Development of a Small-Molecule Serum- and Glucocorticoid-Regulated Kinase-1 Antagonist and Its Evaluation as a Prostate Cancer Therapeutic

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Abstract

Androgens, through their actions on the androgen receptor (AR), are required for the development of the prostate and contribute to the pathologic growth dysregulation observed in prostate cancers. Consequently, androgen ablation has become an essential component of the pharmacotherapy of prostate cancer. In this study, we explored the utility of targeting processes downstream of AR as an alternate approach for therapy. Specifically, we show that the serum and glucocorticoid-regulated kinase 1 (*SGK1*) gene is an androgen-regulated target gene in cellular models of prostate cancer. Furthermore, functional serum- and glucocorticoid-regulated kinase 1 (SGK1) protein, as determined by the phosphorylation of its target Nedd4-2, was also increased with androgen treatment. Importantly, we determined that RNA interference–mediated knockdown of SGK1 expression attenuates the androgen-mediated growth of the prostate cancer cell line LNCaP. Given these findings, we explored the utility of SGK1 as a therapeutic target in prostate cancer by developing and evaluating a small-molecule inhibitor of this enzyme. From these studies emerged GSK650394, a competitive inhibitor that quantitatively blocks the effect of androgens on LNCaP cell growth. Thus, in addition to androgen ablation, inhibition of pathways downstream of AR is likely to have therapeutic utility in prostate cancer. [Cancer Res 2008;68(18):7475–83]

Introduction

One in six men will develop prostate cancer during their lifetime, and with ∼30,000 deaths per year attributed to the disease, it is the second leading cause of cancer death in men (1). The majority of prostate cancers express the androgen receptor (AR) and rely on androgens for growth and survival. For this reason, patients with advanced prostate cancers generally undergo androgen deprivation therapy with chemical and/or surgical castration as a primary intervention. Although 80% of patients with local or metastatic prostate cancer initially respond favorably to androgen ablation therapy, most patients eventually experience a relapse of the disease, which is then considered to be hormone refractory (2, 3). Unfortunately, effective therapeutic options are limited for these patients. Although hormone-refractory disease is no longer responsive to androgen deprivation therapy, AR signaling pathways remain active and necessary for cancer progression (4). Therefore, novel therapeutics that facilitate AR turnover or target signaling pathways downstream of receptor activation may be an effective treatment option for hormone-refractory disease. Consequently, there is renewed interest in the pharmacologic exploitation of AR signaling pathways for prostate cancer therapeutics.

Although the phenotypic responses of the prostate and prostate cancer cells to androgens are well described, the molecular events underlying these activities are not well understood. However, the results of microarray studies done by both our laboratory and others have led to the identification of pathways and specific genes that are likely to be important for AR biology in prostate cancer. Among the genes identified in this manner is that encoding serum- and glucocorticoid-regulated kinase 1 (SGK1; refs. 5–7). SGK1 belongs to the AGC family of serine/threonine protein kinases, which also includes protein kinase C and Akt. As its name implies, *SGK1* expression is regulated by glucocorticoids and serum, in addition to various other types of signals (8–15). The enzymatic activity of SGK1 is regulated by specific phosphorylation events initiated by phosphoinositide-3 kinase (PI3K; refs. 16–20). Specifically, upon PI3K activation, SGK1 is phosphorylated at Ser^{422} by an as yet unidentified kinase, referred to as the phosphoinositide-dependent protein kinase (PDK)-2, followed by the phosphorylation of Thr^{256} within the activation loop of the SGK1 catalytic domain by PDK1 to become fully activated (19).

Many prostate cancers rely on the PI3K signaling pathway for growth and survival. This is reflected by the high incidence of loss-of-function mutations in the gene that encodes the PI3K negative regulator phosphatase and tensin homologue (PTEN) in prostate cancers (21, 22). In fact, the prostate-specific *PTEN* knockout mice develop invasive prostate cancer with 100% penetrance, indicating that within the context of the murine prostate, constitutive PI3K signaling is sufficient for the development of cancer (23).

A considerable number of studies have addressed the role of Akt as the mediator of PI3K-dependent stimulation of growth and survival in prostate cancer. Interestingly, however, both Akt and SGK1 phosphorylate and regulate the activity of many of the same target proteins. Recently, SGK1 has also been implicated in the regulation of cell growth and survival downstream of PI3K activation (24–27). Therefore, considering that most prostate cancers rely on the androgen signaling pathway for growth, and activation of this pathway leads to the induction of *SGK1* expression, we hypothesized that SGK1 is required for androgen-mediated prostate cancer growth. This study shows that inhibition of SGK1 expression or

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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activity antagonizes androgen-induced growth of the prostate cancer cell line LNCaP, suggesting that SGK1 might be a viable target for the treatment of prostate cancer.

Materials and Methods

Reagents and antibodies. All chemicals and reagents were purchased from Sigma unless otherwise specified. Methyltrienolone (R1881) was purchased from Perkin-Elmer and bicalutamide (Casodex) was provided as a gift from P. Turnbull (GlaxoSmithKline, Research Triangle Park, NC). GSK650394 was synthesized by the CVU Medicinal Chemistry Department at GlaxoSmithKline (King of Prussia, PA). The goat polyclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) V-18 antibody was obtained from Santa Cruz Biotechnology and the rabbit polyclonal SGK1 antibody (KAP-PK015) was purchased from Stressgen Bioreagents, Inc. The mouse monoclonal AR441 antibody that recognizes AR was a gift from Dr. Dean Edwards (Baylor College of Medicine, Houston, TX). The Nedd4-2 and phospho-Nedd4-2 antibodies were provided as gifts from Dr. Olivier Staub (University of Lausanne, Lausanne, Switzerland).

Cell culture. The human prostate carcinoma cell line LNCaP-FGC was obtained from American Type Culture Collection (ATCC) and maintained in RPMI medium 1640 (Invitrogen) supplemented with 8% fetal bovine serum (FBS), 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate (NaPyr; Invitrogen). The prostate cancer cell line LAPC4 was a gift from Dr. Charles Sawyers (Memorial Sloan-Kettering Cancer Center, New York, NY) and maintained in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 15% FBS, 0.1 mmol/L nonessential amino acids, 1 mmol/L NaPyr, and 1 nmol/L R1881. The prostate cancer cell line VCaP was provided by K. Pienta (University of Michigan, Ann Arbor, MI) and maintained in DMEM (Invitrogen) supplemented with 8% FBS, 0.1 mmol/L nonessential amino acids, and 1 mmol/L NaPyr. The M-1 cell line (derived from SV40-transformed mouse cortical collecting duct) was obtained from ATCC and maintained in Ham's F-12/DMEM supplemented with 5% FBS; 2 mmol/L L-glutamine; 1% antibiotic/antimycotic; 100 nmol/L dexamethasone; and transferrin, insulin, and sodium selenite (6.25 μg/mL each). The HeLa cell line was obtained from ATCC and maintained in Ham's DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, and 1% antibiotic/antimycotic. The COS-7 cell line was obtained from ATCC and maintained in DMEM (high-glucose) supplemented with 10% FBS, 2 mmol/L L-glutamine, and 1% antibiotic/antimycotic. All cells were maintained in humidified incubators at 37°C with 5% CO_2.

Immunoblotting. Western blots were done as previously described (28). Membranes were probed with the appropriate primary antibody at the indicated dilutions [anti-SGK1, anti-AR, and anti-GAPDH at 1:1,000; anti–Nedd4-2 at 1:500; and anti–phospho-Nedd4-2 (Ser^{328}) at 1:200].

Transient transfection of small interfering RNAs. Chemically synthesized Stealth small interfering RNAs (siRNA) were purchased from Invitrogen and transfected using Dharmafect-1 transfection reagent (Dharmacon) according to the manufacturer's instructions. See Supplementary Table S1 for the sequences of siRNAs used in these studies.

RNA isolation and quantitative PCR. RNA isolation and quantitative PCR were done as previously described (28). See Supplementary Table S1 for sequences of quantitative PCR primers used in these studies. In those studies using actinomycin D and cycloheximide, the compounds were used at concentrations previously shown to inhibit transcription and translation in LNCaP cells (29).

Cell growth assays. LNCaP cells were plated at a density of 5,000 per well (for studies with GSK650394) or 20,000 per well (for siRNA studies) in 96-well plates in 100 μL of phenol red-free (PRF)–RPMI 1640, supplemented with 8% charcoal-stripped FBS, 0.1 mmol/L nonessential amino acids, and 1 mmol/L NaPyr. After a 2-d incubation, cells were transfected with siRNAs as described above. Twenty microliters of medium were removed from each well and replaced with 20 μL of the siRNA/Dharmafect-1 mix to obtain a final concentration of 50 nmol/L of siRNA. The siRNA transfection was repeated on day 6. At day 3, cells were treated with hormone with or without GSK650394 by removing 50 μL of the medium and replacing this with 50 μL of PRF-RPMI 1640 with 8% charcoal-stripped FBS, nonessential amino acids, and NaPyr containing a 2× concentration of the appropriate hormone/inhibitor treatment. At days 5 and 7, the treatment was repeated. On the 10th day, the medium was removed and the relative cell number was measured using the FluoReporter Blue assay (Invitrogen) according to the manufacturer's instructions.

Fluorescence polarization assay. SGK1 S422D cDNA (amino acids 60–431) was subcloned into pFastBAC-HTc (Invitrogen). Protein was expressed in Sf9 insect cells and purified by Ni^{2+}-NTA agarose chromatography. SGK1 was stored in a buffer containing 50 mmol/L Tris-HCl (pH 7.5), 270 mmol/L sucrose, 150 mmol/L NaCl, 0.1 mmol/L EGTA, 0.1% β-mercaptoethanol, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L benzamidine. SGK1 was diluted to 4 μmol/L and activated in 50 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L EGTA, 0.1% β-mercaptoethanol, 10 mmol/L magnesium acetate, and 0.1 mmol/L ATP with 3.3 μg/mL glutathione *S*-transferase-PDK1 at 30°C for 30 min. SGK1 was then repurified by Ni^{2+}-NTA agarose chromatography. To determine the efficiency of the activation reaction, activated SGK1 [diluted in 50 mmol/L Tris-HCl (pH 7.5), 0.1% (v/v) β-mercaptoethanol, 0.1 mmol/L EGTA, and 1 mg/mL bovine serum albumin (BSA)] was assayed for kinase activity using 30 μmol/L of a CROSStide substrate peptide (a synthetic peptide consisting of the amino acids GRPRTSSFAEG; Millipore Corp.) in 50 μL of 50 mmol/L Tris-HCl (pH 7.5), 0.1% (v/v) β-mercaptoethanol, 0.1 mmol/L EGTA, 10 mmol/L magnesium acetate, 100 μmol/L [γ-^{32}P]ATP (50–1,000 cpm/pmol) at 30°C for 10 min. Reactions were stopped by spotting 40 μL of the assay mixture onto Whatman P81 paper, which was washed in 75 mmol/L phosphoric acid, followed by acetone before air-drying and counting. Microscint-20 liquid scintillation fluid (Packard Instruments) was added and the signal was detected by measuring for 30 s per well in a Packard TopCount NXT Scintillation Counter.

A Rhodamine Green–labeled fluorescence polarization ligand (a proprietary ATP mimetic; 0.5 nmol/L final concentration) and SGK1 (1 nmol/L final concentration) were incubated in buffer containing 50 nmol/L HEPES (pH 7.5), 1 mmol/L CHAPS, 10 nmol/L $MgCl_2$, and 1 mmol/L DTT for 15 min at room temperature. GSK650394 was dissolved in DMSO and diluted in buffer for concentration-response curve determination. The fluorescence signal was measured using an Acquest (Molecular Devices) at excitation and emission wavelengths of 485 and 530 nm, respectively. SGK1 inhibition IC_{50} was calculated from these data using GraphPad Prism 3 software (GraphPad Software).

Scintillation proximity assay. SGK1 S422D (60–431 amino acids; 0.275 μg/mL final concentration) or SGK2 (0.875 μg/mL final concentration) was activated by PDK1 (1.1 μg/mL final concentration) in a buffer consisting of 50 mmol/L Tris (pH 7.5), 0.1 mmol/L EGTA, 0.1 mmol/L EDTA, 10 mmol/L $MgCl_2$, 0.1% β-mercaptoethanol, 1 mg/mL BSA, and ATP (final concentration of 0.15 mmol/L) and incubated for 30 min at 30°C. SGK2 was prepared exactly as described for SGK1, except it corresponded to the full-length protein. A solution containing biotinylated CROSStide peptide at a final concentration of 75 μmol/L and [γ-^{32}P]ATP corresponding to 2×10^6 cpm was prepared in the reaction buffer. In a 96-well plate, 5 μL of GSK650394 were added to 25 μL of the activated enzyme mixture. To this, 20 μL of the CROSStide mixture were added and incubated for 1 h at room temperature. Next, 50 μL of a 25 mg/mL slurry of streptavidin-coated scintillation proximity assay beads (Amersham) in PBS with 0.1 mol/L EDTA (pH 8.0) were added. The plate was then sealed and centrifuged for 8 min at 2,000 rpm, and the signal was detected by measuring for 30 s per well in a Packard TopCount NXT Scintillation Counter. The IC_{50} values of the inhibition of SGK1 and SGK2 activities by GSK650394 were calculated from these data using GraphPad Prism 3 software.

Toxicity assays. The toxicity of GSK650394 to M-1 and HeLa cells was assessed using the Cell Proliferation Kit [2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt (XTT)] following the manufacturer's instructions (Roche). Briefly, 10,000 HeLa or M-1 cells per well were plated into 96-well plates in 100 μL of the appropriate maintenance medium. After 48 h, medium was removed and replaced with 100 μL of EMEM with Earle's salts containing 2 mmol/L L-glutamine and 1% antibiotic-antimycotic overnight. M-1 cells were also supplemented with 1 μg/mL insulin, 6.25 μg/mL sodium selenite, and 6.25 μg/mL transferrin. After 24 h, the medium was removed and replaced with 100 μL of medium

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alone or medium containing increasing concentrations of GSK650394. For HeLa cells, 50 μL of activated XTT solution were added after 4 h. For M-1 cells, 50 μL of activated XTT solution were added after 24 h. Following a 2-h incubation, absorbance was measured at 490 nm using a SpectraMAX PLUS spectrophotometer (Molecular Devices) and the data were analyzed to obtain IC_{50} values using GraphPad Prism 3 software.

Short circuit current assay. Short circuit current (SCC) was determined in M-1 cells as previously reported (30). Cells were grown to confluence, trypsinized, and plated onto 12-mm transwell membranes (0.4-μm pore diameter; Corning) at a density of 55,000 per well in EMEM supplemented with 5% FBS, 15 mmol/L HEPES, 2 mmol/L L-glutamine, 1 μg/mL insulin, 6.25 μg/mL sodium selenite, 6.25 μg/mL transferrin, and 1% antibiotic/antimycotic. After the cells reached confluency on the transwell membranes, the medium was replaced with medium lacking FBS. Baseline measurements were obtained to ensure monolayer confluency and viability by showing adequate resistance. The voltage and resistance of the cell monolayer were measured using a EVOMX Epithelial Voltohmmeter with an STX2 electrode (WPI). Voltage was zeroed by measuring V_{te} across a transwell with no cells, then V_{te} was determined from the basolateral chamber. The initial resistance, R_{te}, was determined by applying current across the cell monolayer for a period of <1 s. The equilibrium current, I_{eq}, was calculated by dividing the voltage by the resistance for initial measurement and normalized by surface area of the membrane. Aldosterone (1 μmol/L) was added to the appropriate compartment and I_{eq} was obtained from six wells. After 4 h, GSK650394 plus aldosterone was added and I_{eq} was obtained. At the end of the experiment, amiloride, which inhibits epithelial sodium transport through the inhibition of the epithelial sodium channel, was added to a final concentration of 10 μmol/L and the amiloride-sensitive SCC was measured and subtracted from the total SCC to obtain SGK1-induced SCC. The IC_{50} values were calculated from these data using GraphPad Prism 3 software.

Results

Androgen-activated AR induces the expression of *SGK1*. Microarray analyses done in our laboratory revealed that the *SGK1* gene is regulated by androgens in the LNCaP prostate cancer cell line (5). To validate the microarray results and to determine if *SGK1* is regulated by androgens in other AR-expressing prostate cancer cell lines, the expression levels of *SGK1* were measured in the prostate cancer cell lines LNCaP, VCaP, and LAPC4 in the presence and absence of the synthetic androgen R1881 using quantitative PCR. Similar to the induction observed in our microarray study, SGK1 mRNA levels were up-regulated ∼20-fold in LNCaP cells (Fig. 1*A*). This up-regulation in response to androgens was also apparent in other AR-expressing prostate cancer cell lines, with VCaP cells exhibiting a 10-fold increase and LAPC4 cells a 2.5-fold increase in SGK1 mRNA levels. Although LAPC4 cells showed a less dramatic androgen-mediated induction of *SGK1* expression levels, we did note that the basal level of *SGK1* expression is relatively high in these cells. Given the robust androgen-dependent induction of *SGK1* expression in LNCaP cells, all subsequent studies were done in this cell line.

SGK1 mRNA levels are up-regulated by androgens in a dose-dependent manner, with levels increasing at 100 pmol/L R1881 and reaching maximal levels at 10 to 100 nmol/L, which is the approximate concentration at which ligand saturates the receptor (Fig. 1*B*). The enhancement of SGK1 mRNA levels in response to androgens is evident as early as 3 hours posttreatment, suggesting that the *sgk1* gene is a direct transcriptional target of androgen-bound AR (Fig. 1*C*). This was confirmed by showing that *SGK1* mRNA was not induced in the presence of actinomycin D but was unaffected by cycloheximide (Supplementary Fig. S1). Importantly, the antiandrogen Casodex (1 μmol/L) inhibited R1881 (1 nmol/L)–dependent increases in SGK1 transcript levels (Fig. 2*A*). Furthermore, three AR-targeted siRNAs, each of which effectively suppressed expression of both AR mRNA and protein, suppressed the androgen-mediated up-regulation of SGK1 mRNA expression (Fig. 2*B–D*). Taken together, these studies confirm that *SGK1* is a primary target of AR in prostate cancer cells.

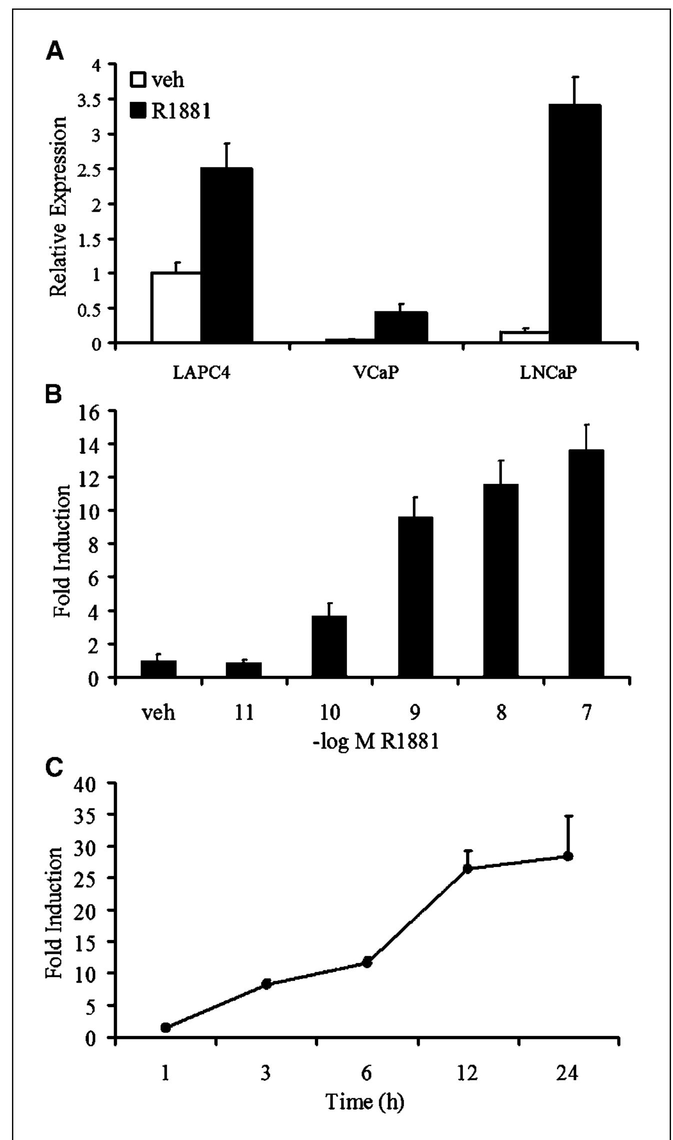

Figure 1. Androgens up-regulate SGK1 transcript levels. LNCaP, VCaP, and LAPC4 cells were grown in medium with charcoal-stripped FBS for 3 d before the addition of ethanol (*veh*) or R1881 [10 nmol/L (*A* and *C*) or at the indicated doses (*B*)]. After a 24-h incubation (*A* and *B*) or the indicated time points (*C*), cells were lysed and RNA was isolated and reverse transcribed. The expression of *SGK1* was assessed with quantitative PCR and normalized to *GAPDH* expression levels. Each experiment was done at least thrice, with a representative experiment shown; *bars,* SD.

Androgen treatment increases SGK1 protein levels and activity. The up-regulation of SGK1 mRNA levels in the presence of androgens was accompanied by a commensurate increase in steady-state SGK1 protein levels (Fig. 3*A*). In the absence of androgens, SGK1 protein levels were undetectable by immunoblotting. In response to R1881 (10 nmol/L), protein levels began to increase within 3 hours of treatment and were sustained up to

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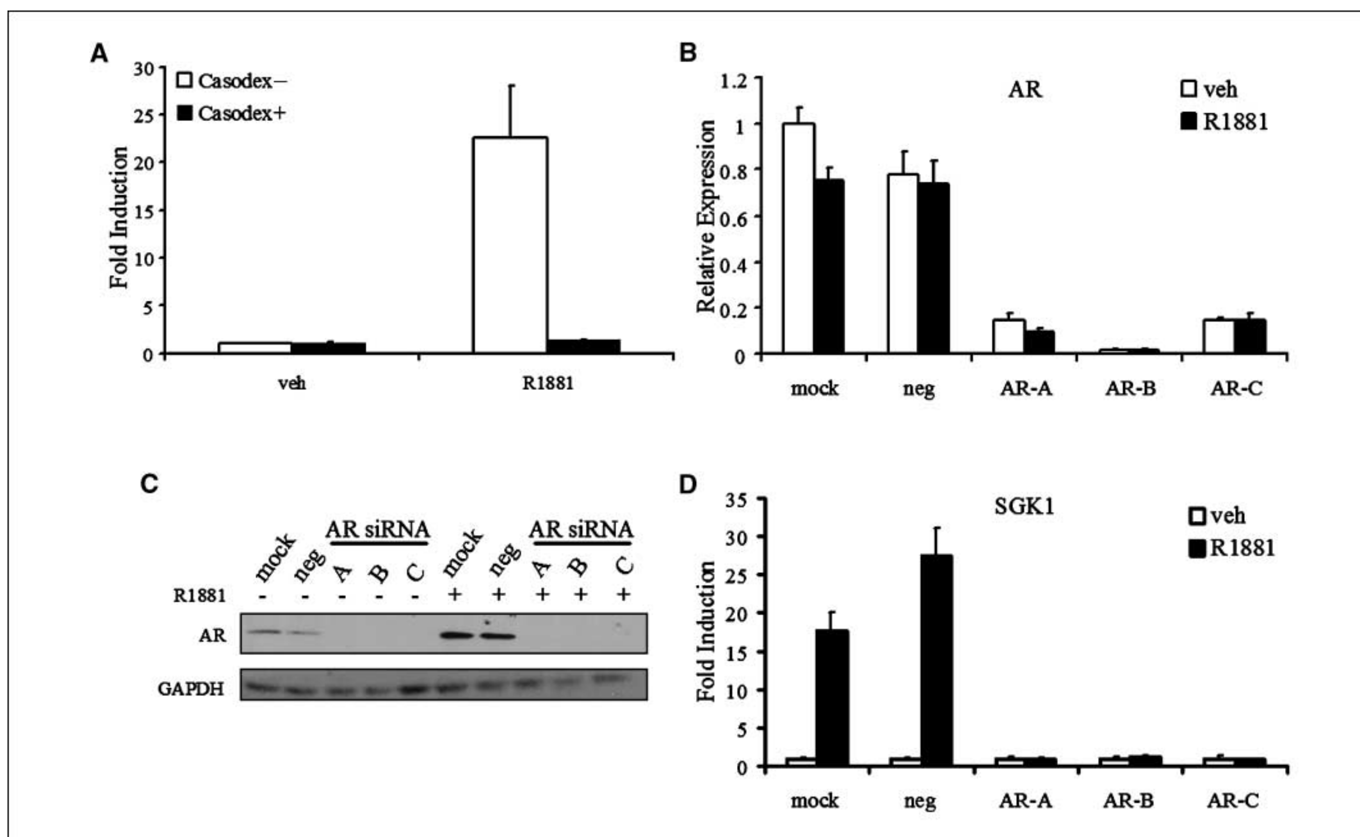

Figure 2. The androgen-mediated up-regulation of *SGK1* is AR dependent. *A,* LNCaP cells were grown in medium with charcoal-stripped FBS for 3 d before the addition of ethanol or R1881 (1 nmol/L) in the presence or absence of Casodex (1 μmol/L). After a 24-h incubation, cells were lysed and RNA was isolated and reverse transcribed. The expression of *SGK1* was assessed with quantitative PCR and normalized to *GAPDH* expression levels. *B* to *D,* LNCaP cells were transiently transfected with Stealth siRNAs targeting AR (AR-A, AR-B, or AR-C) or a negative control (*neg*) Stealth siRNA at a final concentration of 50 nmol/L. Cells were "mock" transfected as an additional negative control. Forty-eight hours later, cells were treated with ethanol or R1881 (10 nmol/L) for 24 h. AR (*B*) and SGK1 (*D*) mRNA levels were detected with quantitative PCR. SGK1 and AR mRNA levels were normalized with GAPDH mRNA levels. Each experiment was done at least thrice, with a representative experiment shown; *bars,* SD. *C,* whole-cell extracts were prepared and proteins were separated on a SDS-PAGE gel and transferred onto a nitrocellulose membrane, which was probed with antibodies against AR and GAPDH (loading control).

48 hours posttreatment (Fig. 3*B*). This is notable considering that others have shown that SGK1 expression, in response to various stimuli, is typically rapid and transient (12). LNCaP cells are *PTEN* null and therefore exhibit constitutive activation of PI3K (31). Not surprisingly therefore, the SGK1 protein is phosphorylated and migrates as multiple bands on immunoblots.

One of the most well-characterized roles for SGK1 is in hypertension where SGK1 phosphorylates and inhibits the ubiquitin ligase neural precursor cell–expressed, developmentally down-regulated protein 4-2 (Nedd4-2) at Ser[468] (S468), which ultimately affects epithelial sodium ion transport (32). Hence, to verify that the SGK1 protein is active in LNCaP cells under our particular growth conditions, we analyzed the phosphorylation state of the SGK1 target protein Nedd4-2. Here, we show an increase in Nedd4-2 S468 phosphorylation in response to androgen treatment, confirming that androgens facilitate an enhancement of SGK1 signaling processes (Fig. 3*A*). Interestingly, the strong enhancement of SGK1 levels in response to androgens did not produce an immediate dramatic elevation of Nedd4-2 phosphorylation. This may suggest that not only does SGK1 need to be expressed and posttranslationally activated but other limiting activities (i.e., translocation to its target) must also occur. Additionally, the constitutive PI3K activity in LNCaP cells due to the *PTEN*-null status may increase the basal phosphorylation of Nedd4-2, dampening the effects of androgens on this end point.

***SGK1* expression is required for androgen-mediated proliferation of prostate cancer cells.** Given the role of SGK1 as a growth factor–responsive kinase and the fact that it is a downstream target of PI3K, we hypothesized that its induction by androgens may be necessary for androgen-dependent growth of LNCaP cells. To test this hypothesis, three siRNAs, each of which effectively reduced SGK1 mRNA and protein expression, were analyzed for their effects on LNCaP cell growth (Fig. 4*A* and *B*). As a positive control, we showed that each of the three different AR siRNAs completely abrogated the androgenic stimulation of growth in LNCaP cells, whereas negative control siRNA had no effect under the same conditions (Fig. 4*C*). More importantly, when any of the three SGK1 siRNAs was transfected into LNCaP cells, we observed a 50% to 60% inhibition of the 2.5-fold stimulation of growth in the presence of androgens, with no effect on the cells in the absence of androgens (Fig. 4*C*). These studies, using RNA interference–mediated knockdown of *SGK1*, support the hypothesis that this protein is required for androgen-dependent growth of LNCaP cells.

Development of a novel SGK1 inhibitor, GSK650394. Given that *SGK1* expression is required for androgen-dependent growth of prostate cancer cells, we hypothesized that SGK1 would be a

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viable target for the development of pharmacologic agents for the treatment of prostate cancer. To test this, we developed a novel compound, GSK650394, which functionally inhibits SGK1, and examined the effects of this compound on cellular models of prostate cancer. The structure of GSK650394 is shown in Fig. 5*A* and its initial characterization is described below and summarized in Supplementary Table S2.

A fluorescence polarization assay was used to determine the affinity of GSK650394 for activated SGK1. The ability of GSK650394 to displace a Rhodamine Green fluorescently labeled small-molecule ATP mimetic bound to purified SGK1 protein corresponding to amino acids 60 to 431 (which harbors a S422D mutation to mimic phosphorylation of the PDK2 site and was fully activated by recombinant PDK1) was measured. In this assay, GSK650394 inhibited activated SGK1 with an IC_{50} of 13 nmol/L (data not shown).

The ability of GSK650394 to inhibit the enzymatic activity of SGK1 and SGK2 was measured using an *in vitro* activity-based scintillation proximity assay. This assay measures SGK1- or SGK2-mediated phosphorylation of a serine residue within a synthetic biotinylated peptide substrate. SGK1 or SGK2 phosphorylates the peptide substrate, thereby incorporating a radiolabeled phosphate, which is subsequently incubated with streptavidin-coated polystyrene beads containing a scintillant. The localization of the radiolabeled peptide within the immediate vicinity of the scintillant-containing bead generates a measurable light signal. GSK650394 inhibited the enzymatic activity of SGK1 and SGK2 in the scintillation proximity assay with IC_{50} values of 62 and 103 nmol/L, respectively (Fig. 5*B*).

The inhibitory effects of GSK650394 on SGK1 activity were further evaluated using cell-based assays. Importantly, GSK650394 is relatively nontoxic, with LC_{50} values of 41 μmol/L in M-1 cells (68 times its activity IC_{50}) and LC_{50} >100 μmol/L in HeLa cells in XTT assays, which measure mitochondrial enzymatic activity (data not shown). SGK1 has been linked to hypertension and mice lacking the *sgk1* gene have higher sodium excretion and lower blood pressure than wild-type mice when fed a low-sodium diet (33, 34). This has been attributed to the regulation of epithelial sodium ion transport by SGK1 in response to aldosterone stimulation. GSK650394 was evaluated for its effects on this well-documented SGK1-mediated biological activity, which was measured using an aldosterone-stimulated SCC cellular assay.

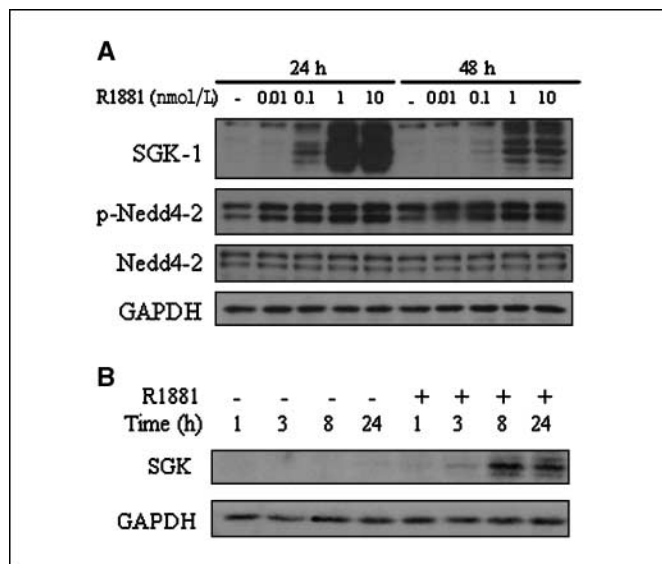

Figure 3. Androgen treatment leads to an increase in SGK1 protein levels and activity in LNCaP cells. *A,* LNCaP cells were grown in medium with charcoal-stripped FBS for 3 d before the addition of ethanol or R1881 at the indicated concentrations (*A*) or 10 nmol/L (*B*). After incubation for 24 or 48 h (*A*) or for the duration indicated (*B*), cells were lysed and proteins were separated on a SDS-PAGE gel and transferred onto a nitrocellulose membrane, which was probed with antibodies against AR, SGK1, Nedd4-2, phospho-Nedd4-2, and GAPDH (loading control). Each experiment was done at least thrice, with a representative experiment shown.

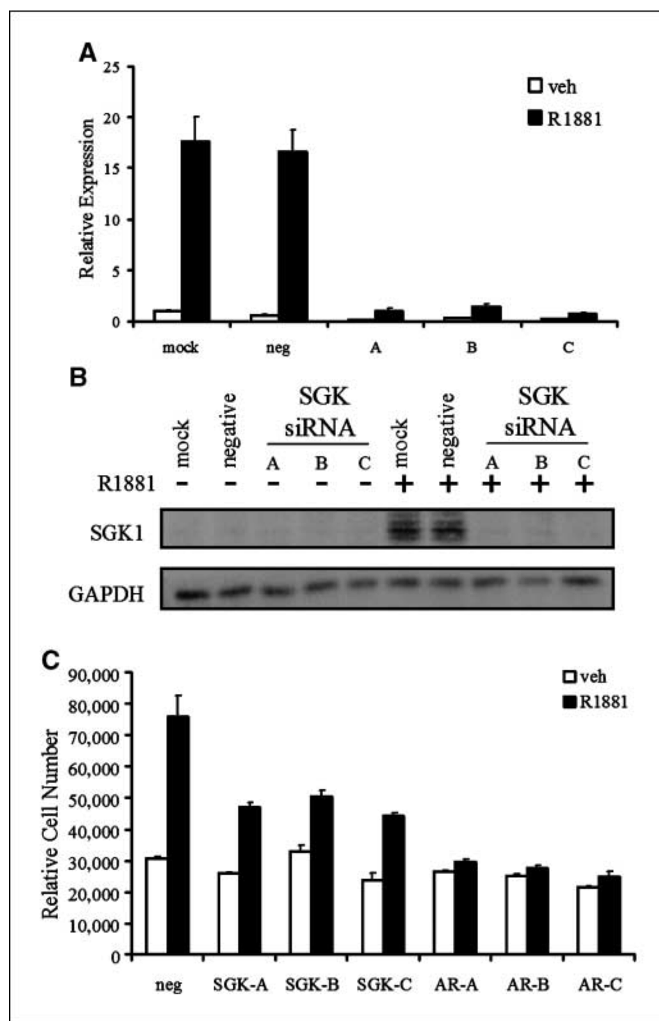

Figure 4. *SGK1* expression is required for androgen-mediated proliferation of LNCaP cells. LNCaP cells were transfected with the Stealth SGK1 A, B, or C siRNAs or a negative control siRNA at a final concentration of 50 nmol/L. Cells were mock transfected as an additional negative control. Two days later, cells were treated with ethanol or R1881 (10 nmol/L). *A,* after a 24-h incubation, cells were lysed and RNA was isolated. RNA was reverse transcribed and transcript levels of SGK1 were measured with quantitative PCR and were normalized to GAPDH mRNA levels; *bars,* SD. *B,* whole-cell extracts were collected and proteins were separated on a SDS-PAGE gel, followed by transfer onto a nitrocellulose membrane. The membrane was probed with antibodies against SGK1 or GAPDH (loading control). *C,* LNCaP cells were incubated in medium with charcoal-stripped FBS for 2 d. Cells were transiently transfected with Stealth SGK1 (*SGK-A*, *SGK-B*, or *SGK-C*), AR (*AR-A*, *AR-B*, or *AR-C*), or negative control siRNAs at a final concentration of 50 nmol/L. An additional transfection of these siRNAs was done 4 d later. Cells were treated with ethanol or R1881 (10 nmol/L) on days 3, 5, and 7. On day 10, cells were lysed and the relative number of cells was measured with the fluorescent DNA-binding dye FluoReporter Blue. Each sample was done in triplicate and the experiment was done at least thrice, with a representative experiment shown; *bars,* SE.

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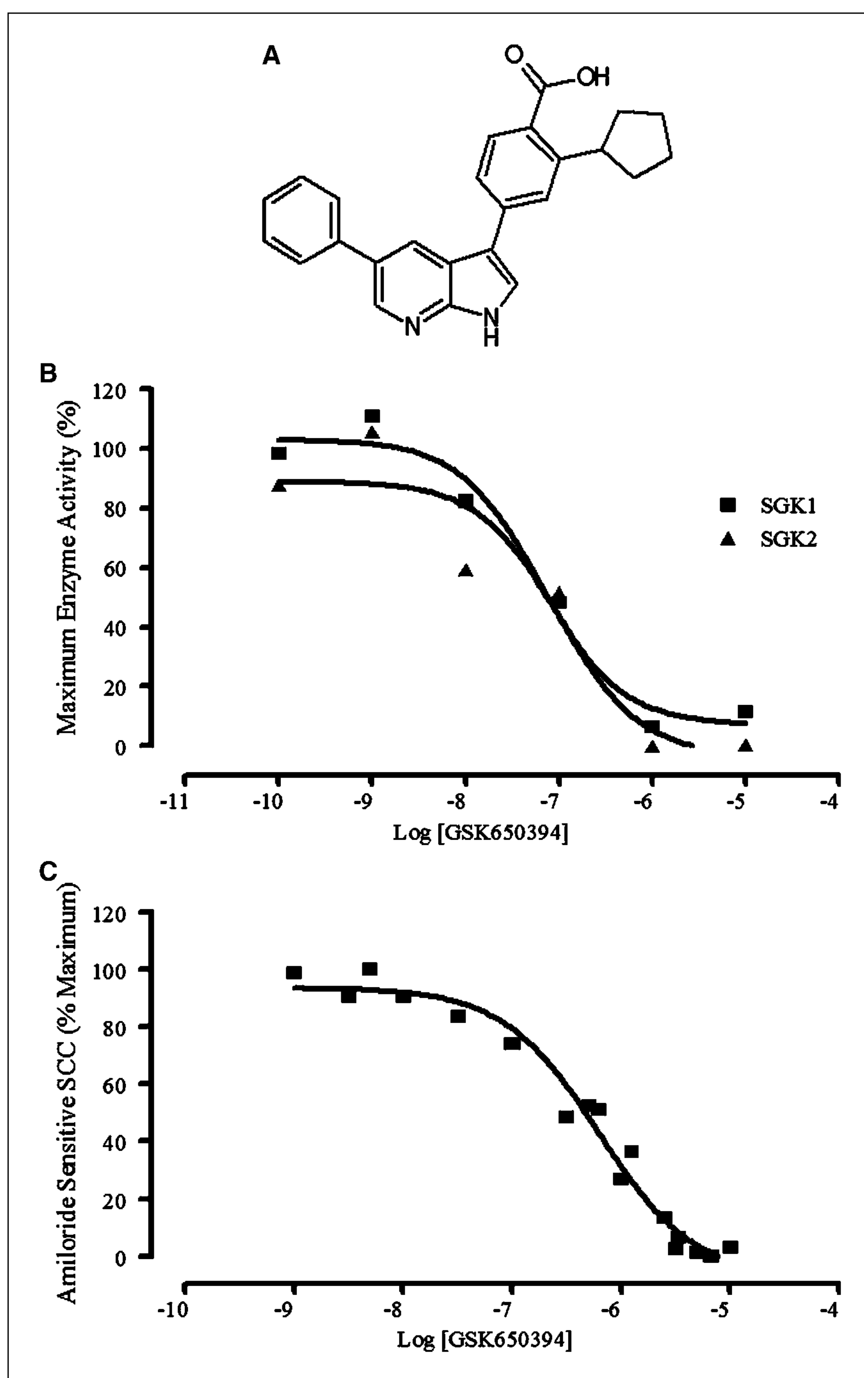

Figure 5. GSK650394 inhibits the activity of SGK1. *A,* chemical structure of GSK650394. *B,* GSK650394 quantitatively inhibits the activity of SGK1 and SGK2 in a scintillation proximity assay. Activated SGK1 or SGK2 proteins were incubated with increasing concentrations of GSK650394 and a synthetic biotinylated peptide substrate (CROSStide). Activity was assessed by detection of the radiolabeled phosphate that was incorporated into the biotinylated CROSStide, when bound to streptavidin-coated polystyrene beads containing a scintillant. The experiment was done twice, and results were normalized to percent maximum enzyme activity, with a representative experiment shown. *C,* GSK650394 fully inhibits amiloride-sensitive SCC in M-1 cells. M-1 cells were plated in EMEM with 5% FBS onto 12-mm transwell membranes with 0.4-μm pore diameter and 1.13-cm^2 active surface area and maintained for 5 to 7 d until confluent. On the day of the experiment, medium was replaced with EMEM with no supplements and basal voltage and resistance measurements were obtained and the baseline was set. Aldosterone (1 μmol/L) was added to the appropriate compartment and I_{eq} was obtained from six wells. After 4 h, GSK650394 plus aldosterone was added and I_{eq} was obtained. At the end of the experiment, amiloride was added to a final concentration of 10 μmol/L and the amiloride-sensitive SCC was measured and subtracted from the total SCC to obtain SGK1-induced SCC. The experiment was done twice, and results were normalized to percent maximum amiloride-sensitive SCC, with a representative experiment shown.

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GSK650394 inhibited SGK1-mediated epithelial transport with an IC_{50} of 0.6 μmol/L in the SCC assay (Fig. 5*C*).

The specificity of GSK650394 for SGK1 over that of the most closely related AGC kinase family member, Akt, and other related kinases was measured using standard *in vitro* kinase assays (University of Dundee, Scotland, United Kingdom). The selectivity of GSK650394 for SGK1 over that of Akt and other related kinases proved to be >30-fold, whereas GSK650394 was >60-fold selective for SGK1 over the upstream AGC kinase PDK1 (Supplementary Table S2).

GSK650394 inhibits SGK1 activity and androgen-mediated LNCaP cell growth. When tested in LNCaP cells, GSK650394 repressed the androgen-mediated enhancement of Nedd4-2 phosphorylation, suggesting that GSK650394 antagonizes SGK1 activity in these cells (Fig. 6*A*). Next, a 10-day growth assay, similar to the one described for the siRNA studies (Fig. 4*C*), was used to measure the effects of GSK650394 on LNCaP cell growth. Of note, GSK650394 had no effect on LNCaP cell growth or survival in the absence of androgens and is therefore not toxic at the levels used in these experiments (Fig. 6*B*). Although GSK650394 had no effect on cell survival or growth in the absence of androgens, the compound dramatically inhibited the androgen-stimulated growth of LNCaP cells. In the LNCaP growth assay, the IC_{50} of GSK650394 was ~1 μmol/L (which is similar to the previously measured IC_{50}

in other cell-based assays). Furthermore, 10 μmol/L GSK650394 completely abrogated androgen-mediated growth, indicating that small-molecule inhibitors of SGK1 may be an effective approach for the treatment of AR-driven prostate cancer.

Discussion

These studies show that androgens up-regulate SGK1 mRNA levels in an AR-dependent fashion. This observation parallels findings presented within a recent report by Shanmugam et al. (6). However, in contrast to the studies by Shanmugam et al. in which cells are grown under serum-free conditions, the experiments presented herein were done in the presence of 8% charcoal-stripped serum, which is more reflective of physiologic conditions. Therefore, our studies suggest that even in the presence of serum, which in and of itself is capable of inducing *SGK1* expression, androgens are able to enhance SGK1 mRNA levels. Our studies further show that the androgen-dependent induction of SGK1 mRNA levels correlates with an increase in functionally active SGK1 protein. This observation, in combination with the demonstration that siRNA-mediated down-regulation of *SGK1* expression inhibits androgen-dependent growth of prostate cancer cells, suggests that the enzymatic activity of SGK1 might be responsible for androgen-stimulated prostate cancer growth and may represent a viable target for the treatment of prostate cancer.

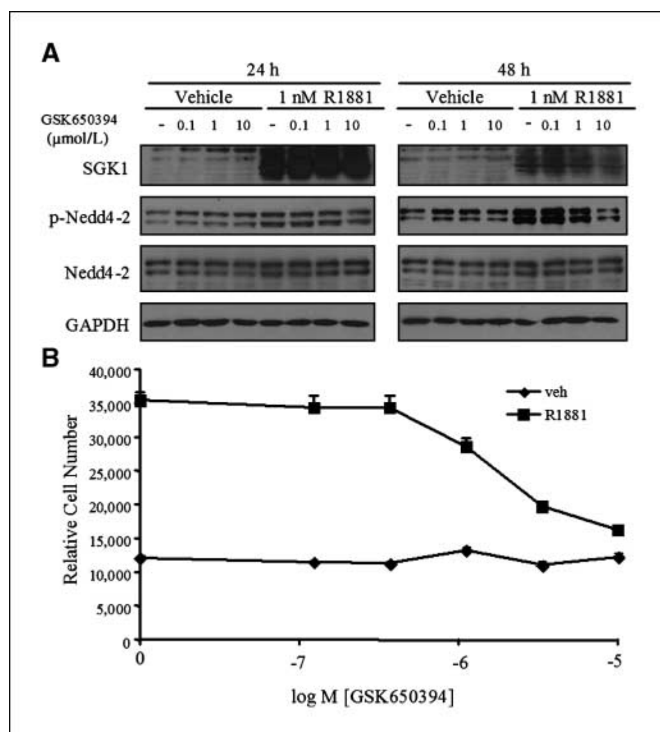

Figure 6. GSK650394 inhibits SGK1 activity and androgen-mediated LNCaP cell growth. *A,* LNCaP cells were grown in medium with charcoal-stripped FBS for 3 d before the addition of ethanol, R1881, DMSO (−), or GSK650394 at the indicated concentrations. Whole-cell extracts were collected and proteins were separated on a SDS-PAGE gel, followed by transfer onto a nitrocellulose membrane. The membrane was probed with antibodies that recognize SGK1, Nedd4-2, phospho-Nedd4-2 (Ser^{328}), or GAPDH (loading control). *B,* LNCaP cells were plated in 96-well plates and grown in medium with charcoal-stripped FBS for 3 d. Cells were treated with ethanol or R1881 (10 nmol/L) and the indicated doses of GSK650394 or DMSO on days 3, 5, and 7. On day 10, cells were lysed and the relative number of cells was measured with the fluorescent DNA binding dye FluoReporter Blue. Each sample was done in triplicate and the experiment was done at least thrice, with a representative experiment shown; *bars,* SE.

A possible mechanism by which SGK1 might control cell cycle progression and proliferation in prostate cancer cells is through the direct or indirect regulation of the activity of the mammalian target of rapamycin (mTOR) protein. Notably, it has been shown by others that androgens stimulate mTOR activity in LNCaP cells (7). Active mTOR contributes to an enhancement of the translation rates of various proteins involved in cell cycle progression, including cyclins D1 and D2 (7). In addition, Xu and colleagues indicated that SGK1 may activate mTOR through the activation of various cellular transporters and the subsequent influx of certain nutrients and amino acids (7, 35). Interestingly, the expression of many of the same cellular transporters is enhanced by androgens (7). Additionally, SGK1 may regulate mTOR activity in a more direct manner through the phosphorylation and inactivation of TSC2, a negative regulator of mTOR, because the expression of constitutively active SGK1 in cardiomyocytes is associated with an enhancement of TSC2 phosphorylation and mTOR activity (24).

It has been suggested that SGK1 may regulate AR transcriptional activity, affecting androgen-mediated prostate cancer growth through a positive feedback mechanism (6). We have not been able to confirm this result and were unable to observe any effects of GSK650394 on the expression level of a panel of androgen-regulated genes in LNCaP cells under conditions where proliferation was completely repressed (data not shown). At this time, however, we cannot exclude the possibility that SGK1 activity is required for the regulation of a subset of androgen-regulated genes.

We have shown that SGK1 expression (siRNA-mediated down-regulation) and activity (GSK650394 studies) are required for the effects of androgens on prostate cancer growth. The growth inhibition observed following treatment with GSK650394 was more dramatic than that observed with the SGK1 siRNAs. This might be due to the residual expression of SGK1 observed in our knockdown experiments. Given its function as an enzyme, even a relatively low level of expression of the protein could have dramatic effects on downstream targets. Alternatively, the effectiveness of GSK650394 as a growth inhibitor might also be due to the nonspecific inhibition of other kinases, particularly other members of the SGK protein family, SGK2 and SGK3. Interestingly, although previous reports have suggested that SGK3 expression is constitutive and is only regulated at the protein level, we have shown that SGK3 expression levels are also induced in response to androgen treatment in all the AR-positive prostate cancer cell lines that were tested (ref. 36; data not shown). Notably, the up-regulation of SGK3 mRNA levels (∼3-fold induction) is much less dramatic than the stimulation of SGK1 expression (∼20-fold induction). SGK2 expression was not affected by androgens in any of the cell lines tested. Whereas SGK1 and SGK3 have been shown to phosphorylate many of the same target proteins *in vitro*, differences in subcellular localization suggest that these proteins regulate distinct signaling pathways (36). SGK3 is localized to endosomes where it has been suggested to play a role in endocytosis (37), unlike the defined role in growth and survival ascribed to SGK1. Therefore, although GSK650394 is able to inhibit SGK2 and possibly also inhibits SGK3, the expression patterns and subcellular localizations of these proteins suggest that inhibition of these kinases by the SGK1 antagonist will probably be of little consequence to the growth of prostate cancer cells.

GSK650394 is relatively selective for SGK1 over Akt, the most closely related AGC kinase family member, as well as a host of

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other related kinases, indicating that it should be a useful tool to dissect the relative roles of Akt and SGK1 in PI3K-regulated mechanisms. SGK1 and Akt share many of the same phosphorylation targets and Akt has been implicated in the control of cell growth and survival downstream of PI3K activation. It is unclear whether Akt activity is required for prostate cancer growth and/or survival. Transgenic mice expressing a prostate-restricted, constitutively active Akt develop prostate intraepithelial neoplasia but never develop prostate cancer (38). Although activation of Akt by androgens via a nongenomic mechanism has been described, this phenomenon has never been reported to occur in LNCaP cells, which exhibit constitutive PI3K and Akt activation due to their PTEN-null status (31, 39, 40). Xu and colleagues (7) were unable to observe changes in the activation state of Akt following androgen treatment of LNCaP cells. Furthermore, in preliminary studies done in our laboratory, we have not observed any changes in the phosphorylation state of Ser^{473} of Akt in response to androgen treatment (data not shown). This suggests that, at least in these cells and possibly in all prostate cancer cells exhibiting constitutive PI3K activity, androgen-stimulated proliferation may not depend on Akt activity but rather on SGK1. Therefore, the SGK1 inhibitor GSK650394 might be especially useful for the treatment of those prostate cancer tumors that have developed mutations in *PTEN* or *PI3K*, leading to constitutive activation of downstream signaling pathways.

The identification and characterization of the novel SGK1 antagonist GSK650394 indicate that it can serve as a model compound to aid in the identification of other small-molecule inhibitors with similar activity and ideal pharmacokinetic characteristics to be used in clinical trials. To date, GSK650394 has been formulated for *in vivo* use and can reach exposure levels in rats above the SCC IC_{50} using a 50 mg/kg twice-daily dosing schedule. Importantly, preliminary results monitoring blood pressure and urinary metabolites as pharmacodynamic markers suggest that GSK650394 is efficacious in rat models of hypertension. However, at present, we have not tested GSK650394 in any animal models of prostate cancer. At a minimum, GSK650394 is a useful pharmacologic tool to further identify pathways regulated by SGK1 that may permit the development of more effective prostate cancer treatments. The clinical success of lapatinib for advanced/metastatic breast cancer and imatinib for chronic myelogenous leukemia and gastrointestinal tumors suggests, by analogy, that kinase activities can be selectively targeted and modulated for the treatment of prostate cancer or hormone-refractory prostate cancer.

Disclosure of Potential Conflicts of Interest

D.P. McDonnell: Commercial research grant, GlaxoSmithKline. The other authors disclosed no potential conflicts of interest.

Acknowledgments

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