Antiandrogenic and Growth Inhibitory Effects of Ring-Substituted Analogs of 3,3′-Diindolylmethane (Ring-DIMs) in Hormone-Responsive LNCaP Human Prostate Cancer Cells

Khalil Abdelbaqi,[1] Nathan Lack,[2] Emma Tomlinson Guns,[2] Leela Kotha,[3,4] Stephen Safe,[3,4] and J. Thomas Sanderson[1]*

[1]*INRS-Institut Armand-Frappier, Université du Québec, Laval, QC, Canada*
[2]*The Prostate Centre at Vancouver General Hospital (VGH), Vancouver, BC, Canada*
[3]*Department of Veterinary Physiology and Pharmacology, A&M University, College Station, Texas*
[4]*Institute for Bioscience and Technology, Houston, Texas*

BACKGROUND. Cruciferous vegetables protect against prostate cancer. Indole-3-carbinol (I3C) and its major metabolite 3,3′-diindolylmethane (DIM), exhibit antitumor activities in vitro and in vivo. Several synthetic ring-substituted dihaloDIMs (ring-DIMs) appear to have increased anticancer activity.

METHODS. Inhibition of LNCaP prostate cancer cell growth was measured by a WST-1 cell viability assay. Cytoplasmic and nuclear proteins were analyzed by immunoblotting and immunofluorescence. Androgen receptor (AR) activation was assessed by measuring prostate-specific antigen (PSA) expression and using LNCaP cells containing human AR and an AR-dependent probasin promoter-green fluorescent protein (GFP) construct.

RESULTS. Like DIM, several ring-substituted dihaloDIM analogs, namely 4,4′-dibromo-, 4,4′-dichloro-, 7,7′-dibromo-, and 7,7′-dichloroDIM, significantly inhibited DHT-stimulated growth of LNCaP cells at concentrations $\geq$1 μM. We observed structure-dependent differences for the effects of the ring-DIMs on AR expression, nuclear AR accumulation and PSA levels in LNCaP cells after 24 hr. Both 4,4′- and 7,7′-dibromoDIM decreased AR protein and mRNA levels, whereas 4,4′- and 7,7′-dichloroDIM had minimal effect. All four dihaloDIMs (10 and 30 μM) significantly decreased PSA protein and mRNA levels. Immuofluorescence studies showed that only the dibromoDIMs increased nuclear localization of AR. All ring-DIMs caused a concentration-dependent decrease in fluorescence induced by the synthetic androgen R1881 in LNCaP cells transfected with wild-type human AR and an androgen-responsive probasin promoter-GFP gene construct, with potencies up to 10-fold greater than that of DIM.

CONCLUSION. The antiandrogenic effects of ring-DIMs suggest they may form the basis for the development of novel agents against hormone-sensitive prostate cancer, alone or in combination with other drugs. *Prostate 71: 1401–1412, 2011.* © 2011 Wiley-Liss, Inc.

KEY WORDS: **ring-DIMs; LNCaP cells; androgen receptor; prostate specific antigen; immunofluorescence; dihydrotestosterone**

INTRODUCTION

Prostate cancer is the most common diagnosed malignancy in Western men and the second leading cause of male death in industrialized countries, with the exception of some Asian countries such as Japan [1,2]. It is generally considered to be a disease of aging men [3]. Epidemiologic studies show that high dietary intake of fruits and vegetables protects against carcinogenesis in many tissues [4,5] Vegetables of the Brassica

Grant sponsor: Canadian Institutes of Health Research (CIHR); Grant number: ISO 93977; Grant sponsor: Natural Sciences and Engineering Council of Canada (NSERC); Grant number: 313313; Grant sponsor: Texas A&M University and Texas Agrilife Research.

*Correspondence to: Dr. Thomas Sanderson, INRS-Institut Armand-Frappier, 531, blv des Prairies, Building 18, Laval, QC, H7V 1B7, Canada. E-mail: thomas.sanderson@iaf.inrs.ca

Received 25 June 2010; Accepted 14 January 2011
DOI 10.1002/pros.21356
Published online 14 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

family, in particular broccoli and Brussels sprouts, exhibit lower incidences of certain cancers in individual consumers, including hormone-dependent cancers such as prostate [4] and breast cancer [5]. Cruciferous vegetables contain various chemopreventative phytochemicals including indole-3-carbinol (13C), which is rapidly hydrolysed in the acidic environment of the stomach and converted into structurally diverse condensation products including 1,1-bis(3′-indolyl)methane (DIM) [6,7].

DIM and I3C inhibit growth of various cancer cells in vitro and in vivo [8]. In vitro studies have shown that DIM and I3C inhibit cancer cell proliferation, inhibit G_o/G_1 to S-phase progression of prostate cancer cells and induce apoptosis through a mitochondrial pathway [7,9–11]. In vivo studies have shown that growth of *N*-methyl-*N*′-nitrosourea- and 7-12-dimethylbenzoanthracene-induced mammary tumors in rats was inhibited by dietary administration of cabbage or broccoli [12] and mice treated with 5–10 mg/kg DIM (3× per week) had significantly smaller prostate tumor volumes and weights compared to untreated animals [13]. DIM also inhibited cell proliferation, migration, invasion, and capillary tube formation of cultured human umbilical vascular endothelial cells (HUVECs) and repressed vascularization in a Matrigel Plus assay and tumorigenesis in athymic mice bearing MCF-7 breast cancer cells as xenografts [14]. Previously, it was reported that a lower concentration of formulated DIM (B-DIM; 50% higher in vivo bioavailability) [15] induced apoptosis and inhibited growth, angiogenesis, and invasion of prostate cancer cells by regulating the Akt and NF-κB signaling pathways [16,17] and their downstream target genes and responses. It has also been suggested that B-DIM could be effective in prevention and/or treatment of hormone-sensitive as well as hormone-refractory prostate cancer (HRPC) [18]. 1,1-Bis-(3′-indolyl)-1-(*p*-substituted-phenyl)methanes (C-DIMs) are synthetic analogs of DIM that act as peroxisome proliferator-activated receptor-gamma (PPARγ) agonists and these C-DIM analogs also inhibit prostate cancer cell growth and androgen responsiveness in AR-positive prostate cancer cells [19–25].

The androgen receptor (AR) is a member of the nuclear receptor superfamily and in healthy tissue acts as a ligand-dependent nuclear transcription factor. Upon binding to the AR, androgens activate the receptor, which dimerizes and, in turn, interacts with androgen responsive elements (AREs) or other transcription factors in promoter regions of target genes, such as prostate-specific antigen (PSA). Le et al. [26] established that DIM at a concentration of 10 and 30 μM acted as a pure AR antagonist, blocking expression of androgen-responsive genes and inhibiting DHT-induced AR translocation and nuclear foci formation. Moreover, they showed that DIM inhibited endogenous PSA transcription and reduced DHT-induced intracellular and secreted PSA protein levels in LNCaP human androgen-dependent prostate cancer cells.

AR-dependent signaling pathways play important roles in the progression of prostate cancer from an androgen-sensitive to a hormone-resistant stage [27]; during this progression, the majority of prostate cancer cells still express AR [28]. However, it has been shown that Akt and NF-κB activate AR signaling through ligand-independent phosphorylation of AR or transcriptional regulation of AR [20,21]. IL-4 increased NF-κB activation of AR [29] through binding cognate response elements in the AR gene promoter [30].

Patients with early-stage prostate cancer are treated with chemotherapies [31], such as androgen-deprivation therapy. However, many patients, particularly those with advanced prostate cancer, eventually develop resistance and progress to HRPC, for which there are few effective curative therapies [32]. This has led investigators to test cytotoxic chemotherapeutic agents for treatments of HRPC. Hormone therapy or androgen-ablation therapy depletes hormones (castration) or blocks their action (LHRH agonists, antiandrogens, steroid 5α-reductase inhibitors) and stops or slows cancer cell growth. New approaches have been developed involving treatments that either block adrenal, testicular, or local androgen production (e.g., with steroid 17α-reducatse inhibitors) to inhibit androgenic effects in the target tissues [31,33,34]. In previous studies, we have shown that several substituted DIMs were more potent inhibitors of breast cancer cell growth than DIM [35,36]. The objective of this study was to examine the antiandrogenic effects of a number of synthetic symmetrically ring-substituted dihaloDIM analogs (ring-DIMs) in an in vitro model, LNCaP human prostate cancer cells, to evaluate their potential as novel therapeutic agents for treatment of hormone-sensitive prostate cancer.

MATERIALS AND METHODS

Cell Lines and Reagents

LNCaP cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) or 10% dextran-coated charcoal-stripped FBS (free from endogenous steroid hormones, growth factors, and cytokines), 2 mM L-glutamine, 1% Hepes, 1% sodium pyruvate, and 10 ml/L of 100× antibiotic-antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of an atmosphere with 5% CO_2. Ring-DIMs (ring-substituted 4,4′- and 7,7′-dihaloDIMs) at >95% purity were provided by Dr S Safe, Texas A&M

University, and dissolved in DMSO to make 100 mM stock solutions (for structures see Fig. 1). The final concentration of DMSO in culture medium was 0.1%. This concentration of DMSO was established as nontoxic to the cells. Dihydrotestosterone (DHT; Steraloids Inc, Newport, RI) was dissolved in DMSO to make 50 mmol/L stock solutions. Primary antibodies for AR and β-actin and appropriate secondary antibodies were purchased from Millipore-Upstate (Chicago, IL). Histone and Cox IV primary antibodies were obtained from Abcam (Cambridge, MA).

Growth Inhibitory Effects of Ring-DIMs

The growth inhibition effects of ring-DIMs on LNCaP cells were tested using 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) cleavage assay (Roche Diagnostics, Laval, QC). LNCaP cells were seeded in 96-well plates at a density of 1×10^3 cells/well in phenol red-free RPMI 1640 medium containing 10% dextran-coated charcoal stripped FBS. After 24 hr of incubation at 37°C in a humidified atmosphere with 5% CO_2, the cells were treated with 0.01, 0.02, 0.1, 0.3 nM DHT, and 1, 10, 30 μM of DIM; 4,4-dibromo-; 4,4′-dichloro-; 7,7′-dibromo-; and 7,7′-dichloroDIM. After 48 hr, a second treatment with the same products was carried out in fresh medium and the cells were incubated for another 48 hr. Then, the cells were incubated with 10 μl/well of WST-1 and incubated for 4 hr. The absorbance of the samples at 480 nm was determined using a SpectroMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

GFP-AR Translational Activity Assay

The effect of ring-DIMs on AR transcription was assayed as previously described [37]. Briefly, stably transfected enhanced green fluorescent protein-(eGFP)-expressing LNCaP cells (LN-ARR_2PB-eGFP) containing an androgen responsive probasin-derived promoter (ARR_2PB) were grown in phenol red-free RPMI 1640 supplemented with 5% stripped serum. After 5 days, the cells were transferred to 96-well plates (3.5×10^4 cells/well) with 0.1 nM R1881 and increasing concentrations (0–30 μM) of DIM and ring-DIMs (n = 3). Bicalutamide (Sigma–Aldrich) was used as positive control for AR antagonism. The cells were incubated for 3 days and fluorescence was then measured (excitation λ = 485 nm, emission λ = 535 nm). The viability of the cells was assayed by MTS cell proliferation assay (CellTiter 96 AQueous One Solution Reagent, Promega, Madison, WI).

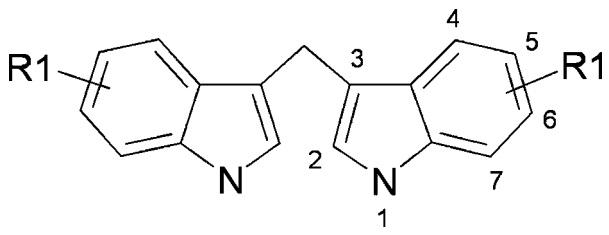

Fig. 1. Chemical structures of DIM (RI = H) and the dihalogenated ring-DIMs (RI = Br or CL) used in this study.

Western Blot Analysis

LNCaP cells were cultured in phenol red-free RPMI 1640 medium containing stripped serum and treated with the ring-DIMs (1, 10, and 30 μM) in the absence or presence of 10 nM DHT (24 hr). Nuclear and cytoplasmic protein fractions were isolated using a NE-PER® Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL). Purity of the fractions was verified using antibodies against histone (nuclear) and Cox IV (cytoplamic). Protein contents were determined using a BCA protein detection kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein (40 μg) were diluted with loading buffer and boiled for 5 min, then loaded onto 10% SDS–PAGE. After electrophoresis, gels were transferred to polyvinylidene diflouride membranes (Bio-Rad, Hercules, CA) overnight at 4°C in transfer buffer. Membranes were blocked using Tris-buffered saline (TBS) plus 5% milk powder (blocking buffer) for 1 hr at room temperature, and then incubated with primary antibody at a 1:2,000 dilution in blocking buffer at 4°C overnight, followed by washes with TBS-T (TBS containing 0.1% Tween) and incubation with the secondary antibody for 1 hr at room temperature. Membranes were then rinsed with TBS-T, TBS 1×, and with water. After rinsing, the membranes were incubated with enhanced chemiluminescence (ECL) reagents for 5 min after which the membranes were sealed in plastic wrap and photographed using ECL hyperfilm to detect immunoreactive bands.

Immunoflourescence Staining and Flourescence Imaging

LNCaP cells were plated on cover slips in 24-well plates containing phenol red-free medium with 10% stripped FBS. Cells were then treated with 10 nM DHT alone or in combination with 30 μM of DIM and the ring-DIMs. After 24 hr, the cells were washed with PBS and fixed with methanol for 10 min at −20°C. Then coverslips were washed with PBS and made permeable with 0.3% Triton X-100 in PBS for 15 min at room temperature. Then they were treated with 5% bovine serum albumine for 45 min, and incubated with AR antibodies (1:50) over night at 4°C. After washing with PBS, cells were incubated with Alexa Fluor® 488-

conjugate goat anti-rabbit antibody (1:500, Invitrogen, ON, Canada) along with 0.1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, ON, Canada) at room temperetaure for 30 min. Cell images were captured on a fourescence microscopy system (TE2000, Nikon, Mississauga, ON), using camera COOL SNAP (Photometrics, Tuscon, AZ) 60 × 1.4 objective and 488/364 nm laser wavelenghs to detect Alexa Fluor® 488 and DAPI, respectively.

PSA Assay

Cells were grown in six-well plates. After 24 hr; the cells were washed with PBS and maintained in phenol red-free RPMI 1640 medium containing 10% stripped FBS and were treated with 0.3 nM DHT alone or in combination with 1, 10, and 30 μM of the ring-DIMs. The cells were incubated for 24 hr. After incubation, the conditioned medium was collected and its PSA concentration (ng/100 μg cellular protein) was determined using a human PSA ELISA kit (Anogen, Mississauga, ON, Canada).

RNA Extraction and RT-PCR

Total RNA was extracted from LNCaP cells using the RNAeasy mini kit with DNAase treatment to remove any traces of genomic DNA (Roche Diagnostics). One microgram of total RNA was used per reaction in a 25 μl reaction volume. Each sample was subjected to reverse transcription using Access RT-PCR Core reagents. The thermal cycler conditions were 48°C for 30 min, 95°C for 10 min, followed by 35 cycles at 95°C for 2 min, 58°C for 1 min, and 72°C for 2 min, and finally 1 cycle at 72°C for 7 min. The following primers were used: for AR, fw: 5′-gcc-att-gag-cca-ggt-gta-gt-3′ and rv: 5′ aga-agg-gga-agg-gat-cac-ac-3′, to produce a 600-bp PCR product. For PSA, fw: 5′-gcc-tct-cgt-ggc-agg-gca-gt-3′ and rv: 5′-gtg-cgc-aag-ttc-acc-ctc-ag-3′) producing a 444-bp PCR product. β-Actin was used as reference gene. RT-PCR analysis of each gene was carried out in the exponential amplification phase.

Statistics

Experiments were replicated at three independent times. The data are presented as means ± SD. Statistical analyses of differences between controls and treated groups were performed using a Student's *t*-test with Bonferroni correction for multiple comparisons. $P < 0.05$ was considered statistically significant. IC_{50} values for growth inhibition and inhibition of AR translational activation were calculated using nonlinear curve-fit analysis (IC_{50} values for growth inhibition were determined at 0.1 nM DHT) and they are presented as means ± SD (n = 4). All analyses were performed using GraphPad Prism v5.03 (GraphPad Software, San Diego, CA).

RESULTS

Ring-DIMs Inhibit Androgen-Stimulated LNCaP Cell Growth

To characterize the response of LNCaP cells to a potent androgen under our culture conditions, we tested the effect of DHT on LNCaP cell growth. Cells treated with DHT showed an increase in growth of about 3 to 5-fold between 0.01 and 0.3 nM. Our results show that 30 μM DIM decreased growth of LNCaP cells (Fig. 2A). The 4,4′- and 7,7′-dibromoDIMs (10 and 30 μM) significantly inhibited DHT-stimulated LNCaP cell growth, whereas a 1 μM concentration of these compounds had minimal effects (Fig. 2B,C). Similarly, 10 and 30 μM 4,4′- and 7,7′-dichloroDIM also inhibited LNCaP cell growth, whereas a 1 μM concentration exhibited a lesser effect (Fig. 2D,E). IC_{50} values (μM) were 34.4 ± 5.1 for DIM, 11.2 ± 2.4 and 15.3 ± 3.9 for 4,4′- and 7,7′-dibromoDIM, and 14.2 ± 4.1 and 10.6 ± 3.4 for 4,4′- and 7,7′-dichloroDIM, respectively. Cytotoxic effects of the ring-DIMs in the human prostate carcinoma cell line were excluded by measuring levels of intracellular lactate dehydrogenase (LDH). Our results showed that ≤30 μM of the ring-DIMs were not overtly cytotoxic and did not cause increased LDH leakage or decreased cell viability by WST-1 assay compared to controls but concentrations ≥50 μM caused a significant increase in LDH leakage (not shown).

Ring-DIMs Inhibit AR-Dependent GFP Expression

The effects of DIM and ring-DIMs on AR transcriptional activity was assayed using an LNCaP cell line that stably expresses an AR-responsive promoter linked to an eGFP reporter (Fig. 3). DIM and the ring-DIMs all decreased R1881-induced AR transcriptional activity in a concentration-dependent manner. Time-course experiments showed that this decrease was also time-dependent between 0 and 72 hr (not shown). The 4,4′- and 7,7′-dichloro- and -dibromoDIMs displayed greater potency than DIM itself and the most potent compound was 7,7′-dichloroDIM. IC_{50} values (μM) were 19.6 ± 3.6 for DIM, 2.3 ± 1.3 and 1.9 ± 0.9 for 4,4′- and 7,7′-dibromoDIM, and 3.8 ± 1.4 and 0.7 ± 0.6 for 4,4′- and 7,7′-dichloroDIM, respectively. Bicalutamide at 10 μM completely inhibited AR-mediated transcriptional activation (not shown).

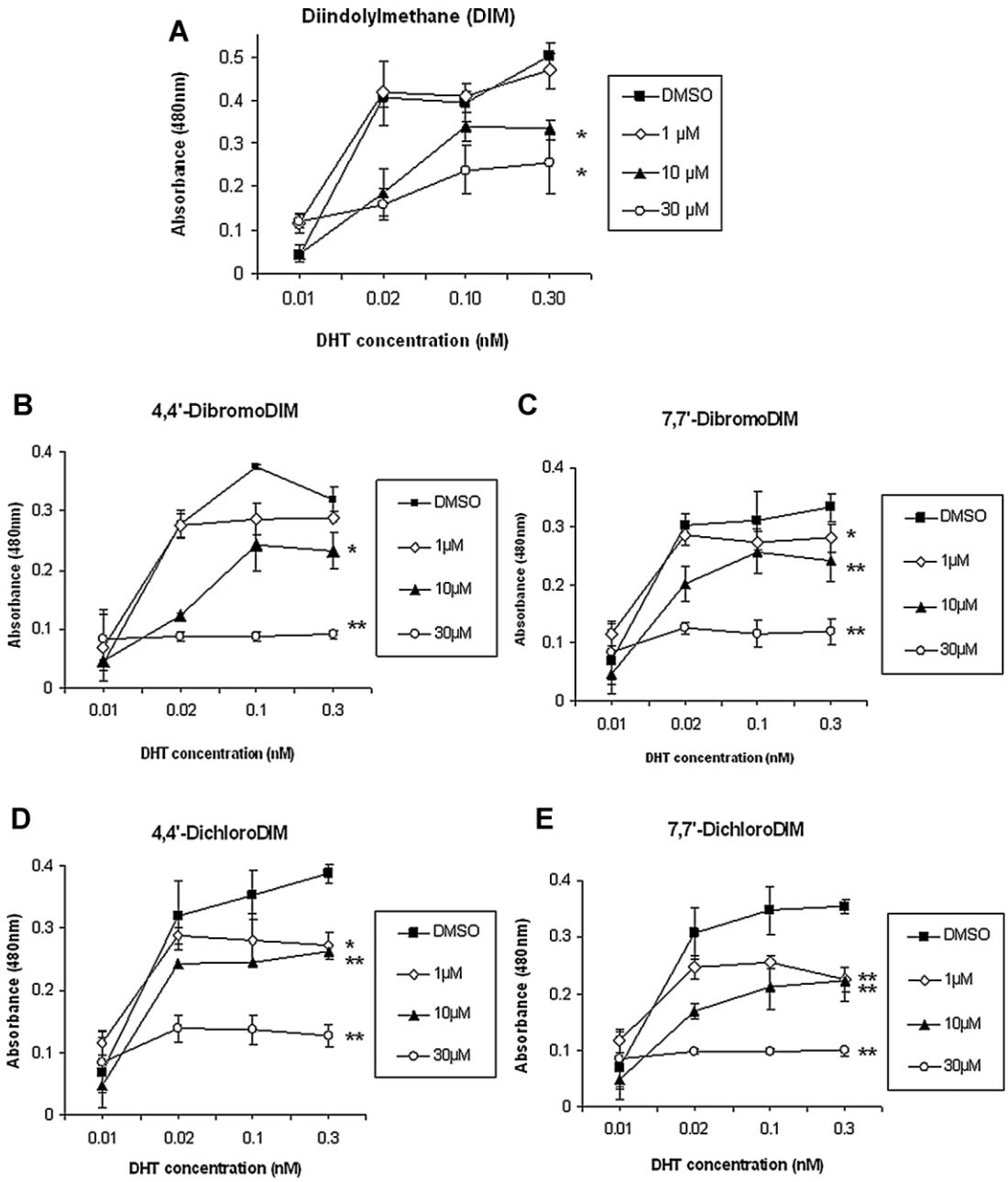

Fig. 2. Growth inhibitory activity of (**A**) diindolylmethane (DIM); (**B**) 4,4′-dibromo-; (**C**) 7,7′-dibromo-; (**D**) 4,4′-dichloro-; and (**E**) 7,7′-dichloroDIM in LNCaP cells in the presence of increasing concentrations of DHT. Results are expressed as mean ± SD of four determinations for each treatment group; statistically significant differences are indicated as ($^{*}P < 0.05$, $^{**}P < 0.01$) for all treated groups.

Differential Effects of Ring-DIMs on AR Expression in LNCaP Cells

Western blot analysis showed that 30 μM 4,4′- and 7,7′-dibromoDIM reduced AR protein levels in both cytoplasmic and nuclear extracts of LNCaP cells treated with 10 nM DHT, whereas, 4,4′- and 7,7′-dichloroDIM had minimal effect on AR expression (Fig. 4A,B). The effects of these compounds on AR mRNA levels were determined by RT-PCR. A 30 μM concentration of 4,4′-dibromo- or 7,7′-dibromoDIMs (24 hr exposure) down-regulated AR mRNA levels, whereas AR mRNA levels were unchanged in cells treated with 30 μM 4,4′- or 7,7′-dichloroDIM (Fig. 5), and these results are consistent with the effects observed on AR protein expression. In contrast to previous studies [26] 30 μM DIM did not affect DHT-induced nuclear uptake of AR; nor did it alter AR mRNA levels. Furthermore, we conducted immunoflourescence experiments to examine the effects of

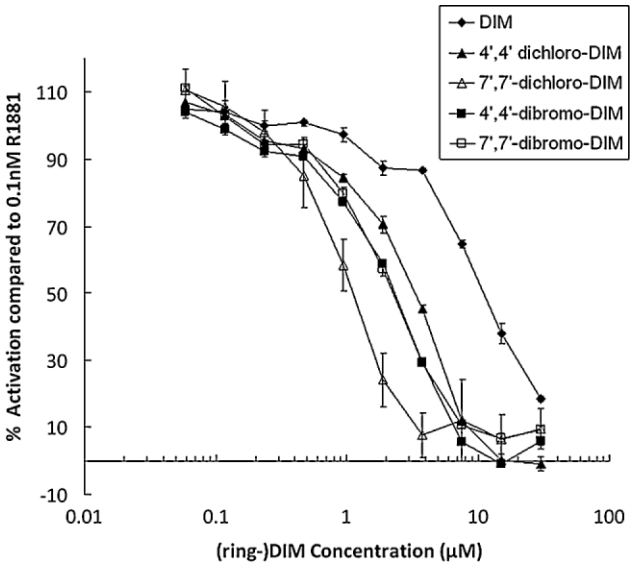

Fig. 3. Androgen receptor-mediated transcriptional activity of stably transfected eGFP-expressing LNCaP cells in the presence of increasing concentrations (0–30 μM) of DIM (◆), 4,4′-dichloro- (▲), 4,4′-dibromo- (△), 7,7′-dichloro (■), and 7,7′-dibromoDIM (□). The % activation was calculated by normalizing the data to the positive (0.1 nM R1881) and negative (DMSO) controls.

ring-DIMs (30 μM for 24 hr). on AR translocation in LNCaP cells. Less nuclear AR staining was observed in ring-DIM-treated cells compared to cells treated with DHT alone, with the exception of 4,4-dichloroDIM. The brominated ring-DIMs were most effective in decreasing DHT-stimulated accumulation of AR in the nucleus (Fig. 6).

Ring-DIMs Inhibit PSA Release and Expression in LNCaP Cells

Release of endogenous PSA protein in LNCaP cells was measured in culture medium after treatment with 0.3 nM DHT alone or in combination with 1, 10 or 30 μM 4,4-dibromo-; 4,4′-dichloro-; 7,7′-dibromo-; and 7,7′-dichloroDIM. The ring-DIMs (10 and 30 μM) significantly inhibited release of PSA protein in the presence or absence of DHT (Fig. 7), and significant inhibition of DHT-induced PSA secretion was observed in all cells treated with 1 μM 4,4′- and 7,7′-dibromoDIM and 4,4′-dichloroDIM. Furthermore, RT-PCR analysis showed that ring-DIMs decreased the levels of PSA mRNA expression (Fig. 8), confirming the potent antiandrogenic activity of these compounds.

DISCUSSION

One of the current strategies for treatment of prostate cancer is the use of steroidal and non-steroidal antihormone therapy [33]. The mechanisms of action of these agents may be different, but they all inhibit the effects of endogenous hormones or their metabolites on the growth of prostate cancer cells [38]. These chemical agents have defined half-lives and the maintenance of therapeutically active tissue concentrations is a challenge. Moreover, long-term side-effects for some of these compounds, such as flutamide or bicalutamide, are a serious issue for patients undergoing antihormonal chemotherapy [39–42].

I3C and DIM inhibit growth of various cancer cell lines through multiple mechanisms and these compounds also directly activate or inactivate a number of receptors. The antiproliferative effects of DIM on cancer cells in vitro suggest that this diet-derived phytochemical might have clinical utility as a therapeutic agent for certain types of cancer. The range of cancer cell lines that are sensitive to growth inhibition by I3C and DIM is broad, and includes carcinomas of prostate, cervix, breast, and liver. Antiproliferative effects of DIM in cell lines from ovarian and endometrial cancers, myelomas and gliomas have also been observed [13], and there is clinical evidence regarding the efficacy of I3C for treating cervical cancer [43].

Previous studies have shown that DIM competitively binds the AR and decreases DHT-induced transactivation/gene expression in LNCaP cells [26]. It has been reported that DIM modulates the expression of a large number of genes important for cell growth, cell cycle progression, and apoptosis. The mechanism of the antiandrogenic activity of DIM in LNCaP cells was novel since this compound inhibited DHT-induced nuclear translocation of cytoplasmic AR [21], whereas most other antiandrogens do not block nuclear translocation of the AR, but form transcriptionally inactive nuclear AR complex [44,45]. Ring-substituted DIMs exhibit potent anti-breast cancer activities [35,36]; however, the structure-dependent effects of these compounds as antiandrogens have not previously been reported. In the present study, 4,4′- and 7,7′-dibromoDIM decreased levels of cytoplasmic and nuclear AR protein as well as AR mRNA, whereas the chlorinated analogs had minimal effects on AR levels (Figs. 4 and 5). Our immunofluorescence experiments corroborate these observations; treatment with the dibromoDIMs decreased nuclear AR staining (accumulation) more effectively than the dichloroDIMs, which had minimal to non-detectable effect on DHT-stimulated nuclear AR accumulation. The results obtained with DIM were similar to those observed for the chlorinated ring-DIMs and did not correspond to a previous report showing that 1, 10, and 50 μM DIM decreased DHT-induced nuclear AR protein [26]. A recent study also showed that the B-DIM formulation alone decreased AR protein and mRNA levels; however, in cells co-treated with DHT and B-DIM the effects on AR protein levels were not dramatic [16]. An explanation for the

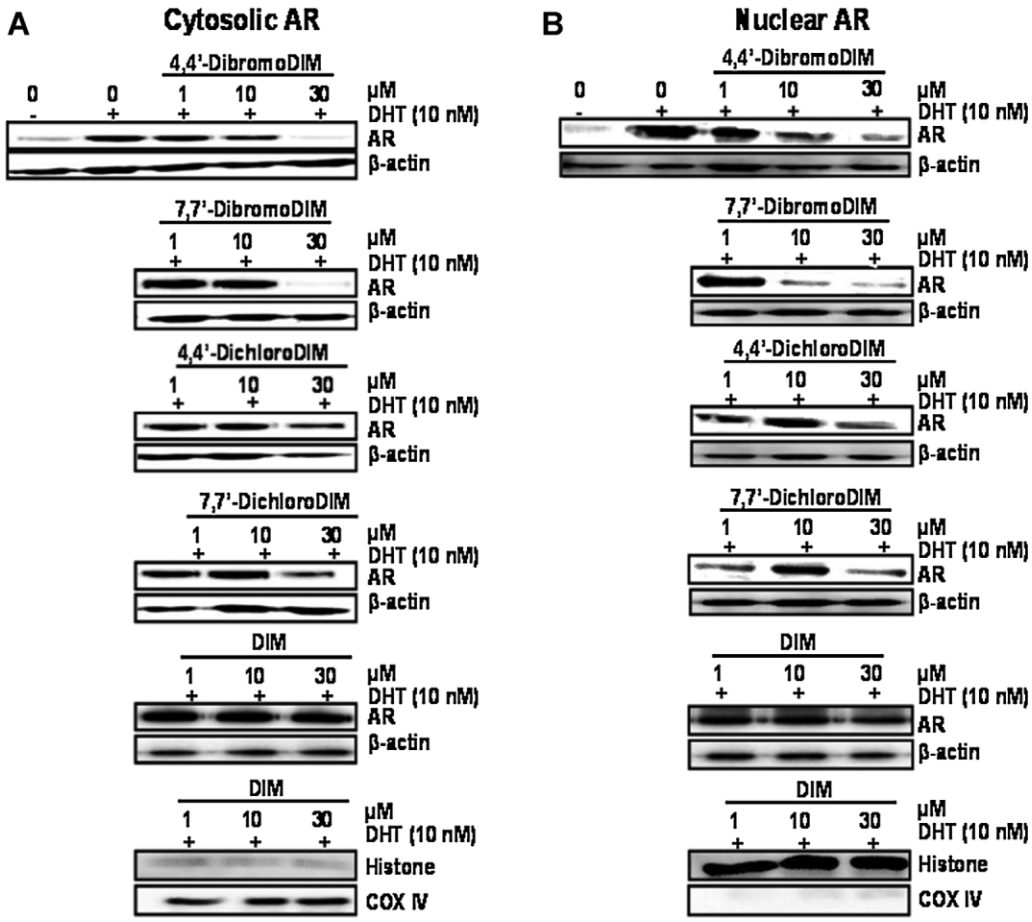

Fig. 4. Antiandrogenic effect of ring-DIMs on AR protein levels in the cytoplasmic (**A**) and nuclear (**B**) fractions of LNCaP cells. LNCaP cells were exposed to 4,4′-dibromo; 4,4′-dichloro; 7,7′-dibromo; and 7,7′-dichloroDIM in the absence or presence of 10 nM DHT for 24 hr in phenol red-free RPMI 1640 medium containing 10% stripped FBS. Histone and COX IV were detected to show the purity the nuclear and cytoplasmic preparations.

differences between these studies is currently being investigated. Our current data demonstrate that dibromoDIMs, but not dichloroDIMs, decreased AR protein expression in LNCaP cells and the activity of the dibrominated analogs was similar to that previously described for C-DIM analogs [46]. The potential mechanisms of dibromoDIM-mediated decreased expression of AR in both cytoplasm and nucleus could be due to down-regulation of AR transcription (as shown in Fig. 5) or enhanced degradation by other mechanisms that have not yet been identified.

The present study has characterized the growth inhibitory effects of DIM and ring-DIMs in LNCaP human prostate cancer cells and has shown that the ring-DIMs are more potent inhibitors of LNCaP cell growth than DIM (Fig. 2). The effects of ring-DIMs on androgen-induced transcriptional activity were investigated in LNCaP cells transfected with wild-type human AR and an AR-responsive promoter (probasin) linked to the GFP gene. The results indicate that DIM and the ring-DIM compounds act as AR antagonists (not necessarily by direct competition for the ligand-binding site) and decrease the AR-dependent expression of intracellular GFP stimulated by the AR agonist R1881.

It has previously been reported that DHT stabilizes the AR in LNCaP cells and increased levels are observed after 24 hr of treatment, in part, due to increased protein synthesis [47] as well as decreased protein degradation [48]; we also find that DHT increases AR protein levels in LNCaP cells (Fig. 4A,B). Our studies with 4,4′- and 7,7′-dibromo- and -dichloroDIM analogs of DIM show that some of the effects of these compounds in LNCaP cells are structure-dependent. A 30 μM concentration of 4,4′- and 7,7′-dibromoDIM significantly inhibited AR mRNA and protein expression, whereas minimal (non-detectable) inhibition was observed in cells treated with the same concentrations of 4,4′ and 7,7′-dichloroDIM. Nevertheless, all four dihaloDIM

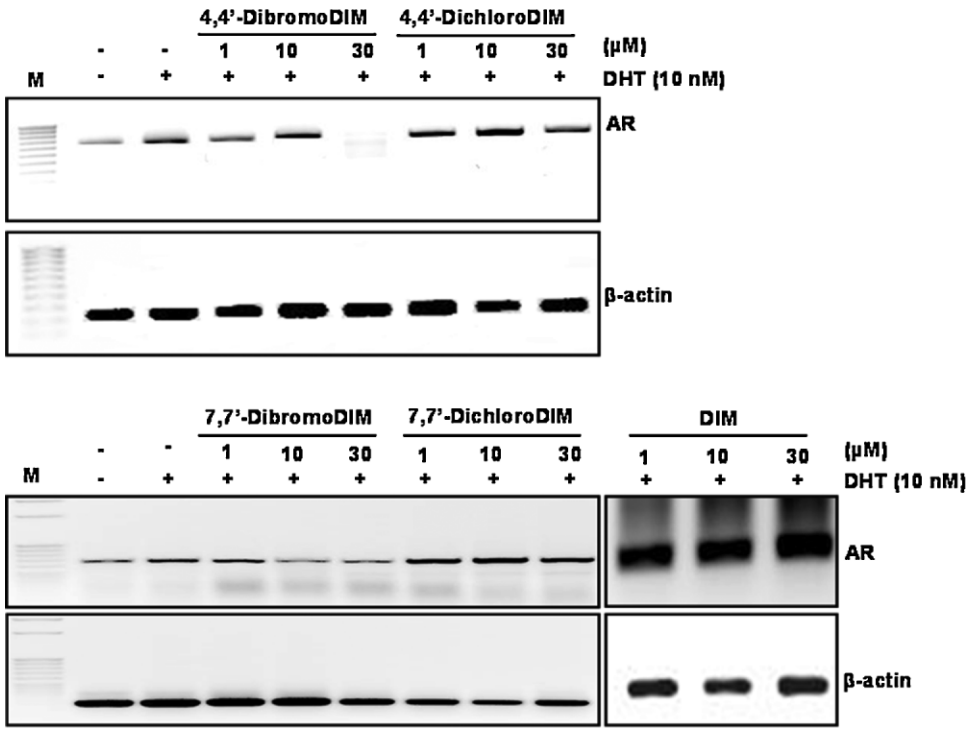

Fig. 5. Effects of ring-DIMs on AR mRNA levels in LNCaP cells treated with 4,4′-dibromo; 4,4′-dichloro; 7,7′-dibromo; and 7,7′-dichloroDIM. LNCaP cells were exposed to the compounds (1, 10, and 30 μM) in the absence or presence of 10 nM DHT for 24 hr in phenol red-free RPMI 1640 medium containing 10% stripped FBS. One of 3 experiments is shown.

congeners exhibited similar growth inhibitory and antiandrogenic activity (Figs. 2,3,7 and 8). These results suggest that the dibromoDIMs exhibit antiandrogenic activity through down-regulation of AR mRNA and protein synthesis resulting in decreased nuclear (as well as cytoplasmic) AR. In addition, the dibromoDIMs may be abrogating the AR protein-stabilizing effect of DHT as has been shown with the antiandrogens hydroxyflutamide and bicalutamide [48]. Although the dichloroDIM isomers also exhibit AR antagonist activity, their effects appear to be independent of AR down-regulation or decreased nuclear uptake and the mechanisms of this antiandrogenic activity are currently being investigated. Our preliminary results using AR-negative PC-3 cells show growth inhibitory effects of all four halogenated ring-DIM congeners, indicating that non-AR-mediated effects also play a role.

A previous study showed that B-DIM (10 and 50 μM) inhibited nuclear AR translocation in LNCaP and in AR-positive, but androgen-independent C4-2B cells [16]. However, our results show that although 30 μM 4,4′- and 7,7′-dibromoDIM blocked the androgen-stimulated AR translocation from the cytoplasm to the nucleus (Fig. 6B,C), minimal (or no) inhibition of AR translocation was observed after treatment with 4,4′- and 7,7′-dichloroDIM (Fig. 6D,E).

The observed down-regulation of PSA expression and secretion in LNCaP cells treated with ring-DIMs (Figs. 7 and 8) is important since PSA is a biochemical marker for detection and monitoring of prostate cancer, and decreased PSA levels are usually associated with an improved prognosis for cancer patients [49,50]. PSA expression is regulated by the AR and is thought to function as a growth factor in LNCaP cells [51,52]. PSA promotes 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 [53,54]. In this study, we observed that 4,4′/7,7′-dibromo- and 4,4′/7,7′-dichloroDIMs significantly decreased DHT-induced secretion of PSA protein by LNCaP cells (Fig. 7) and intracellular PSA mRNA levels (Fig. 8), and this is consistent with the antiandrogenic effects of ring-DIMs observed on AR-mediated translational activation and on cell growth. We are currently examining the effects of ring-DIMs on the cell cycle and their pro-apoptotic effects in LNCaP and PC-3 cells, since these responses may also contribute to the observed inhibition of LNCaP cell growth (Fig. 2) and may be important for activity against androgen-independent prostate cancers.

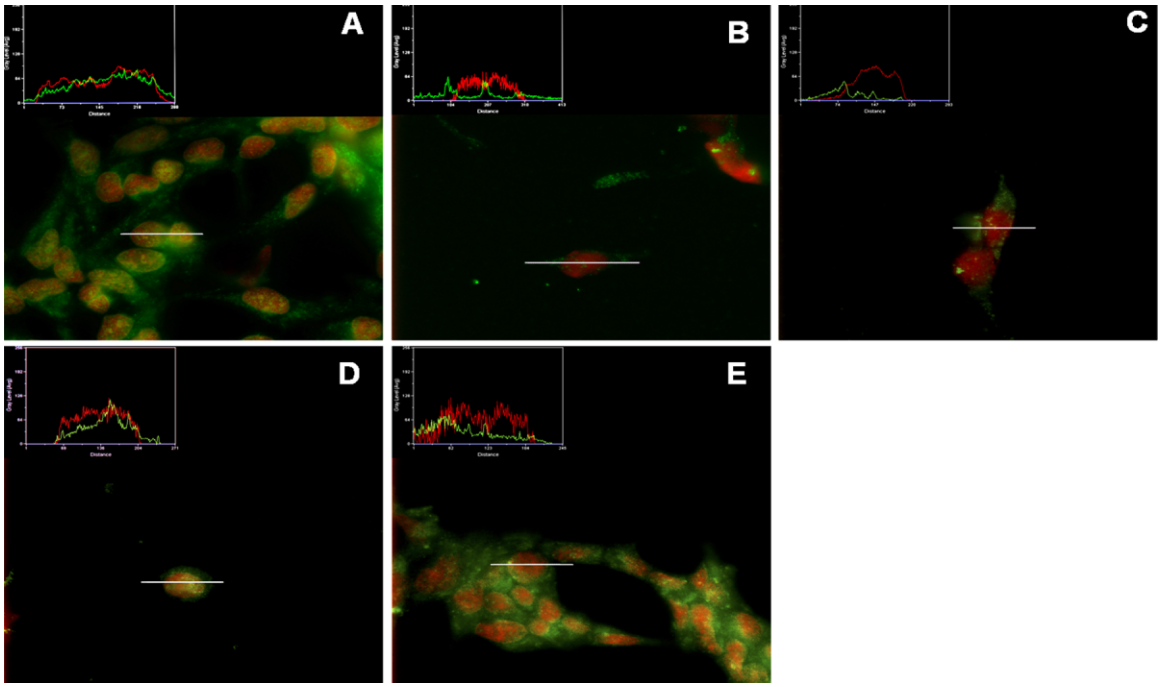

Fig. 6. Effect of ring-DIMs on androgen-stimulated AR translocation into the nucleus of LNCaP cells. LNCaP cells were treated with 10 nM DHT (**A**) as positive control, and with 30 μM of 4,4′-dibromo (**B**); 7,7′-dibromo (**C**); 4,4′-dichloro (**D**); and 7,7′-dichloroDIM (**E**) for 24 hr in phenol red-free RPMI 1640 medium containing 10% stripped FBS. Cells were then incubated with anti-AR antibody followed by the incubation with Fluor® 488-conjugate goat anti-rabbit antibody along with 0.1 μg/ml of DAPI. Cell images were captured on a flourescence microscopy system using 60 × 1.4 objective and 488/364 nm laser wavelengths to detect Fluor® 488 and DAPI, respectively. The blue color of DAPI staining was changed to red for greater visibilty. The translocated AR is colored in green and the nucleus in red. Analysis of a cross-section (white bar) of the stains appear at the upper-side of each image showing the proportional intensity between green (presence of AR) and red (the nucleus, DAPI) color. One of 3 experiments is shown.

This study shows that ring-DIMs, like C-DIMs [46], exhibit antiandrogenic activities in LNCaP cells and inhibit androgen-induced growth (Fig. 2), AR activation (Fig. 3), and PSA secretion (Fig. 7) and expression (Fig. 8). Despite the similarities in their activities in LNCaP cells, ring-DIMs exhibit significant differences in their effects on AR expression and nuclear accumulation, where the dibromoDIMs down-regulate AR at the protein and transcriptional levels, but the dichloroDIMs have minimal effect on AR expression and this was also observed for DIM. These results indicate that dihaloDIMs are more effective than DIM; we also suggest that the cellular uptake of dibromoDIMs and dichloroDIMs are greater than that of DIM, due to their greater hydrophobicity. The observed differences in the mechanisms of action of dibromoDIMs versus dichloroDIMs are currently under study. We are also examining how the effects of various ring substituents and their positions modulate the mechanisms of action of ring-DIMs differently from DIM in LNCaP cells [55]. It is known that the interaction of DHT with the ligand-binding pocket of the AR is driven predominantly by electrostatic bonds [55,56]. Differences in the activities of dibromoDIMs versus dichloroDIMs could be due to several factors including their electronegativity (Br < Cl), size/volume (Br > Cl), and current studies with various ring-substituted DIMs are using QSAR procedures to determine the structural parameters that influence the antiandrogenic activity of these compounds [57–59].

Our results suggest that the antiandrogenic activity of dihaloDIMs are complex and involve multiple pathways including inhibition of nuclear transactivation. Current studies are further investigating their effects on cell proliferation and apoptosis in vitro in several prostate cancer cell lines. Results of this study provide strong evidence that the antiandrogenic effects of the ring-DIMs may form the basis for the development of novel agents for clinical treatment of hormone-sensitive prostate cancer either alone or in combination with other drugs.

ACKNOWLEDGMENTS

This study was financially supported by grants from the Canadian Institutes of Health Research (CIHR, grant number ISO 93977) grant and Natural Sciences and Engineering Research Council of Canada (NSERC,

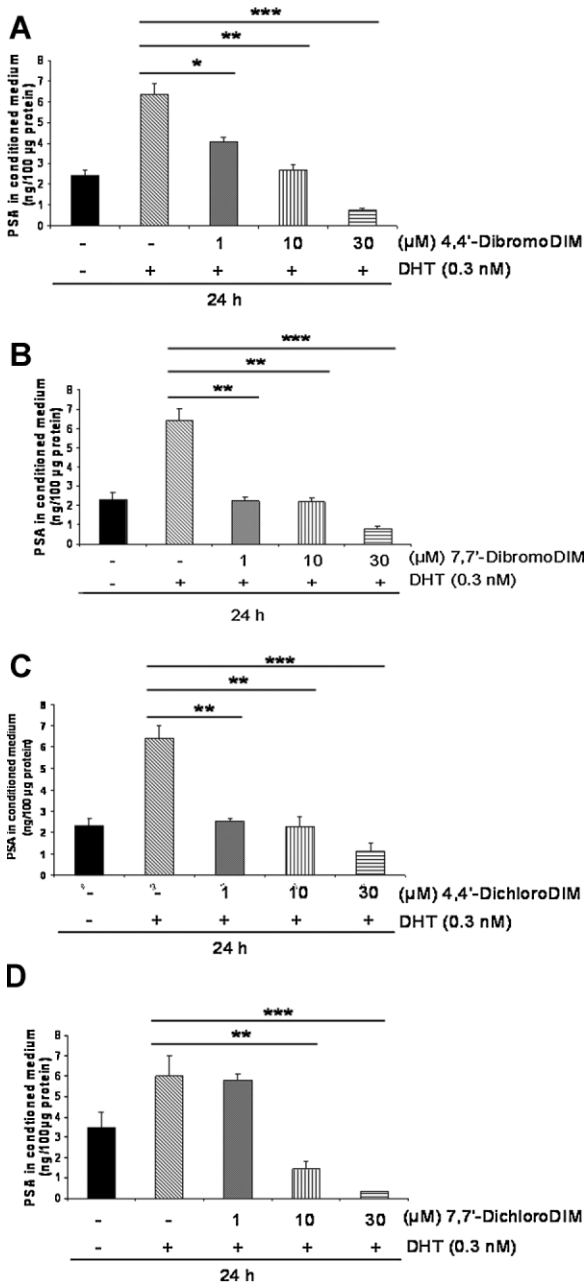

Fig. 7. Effect of (**A**) 4,4′-dibromo-, (**B**) 7,7′-dibromo-, (**C**) 4,4′-dichloro-, and (**D**) 7,7′-dichloroDIM on PSA release in DHT-stimulated LNCaP cells. Exposures were for 24 hr. Results are expressed as mean ± SD of four determinations per treatment group, and significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) differences compared to control (0.3 nM DHT) are indicated.

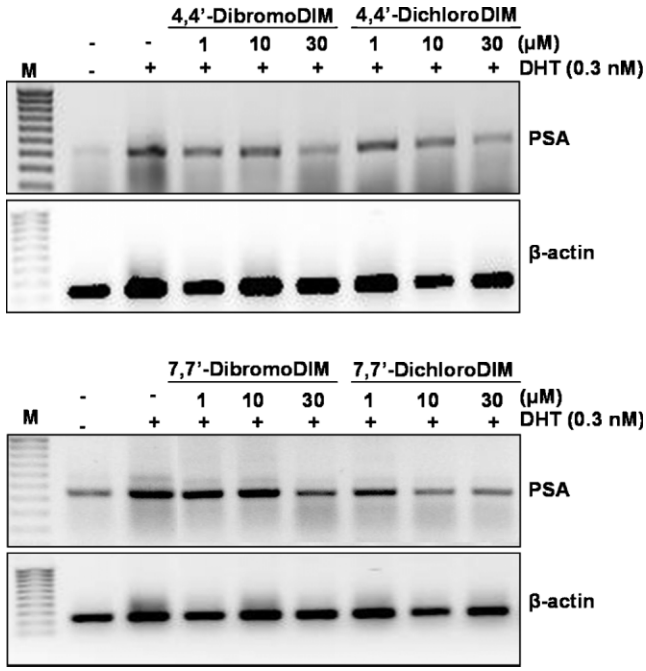

Fig. 8. Ring-DIMs inhibit PSA mRNA expression level in LNCaP cells treated with 4,4′-dibromo; 4,4′-dichloro; 7,7′-dibromo; and 7,7′-dichloroDIM. LNCaP cells were exposed to the compounds (1, 10, and 30 µM) in the absence or presence of 0.3 nM DHT for 24 hr in phenol red-free RPMI 1640 medium containing 10% stripped FBS. One of 3 experiments is shown.

grant number 313313) to Thomas Sanderson as well as support from Texas A&M University and Texas Agrilife Research. We thank Julie Dufresne, Marcel Desrosiers, Marlène Fortier, Talal Oufkir, and Jihane Gasmi for their technical assistance.

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