



Effect of anti-histone acetyltransferase activity from *Rosa rugosa* Thunb. (Rosaceae) extracts on androgen receptor-mediated transcriptional regulation

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ARTICLE INFO

Article history:

Received 30 October 2007

Received in revised form 5 May 2008

Accepted 6 May 2008

Available online 13 May 2008

Keywords:

Rosa rugosa Thunb.
Histone acetyltransferase
Androgen receptor
Prostate cancer

ABSTRACT

Ethnopharmacological relevance: *Rosa rugosa* Thunb. (Rosaceae) has been traditionally used for treatments of diabetes, chronic inflammatory diseases, pain, and anticancer in Korea.

Aim of study: We investigate the inhibitory effect of histone acetyltransferase activity from the methanol extract of stems of *Rosa rugosa* on androgen receptor-mediated transcriptional regulation.

Materials and methods: For the present study, *Rosa rugosa* methanol extract (RRME) was obtained from stem part of *Rosa rugosa* using methanol extraction. Histone acetyltransferase assay were performed to measure the inhibitory effect on acetylation, reporter assay, real-time PCR and ChIP assay were performed to measure androgen receptor-mediated transcriptional regulation, and MTT test were performed to measure cell viability.

Results: RRME inhibited both p300 and CBP (60–70% at 100 µg/ml) activity. We show RRME mediates agonist-dependent androgen receptor (AR) activation and suppresses antagonist-dependent inhibition. RRME treatment also decreased transcription of AR regulated genes and also reduced histone H3 and AR acetylation in the promoters of prostate-specific antigen (PSA) and β-2-microglobulin (B2M). Finally, RRME treatment reduced the growth of LNCaP, a human prostate cancer cell line.

Conclusion: These results demonstrate RRME is a potent HAT inhibitor, which reduced AR and histone acetylation leading to decreased AR-mediated transcription and reduced LNCaP cell growth.

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1. Introduction

Prostate cancer is the second leading cause of male-death worldwide and is typically treated with hormone therapy which blocks testosterone signaling through the androgen receptor (AR) (Yoon and Wong, 2006). Although hormone blockade therapy is initially effective, most patients eventually progress into hormone refractory or androgen-independent cancer (Lu et al., 2007). AR,

a member of the steroid receptor superfamily, is a transcription factor that mediates androgen action in cells (Lu et al., 2007). Post-translational AR modifications, including acetylation and phosphorylation, are key post-translational modifications that influence human prostate cancer cell growth (Daskivich and Oh, 2006). TIP60, P300, and PCAF enhance AR transcriptional activity by directly acetylating the receptor and also by up-regulating histone acetylation of AR target genes. AR acetylation regulates co-regulator recruitment and growth properties of the receptors in cultured cells and *in vivo*. AR acetylation mimic mutants results in reduced apoptosis and enhanced growth, properties that correlate with altered promoter specificity for cell-cycle genes (Fu et al., 2004; Manoharan et al., 2007).

Recent studies implicate alteration in chromatin structure by histone hyperacetylation/deacetylation is important in eukaryotic

Abbreviations: HAT, histone acetyltransferase; RRME, *Rosa rugosa* methanol extract; AR, androgen receptor; CBP, CREB-binding protein; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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gene transcription, carcinogenesis, and cancer therapy (Yoon et al., 2003). The reversible histone acetylation is controlled by histone acetyltransferases (HAT) and histone deacetylases (HDAC), which catalyze the addition or removal of acetyl groups from lysine residues (Inche and La Thangue, 2006; Stimson et al., 2005).

Translocation, amplification, overexpression, or mutation of HAT genes occurs in a variety of cancers, especially those of epithelial origin (Davis and Ross, 2007). The two closely related HATS, p300 and CBP function as transcriptional cofactors for a number of nuclear proteins including oncoproteins (e.g. myc, jun and fos), transforming viral proteins (e.g. E1A and E6), and tumor suppressors (e.g. p53, E2F, Rb, and BRCA1) (Iyer et al., 2004). HAT activity directly interacts with tumor suppressors to enhance their transcriptional activity. Mutation in the HAT active site abolishes transactivation capability of HAT proteins like p300/CBP (Balasubramanyam et al., 2004a,b). CBP and p300 are found disrupted by translocations in leukemia with translocation partners including MLL, MOZ, and MORF (Borrow et al., 1996; Panagopoulos et al., 2001; Sobulo et al., 1997). A developmental disorder, Rubenstein-Taybi syndrome (RTS), is caused by germ-line mutations in *CBP*, and patients with RTS are predisposed to cancer, usually childhood tumors (Iyer et al., 2004). These enzymes are current targets of cancer therapy.

HAT inhibitors as the anticancer effects were relatively less documented to HDAC inhibitors. HAT inhibitors, garcinol (Balasubramanyam et al., 2004a), curcumin (Balasubramanyam et al., 2004b), and anacardic acid (Sun et al., 2006), are isolated from natural products. These compounds have aided investigations into HAT functions, although their potential for development as clinical drug candidates remains to be determined. In the present study, we screened approximately 500 edible plant extracts for the ability to inhibit HAT activity. *Rosa rugosa* methanol extract (herein referred to as RRME) was an effective HAT inhibitor, especially p300 and CBP.

Rosa rugosa Thunb. (Rosaceae) has been used as folk medicines to treat several disorders, such as diabetes, chronic inflammatory diseases, pain, and anticancer in Korea (Jung et al., 2005; Yoshizawa et al., 2000; Ng et al., 2004; Park et al., 2005). Both aerial and underground parts of *Rosa rugosa* including flower, stem, fruit, and root contain high amount of phenolic compounds (Hashidoko, 1996; Park et al., 2005).

We demonstrate RRME is able to inhibit AR and HAT acetylation. We also show RRME can induce prostate cancer cell death. Therefore, the aim of the present study was to investigate an effect of anti-histone acetyltransferase activity from RRME on androgen receptor-mediated transcriptional regulation and suggest the possible mechanisms of natural compounds to support the further study of the candidate in anti-prostate cancer treatment.

2. Materials and methods

2.1. Sample and reagents

The stem parts of *Rosa rugosa* Thunb. (Rosaceae) were collected from Goseong-gun, Gangwon-do, Korea, and were authenticated by Dr. Sang In Shim, at Gyeongsang National University. The voucher specimen was deposited in the same institute. RPMI-1640 medium, antibiotics, and fetal bovine serum (FBS) purchased from Gibco BRL (Grand Island, NY, USA). HAT activity colorimetric assay kit was obtained from Biovision (Mountain View, CA, USA). Easy-spin total RNA extraction kit and Maxime RT premix kit was from Intron (Seoul, Korea). Antibodies were purchased from Upstate Biotechnology (Charlottesville, VA, USA) and Santa Cruz (Santa Cruz, CA, USA). Lipofectamine 2000

transfection reagent was from Invitrogen (Carlsbad, CA, USA). PGL3-PSA construct was a kind gift from Dr. Kyung-Sup Kim (Yonsei Univ.). Protein A/G PLUS agarose bead was from Santa Cruz.

2.2. Preparation of extracts

The stems of *Rosa rugosa* (1 kg) were dried and extracted with methanol (3 × 4.0 l) for 3 days at room temperature. The extract was filtered, concentrated under reduced pressure and lyophilized to yield 63 g from 1 kg of stems, which was stored at –20 °C until used.

2.3. Cell culture

Human prostate cancer cell line LNCaP and human breast cancer cell line MCF-7 were obtained from American Type Culture Collection (Manassas, VA, USA). LNCaP Cells were grown in RPMI 1640 and MCF-7 were grown in DMEM with L-glutamine supplemented with 10% FBS, 1% antibiotics, and antimycotics. All cultures were maintained in humidified chamber at 37 °C with 5% CO₂.

2.4. HAT activity assay

LNCaP cell nuclear extract was prepared as described previously (Dignam et al., 1983). HAT activity assays were performed using nuclear extracts following the manufacturer's protocol (Biovision). Nuclear extracts were immunoprecipitated (IP) using anti-p300 and anti-CBP. Pre-cleared nuclear extract (150 µg) was incubated with antibodies overnight with Protein A/G PLUS agarose beads at 4 °C. Afterwards, beads were washed with HAT assay buffer (50 mM Tris, pH 8.0, 10% glycerol, 0.1 mM EDTA) and assayed for HAT activity.

2.5. Reporter assay

LNCaP cells were seeded at 5 × 10⁵ per well in six-well culture plates and incubated overnight with RPMI 1640 containing 10% FBS. At 90% confluence, a 0.5 µg of pGL3-PSA and 1 ng of pGL-5 SV40 per six wells were transfected into LNCaP cells to perform a dual luciferase assays (Promega, Madison, WI). Medium was replaced with phenol red-free RPMI 1640 media containing 5% charcoal-stripped fetal bovine serum. After 48 h, 50 nM of the synthetic androgen R1881 treatment, 20 µM flutamide (Sigma-Aldrich) or RRME (100 µg/ml) was added. Cell were lysed and analyzed after 18 h by MicroLumat Plus (EG & G BRTHOLD, Germany).

2.6. Real-time PCR analysis

LNCaP cells were seeded at 5 × 10⁵ per well in six-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. After 48 h, 50 nM of the synthetic androgen R1881 treatment and RRME (100 µg/ml) was added. After overnight incubation, total RNA was isolated by Easy-spin total RNA extraction kit. Real-time PCR analysis and quantification were performed with SYBR Green PCR Master mix Reagents using an ABI Prism 7700 sequence detection system (Applied biosystems, Foster city, CA, USA). The singularity and specificity of amplifications were checked by Dissociation Analysis Software. All samples were normalized to GAPDH. Primer sequences used were for PSA, 5'-AGTCTGAGGAGGTCTTCTGGTG-3' and 5'-GAGGTCGTGGCTGGAGTCATCA-3' and 5'-GACTTGATAATAGCT

CCTCTGGT-3'; for the NKX-3.1, 5'-CTGTCAGCCCCTGAACGG-3' and 5'-AACCATATCTTCACCTGGGTCTCC-3'. Primers for the GAPDH amplification were 5'-CGCGGGGCTCTCCAGAACATCATCC-3' and 5'-CTCCGACGCC TGCTTACCACCTTCTT-3'. PCR was performed as follows: 94°C, 2 min, 40 cycles, 94°C, 30 s, 55°C, 30 s, 72°C, 30 s, and an extension at 94°C for 5 min. All reactions were performed in triplicate. Relative expression levels and S.D. values were calculated using the comparative method.

2.7. 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 μ l of anti-Ac-lysine (Santa Cruz) overnight with Protein A/G PLUS agarose beads at 4°C. Western analysis was performed with anti-AR (Upstate) and was visualized by chemiluminescence.

2.8. ChIP assays

For ChIP assays, we first isolated chromatin as described (Fu et al., 2000). In brief, approximately 2×10^9 LNCaP cells in 150-mm dishes were first treated with PBS containing 1% formaldehyde for 10 min, washed twice with PBS, and incubated with 100 mM Tris, pH 9.4, 10 mM dithiothreitol (DTT) at 30°C for 15 min. The cells were rinsed twice with PBS and resuspended in 600 μ l of SolA buffer (10 mM Hepes pH 7.9, 0.5% NP-40, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) by pipetting. After a short spin (5 min at $800 \times g$), pellets were resuspended in SolB (20 mM Hepes pH 7.9, 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA) containing protease inhibitors by vigorous pipetting to extract nuclear proteins. After centrifugation at $15,700 \times g$ 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 chromatin into fragments with an average length of 0.5–1 kb. The ChIP assays were then performed with indicated antibodies essentially as described but omitting SDS in all buffers (Shang et al., 2000). Primers used for ChIP analysis: for PSA, 5'-CATGTTACATTAGT ACACCTGGCC-3' and 5'-TCTCAGATCCAGGCTTGCTTACTGRC-3'; for B2M, 5'-AGACTTCCCAATTTT GCCATCCTA-3', 5'-AAAGGCCTGAAATGTT-AGT-GTTGAGT-3'.

2.9. Cell viability test

LNCaP and MCF-7 cells were seeded at 1×10^4 cells per well in 96-well culture plates and incubated overnight with RPMI 1640 containing 10% FBS. Cells were then treated with serum-free medium-containing RRME (0–200 μ g/ml) for 48 h. After 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) solution in PBS was added and the plates were incubated for 2 h. Afterwards, the medium was removed and the formed blue formazan was solubilized with DMSO. Absorbance was measured at 570 nm with background subtraction at 630 nm.

3. Results and discussion

3.1. Anti-HAT activity from RRME

In the present study, we used a prostate cancer model to screen for medicinal plant extracts which could influence histone and non-histone acetylation. We screened approximately 500 medicinal plant extracts to select a candidate with a high HAT inhibitory effect. RRME exhibited one of the highest HAT inhibitory effects among

the library, in the concentration-dependent manner (0–200 μ g/ml) (Fig. 1A). To further examine the enzyme-specific anti-HAT activity RRME, we immunoprecipitated p300 and CBP and incubated them with increasing RRME concentrations. RRME (100 μ g/ml) inhibited the activity of p300 and CBP by approximately 60–70%, whereas the HAT activity with IgG was not affected (Fig. 1B). Recent investigations suggest that alterations in the activities of HDACs and HATs are associated with cancer development (Stimson et al., 2005; Davis and Ross, 2007). In prostate cancer, AR function is a critical determinant of cancer pathogenesis and progression, and recent studies have shown alterations in histone acetylation occur in patients with prostate cancer (Popov et al., 2007). Previous studies also demonstrate AR is acetylated *in vitro* and *in vivo* by p300, p/CAF, and TIP60. As suggested in recent studies, development of HAT inhibitors from dietary compounds is the next therapeutic target (Davis and Ross, 2007; Stimson et al., 2005). Our results suggest RRME may possess natural compounds to manage prostate cancer cell growth by inhibiting HAT activity.

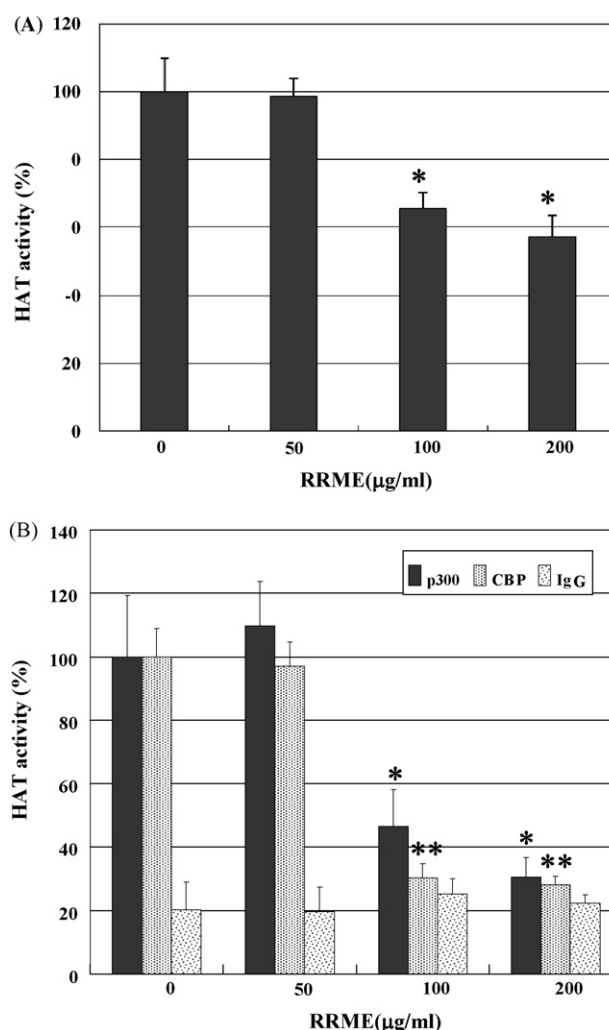


Fig. 1. Inhibitory effect of RRME on HAT activity. (A) Histone acetyltransferase assays were performed using varying concentration of RRME. Data are expressed as the mean \pm S.D. of three-independent experiments, and presented as the percentage of HAT activity. * $p < 0.05$ is assessed as significant differences between the untreated group and RRME treated group. (B) Performed with immunoprecipitated product using anti-p300, CBP, and IgG. Data are expressed as the mean \pm S.D. of three-independent experiments, and presented as the percentage of HAT activity by p300 or CBP. * or ** $p < 0.05$ is assessed as significant differences between the untreated group and RRME treated group.

3.2. Repression of AR-mediated transcriptional by RRME

Based on anti-HAT results, further examination of RRME revealed a significant reduction of transcription activity of a PSA promoter luciferase reporter gene. To test whether RRME modulate AR-mediated transcription in an androgen-sensitive prostate cancer cell, we measured the luciferase activity using an androgen-dependent reporter construct (pGL3-PSA) bearing ARE (androgen responsive element) in the LNCaP cell line. Cells were incubated with or without synthetic androgen R1881, and then were treated with 100 $\mu\text{g}/\text{ml}$ of RRME (final concentration). Results show RRME decreased luciferase activity by 50% at 100 $\mu\text{g}/\text{ml}$ concentration, compared with R1881 alone. This suggests RRMEs containing anti-HAT activity repress AR-mediated transcription (Fig. 2A). To investigate the effect of RRME treatment on antagonist-bound AR-mediated transcription, we examined if AR activation of target genes by R1881 were challenged by addition of flutamide. Flutamide (20 μM) partially repressed approximately 50% of the transcription activity of androgen responsive PSA promoter; however, RRME treatment synergisti-

cally enhanced the antagonistic effects on reporter gene activity (Fig. 2A).

To investigate the effects of RRME on the expression of endogenous AR target genes, we next selected three AR responsive genes including NKX-3.1 and PSA (Yoon et al., 2006). Total RNA was extracted 18 h after co-treatment with RRME and R1881. Consistent with the reporter assay, R1881 treatment increased AR target gene transcription. However, RRME (100 $\mu\text{g}/\text{ml}$) treatment repressed mRNA expression of NKX-3.1 and PSA (Fig. 2B). Thus, RRME repressed the expression of AR target genes probably by reducing HAT activity.

AR expression in primary prostate cancer can be detected throughout hormone sensitive and hormone refractory cancers (Yoon et al., 2006). In prostate cancer therapy, the first-line of treatment is to specifically inhibit AR activity through either ligand depletion and/or through antagonists that bind directly AR (Burd et al., 2006). In this experiment, the combinatorial effect of flutamide and RRME was a more effective than either one individually. This is significant because AR antagonists, such as flutamide and bicalutamide, are broadly used in the treatment of prostate cancer

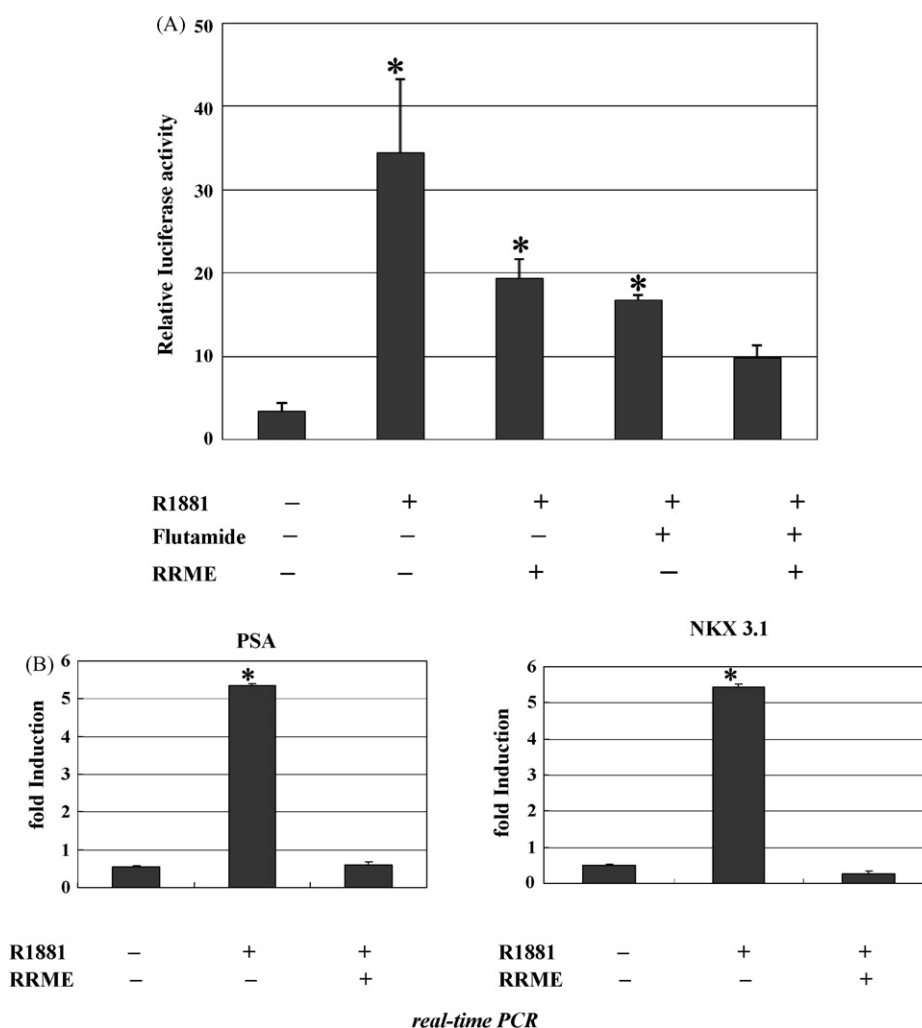


Fig. 2. Repression of AR-mediated transcriptional activation by RRME. (A) LNCaP cells were transiently transfected with pGL3-PSA and pGL3 SV40. Cells were treated with or without R1881, and then flutamide (20 μM) and RRME (100 $\mu\text{g}/\text{ml}$). Data are expressed as the mean \pm S.D. of three-independent experiments, and presented as the percentage of relative luciferase activity. * $p < 0.05$ is assessed as significant differences between the untreated group and R1881 only or between the untreated group and R1881/RRME treated or between the untreated group and R1881/flutamide or between the untreated group and R1881/RRME/flutamide group. (B) Real-time PCR analysis of PSA and NKX-3.1 was performed 16 h after treatment with RRME (100 $\mu\text{g}/\text{ml}$), with or without R1881. Data are expressed as the mean \pm S.D. of three-independent experiments, and presented as fold induction. * $p < 0.05$ is assessed as significant differences between the untreated group and R1881 treated or between the untreated group and R1881/RRME treated group.

(Daskivich and Oh, 2006). Developing drugs with increased capacity to promote antagonist activity is likely to improve the efficacy of current anti-hormone therapy.

3.3. Reduction of AR and histone acetylation by RRME

Our findings that repression of AR-mediated transcription was mediated by decreased HAT activity, led us to examine whether unregulated AR acetylation by R1881 treatment would be modulated by RRME. To determine whether the treatment of RRME led to reduced AR and histone acetylation, we performed IP and ChIP assays in cells treated with or without RRME. LNCaP cells were treated with or without R1881 or RRME, either individually or in combination. After 3 days, cells were analyzed via IP Western with anti-AR and anti-acetyl-lysine antibodies. In presence or absence of RRME, AR was equally detected in the immunoprecipitate by acetyl-lysine antibody (Fig. 3A). Acetylated AR increased by R1881 treatment, whereas RRME reduced AR acetylation. These results demonstrated that RRME suppressed AR acetylation in LNCaP cells. Next we examined the acetylation status via a ChIP assay using anti-AR, AcH3, and AcH4. In AR responsive genes (prostate-specific antigen (PSA) and β -2-microglobulin (B2M) genes), AR acetylation was dramatically increased by treatment with R1881 or by the combination of R1881 and RRME (Fig. 3B). Histone H3 and H4 acetylation only increased with R1881 treatment; however, there was a significant decrease of histone H3 and H4 acetylation with RRME treatment. Together, these experiments suggest that repression of AR-mediated transcription resulted from histone and AR hypoacetylation. Thus, RRME might

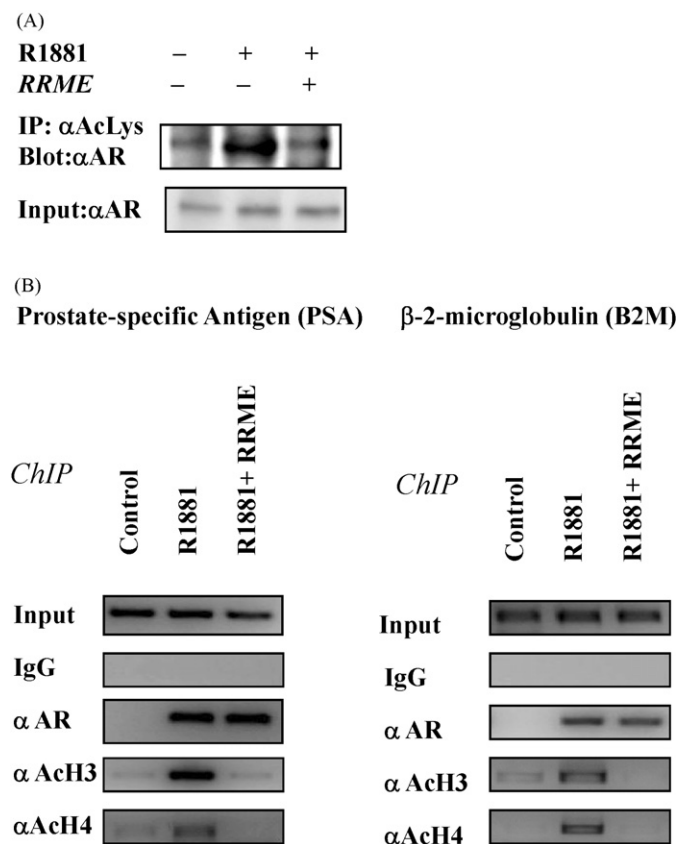


Fig. 3. RRME reduced AR and histone acetylation. (A) LNCaP cells were treated with or without R1881, alone or in combination. Cell lysates were immunoprecipitated with anti-ac-lysine and blotted with anti-AR. (B) ChIP assays were processed with anti-AcH3 and AcH4.

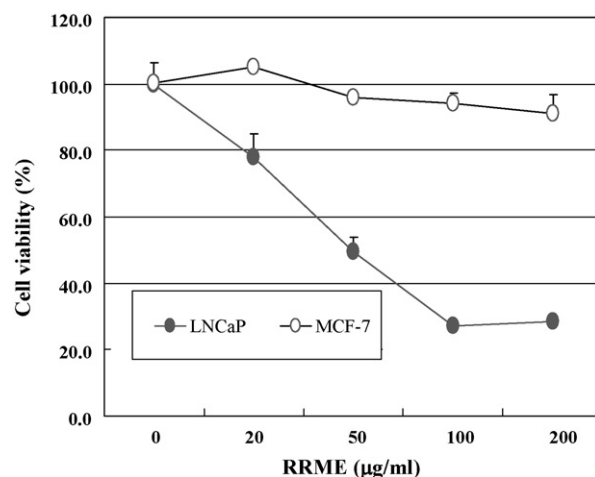


Fig. 4. The effect of RRME on prostate cancer cell growth. LNCaP and MCF-7 were treated with serum-free medium containing RRME (0–200 μ g/ml) for 48 h. Results represent the mean value with S.D. from five-independent experiments.

inhibit hyperacetylation of both histone and AR through anti-HAT activity.

3.4. RRME induces cancer cell death

To examine the effect of RRME on cancer cell viability, we performed the MTT test in LNCaP and MCF-7 cell lines. AR-mediated transcriptional activation is correlated with prostate cancer cell growth. RRME treatment increased LNCaP cell death by approximately 70%, but did not affect MCF-7 cells, a breast cancer cell (Fig. 4). These results confirm the recent findings of Fu et al. (2003) who show increased prostate cancer cell growth induced by androgen receptor acetylation. Breast cancer is also affected by nuclear receptor activity, namely the estrogen receptor (ER) but RRME treatment does not affect to MCF-7 cells in this range of concentrations. Our data suggest modulation of AR acetylation, by HAT inhibitors, control aberrant cellular growth in the LNCaP cell line. Furthermore, the results suggest RRME may have potent antitumor activity against prostate cancer culture model, supporting HAT as a molecular target for anti-prostate cancer therapy.

4. Conclusion

Recent investigations suggest altering activities of HDACs and HATs are associated with cancer development. In prostate cancer, AR function is a critical determinant of cancer pathogenesis and progression, and alterations in histone acetylation occur in patients with prostate cancer (Popov et al., 2007). We used a prostate cancer model to investigate how HAT inhibitors influence histone and androgen receptor (AR) acetylation and also affect cell growth. We identified a candidate extract from *Rosa rugosa* (RRME) that contained HAT inhibitory activity. The anti-HAT activity of RRME has potential as an anti-prostate cancer agent by inhibiting HAT activity. Currently, there is a lack of effective second line therapies when prostate cancer becomes androgen insensitive and longer responds to androgen. Interestingly, our results suggest that RRME synergistically enhanced the antagonistic effects with flutamide, and it may be a potent candidate to develop a novel therapeutic drug or drug combination for prostate cancer treatment.

Acknowledgments

This study was supported by a grant (Code #20070301034007) from BioGreen 21 Program, Rural Development Administration,

Republic of Korea; a faculty grant of Yonsei University College of Medicine for 2007 (H.G. Yoon). We thank Goseong-gun for providing the stem of *Rosa rugosa*.

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