De-repression of the p21 promoter in prostate cancer cells by an isothiocyanate via inhibition of HDACs and c-Myc

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Abstract. Natural isothiocyanates from cruciferous vegetables have been described as important dietary factors for prostate cancer prevention. Phenethyl isothiocyanate (PEITC), found rich in watercress, induces growth arrest and apoptosis in prostate cancer cells, and also inhibits the testosterone-mediated growth of prostates by regulating the androgen receptor and cell cycle progression in rats. PEITC has been recently identified as an inhibitor of histone deacetylases (HDACs). Herein we describe the mechanism of PEITC-mediated growth attenuation in relation to HDAC inhibition in human prostate cancer cells. Exposure of androgen-dependent prostate cancer cells LNCaP to PEITC resulted in cell cycle arrest and a p53-independent up-regulation of the inhibitors of cyclin-dependent kinases, including $p21^{WAF1}$ and p27. The mechanism of p21 activation was investigated. PEITC significantly enhanced histone acetylation and induced selective modification of histone methylation for chromatin remodeling. Chromatin immunoprecipitation revealed that the p21 gene was associated with the PEITC-induced hyper-acetylated histones. As a result, the chromatin unfolding permitted the transcription activation of the p21 gene. PEITC also significantly reduced the expression of c-Myc which represses p21. Pull-down assays using Sp1 affinity oligo beads of the p21 promoter, showed decreased c-Myc binding to the Sp1 transcriptional complexes in the p21 promoter, resulting in reduced p21 repression. The quantity of PEITC (0.5-1 μM) effective to mediate cell cycle arrest was less than that for inhibiting c-Myc (2-5 μM), suggesting that the inhibition of HDACs may be the primary mechanism for p21 activation. The PEITC-mediated growth attenuation of prostate cancer cells includes an interactive mechanism involving HDAC and c-Myc inhibition.

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Introduction

There is epidemiological evidence that the incidence of prostate cancer varies in different regions of the world. As populations move to a high-risk area, they assume the risk of that geographic region (1-3). These observations have revealed the relationship of diet and the risk of prostate cancer. The effectiveness of dietary interventions such as vitamin E (4), selenium (5) and low fat diets (6) for preventing prostate cancer has been demonstrated. Epidemiological surveys indicate that the intake of cruciferous vegetables such as broccoli, cabbage, Brussels sprouts, cauliflower and watercress, is inversely related to the incidence of prostate cancer, especially advanced cases (7). We demonstrated that isothiocyanates from cruciferous vegetables may be an important dietary factor responsible for prostate cancer prevention (8,9). Isothiocyanates occur naturally as thioglucoside conjugates, i.e., glucosinolates, in a wide variety of cruciferous vegetables (10). They are released from glucosinolates by hydrolytic action as vegetable tissues are cut or masticated (11). Hydrolysis of the glucosinolate gluconasturtin, which is abundant in watercress, yields phenethyl isothiocyanate (PEITC). PEITC significantly down-regulates the androgen receptor (AR) in both androgen-dependent and -independent prostate cancer cells (12) and in the testosterone-stimulated prostate organ of rats (13). The transcription of the AR was reduced via inhibition of the transcription factor Sp1, as well as by accelerating protein degradation. PEITC and its major metabolite have been demonstrated to induce growth arrest and apoptosis in human prostate cancer cells in culture and in xenografts (9,12,14).

Recently, we demonstrated that PEITC is an inhibitor of the activity and level of histone deacetylases (HDACs) (15). The mechanism of PEITC-mediated growth inhibition of prostate cancer cells, in relation to HDAC inhibition, however has not been clarified. In this study, we showed that the p21 gene, the cyclin-dependent kinase inhibitor of the cell cycle, was activated due to PEITC mediated-histone acetylation and

chromatin unfolding, leading to cell cycle arrest. Additionally, PEITC down-regulated c-Myc, thus alleviating the repressive effect of c-Myc on the p21 promoter. These results revealed an interactive mechanism inhibiting the growth of prostate cancer cells.

Materials and methods

Cell culture and cell cycle phases. PEITC was purchased from LKT Labs (St. Paul, MN) with 98% purity determined by HPLC and NMR. The androgen-dependent (AD) human prostate cancer cell line LNCaP was maintained in RPMI-1640 with 10% heat-inactivated bovine serum (FBS). The androgen-independent (AI) sub-line derived from the LNCaP cell line, was maintained in RPMI-1640 containing 10% charcoal-stripped FBS (Hyclone Laboratories, Logan, UT) and 5 μg/ml insulin (12). LNCaP cells in exponential growth phase were prepared in cultures at $0.4x10^6$ cells/ml and exposed to various concentrations of PEITC prepared in 30% DMSO and 20 mM phosphate buffer, pH 5. Cultures exposed to the vehicle without PEITC were used as controls. The cell cycle phases were determined with a flow cytometric method using a Becton-Dickinson FACScan (9). Cells were prepared, and the DNA was stained with propidium iodide (50 μg/ml) according to a previously described method (9).

Histone modification. The status of acetylated or methylated histones from the prostate cancer cells was determined by Western blot analysis. Essentially, total proteins from LNCaP cells exposed to PEITC or to 2 mM sodium butyrate were extracted with a lysis buffer and incubated on ice (15). The acid soluble proteins were collected by centrifugation at 4˚C, and dialyzed twice against 0.1 N acetic acid, and then distilled water. Twenty micrograms of the acid soluble traction (histone extracts) or the total protein preparation was subjected to SDS-PAGE and immunoblotted with a site-specific antibody against acetylated or methylated histones (Upstate Biotechnology, Lake Placid, NY). Monoclonal antibodies against p21, p27, or p53 were purchased from Dako (Carpinteria, CA). The reaction was visualized with an ECL Western blot detection reagent (Amersham Biosciences, Pittsburgh, PA).

Chromatin immunoprecipitation (ChIP) assay. The LNCaP cells were cultured for 24 h with PEITC at 10 μM or with the vehicle control medium. ChIP analyses were performed according to the protocol of a ChIP assay kit (Upstate Biotechnology) (16). Essentially, the DNA and histones were cross-linked by adding formaldehyde to the cultures to a final concentration of 1%. After incubation for 10 min, the cell lysates were prepared. After sonication, the cell lysates (200 μl) in a ChIP dilution buffer were incubated with a rabbit antibody against acetylated histone H3 or with a non-specific control Ig overnight. The mixtures were incubated with salmon sperm DNA/protein A agarose slurry for 1 h, then the beads were washed and the bound molecules eluted. After de-cross-linking, the DNA molecules in the precipitates were recovered. The p21 gene fragments, TATA area (-33 to +47), down TATA area (+43 to +122) and D (+3267 to +3366), were selected for amplification by PCR as described (17).

Pull-down assays. Three repeats of the Sp1-binding site oligo sequence within the p21 promoter: sense [BioTEG]GGG CGG TCC CGG GCG GGG GCG GTC CCG GGC GGG GGC GGT CCC GGG CGG-3' and antisense 5'-CCG CCC GGG ACC GCC CCC GCC CGG GAC CGC CCC CGC CCG GGA CCG CCC-3' were bound to streptavidin/magnetic beads (BioLab, Boston, MA). Nuclear extracts from AD cells (2 mg) treated or untreated with 10 μM PEITC for 24 h were incubated with the beads for 30 min in binding buffer at room temperature, and after extensive washes with the binding buffer, the proteins were eluted with binding buffer containing indicated concentrations of KCl, and the fractions were subjected for Western blotting using antibodies against c-Myc and Sp1, respectively.

Results

Up-regulation of p21 by PEITC. We previously demonstrated that exposure of human prostate cancer LNCaP cells in culture to PEITC resulted in significant growth inhibition as determined by MTT assay (12). To evaluate the effects of PEITC on cell cycle progression as a mechanism of growth inhibition, LNCaP cells were exposed to PEITC, and the cell cycle phase progression was analyzed. The cell cycle phase distribution was determined from the DNA frequency histograms of cells analyzed using the flow cytometric method. After exposure to PEITC for 24 h, a regular decrease in the proportion of cells in S-phase was observed, as compared to untreated control cells. A minimal decrease in S-phase cells was observed after exposure to 0.5 μM (Fig. 1A). PEITC also modulated a decrease in the proportions of cells in G_2M phase (Fig. 1A). At 10 μM, the replicating cells in S and G_2M phases from the untreated cells (~62.5%) were decreased to ~45%. The G_1 phase showed a concomitant increase (Fig. 1A), indicating a G_1 block of the cell cycle.

The potential activation of the inhibitors of cyclin-dependent kinases such as p21 and p27 which regulate cell cycle progression was examined. Representative results in Fig. 1B show a significant up-regulation of p21 and p27, observed at 0.5 μM or more PEITC, as compared to untreated control cells. In contrast, the expression of p53 was unchanged after exposure to PEITC (Fig. 1B), indicating that the up-regulation of p21 and p27 was p53 independent.

Epigenetic regulation by PEITC and p21 activation. PEITC has been described as an inhibitor of histone deacetylases (HDACs), which results in enhancing covalent acetylation of histones (15). Subsequently, chromatin unfolding would allow more accessibility for the regulators for transcription of genes associated with the hyperacetylated histones. Compared to the untreated control cells, Fig. 2A shows that PEITC mediated a concentration-dependent enhancement of acetylation of histone H3. The results were similar to those mediated by sodium butyrate, a known HDAC inhibitor, used as a control. Fig. 2A also shows that PEITC selectively increased the level of mono/di/trimethylation at lysine 4 of histone H3 while it decreased the level of trimethylated lysine 9 of H3. Methylated histone H3 lysine 4 is a marker of activated chromatin, whereas methylation of histone H3 lysine 9 signifies hetero-chromatin (18). The results are consistent with the interpretation

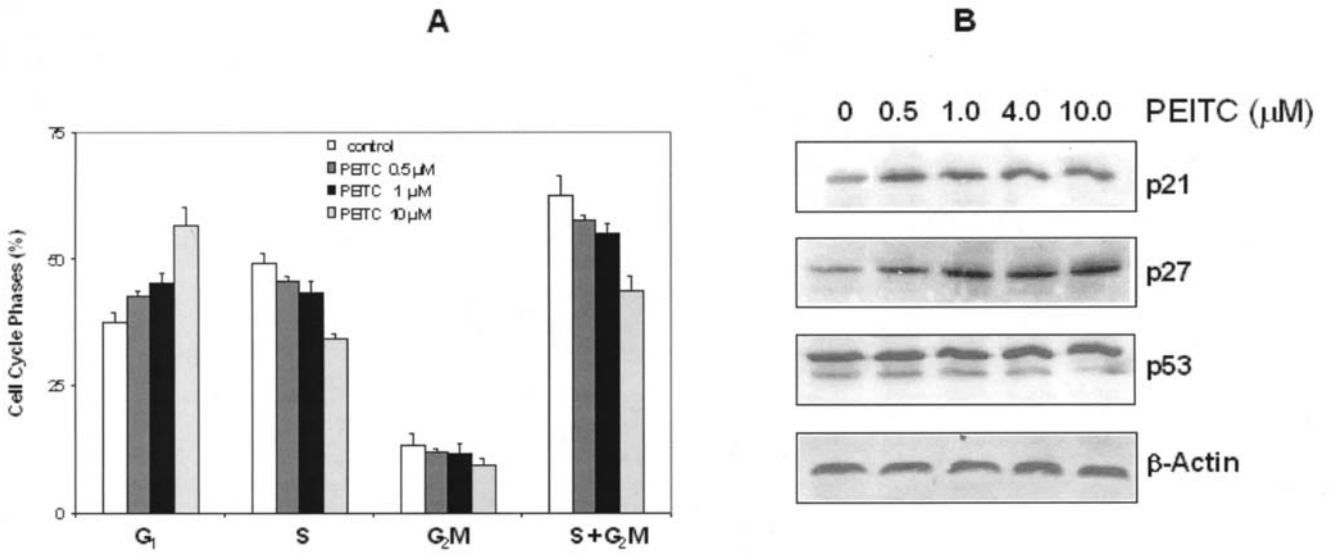

Figure 1. Regulation of p21 and cell cycle by PEITC. Human androgen-dependent prostate LNCaP cells grown exponentially were exposed to PEITC for 24 h. (A) Cell cycle phase analyses revealed a concentration-related decrease in replicating S- and G_2M-phase cells after exposure to PEITC at the indicated concentrations. Vertical bars indicate the means ± SD of three separate experiments. (B) Cells were harvested, total proteins were extracted, and 50 μg proteins was subjected to Western blotting using monoclonal antibodies against p21, p53, or p27, or an antibody against ß-actin as a loading control.

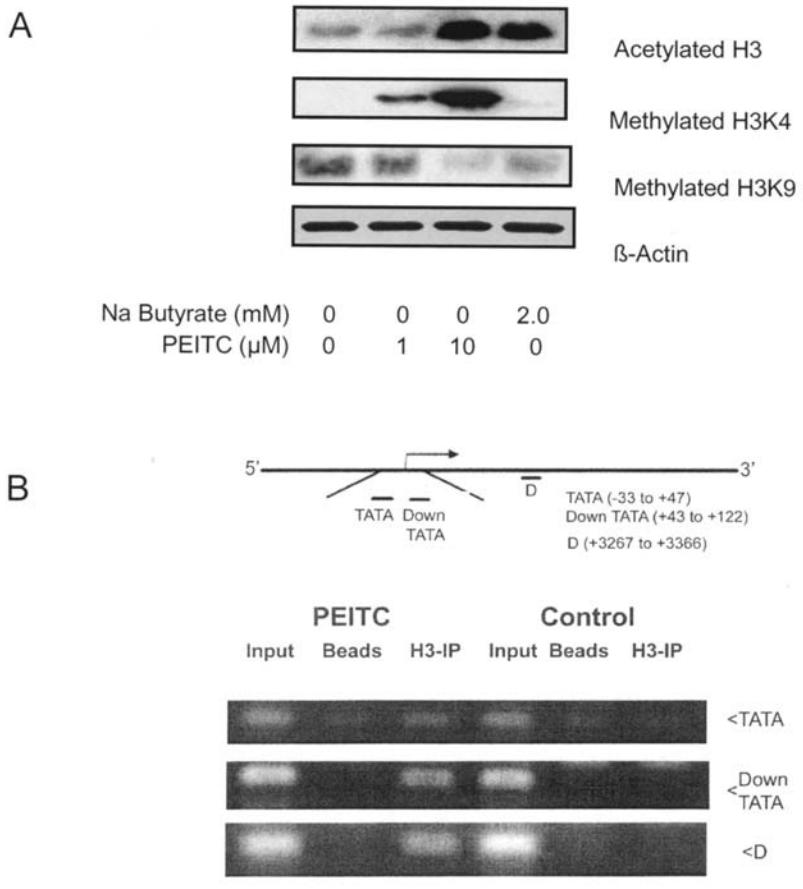

Figure 2. (A) Status of histone acetylation and methylation mediated by PEITC. Western blots showing histone modification of LNCaP AD cells after exposure for 30 h to PEITC, or to sodium butyrate, a known HDAC inhibitor. Antibody against ß-actin was used as a loading control. PEITC enhanced histone acetylation and selectively modified histone methylation, consistent with the marks of chromatin unfolding and transcription activation. (B) ChIP assay shows association of the p21 gene with acetylated histones accumulated by PEITC induction. Upper figure: schematic presentation of PCR primer sets TATA, down TATA, and D for the p21 gene. Lower figure: chromatin fragments from LNCaP AD cells after exposure for 24 h to medium without PEITC (control), or to 10 μM PEITC, were precipitated with an anti-acetylated histone H3 antibody (H3-IP), or with a non-specific Ig as a control (Beads). Indicated primers for areas of the p21 gene were used for PCR amplification of the DNA isolated from the immunoprecipitated chromatin. Input: DNA extracted from equal volumes of cell lysates was used as templates, and the PCR product served as input.

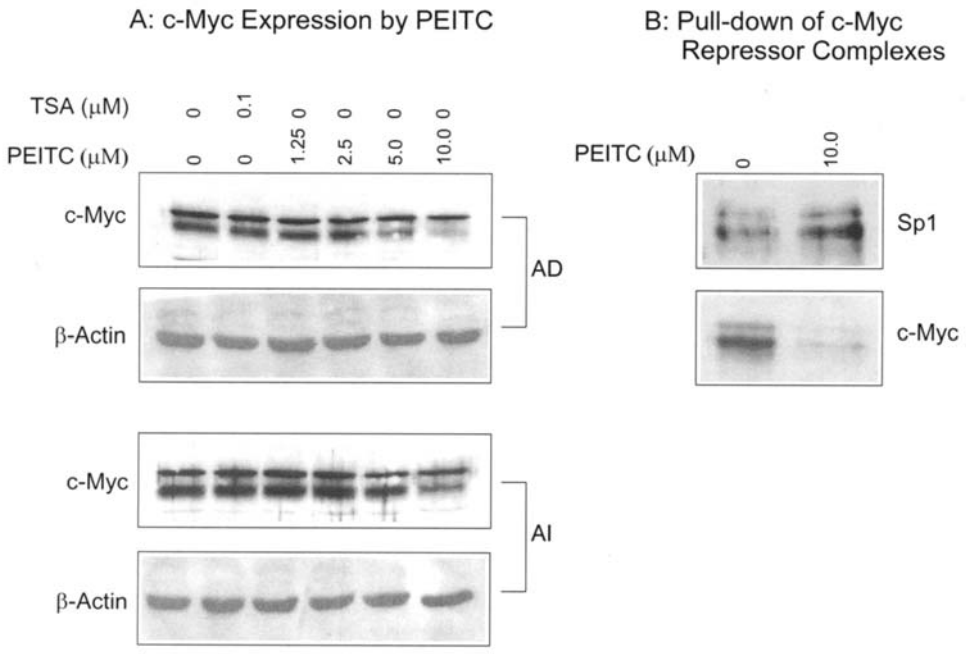

Figure 3. (A) Reduction of c-Myc expression by PEITC. LNCaP AD and LNCaP AI cells at exponential growth phase were exposed to PEITC or TSA for 24 h. The cells were harvested, total proteins were extracted, and 50 μg proteins was subjected to Western blotting with antibodies against c-Myc, or ß-actin as a loading control. (B) Affinity pull-down assays show the level of c-Myc repressor complexes. LNCaP AD cells at exponential growth phase were exposed to 10 μM PEITC for 24 h, and the cell proteins were extracted. The proteins (2 mg) were incubated for 30 min at room temperature with affinity beads of 3 repeats of biotin-labeled double-stranded Sp1-binding sites of the p21 promoter, close to the transcription initiation site: [BioTEG]-GGGCGGTCCCGGG CGG. The beads were collected and washed with binding buffer and eluted using the binding buffer containing 0.25 M of KCl. The eluted proteins were subjected to Western blotting.

that PEITC induces permanent histone modifications, which provided the basis for examining the activation of p21 by chromatin remodeling.

To examine the relationship between acetylated histones and transcriptional activation of p21, the chromatin immunoprecipitation (ChIP) assay was performed. An anti-acetylated histone H3 antibody was used to precipitate the chromatin fragments from untreated LNCaP cells, or from cells exposed to PEITC. The DNA from the precipitates was amplified according to three primers complementary to the p21 gene fragments, including the promoter region as schematically presented in Fig. 2B. As demonstrated, the chromatin from cells exposed to PEITC clearly contained the p21 gene fragments covering the promoter area TATA, down TATA, and D as compared to cells not exposed to PEITC, where p21 was nearly undetectable (Fig. 3). Immunoprecipitation using a non-specific Ig as control showed a negative background (Fig. 2B). The results demonstrated that more p21 DNA was present in the precipitates of hyperacetylated histones, indicating the association of the p21 gene with the highly acetylated histones and thereafter its transcriptional activation with the unfolded chromatins.

Down-regulation of c-Myc and de-repression on the p21 promoter. Transcription of p21 is known to be negatively regulated by c-Myc, which serves as a repressor on the Sp1 site within the p21 promoter (19,20). The possibility that PEITC also affects the c-Myc-mediated repression of p21 transcription was examined. The androgen-dependent (AD) LNCaP cells and an androgen-independent (AI) sub-line of LNCaP prostate cancer cells, grown at exponential phase, were exposed to PEITC, or to a known HDAC inhibitor trichostatin A (TSA). After 24 h, cells were examined for c-Myc protein expression by Western blotting. Compared to untreated control cells, the PEITC exposure resulted in a concentration-related decrease in c-Myc protein, in both LNCaP AD and AI cells (Fig. 3A). A significant decrease was observed with PEITC used at 2-5 μM, while TSA exposure showed no effect on the c-Myc expression.

To further examine the relationship of decreased c-Myc expression with p21 transcription, an affinity pull-down assay was performed. LNCaP AD cells, after exposure to 10 μM PEITC for 24 h, were harvested, and the total proteins were extracted. They were incubated with affinity beads which contained biotin-labeled double-stranded Sp1-binding sites of the p21 promoter, located close to the transcription initiation site. After binding, the beads were washed, and the proteins were eluted for Western blot analysis. Fig. 3B illlustrates the levels of c-Myc repressor complexes from representative pull-down experiments. Exposure to PEITC resulted in a significant decrease in the c-Myc protein level, which in turn reduced its amount of binding to the Sp1-transcriptional complexes in the promoter of the p21 gene. The results showed that a down-regulation of c-Myc, as mediated by PEITC, reduced the repressive effect of c-Myc on the p21 promoter.

Discussion

This study demonstrated that PEITC, a natural isothiocyanate from cruciferous vegetables, modulates proliferation of prostate cancer cells by activating the inhibitors of cyclin-dependent kinases including p21, leading to cell cycle arrest

(21). The activation of p21 was demonstrated to be p53 independent, and the experimental results described two interactive mechanisms responsible for p21 activation.

One mechanism involved PEITC as an HDAC inhibitor. PEITC enhanced histone acetylation, allowing chromatin unfolding and increased the accessibility of regulators to the DNA for transcription activation of p21. Chromatin unfolding was further supported by selective histone methylation, including the enhancement of methylated histone H3 lysine 4 and the decrease in methylated H3 lysine 9, which are consistent with markers of transcriptional competent chromatins (22). The ChIP analyses clearly demonstrated that more DNA of the p21 promoter was present in the precipitates of hyperacetylated histones, mediated by PEITC, indicating the association of the p21 gene with the highly acetylated histones. As a result, the unfolded chromatins permitted activation of p21 transcription. The results suggest that there is a direct relationship between the activation of growth regulatory genes, such as p21, and chromatin remodeling by PEITC.

The experimental results clearly demonstrated that PEITC mediated a down-regulation of c-Myc in both the androgen-dependent and -independent prostate cancer cells, leading to a reduced repressive effect on the p21 promoter. TSA used in place of PEITC did not alter the level of c-Myc and the repressive effect on p21. This observation is consistent with a previous study by Claassen and Hann (26) showing that transcription repression by c-Myc is independent of TSA as seen with transforming growth factor ß-mediated cell cycle arrest. Some studies showed that c-Myc interacts with transcription factor Miz-1, which binds the transcriptional start site of p21 and initiates repression (23,24). Another mechanism indicates that c-Myc represses p21 by forming a complex with Sp1-Smad, which is a transcription factor of a number of cell cycle regulators (20,25). Whether or not the PEITC-mediated reduction of the c-Myc repressive effect on the p21 promoter requires prior chromatin remodeling for more access to the p21 promoter is currently under investigation. Our experimental results indicated that the PEITC concentrations required for de-repressing c-Myc, i.e. 2-5 μM, are higher than the 0.5-1 μM range effective for cell cycle inhibition. This indicates that at low concentrations of PEITC the HDAC inhibition and chromatin remodeling may be the primary mechanism for p21 activation, while at higher concentrations PEITC also de-represses c-Myc.

The transcriptional activation of p21 by PEITC, via inhibition of HDACs, implies the presence of a dysregulated epigenetic mechanism in prostate cancer cells. This may include an aberrant formation of heterochromatins due to the architecture of local chromatins, as well as the status of histone modification (27,28). These manifestations could contribute to the pathogenesis of inactivating important regulatory genes in prostate carcinogenesis, such as p21. A lack of proper functioning of the regulator proteins of the cell cycle could cause a loss of cell cycle control, leading to uncontrolled replication of cancer cells.

We previously demonstrated that PEITC is an effective repressor for the transcription of the androgen receptor in prostate cancer cells (12). As a result, the growth of prostate cancer cells with functional AR was significantly inhibited. PEITC was also effective in inhibiting the growth of androgen-independent prostate cancer cells, such as the PC-3 cell line (29,30). Judged together with our current experimental results, PEITC may target both the AR and cell cycle regulators for attenuating the growth of androgen-dependent and -independent prostate cancer cells. In AR-positive prostate cancer cells, the growth regulation could be achieved by a combination of inhibiting c-Myc and HDACs for activating cell cycle regulators, and also by regulating the AR. In AR-negative prostate cancer cells, on the other hand, up-regulating the cell cycle regulators, via an inhibition of HDACs and c-Myc, may represent the predominant mechanism for regulating their growth.

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