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# Mahanine reverses an epigenetically silenced tumor suppressor gene RASSF1A in human prostate cancer cells

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**Abstract**

It is becoming clear that frequent epigenetic silencing of tumor suppressor genes could be responsible for the development of cancer in various organs. Several recent reports suggest that suppression of RASSF1A is associated with the advanced grade and stage of prostate cancer and many other cancers. In this investigation, we demonstrated that, mahanine, a plant derived carbazole alkaloid, induced RASSF1A expression in both androgen-responsive (LNCaP) and androgen-negative (PC3) prostate cancer cells by inhibiting DNA methyltransferase (DNMT) activity. Mahanine-induced expression of RASSF1A in turn significantly reduced cyclin D1 but not other cyclins. To understand the inverse relationship between RASSF1A and cyclin D1, we observed that mahanine treatment down-regulates cyclin D1 and addition of RASSF1A siRNA prevented this inhibition. This study show for the first time that mahanine can reverse an epigenetically silenced gene, RASSF1A in prostate cancer cells by inhibiting DNMT activity that in turn down-regulates a key cell cycle regulator, cyclin D1. Mahanine therefore, promises an encouraging therapeutic choice for advanced prostatic cancer.


*Keywords:* Mahanine; Carbazole alkaloid; Prostate cancer; RASSF1A; Cyclin D1; Epigenetic silencing

The etiology of human prostatic carcinoma remains largely undefined. However, it is becoming clear that epigenetic inactivation of various tumor suppressor genes could play a pivotal role in the development of various cancers including prostate cancer. One such tumor suppressor is the Ras-association domain family1 (RASSF1) gene. Two major isoforms of RASSF1, A and C, are produced from the human RASSF1 gene on chromosome 3p21.3 [1,2]. A diacylglycerol-binding domain is present at the amino-terminus of RASSF1A, whereas its carboxy-terminus contains a Ras-association domain. RASSF1C is a smaller protein (50 amino acids) that lacks the amino-terminal C1 domain and thought to play a role in RAS-mediated cellular activities [3]. However, biological function of RASSF1A is largely unknown. RASSF1A is probably the most frequently methylated gene described thus far in human cancers [4,5]. RASSF1A gene methylation has been reported in at least 37 tumor types. For example, methylation of RASSF1A is found in 80% of small cell lung cancers [2,6], over 60% of breast tumors [2,7], in 90% of liver cancers [8], 63% of pancreatic tumor [9], 40% of non-ileal tumors [9], 69% of ileal tumors [9], 70% of primary nasopharyngeal cancers [10], 91% of primary renal cell carcinomas [11], 62% bladder tumor [12], and over 70% of prostate cancers [13,14]. Ectopic expression of RASSF1A in cancer cell lines that lack endogenous RASSF1A transcripts resulted in reduced growth of the cells in vitro and in nude mice supporting a role for RASSF1A as a tumor

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suppressor gene [1,2,11,13,15]. However, the mechanism underlying this tumor suppression is unclear. RASSF1A KO-mice were viable and fertile, however as expected were prone to spontaneous tumorigenesis (lymphoma, leukemia, lung adenoma, breast adenocarcinoma, rectal papiloma) in advanced age (18–20 months) [16]. Shivakumar and his associates have shown that the exogenous expression of RASSF1A induced cell cycle arrest in human lung cancer cells (H1299) at the G1 phase which was associated with the down-regulation of cyclin D1 [17]. All these evidences indicate that RASSF1A might have a role in the regulation of cell cycle. Since the restoration of RASSF1A expression in tumor cell lines impairs their tumorigenicity [7,15], factors that can restore RASSF1A expression have immense potential in inhibiting tumor growth.

In a recent study, we have evaluated the anti-proliferative activity of mahanine, isolated and purified from *Murraya koenigii*, in human prostate cancer cells. We demonstrated that mahanine inhibits growth dose-dependently in both androgen-responsive, LNCaP and androgen-independent, PC3 cells [18]. In the present study, we show that mahanine inhibits DNA methyltransferase (DNMT) activity and induces the expression of an epigenetically silenced tumor suppressor gene, RASSF1A in human prostate cancer cells and expression of RASSF1A down-regulates cyclin D1 to inhibit growth.

## Materials and methods

*Mahanine purification and cell culture*. The purification of mahanine and its structure has been described previously by us [18]. PC3, LNCaP, A431, A549, ASPC-1, HT-29, MCF7, and SKOV-3 cells (ATCC, Manassas, VA) were grown in IMEM without phenol red (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Quality Biologicals, Gaithersburg, MD), 2 mM glutamine, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (Sigma, St. Louis, MO) in the presence of 5% $CO_2$ at 37 °C. Twenty-four hours after seeding, cells were treated with vehicle (DMSO) or 1, 2, and 3 μg/ml mahanine and the media was replenished every 24 h.

*Western blot analysis*. Protein lysates from PC3 and LNCaP cells treated with or without mahanine were resolved on 12% SDS–PAGE and transferred to nitrocellulose membranes and were probed with 1:1000 dilution of cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Each blot was re-probed with 1:10,000 dilution of β-actin (Sigma). Images of the membranes were captured using a Fuji LAS-1000 Imager (Tokyo, Japan) and imported into Adobe Photoshop. Band intensities were quantified by utilizing ImageJ software (NIH, Bethesda, MD).

*DNA methyltransferase activity assay*. Cells were plated in complete growth media, then treated with or without various concentrations of mahanine (1, 2, and 3 μg/ml) for 3 days and then were harvested and nuclear extracts were prepared according to manufacturer's protocol (Nuclear extraction kit, Epigentek, Brooklyn, NY). DNMT activity was measured using an EpiQuik DNA methyltransferase activity assay kit (Epigentek). Results were expressed as percent DNMT activity compared to the positive control (provided by the kit) as 100%.

*Reverse transcriptase-polymerase chain reaction (RT-PCR)*. RNA was extracted from PC3, LNCaP, A431, A549, ASPC-1, HT-29, MCF7, and SKOV-3 cells with TRIzol solution (Invitrogen, Carlsbad, CA) and genes of interest were amplified using 500 ng of total RNA reverse-transcribed to cDNA using a Superscript II kit (Invitrogen) with random hexamers. Human-specific primers were designed using the Primer Quest program and purchased from Integrated DNA Technologies, Inc (Coralville, IA). Their sequences and product band sizes are: cyclin D1 forward primer 5′-CACACGGACTACAGGGGAGT-3′, cyclin D1 reverse primer 5′-AGGAAGCGGTCCAGGTAGTT-3′ (475 bp) and GAPDH forward primer: 5′-CCA CCCATGGCAAATTCCATGGCA-3′, GAPDH reverse primer: 5′-TCTAGACGGCAG GTCAGGTCCACC-3′ (598 bp). PCRs were initiated at 94 °C for 2 min, followed by 28 cycles of 94 °C for 1 min, 1 min annealing temperature, 72 °C for 1 min, and final extension at 72 °C for 5 min. The annealing temperature for cyclin D1 and GAPDH was 60 °C. Primers and PCR conditions for RASSF1A are used as described by Rong and associates [19]. After amplification, PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide fluorescence using the Fuji LAS-1000 Imager. Images were captured and imported to Adobe Photoshop. Band intensities were quantified by using ImageJ software (NIH, Bethesda, MD).

*Statistical analyses*. All data were derived from at least three independent experiments and statistical analyses were conducted using Prism 3 GraphPad software. Values were presented as means ± SEM. Significance level was calculated using the one-way analysis of variance (ANOVA) followed by the Dunnett's post-test with an assigned confidence interval of 95%. $p$-value $<0.05$ was considered significant.

## Results

### *Mahanine induces the expression of an epigenetically silenced gene RASSF1A in human prostate and various other cancer cells*

We previously demonstrated that mahanine inhibits growth in prostate cancer cells [18] however the underlying mechanism of this growth inhibition was not entirely understood. Since RASSF1A gene silencing occurs in 37 different cancer types, we compared the expression of RASSF1A in normal epithelial cells (PrEC) and in prostate cancer cells (PC3 and LNCaP). As expected, a robust expression was observed in PrEC while it could not be detected in PC3 and LNCaP cells (Fig. 1A). Addition of mahanine to prostate cancer cells (PC3 and LNCaP) induced the expression of RASSF1A dose-dependently (20–80-folds) and significantly (Fig. 1B and C). To determine the effect of mahanine is not unique to prostate cancer cells, we evaluated the effects of mahanine in various non-prostatic human cancer cell lines. Similar to prostate cancer cells, RASSF1A was not expressed in epidermoid (A431), lung (A549), pancreatic (ASPC-1), colon (HT-29), breast (MCF7), and ovarian (SKOV-3) cells and mahanine treatment for 2 days induced RASSF1A expression in all of them (Fig. 1D). These results suggest that mahanine is capable to induce the epigenetically silenced gene RASSF1A, not only in prostate but also in various non-prostatic cancer cells.

### *Mahanine restores the expression of RASSF1A by inhibiting DNA methyltransferases activity in human prostate cancer cells*

Since DNA methyltransferases (DNMTs) hypermethylate the DNA which results in silencing of gene expression and since mahanine restores the expression of RASSF1A, it is tempting to speculate that mahanine

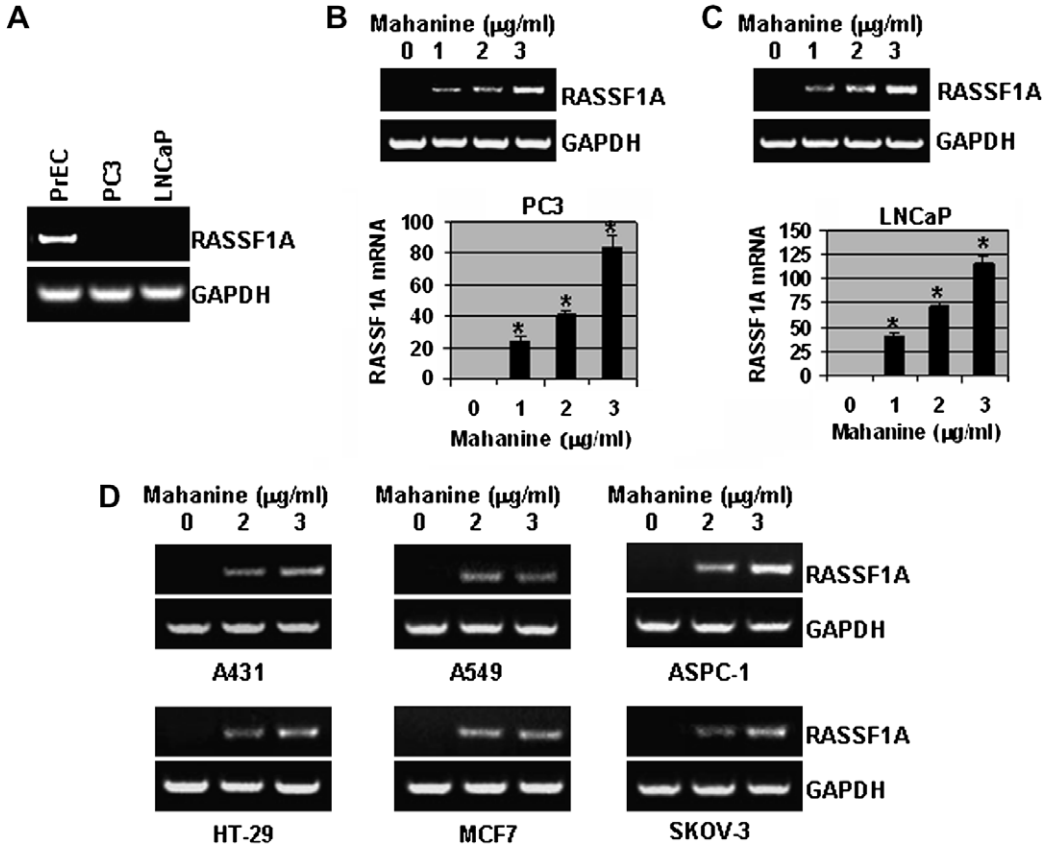


Fig. 1. Mahanine induces RASSF1A in prostate and other cancer cells. (A) Expression of RASSF1A and GAPDH in untreated normal prostate epithelial cells (PrEC) and human prostate cancer cells (PC3 and LNCaP) by RT-PCR. (B and C) Expression of RASSF1A and GAPDH in PC3 and LNCaP cells treated with 0, 1, 2, and 3 μg/ml mahanine for 3 days. (D) Expression of RASSF1A and GAPDH in various non-prostate cancer cells. Representative photograph from an experiment that was repeated thrice. Quantitative estimations of relative levels of RASSF1A mRNAs (lower panels) were determined by densitometric measurements of RT-PCR gels from three independent experiments after normalization with GAPDH. Columns, mean; bars, SEM. $^{*}p < 0.001$, significantly different from control.

might inhibits DNMT activity to prevent DNA methylation and thus induces the expression of RASSF1A. Similarly, histone deacetylation (HDAC) also involved in alteration in gene expression. Therefore, our intent was to find out whether DNMTs or HDACs or both could be involved in the re-expression of RASSF1A in prostate cancer cells. To investigate this, prostate cancer cells were treated with DNMT inhibitor, Aza (5 μM of 5-Aza-2′-deoxycytidine, Sigma) and HDAC inhibitor, TSA (100 nM of trichostatin A, Sigma) for 3 days and then the expression of RASSF1A were analysed by RT-PCR. As seen in Fig. 2A, methylation inhibitor induces the expression of RASSF1A in prostate cancer cells, whereas histone deacetylase inhibitor had no effect. Results therefore indicate that silencing of RASSF1A gene in PC3 and LNCaP cells is by DNA methylation and not due to histone acetylation. Considering demethylation of RASSF1A gene may be due to the inhibition of DNMTs, we examined the total DNMT activity in PC3 and LNCaP cells with or without the treatments of various concentrations of mahanine for 3 days. Fig. 2B shows that total DNMT activity in mahanine-untreated PC3 and LNCaP cells were substantial when compared to DNMT positive control (provided by the kit), however mahanine treatment dose-dependently and significantly ($p < 0.001$) decreased DNMT activity. These results show that the restoration of RASSF1A could be the result of inhibition of DNMT activity by mahanine.

### *RASSF1A down-regulates cyclin D1 in human prostate cancer cells*

It was previously reported that exogenous expression of RASSF1A arrested the progression of cell cycle in cancer cells which is related to the reduction of cyclin D1 [17]. Since mahanine-induced RASSF1A expression in our experiment, it was our intention to examine whether RASSF1A is involved in the regulation of cyclin D1. Transient transfection of RASSF1A in PC3 cells for 3 days increased its expression (Fig. 3A). This led to the significant decrease ($p < 0.001$) of cyclin D1 protein (Fig. 3B) but not cyclin A1, B1, or E1 (Fig. 3C). Results of this experiment demonstrate that up-regulation of RASSF1A causes down-regulation of cyclin D1 protein and this is expected to have an impact on cell proliferation.

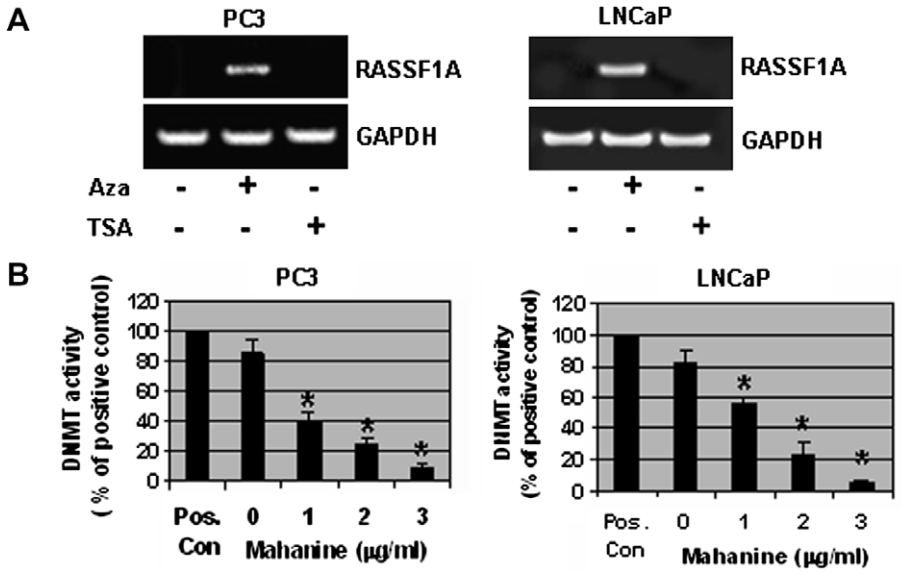


Fig. 2. Mahanine inhibits DNA methyltransferase activity in human prostate cancer cells. (A) RT-PCR analyses showing the expression of RASSF1A and GAPDH in PC3 and LNCaP cells treated with 5 μM of 5-Aza-2′-deoxycytidine or 100 nM of trichostatin A or vehicle (DMSO) for 3 days. (B) Total DNMT activity in PC3 and LNCaP cells were treated with 0, 1, 2, and 3 μg/ml mahanine for 3 days. Data are means of three independent experiments. Columns, mean; bars, SEM. $^{*}p < 0.001$, significantly different from control.

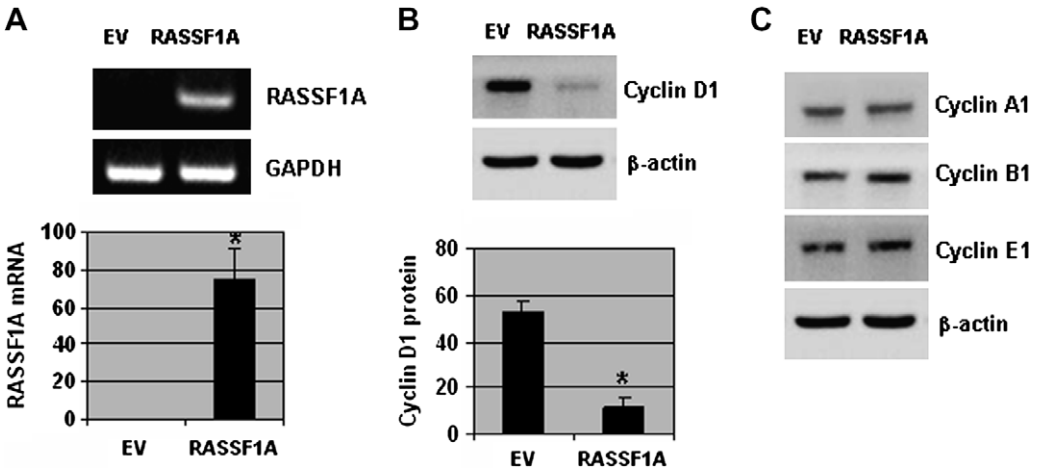


Fig. 3. RASSF1A down-regulates cyclin D1 protein in prostate cancer cells but not other cyclins. PC3 cells were transiently transfected with 200 ng/ml empty vector (EV) or RASSF1A expression vector for 3 days. RNA was extracted, and RT-PCR assays were performed to detect RASSF1A and GAPDH mRNAs (A). (B and C) Western blot analyses of cyclin D1, A1, B1, E1, and β-actin. Representative photograph from an experiment that was repeated thrice. Quantitative estimations of relative levels of RASSF1A mRNA and cyclin D1 protein were determined by densitometric measurements of RT-PCR gels and immunoblots from three independent experiments after normalization with GAPDH and β-actin, respectively. Columns, mean; bars, SEM. $^{*}p < 0.001$, significantly different from control.

*Mahanine down-regulates cyclin D1 and RASSF1A siRNA prevents this decrease in human prostate cancer cells*

Results of the above mentioned experiments emphasized the need to study the levels of cyclin D1 in PC3 and LNCaP cells with or without various concentrations of mahanine treatments. Mahanine dose-dependently and significantly ($p < 0.001$) decreased cyclin D1 protein levels (Fig. 4A). However, the levels of cyclin A1, B1, and E1 were unchanged in PC3 (Fig. 4B) and LNCaP (data not shown) cells after mahanine treatments. To show that mahanine-induced RASSF1A expression is directly associated with cyclin D1 down-regulation, RASSF1A siRNA containing plasmid was transiently transfected in PC3 cells for 48 h and then cells were treated for another 48 h with or without 2 μg/ml mahanine. As seen in Fig. 4C, RASSF1A message was induced with 2 μg/ml mahanine and cyclin D1 message was decreased almost to a non-detectable level compared to the vehicle-treated cells. When RASSF1A siRNA was transfected to PC3 cells, it prevented the mahanine-induced decrease in cyclin D1 protein level. These results show that mahanine induces the expression of

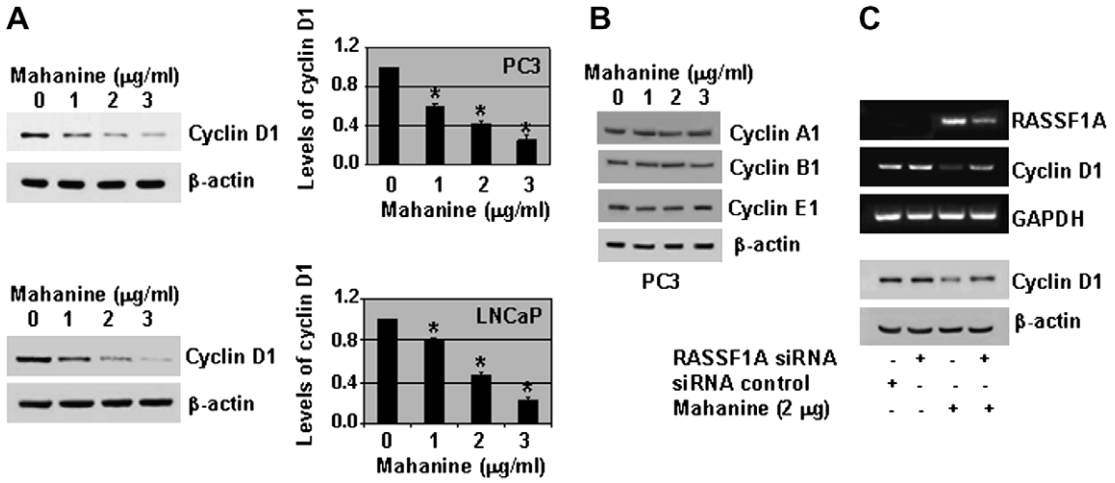


Fig. 4. Mahanine down-regulates cyclin D1 protein and RASSF1A siRNA prevents mahanine-induced repression of cyclin D1 in prostate cancer cells. (A) Western blots showing cyclin D1 protein levels in PC3 and LNCaP cells treated with 0, 1, 2, and 3 μg/ml mahanine for 3 days. All immunoblots were re-probed with β-actin antibodies to ensure equal loading. Quantitative analyses of relative levels of cyclin D1 proteins are shown on right. Columns, mean of three independent experiments with quadruplicate samples; bars, SEM. $^{*}p < 0.001$, significantly different from control. (B) Western blots showing cyclin A1, B1, and E1 protein levels in PC3 cells treated with 0, 1, 2, and 3 μg/ml mahanine for 3 days. (C) PC3 cells were transfected with 200 ng of siRNA control plasmids or RASSF1A siRNA plasmids with or without 2 μg/ml mahanine for 48 h in normal growth media. After treatment, cells were harvested for immunoblot and RT-PCR analyses.

RASSF1A and that in turn down-regulates cyclin D1 in prostate cancer cells.

## Discussion

We have recently demonstrated that mahanine inhibits growth in human prostate cancer cells [18] however the underlying mechanism involved in the reduction of growth was not clear. In the present study, we show that mahanine induces the expression of an epigenetically silenced gene, RASSF1A in prostate cancer cells. Although RASSF1A is epigenetically silenced in many carcinomas, the epigenetic mechanism of RASSF1A silencing is largely unknown. It has been demonstrated that promoter hypermethylation could be one of the major causes of RASSF1A gene silencing in variety of human cancers [2,4–15]. We have found that mahanine inhibits DNMT activity and concomitantly expresses RASSF1A. It is therefore very likely that mahanine induces the expression of RASSF1A by inhibiting DNMT. However, further investigations would be required to determine whether one or more DNMTs are involved in this process.

Using two human prostate cancer cell lines, PC3 and LNCaP, we demonstrated that mahanine dose-dependently decreased cyclin D1 message and protein levels and eventually arrested the cell at G0/G1 phase (data not shown). The down-regulation of cyclin D1 in prostate cancer cells is consistent with previous reports that have shown G1 arrest induced by exogenous RASSF1A. Shivakumar and associates [17] have described that RASSF1A induces a G1 growth arrest by inhibiting cyclin D1 protein accumulation in RASSF1A expressing cells (H1229). Our present study with PC3 and LNCaP cells adds a new domain through the use of mahanine. Cyclin D1 expression is significantly reduced by mahanine and this is possible because mahanine induced the expression of a silenced tumor supppressor gene, RASSF1A.

Overexpression of cyclin D1 is a common event in various forms of cancer including prostate cancer [20–22]. The overexpression of cyclin D1 leads to enhanced organ growth in mice [23]. Transient transfection of hepatocytes with cyclin D1 leads to vigorous proliferation and more than 50% increase in liver mass within 6 days [24]. Conversely, cyclin D1 knockout mice are smaller than wild-type mice, and mice with the homozygous deletion of the p27 gene (which inhibits cyclin D1/Cdk4/6 complexes) show gigantism and enhanced organ size [25]. Moreover, the expression of cyclin D1 modulates invasive ability by increasing matrix metalloproteinase (MMP-2 and MMP-9) activity and motility in glioma cells [26]. Furthermore, some studies have shown that overexpression of cyclin D1 is associated with metastatic prostate cancer to bone [20].

In summary, our data demonstrate that mahanine is a potent inducer of RASSF1A expression and most likely via the inhibition of DNMT activity. RASSF1A in turn inhibits a key cell cycle modulator, cyclin D1 that would eventually repress cell proliferation and its invasive potential in prostate and other cancer cells. For this reason mahanine offers a novel and potential therapeutic choice for treating advanced prostate cancer.

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