase 1 in Nonmetastatic Prostate Cancer Reflects Its Role in Facilitating Androgen Receptor Nuclear Translocation

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Abstract

Prostate cancer affects a large proportion of the male population, and is primarily driven by androgen receptor (AR) activity. First-line treatment typically consists of reducing AR signaling by hormone depletion, but resistance inevitably develops over time. One way to overcome this issue is to block AR function via alternative means, preferably by inhibiting protein targets that are more active in tumors than in normal tissue. By staining prostate cancer tumor sections, elevated LIM kinase 1 (LIMK1) expression and increased phosphorylation of its substrate Cofilin were found to be associated with poor outcome and reduced survival in patients with nonmetastatic prostate cancer. A LIMK-selective small molecule inhibitor (LIMKi) was used to determine whether targeted LIMK inhibition was a potential prostate cancer therapy. LIMKi reduced prostate cancer cell motility, as well as inhibiting proliferation and increasing apoptosis in androgen-dependent prostate cancer cells more effectively than in androgen-independent prostate cancer cells. LIMK inhibition blocked ligand-induced AR nuclear translocation, reduced AR protein stability and transcriptional activity, consistent with its effects on proliferation and survival acting via inhibition of AR activity. Furthermore, inhibition of LIMK activity increased γ-tubulin acetylation and decreased AR interactions with γ-tubulin, indicating that the role of LIMK in regulating microtubule dynamics contributes to AR function. These results indicate that LIMK inhibitors could be beneficial for the treatment of prostate cancer both by reducing nuclear AR translocation, leading to reduced proliferation and survival, and by inhibiting prostate cancer cell dissemination. Mol Cancer Ther; 14(1) January 2015

Introduction

Prostate cancer is the most commonly diagnosed malignancy and second leading cause of cancer-related deaths in American men (1). At the molecular level, prostate cancer development and progression are driven primarily by activity of the androgen receptor (AR), a steroid hormone receptor typically localized in the cytoplasm in the absence of hormone stimulation (2). In the presence of ligand, ARs translocate to the nucleus to activate the transcription of target genes that control cell-cycle progression, cell growth, and survival. As a result, the first line of therapy in prostate cancer has been to decrease AR activity by hormone depletion (3). Unfortunately, hormone-ablation therapy often leads to the development of castration-resistant prostate cancer (CRPC) that may quickly progress to metastatic disease with high mortality rates (4). Therefore, a major goal is to identify potential targets for the development of prostate cancer therapies that target AR function in a hormone-independent manner. Such treatments might not only delay the progression of prostate cancer to CRPC, but could possibly also be used for the treatment of CRPC, which maintains and relies upon active AR (4).

Targeting the microtubule cytoskeletal network is one approach that has been used to achieve the goal of reducing AR signaling. Docetaxel, a microtubule-stabilizing drug commonly used for the treatment of prostate cancer, has been shown to exert its cytotoxic effect on prostate cancer cells by inhibiting microtubule-mediated AR nuclear translocation in addition to its direct antimitotic activity (5, 6). Two major issues with docetaxel treatment are that resistance develops over time, and its general antimitotic and microtubule-stabilizing actions result in strong side effects, including alopecia, neutropenia, and anemia. Therefore, an appealing objective for future prostate cancer drug development is to identify alternative microtubule regulators, which if inhibited would affect AR function with low nonspecific cytotoxicity. In particular, if this target was more active in prostate cancer, its inhibition would improve drug selectivity for prostate cancer tumors over normal tissue and contribute to a greater therapeutic window.

Although best known as regulators of actin–myosin cytoskeletal dynamics (7), LIM kinases 1 and 2 (LIMK1 and LIMK2) also contribute to the regulation of the microtubule cytoskeleton (8–10). LIM kinases are highly homologous serine/threonine kinases that are activated by RhoA/ROCK, Rac/PAK, and Cdc42/MRCK signaling pathways (7). The most well-characterized LIMK
substances are Cofilin proteins, which are inhibited for their actin-severing activities when phosphorylated on serine 3 (11). There have been previous reports of elevated LIMK1 expression in prostate cancer (12–14), in which it was postulated to have a role in promoting metastasis (15). However, there have been no previous studies that systematically evaluated the expression levels of LIMK1, LIMK2, or phosphorylation of their common substrate Cofilin as an indicator of kinase activity in primary prostate cancer tumor samples accompanied by analysis of prostate cancer clinical outcomes.

We undertook immunohistochemical analysis of a prostate cancer tissue microarray (TMA) comprised of 164 primary prostate cancer and 23 benign hyperplasia samples from 94 individual patients (16), and identified significant associations of elevated LIMK1 expression and phosphorylation of nuclear Cofilin with reduced survival of patients with nonmetastatic prostate cancer. Moreover, elevated levels of LIMK1 and cytoplasmic phospho-Cofilin were both associated with significantly higher lymphovascular invasion. To pharmacologically evaluate whether LIMK could be a potential prostate cancer drug target, we tested a potent and selective LIMK inhibitor (LIMKi; ref 17). LIMK inhibition reduced prostate cancer cell motility, suggesting that blocking LIMK activity could be beneficial as an antimetastatic therapeutic target in prostate cancer. Interestingly, we observed a cytotoxic effect of LIMK inhibition that was significantly greater in androgen-dependent prostate cancer cells than in androgen-independend cells. Treatment of androgen-dependent prostate cancer cells with LIMKi reduced AR nuclear translocation and transcriptional activity by altering microtubule dynamics that facilitate AR interactions with αTubulin, thus inhibiting cell proliferation. Therefore, in addition to a potential role in promoting metastasis, changes in LIMK1 and LIMK2 expression and/or activity might contribute to AR function in prostate cancer via regulation of microtubule cytoskeletal dynamics. These results justify further investigation of LIM kinases as potential targets for prostate cancer therapy.

Materials and Methods

TMA and immunohistochemistry

The prostate cancer TMA, previously described in (16), was comprised of primary prostate cancer tumor samples. Within the TMA cohort, 49 samples had available metastasis data, which were typically identified by a bone scan. Survival was defined as disease-specific, patients who died from intercurrent disease were censored in the analysis. Immunohistochemical staining of TMA slides was performed as described previously (18), using antibodies against LIMK1, LIMK2, and p-Cofilin (18, 19). TMA slides were scanned and staining intensities were scored using the SlidePath application (Leica Biosystems). The staining was scored low if the sample histoscore was below or equal to the median histoscore for the entire cohort, or high, if above.

Statistical analysis

Survival differences were determined using the log-rank (Mantel–Cox) test. The Mann–Whitney test was used to compare correlation between protein expression and lymphovascular invasion, in Statistical Package for Social Sciences software (SPSS, Version19). All other indicated statistical analyses were performed in Prism5 (GraphPad) software. The F test was used to compare LIMKi EC50 values.

Cell lines and antibodies

Cell lines were cultured in RPMI media, supplemented with 10% FBS and 2 mmol/L L-glutamine (GIBCO). LNCaP-AI cells were cultured in phenol red-free RPMI with 10% charcoal-stripped serum (CSS) and 2 mmol/L L-glutamine (GIBCO). The LNCaP, DU145, PC3, RWPE-1 cell lines were from the ATCC, LNCaP-AI was made in the laboratory of Hing Leung (Beatson Institute for Cancer Research, Glasgow, UK) and gifted to us, CWR22 cells were from Thomas Prettow (Case Western Reserve University, Cleveland OH). All cell lines were obtained at the beginning of the study in April 2012 and authenticated using the GenePrint 10 system STR multiplex assay (Promega) that amplifies 9 tetranucleotide repeat loci and Amelogenin gender determining marker. Antibodies: Santa Cruz Biotechnology, AR (N-20 and sc-816), LIMK2 (H-78 and sc-5577); Cell Signaling Technology, p-Cofilin (Ser3, 3311), LIMK1 (3842); Abcam, Cofilin (ab54532), LIMK1 (ab55414); Sigma, αTubulin (clone DM1A, T9026); Millipore, MMP-1 (04–1112); Leica, MMP-10 (NCL-MMP10); Novus Biologicals, acetylated-α Tubulin (NB600-567).

Cytotoxicity assays

Cells were plated in 96-well plates at 2,000 cells per well in triplicate and treated the next day without changing the media. Drugs were serial diluted in DMSO, before diluting equal amounts in media at 2× of the final concentration. Then 100 μL of drug-containing media was added to each well that already contained 100 μL of cells. Cells were treated for 72 hours, fixed with 4% paraformaldehyde, and stained with 250 ng/ml DAPI. Plates were imaged on a High Content Imaging Operetta system (PerkinElmer) and nuclei in each well were quantified using ImageJ High Content Imaging and Analysis Software (PerkinElmer). For cytotoxicity of RWPE-1 cells, CellTiter-Glo (Promega) Luminescent Cell Viability assay was used according to the manufacturer's instructions. The effect of each treatment was calculated as the percentage of change in cell number relative to DMSO-treated control. EC50 values were calculated from dose–response curves, constrained between 0% and 100%, using Prism 5 (GraphPad) software.

Caspase 3/7 activity

Cells were plated and treated as for the cytotoxicity assay, or transfected with siRNA as in colony formation assay, and plated in 96-well plates. Caspase activity was measured 72 hours after inhibitor treatment or siRNA transfection using the Caspase-Glo3/7 Assay system (Promega) following the manufacturer's instructions.

Sub-G1 quantification

Cells were plated in 6-well plates at 5 × 105 cells per well and treated the next day with DMSO or 10 μmol/L LIMKi for 24 hours. The percentage of cells with sub-G1 DNA content were measured and analyzed as described previously (20).

siRNA knockdown and colony formation assay

LNCaP or CWR22 cells were transiently transfected with ON-TARGETplus SMART pool siRNAs (Thermo Scientific) against LIMK1 or LIMK2, or a nontargeting control (NTC) siRNA, using DreamFect Gold transfection reagent (OZ Biosciences) and plated in 24-well plates at 105 cells per well in duplicate. Twenty-four hours after transfection, media were changed to 1 mL per well of growing media and cells were incubated for 24 hours for lysis and knockdown analysis by Western blotting, or incubated for 7 days for colony formation. To quantify colony formation, cells were

www.aacrjournals.org Mol Cancer Ther; 14(1) January 2015 247
fixed in methanol and stained with 0.2% (v/v) Crystal Violet solution. Staining was quantified with an ODYSSEY infrared imaging system (LI-COR).

**Commmunoprecipitation and Western blotting**

LNCaP cells were incubated in phenol red-free RPMI with CSS for 24 hours, and then treated with LIMKi, docetaxel, or DMSO in the presence of 1 nmol/L dihydrotestosterone (DHT) for 24 hours. Cells were lysed with 500 μL of lysis buffer [1 x TBS, 1% Triton-X, 1 nmol/L EDTA, 0.2 mmol/L Na3VO4, 20 mmol/L NaF, 1 mmol/L PMSF, and eComplete Mini protease inhibitor cocktail (Roche)] per 10-cm plate. Lysates were cleared by 10-minute centrifugation and precleared with Protein-A agarose beads (Life Technologies). After preclearing, beads were removed, and then lysates were incubated with anti-AR antibody at 1:25 dilution for 2 hours at 4°C, then 30 μL Protein-A agarose beads were added for 1 additional hour. Beads were washed three times with TBS and boiled with warmed 1% SDS. To quantify the amount of immunoprecipitated protein, immunoprecipitated samples and total cell lysates (1% of immunoprecipitated) were boiled with loading dye and Western blotted (20) with anti-AR and α-Tubulin antibodies. Quantification of Western blots was performed directly without signal amplification or X-ray film using infrared emitting secondary antibodies and detection with an ODYSSEY infrared imaging system (LI-COR).

**LIMK1 antibody validation**

LIMK1 peptide (ab158818 (Abcam)) was spotted on PVDF membranes at 50, 25, or 5 ng in duplicate, and membranes were blocked and blotted as for Western Blotting with LIMK1 primary antibody ab55414 (Abcam), LIMK1 antibody that has been preincubated with 4 μg/mL LIMK1 peptide or nonspecific control Cofilin peptide (amino acids 1–20). After incubation, membrane was developed as for a Western blot analysis. Immunohistochemistry was performed as described above using LIMK1 antibody with or without preincubation with LIMK1 competitor peptide or Cofilin peptide as a nonspecific control.

**Luciferase assay**

CWR22 cells were plated in 96-well plates at 2 × 104 cells per well in triplicates and transfected the next day with p(ARE)_3-Luc (21) and CMV (cytomegalovirus)-driven Renilla luciferase plasmids at the 10:1 ratio using X-tremeGENE HP transfection reagent (Roche) following the manufacturer’s instructions. Next day, cells were incubated in phenol red-free RPMI with CSS with DHT or vehicle control (ethanol), in the presence of indicated drugs for 24 hours. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega), following the manufacturer’s protocol. Firefly Luciferase measurements were normalized to Renilla Luciferase values for transfection control and plotted as fold change of DMSO-treated control.

**Real-time PCR**

CWR22 cells were pretreated with 3 μmol/L LIMKi, or DMSO vehicle control in CSS RPMI without phenol-red for 24 hours, then treated with 1 nmol/L DHT in the presence of the drugs for additional 24 hours. RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed using the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. PSA gene expression was quantified using the DyNAMO HS SYBR Green qPCR Kit (Thermo Scientific) and the Applied Biosystems 7500 Fast Real-Time System (Life Technologies) using primers against PSA (F: 5′-GGCGCATTTAAGCAGAGGAG-3′; R: 5′-GAACCTGGGGGCTTGATTGAG-3′) and 18S (F: 5′-GTAACCC-GTGAACCCCCATT-3′; R: 5′-CCATCCAATCGGTAGCGG-3′) as an internal control.

**Immunofluorescence**

LNCaP cells grown on coverslips were incubated in phenol red-free RPMI with CSS for 24 hours, then treated with indicated drugs for 24 hours before 2-hour stimulation with 1 nmol/L DHT. Cells were fixed and stained with specific antibodies as described previously (22). Images were taken using a Zeiss710 laser-scanning confocal microscope and analyzed with ZEN2010 (Zeiss) software.

**Nuclear AR quantification**

Nuclear AR (volume per nucleus and the percentage of cells with nuclear AR) were quantified using Volocity 3D Image Analysis software (PerkinElmer).

**Acetylated tubulin quantification**

Immunofluorescence staining intensity of acetylated α-Tubulin was quantified in ImageJ software using fixed intensity threshold, and normalized to total α-Tubulin immunofluorescence intensity levels.

**Cell fractionation**

LNCaP cells were incubated in phenol-red-free RPMI with CSS for 24 hours, then treated with 1 nmol/L DHT in the presence of indicated drugs for 24 hours. After treatment, cells were lysed and fractionated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions.

**Migration assays**

PC3 and DU145 cells were plated in 96-well plates in quadruplicates at 4 × 103 cells per well and treated the next day as in cytotoxicity assay, for 24 hours. After treatment, cell monolayers were scratched, washed, and monitored on the INCELLiTE Kinetic Imaging System (ESSEN BioScience) by continuous imaging every 2 hours for 24 hours. The percentage of wound confluence was quantified using INCELLiTE software analysis (ESSEN BioScience). LNCaP cells were plated in glass-bottom 6-well plates,
Results

LIMK expression and activity in prostate cancer

LIMK1 has previously been reported to be elevated in limited samples of human prostate cancer (12–14); however, its association with patient outcome had not been determined. In addition, neither LIMK2 expression nor phosphorylation of the common substrate Cofilin had been previously characterized in prostate cancer. Therefore, we investigated how LIMK1 and LIMK2 expression and activity varied in prostate cancer, and determined how observed variations were associated with patient outcomes using a TMA comprised of 164 primary prostate cancer samples and 23 benign hyperplasia samples from 92 individual patients (demographic, clinicopathologic, and outcome characteristics of the patients detailed in Supplementary Table S1; ref. 16). Using antibodies validated by peptide competition for LIMK1 (Supplementary Fig. S1A) or by staining tissues from knockout mice for LIMK2 (18) or Serine 3 phosphorylated Cofilin (p-Cofilin; ref. 19), TMA samples were stained, scanned, and scored for staining intensities using an automated algorithm (23). Staining intensities of each protein were correlated with the histopathologic data of the tumor samples. Examples of low and high staining for LIMK1, LIMK2, nuclear, and cytoplasmic p-Cofilin are shown in Fig. 1A. There was a significant association between high LIMK1 expression and poor survival in patients diagnosed with nonmetastatic prostate cancer (P = 0.035; Fig. 1B; Supplementary Table S2), but not in the metastatic disease group (Fig. 1C; Supplementary Table S2). Analysis of LIMK2 levels showed a similar trend of high LIMK2 expression associated with poor patient survival in the nonmetastatic group (P = 0.151; Fig. 1B; Supplementary Table S3). However, high LIMK2 was significantly associated with increased survival in the metastatic disease group (P = 0.048; Fig. 1C; Supplementary Table S3). Because Cofilin has both nuclear import and export signals to facilitate nuclear-cytoplasmic shuttling (24), we analyzed nuclear or cytoplasmic p-Cofilin levels in the TMA samples. Similar to LIMK1, high nuclear p-Cofilin levels showed a significant association with poor patient survival in the nonmetastatic group (P = 0.034; Fig. 1B; Supplementary Table S4). Although the role of cytoplasmic Cofilin has largely been associated with regulation of cytoskeleton dynamics (25), nuclear Cofilin may contribute to nuclear actin regulation, gene transcription and mitosis (26, 27). Together, these results indicate that elevated LIMK expression and activity are associated with increased mortality in nonmetastatic prostate cancer. Although LIMK1 and Cofilin phosphorylation was not associated with patient mortality in metastatic prostate cancer (Supplementary Tables S2, S4, and S5), elevated LIMK1 and cytoplasmic p-Cofilin were significantly associated with increased lymphovascular invasion (Fig. 1D), a clinical characteristic of more aggressive tumors and potential marker of progression to metastatic disease (28). There were no significant associations between LIMK1, LIMK2, or Cofilin phosphorylation with patient age, Gleason score, PSA elevation or relapse (Supplementary Table S6).

Analysis of publicly available gene-expression data using Oncomine (29) revealed significantly elevated LIMK1 mRNA expression levels in prostate carcinoma relative to normal gland tissue (Fig. 1E; ref. 30). Furthermore, analysis using the cbio Cancer Genomics Portal (31) of 85 prostate adenocarcinoma tumor samples revealed significantly increased LIMK1 expression or amplification in 14 cases (16%), with 5 of 12 patients (42%) having elevated LIMK1 expression undergoing biochemical recurrence in contrast with 16 of 68 patients (24%) without alterations in LIMK1 expression (32). In addition, disease-free survival trended toward being worse for patients with altered LIMK1 expression (Supplementary Fig. S1B). Analysis of LIMK1 gene copy number by The Cancer Genome Atlas (TCGA; http://tcga-data.nci.nih.gov/tcga/) indicated that there was a significant increase in prostate adenocarcinoma samples relative to normal prostate tissue (Fig. 1F). These results are consistent with increased mRNA contributing to elevated LIMK1 protein in prostate cancer. Together, our findings indicate that elevated LIMK expression and activity are associated with early-stage prostate cancer growth and progression.

LIMK inhibitor decreases prostate cancer cell motility

Given the previous reports of elevated LIMK1 expression in metastatic prostate cancer (12–15) and our findings that blocking LIMK activity reduces breast cancer cell invasiveness (33), we sought to determine whether pharmacologic inhibition of LIMK activity reduces prostate cancer cell motility. We initially compared LIMK1, LIMK2, and Cofilin Serine3 phosphorylation in several prostate cancer cell lines, and found that Cofilin phosphorylation was highest in parental androgen-dependent LNCaP cells, with lower levels in an androgen-independent LNCaP variant (LNCaP-AL), derived by continuous culture in hormone-depleted media (16), as well as AR-negative PC3 and DU145 cells (Fig. 2A). Combined LIMK1 and LIMK2 levels were highest in LNCaP cells, with lower levels in the androgen-independent LNCaP-AL, PC3, and DU145 prostate cancer cells. Treatment of LNCaP cells with 1 or 10 nmol/L DHT did not further increase ILMK1 or LIMK2 protein levels (Supplementary Fig. S1C).

To investigate the role of LIMK activity in prostate cancer cell motility, we used a potent selective small molecule inhibitor [N-[5-\{2-[2,6-Dichloro-phenyl]-5-difluoromethyl-2H-pyrrole-3-yl\}-thiazol-2-yl]-isobutyramide [compound 3 in (ref. 17); hereafter termed LIMKi] that equipotently inhibits LIMK1 and LIMK2. We confirmed that LIMKi effectively reduced p-Cofilin in LNCaP cells after 24-hour treatment (Fig. 2B), as we previously observed for other cell lines, including MDAMB231 breast cancer cells (22), NMuMG mouse mammary epithelial cells (34), and U2OS human osteosarcoma cells (20).

As a major regulator of cytoskeleton dynamics, LIMK1 has been implicated in prostate cancer cell migration and invasion (12, 14, 15). Given that maximal inhibition of Cofilin phosphorylation was achieved at 10 nmol/L LIMKi (Fig. 2B), we tested this concentration on DU145 and PC3 cell migration. Cells were plated in a dense monolayer in 96-well plates, then treated the next day with 10 nmol/L LIMKi for 24 hours. After treatment, PC3 cell migration was analyzed in an automated scratch wound-healing assay, with images acquired every 2...
hours for 24 hours (Fig. 2C). After creating an initial mask, wound confluence was measured by determining the percentage of wound area that is occupied by cells over time. There was a marked effect of LIMKi on 2D migration of PC3 cells over time (Fig. 2D, left graph) and at the 24-hour experimental endpoint (Fig. 2D, right graph). LIMKi had a more moderate inhibitory effect on the 2D migration of DU145 cells over time (Fig. 2E, left graph), which was significant at the 24-hour experimental endpoint (Fig. 2E, right graph). These results suggest that LIMK inhibition could have the therapeutic benefit of reducing prostate cancer dissemination.

Sensitivity of androgen-dependent prostate cancer cells to LIMK inhibition

In addition to testing how LIMKi affected DU145 and PC3 scratch wound closure, we initially tested LNCaP cells but found that they did not migrate in this assay format. As an alternative, we used single-cell tracking to measure random migration velocity. Treatment with 1 nmol/L DHT significantly increased migration velocity, which was significantly reversed by 5 µmol/L LIMKi (Fig. 2F), indicating a role for LIMK activity in androgen-induced motility. Expression of the matrix metalloproteinases MMP-10 (35) and MMP-1 (36), which had previously been implicated in
Figure 3.
The LIMK inhibitor blocks survival of androgen-dependent prostate cancer cell lines. A, LNCaP (red) and LNCaP-AI (green) cell lines were treated with half-log serial dilutions of LIMKi, adriamycin, actinomycin D, and camptothecin (left to right) for 72 hours. Drug cytotoxicity was measured as the percentage of survival relative to DMSO-treated control (100%). Graphs, combined results of three independent experiments performed in triplicate ± SEM. B, DNA content of LNCaP or LNCaP-AI cells treated with 10 μmol/L LIMKi (red line) or DMSO vehicle (black line) for 72 hours as analyzed by propidium iodide staining followed by flow cytometry. Graph, the results from four independent experiments ± SEM. Statistical significance was tested by two-way ANOVA; the P value, significant effect of cell type on drug response. C, caspase3/7 activity for each treatment was measured and normalized to DMSO alone–treated control. (Continued on the following page.)
prostate tumor growth, was not affected by DHT or LIMKi treatment (Supplementary Fig. S1D). Interestingly, when LNCaP cells were treated with 10 μmol/L LIMKi, we readily observed reduced LNCaP cell numbers. To quantify the effect of LIMKi on proliferation, we treated LNCaP or LNCaP-AI cells in 96-well plates with half-log serial dilutions of LIMKi. After 72 hours, surviving cells were fixed, stained with DAPI, and nuclei numbers quantified with an Operetta High Content Imaging System. LNCaP cells were significantly more sensitive to LIMKi than LNCaP-AI cells (Fig. 3A, left). However, the two cell lines did not differ in their sensitivities to adriamycin, actinomycin D, or camptothecin (Fig. 3A), all of which exert their cytotoxicity independent of LIMK inhibition. In addition, 10 μmol/L LIMKi treatment induced a substantial increase in the percentage of LNCaP cells with sub-G1 DNA content (i.e., induced a substantial increase in the percentage of LNCaP cells with sub-G1 DNA content (i.e., <2N), indicative of the induction of apoptosis, but not in LNCaP-AI cells (Fig. 3B). Moreover, evaluation of the LIMKi rank order of potency on cell number for various prostate cancer cell lines revealed androgen-dependent LNCaP and CWR22 cells to be >2 to 8 times more sensitive than androgen-independent LNCaP-AI, DU145, and PC3 cells (Table 1). Similarly, using a cell viability assay that measures ATP levels, immortalized RWPE-1 normal prostate epithelial cells had an EC50 value for LIMKi of 6.67 μmol/L, similar to the values determined for androgen-dependent CWR22 cells (Table 1). Consistent with these results, activity of the apoptosis executioner caspases 3 and 7 was induced by 10 μmol/L LIMKi in LNCaP and CWR22 cells over 3-fold relative to untreated control cells, but not in androgen-independent LNCaP-AI, DU145, and PC3 cells (Fig. 3C). These results indicate that androgen-dependent prostate cancer cells, which had the highest relative levels of LIMK expression and Cofilin phosphorylation, were more sensitive to LIMK inhibition than androgen-independent prostate cancer cells, suggesting that LIMK activity may contribute to AR function and activity.

To validate the on-target effects of LIMKi (17), we analyzed the effect of individual or combined LIMK1 and LIMK2 siRNA-mediated knockdown (Fig. 3D) on the induction of apoptosis by measuring caspase 3/7 activity. In both LNCaP and CWR22 cells, LIMK2 or combined LIMK1 + LIMK2 knockdown induced significant caspase activity (Fig. 3E). Furthermore, proliferation of androgen-dependent LNCaP and CWR22 cell lines was strongly inhibited by the simultaneous knockdown of both LIMK1 and LIMK2 in a colony formation assay (Fig. 3F), similar to the effect of LIMKi. These results indicate that LIM kinases have roles in supporting proliferation of androgen-dependent prostate cancer cells.

**LIMK inhibition targets AR activity in androgen-dependent prostate cancer cells**

AR is a steroid hormone receptor that translocates into the nucleus upon ligand binding to regulate transcription of downstream target genes that promote prostate cell growth, proliferation, and survival. Given the observed effects of LIMKi on androgen-dependent prostate cancer cell number and apoptosis, we sought to determine whether LIMKi treatment affected AR subcellular localization. Following 24-hour incubation in hormone-depleted media (37) to reduce background AR signaling, LNCaP cells were stimulated with 1 nmol/L DHT for 2 hours. Representative confocal immunofluorescence images show increased nuclear AR staining following DHT stimulation, quantified either by determining the percentage of cells with detectable nuclear AR (Fig. 4A, top) or by measuring the mean volume of AR staining per nucleus (Fig. 4A, bottom), both of which were reduced by treatment with 3 μmol/L LIMKi or 4 nmol/L docetaxel, a standard prostate cancer chemotherapy drug previously shown to inhibit AR nuclear translocation (38). Furthermore, the effect of LIMKi and docetaxel on DHT-induced AR nuclear translocation was supported by cellular fractionation following the same treatment used for immunofluorescence experiments. A representative Western blot analysis shows that DHT-induced nuclear AR accumulation was reduced by 1 and 3 μmol/L LIMKi or 4 nmol/L docetaxel (Fig. 4B). Combined results from three independent experiments revealed that 3 μmol/L LIMKi had a comparable inhibitory effect on DHT-induced nuclear accumulation of AR as 4 nmol/L docetaxel (Fig. 4B). Interestingly, inhibition of p-Coﬁlin by 4 nmol/L docetaxel was comparable with that observed for 3 μmol/L LIMKi, suggesting that docetaxel inhibits LIMK activity as a direct or indirect off-target, which may additionally contribute to its antiproliferative mechanism of action in prostate cancer.

Because of the inhibitory effect of LIMKi on AR nuclear accumulation, we tested the possibility that LIMKi inhibition might affect AR protein stability. LNCaP cells were treated with 3 μmol/L LIMKi or vehicle (DMSO) control for 24 hours, followed by inhibition of de novo protein translation with 20 ng/mL cycloheximide (CHX) for the indicated times. By measuring AR protein levels at each time point by Western blotting, we observed less remaining AR protein in the presence of LIMKi compared with DMSO-treated controls, consistent with LIMKi treatment reducing AR protein stability (Fig. 4C).

To test the effect of LIMKi on AR transcriptional activity, we used a Luciferase reporter construct comprised of three consensus AR-response element (ARE) repeats (21) in a transcriptional reporter system. Given the observed effects of LIMKi on androgen-dependent prostate cancer cell number and apoptosis, we sought to determine whether LIMKi treatment affected AR subcellular localization. Following 24-hour incubation in hormone-depleted media (37) to reduce background AR signaling, immortalized RWPE-1 normal prostate epithelial cells had an EC50 value for LIMKi of 6.67 μmol/L, similar to the values determined for androgen-dependent CWR22 cells (Table 1). Similarly, using a cell viability assay that measures ATP levels, immortalized RWPE-1 normal prostate epithelial cells had an EC50 value for LIMKi of 6.67 μmol/L, similar to the values determined for androgen-dependent CWR22 cells (Table 1). Consistent with these results, activity of the apoptosis executioner caspases 3 and 7 was induced by 10 μmol/L LIMKi in LNCaP and CWR22 cells over 3-fold relative to untreated control cells, but not in androgen-independent LNCaP-AI, DU145, and PC3 cells (Fig. 3C). These results indicate that androgen-dependent prostate cancer cells, which had the highest relative levels of LIMK expression and Cofilin phosphorylation, were more sensitive to LIMK inhibition than androgen-independent prostate cancer cells, suggesting that LIMK activity may contribute to AR function and activity.

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Figure 4.
Treatment with LIMKi reduces AR function. A, LNCaP cells were stimulated with 1 nmol/L DHT for 2 hours after being hormone starved and treated with DMSO vehicle, 3 µmol/L LIMKi, or 4 nmol/L docetaxel for 24 hours. Cells were fixed and stained with fluorescent antibodies against AR (green), α Tubulin (red), or stained with DAPI (blue). Representative confocal immunofluorescence images (top) and 3-dimensional reconstructions from z-stacks (bottom) are shown. The percentage of cells with nuclear AR (top graph) and mean AR volume (µm³) per nucleus (bottom graph) were quantified with Volocity software. Combined results from three independent experiments ± SEM are shown. Statistical significance was tested by the unpaired Student’s t test between indicated conditions. B, LNCaP cells were treated as above. Cells were fractionated and nuclear lysates analyzed by Western blot analysis for changes in nuclear AR. Total cell lysates of the same cells were analyzed by Western blot analysis for changes in p-Cofilin. Graph, quantification results of nuclear AR levels normalized to nuclear fraction loading control, plotted as the percentage of change compared with unstimulated cells. Average results of three independent experiments ± SEM are shown. (Continued on the following page.)
Cells were transfected with p(ARE) for the assay, so androgen-dependent CWR22 cells were used. Cells were transfected with p(ARE)3-luciferase reporter and Renilla luciferase control plasmids and then treated with 1 or 10 nmol/L DHT for 24 hours in the presence of 3 or 10 µmol/L LIMKi, DMSO vehicle control, or 10 µmol/L of the antiandrogen bicalutamide. These experiments revealed that 10 µmol/L LIMKi reduced DHT-induced AR transcriptional activity to a similar extent as 10 µmol/L bicalutamide. These results indicate that inhibition of LIMK activity reduced AR nuclear accumulation, stability, and transcriptional activity in androgen-dependent prostate cancer cells, which would contribute to the antiproliferative and proapoptotic effects of LIMKi treatment (Fig. 3).

LIMK inhibition affects microtubule stability and AR-αTubulin interactions

AR has been previously shown to interact directly with microtubules, whereas disruption of microtubule dynamics leads to reduced nuclear AR translocation (38). In addition to being a major regulator of actin dynamics, LIMK has also been shown to regulate microtubule dynamics (8–10). Therefore, we tested the effect of LIMKi on the levels and distribution of acetylated αTubulin (Ace-αTub), which is associated with increased stabilization and altered dynamics of the microtubule network (39). To visualize changes in microtubule structure and αTubulin acetylation, we performed confocal immunofluorescence microscopy on LNCaP cells treated with 10 µmol/L LIMKi for 24 hours. Representative Z-plane optical slices as well as maximum projection images show a noticeable change in overall microtubule structure (Fig. 5A). Acetylation of αTubulin with increasing concentration of LIMKi was also detected by immunofluorescence treatment (Fig. 5B), which, when combined results from three independent experiments were quantified and normalized to total αTubulin, revealed increased tubulin acetylation with increasing LIMKi concentrations (Fig. 5B). Western blot analysis showed a similar trend of increased acetylated αTubulin over total αTubulin with increasing LIMKi concentrations (Fig. 5C). To determine whether the LIMKi-induced increase in αTubulin acetylation affected AR–αTubulin association, LNCaP cells were treated identically as for AR nuclear localization analysis in Fig. 4, and AR–αTubulin interactions analyzed by communoprecipitation. There was a reduction in αTubulin associated with AR immunoprecipitated from cells treated with LIMKi (Fig. 5D); 3 µmol/L LIMKi had a similar effect to 4 nmol/L docetaxel treatment (Fig. 5D). These results indicate that LIMK inhibition attenuates AR nuclear translocation by decreasing αTubulin acetylation with consequent effects on microtubule dynamics, and by reducing AR interaction with αTubulin.

Discussion

In this report, we established a correlation between poor survival in patients with nonmetastatic prostate cancer and increased LIMK1 and nuclear phospho-Coilin levels (Fig. 1B; Supplementary Tables S2 and S4). In addition, elevated LIMK1 and phosphorylated cytoplasmic Coilin were associated with increased lymphovascular invasion (Fig. 1D). Associated with these expression patterns, we identified a role for LIM kinases in metastatic prostate cancer cell motility and in promoting AR function in prostate cancer via their contribution to regulating microtubule dynamics. Intriguingly, inhibition of LIMK activity with a selective small molecule inhibitor had greater cytotoxic effects on androgen-dependent than on androgen-independent prostate cancer cells (Fig. 3 and Table 1). Treatment of androgen-responsive cells with LIMKi decreased DHT-induced AR nuclear translocation, protein stability, and transcriptional activity (Fig. 4). Impaired nuclear translocation of AR in the presence of LIMKi was likely the product of decreased AR–αTubulin interactions and increased αTubulin acetylation, indicative of increased microtubule stability (Fig. 5). Taken together, our results indicate that LIM kinases positively regulate AR function in AR-dependent prostate cancer, promoting disease development and progression to an early locally invasive state. In addition, these studies provide pharmacologic evidence, with mechanism-of-action detail, showing that LIMK inhibition has potential as a therapeutic approach for the treatment of prostate cancer.

The finding of LIMK1 association with poor patient survival in the nonmetastatic prostate cancer group is consistent with a previous observation of correlation between expression of the LIMK upstream regulator Rhoa with poor patient survival at early stages of prostate cancer and prostate cancer lymph node invasion (40). LIMK1 has been previously reported to be upregulated in prostate cancer tumors and cell lines, in which it was postulated to have a role in metastasis largely due to the generally held view of LIMK1 as a promoter of cell motility and migration (12–14). These studies, however, did not examine LIMK1 expression levels in nonmetastatic versus metastatic tumor samples, nor was the link between LIMK1 expression with patient outcome determined for either group.

The role of LIMK2 in prostate cancer has largely been overlooked. Our results indicate that LIMK2 expression in nonmetastatic prostate cancer is similar to that of LIMK1, with a trend of elevated LIMK2 associated with poor patient survival and increased lymphovascular invasion. However, these observations did not achieve statistical significance in the number of samples in the TMA dataset. However, the strong trend of association with poor survival in the nonmetastatic patient cohort and lymphovascular invasion for all three markers,
Figure 5.
LIMKi increases αTubulin acetylation and reduces AR–αTubulin interactions. A, LNCaP cells were treated with DMSO vehicle, or 10 µmol/L LIMKi for 24 hours, fixed, and stained with fluorescent antibodies against total αTubulin. Narrow Z-plane images were taken at 0.5-µm intervals, and distance of each image from the starting point is indicated. B, fluorescence staining intensity of acetylated αTubulin (Ace-αTub, green) was quantified and normalized to total αTubulin (red) for each field. Graph, average quantification results for three independent experiments (each using three fields/treatment) ± SEM. Statistical significance was tested by one-way ANOVA and the post hoc Newman–Keuls multiple comparison test, conditions differing significantly from each other as indicated. C, LNCaP cells were treated with DMSO vehicle or increasing LIMKi concentrations for 24 hours. Cells were lysed and αTubulin acetylation levels determined by Western blot analysis. Acetylated αTubulin (Ace-αTub) was normalized to total αTubulin levels and plotted as fold change over DMSO-treated control. Graph, results of three independent experiments ± SEM. Statistical significance was tested by one-way ANOVA and the post hoc Dunnett multiple comparison test, condition differing significantly from DMSO only control as indicated. D, LNCaP cells were treated with DMSO vehicle, 3 µmol/L or 10 µmol/L LIMKi, or 4 nmol/L docetaxel (Dcxl) for 24 hours. Cells were lysed and AR was immunoprecipitated (IP). Amount of communoprecipitated αTubulin was determined by Western blot analysis. Total cell lysates (1% of the IP amount) were analyzed by Western blot analysis. Graph, αTubulin levels normalized to AR levels in each IP sample from three independent experiments ± SEM.
LIMK1, LIMK2, and p-Cofilin, and the requirement for combined LIMK1 and LIMK2 knockdown by siRNA for full antiproliferative effects (Fig. 3E) leads us to propose that both LIMK1 and LIMK2 contribute to development and progression of early-stage prostate cancer, which is often characterized as AR positive and hormone dependent. Indeed, a role of LIM kinase signaling in early AR-positive and hormone-dependent prostate cancer is consistent with the greater sensitivity of androgen-dependent than androgen-independent prostate cancer cell lines to LIMKi in cell proliferation and apoptosis detection assays (Fig. 3 and Table 1).

The greater sensitivity of androgen-dependent prostate cancer cell lines and RWPE-1 prostate epithelial cells to LIMKi supports the conclusion that LIMKi targets AR function (Fig. 4 and Table 1). We observed an inhibitory effect of LIMKi on AR nuclear translocation (Fig. 4A and B) and decreased AR–αtubulin interaction that were associated with increased tubulin acetylation (Fig. 5). LIM kinases are important regulators of actin cytoskeleton dynamics (7). Given that AR nuclear translocation has been previously reported to depend on the filamentous actin cross-linking protein filamin (21), an additional possibility is that LIMKi inhibition may affect AR translocation via effects on the actin cytoskeleton. This double effect on both actin and microtubule cytoskeletal networks that contribute to AR nuclear accumulation may explain the apparent selective advantage for elevated LIMKi expression in the patients with poor-outcome nonmetastatic prostate cancer and the increased sensitivity of androgen-dependent prostate cancer cell lines to LIMKi.

Ligand-bound AR is rapidly degraded by a proteasome-mediated degradation pathway if sequestered in the cytoplasm (41, 42). The inhibition of nuclear AR accumulation in the presence of LIMKi was accompanied by a more rapid decrease in AR protein levels (Fig. 4C), possibly due to the accumulation of ligand-bound AR in the cytoplasm, which would subsequently be targeted for degradation. This inhibitory effect on protein stability likely contributes to the LIMKi cytotoxic mechanism of action in androgen-dependent prostate cancer cells.

We found a significant association between elevated LIMKi expression and poor survival in nonmetastatic prostate cancer, whereas LIMK2 expression had a similar trend in the same patient group (Fig. 1). However, we also observed that elevated LIMKi expression in metastatic prostate cancer was associated with better patient outcome. These differences could indicate a unique role for LIMKi in cancer. We previously reported that progressively decreased LIMK2 expression due to promoter methylation was associated with poor prognosis in patients with colorectal cancer, whereas LIMK2 deletion increased tumor burden in a colitis-associated colorectal cancer mouse model (18). We determined that was due to a role of LIMK2 in restraining gastrointestinal stem cell proliferation. On the basis of these observations, a possibility is that there is selection for decreased LIMK2, but not LIMKi, expression in advanced prostate cancer to promote the expansion of stem-like tumor cells. Consistent with our observations, analysis using the cbio Cancer Genomics Portal (31) of mRNA levels in 85 prostate adenocarcinoma tumor samples revealed significantly decreased LIMK2 relative to normal tissue in 27 cases (31% of ref. 32).

Our results suggest that there may be potential for the use of LIMKi inhibitors as an AR-dependent prostate cancer–targeted therapy. Moreover, the effect of LIMKi on prostate cancer cell migration (Fig. 2) suggests that LIMKi inhibitors might have an additional benefit of limiting cancer spread as well as targeting tumor growth. Although AR-negative prostate cancer cell lines showed a relatively weak response to LIMKi in cytotoxicity assays (Fig. 3 and Table 1), LIMKi inhibitors may target CRPC cells that still rely on AR for survival. Recent research has shown that CRPC cells are reliant on AR signaling, and adapt to hormone depletion therapy by increasing AR expression and/or upregulating androgen production (43, 44). This hypothesis is supported by the successful treatment of CRPC with taxanes, such as docetaxel that target AR function or abiraterone, which inhibits 17α-hydroxylase activity to reduce androgen production (45). These findings support the notion that blocking AR activity is the prime target for treatment of both drug-naïve prostate cancer and CRPC. As general microtubule-targeting drugs, taxanes have high overall toxicity because their target is essential in normal cells, and is not differently expressed in tumor cells. Given the elevated expression of LIM kinases in the nonmetastatic poor outcome patient group (Fig. 1), our data support the idea that inhibition of LIM kinases could have tumor selective effects while leaving normal tissues relatively unaffected. In addition, the finding that LIMK2 knockdown sensitized neuroblastoma cells to taxane treatment (46) suggests that the treatment of CRPC with docetaxel could be made more effective through combination therapy with LIMKi inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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References


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