A Naturally Derived Small Molecule Disrupts Ligand-Dependent and Ligand-Independent Androgen Receptor Signaling in Human Prostate Cancer Cells

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Abstract

Continued reliance on androgen receptor (AR) signaling is a hallmark of prostate cancer, including the development of castration-resistant prostate cancer (CRPC), making it an attractive therapeutic target for prostate cancer treatment. Mahanine is a novel carbazole alkaloid derived from the leaves of Murraya koenigii, commonly known as the curry leaf plant, which grows widely across East-Asia. We show here that mahanine possesses the ability to inhibit ligand-dependent and -independent AR transactivation, leading to a prominent decline in AR target gene expression. Mahanine treatment causes a time- and dose-dependent decline in AR protein levels, including truncated AR splice variants, in a panel of androgen-responsive and -independent prostate cancer cells. The decrease in AR levels induced by mahanine occurs posttranslationally by proteasomal degradation, without any change in the AR gene expression. Mahanine treatment induces an outward movement of the AR from the nucleus to the cytoplasm, leading to an initial increase in cytoplasmic AR levels, followed by a gradual decline in the AR levels in both cellular compartments. Ligand-induced AR phosphorylation at Ser-81, a phospho-site associated with prostate cancer cell growth and AR transactivity, is greatly diminished in the presence of mahanine. The decline in AR phosphorylation at Ser-81 by mahanine occurs via the inactivation of mitotic kinase CDK1. Collectively, our data demonstrate that mahanine strongly disrupts AR signaling and inhibits the growth of androgen-dependent and -independent prostate cancer cells, thereby implicating a therapeutic role of mahanine in prostate cancer treatment. Mol Cancer Ther; 13(2); 1–12. ©2013 AACR.

Introduction

Prostate cancer is estimated to represent 28% of the new cancer cases diagnosed in men in the year 2013, making it the most widely diagnosed cancer in men (1). Radio- or hormonal therapy is the first line of treatment used for early stages of prostate cancer when the tumor is still confined within the prostatic capsule. Although this approach is successful in causing initial tumor regression, the cancer is often found to reoccur, adopting an androgen-independent phenotype known as castration-resistant prostate cancer (CRPC).

The androgen receptor (AR) signaling axis remains intact in CRPC despite the low levels of circulating androgen in these patients. The illicit activity of the AR can be attributed to several different molecular mechanisms, including: intracrine steroidogenesis within the tumor microenvironment, AR gene amplification leading to increased sensitivity to low levels of circulating androgens, AR mutations resulting in a gain-of-function or an ability to promiscuously respond to other steroids and growth factors, and androgen-independent AR activation by various cellular kinases such as mitogen-activated protein kinase (MAPK), Akt, and Src (2–5). Although there may not be a single definitive mechanism to explain the transition of a hormone-sensitive state to a hormone-refractory one, it is evident that uninterrupted AR signaling plays a central role in the development of CRPC.

The AR is a steroid receptor that exists in the cytosol in an inactive conformation bound to chaperone proteins such as Hsp90 (6). Ligand binding causes the AR to adopt an altered conformation, allowing it to disengage from its interaction with chaperones, and undergo various post-translational modifications which facilitate its export to the nucleus, where it executes its transcriptional effects in
conjunction with coactivator proteins. In addition to androgen, various growth factors such as EGF and interleukin-6 (IL-6) are known to modulate the activity and stability of the AR via diverse signaling cascades (7–9). Phosphorylation is the most common posttranslational modification associated with AR in response to stimulation by androgen and other growth factors (10). Majority of the known phospho-sites are concentrated in the N-terminal domain (NTD) of the AR, which also contains the activation of function-1 (AF-1) domain, although residues within the DNA-binding domain (DBD) and C-terminal ligand-binding domain (LBD) have also been reported to undergo phosphorylation (3, 11, 12). The effects of AR phosphorylation are varied, and can result in AR transactivation, nuclear translocation, and DNA binding and degradation, depending on the kinase cascade and site of phosphorylation involved (11, 13–15). Phosphorylation of the AR on its Ser-81 site occurs in a time-dependent manner in response to androgen, with maximal phosphorylation taking place after 6 to 8 hours, and has been reported to be the most abundantly phosphorylated site following ligand stimulation (16, 17). Phosphorylation of Ser-81 is associated with nuclear localization and chromatin binding of the AR, thereby influencing the transcriptional output in androgen-stimulated cells (14). Furthermore, Ser-81 phosphorylation governs the stability of the AR and prevents its proteasomal degradation (18). Independent studies have demonstrated the importance of AR Ser-81 phosphorylation in regulating prostate cancer cell growth in response to ligand and soluble stromal factors (19, 20). Taken together, these findings suggest that Ser-81 phosphorylation is an important determinant of AR stability and activity, and its inhibition alters AR transcriptional output and affects prostate cancer cell growth.

CDK1, in conjunction with its binding partner, cyclin B1, is essential to drive the G2–M transition of the cell cycle and was the first kinase identified to phosphorylate AR Ser-81. CDK1 overexpression has been reported in prostate cancer, and is associated with disease recurrence and resistance to therapy (21, 22). Recent evidence indicates that CDK1 also plays a role in promoting prostate cancer cell migration, suggesting its involvement in disease progression (23). Several naturally derived, synthetic, and molecular agents targeting CDK1 have been assessed for use in prostate cancer therapy and are found to significantly decrease growth and proliferation (24–27). The elevated activity of CDK1 in CRPC and its ability to phosphorylate multiple sites on the AR, such as Ser-81 and Ser-515 (28), implicate the possibility of cross-talk between CDK1 and AR signaling pathways, resulting in increased AR activity and cell proliferation.

In the present study, we investigated the effects of a novel anticancer compound, mahanine, on the AR signaling pathway in human prostate cancer cells. Mahanine is a carbazole alkaloid drug derived from the leaves of *Murraya koenigii*, also known as the curry leaf plant, which grows widely across East-Asia (29). Mahanine has been shown to possess cytotoxic, antioxidative, and antiproliferative properties (30). Studies using human leukemic cell lines have shown that treatment with mahanine results in the apoptotic death of these cells via mitochondrial pathways (31, 32). Our prior work has shown that mahanine treatment restores the expression of the epigenetically silenced tumor suppressor gene, RASSF1A, in a panel of cancer cell lines, including prostate cancer (33). A recent report demonstrates the ability of mahanine to induce cell death in the pancreatic cancer cells via the induction of reactive oxygen species production (34).

We show here that mahanine inhibits ligand-dependent and -independent transactivation of the AR, leading to a significant decrease in the expression of androgen-regulated genes. Furthermore, mahanine compromises the stability of the AR, resulting in its proteasomal degradation. Mahanine prevents ligand-induced phosphorylation of the AR on its Ser-81 residue via the inactivation of CDK1. Taken together, our results suggest a potential therapeutic role for mahanine in the treatment of prostate cancer based on its ability to disrupt AR signaling, induce AR degradation, and inactive CDK1 in prostate cancer cells.

Materials and Methods

Cell culture and reagents

Prostate cancer cell lines (LNCaP, VCaP, LNCaP C4-2B, and 22Rv1) were obtained from the American Type Culture Collection and were cultured in phenol red–free Improved Minimum Essential Medium (IMEM; Invitrogen) containing 10% FBS (Atlanta Biologicals), 2 mmol/L glutamine, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Sigma), unless otherwise specified. All cell lines used were tested and authenticated at the Tissue Culture Shared Resource in Lombardi Comprehensive Cancer Center by DNA fingerprinting short-tandem repeat (STR) analysis. The chemical structure of mahanine and its purification protocol have been described previously (Supplementary Fig. S1; refs. 30, 35). Dihydrotestosterone (DHT) (Steraloids), forskolin (Sigma), and IL-6 (Invitrogen) were used in the concentrations indicated below. Cyclohexamide and MG132 were obtained from Tocris Biosciences. CDK1-AF and cyclin B1 expression vectors were obtained from Dr. Steven Balk from Harvard University (Cambridge, MA; ref. 17).

Quantitative real-time PCR

Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Of note, 1 μg of RNA was converted to cDNA using the RT² First Strand Kit (Qiagen), following which quantitative real-time PCR (qRT-PCR) analysis was carried out on the ABI 7900HT (Applied Biosystems) using RT² SYBR Green qPCR Master mix from Qiagen. Primer sets were obtained from Integrated DNA Technologies and primer sequences are provided in Supplementary Tables S1 and S2. Prostate-specific antigen (PSA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and AR7 qPCR primer sequences have been previously reported (36, 37).
Luciferase assay

Cells were transiently transfected with an androgen response element luciferase reporter (ARR3-TK–Luc) construct (from Dr. Robert Matusik, Vanderbilt University, Nashville, TN) or a PSA promoter luciferase reporter construct (from Dr. Donald Tindall, Mayo Clinic, Rochester, MN; 200 ng) using GeneJET II transfection reagent (Signagen), following which they were treated with DHT (1 nmol/L) and the indicated concentrations of mahaine. The Dual Luciferase Assay Kit (Promega) was used to measure luciferase activity, which was read using a microplate luminometer (Harta Instruments). Preparations were normalized to Renilla luciferase activity by cotransfection of pRL-TK-luciferase plasmid (50 ng). AR-NTD-Gal4DBD and Gal4UAS-TATA-luciferase vectors were obtained from Dr. Marianne Sadar (University of British Columbia, Vancouver, Canada; ref. 38) and VP16-GaIDBD was obtained from Dr. Winship Herr (Université de Lausanne, Lausanne, Switzerland; ref. 39), and were transfected into the LNCaP cells as described above.

Western blot analysis

Cells were harvested to extract total cellular protein, which was resolved using 8% to 12% SDS–PAGE and electrotransferred to nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with a 5% solution of nonfat milk powder in TBS containing 0.1% Tween-20, and were incubated with respective primary antibodies overnight at 4°C. Following an hour-long incubation with horseradish peroxidase–conjugated secondary antibodies (1:5,000 dilution; Santa Cruz Biotechnology), membranes were visualized using a chemiluminescent reagent (Santa Cruz Biotechnology). Images of the membranes were captured using a Fuji LAS-1000 Imager. The membranes were stripped (CS) media for 4 days and transfected with an androgen response element–luciferase reporter construct (ARR3-TK–Luc). Expectedly, the addition of DHT (1 nmol/L) significantly induced luciferase activity; however, this induction was inhibited in the presence of mahaine in a dose-dependent manner, suggesting that mahaine inhibits DHT-induced AR transactivation (Fig. 1A). A similar effect was observed when a PSA promoter–luciferase reporter construct was used (Fig. 1B). In addition, we transfected LNCaP cells with human telomerase reverse transcriptase (hTERT)-promoter luciferase construct and c-myc-promoter luciferase construct and assessed whether mahaine treatment altered promoter activity of these genes. We did not observe any significant change in the luciferase activity upon mahaine treatment, indicating that mahaine does not ubiquitously

Immunoprecipitation

Cells were washed with ice-cold PBS following which total protein was extracted, quantified, and diluted to obtain a concentration of 1 μg/μL. Of note, 500 μL of cell lysate was subjected to preclearance using 50 μL Protein A/G Plus Agarose beads (Santa Cruz Biotechnology) for 15 minutes. The lysate was incubated at 4°C with an AR antibody (441; Santa Cruz Biotechnology) for a period of 16 hours with constant rotation. The antibody–protein complexes were pulled down using 50 μL Protein A/G Plus Agarose beads for 5 hours, following which the beads were separated by centrifugation at 14,000 x g for 1 minute at 4°C. The beads were washed three times with a cell lysis buffer after which they were boiled with the Laemmlı buffer to detach the adsorbed protein. The protein was resolved using SDS–PAGE as described above.

Immunofluorescence staining

LNCaP cells were grown on enhanced chemiluminescence (ECL) (Upstate Biotechnology Inc.)–coated chamber slides in phenol red–free IMEM. After the indicated treatments, cells were fixed in methanol, air-dried, and rehydrated with PBS. Of note, 0.2% bovine serum albumin (BSA) was used to block the cells, following which they were incubated overnight at 4°C with primary antibody. Cells were washed three times with PBS and incubated with 4 μg/mL Alexa Fluor 488 or 594–conjugated secondary antibody (Invitrogen) for 1 hour. Cells were washed three times with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) for 5 minutes. Cells were washed once again with PBS and mounted using 50% glycerol and were viewed using a fluorescent microscope (Olympus BX, Olympus Corp.). Images were imported into Adobe Photoshop.

Nuclear–cytoplasmic separation

LNCaP cells were treated with mahaine or dimethyl sulfoxide (DMSO) as described below, and the cytoplasmic and nuclear fractions were obtained using the Nuclear Extract Kit (Active Motif) according to the manufacturer’s protocol.

Statistical analysis

All data were derived from at least three independent experiments and statistical analysis was conducted using the Prism 3 GraphPad software. Values were presented as means ± SEM. Significance level was calculated using one-way ANOVA or Student t test as applicable. P value <0.05 was considered significant.

Results

Mahanine inhibits ligand-dependent AR transactivation

Prostate cancer cells often exhibit increased AR activity, leading to elevated expression of androgen-regulated genes (40). To assess the ability of mahaine to attenuate ligand-induced transcriptional activity of the AR, LNCaP cells were cultured in the absence of androgen [charcoal-stripped (CS) media] for 4 days and transfected with an androgen response element–luciferase reporter construct (ARR3-TK–Luc). Expectedly, the addition of DHT (1 nmol/L) significantly induced luciferase activity; however, this induction was inhibited in the presence of mahaine in a dose-dependent manner, suggesting that mahaine inhibits DHT-induced AR transactivation (Fig. 1A). A similar effect was observed when a PSA promoter–luciferase reporter construct was used (Fig. 1B). In addition, we transfected LNCaP cells with human telomerase reverse transcriptase (hTERT)-promoter luciferase construct and c-myc-promoter luciferase construct and assessed whether mahaine treatment altered promoter activity of these genes. We did not observe any significant change in the luciferase activity upon mahaine treatment, indicating that mahaine does not ubiquitously
decrease the promoter-luciferase activity of all genes (Supplementary Fig. S2A and S2B).

To confirm that the observed decrease in ARR3-TK-luciferase and PSA promoter-luciferase activity could be attributed to the ability of mahanine to inhibit DHT-induced AR transactivation, and was not a result of AR degradation in the presence of mahanine, we measured the cellular levels of AR and PSA in LNCaP cells that had been cultured in CS media and subsequently treated with DHT (1 nmol/L) with or without mahanine (10 μmol/L). Our results show that although PSA levels decline in a manner that mimics the effect of mahanine on the PSA promoter-luciferase activity, the AR levels remain mostly unchanged during this time (Fig. 1C). This suggests that the decline in AR transcriptional activity can be attributed to the inactivation of the AR in the presence of mahanine, and is not because of a decline in the cellular AR levels.

To further confirm the inhibitory effects of mahanine on AR signaling, the expression levels of a number of well-established androgen-regulated genes (GREB1, PSA, NDRG1, PMEPA1, and SGK1) was measured in the LNCaP cells using qRT-PCR and in LNCaP and VCaP cells using semi-qRT-PCR. Cells were cultured in CS media to ensure low basal AR transcriptional activity and target gene expression. The increase in AR target gene expression upon the addition of DHT was effectively repressed in the presence of mahanine, further confirming the ability of mahanine to disrupt ligand-induced AR signaling (Fig. 1D–H and Supplementary Fig. S3).

Mahanine inhibits ligand-independent AR transactivation

In addition to androgen, the AR can be activated by various growth factors and steroids (41). Although...
androgen binds to the LBD located in the C-terminus of the AR, growth factors activate the NTD of the AR, which contains the AF-1 domain, via various signaling cascades. To assess the ability of mahanine to disrupt ligand-independent activation of the AR, we cotransfected LNCaP and VCaP cells with an expression vector encoding a fusion protein of the Gal4-DBD and the NTD of the AR (AR-NTD) and a luciferase reporter vector containing the Gal4-binding site (Gal4UAS-TATA-luciferase). The addition of forskolin (50 μmol/L) or IL-6 (50 ng/mL) caused a robust increase in the luciferase activity, indicating that these growth factors activate the AR-NTD. However, the presence of mahanine significantly inhibited the induction of luciferase activity by these growth factors, without affecting the levels of AR-NTD-Gal4DBD fusion protein, indicating that mahanine possesses the ability to inhibit ligand-independent transactivation of the AR (Fig. 2A and B and Supplementary Fig. S4A). Mahanine did not suppress the luciferase activity of a constitutively active VP16-Gal4DBD fusion protein, confirming that the inhibitory effect of mahanine on the AR-NTD does not involve Gal4-DBD (Fig. 2C). To further confirm this effect of mahanine, LNCaP and VCaP cells were transfected with an ARR3-TK-luciferase reporter construct and treated as described earlier with forskolin or IL-6 in the absence and presence of mahanine. Although both forskolin and IL-6 significantly induced endogenous AR activation, this effect was significantly diminished in the presence of mahanine, thereby confirming the ability of mahanine to disrupt ligand-independent AR transactivation, without altering the endogenous AR levels (Fig. 2D and E and Supplementary Fig. S4B).

Mahanine causes a dose- and time-dependent decline in cellular AR levels

Our experiments so far have involved mahanine treatment for a period of 24 hours, during which we established...
that mahanine treatment at a dose of 10 μmol/L does not alter AR cellular levels (Figs. 1C and 2), but significantly disrupts ligand-dependent and -independent AR signaling. Next, we sought to determine whether longer periods of mahanine treatment affected cellular AR levels. To this end, we treated a panel of prostate cancer cells (LNCaP, VCaP, LNCaP C4-2B, and 22Rv1) with various doses of mahanine (2.5, 5, and 10 μmol/L) for 3 days and measured AR levels by Western blot analysis. We found that the AR protein levels decreased in all cell lines in a dose-dependent manner. A similar decrease in AR levels was observed in LNCaP and LNCaP C4-2B cells treated for 1 to 3 days with mahanine (10 μmol/L), indicating that mahanine also works to decrease AR levels in a time-dependent fashion (Fig. 3A–C and Supplementary Figs. S5A–SSC). It is worth noting that mahanine treatment also decreased the levels of the 80-kDa splice variant, ARv7, of the AR expressed in 22Rv1 cells (Fig. 3C), indicating that mahanine induces degradation of not only full-length AR, but also decreases the levels of AR splice variants that are associated with aggressive forms of prostate cancer.

To determine whether the observed decrease in AR protein levels was due to decreased AR gene expression, we assessed AR message levels in LNCaP and VCaP cells treated with mahanine (10 μmol/L) for 3 days. Our data showed that AR message levels remain mostly unchanged upon mahanine treatment (Fig. 3D and Supplementary Fig. S5D). In addition, we assessed AR and ARv7 message levels in 22Rv1 cells treated in the same manner as described above and once again we did not observe any significant changes in the message levels of these genes (Fig. 3E), which indicates that mahanine decreases AR and ARv7 protein levels posttranslationally, without affecting AR gene transcription.

Figure 3. Mahanine induces proteasomal degradation of AR. LNCaP cells were treated with (A) indicated doses of mahanine for 3 days or (B) mahanine (10 μmol/L) for 1 to 3 days and the cellular levels of AR were measured by Western blotting. β-Actin was used as a loading control. C, 22Rv1 cells were used to assess AR and Arv7 levels upon mahanine treatment as described in A. D, AR message levels were assessed using qRT-PCR in LNCaP cells treated with mahanine (10 μmol/L) for 3 days. E, AR and Arv7 message levels were measured in 22Rv1 cells treated with mahanine (10 μmol/L) for 3 days using qRT-PCR. F, LNCaP cells were treated as shown with mahanine (20 μmol/L) and MG132 (5 μmol/L) for a period of 12 hours. AR cellular levels were measured using Western blot analysis. H, AR was immunoprecipitated from LNCaP cells treated with mahanine (20 μmol/L) and MG132 (5 μmol/L) as indicated, following which Western blot analysis was carried out using anti-ubiquitin and anti-AR antibody.
To assess the susceptibility of AR to degradation in the presence of mahanine, we treated LNCaP cells with cyclohexamide (10 μg/mL) over a period of 48 hours, in the absence and presence of mahanine. The presence of mahanine accelerated AR degradation, confirming that mahanine compromises the stability of the AR (Fig. 3F).

Mahanine induces proteasomal degradation of the AR

The ubiquitin–proteasome pathway is the predominant mechanism for AR degradation. To explore the mechanism by which mahanine induces AR degradation, LNCaP cells were treated with mahanine (20 μmol/L), with or without a proteasome inhibitor, MG132 (5 μmol/L), for 12 hours. The decrease in AR levels upon mahanine treatment was significantly rescued when the proteasome mechanism for AR degradation. To explore the mechanism by which mahanine induces AR degradation, LNCaP cells were treated with mahanine (20 μmol/L) for a 12-hour window, during which the AR localization was monitored at specific intervals of time by immunofluorescence staining. Over a 12-hour treatment time, the AR content of the nucleus was progressively depleted, accompanied by an initial increase in the cytoplasmic levels of the AR after 3 hours of mahanine treatment, followed by a gradual decline at 6 and 12 hours (Fig. 4C). To confirm the above pattern of subcellular movement of the AR, the nuclear and cytoplasmic fractions of LNCaP cells treated with mahanine (20 μmol/L) over a 12-hour time frame were collected. The AR subcellular distribution patterns mimicked those observed by immunofluorescence staining, with a noticeable dip in the AR levels after 3 hours of mahanine treatment, followed by a gradual decline (Fig. 4D).

To clarify that the observed increase in cytoplasmic AR levels following mahanine treatment is the result of AR shuttling out of the nucleus and not due to increased AR synthesis in the cytoplasm, LNCaP cells were pretreated with cyclohexamide (10 μg/mL) and MG132 (5 μmol/L) for 1 hour, after which mahanine (20 μmol/L) was administered for 3 hours. The presence of cyclohexamide and MG132 ensured that no further AR was synthesized in the cell and the existing AR was not degraded by endogenous mechanisms or the presence of mahanine. Under these conditions, a distinct elevation in the cytoplasmic AR was still observed upon mahanine treatment, along with a decline in nuclear AR levels. These data confirm the ability of mahanine to cause a subcellular shift in the localization of AR, inducing its movement out of the nucleus and into the cytoplasm, and subsequently resulting in a decrease in the AR levels in both the cellular compartments (Fig. 4E). Interestingly, in LNCaP cells cultured in CS media, which caused the AR to localize predominantly in the cytoplasm, mahanine did not prevent the shuttling of the AR from the cytoplasm to the nucleus upon stimulation with DHT. However, although the AR did localize in the nucleus in cells treated with DHT and mahanine, PSA expression was not induced in the mahanine-treated cells and remained at the control levels, suggesting that the AR which translocated to the nucleus in the presence of mahanine was not transcriptionally active (Fig. 4F).

Mahanine prevents AR nuclear translocation and causes it to accumulate in the cytoplasm

Nuclear localization is an important prerequisite for the AR to exert its transcriptional effects. To evaluate the effects of mahanine on the cellular distribution of the AR, LNCaP cells were treated with DHT (1 nmol/L), with or without mahanine (10 μmol/L), and immunostained for AR. In untreated control cells, although the AR was found to exist predominantly in the nucleus, it was also detectable in the cytoplasm. The nuclear localization of the AR in control cells can be attributed to steroids and other growth factors present in the serum-supplemented media. Upon stimulation with DHT, all of the AR was found to localize in the cell nucleus; however, when the cells were treated with both, DHT and mahanine, the nuclear localization of the AR was greatly diminished, with a large proportion of the AR present in the cytoplasm (Fig. 4A). To determine whether mahanine had a similar effect on the cellular distribution of AR splice variant, ARv7, 22Rv1 cells were treated with mahanine for 24 hours and then immunostained using an ARv7-specific antibody. We found that mahanine treatment induced the relocalization of ARv7 from the nucleus to the cytoplasm, suggesting that mahanine affects the cellular distribution of not only full-length AR, but also its splice variant ARv7 (Fig. 4B).

To further understand how mahanine affects the intracellular distribution of the AR, LNCaP cells were treated with mahanine (20 μmol/L) for a 12-hour window, during which the AR localization was monitored at specific intervals of time by immunofluorescence staining. Over a 12-hour treatment time, the AR content of the nucleus was progressively depleted, accompanied by an initial increase in the cytoplasmic levels of the AR after 3 hours of mahanine treatment, followed by a gradual decline at 6 and 12 hours (Fig. 4C). To confirm the above pattern of subcellular movement of the AR, the nuclear and cytoplasmic fractions of LNCaP cells treated with mahanine (20 μmol/L) over a 12-hour time frame were collected. The AR subcellular distribution patterns mimicked those observed by immunofluorescence staining, with a noticeable dip in the nuclear AR levels after 3 hours of mahanine treatment, followed by a gradual decline (Fig. 4D).

To clarify that the observed increase in cytoplasmic AR levels following mahanine treatment is the result of AR shuttling out of the nucleus and not due to increased AR synthesis in the cytoplasm, LNCaP cells were pretreated with cyclohexamide (10 μg/mL) and MG132 (5 μmol/L) for 1 hour, after which mahanine (20 μmol/L) was administered for 3 hours. The presence of cyclohexamide and MG132 ensured that no further AR was synthesized in the cell and the existing AR was not degraded by endogenous mechanisms or the presence of mahanine. Under these conditions, a distinct elevation in the cytoplasmic AR was still observed upon mahanine treatment, along with a decline in nuclear AR levels. These data confirm the ability of mahanine to cause a subcellular shift in the localization of AR, inducing its movement out of the nucleus and into the cytoplasm, and subsequently resulting in a decrease in the AR levels in both the cellular compartments (Fig. 4E). Interestingly, in LNCaP cells cultured in CS media, which caused the AR to localize predominantly in the cytoplasm, mahanine did not prevent the shuttling of the AR from the cytoplasm to the nucleus upon stimulation with DHT. However, although the AR did localize in the nucleus in cells treated with DHT and mahanine, PSA expression was not induced in the mahanine-treated cells and remained at the control levels, suggesting that the AR which translocated to the nucleus in the presence of mahanine was not transcriptionally active (Fig. 4F).

Mahanine inhibits DHT-induced phosphorylation of AR Ser-81

Phosphorylation is the most common posttranslational modification associated with AR in response to stimulation by androgen and other growth factors. Prior studies have found that the cell-cycle kinases such as CDK1 and CDK9 stimulate phosphorylation at AR Ser-81, thereby mediating cell growth, chromatin binding, and transcriptional activity of the AR (17, 19). Our findings that mahanine regulates prostate cancer cell growth and AR transcriptional activity in response to DHT, led us to explore whether it modulated AR Ser-81 phosphorylation. Time-course studies were carried out in LNCaP cells cultured in CS media for 4 days, following which DHT was added at regular time intervals for up to 24 hours. In
accordance with the findings of others, AR Ser-81 phosphorylation was maximally induced between 6 and 12 hours of DHT treatment. Interestingly, in the presence of mahanine, the induction of AR Ser-81 phosphorylation was completely inhibited, with only a very slight induction noticeable at 24 hours (Fig. 5A). This indicates that mahanine inhibits DHT-induced phosphorylation of AR Ser-81. The prevention of AR Ser-81 phosphorylation by mahanine was accompanied by an inhibition in the induction of PSA expression by DHT, which is consistent with the prior reports demonstrating the importance of AR Ser-81 phosphorylation for AR transcriptional activity (14).

Mahanine inhibits AR Ser-81 phosphorylation via the inactivation of CDK1

CDK1 has maximal activity at G2–M phase of the cell cycle, in which it mediates entry into mitotic prophase in conjunction with its binding partner, cyclin B1. In addition to mitotic protein targets, CDK1 is known to phosphorylate the AR at its Ser-81 site in an androgen-dependent manner. The ability of mahanine to modulate prostate cancer cell growth (Supplementary Fig. S6A and S6B) and cell-cycle distribution (data not shown) led us to explore whether mahanine inhibits AR Ser-81 phosphorylation via CDK1 inactivation. We
did not observe a direct inhibition of CDK1 activity by mahanine using an *in vitro* kinase assay (Supplementary Fig. S7). Next, LNCaP cells were synchronized to the G2–M phase by a 24-hour nocodazole treatment (100 ng/mL) to attain maximal CDK1 activity, as indicated by an increase in activating T161 phosphorylation and a decrease in inhibitory Y15 phosphorylation in the synchronized cells compared with the asynchronized control cells. However, the activation of CDK1 by nocodazole was inhibited in the presence of mahanine. Furthermore, although nocodazole caused an induction in AR Ser-81 phosphorylation, this effect was significantly diminished in the presence of mahanine (Fig. 5B).

To confirm that mahanine inhibits AR Ser-81 phosphorylation via CDK1 inactivation, we cotransfected LNCaP cells with an expression vector encoding constitutively active CDK1 (CDK1-AF) and an expression vector for its binding partner, cyclin B1. The cells were treated with DHT in the absence and presence of mahanine. Although mahanine completely inhibited DHT-induced AR Ser-81 phosphorylation, the presence of constitutively active CDK1 rescued the decline in AR Ser-81 phosphorylation induced by mahanine, demonstrating that mahanine inactivates CDK1 and thereby prevents DHT-induced AR Ser-81 phosphorylation (Fig. 5C).

**Discussion**

The central role of the AR in the progression of CRPC has become increasingly evident, making it a highly attractive therapeutic target for CRPC therapy. Recent approaches in CRPC therapy include the use of new-generation antiandrogen drugs, such as MDV3100 and ARN509, which specifically target the LBD of the AR, or drugs which block intratumoral androgen synthesis, such as abiraterone (42, 43). Although these newly approved drugs improved median overall survival by 3 to 5 months in phase III clinical trials, their mechanism of action is limited to targeting the canonical, ligand-dependent activation of the AR (44). However, AR signaling is not solely mediated by androgen; cross-talk of the AR axis with various signaling cascades triggered by growth factors activates the AR even in the absence of circulating androgens. In addition, the generation of AR splice variants, majority of which lack the LBD and are thereby androgen-independent, is associated with disease progression and resistance to the newly approved drugs, MDV3100 and abiraterone, and adds another layer of complexity in the development of therapeutic agents that are effective in CRPC. Drugs that disrupt AR signaling, irrespective of its manner of activation, and thereby inhibit its downstream effects such as prostate cancer growth and progression, would more effectively prevent growth of the androgen-dependent and -independent cell types of which the prostatic tumor is comprised. To this end, recent reports have described the use of agents that downregulate the levels of AR splice variants and effectively prevent the growth of drug-resistant prostate cancer cells for CRPC therapy (45, 46).

Naturally derived, phytochemical compounds from diverse sources have demonstrated antitumorogenic properties in prostate cancer (47–50). We show here that mahanine, a carbazole alkaloid derived from the curry leaf plant, possesses the ability to inhibit the growth of androgen-dependent and -independent prostate cancer cells (Supplementary Fig. S6A and S6B). Interestingly, mahanine did not demonstrate growth-suppressive effects on RWPE1 cells, a nontumorigenic prostatic epithelial cell line, and although it did decrease slightly the AR levels in these cells, it did not have a pronounced effect on AR nuclear localization as seen in prostate cancer cell lines (LNCaP and 22Rv1), suggesting a differential effect of mahanine on normal versus cancerous cells of the prostate (Supplementary Figs. S6A S8).

Mahanine blocked ligand-dependent activation of AR signaling, effectively inhibiting the expression of downstream target genes of the AR, including PSA. Importantly, the activation of the NTD of the AR was prevented in the presence of mahanine, suggesting that its inhibitory effects on AR signaling include the segment involved in androgen-independent activation of the AR. The ability to prevent ligand-independent activation of...
the AR, specifically by inhibiting the AR NTD, is of importance in patients with CRPC who have previously undergone androgen deprivation therapy but continue to retain high AR activity due to the promiscuous activation of the AR by paracrine or autocrine growth factors, or due to splice variants that lack the LBD and are constitutively active.

Our findings indicate that mahanine inactivates the AR at first, within 24 hours of treatment with 10 μmol/L of mahanine, following which the AR undergoes proteasomal degradation after about 48 hours and continues to decline in the cells up to 72 hours after treatment. The ability of mahanine to inactivate ligand-dependent and -independent activity of the AR, prevent its nuclear localization, and subsequently induce its degradation highlights the various levels at which mahanine intercepts the AR axis. Most importantly, mahanine also induces degradation of truncated AR splice variants, demonstrating its effectiveness against a key mediator of castration resistance and aggressive prostate cancer.

Another interesting effect of mahanine on the AR signaling pathway is its ability to completely inhibit AR Ser-81 phosphorylation. The Ser-81 site is the most abundantly phosphorylated site in response to androgen and has been shown to be imperative for AR nuclear localization, chromatin binding, transcriptional activation, and growth. Our data demonstrate a striking inhibition of DHT-induced phosphorylation at AR Ser-81 by mahanine, accompanied by a decline in transcriptional activation of the AR, exemplified by decreased PSA expression. In addition, we found that although mahanine does not prevent DHT-induced nuclear translocation of the AR (Fig. 4E), it inhibits AR Ser-81 phosphorylation to a large extent and thereby prevents phosphorylated AR Ser-81 from accumulating in the nucleus (Supplementary Fig. S9). Despite the AR localizing in the nucleus, PSA expression was not induced in the presence of mahanine, which correlates well with the findings of others that phosphorylation at the AR Ser-81 site is important for AR transactivation in response to the ligand (14). Although phosphorylation at this site can be induced by androgen at any phase of the cell cycle, mahanine-mediated inhibition of AR Ser-81 phosphorylation depends on the inactivation of mitotic kinase CDK1, which is maximally active at the G2-M transition and was the first kinase reported to phosphorylate this site. This suggests a potential link between the growth inhibitory effects of mahanine and the disruption of AR signaling. However, because several different signaling cascades are known to mediate the growth inhibitory effects of mahanine, it is likely that the inhibition of AR signaling is also a result of the combined effect of mahanine on various pathways involved in AR activation, and does not solely depend on CDK1 inactivation. Nevertheless, because AR Ser-81 is known to be important for AR nuclear localization and transcriptional activity, and CDK1 activity is found to be elevated in CRPC, this finding is of importance in establishing the potential of mahanine for prostate cancer therapy.

The current lack of epidemiologic data on mahanine makes it difficult to estimate physiologically achievable concentrations of mahanine. Further studies on animal models would be essential to evaluate the in vivo effectiveness of mahanine in disrupting AR signaling. Taken together, our findings demonstrate that mahanine intercepts the AR signaling pathway at multiple levels, prevents phosphorylation at the most abundantly phosphorylated site on the AR, and ultimately induces the degradation of both full-length AR and its splice variants, thereby highlighting the therapeutic potential of mahanine in patients with prostate cancer and CRPC, in which the AR plays a central role in tumor growth and progression.

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

Authors’ Contributions
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.S. Amin, S. Jagadeesh, P.P. Banerjee
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.S. Amin, P.P. Banerjee
Writing, review, and/or revision of the manuscript: K.S. Amin, P.P. Banerjee
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.S. Amin, P.G. Rao, P.P. Banerjee
Study supervision: K.S. Amin, P.P. Banerjee
Isolation of the naturally derived small-molecule mahanine from Murraya koenigii by using different purification methods such as TLC, column chromatography, and recrystallization and its structure determination by using NMR, IR, and mass spectroscopy: G. Baishya
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References


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