Suppression of Androgen Receptor Expression by Dibenzoylmethane as a Therapeutic Objective in Advanced Prostate Cancer

KIMBERLY M. JACKSON[1], MONICA C. FRAZIER[2] and WAYNE B. HARRIS[3]

[1]*Chemistry Department, Spelman College, Atlanta, GA;*
[2]*Department of Biology, Columbus State University, Columbus, GA;*
[3]*Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA, U.S.A.*

Abstract. *Background: The androgen receptor (AR) plays an important role in the development and progression of prostate cancer. Functional AR expression persists in most cases of hormone-refractory prostate cancer and may play a role clinically in the progression from hormone-responsive to hormone-refractory or advanced prostate cancer. In order to combat the progression of this disease, one needs to identify new chemotherapeutic agents with novel mechanisms of action. Materials and Methods: In this study, we attempt to clarify the molecular mechanism by which dibenzoylmethane (DBM), a β-diketone, inhibits the growth of androgen-responsive human LNCaP prostate cancer cells and down-regulates expression of the AR. To this end, we treated LNCaP cells with different concentrations of DBM to monitor function and expression of AR and an AR-associated protein. Results: Previous studies showed that DBM could inhibit cell proliferation in LNCaP cells by arresting the cells at the G1 phase without causing cell death. Western blot and RT-PCR/Northern blot analyses showed a reduction in AR protein and mRNA expression by DBM in a dose-dependent manner. Furthermore, stable transfections of an androgen-independent human prostate cancer cell line, transfected with a full-length human AR cDNA sequence, showed that DBM down-regulated AR protein levels. DBM also inhibited the secretion of the AR-regulated tumor marker, prostate-specific antigen (PSA). Moreover, the relative binding affinity of DBM to AR was lower than that of the synthetic androgen R1881 (methyltrienolone) suggesting that DBM must suppress AR expression independent of an AR-DBM bound interaction. Conclusion: These data provide new insights into how DBM regulates AR function and cell growth, as well as providing promising evidence to support DBM as a chemotherapeutic agent for prostate cancer through suppression of the function of the androgen receptor.*

Prostate cancer growth begins as an androgen-dependent tumor (1). Over time, the cancer advances, eventually progressing to an androgen-independent, hormone-refractory state, whereby there is no effective treatment (2, 3). The molecular basis for androgen-independent prostate cancer is poorly understood. Since androgens and the androgen receptor (AR) are essential for the development and maintenance of the normal prostate and influence the growth of prostatic neoplasms, androgen deprivation or androgen blockage at the AR remains the initial treatment of choice for prostate cancer (4-6). Most androgen-independent prostate tumors continue to express AR as well as the androgen-dependent gene prostate-specific antigen (PSA), which indicates that these cells maintain a functional AR signaling pathway (7). Alterations in the expression and function of the AR are thought to be responsible for the progression of some prostate cancers to androgen independence (8-10). Thus, the AR plays a critical role in prostate cancer growth and progression; therefore, understanding and modulating its function may be essential to the development of new treatment modalities.

Dibenzoylmethane (DBM), a small β-diketone compound, is a minor constituent of licorice that is widely used in sunscreens as an ultraviolet blocking agent (11). Dietary DBM has been reported to inhibit growth in mammary-induced 7,12-dimethylbenz[a]anthracene (DMBA) tumors and lymphomas/leukemias, and 7,12-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumors in mice (11, 12). DBM has

Abbreviations: DBM, dibenzoylmethane; AR, androgen receptor; PSA, prostate-specific antigen.

Correspondence to: Kimberly M. Jackson, Ph.D., Spelman College, Department of Chemistry, Box 1134, 350 Spelman Lane, SW, Atlanta, GA 30314, U.S.A. Tel: +1 404 270 5792, e-mail: kjackson@spelman.edu

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also been reported to inhibit tumor formation in a rat mammary tumor model system (13). Although DBM exhibits antineoplastic activity, preliminary reports on potential mechanisms of action for DBM are non-existent with respect to tumor growth inhibition. Carcinogen detoxification has been proposed as a possible mechanism of action since DBM has been reported to potently induce phase 2 hepatic detoxification enzymes (14). DBM has also been reported to inhibit the binding of [^{3}H]-estradiol to the estrogen receptor *in vitro* (12), suggesting a possible mechanism for the inhibitory actions of DBM on murine breast tumorigenesis. Recently, our laboratory reported that DBM induced pronounced changes in the expression of cell cycle regulating proteins, valosin-containing protein (VCP), phospho-enolpyruvate carboxykinase (PEPCK), and actin (15), and induced cell cycle arrest during the G1 phase of the cell cycle (16). It has also been reported that DBM induced HIF-1α (a transcription factor which controls hypoxia and ultimately tumor behavior) under low toxicity measures, not only in the prostate cancer cell lines LNCaP and PC-3, but also in cardiomyocytes which suggests a potential chemotherapeutic benefit in ischemic diseases (17). In this present study, we describe for the first time a novel action of DBM in the androgen-responsive LNCaP prostate cancer cell line resulting in a decrease in the expression and function of the AR.

Materials and Methods

Cell culture and treatments. The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and cultured in Hepes-buffered RPMI-1640 medium (Cellgro, Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA), 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 2 mM L-glutamine in a humidified 5% CO_2 atmosphere at 37˚C. Cells were propagated in 96-well, 6-well or 100 mm culture dishes at the desired density in the above growth medium until reaching 50-70% confluence. A 50 mM stock solution of dibenzoylmethane (Sigma-Aldrich, St. Louis, MO, USA) was prepared in dimethyl sulfoxide (DMSO), stored in amber-colored microfuge tubes at –20˚C and used within one week. Final concentrations of DBM were prepared by diluting the stock solution with RPMI-1640.

Protein isolation and Western blot analysis. LNCaP cells were seeded in 100 mm plates and allowed to adhere for 24 h. They were then cultured for 72 h with DBM. Cell monolayers were detached by trypsinization. Whole cell lysates were prepared and protein content quantified using the BCA protein assay kit (Pierce-Endogen, Rockville, IL, USA). Cell extracts containing 10-30 μg of protein were separated on 10% SDS-PAGE gels and transferred to a nitrocellulose filter. Nitrocellulose filters were blocked for 60 min with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 and were probed with specific antibodies [monoclonal anti-AR (1:1000 dilution; Biogenex, San Ramon, CA, USA) or anti-PSA (1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)]. Filters were washed and incubated with a secondary antibody, horseradish-peroxidase-conjugated goat anti-mouse Ig (Santa Cruz Biotechnology Inc.). Bands were visualized by fluorography with the SuperSignal West Pico system (Pierce-Endogen).

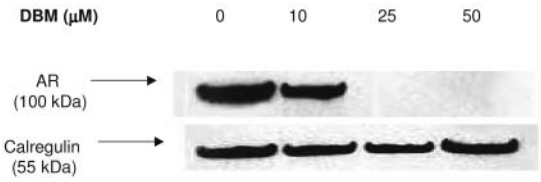

Figure 1. *DBM inhibits androgen receptor (AR) protein levels in LNCaP cells. LNCaP cells were treated with different amounts of DBM for 72 h. Whole-cell lysates were extracted from these cells and 15 μg of protein was analyzed by Western blotting. Calregulin was used for normalization.*

Northern blot analysis. Total RNA was extracted using TRIzol Reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. The RNA was dissolved in DEPC water and quantitated by measuring absorbance at 260 nm. The RNA (10 μg) was denatured at 50˚C for 30 min in loading buffer (Ambion, Austin, TX, USA) and run on a 1% Agarose-LE gel (Ambion). RNA was transferred onto a positively charged nylon membrane (Boeheringer-Mannheim, Mannheim, Germany) and hybridized with AR and β-actin probes. Membranes were stripped for 5 min in boiling stripping buffer (0.1 SSC, 1% SDS) before hybridization with the second probe. The AR probe was constructed from a 564 bp PCR fragment of human AR cDNA (ATCC) using the following primers: AR forward, 5'-TCACC GCACCTGATGTGTGGTACC-3', and AR reverse, 5'-ACACCT GGCTCAATGGCTTCCAGG-3'. The β-actin probe was generated from DECA template DNA purchased from Ambion. Each probe was labeled with α-^{32}P-dCTP (ICN, Costa Mesa, CA, USA) using Prime-It II Random Primer Labeling Kit (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's instructions.

Immunoprecipitation. Treated and untreated cells were starved in RPMI-1640 –methionine, –cysteine media (ICN) for 30 min at 37˚C. Cells were labeled with 100 μCi [^{35}S]Translabel –methionine (ICN) for 1 h at 37˚C and then washed 3 times with cold 1X PBS. Cells were then lysed in lysis buffer (1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 0.1 mM PMSF and 1X PBS) and lysates pelleted at 13,000 xg, 15 min, 4˚C. Lysates were placed into fresh tubes and rotated overnight at 4˚C with an anti-AR antibody (Pharmingen, San Diego, CA, USA). Protein A agarose beads (Sigma) (100 μl of a 0.1 μg/ml slurry) were added to each lysate and allowed to rotate for 2 h at room temperature. Beads were collected by centrifugation (13,000 xg, 0.5 min) and washed 3 times in lysis buffer. Beads were resuspended in protein sample buffer, boiled for 5 min and run out on a 7.5% SDS-PAGE gel, which was fixed and dried for analysis by autoradiography.

Androgen receptor binding assay. LNCaP cells were plated at a density of 5x10^5 cells/well in 6-well plates for 2 days. The growth media was removed and replaced with RPMI-1640 phenol-red free media (Cellgro, Mediatech) containing 10% charcoal stripped FBS (Hyclone, Logan, UT, USA) and allowed to incubate overnight. Cells were then incubated in RPMI-1640 phenol-red free charcoal stripped media containing [^{3}H] R1881 methyltrienolone (Perkin

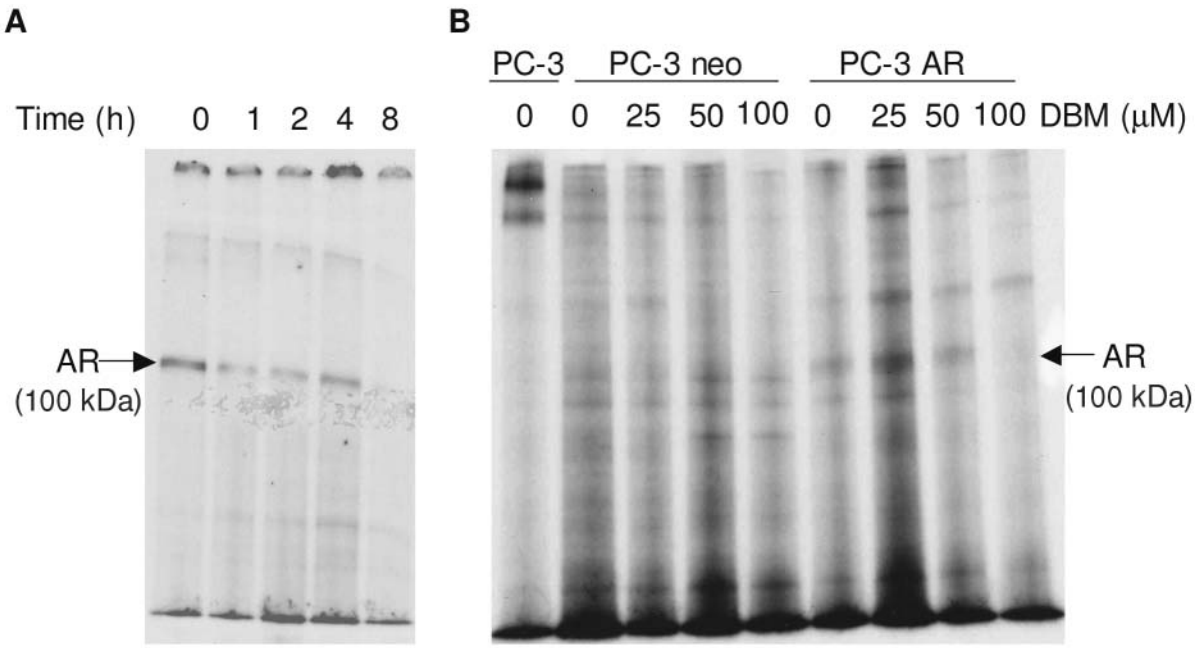

Figure 2. *DBM inhibition of AR protein synthesis as seen in LNCaP and stably transfected PC-3 prostate cancer cells with full length AR. (A) LNCaP cells treated with 50 μM DBM and pulse-closed for different times, and (B) PC-3, PC-3 neo, and PC-3 AR cells treated with 0, 25, and 50 μM DBM for 72 h, were starved for 30 min in methionine-deficient media followed by labeling with Translabel ^{35}S methionine for 1 hr. After labeling, lysates were run out on SDS-PAGE electrophoresis and analyzed by immunoprecipitation. Results were analyzed by autoradiography.*

Elmer, Boston, MA, USA) at concentrations of 0.5-50 nM in the presence and absence of 0.05-50 mM DBM for 30 min at 37˚C. Non-specific binding was measured in the presence of a 2000-fold molar excess of unlabeled R1881. Bound and free fractions were separated by centrifugation and radioactivity measured in a liquid scintillation counter. The data were plotted according to the Scatchard method (18), with the appropriate regression line.

Measurement of intracellular and secreted PSA expression. The TANDEM®-MP PSA Immunoradiometric Assay (Hybritech Inc., Beckman Coulter, Chaska, MN, USA) was used to measure the total levels of PSA (intracellular and free). This assay is based on the quantitative binding of PSA by the cognate (biotin-labeled) antibody on a solid matrix, followed by the retention of the PSA:antibody complex with an alkaline-phosphatase-tagged IgG, and the cleavage of the substrate *p*-nitrophenyl phosphate by the IgG-tagged alkaline phosphatase. Color products were quantified by measuring the absorbance of the quenched reaction at 450 nm in a microplate reader. Absorbance or product formation is proportional to the concentration of PSA present in the samples.

Results

Regulation of AR protein expression by DBM. To determine the effect of DBM on the expression levels of AR protein, Western blot analysis was employed. LNCaP cells were treated for 72 h with 0, 10, 25 or 50 μM DBM. Cells were lysed and Western blot analysis was performed using the monoclonal mouse anti-AR antibody F39.4.1, which recognizes a unique immunogenic *N*-terminal transactivation domain of the AR that has low sequence homology with other steroid receptors. It recognizes a 100 kDa human AR protein in Western blot analysis. The antibody does not cross-react with human estrogen, progesterone or glucocorticoid receptors. Results indicated a dose-dependent decrease in AR protein when cells were treated with increasing concentrations of DBM (Figure 1). To further characterize this decrease in AR protein, assays to investigate the effects of DBM on AR protein synthesis were investigated. Immunoprecipitation assays demonstrated a dose-dependent loss in synthesis of AR protein within 8 h of treatment of LNCaP cells with DBM (Figure 2A). Furthermore, stable transfections of an androgen-independent human prostate cancer cell line, which lacks AR expression (PC-3 neo), and one transfected with a full-length human AR cDNA sequence (PC-3 AR) also showed a dose-dependent loss of AR protein synthesis after a 72 h treatment with DBM (Figure 2B) (19). Unlike LNCaP cells, the IC_{50} of PC-3 cells is 100 μM DBM (16); hence, the concentrations of DBM used are higher for the PC-3 cells in comparison to the LNCaP. The PC-3 AR cell line was kindly provided by Dr. Leland Chung, Winship Cancer Institute, Emory University, Atlanta, Georgia.

Analysis of AR mRNA levels and DBM binding studies. Because of the loss of AR protein expression and synthesis, we speculated that this loss resulted from a loss of AR mRNA.

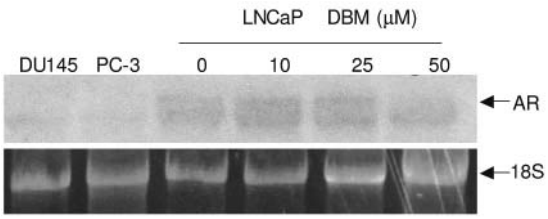

Figure 3. *Effects of DBM on the expression of AR mRNA. LNCaP cells were treated with or without the indicated concentrations of DBM. Total RNA was extracted 72 h after DBM treatment and analyzed by Northern blot analysis. Total RNA (10 μg/lane) was run out on a 1% agarose gel and transferred to an nylon membrane that was hybridized to an hAR cDNA probe. 18S rRNA is shown as a loading control. DU145 and PC-3 prostate cancer cells were used as negative controls. A representative blot is shown.*

Therefore, in order to illustrate the mechanism proposed for the down-regulation of AR by DBM, we used Northern blot analyses to assess alterations in the steady-state levels of the corresponding mRNAs in DBM-treated LNCaP cells. As predicted, mRNA levels of the 10.8 kb AR decreased in LNCaP cells following a 72 h treatment with DBM (Figure 3).

The establishment of a down-regulation of AR expression by DBM due to loss of AR mRNA and synthesis of AR protein led to questions on the binding potential between DBM and AR. In order for there to be a binding potential, there must be an association between DBM and AR. Binding studies showed non-competitive binding of DBM to AR. This finding was confirmed by measurement of specific binding of ^{3}H-labelled R1881 (Figure 4).

Analysis of PSA levels. Studies on the effects of DBM on AR expression showed a down-regulation in levels of AR protein and mRNA. Whereas data up to this point addressed the effects of DBM on AR expression, none addressed the effects of DBM on AR function. To test for possible effects on AR function, assays were performed which would analyze the effects of DBM on levels of secreted and intracellular PSA. Because PSA levels are regulated through the AR signaling pathway, our data would suggest that PSA levels would decrease due to the decrease in AR expression and synthesis. As shown in Figure 5, DBM did cause a decrease in levels of intracellular PSA (inset), as well as a decrease in secreted PSA levels. Together, these data indicate that DBM not only causes a decrease in the levels of AR protein and mRNA, but also may inhibit the function of AR as indicated *via* the loss of PSA expression and secretion.

Discussion

There is mounting evidence that expression of functional AR persists in most cases of hormone-refractory prostate cancer. Current evidence supports the hypothesis that activation of AR by alternative ligands is an important mechanism of progression from hormone-responsive to hormone-refractory prostate cancer (10). Two common therapies for the initial treatment of metastatic prostate cancer are the removal of androgen by orchiectomy or by the use of luteinizing hormone releasing hormone (LHRH) agonists. These procedures eliminate the possibility of any interaction between androgens and the AR by blocking androgen production in the testes. The use of nonsteroidal antiandrogens, another common therapy, prevents nuclear uptake of androgen by competitively interfering with the binding of androgens to the AR. However, neither of the current approaches prevents activation of the AR by alternative ligands. Thus, therapeutic strategies must be developed to eliminate the expression and transcriptional activation of the AR from prostate cancer cells.

In this report, we provide evidence that the curcumin-analogue, DBM, suppresses AR protein and mRNA expression in hormone-refractory human prostate cancer cells. Our previous work has shown the antiproliferative effects of DBM in prostate cancer cells to cause deregulation of the G1-phase of the cell cycle (16) . However, the mechanistic bases for these antineoplastic effects are unknown. To examine the outcome of AR activity in AR-positive, hormone-refractory prostate cancer cells upon treatment with DBM, we monitored AR functionality by the regulation of AR protein and mRNA expression and downstream events.

The expression of the AR mRNA is regulated by various factors including hypermethylation of the AR gene promoter (20), growth factor-dependent pathways (21) and auto-regulation based on ligand concentration (22). Growth factor regulation and autoregulation of AR gene expression have been reported in LNCaP cells (21, 23). There is some evidence that AR protein expression does not invariably correlate with mRNA expression. Basic fibroblast growth factor (bFGF), the prototype of the heparin-binding growth factors, is commonly expressed in prostate cancer. AR protein expression is suppressed in a dose-dependent manner in LNCaP cells exposed to bFGF without affecting AR mRNA levels (9). In contrast, the negative regulation of AR protein expression by epidermal growth factor (EGF) is associated with diminished AR mRNA levels (21). Therefore, it was important to determine the level at which DBM suppresses AR protein. In our studies, DBM suppressed expression of AR mRNA and protein synthesis in a dose-dependent fashion in LNCaP cells. DBM follows a series of compounds that have now shown suppression of AR protein and transcriptional activity. More recently, other anticarcinogenic agents such as curcumin, selenium, and 3,3'-diinolylmethane (DIM) have shown similar results of reduced AR transcript and protein expression in LNCaP cells (24-26).

AR signaling regulates PSA expression as a downstream event upon AR transactivation. PSA protein is an AR target and widely used diagnostic biomarker for prostate cancer. To further confirm the effect of DBM on AR nuclear

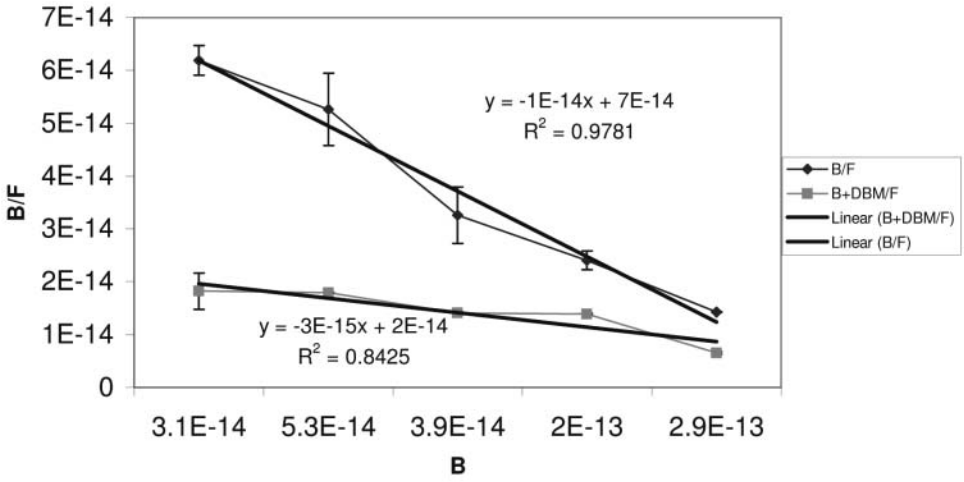

Figure 4. *Scatchard analysis of ligand (^{3}H-R1881 and DBM) binding to AR in LNCaP cells. Whole cell binding assays using ^{3}H-R1881 and DBM were performed on LNCaP cells in triplicate in the presence or absence of 400-fold excess of unlabeled R1881. The abscissa indicates concentrations of receptor-bound ligand, while the bound ligand to free ligand ratio (B/F) is placed on the ordinate.*

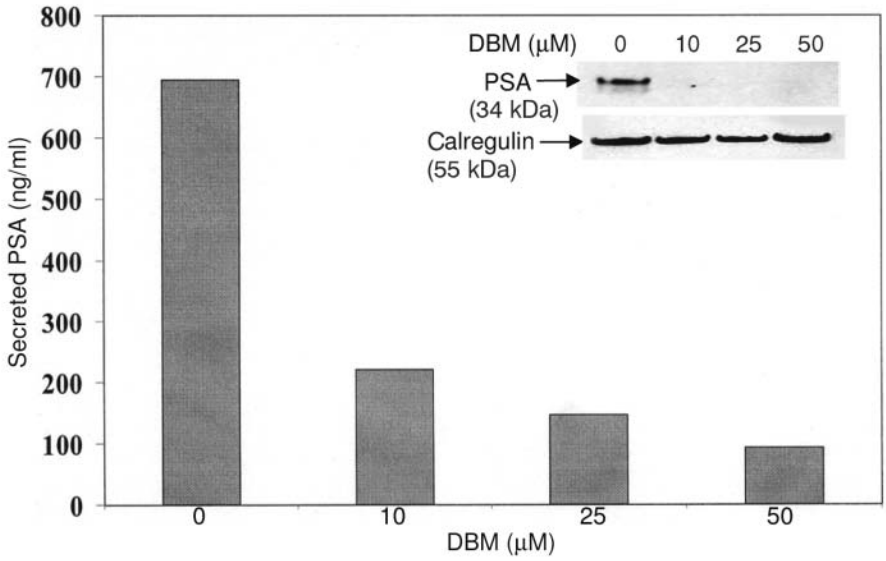

Figure 5. *Effects of DBM on intracellular and secreted PSA protein levels in LNCaP cells. LNCaP cells were treated with different concentrations of DBM for 72 h. Following treatment, PSA levels in the supernatants were measured by an enzyme immunoassay. Cells were also harvested and whole cell lysates were prepared for analysis of PSA levels using Western blotting (see inset). Calregulin was used for normalization.*

translocation, PSA expression was assessed in DBM-treated LNCaP cells. Subsequently, DBM inhibited PSA secretion and expression in a dose-dependent manner similar to the AR protein. This down-regulatory event by PSA has significant clinical implication, since PSA can serve to monitor patients undergoing treatment.

In conclusion, it is clear that AR expression and functionality in prostate cancer initiates a multitude of signal transduction pathways (7). The ability of the AR to induce growth in response to a wide variety of stimuli suggests that targeted inhibition of specific pathways may not be a successful strategy. Suppression of AR expression, however, could potentially be more effective. In the absence of expressed AR, disease progression based on activation by alternative ligands could not occur. This work allowed for specific evaluation of the effect of DBM on AR protein and transcript expression while also evaluating the effect of DBM on the expression levels of PSA, a downstream event involved in multiple AR signaling pathways. The molecular mechanism of action of DBM in prostate cancer, although not fully understood, has clinical applications worth exploring. The work described here will lay the groundwork for an alternative

treatment strategy for prostate cancer with the objective of developing AR suppression therapy for clinical practice.

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