Plant-derived 3,3'-Diindolylmethane Is a Strong Androgen Antagonist in Human Prostate Cancer Cells*

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Prostate cancer is the second leading cause of cancer-related mortality in American men, with more than 40,000 deaths in 1997 (1). One of every four cancers diagnosed is of prostatic origin, making prostate cancer the most commonly diagnosed cancer (2). Although the incidence of prostate cancer in Japanese and Chinese men is remarkably low compared with the incidence in American males, after migration to the US, the risk of later generations of Asian immigrants rises to levels that are similar to American males (3, 4). The differences in prostate cancer diagnosed among various population groups suggest that factors in the environment, lifestyles, and diet play a role in prostate cancer initiation and/or progression.

One possible contributor to the lower prostate cancer rates in Asian men is the higher consumption of phytochemical-rich vegetables that is typical of this population (5, 6). Consumption of cruciferous vegetables, including broccoli, Brussels sprouts, kale, and cauliflower, has been associated with a decreased risk of various human cancers. The strongest associations are with cancers of the breast, endometrium, colon, and prostate (7–10).

Incorporation of Brassica plants in feed reduces spontaneous and carcinogen-induced tumorigenesis in experimental animals, with the greatest protective effects seen in mammary tumors (11–13). A major active compound in cruciferous vegetables, indole-3-carbinol, along with its primary digestive derivative, 3,3'-diindolylmethane (DIM),1 exhibit promising cancer-protective properties in vivo and in vitro. These compounds reduced the incidence of dimethylbenzanthracene-induced mammary tumors in rats, benzo[a]pyrene-induced tumors of the forestomach in mice, and benzo[a]pyrene-induced pulmonary adenomas in mice (14, 15). Indole-3-carbinol has been shown to inhibit proliferation of both breast (16, 17) and prostate cancer cells (18, 19) by blocking the cell cycle and inducing apoptosis. In addition, DIM inhibited proliferation and induced programmed cell death in human breast tumor cells in culture (20, 21). The cancer-preventive effects of DIM, especially on hormone-mediated breast cancer, and the effects of indole-3-carbinol on prostate cancer cells led us to investigate the effects and mechanism of action of DIM against proliferation of prostate tumor cells.

To examine the androgen antagonist effects of DIM, we conducted a series of cell proliferation and gene activation studies in androgen-dependent (LNCaP) and androgen-independent (PC-3) human prostate cancer cell lines. LNCaP cells were derived from lymph node metastasis, and PC-3 cells were derived from bone metastasis (22–25). We found that DIM is a strong antiandrogen that inhibited androgen-dependent tumor cells and that these cells were co-transfected with a wild-type androgen receptor expression plasmid. Using fluorescence imaging with green fluorescent protein androgen receptor and Western blot analysis, we demonstrated that DIM inhibited androgen-induced androgen receptor (AR) translocation into the nucleus. Results of receptor binding assays indicated further that DIM is a strong competitive inhibitor of DHT binding to the AR. Results of structural modeling studies showed that DIM is remarkably similar in conformational geometry and surface charge distribution to an established synthetic AR antagonist, although the atomic compositions of the two substances are quite different. Taken together with our published reports of the estrogen agonist activities of DIM, the present results establish DIM as a unique bifunctional hormone disrupter. To our knowledge, DIM is the first example of a pure androgen receptor antagonist from plants.

3,3'-Diindolylmethane (DIM) is a major digestive product of indole-3-carbinol, a potential anticancer component of cruciferous vegetables. Our results indicate that DIM exhibits potent antiproliferative and antiandrogenic properties in androgen-dependent human prostate cancer cells. DIM suppresses cell proliferation of LNCaP cells and inhibits dihydrotestosterone (DHT) stimulation of DNA synthesis. These activities were not produced in androgen-independent PC-3 cells. Moreover, DIM inhibited endogenous PSA transcription and reduced intracellular and secreted PSA protein levels induced by DHT in LNCaP cells. Also, DIM inhibited, in a concentration-dependent manner, the DHT-induced expression of a prostate-specific antigen promoter-regulated reporter gene construct in transiently transfected LNCaP cells. Similar effects of DIM were observed in PC-3 cells only when these cells were co-transfected with a wild-type androgen receptor expression plasmid. Using fluorescence imaging with green fluorescent protein androgen receptor and Western blot analysis, we demonstrated that DIM inhibited androgen-induced androgen receptor (AR) translocation into the nucleus. Results of receptor binding assays indicated further that DIM is a strong competitive inhibitor of DHT binding to the AR. Results of structural modeling studies showed that DIM is remarkably similar in conformational geometry and surface charge distribution to an established synthetic AR antagonist, although the atomic compositions of the two substances are quite different. Taken together with our published reports of the estrogen agonist activities of DIM, the present results establish DIM as a unique bifunctional hormone disrupter. To our knowledge, DIM is the first example of a pure androgen receptor antagonist from plants.

1 The abbreviations used are: DIM, 3,3'-diindolylmethane; AR, androgen receptor; ARE, androgen response element; DHT, 5α-dihydrotestosterone; GFP, green fluorescent protein; MMTV-Luc, murine mammary tumor virus-luciferase; PSA, prostate-specific antigen; CREB, cAMP-response element-binding protein; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; DCC, dextran-coated charcoal.
petitive binding assays, nuclear translocation studies, and structural modeling computations suggest that DIM disrupts AR function in a manner similar to a chemically dissimilar synthetic antiandrogen, Casodex. Our results identify DIM as a structurally novel, naturally occurring, pure androgen antagonist of potential cancer preventive and therapeutic usefulness for prostate cancer.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM, and LipofectAMINE reagent were supplied by Invitrogen. Phenol red-free DMEM base, fetal bovine serum (FBS), calf serum, cytochrome c, 2-bromo-1-6-triptycene-3,20-dione, and S-methylisothiourea sulfate were purchased from Sigma. CellTiter 96 Aqueous One Solution Assay was obtained from Promega. Casodex was provided by AstraZeneca. Dextran-coated charcoal-FBS (DCC-FBS) was from Hyclone (Logan, UT). [3H]-DHT and [3H]thymidine were supplied by PerkinElmer Life Sciences. AR rabbit (sc-816, sc-815) polyclonal IgGs and PSA mouse (sc-7316) and goat (sc-7638) monoclonal IgGs were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PSA total (M86506M) and free (M86806M) monoclonal antibodies were from Biodesign International (Saco, ME). DIM was prepared from indole-3-carbinol as described (26–28) and recrystallized in toluene. All other reagents were of the highest grade available.

Cell Culture—The human prostate adenocarcinoma cell lines LNCaP, PC-3, and DU145 were obtained from the American Type Culture Collection (Manassas, VA). They were grown as adherent monolayers in 10% FBS-DMEM, supplemented with 4.0 g/liter glucose and 3.7 g/liter sodium bicarbonate in a humidified incubator at 37°C and 5% CO₂, and passaged at ~80% confluency. Cultures used in subsequent experiments were at less than 40 passages. Cells grown in stripped conditions were in 5% DCC-FBS-DMEM base supplemented with 4.0 g/liter glucose, 3.7 g/liter sodium bicarbonate, and 0.293 g/liter L-glutamine.

Cell Growth—Before the beginning of the treatments, cells were depleted of androgen for 4–7 days in medium composed of DMEM base without phenol red and with 4.0 g/liter glucose and 3.7 g/liter sodium bicarbonate. During the depletion period, medium was changed every 48 h. Treatments were administered by the addition of 1 μM of a 1,000-fold concentrated solution of DIM in Me₂SO/ml of medium. Once the treatment period started, medium was changed daily to counter possible loss of readily metabolized compounds.

Cell Counting—Cells were harvested by trypsinization and resuspended in culture medium. Aliquots were diluted 50-fold in Isoton II (Coulter Corp., Miami, FL), and 200-μl duplicates were counted in a model Z1 Coulter particle counter and averaged.

[3H]Thymidine Incorporation—LNCaP cells were plated onto 24-well plates (Corning) with 2 × 10⁵ cells/well and treated with varying concentrations of DIM with and without 1 nM DHT for 24–48 h. [3H]Thymidine (3 μCi) was added to each well and incubated at 37°C for 2–3 h. Medium was removed, and the cells were washed three times with 2 ml of ice-cold 10% trichloroacetic acid followed by the addition of 300 μl of 0.3 N NaOH to each well and then incubated at room temperature for 30 min. Aliquots (150 μl) were transferred into the scintillation vials with 4 ml of ScintiVerse BD scintillation fluid (Fisher) and counted for radioactivity by a Beckman liquid scintillation counter.

Plasmid Reporters and Expression Vectors—The ARE-responsive luciferase reporter plasmid, pPSA-630 luciferase (pPSA-Luc), was a gift from Dr. M. D. Sadar (29). pPSA-Luc contains the PSA promoter region 630 to 12 with three AREs, all of which are critical to the activity of AR function in a manner similar to a chemically dissimilar antiandrogen, Casodex. Our results identify DIM as a structurally novel, naturally occurring, pure androgen antagonist of potential cancer preventive and therapeutic usefulness for prostate cancer.

**Analysis of Intracellular and Secreted PSA—** LNCaP cells growing on 100-mm plates were treated as indicated for 24 h. Cells were lysed as precipitated binding assays, nuclear translocation studies, and structural modeling computations suggest that DIM disrupts AR function in a manner similar to a chemically dissimilar synthetic antiandrogen, Casodex. Our results identify DIM as a structurally novel, naturally occurring, pure androgen antagonist of potential cancer preventive and therapeutic usefulness for prostate cancer.

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**Plasmid Reporters and Expression Vectors**—The ARE-responsive luciferase reporter plasmid, pPSA-630 luciferase (pPSA-Luc), was a gift from Dr. M. D. Sadar (29). pPSA-Luc contains the PSA promoter region (~630 to 12) with three AREs, all of which are critical to the activity of the PSA-Luc promoter. The MMTV-Luc, containing one consensus ARE, and the expression vector, pCMV-hAR, which constitutively expresses a fully functional human androgen receptor, were also generously provided by Dr. M. D. Sadar. The pCMV-GFP-rAR was a gift from Dr. A. K. Roy (30).

**RNA Extraction, mRNA Purification, and Northern Hybridization**—mRNA isolation and Northern blot analyses were conducted as described previously (20, 31). PSA cDNA was generously provided by Dr. M. D. Sadar, and the cDNA probes were biotinylated using NEBLOTE Photoprobe kit (New England Biolabs, Beverly, MA), purified via precipitation with 3 M sodium acetate, pH 5.2, and washed with 70% ethanol. After hybridization with cDNA probes, the membrane was incubated with alkaline phosphatase then biotinylated with alkaline phosphatase followed by the Phototope-CDP-Star assay (New England Biolabs) and autoradiographed. The amount of mRNA was quantified by Gel Densitometer and averaged.

**Analysis of Intracellular and Secreted PSA—** LNCaP cells growing on 100-mm plates were treated as indicated for 24 h. Cells were lysed as previously described (20) for intracellular PSA analysis. For secreted proteins, spent medium was collected and concentrated 15-fold using Millipore Centriprep YM-10 following the manufacturer’s protocol (Bedford, MA). Protease inhibitors (10 μg/ml aprotenin, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 50 μg/ml phenylmethylsulfonyl fluoride) were added, and the proteins were immunoprecipitated with 3 μg/ml monoclonal free PSA antibody (Biodesign International) for 2 h and co-immunoprecipitated overnight at 4°C with protein A/G-agarose (Santa Cruz Biotechnology) on a rotator. The samples were then subjected to Western blot analysis as described previously (20) using a monoclonal PSA (sc-7316) primary antibody and a goat anti-mouse-IgG-AP secondary antibody (sc-208) from Santa Cruz Biotechnology.

**Transient Transfections with Reporters and Luciferase Assay—** LNCaP and PC-3 cells were transfected with some modifications, as previously described (20). For AR transactivation, cells were transfected with 0.1 μg of MMTV-Luc or pPSA-Luc per plate. Co-transfection experiments with pCMV-hAR or pCMV-GFP-rAR, 0.1 μg/plate, was also used. For experiments involving GFP fluorescence imaging, treatments were not added until 30 h after transfection.

**Hormone Binding Assay—** LNCaP cells were grown in 5% DCC-FBS-
DMEM medium supplemented with 4.0 g/liter glucose and 3.7 g/liter sodium bicarbonate and harvested in Hepes-buffered saline containing 1.5 mM EDTA by scraping with a rubber policeman. The cells were placed on ice, collected by centrifugation, washed with ice-cold TKEG buffer (20 mM Tris-HCl, pH 7.4, 50 mM KCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) and resuspended in 250 μl/plate homogenization buffer (50 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 10 mM sodium molybdate, 2.5 mM β-mercaptoethanol, 50 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol). Cells were homogenized using a Polytron apparatus at medium speed for 1 min on ice. The homogenates were centrifuged at 50,000 rpm in 4 °C for 60 min. The supernatant solution was divided into 1.0-ml aliquots, quickly frozen in a dry-ice/ethanol bath, and stored at −80 °C. Protein concentration was determined by the Bradford assay using bovine serum albumin as the standard. For each competitive binding assay, 50 μl of 20 nM [3H]DHT in 50% ethanol, 10 mM Tris, pH 7.5, 10% glycerol, 1 mg/ml BSA, and 1 mM dithiothreitol was placed in a 1.5-ml microcentrifuge tube. Competitive ligands were added as 1.0 μl of 100-fold dilution in Me2SO. After mixing, 95 μl of either LNCaP cell extracts or recombinant AR protein (PanVera, Madison, WI) was added, and the solutions were vortexed and incubated at room temperature for 2–3 h. Proteins were precipitated by the addition of 100 μl of 50% hydroxylapatite slurry equilibrated in TE (50 mM Tris, pH 7.4, 1 mM EDTA) and incubated on ice for 15 min with vortexing every 5 min to resuspend the hydroxyapatite. The pellet was washed with 1.0 ml of ice-cold wash buffer (40 mM Tris, pH 7.4, 100 mM KCl) and centrifuged for 5 min at 10,000 × g at 4 °C. The supernatant was carefully aspirated, and the pellet was washed 2 more times with 1.0 ml of wash buffer. The final pellet was resuspended in 200 μl of ethanol and transferred to a scintillation vial. A negative control was used for each reaction. All negatives contained no protein, and nonspecific binding was determined using 100-fold (0.1 μM) excess unlabeled DHT.

**Fig. 2.** The effect of DIM on expression of endogenous PSA gene. A, Northern blot of PSA mRNA in LNCaP cells treated with 1, 10, and 50 μM DIM for 24 h. B, time course of PSA down-regulation by treatment with 10 μM DIM. C, cells grown in either complete (C) or stripped (S) medium were treated with 1 mM DHT for increasing times. Greatest induction of PSA mRNA by 1 mM DHT at 72 h was inhibited with 24 h of 50 μM DIM co-treatment. β-Actin was used as the internal control for Northern blot analysis. Regulation of PSA gene expression was quantified by scanning with a gel densitometer and is represented as -fold change over control.
HDK buffer (25 mM Hepes, pH 7.5, 1 mM dithiothreitol, 0.4 M KCl). Cytosolic and nuclear extracts were subsequently analyzed by Western blot analysis.

**Western Blot Analysis**—After the indicated treatment, Western immunoblot analyses of androgen receptor from LNCaP cells were performed as described previously (20). In short, polyclonal AR antibodies, sc-816 and sc-815, from Santa Cruz Biotechnology were used as primary antibodies with a chemiluminescence protein detection method. Blotted membranes were stained with Coomassie Blue to determine protein loading, or β-actin (sc-8432, Santa Cruz Biotechnology) was used as an internal control. The amount of protein was quantified by Gel Densitometer (Bio-Rad) and normalized with β-actin when used as an internal control.

**Fluorescence Imaging**—PC-3 cells were plated on cover slips in 6-well culture plates at 1.5 × 10⁵ cells/well in 5% DCC-FBS-DMEM medium. Cells were co-transfected with pCMV-GFP-rAR and pPSA-Luc or MMTV-Luc as indicated above. Cover slips were placed on microscope slides, and images were taken at 1000X. Fluorescence imaging of GFP was performed using a Zeiss Axiopt 381 and Q-imaging MicroPublisher at the College of Natural Resources Biological Imaging Facility of the University of California, Berkeley, CA.

**Modeling of DIM Binding to the AR Ligand Binding Domain**—Quantum mechanical geometry optimizations were performed at a high level of theory, 6-31G**/MP2, for DHT, DIM, Casodex, and R1881. Using these molecular coordinates, a solvent-accessible surface was constructed surrounding each molecule; such a surface enables coupling of the ab initio electronic structure calculations to the solution of the Poisson-Boltzmann equation (34). The coupling was accomplished through the single and double layers of charge at the boundary and allowed for relaxation of the quantum electronic charge distribution in response to these surrounding layers. This first principles approach eliminated the need to assign fractional charges to the atoms. The induced polarization charge at the interface was then mapped onto the nodes ("dots") of the elements of the solvent accessible surface. A comparison was then made between these molecules. The atomic configuration of DHT determined experimentally, i.e. obtained from the crystal structure of the molecule in the androgen receptor, provided a template for comparison of the feasibility of the androgen receptor binding a different ligand (35).

**FIG. 3.** DIM inhibits expression of secreted and intracellular PSA protein. A, Western blot analysis of intracellular PSA level in cells treated with Me₂SO (DMSO) control and 50 μM DIM in the presence and absence of 1 nM DHT. B, DHT (1 nM) induced the expression of secreted PSA, and co-treatment with 50 μM DIM inhibited the expression of the secreted protein. Coomassie Blue staining was used to verify equal protein loading.

**FIG. 4.** Transcriptional activation of reporter genes in the presence of DIM and DHT. A, LNCaP cells were transiently transfected with the MMTV-Luc promoter containing a single androgen response element. After transfection, the cells were treated with increasing concentrations of DIM with and without 1 nM DHT for 24 h. B, LNCaP cells were transiently transfected with the pPSA-Luc promoter containing three androgen response elements. After transfection, the cells were treated with increasing concentrations of DIM with and without 1 nM DHT for 24 h. For both experiments at the completion of treatment luciferase analysis was performed, and luciferase activity in cytosol preparations from individual plates was normalized for protein concentration as determined by Bradford assay. The graph is representative of three different experiments.
constructed a solvent-accessible surface, SR, surrounding the DHT molecule (crystal structure) and used this surface as a reference “standard” or template for the androgen receptor’s ligand binding site. The center-of-mass of each androgen receptor ligand, DHT, Casodex, R1881 (optimized coordinates), and DIM, was translated to the center-of-mass of the template (crystal structure coordinates) and then rotated about the x, y, and z axes through the center-of-mass. We calculated the fractional surface area of the ligand, which did not fit into the binding site template, as follows. For each element, i, of the ligand surface, SL, we then found its nearest neighbor element j on SR, allowing us to form the vector $\mathbf{r}_{ij} = \mathbf{r}_i - \mathbf{r}_j$ from element j (nearest neighbor to i of SL) on SR to element i. By forming the dot product of $\mathbf{r}_{ij}$ with the normal to the element at j on SR, $\mathbf{r}_{ij} \cdot \mathbf{n}_j$, we could determine whether element i of SL is inside or outside of SR. In this way we calculated a $\Delta SL$, the fractional surface area of each ligand that lies outside the template DHT surface SR. This method was repeated using the crystal structure of R1881 as the binding site template (36).

RESULTS

DIM Inhibits the Proliferation and DNA Synthesis of Uninduced and DHT-induced LNCaP Cells—The effects of DIM on human prostate cancer cell growth were examined using LNCaP and PC-3 cells. After a 96-h treatment, DIM produced a concentration-dependent inhibition of LNCaP cell proliferation with maximal inhibition of 70% at 50 μM. At these concentrations, DIM had no observable effects on the growth of PC-3 cells (Fig. 1A). In addition, we examined the effects of varying con-

![Fig. 5. Androgen receptor-mediated transcriptional activation of the MMTV-Luc reporter gene in PC-3 cells.](http://www.jbc.org/)

DIM Is a Potent Androgen Antagonist in Prostate Cancer Cells

![Fig. 6. Relative binding affinity to the androgen receptor.](http://www.jbc.org/)
centrations of DIM with and without 1 nM DHT on DNA synthesis in LNCaP cells (Fig. 1B). Our results showed a concentration-dependent inhibition of DNA synthesis of these cells of up to 90% under both uninduced and androgen-induced growth conditions.

**Inhibition of Endogenous PSA Expression by DIM—**Northern blot analysis was used to examine the effect of DIM on endogenous PSA gene expression. Fig. 2 shows concentration-dependent (A) and time-dependent (B) decreases of up to 70% in PSA mRNA levels after DIM treatments. In addition, PSA mRNA induction by DHT with increasing time of treatment was inhibited by up to 80% by 24 h of co-treatment with DIM (Fig. 2C). Furthermore, Western immunoblot analysis showed that DIM reduced levels of intracellular and secreted PSA protein to background concentrations (Figs. 3, A and B) after DHT co-treatment. The reduction of PSA expression was comparable with the reduction in DHT-induced mRNA expression determined by Northern blot analysis. These results are consistent with DIM regulation of PSA expression occurring at the transcriptional level and consistent with the antiandrogenic activity of DIM observed in the cell proliferation experiments.

**DIM Down-regulates the Activities of DHT-induced Reporter Genes—**The antiandrogenic effects of DIM were further examined with reporter assays using a MMTV-Luc promoter construct that contains one ARE and a pPSA-Luc promoter construct containing three AREs. These plasmids were transiently transfected into LNCaP cells and, by luciferase analysis, showed that DIM strongly inhibited DHT induction of androgen-responsive genes by more than 50% at 1 μM and more than 90% at 10 μM in both promoter constructs (Fig. 4, A and B). Treatment with DIM alone failed to induce transactivation of these reporter genes. These results further confirm that DIM inhibition of AR-responsive gene expression occurs at the transcriptional level.

**The AR Is the Central Modulator of DIM Inhibitory Effects on Androgen-regulated Gene Expression—**To confirm the importance of the AR in the transcriptional activation of the ARE promoters, we employed PC-3 cells, which exhibit little or no AR expression. We transfected these cells with the pPSA-Luc promoter and performed luciferase analysis to show that without co-transfection of an AR expression vector, DIM has no effect (data not shown). In contrast, co-transfection of an AR expression vector with the pPSA-Luc reporter construct led to a concentration-dependent inhibition of DHT-induced transactivation by DIM that was similar to the effect we had observed in LNCaP cells (Fig. 5). The same results were seen with the MMTV-Luc promoter (data not shown). Moreover, DIM by itself did not induce transactivation of these reporter genes in either cell line with or without co-transfection of the wild-type androgen receptor.

**DIM Competes with Androgen for Binding to the AR in LNCaP Cells and in Recombinant AR Protein—**Because our results strongly implicate the AR as the focus of the DIM mode of action in prostate cells, we assessed directly the ability of DIM to bind to this receptor. Our results of competitive binding assays with both the mutant AR of LNCaP cells and a wild-type recombinant human AR demonstrate that DIM, in the micromolar concentration range, competes with labeled DHT for binding to the AR (Fig. 6). Cyproterone acetate and Casodex, two well-known antiandrogens, were used as positive controls. DIM and Casodex exhibited similar binding affinity for the AR.

**Biochemical Analysis of AR Cytoplasmic/Nuclear Distribution in Cells Treated with DHT and DIM—**To examine the effect of DIM on nuclear translocation of the AR, both Western blot analysis and fluorescence imaging of tagged AR were conducted. LNCaP cells were treated with DIM in the presence and absence of 1 nM DHT. Cytoplasmic and nuclear protein fractions were extracted and subjected to Western blot analysis for the AR. The results show that DIM by itself had no effect on nuclear translocation and that 1 nM DHT produced a strong translocation of the AR into the nucleus. However, DHT-induced AR translocation was blocked up to 75% when cells were co-treated with DIM (Fig. 7).

Fluorescence imaging using a pCMV-GFP-rAR co-transfected with pPSA-Luc was used to confirm and extend the results of our Western blot analyses of endogenous AR translocation. Cells treated with 1 nM DHT showed hormone-induced trafficking of the AR to be predominantly nuclear within 1 h of treatment (Fig. 8A). However, co-treatment with 50 μM DIM partially inhibited the translocation of AR induced by

![Image](http://www.jbc.org/)

**Fig. 7. Cellular localization of the AR in LNCaP cells treated with DIM and DHT.** LNCaP cells grown in 5% DCC-FBS DMEM for 5 days were treated for 24 h before extraction of cytosolic and nuclear proteins with increasing concentrations of DIM in the presence and absence of 1 nM DHT. Western blot analysis was performed using an antibody to the AR (Santa Cruz Biotechnology).
co-treating cells with 50 PC-3 cells were co-transfected with ApcMV-GFP-rAR and pPSA-Luc and treated as indicated. Because DIM is a strong and prevents the formation of nuclear AR foci. DIM both inhibits the nuclear translocation of the liganded AR DHT and DIM was similar to activity of the simple pCMV-AR-activity of the chimeric receptor construct in the presence of 50 μM DIM and investigated the mechanism of its action in human prostate cancer cells. The present study characterized the antiandrogenic activity of DIM and investigated the mechanism of its action in human prostate tumor cells. This study is the first to reveal that (a) DIM suppresses DHT-induced cell growth and PSA expression and exhibits no AR agonist activity, (b) DIM has a strong affinity for both the mutant AR inLNCaP cells and for recombinant wild-type human AR, (c) nuclear translocation and foci formation of DHT-bound AR are inhibited by DIM, and (d) modeling studies showed that DIM is remarkably similar in molecular geometry and surface charge distribution to the well established synthetic antiandrogen, Casodex. Our investigation, leads to the conclusion that DIM is a strong, pure androgen antagonist.

Considerable progress has been made in recent years in elucidating the sub-cellular mode of action of the AR. The unliganded AR resides predominantly in the cytoplasm where it is sequestered as a multiprotein complex with heat shock proteins and immunophilins. Upon ligand binding, the AR dissociates from the multiprotein complex, homodimerizes, and is transported into the nucleus, resulting in stimulation or inhibition of androgen receptor-mediated gene expression (30, 37). Our Western blot analysis showed that treatment with DHT alone induced nuclear translocation of the AR and that co-treatment with DIM inhibited DHT-induced translocation in a concentration-dependent manner. To confirm our Western analysis, we used fluorescence imaging with GFP-AR to show that unliganded GFP-AR is primarily localized in the cytoplasm, and upon androgen treatment, it migrates into the nucleus. Translocation of AR induced by DHT is inhibited by DIM co-treatment.

**DISCUSSION**

All of the ligands exhibit the same width, but DIM and Casodex are twice the height of the other ligands. In addition, comparison of the crystal structure of DHT with its computationally optimized conformation showed a slight bending upward of the 3-OH end in the optimized molecule versus a more planar, slightly downward-pointing 3-OH end in the crystal structure. The same change is seen in R1881 (data not shown). This result suggests a slight conformational change in the ligand when it binds to the receptor binding site. We then compared the solvent-induced polarization charges for the AR ligands. We compared solvent-accessible surfaces for DIM, DHT, R1881, and Casodex. The results indicated a similar charge pattern and ellipsoid shapes for all of the ligands, with positive surface charge above the oxygen, fluorine, or nitrogen atoms on both ends of the molecules (data not shown).

Because both DIM and Casodex act as pure antiandrogens, we compared the structures of these ligands more closely. As shown in Fig. 10, the two ligands are remarkably similar in conformation despite their considerable difference in atomic compositions. Both molecules have a planar region (Fig. 10A) containing a polar atom (nitrogen for DIM, fluorine for Casodex) that can bind into the known AR binding site in a manner comparable with the 3-OH group of DHT, combined with a bulky region at the opposite end of the ligand. When rotated by 90 °, to look directly down the bulky end (Fig. 10B), we observe that this end of each of the molecules tilts 30–45 ° relative to the distal aromatic rings, suggesting a similar fit into the androgen receptor ligand binding site. These conformations are in contrast to the more planar structures of the AR agonists, DHT and R1881.

**DISCUSSION**

The present study characterized the antiandrogenic activity of DIM and investigated the mechanism of its action in human prostate tumor cells. This study is the first to reveal that (a) DIM suppresses DHT-induced cell growth and PSA expression and exhibits no AR agonist activity, (b) DIM has a strong affinity for both the mutant AR inLNCaP cells and for recombinant wild-type human AR, (c) nuclear translocation and foci formation of DHT-bound AR are inhibited by DIM, and (d) modeling studies showed that DIM is remarkably similar in molecular geometry and surface charge distribution to the well established synthetic antiandrogen, Casodex. Our investigation, leads to the conclusion that DIM is a strong, pure androgen antagonist.

Considerable progress has been made in recent years in elucidating the sub-cellular mode of action of the AR. The unliganded AR resides predominantly in the cytoplasm where it is sequestered as a multiprotein complex with heat shock proteins and immunophilins. Upon ligand binding, the AR dissociates from the multiprotein complex, homodimerizes, and is transported into the nucleus, resulting in stimulation or inhibition of androgen receptor-mediated gene expression (30, 37). Our Western blot analysis showed that treatment with DHT alone induced nuclear translocation of the AR and that co-treatment with DIM inhibited DHT-induced translocation in a concentration-dependent manner. To confirm our Western analysis, we used fluorescence imaging with GFP-AR to show that unliganded GFP-AR is primarily localized in the cytoplasm, and upon androgen treatment, it migrates into the nucleus. Translocation of AR induced by DHT is inhibited by DIM co-treatment.
Distinct patterns of nuclear distribution of the GFP-AR exist for different ligands, and these patterns correlate with the transactivation activity of the ligand of the AR. Exposure to DHT causes a punctate distribution pattern that is indicative of the association of the translocated receptor within a subnuclear compartment. The formation of these nuclear foci is thought to provide platforms for the interaction of nuclear receptor and co-activators (38). Liganded steroid hormone receptors are transferred to common compartments located in the euchromatin region and form a complex with co-activators, such as steroid receptor coactivator 1, transcriptional intermediary factor 2, and CREB-binding protein, which are also accumulated in the same subnuclear compartments. For the AR, CREB-binding protein was found to be essential for foci formation, and the process of compartmentalization is essential for full transactivation (39). Furthermore, foci formation was shown to be closely linked to transcriptional activation by the AR. It has been reported that a homogeneous pattern of nuclear distribution correlates with an inactive receptor (30, 39). However, patterns of subnuclear compartmentalization vary among different antiandrogens. Well known antiandrogens such as cyproterone acetate and hydroxyflutamide can inhibit androgen activity at relatively high concentrations, whereas they exhibit AR agonist activity at low concentrations (40). Cyproterone acetate induces formation of nuclear foci at low concentrations, also. However, in cells treated with the pure AR antagonist, Casodex, the translocated receptor showed an evenly distributed pattern (37, 41). Other chemicals that have antiandrogen activities and that are well known environmental endocrine disrupters, were also reported to follow this correlation of AR transactivation function and subnuclear clustering. The agricultural fungicide vinclozolin and the insecticide nitrofen have been shown to disrupt formation of intranuclear fluorescence foci while inhibiting AR transactivation (30, 42). Similarly, we show that DIM exerts no agonistic activity while strongly inhibiting DHT-mediated AR transactivation and DHT-induced formation of nuclear foci. Co-treatment with DIM and DHT inhibited AR translocation and produced a homogeneous pattern of fluorescence distribution.

Further comparisons of DIM to known antiandrogens show clear differences in modes of action. The partial androgen agonists, cyproterone acetate and hydroxyflutamide, have been shown to induce partial nuclear translocation of AR at concentrations as low as 1 μM (43). Casodex, which is reported to be a pure AR antagonist, could also stimulate AR nuclear translocation (41). In contrast, DIM did not stimulate AR nuclear
translocation, even at the highest concentration used in the present studies. These results indicate that DIM might mediate an early block in androgen action, including the inhibition of heat shock protein dissociation from the AR and/or a masking of the nuclear translocation signal.

A comparison of the expected lowest energy conformation of DIM with conformations of other androgen receptor ligands, DHT, R1881 and Casodex, indicated several similarities. Quantum mechanical comparisons showed that all of these ligands exhibit similar solvent-induced polarization charge distributions around the region of the molecule that fits into the 3-OH end of the AR binding site, thought to be important for ligand stabilization (35). Although generally comparable in overall size, DIM is a bulkier ligand than either of the two agonists DHT or R1881, which may increase the pocket volume, decreasing hydrogen bonding at the 17β-OH end of the binding site. This disturbance could interfere with helix positioning or orientation of the bound AR, ultimately affecting downstream actions of the AR. Because it has been suggested that the precise positioning of helix 12 is required for the activation of the AR (35), the possibility exists that DIM causes a misplacement of this helix, which contributes to DIM antagonist activity. Furthermore, the structural similarities of DIM and Casodex support the notion that these two ligands may affect their antagonistic effects through a similar steric mechanism.

The down-regulation of PSA by DIM is important because of the association of PSA expression with prostate cancer. PSA is a 240-amino acid glycoprotein with a molecular mass of ~34 kDa that is secreted by prostatic epithelial cells. PSA has been reported to promote the proliferation, migration, and metastasis of prostate cancer cells through several mechanisms, including cleavage of insulin-like growth factor-binding protein-3 and degradation of extracellular matrix proteins fibronectin and laminin (44, 45). PSA expression is regulated by the AR and is thought to function as a growth factor in LNCaP cells (46–49). Thus, down-regulation of PSA expression may be important in the antiproliferative effects of DIM in LNCaP cells. In addition, PSA is the most commonly used biochemical marker for detection and monitoring of prostate cancer, and decreases in PSA levels are associated with better prostate cancer prognosis (50, 51). Thus, these results indicate a possible role of DIM in prostate cancer therapy.

It is interesting to note that the antiproliferative and antiandrogenic activity of DIM in LNCaP cells were observed at physiologically relevant concentrations. A man of average weight who consumes 200 g of broccoli daily will obtain ~12 mg of DIM. With maximum absorption of DIM, the blood concentration of DIM would be as high as ~10 μM. Therefore, in vitro concentrations of DIM from dietary Brassica vegetables represent the effective levels of DIM in vivo.

In conclusion, our study establishes DIM as a pure androgen antagonist that blocks expression of androgen-responsive genes and inhibits AR nuclear translocation and nuclear foci formation. The discovery of DIM as the first pure androgen receptor antagonist from plants establishes this substance as a new class of hormonally active agents with potential both as environmental androgen disrupters and as prostate tumor preventive and therapeutic agents.

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