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Anti-Histone Acetyltransferase Activity from Allspice Extracts Inhibits Androgen Receptor-Dependent Prostate Cancer Cell Growth

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Histone acetylation depends on the activity of two enzyme families, histone acetyltransferase (HAT) and deacetylase (HDAC). In this study, we screened various plant extracts to find potent HAT inhibitors. Hot water extracts of allspice inhibited HAT activity, especially p300 and CBP (40% at 100 μg/ml). The mRNA levels of two androgen receptor (AR) regulated genes, PSA and TSC22, decreased with allspice treatment (100 μg/ml). Importantly, in IP western analysis, AR acetylation was dramatically decreased by allspice treatment.

Furthermore, chromatin immunoprecipitation indicated that the acetylation of histone H3 in the PSA and B2M promoter regions was also repressed. Finally, allspice treatment reduced the growth of human prostate cancer cells, LNCaP (50% growth inhibition at 200 μg/ml). Taken together, our data indicate that the potent HAT inhibitory activity of allspice reduced AR and histone acetylation and led to decreased transcription of AR target genes, resulting in inhibition of prostate cancer cell growth.

Key words: allspice; histone acetyltransferase; androgen receptor; acetylation; prostate cancer

The androgen receptor (AR) function has been found to play a crucial role in the onset and progression of prostate cancer.[1,2] Prostate cancers are typically treated with hormone therapy aimed at blocking testosterone signaling at the AR.[3,4] The effects of androgens are mediated through the AR, a member of a large family of ligand-dependent transcriptional factors that belong to the steroid receptor superfamily. AR acetylation is a key posttranslational modification regulating growth control in human prostate cancer.[1,5,6] The AR is acetylated by p300, P/CAF, and TIP60, and acetylation of the AR regulates the recruitment of co-regulators to the basal transcriptional machinery of AR target genes and growth properties of the receptors in cultured cells and *in vivo*. AR acetylation-mimicking mutants convey reduced apoptosis and enhanced growth properties, correlating with altered promoter specificity for cell-cycle target genes.[6,7]

Recent studies have shown that alterations in chromatin structure due to histone hyperacetylation/deacetylation play an important role in eukaryotic gene transcription, carcinogenesis, and cancer therapy.[8,9] The reversible process of histone acetylation is controlled by two classes of enzymes, histone acetyltransferases (HAT) and histone deacetylases (HDAC), which catalyze the addition and removal respectively of acetyl groups of key lysine residues on proteins.[10,11] Dysfunction in the balance between the acetylation and deacetylation states of histones is often associated with manifestation of cancer.[8,10,12,13]

The HDAC inhibitors have been relatively well-documented in comparison with HAT inhibitors, but,

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Abbreviations: AR, androgen receptor; HAT, histone acetyltransferase; HDAC, histone deacetylase; ARE, androgen responsive element

there is increasing evidence that HAT function is associated with cancer causation and progression. The genes that have been identified as having HAT activity are the P300/cAMP response element binding protein (CREB)-binding protein (CBP) family, TAF250, p300/CBP-associated factor (PCAF), hGCN5, and TIP60.[11,13,14] Of these co-activators, TIP60, P300, and PCAF enhance the inherent transcriptional activity of the AR by direct receptor acetylation, thereby up-regulating the histone acetylation rate of AR target genes. A limited number of HAT inhibitors have been described: synthetic peptide-CoA conjugate (p300 and PCAF),[15] isothiazolone (PCAF),[11] polyprenylated benzophenone from *Garcinia indica* fruit rind,[14] curcumin from *Curcuma longa* (p300),[16] and anacardic acid from cashew nut shell liquid.[17]

Allspice is the dried berry of *Pimenta dioica*, which belongs to the Myrtaceae family. Allspice berries contain 2–5% essential oils, of which the major constituents are eugenol (60–75%), eugenol methyl ether, cineole, phellantrene, and caryophyllenes.[18,19] Allspice extract exhibits the highest content of redox-active compounds and up-regulation of the cytochrome P450 3A4 promoter.[19] In the present study, we found that allspice hot water extract is a potent inhibitor of HAT activity, especially for p300 and CBP, and that it can be developed for potential use as an antineoplastic therapeutics *via* inhibition of prostate cancer cell growth.

Materials and Methods

Reagents and construction. Allspice was purchased from Youyang Spice (Seoul, Korea). RPMI-1640 and Dulbecco's Modified Eagle medium, antibiotics and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY). A HAT activity colorimetric assay kit was from Biovision (Mountain View, CA). An Easy-spin total RNA extraction kit and a Maxime RT premix kit were from Intron (Seoul, Korea). The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Charlottesville, VA). Lipofectamine2000 transfection reagent was from Invitrogen (Carlsbad, CA, USA). PGL3-PSA construct was the kind gift from Dr. Kyung-Sup Kim (Yonsei University). Protein A/G PLUS agarose beads were from Santa Cruz Biotechnology.

Preparation of aqueous extract. To prepare the sample, allspice powder (5 g dry weight) was refluxed with 4 volumes of distilled water for 2 h. The extraction was centrifuged at 3,000 × *g*, evaporated in a vacuum, and then lyophilized.

HAT activity assay. LNCaP cell nuclear extract was prepared as described previously.[20] HAT activity assays were performed using nuclear extracts following the manufacturer's protocol (Biovision Biotechnology). Immunoprecipitations (IP) were performed using anti-p300 (Upstate Biotechnology) and anti-CBP (Santa Cruz Biotechnology) with LNCaP nuclear extracts. Precleared nuclear extract (50 µl) was incubated with 2 µl of antibodies overnight with Protein A/G PLUS agarose beads at 4 °C. Immunoprecipitations were collected and washed with HAT assay buffer (50 mM Tris, pH 8.0, 10% glycerol, 0.1 mM EDTA), and then used in a HAT activity assay.

Cell culture. Human prostate cancer cell lines, LNCaP and DU145, and cervical epithelial carcinoma HeLa were obtained from the American Type Culture Collection (ATCC, Manassas, VA). LNCaP and Du145 were grown in RPMI 1640 with L-glutamine, and HeLa was in DMEM, supplemented with 10% heat-inactivated FBS, and 1% antibiotics and antimycotics. All cultures were maintained in a humidified chamber at 37 °C under 5% CO_2.

Reporter assay. LNCaP cells were seeded at 5×10^5 per well in 6-well culture plates and incubated overnight with RPMI 1640 containing 10% FBS. At 90% confluence, 4 µg of pGL3-PSA and 1 ng of pRL-SV40 DNA were used in each well to transfect LNCaP cells for luciferase assay. The pRL-SV40 DNA was transfected for internal control as the baseline response in the dual luciferase reporter assay system. The medium was replaced with phenol-free RPMI 1640 containing 1% charcoal-stripped fetal bovine serum. The medium was then changed by 50 nM of synthetic androgen R1881 treatment, and allspice extracts (50 and 100 µg/ml) were added. The cells were lysed after 60 h incubation and analyzed by the Dual-Luciferase Reporter assay system (Promega, Madison, WI) by MicroLumat Plus (EG and G Berthold, Wildbad, Germany).

Reverse transcription and polymerase chain reaction (RT-PCR). LNCaP cells were seeded at 5×10^5 per well in 6-well culture plates and incubated with RPMI 1640 containing 10% FBS. At 90% confluence, the medium was replaced with phenol-free RPMI 1640 containing 10% charcoal-stripped fetal bovine serum for 48 h. The medium was then changed by 50 nM of synthetic androgen R1881 treatment, and allspice extracts (100 µg/ml) were added 6 h after R1881 treatment. After overnight incubation, total RNA was isolated with an Easy-spin total RNA extraction kit, and RT-PCR was performed with a Maxime RT-premix kit. GAPDH was used as an internal control. The primer sequences used in this study were as follows: for the PSA, 5′-GCCCACCCAGGAGCCAGCACT-3′ and 5′-GGCCCCCAGAATCACCCGAG CAG-3′; for the TSC22, 5′-GACTTGATAATAGCTCCTCTGGT-3′ and 5′-ATTTTTCTCTATTAGTTCTTTGATTTG-3′. The primers for GAPDH amplification were 5′-CGCGGGGCTCTCCAGAACATCATCC-3′ and 5′-CTCCGACGCCTGCTTCACCACCTTCTT-3′. The reaction mixture was heated at 94 °C for 2 min, then 27 cycles at 94 °C for 30 s, 55 °C

for 30 s, and 72 °C for 30 s, and extension at 94 °C for 5 min. Amplification products were electrophoresed through 1.5% agarose gels.

Immunoprecipitation and western blotting. LNCaP cells (1×10^6) on 100-mm plates were washed twice with PBS and lysed with RIPA buffer. Cell lysates were incubated with 1 µl of anti-Ac-lysine (Upstate Biotechnology) overnight with Protein A/G PLUS agarose beads at 4 °C. Immunoprecipitates were collected, washed with HAT assay buffer, and then resuspended with 2× sample buffer. Western analysis was performed with anti-AR (Santa Cruz Biotechnology) and protein was visualized by chemiluminescence (PerkinElmer LAS, Boston, MA, USA).

ChIP assays. For ChIP assays, we first isolated chromatin as described previously.[21] In brief, approximately 2×10^9 LNCaP cells in 150-mm dishes were treated with PBS containing 1% formaldehyde for 10 min, washed twice with PBS, and then incubated with 100 mM Tris (pH 9.4) and 10 mM dithiothreitol (DTT) at 30 °C for 15 min. The cells were then rinsed twice with PBS and resuspended in 600 µl of SolA buffer (10 mM HEPES, pH 7.9, 0.5% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) by pipetting. After a short spin (5 min at 800 × *g*), the pellets were resuspended in SolB (20 mM HEPES, pH 7.9, 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) containing protease inhibitors by vigorous pipetting to extract nuclear proteins. After centrifugation at 13,000 rpm for 30 min, the nuclear pellets were resuspended in IP buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, and protease inhibitors) and sonicated to break the chromatin into fragments with an average length of 0.5–1 kb. The ChIP assays were then performed with the indicated antibodies (anti-AR, anti-acetyl histone H3, and H4 from Upstate and IgG from Gymed, Boston, MA) essentially as described, but omitting SDS in all buffers.[15] The primers used for ChIP analysis were: for PSA, 5′-CATGTTCACATTAGTACACCTTGCC-3′ and 5′-TCTCAGATCCAGGCTTGCTTACTGTC-3′ and for B2M, 5′-AGACTTCCCAAATTTTGCCATCCTA-3′ and 5′-AAAGGCCTGAAATGTTAGTGTTGAGT-3′.

Cell viability test. Cells were plated at the following densities for the experiments in 96-well culture plates: 1×10^4 cells/well for LNCaP, 1×10^4 cells/well for DU145, and 3×10^3 per well for HeLa cells. All plates were incubated overnight with RPMI 1640 or DMEM containing 10% FBS. The cells were then treated with serum-free medium containing allspice extracts for 48 h. After 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in PBS was added, and the plates were incubated for 2 h. At the end of incubation, the medium was removed and the resulting formazan was solubilized with DMSO. Absorbance was measured at 570 nm with background subtraction at 630 nm.

Results

Identification of anti-HAT activity from the allspice extract

We screened 500 plant extracts to examine inhibitory effect on HAT at a concentration of 100 µg/ml (data not shown) with LNCaP nuclear extracts. The allspice hot water extracts exhibited one of the highest HAT inhibitory effects among the plant extracts. As Fig. 1A shows, HAT activity was inhibited by allspice extracts in a concentration-dependent manner (50–200 µg/ml). To examine further the enzyme-specific anti-HAT activity of allspice, immunoprecipitated p300 and CBP were incubated with increasing concentrations of extracts. We observed that the specific enzyme activities of p300 and CBP were strongly inhibited, approximately 50–60%, by allspice extract at a concentration of 100 µg/ml, whereas HAT activity with IgG was unchanged in the presence of allspice extracts (Fig. 1B). These results led us to believe that the hot water extract of allspice possessed inhibitory effects on p300 and CBP.

Repression of AR-mediated transcriptional activation by allspice extract

Androgen receptor (AR) expression is observed in primary prostate cancer and can be detected throughout hormone-sensitive and hormone refractory cancers.[22,23] To determine whether allspice extracts modulate AR-mediated transcription in androgen-sensitive prostate cancer cells, we measured luciferase activity using an androgen-dependent reporter construct (pGL3-PSA) bearing the ARE (androgen responsive element) in the LNCaP cell line. The cells were incubated with or without synthetic androgen R1881, and then 50 or 100 µg/ml of allspice extract (final concentration) was added. Representative experiments showed that treatment with allspice extracts decreased luciferase activity by 70% at 100 µg/ml concentration as compared with R1881 alone, suggesting that allspice contains anti-HAT activity that represses AR-mediated transcriptional activation (Fig. 2A).

To determine the effect of allspice extracts on the expression of the endogenous AR target gene, we next selected two AR target genes, PSA and TSC22, about which our group has reported.[3] Total RNA was extracted 18 h later in the absence or presence of allspice extract treatment with R1881. Consistently with the reporter assay, mRNA expression of AR target genes was robustly enhanced by R1881 treatment and allspice extract (100 µg/ml), and mRNA expression of PSA and TSC22 was repressed (Fig. 2B). Thus treatments with allspice extracts containing anti-HAT activity led to repression of AR target gene expression levels by inhibition of co-activator dynamics, which are essential for AR-mediated transcriptional activation.

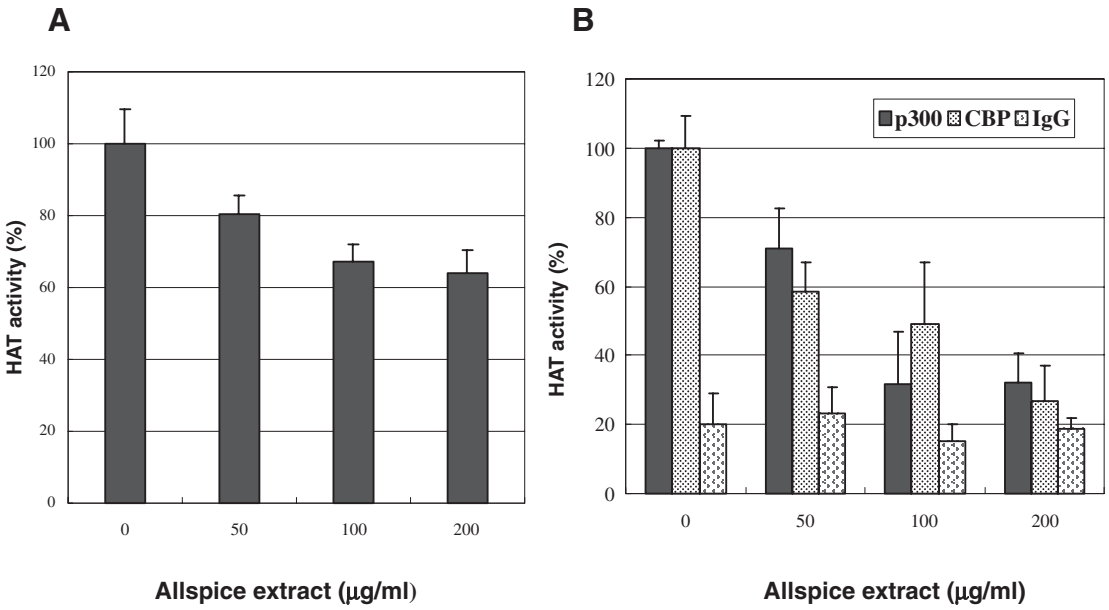

Fig. 1. Inhibition of HAT Activity by Allspice.

Histone acetyltrasferase assays were performed at various concentrations of allspice (A), and with IP product using anti-p300, CBP, and IgG (B). The results represent the mean values with S.D.

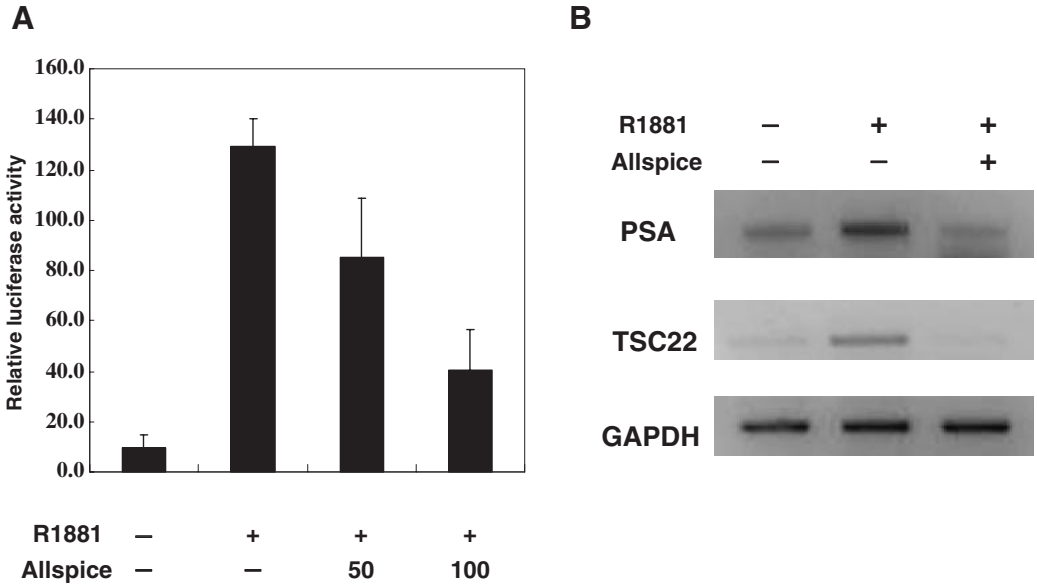

Fig. 2. Repression of AR-Mediated Transcriptional Activation by Allspice Hot Water Extract.

LNCaP cells were transiently transfected with pGL3-PSA and 1 ng of SV40 DNA. After 24 h, the cells were treated in the absence and presence of R1881, and then allspice extract was added at 100 μg/ml to samples 6 h later. Cells were lysed and analyzed after 60 h of incubation. Results are presented as relative luciferase activity (A). RT-PCR analysis of PSA and TSC22 was performed 16 h after treatment with allspice (100 μg/ml) and with or without R1881. RT-PCR analysis of PSA and TSC22 was performed 16 h after allspice treatment (100 μg/ml) with or without R1881 (B). Mean values were calculated from three independent experiments.

Treatment with allspice led to reduced AR and histone acetylation

To determine whether the allspice treatment led to reduced AR and histone acetylation, we performed IP western analysis and ChIP assays with or without allspice-treated LNCaP cells. For this purpose, LNCaP cells were treated with or without R1881 or allspice, either individually or in combination. Three days later, the cells were lysed for IP western analysis with anti-AR and anti-ac-lysin. In the presence or absence of allspice, AR was equally detected in immunoprecipitate by anti-Ac-lys antibody (Fig. 4A). Acetylated AR was increased

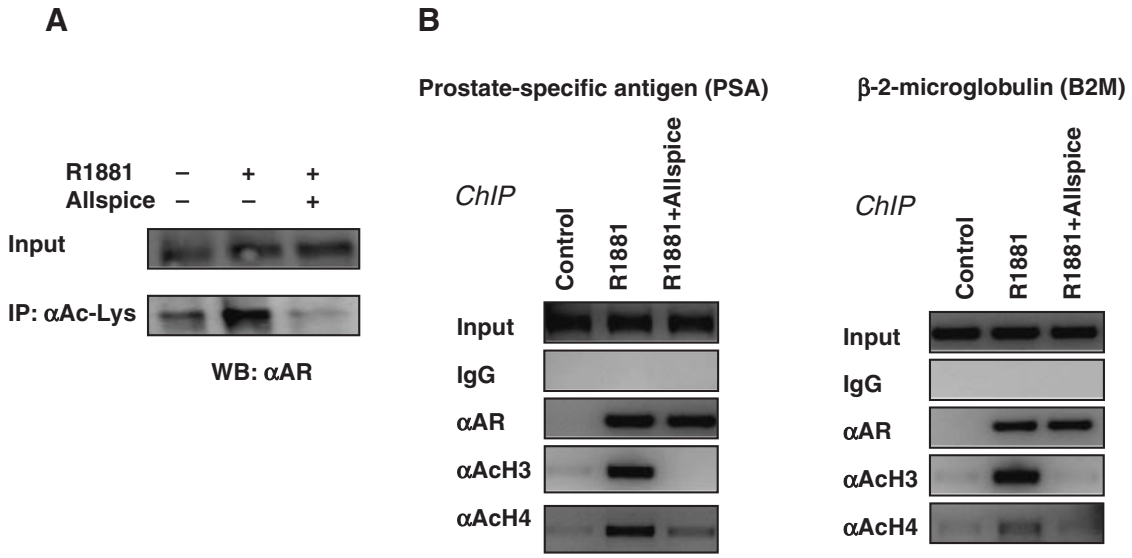

Fig. 3. Allspice Treatment Led to Reduced AR and Histone Acetylation.

LNCaP cells were treated in the absence and presence of R1881 or with allspice, either individually or in combination. Three days later, cell lysates were immunoprecipitated with anti-ac-lysin and blotted with anti-AR (A). For ChIP assays, cells were processed with anti-AcH3 and AcH4 (B).

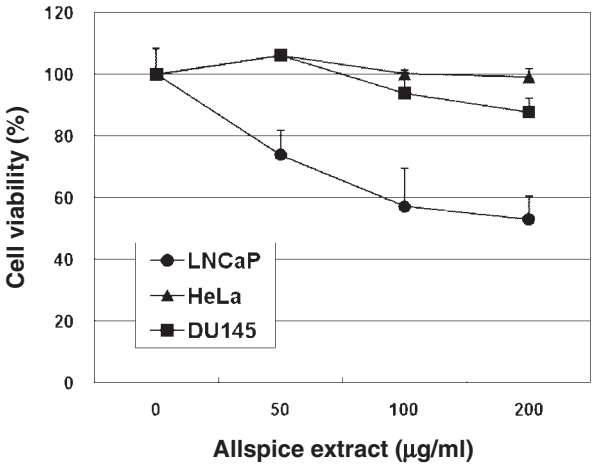

Fig. 4. The Effect of Allspice Extract on Various Cancer Cell Growths.

LNCaP, DU145, and HeLa were seeded in 96-well culture plates and incubated overnight with RPMI 1640 or DMEM containing 10% FBS. The cells were then treated with serum-free medium containing allspice extracts (final concentration, 100 μg/ml) for 48 h. The results represent mean values with S.D.

by R1881, whereas allspice treatment reduced AR acetylation. These results indicate that allspice suppressed AR acetylation in LNCaP cells. For ChIP assays, the cells were processed with anti-AR, AcH3, and AcH4. In Fig. 4B, as a graphic representation shows, AR was dramatically increased by R1881 or the combination of R1881 and allspice in the AR target genes, prostate specific antigen (PSA), and β-2-microglobulin (B2M) genes. Acetylation of histone H3 and H4 increased with treatment by R1881 alone; however, significant decreases of histone H3 and H4 acetylation were observed with allspice treatment. Together, these experiments indicate that repression of AR-mediated transcription was induced due to regulation of histone and non-histone acetylation, after inhibition of co-activator recruitment.

Allspice induces cancer cell death

To determine the effect of allspice on cancer cell viability, we carried out an MTT test on LNCaP, DU145 and HeLa cells. Each cell was incubated with various concentrations of allspice extract for 48 h, and viable cells were measured by MTT assay. Formazan production decreased in a dose-dependent manner with the addition of allspice extracts in LNCaP cells, and cell viability was reduced approximately 52% at 200 μg/ml, whereas allspice extract did not affect cell viability in DU145 or HeLa cells (Fig. 4). This result suggests that allspice extract has potent antitumor activity against prostate cancer in a culture model, supporting the thesis that HAT is a molecular target for anticancer therapy. Our data suggest that modulation of AR acetylation by HAT inhibitors controls aberrant cellular growth in the LNCaP cell line.

Discussion

There is growing evidence suggesting a link between alterations in chromatin structure and the development of cancer by histone hyperacetylation and hypoacetylation.[8,9,11,12] In prostate cancer, AR function is critical to the development and progression of cancer.[3] Co-activators associate with AR in an androgen-dependent manner, and co-activator proteins augment AR activity

through several functions. These co-activators, especially p/CAF, p300, and Tip60, contain intrinsic HAT activity directly acetylating both histones and non-histones, so as to regulate AR activity.[1,6,11,13] In the present study, we used a prostate cancer model to investigate the connection between histone and non-histone (AR) acetylation and HAT inhibitors. First, we screened approximately 500 edible plant extracts to select a candidate with a high HAT inhibitory effect. The hot water extract of allspice exhibited the highest HAT inhibitory effects and also enzyme specific inhibitory activities on p300 and CBP in a dose-dependent manner. As suggested in recent studies, the development of HAT inhibitors from dietary compounds is the next therapeutic target, following HDAC inhibitors.[5,11] Recent investigations have suggested that several dietary factors, *viz.*, garcinol, curcumin, and anacardic acid, have the ability to inhibit HAT activities. Garcinol[14] has been found to inhibit p300 and PCAF *in vitro* and *in vivo*, anarcardic acid[17] to inhibit TIP60 as well as p300 and PCAF, and curcumin[16] to inhibit p300 and PCAF. These dietary compounds are associated with the prevention of cancer and other diseases. There have been several reports on phenolics found to be effective against prostate cancer from grape seeds,[24–26] green tea extracts[27] and blueberry extracts.[28] Allspice, consisting of dried pimento berries, is known to have antioxidant activity and contains eugenol as its main active component, cinnamaldehydes, vanillin, and many phenolic compounds.[29] Our present results suggest that the phenolic compounds of allspice have a potentially important role in managing prostate cancer cell growth *via* the inhibition of HAT activity. Hence, we hypothesize that HAT inhibitors from allspice can inhibit AR-dependent prostate cancer cell growth by repressing AR-mediated transcription activation. Importantly, allspice extracts repressed the activity of the PSA-LUC reporter in a dose-dependent manner, by up to 70%. RT-PCR analysis further substantiated that allspice extract can repress AR-mediated transcriptional regulation. These data together suggest that anti-HAT activity from allspice represses AR-mediated transcriptional activation by inhibiting co-activator function.

CBP/P300 acetylates both histones and non-histone proteins, regulating their activity.[23] Several studies have reported that AR can be acetylated *in vitro* by p300, P/CAF, and TIP60.[6,30] A recent study indicates that AR is also acetylated in the presence of the HDAC inhibitor TSA in a prostate cancer cell line.[31] These findings prompted us to test whether AR acetylation would be regulated by allspice extract, a potent HAT inhibitor. Treatment with allspice extract dramatically decreased the acetylation of AR in IP western analysis; furthermore, a decrease in histone H3 and H4 acetylation within AR target genes was induced by allspice extract in ChIP experiments. Taken together, our data indicate that anti-HAT activity prevents hyper-acetylation of AR and histones through inhibition of p300/CBP HAT activities, resulting in repression of AR-mediated transcription.

AR is also involved in the development and progression of prostate cancer, one of the most frequently diagnosed cancers in males. It has been reported that AR-mediated transcriptional activation is correlated with prostate cancer cell growth. According to an MTT assay, cell viability was reduced approximately 52% by allspice treatment in an androgen-sensitive LNCaP cell line, but cell viability was not changed in androgen insensitive cells, DU145 and HeLa. This result is in accordance with the recent findings of Fu *et al.* (2003),[31] who have studied the promotion of prostate cancer cell growth by androgen receptor acetylation. Kang *et al.* (2006)[32] studied enhancement of caspase-3-dependent brain cancer cell death induced by a HAT inhibitor, curcumin. This resulted in suppression of AR translation activities, leading to expression of AR-regulated genes.

In summary, we conclude that the water extract of allspice is potentially an HAT inhibitor. The fact that mimicking mutants of AR acetylation enhances prostate cancer cell growth and the fact that expression of AR co-activators in prostate cancer tissues correlates with AR expression, prostate cancer progression, and recurrence suggest that AR acetylation contributes to the development of androgen-independent prostate cancer.[6,33] Inhibition of the AR acetylation process and AR co-activator binding is likely to lead to the development of new therapeutic drugs for prostate cancer.

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