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Biochemical and Biophysical Research Communications 351 (2006) 26–32
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Hinokitiol, a metal chelator derived from natural plants, suppresses cell growth and disrupts androgen receptor signaling in prostate carcinoma cell lines

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Received 22 September 2006
Available online 10 October 2006

Abstract

Hinokitiol (β-thujaplicin), a troplone-related compound found in the heartwood of cupressaceous plants, strongly inhibits the proliferation of a broad range of tumor cell lines. This is the first report to demonstrate that hinokitiol, a metal chelator derived from natural plants, suppresses cell growth and disrupts AR signaling in prostate carcinoma cell lines. Our present studies indicate that hinokitiol suppresses androgen/AR-mediated cell growth and androgen-stimulated DNA synthesis by [^{3}H]thymidine incorporation in a dose- and time-dependent manner. Hinokitiol simultaneously suppresses the intracellular and secreted PSA levels, a marker for the progression of prostate cancer. Hinokitiol significantly represses the AR mRNA and protein expression in a dose- and time-dependent manner. Additionally, the ligand-binding assay shows that hinokitiol blocks binding of the synthetic androgen [^{3}H]R1881 to AR in LNCaP cells. These findings collectively suggest that hinokitiol is potentially effective against prostate cancer *in vitro*, and thus it might become a novel chemopreventive or chemotherapeutic agent for prostate cancer.

Keywords: Hinokitiol; Prostate cancer; Androgen receptor; PSA; R1881

The androgen receptor (AR) has been studied extensively since the cloning of its cDNA structure in 1988 [1], to elucidate its biological role in the development and progression of prostate cancer. Over-expression of the AR signaling pathway may be linked to the progression of prostate cancer [2]. At present, prostate cancer remains the most commonly diagnosed malignancy and is second only to lung cancer as the leading cause of cancer-related deaths in American men [3]. Since metastatic prostate cancer is an androgen-dependent disease, at least initially for a variable period, androgen deprivation and the blocking of androgen at the AR pathway usually result in a favorable clinical response and dramatic tumor regression [4,5].

AR belongs to the nuclear steroid hormone receptor superfamily and is the essential mediator for androgen action. It is complexed in the cytoplasm to chaperone proteins that keep the receptor in a transcriptionally inactive form. In principle, testosterone synthesized primarily in the testes is further converted to the more potent androgen dihydrotestosterone (DHT) by the enzyme 5α-reductase (5α-R), which is localized primarily in the prostate. Upon binding of the native ligand, DHT, AR dissociates from the chaperone and rapidly translocates into the nucleus where it binds to androgen response elements (AREs) and activates target genes such as prostate-specific antigen (PSA) gene [6,7]. PSA belongs to an androgen-regulated kallikrien-like serine protease produced by both prostate epithelial cells and prostate cancer, and is the most commonly used serum marker for the diagnosis and progression of prostate cancer. PSA gene expression is regulated by androgen through several well-characterized AREs to which the AR binds to initiate transcription [8].

Hinoktiol (β-thujaplicin) is a troplone-related compound found in the heartwood of cupressaceous plants and has various biological actions, including anti-bacterial,

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.09.166

anti-tumor, antioxidant, inducing of cell differentiation, and apoptosis [9–12]. Its anti-tumor action is attributable to a β-diketone structure in its molecule, which facilitates the formation of metal chelates in the presence of various metal ions [13].

The LNCaP cell line is one of the best *in vitro* models for human prostate cancer studies because it possesses several features of human prostate carcinoma, including (i) a high response to androgen for cell growth and the expression of a mutant AR and (ii) the production of PSA, which is a sensitive and specific tumor marker for prostate cancer screening and assessment [14]. Using this cell model, we designed a series of experiments to evaluate the impact of hinokitiol on cell growth and the expression of AR and PSA in prostate cancer cells.

To date, the effect of hinokitiol on prostate cancer has not been reported, despite a few sporadic studies dealing with its action in several other kinds of tumor cells. The present study is the first to demonstrate that hinokitiol, a metal chelator derived from natural plants, suppresses cell growth and disrupts AR signaling in prostate cancer cell lines. Our main findings may be summarized as follows: (1) hinokitiol suppresses cell growth and DNA synthesis, (2) hinokitiol down-regulates the expression of AR and PSA, and (3) hinokitiol blocks AR binding to androgens in prostate cancer cells.

Materials and methods

Materials. Hinokitiol was obtained from Anan Inc. (Japan). Fetal bovine serum and tissue culture media were obtained from Invitrogen (Grand Island, NY). The cell proliferation kit (XTT) and CytElisa™ Human PSA kit were obtained from Roche Diagnostics (Mannheim, Germany) and Cytimmune Science (Rockville, ML). Anti-AR and PSA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cyproterone acetate (CPA) and anti-β-actin antibody were obtained from SIGMA Chemical Co. (St. Louis, MO). R1881 was obtained from Daiichi Pure Chemicals (Japan), [^{3}H]R1881, [^{3}H]thymidine was obtained from NEN Life Science Products (Boston, MA).

Cell culture and XTT assay. LNCaP and PC-3 cells were obtained from the American Type Culture Collection and cultured routinely in 96-well culture plates at 5×10^3 cells/well in phenol red-free RPMI 1640 containing 5% normal FBS or dextran-charcoal-stripped FBS (CSS) with or without 1 nM R1881. After treatment with hinokitiol at different concentrations for 96 h, 50 μl of XTT labeling mixture was added to each well and the cells were incubated for an additional 6 h. The absorbance was measured at 450 nm with a reference wavelength of 650 nm on a 96-well microplate reader.

[^{3}H]Thymidine incorporation. LNCaP cells were grown in 96-well plates at 5×10^3 cells/well in phenol red-free RPMI 1640 containing 5% normal FBS or CSS with or without 1 nM R1881. After the treatment with hinokitiol at different concentrations for 24 h, the cells were pulsed with 1 μCi/well [^{3}H]thymidine for 4 h at 37 °C. After incubation, the cells were washed with PBS and collected to a multiwell UniFilter using an automatic multiple cell harvester (Filtermate 196, Packard). Fifty microliters of liquid scintillation fluid (Microscint-O, Packard) was added to each well and the radioactivity was recorded using a microplate scintillation counter (Packard Topcount).

RNA extraction and real-time RT-PCR. The total cellular mRNA from LNCaP cells was isolated using the Trizol reagent (Invitrogen, CA) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed on the ABI Prism 7700 Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems; Foster City, CA). The PCR primers and TaqMan probes for AR and β-actin were obtained from Applied Biosystems (Foster City, CA). The PCR conditions were as follows: an initial incubation at 50 °C for 2 min, then a denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative quantification of gene expression was carried out using the comparative C_T method.

PSA ELISA. LNCaP cells were seeded at 2×10^4 cells/well in 24-well plates. After 72 h the medium was changed to an androgen-free medium for an additional 24 h to deplete endogenous steroids prior to assay. The cells were then treated with hinokitiol at different concentrations, with or without 1 nM R1881, for 24 h. The supernatants were collected and subjected to low-speed centrifugation to remove cell debris. The PSA protein level in the culture medium was determined using the CytElisa™ Human PSA kit and normalized according to the total number of cells.

Western blot. LNCaP cells were lysed in ice-cold lysis buffer (50 mM Tris–HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). Lysate was sonicated and protein concentration was determined. An equal amount of protein from each lysate was separated by SDS–PAGE and then transferred onto PVDP membrane. After blocking with 5% nonfat dried milk/TBST, individual membranes were probed with antibodies specific to AR, PSA, and β-actin overnight at 4 °C. After incubation with horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands on the membrane were detected by chemiluminescence using an enhanced ECL detection kit (Amersham).

Competitive-binding assay for AR. LNCaP cells were plated at 2×10^5 in polylysine-coated dishes and grown to subconfluence in 5% CSS medium. After starvation in basal medium for 24 h, the cells were treated with 2 ml of serum-free RPMI 1640 containing 10 nM [^{3}H]R1881 and 1 μM triamcinolone acetonide to saturate progesterone and glucocorticoid receptors, in the presence or absence of 200-fold excess of cold competitors (R1881, CPA) or the indicated concentrations of hinokitiol. After additional incubation at 37 °C for 2 h, each sample was collected using a sampling harvester and unbound ligand was removed by extensive washes. Fiber filters that contained bound ligand were transferred to counting vials containing 5 ml of liquid scintillation fluid and counted with a multipurpose scintillation counter (Aloka LSC-5700).

Results

Hinokitiol suppresses the cell growth and DNA synthesis

To explore the novel therapeutic possibilities for prostate cancer of agents originating in natural plants, we first examined the cell viability by XTT assay to evaluate the effect of hinokitiol on prostate cancer. As shown in Fig. 1A, inhibition of cell growth occurred initially at about 5 μM, increased as the dose was increased, and maximal inhibition was achieved at 100 μM in LNCaP cells after treatment with hinokitiol for 96 h. In contrast, under the same conditions, there was no measurable increase in cell death in androgen-independent PC-3 cells, which suggests the existence of a selectively inhibitory mechanism mediated by AR signaling in prostate cancer.

The ability of hinokitiol to inhibit proliferation stimulated by androgen in LNCaP cells was also examined and results are presented in Fig. 1B. Androgen-enhanced LNCaP cell proliferation in medium with 1 nM R1881 is 1.5 times greater than that of vehicle-treated cells in androgen-free medium, which confirms the cell-growth response to androgen stimulation. Similarly, this androgen-stimu-

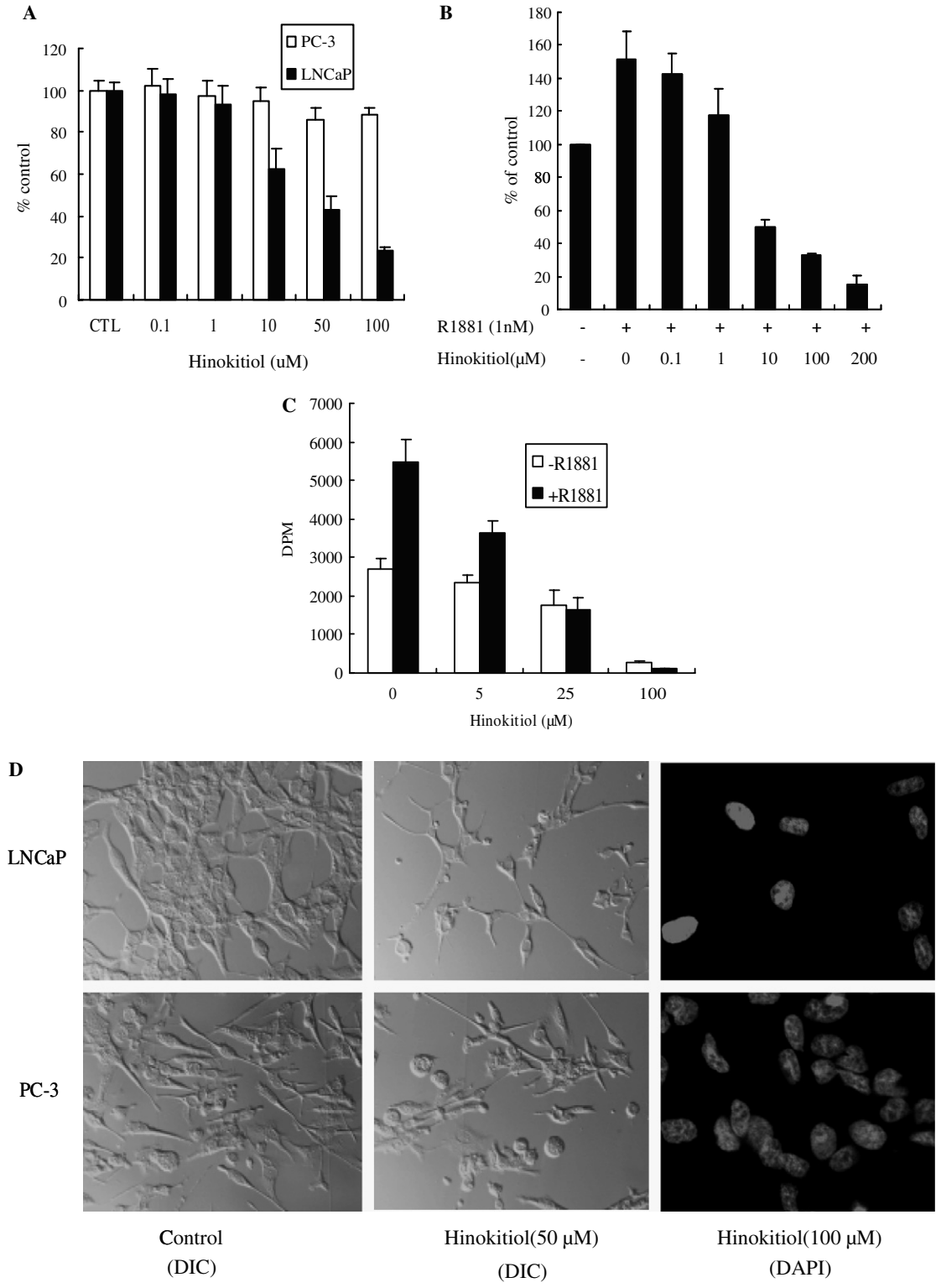

Fig. 1. Hinokitiol suppresses cell growth and DNA synthesis in prostate carcinoma cell lines. (A) LNCaP and PC-3 cells grown in normal 5% FBS medium were treated with vehicle (DMSO) or various concentrations of hinokitiol (0.1–100 μM) for 96 h. Cell viability was determined by XTT assay. (B) LNCaP cells grown in androgen-free 5% CSS medium were treated with vehicle or various concentrations of hinokitiol (0.1–200 μM) in the presence or absence of 1 nM R1881 for 96 h. Cell viability was determined by XTT assay. (C) LNCaP cells grown in 5% FBS or CSS medium in the presence or absence of 1 nM R1881 were treated with vehicle or various concentrations of hinokitiol (0.1–100 μM) for 24 h. The cells were pulsed with 1 μCi/well [^{3}H]thymidine, followed by incubation for an additional 4 h, and then the radioactivity was counted using a microplate scintillation counter. (D) the morphological changes of LNCaP and PC-3 cells were examined using light and fluorescent microscopes after treatment with vehicle, 50 or 100 μM hinokitiol for 72 h. The values are expressed as percentage of control and presented in means ± SD of at least four individual samples.

lated proliferation was inhibited markedly by hinokitiol in a dose-dependent manner.

To assess the effect of hinokitiol on DNA synthesis, the cells were pulse-labeled with [^{3}H]thymidine incorporation under the same conditions as described above. Androgen caused a striking increase in [^{3}H]thymidine uptake after the cells were exposed to 1 nM R1881 (Fig. 1C). However, the androgen-stimulated DNA synthesis was suppressed appreciably by hinokitiol in a dose-dependent manner. These data agree well with our results from cell proliferation, which indicates that hinokitiol strongly suppresses cell growth and DNA synthesis in prostate cancer cells.

Normal LNCaP cells are spindle-shaped and adhered loosely to the culture surface. After exposure of the cells to hinokitiol over three days, a typical neuron-like feature with fine and branched long cytoplasmic processes was observed in some LNCaP cells, but this failed to occur in PC-3 cells (Fig. 1D). However, apoptotic nuclear changes were not observed by fluorescent microscopy in either cell line, even when treated with higher doses. This observation is consistent with a previous report and most likely indicates cell differentiation rather than apoptosis mediated by hinokitiol in prostate cancer [12].

Hinokitiol inhibits secreted and intracellular level of PSA

To verify the effect of hinokitiol on the androgen-regulated expression of PSA, the intracellular PSA protein expression was analyzed by Western blot (see Fig. 2A). Control cells cultured in androgen-free medium constitutively expressed a significantly low level of PSA protein. With synthetic androgen R1881, the intracellular PSA level in these cells increased about 6-fold. This androgen-regulated PSA protein level was suppressed markedly by coculture with hinokitiol for 24 h.

Next, the secreted PSA stimulated by androgen was determined using an ELISA kit. In agreement with the results above, androgen pronouncedly enhanced the amount of PSA protein secreted into media. Similarly, hinokitiol reduced markedly the amount of secreted PSA stimulated by androgen after coculture with androgen (Fig. 2B). These findings are consistent with our hypothesis that the cell growth inhibited by hinokitiol is possibly involved in impairing the AR function by blocking AR signaling transduction.

Hinokitiol down-regulates AR mRNA and protein expression

To determine the effect of hinokitiol on the expression of AR mRNA, the steady-state level of AR mRNA was analyzed by real-time reverse transcription-PCR after treatment with hinokitiol at different concentrations.

As shown in Fig. 3A, the reduction of AR mRNA expression occurred initially at 5 μM, increased as the dose was increased, and the amount of inhibition rose to 80% at 100 μM by 8 h after exposure of LNCaP cells to hinokitiol. It is most likely that the decrease in AR mRNA level by

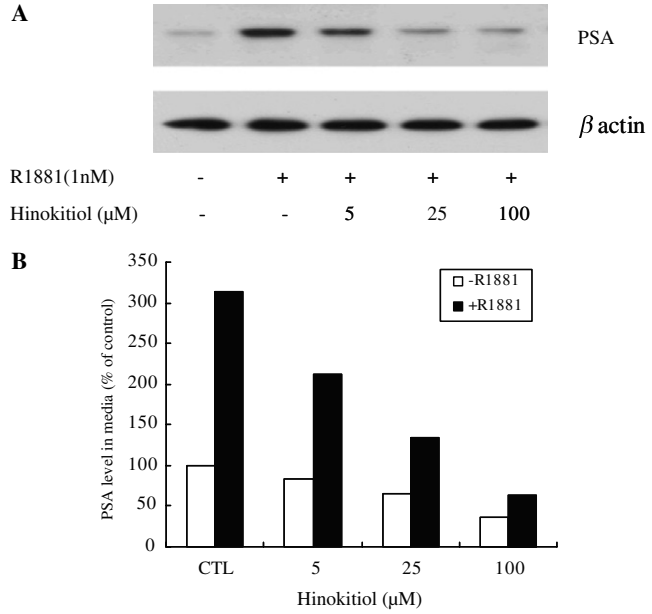

Fig. 2. Hinokitiol inhibits the intracellular and secreted PSA level. (A) the intracellular PSA protein level was determined by Western blot analysis after exposure of the cells for 24 h to various concentrations of hinokitiol in medium, with or without 1 nM R1881. β-Actin was probed as an internal control. (B) The secreted PSA in medium was determined using an ELISA kit after exposure of the cells for 24 h to various concentrations of hinokitiol in medium, with or without 1 nM R1881.

hinokitiol is attributable to the blocking of AR transcription. However, it cannot be ruled out that another factor is involved in the increased mRNA degradation.

To determine whether hinokitiol affects AR protein expression, Western blot analysis was performed after the cells were exposed to hinokitiol at different concentrations in 5% FBS medium. As shown in Fig. 3B, treatment of the cells with hinokitiol at low concentration resulted in a modest reduction of AR expression. As the dose was increased over a period of 48 h, AR expression stimulated by androgen was eliminated almost completely at concentrations higher than 25 μM, which demonstrates clearly that hinokitiol impairs AR function by interfering with AR transcription and translation.

To further confirm the ability of hinokitiol to suppress AR expression in androgen-stimulated LNCaP cells, androgen-stimulated AR expression was analyzed by Western blot following the coculture of hinokitiol with androgen in CSS medium. A similar pattern of the inhibition of AR protein expression was observed (see Fig. 3C). In androgen-stimulated cells, the amount of inhibition caused by hinokitiol is slightly lower than that found in normal serum at the same concentration. These findings indicate that hinokitiol not only suppresses AR mRNA expression, but also represses AR protein expression. However, the reduction of AR in protein seems to slightly lag behind the reduction in its corresponding transcript, possibly because of the time needed for protein turnover.

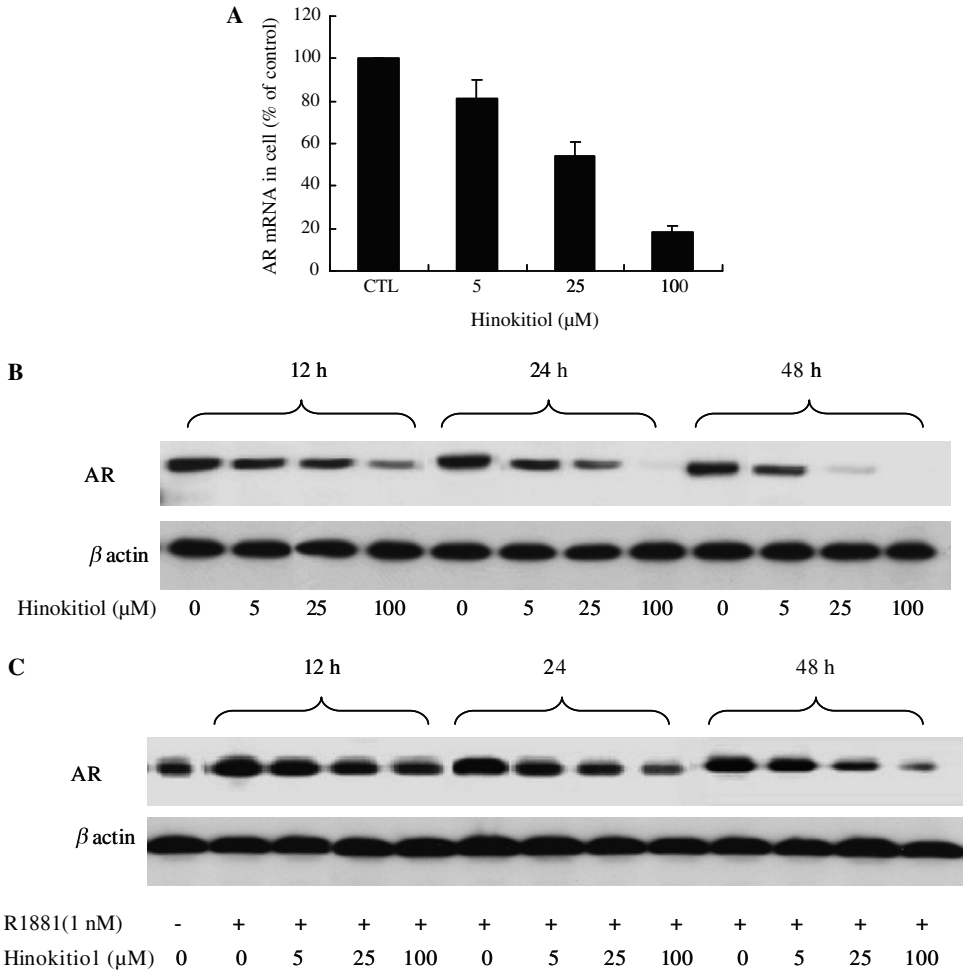

Fig. 3. Hinokitiol down-regulates AR mRNA and protein expression (A) AR mRNA expression in LNCaP cells was determined by quantitative real-time RT-PCR after exposure of the cells for 8 h to various concentrations of hinokitiol. (B) The cellular AR protein level was determined by Western blot analysis after exposure of the cells to various concentrations of hinokitiol in 5% FBS medium, (C) and in 5% CSS medium with or without 1 nM R1881. β-Actin was probed as an internal control.

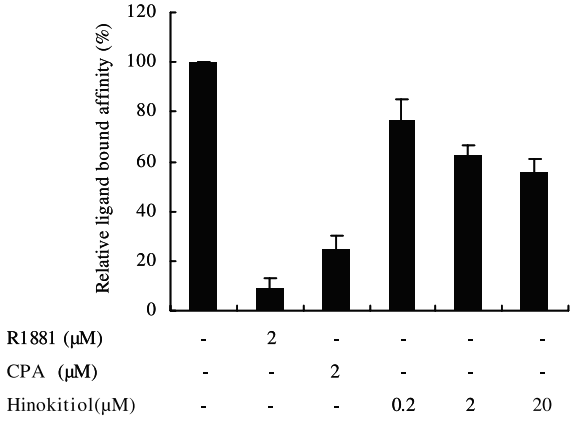

Fig. 4. Effect of hinokitiol on the ligand binding of AR. LNCaP cells grown to subconfluence were treated in serum-free medium containing 10 nM [^{3}H]R1881 in the presence or absence of 200-fold excess of cold competitor (R1881, CPA) or the indicated concentrations of hinokitiol for 2 h. The cells were harvested on the filters and the radioactivity was determined with a scintillation counter. [^{3}H]R1881 binding without competition was set as 100% and data were represented in means ± SD from three individual samples.

Hinokitiol blocks androgen binding to AR

To determine whether hinokitiol has a specific affinity to AR in LNCaP cells, the ability of hinokitiol to block the binding of [^{3}H]R1881 to LNCaP AR was examined by competitive radioligand-binding assay. LNCaP cells were incubated in steroid-free media containing 10 nM [^{3}H]R1881 with various concentrations of hinokitiol and reference drugs. As shown in Fig. 4, unlabeled R1881 bound to LNCaP AR at almost 90%, while the well-known antagonist CPA displaced 75% of [^{3}H]R1881 at over 200-fold concentrations of labeling [^{3}H]R1881. Hinokitiol bound to AR at around 40%, which shows that hinokitiol, acting as an antagonist, effectively blocks androgen binding to AR in LNCaP cells.

Discussion

Androgen ablation therapy is aimed at reducing the level of circulating androgens, or blocking agonist activation with antagonist, or both. Steroidal anti-androgen,

which acts by competing with androgen for occupation of AR, suppresses LH release and reduces testosterone levels [15,16]. However, the resistance to androgen ablation acquired by tumor cells during androgen deprivation remains a severe obstacle to effective therapy. Thus, the development of novel therapeutic agents and alternative therapies for prostate cancer is of critical importance.

In general, iron is critically important for cell proliferation and survival. Tumor cells are frequently more sensitive to Fe than normal cells because cancer cells express higher levels of the Fe-containing enzyme RR, a critical rate-limiting step in DNA synthesis [17,18]. In the present study, we first reveal that hinokitiol suppresses cell growth and induces cell differentiation in prostate cancer cells. Similarly, hinokitiol also suppresses DNA synthesis, which suggests that hinokitiol suppresses the activity of RR via chelating with iron present in catalytic moiety of the enzyme and thus interfering with DNA synthesis in cells.

Our present studies show that hinokitiol inhibits AR mRNA and protein expression by interfering with its transcription and translation. It is usually assumed that unbound AR localizes predominantly in cytoplasm, where it is sequestered as a multiprotein complex with heat shock proteins [19–21]. Hinokitiol may exert its effect through multiple mechanisms by which inactivation of the heat shock protein-90 molecular chaperone is involved in hinokitiol-induced androgen receptor depletion.

Another interesting point relates to zinc, which is an essential component of all cells and required for a variety of cellular activities, such as metalloenzyme activity, nucleoprotein and nucleic acid structure, and transcription interaction. Miyamoto et al. reported that hinokitiol and its zinc chelate inhibited cell growth in various tumor cell lines by inducing apoptosis [22]. A study revealed that LNCaP and PC-3 cells are able to accumulate high levels of zinc, which implies that zinc mediates the progression of prostate cancer [23]. However, the precise mechanism of the AR signaling pathway mediated by zinc is not well understood. At present, we speculate that there are at least two plausible candidates for the mechanism by which hinokitiol mediates AR expression and disrupts AR functions: (1) hinokitiol may chelate with intracellular zinc ion present in the first zinc finger and disrupt the DNA-binding specificity dictated by the P-box, a stretch of five amino acids in the first Zn finger of the DBD; (2) hinokitiol possibly affects the DNA-receptor complex by chelating with zinc localized in the second zinc finger bind, thereby leading to interference with the transcriptional function of AR and the ability of androgen to bind to AR in prostate cancer.

Taken together, unlike the synthetic androgen antagonists, hinokitiol as a metal chelator derived from natural plants is potentially therapeutic against prostate cancer *in vitro*. Thus, hinokitiol may become a novel chemopreventive or chemotherapeutic agent for prostate cancer.

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