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 ligand-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 androgen-independent prostate cancer cells. The decrease in AR levels induced by mahanine occurs post-translationally by proteasomal degradation, without any change in AR gene expression. Mahanine treatment induces an outward movement of the AR from the nucleus to the cytoplasm, leading to an initial rise 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 androgen receptor signaling and inhibits the growth of androgen-dependent and –independent prostate cancer cells, thereby implicating a therapeutic role for mahanine in prostate cancer treatment.
Introduction

Prostate cancer is estimated to represent 28% of new cancer cases diagnosed in men in the year 2013, making it the most widely diagnosed cancer in men (1). Radiation or hormonal therapy is the first line of treatment employed for early stages of prostate cancer, when the tumor is still confined within the prostatic capsule. While 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, or 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 MAPK, Akt, Src (2-5). While 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 steroidal receptor which 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 co-activator proteins. In addition to androgen, various growth factors such as EGF and IL-6 are known to modulate the activity and stability of the AR via diverse signaling cascades (7-9).
Phosphorylation is the most common post-translational 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, 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 after 6-8 hours, and has been reported to be the mostly 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 over-expression 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 crosstalk 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 anti-cancer 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, anti-oxidative and anti-proliferative 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 pancreatic cancer cells via the induction of ROS 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 DHT-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 inactivate CDK1 in prostate cancer cells.
Materials and Methods

Cell culture and reagents- Prostate cancer cell lines (LNCaP, VCaP, LNCaP C4-2B, 22Rv1) were obtained from American Type Culture Collection (Manassas, VA) and were cultured in phenol-red free IMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM glutamine, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (Sigma, St Louis, MO) 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 (30, 35) (Supplementary Fig. 1). DHT (Steraloids, Newport, RI), forskolin (Sigma) and IL6 (Invitrogen) were used in the concentrations indicated below. Cyclohexamide and MG132 were obtained from Tocris Biosciences (Bristol, UK). CDK1-AF and Cyclin B1 expression vectors were obtained from Dr. Steven Balk from Harvard University (17).

Quantitative Real Time PCR (qRT-PCR) - Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. 1 μg of RNA was converted to cDNA using RT² First Strand kit (Qiagen), following which 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 (Coralville, IA) and primer sequences are provided in the supplementary information (Supplementary Table 1 and Supplementary Table 2). PSA, GAPDH and ARv7 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) (200ng) using Genejet vII transfection reagent (Signagen, Gaithersburg, MD), following which they were treated with DHT (1nM) and the indicated concentrations of mahanine. The Dual Luciferase Assay kit (Promega, Madison, WI) was used to measure luciferase activity, which was read using a microplate luminometer (Harta Instruments, Gaithersburg, MD). Readings were normalized to Renilla luciferase activity by co-transfection of pRL-TK-luciferase plasmid (50ng). AR-NTD-Gal4DBD and Gal4UAS-TATA-luciferase vectors were obtained from Dr. Marianne Sadar, University of British Columbia, Vancouver, Canada (38) and VP16-Gal4DBD was obtained from Dr. Winship Herr, Université de Lausanne, Switzerland (39) and transfected into LNCaP cells as described above.

**Western blot analysis**- Cells were harvested to extract total cellular protein which was resolved using 8-12% SDS-PAGE and electro-transferred to nitrocellulose membranes (Amersham Biosciences, Pittsburg, PA). The membranes were blocked with a 5% solution of nonfat milk powder in Tris buffered saline (TBS) containing 0.1% Tween-20, and were incubated with respective primary antibodies overnight at 4°C. Following an hour long incubation with horseradish peroxide-conjugated secondary antibodies (1:5000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), membranes were visualized using chemiluminescent reagent (Santa Cruz Biotechnology). Images of the membranes were captured using a Fuji LAS-1000 Imager (Tokyo, Japan). Androgen receptor (Epitomics, Burlingame, CA and Santa Cruz
Biotechnology), PSA (Dako, Carpinteria, CA), pAR$^{S81}$ (Millipore, Billerica, MA), β-actin, nucleolin, Gal4DBD (Santa Cruz Biotechnology), tubulin (Sigma), pCDK1$^{T161}$, pCDK1$^{Y15}$, CDK1 (Cell Signaling Technologies, Danvers, MA) antibodies were used at concentrations recommended by the respective manufacturer.

**Immunoprecipitation-** Cells were washed with ice-cold PBS following which total protein was extracted and quantified and diluted to obtain a concentration of 1µg/µl. 500µl of cell lysate was subjected to pre-clearance using 50µl Protein A/G Plus Agarose beads (Santa Cruz Biotechnology) for 15 mins. The lysate was incubated at 4°C with androgen receptor antibody (441) (Santa Cruz Biotechnology) for a period of 16 hours with constant rotation. The antibody-protein complexes were pulled down using 50µl Protein A/G Plus Agarose beads for 5 hours, following which the beads were separated by centrifugation at 14,000g for 1min at 4°C. The beads were washed three times with cell lysis buffer after which they were boiled with Laemmli buffer to detach the adsorbed protein. The protein was resolved using SDS-PAGE as described above.

**Immunofluorescence staining-** LNCaP cells were grown on ECL (Upstate Biotechnology Inc, Lake Placid, NY) coated chamber slides in phenol-red free IMEM. After the indicated treatments, cells were fixed in methanol, air-dried and re-hydrated with phosphate buffered saline (PBS). 0.2% 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 4µg/ml Alexa Fluor 488 or 594 conjugated secondary antibody (Invitrogen) for 1hr. Cells were washed three times with PBS and incubated with DAPI or propidium iodide for 5 min.
Cells were washed once again PBS and mounted using 50% glycerol and were viewed using a fluorescent microscope (Olympus BX, Olympus Corp, Tokyo, Japan). Images were imported into Adobe Photoshop.

**Nuclear-Cytoplasmic Separation** - LNCaP cells were treated with mahanine or DMSO as described below and cytoplasmic and nuclear fractions were obtained using Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to manufacturer’s protocol.

**Statistical Analysis** - All data were derived from at least three independent experiments and statistical analysis were conducted using the Prism 3 GraphPad software. Values were presented as means ± SEM. Significance level was calculated using the one-way analysis of variance (ANOVA) or Student’s t test as applicable. p value<0.05 was considered significant.

**Results**

**Mahanine inhibits ligand-dependent AR transactivation**

Prostate cancer cells often exhibit increased androgen receptor activity, leading to elevated expression of androgen regulated genes (40). To assess the ability of mahanine to attenuate ligand induced transcriptional activity of the AR, LNCaP cells were cultured in the absence of androgen (charcoal-stripped or CS media) for 4 days and transfected with an androgen response element-luciferase reporter construct (ARR3-TK-luc). Expectedly, the addition of DHT (1nM) significantly induced luciferase activity; however, this induction was inhibited in the presence of mahanine in a dose-dependent manner, suggesting that mahanine inhibits DHT- induced AR transactivation (Fig. 1A). A similar effect was observed when a PSA
promoter-luciferase reporter construct was used (Fig. 1B). Additionally, we transfected LNCaP cells with hTERT-promoter luciferase construct and c-myc-promoter luciferase construct and assessed whether mahanine treatment altered promoter activity of these genes. We did not observe any significant change in luciferase activity upon mahanine treatment, indicating that mahanine does not ubiquitously decrease promoter-luciferase activity of all genes (Supplementary Fig. 2A and 2B).

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 which had been cultured in CS media and subsequently treated with DHT (1nM), with or without mahanine (10µM). Our results show that while PSA levels decline in a manner that mimics the effect of mahanine on 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 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, SGK1) was measured in LNCaP using quantitative real time PCR and in LNCaP and VCaP cells using semi-quantitative RT-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 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. 3).
Mahanine inhibits ligand-independent AR transactivation

In addition to androgen, the AR can be activated by various growth factors and steroids (41). While androgen binds to the ligand binding domain located in the C-terminus of the AR, growth factors activate the N-terminal domain of the AR, which contains the activation of function-1 domain, via various signaling cascades. To assess the ability of mahanine to disrupt ligand-independent activation of the AR, we co-transfected LNCaP and VCaP cells with an expression vector encoding a fusion protein of the Gal4-DNA binding domain (Gal4DBD) and the N-terminal domain of the AR (AR-NTD) and a luciferase reporter vector containing the Gal4 binding site (Gal4UAS-TATA-luciferase). The addition of forskolin (50µM) or IL6 (50ng/ml) caused a robust increase in 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 2B and Supplementary Fig. 4A). 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 the Gal4DBD (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 above with forskolin or IL6 in the absence and presence of mahanine. While both forskolin and IL6 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 endogenous AR levels (Fig. 2D and 2E and Supplementary Fig. 4B).

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

Our experiments thus far have involved mahanine treatment for a period of 24 hours, during which we established that mahanine treatment at a dose of 10µM does not alter AR cellular levels (Fig. 1C and Fig. 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, 10µM) for 3 days and measured AR levels by Western blot. 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 1-3 days with mahanine (10µM), indicating that mahanine also works to decrease AR levels in a time-dependent fashion (Fig. 3A-C and Supplementary Fig. 5A-C). It is worth noting that mahanine treatment also decreased the levels of the 80 kDa splice variant, ARv7, of the androgen receptor expressed in 22Rv1 cells (Fig. 3C), indicating that mahanine induces degradation of not only full-length androgen receptor, but also decreases the levels of androgen receptor splice variants which 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 (10µM) for 3 days. Our data showed that AR message levels remain mostly unchanged upon mahanine treatment (Fig. 3D and Supplementary Fig. 5D). 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 post-translationally, without affecting AR gene transcription.

To assess the susceptibility of AR to degradation in the presence of mahanine, we treated LNCaP cells with cyclohexamide (CHX) (10μg/ml) over a period of 48 hrs, 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 mahanine (20μM), with or without a proteasome inhibitor, MG132 (5μM), for 12 hrs. The decrease in AR levels upon mahanine treatment was significantly rescued when the proteasome was inhibited by MG132 (Fig. 3G), indicating that AR degradation in the presence of mahanine is mediated by the proteasome. In addition, a two-fold increase in ubiquitinated AR was observed in LNCaP cells treated with MG132 (5μM) and mahanine (20μM) for 12 hours, indicating that mahanine causes an increase in AR ubiquitination, leading to its proteasomal degradation (Fig. 3H). The interaction of AR with chaperone protein Hsp90 was unaffected by mahanine, suggesting that mahanine could be degrading a pool of AR which already exists in an unbound state (Supplementary Fig. 5E).
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 (1nM), with or without mahanine (10µM) 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). In order to determine whether mahanine had a similar effect on the cellular distribution of androgen receptor 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 (20µM) 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 sub-cellular movement of the AR, the nuclear and cytoplasmic fractions of LNCaP cells treated in a similar manner with mahanine (20µM) over a 12 hour time frame were collected. The AR sub-
cellular distribution patterns mimicked those observed by immunofluorescence staining, with a noticeable dip in the nuclear AR levels over a period of 12 hours, and a concomitant rise in the cytoplasmic 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 mahamine treatment is the result of AR shuttling out of the nucleus, and not due to increased AR synthesis in the cytoplasm, LNCaP cells were pre-treated with cyclohexamide (10μg/ml) and MG132 (5μM) for 1 hour, after which mahamine (20μM) 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 mahamine. Under these conditions, a distinct elevation in the cytoplasmic AR was still observed upon mahamine treatment, along with a decline in nuclear AR levels. These data confirm the ability of mahamine to cause a sub-cellular 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 cellular compartments (Fig. 4E). Interestingly, in LNCaP cells cultured in CS media, which caused the AR to localize predominantly in the cytoplasm, mahamine did not prevent the shuttling of the AR from the cytoplasm to the nucleus upon stimulation with DHT. However, although the AR did localize to the nucleus in cells treated with DHT and mahamine, PSA expression was not induced in the mahamine treated cells and remained at the control levels, suggesting that the AR which translocated to the nucleus in the presence of mahamine was not transcriptionally active (Fig. 4F).
Mahanine inhibits DHT-induced phosphorylation of AR Ser-81

Phosphorylation is the most common post-translational 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 medium for 4 days, following which DHT was added for regular time intervals up to 24 hrs. In accordance with others’ findings, 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, where 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. 6A and 6B) 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. 7). Next, LNCaP cells were synchronized to the G2/M phase by 24 hour nocodazole treatment (100ng/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 to the asynchronized control cells. However, the activation of CDK1 by nocodazole was inhibited in the presence of mahanine. Furthermore, while 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 co-transfected 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. While 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 anti-androgen drugs such as MDV3100 and ARN509, which specifically target the ligand-binding domain of the AR, or drugs which block intratumoral androgen synthesis, such as abiraterone (42, 43). While 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; crosstalk 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 ligand binding domain 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 which are effective in CRPC. Drugs which 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 which down-regulate 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 anti-tumorigenic 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 androgen-independent prostate cancer cells (Supplementary Fig. 6A and 6B). Interestingly, mahanine did not demonstrate growth suppressive effects on RWPE1 cells, a nontumorigenic prostatic epithelial cell line, and while 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 Fig. 6A and Supplementary Fig. 8).
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 N-terminal domain 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 CRPC patients who have previously undergone androgen deprivation therapy but continue to retain high AR activity due to promiscuous activation of the AR by paracrine or autocrine growth factors, or due to splice variants which lack the LBD and are constitutively active.

Our findings indicate that mahanine inactivates the AR at first, within 24 hours of treatment with 10μM 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 highlight 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 demonstrates 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. 9). In spite of the AR localizing in the nucleus, PSA expression was not induced in the presence of mahanine, which correlates well with others’ findings that phosphorylation at the AR Ser-81 site is important for AR transactivation in response to 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, since 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, since 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 epidemiological data on mahanine makes it difficult to estimate physiologically achievable concentrations of mahanine. Further studies in 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, where the AR plays a central role in tumor growth and progression.

**Authors’ Contributions**

**Conception and design:** KS. Amin and PP. Banerjee

**Development of methodology:** KS. Amin, S. Jagadeesh, G. Baishya, PG Rao, NC Barua, S. Bhattacharya and PP. Banerjee

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** KS. Amin, and PP. Banerjee

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** KS. Amin and PP. Banerjee

**Writing, review, and/or revision of the manuscript:** KS. Amin and PP. Banerjee

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** KS. Amin and PP. Banerjee

**Study supervision:** PP. Banerjee

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References


Figure Legends

**Figure 1: Mahanine inhibits DHT induced AR transactivation.** LNCaP cells grown in CS media for 4 days were transfected with (A) ARR3-TK-luciferase reporter construct or (B) PSA-promoter luciferase reporter construct and Renilla luciferase plasmids (pRL-TK-Luc) and treated with DHT (1nM) for 24 hours with or without indicated doses of mahanine. Promoter activity was determined after normalization with Renilla luciferase activity. Columns, mean of three independent experiments with quadruplicate samples; bars, SEM. (C) LNCaP cells were grown in CS medium for 4 days and treated as indicated with DHT and mahanine (Mah). AR and PSA levels were assessed using Western blot. β-actin was used as a loading control. (D-H) Expression levels of androgen regulated genes in LNCaP cells treated with DHT in the presence of mahanine were measured by qRT-PCR and normalized to GAPDH levels. Columns, mean of three independent experiments with quadruplicate samples; bars, SD. *p <0.05 ***p value<0.0001.

**Figure 2: Mahanine inhibits ligand-independent AR transactivation.** LNCaP cells were co-transfected with AR-NTD-Gal4DBD expression vector and Gal4UAS-TATA-luciferase reporter construct and treated with (A) Forskolin (FSK) (50μM) or (B) IL6 (50ng/ml) in the absence and presence of mahanine (10μM) for 24 hours. Luciferase activity was measured after normalization with Renilla luciferase. Western blot analysis was performed to show the levels of the AR-NTD-Gal4DBD fusion protein. (C) LNCaP cells were co-transfected with VP16 activation domain-Gal4DBD fusion protein and Gal4UAS-TATA-luciferase reporter and treated with mahanine (10μM) for 24 hours. Luciferase activity was measured after normalization with Renilla luciferase. LNCaP cells were transfected with ARR3-TK-luciferase reporter construct and
treated with (D) Forskolin (FSK) (50μM) or (E) IL6 (50ng/ml) in the absence and presence of mahanine (10μM) for 24 hours. Luciferase activity was measured after normalization with Renilla luciferase. Western blot analysis was performed to show the levels of the endogenous AR. Columns, mean of three independent experiments with quadruplicate samples; bars, SEM. ***p value<0.0001.

**Figure 3: Mahanine induces proteasomal degradation of AR.** LNCaP cells were treated with (A) indicated doses of mahanine for 3 days or (B) mahanine (10μM) for 1-3 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 (10μM) for 3 days. (E) AR and ARv7 message levels were measured in 22Rv1 cells treated with mahanine (10μM) for 3 days using qRT-PCR. (F) LNCaP cells were treated with cyclohexamide (CHX) (10μg/ml) in the absence and presence of mahanine (10μM). The cellular levels of AR were measured by Western blotting. β-actin was used as a loading control. (G) LNCaP cells were treated as shown with mahanine (20μM) and MG132 (5μM) for a period of 12 hours. AR cellular levels were measured using Western blot. (H) AR was immunoprecipitated from LNCaP cells treated with mahanine (20μM) and MG132 (5μM) as indicated, following which Western blot analysis was carried out using anti-ubiquitin and anti-AR antibody.

**Figure 4: Mahanine decreases AR nuclear localization, leading to its cytoplasmic accumulation and subsequent degradation.** (A) LNCaP cells were treated with DHT (1nM) with or without mahanine (10μM) for 24 hours. Immunofluorescence analysis was carried out to
assess AR cellular localization. (B) 22Rv1 cells were treated with mahanine (10μM) for 24 hours. Immunofluorescence analysis was carried out to assess ARv7 localization. (C) Immunofluorescence analysis for AR localization was performed at intervals in LNCaP cells treated with mahanine (20μM) over a 12 hour time frame. (D) Nuclear and cytoplasmic fractions were separated at the indicated intervals of time from LNCaP cells treated with mahanine (20μM) over a 12 hour time frame. AR levels were assessed in each fraction by Western Blot. β-actin and nucleolin were used as cytoplasmic and nuclear loading controls, respectively. (E) LNCaP cells were treated as indicated with CHX, MG132 and mahanine. Nuclear and cytoplasmic fractions were separated after 3 hours treatment and analyzed by Western blotting for AR levels. β-actin and nucleolin were used as cytoplasmic and nuclear loading controls, respectively. (F) LNCaP cells which were cultured in CS media for 4 days were treated with DHT (1nM) for 6 hours in the absence and presence of mahanine (10μM). Nuclear and cytoplasmic fractions were separated and assessed for AR levels by Western Blot. β-actin and nucleolin were used as cytoplasmic and nuclear loading controls, respectively. *p <0.05

**Figure 5: Mahanine inhibits DHT induced AR Ser-81 phosphorylation via the inactivation of CDK1** (A) LNCaP cells were cultured in CS media and subsequently treated with DHT with or without mahanine (10μM) for the indicated time periods. pAR^{S81}, AR and PSA levels were assessed by Western blotting. Tubulin was used as a loading control. (B) LNCaP cells were treated with nocodazole (100ng/ml) in the absence and presence of mahanine (10μM). pAR^{S81}, pCDK1^{T161}, pCDK1^{Y15} and total CDK1 levels were assessed by Western blotting. β-actin was used as a loading control. (C) LNCaP cells were co-transfected with a constitutively active CDK1-AF expression vector and a cyclin B1 expression vector and treated with DHT and
mahanine as indicated. pAR$_{\text{S81}}$, AR and PSA levels were assessed by Western blotting. Tubulin was used as a loading control.
Figure 1.
Figure 4.
Figure 5.
Molecular Cancer Therapeutics

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

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