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1,1-BIS(3'-INDOLYL)-1-(*p*-SUBSTITUTEDPHENYL)METHANES INHIBIT GROWTH, INDUCE APOPTOSIS, AND DECREASE THE ANDROGEN RECEPTOR IN LNCaP PROSTATE CANCER CELLS THROUGH PPARγ-INDEPENDENT PATHWAYS

Sudhakar Chintharlapalli, Sabitha Papineni and Stephen Safe

Department of Veterinary Physiology and Pharmacology (S.C., S.P., S.S.)
Texas A&M University
4466 TAMU, Vet. Res. Bldg. 410
College Station, TX 77843-4466

and

Institute of Biosciences and Technology (S.C., S.P., S.S.)
Texas A&M University Health Science Center
2121 W. Holcombe Blvd.
Houston, TX 77030-3303

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Running Title: C-DIMs decrease androgen-responsiveness in LNCaP cells

Correspondence should be sent to:
Stephen Safe
Department of Veterinary Physiology and Pharmacology
Texas A&M University
4466 TAMU, Vet. Res. Bldg. 409
College Station, TX 77843-4466
Tel: 979-845-5988 / Fax: 979-862-4929
Email: ssafe@cvm.tamu.edu

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Abbreviations:
β-Gal , β-galactosidase; AR, androgen receptor; CDDO, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; C-DIMs, methylene-substituted DIMs; DIM-C-pPhC_6H_5, 1,1-bis(3'-indolyl)-1-(*p*-phenyl)methane; DIM-C-pPhCF_3, 1,1-bis(3'-indolyl)-1-(*p*-trifluoromethyl)-methane; DIM-C-pPhtBu, 1,1-bis(3'-indolyl)-1-(*p*-*t*-butyl)methane; DIM-C-pPhOCH_3, 1,1-bis(3'-indolyl)-1-(*p*-methyl)methane; DIM-C-pPhOH, 1,1-bis(3'-indolyl)-1-(*p*-hydroxyl)-methane; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; NAG-1, nonsteroidal anti-inflammatory drug activated gene-1; NSAID, nonsteroidal anti-inflammatory drug; PI3K, phosphatidylinositol-3-kinase; PPARγ, peroxisome proliferator-activated receptor γ; PSA, prostate specific antigen; TGFβ, transforming growth factor β; TZDs, thiazolidinediones

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ABSTRACT

1,1-Bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes (C-DIMs) containing *para*-trifluoromethyl (DIM-C-pPhCF_3), *t*-butyl (DIM-C-pPhtBu), and phenyl (DIM-C-pPhC_6H_5) groups are a novel class of peroxisome proliferator-activated receptor γ (PPARγ) agonists. In LNCaP prostate cancer cells, these compounds induce PPARγ-dependent transactivation, inhibit cell proliferation, and induce apoptosis. In addition, these PPARγ agonists modulate a number of antiproliferative and proapoptotic responses including induction of p27, ATF3 and nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) and downregulation of cyclin D1 and caveolin-1. Moreover, these effects are not inhibited by the PPARγ antagonist GW9662. The C-DIM compounds also abrogate androgen receptor (AR)-mediated signaling, and decrease prostate specific antigen (PSA) and AR protein expression, and these responses were PPARγ-independent. The effects of C-DIMs on AR and PSA were due to decreased AR and PSA mRNA expression in LNCaP cells. Thus, this series of methylene-substituted diindolylmethane derivatives simultaneously activate multiple pathways in LNCaP cells, including ablation of androgen-responsiveness and downregulation of caveolin-1. Both of these responses are associated with activation of proapoptotic pathways in this cell line.

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INTRODUCTION

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the PPAR subfamily of nuclear receptors that bind and are activated by lipids, prostaglandins, and structurally-diverse synthetic compounds (Rosen and Spiegelman, 2001; Willson et al., 2001; Lee et al., 2003). 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2, fatty acids, and stress-induced nitrolinoleic acid are biochemicals that activate PPARγ; however, their role as endogenous ligands for this receptor is unknown. The synthetic thiazolidinediones (TZD), troglitazone, rosiglitazone and proglitazone are PPARγ agonists, and the latter two compounds are currently being used as insulin-sensitizing drugs for the treatment of type II diabetes (Staels and Fruchart, 2005; Boden and Zhang, 2006). PPARγ agonists are structurally diverse and include flavonoids, phosphonophosphates, chromane carboxylic acids, indole derivatives, and triterpenoids such as 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and related compounds (Suh et al., 1999; Rieusset et al., 2002; Qin et al., 2003; Place et al., 2003; Berger et al., 2003; Koyama et al., 2004; Schopfer et al., 2005; Acton, III et al., 2005; Liu et al., 2005). PPARγ is overexpressed in tumors compared to non-tumor tissues, and this receptor is also expressed in several different cancer cell lines derived from hematopoietic and non-hematopoietic tumors (Ikezoe et al., 2001). Laboratory animal studies demonstrate that PPARγ agonists are highly effective antitumor agents with potential for their development as clinical drugs for cancer chemotherapy (Grommes et al., 2004).

Research in this laboratory has identified 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes containing *para*-trifluoromethyl (DIM-C-pPhCF_3), *t*-butyl (DIM-C-pPhtBu), and phenyl (DIM-C-pPhC_6H_5) substituents as a new class of PPARγ

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agonists that inhibit cancer cell proliferation and tumor growth *in vivo* (Chintharlapalli et al., 2004; Hong et al., 2004; Contractor et al., 2005; Chintharlapalli et al., 2005a; Abdelrahim et al., 2006; Kassouf et al., 2006; Chintharlapalli et al., 2006). These PPARγ-active methylene-substituted diindolylmethanes (C-DIMs) induce PPARγ-dependent transactivation in breast, colon, pancreatic and bladder cancer lines; however, their growth inhibitory and proapoptotic effects are cell context-dependent. For example, some growth inhibitory responses including induction of p21 in pancreatic cancer cells and induction of the tumor suppressor gene caveolin-1 in colon and bladder cancer were PPARγ-dependent and inhibited by PPARγ antagonists or small inhibitory RNA for PPARγ (Chintharlapalli et al., 2004; Hong et al., 2004; Kassouf et al., 2006; Chintharlapalli et al., 2006). In contrast, several proapoptotic responses induced by PPARγ-active C-DIMs were PPARγ-independent, and these include endoplasmic reticulum (ER) stress-induced activation of death receptor 5 (DR5) and induction of non-steroidal antiinflammatory drug-activated gene-1 (NAG-1), a member of the transforming growth factor β (TGFβ) family (Chintharlapalli et al., 2005a; Abdelrahim et al., 2006; Chintharlapalli et al., 2006). Other structural classes of PPARγ agonists induce receptor-dependent and -independent effects, and these multiple modes of action can be advantagous for development of anticancer drugs.

PPARγ agonists inhibit growth and induce apoptosis in prostate cancer cells, and they also affect androgenic responsiveness in androgen receptor (AR)-positive prostate cancer cells (Kubota et al., 1998; Mueller et al., 2000; Moretti et al., 2001; Segawa et al., 2002; Jiang et al., 2004; Jarvis et al., 2005; Yang et al., 2006). For example, troglitazone decreases basal and androgen-induced prostate specific antigen (PSA)

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expression in LNCaP cells at concentrations ≤ 10 μM, and AR protein levels are decreased at higher concentrations (> 20 μM) (Yang et al., 2006). Although the mechanisms of these troglitazone-induced responses are unknown, other PPARγ-inactive analogs of troglitazone were active, indicating that these responses were PPARγ-independent. In this study, we show that PPARγ-active C-DIMs induce growth inhibitory and proapoptotic responses in LNCaP cells and also decrease PSA and AR expression through PPARγ-independent pathways. The antiandrogenic effects are due, in part, to decreased PSA and AR mRNA levels and reporter gene activity in cells transfected with constructs containing PSA (pPSA luc) and AR (pAR-luc) promoter inserts. The C-DIM compounds offer important clinical advantages for treatment of prostate cancer through their activation of multiple responses linked to antiandrogenicity, growth inhibition, and cell death.

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MATERIALS AND METHODS

Cell Lines, Antibodies and Reagent. Human prostate cancer cell line LNCaP was obtained from American Type Culture Collection (Manassas, VA). LNCaP cells were maintained in RPMI 1640 (Sigma) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 μL/L of 100x antibiotic antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO_2. Rosiglitazone was purchased from LKT Laboratories, Inc. (St. Paul, MN). Antibodies for Sp1, poly (ADP-ribose) polymerase, cyclin D1, p27, AR, pERK, ERK, ATF3 and caveolin 1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PSA was obtained from Dakocytomation (Glostrup, Denmark). NAG-1 was from Upstate Biotechnology (Charlottesville, VA), and EGR-1 was from Cell Signaling Technology Inc. (Beverly, MA). Monoclonal β-actin antibody was purchased from Sigma-Aldrich. Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA). LipofectAMINE reagent was supplied by Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagents were from Perkin-Elmer Life Sciences (Boston, MA). The C-substituted DIMs were prepared in this laboratory by condensation of indole with *p*-substituted benzaldehydes, and compounds were > 95% pure by gas chromatography-mass spectrometry.

Plasmids. The Gal4 reporter containing 5X Gal4 response elements (pGal4) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4DBD-PPARγ construct (gPPARγ) was a gift of Dr. Jennifer L. Oberfield (Glaxo Wellcome Research and Development, Research Triangle Park, NC). The PPRE-luc

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construct contains three tandem PPREs with a minimal TATA sequence in pGL2. The AR-Luc construct containing the -5400 to + 580 region of the androgen receptor promoter was provided by Dr. Donald J. Tindall (Mayo Clinic, Rochester, MN) and the PSA-Luc construct containing the 5.8 kb region of the PSA promoter was provided by Dr. Hong-Wu Cheng (University of California, Davis, CA).

Transfection and Luciferase Assay: Prostate cancer cells (1 x 10^5) were seeded in 24-well plates in DMEM/F-12 media supplemented with 2.5% charcoal-stripped FBS and grown overnight. Transient transfections were performed using LipofectAMINE reagent (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. Transfection studies were performed using Gal4Luc (0.4 μg), β-gal (0.04 μg), Gal4DBD-PPARγ (0.04 μg), AR-Luc (0.4 μg), PSA-Luc (0.4 μg). Five to six hr after transfection, the transfection mix was replaced with complete media containing either vehicle (DMSO) or the indicated ligand for 20 to 22 hr. Cells were then lysed with 100 μL of 1X reporter lysis buffer, and 30 μL of cell extract was used for luciferase and β-galactosidase assays. A Lumicount luminometer (PerkinElmer Life and Analytical Sciences) was used to quantify luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

Cell Proliferation Assay: LNCaP prostate cancer cells (2 x 10^4 per well) were plated in 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or the indicated C-DIMs were added. Fresh medium and C-DIMs were added every 48 hr and cells were then trypsinized and counted after 2, 4 and 6 days

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using a Coulter Z1 cell counter. Each experiment was done in triplicate and results are expressed as means ± SE for each set of three experiments.

Western Blot Analysis: LNCaP cells were seeded in DMEM/Ham's F-12 media containing 2.5% charcoal-stripped FBS for 24 hr and then treated with either the vehicle (DMSO) or the compounds for different times as indicated. Cells were collected by scraping in 150 μL of high salt lysis buffer [50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and 10 μL/ml Protease Inhibitor Cocktail (Sigma-Aldrich)]. The lysates were incubated on ice for 1 hr with intermittent vortexing followed by centrifugation at 40,000 *g* for 10 min at 4°C. Before electrophoresis, the samples were boiled for 3 min at 100°C, the amounts of protein were determined, and 60 μg of protein was applied per lane. Samples were subjected to SDS-polyacrylamide gel electrophoresis on 10% gel at 120 V for 3 to 4 hr. Proteins were transferred onto polyvinylidene membranes (polyvinylidene difluoride; Bio-Rad, Hercules, CA) by wet electroblotting in a buffer containing 25 mM Tris, 192 mM glycine, and 15% methanol for 1.5 hr at 0.9 A. The membranes were blocked for 30 min with 5% TBST-BLOTTO (10 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.05% Triton X-100, and 5% nonfat dry milk) and incubated in fresh 5% TBST-BLOTTO with 1:1000 (for SP1, AR, ATF-3, caveolin 1, p27, and cyclin D1), 1:250 (for PSA and PARP), 1:500 (for p-ERK, ERK, NAG-1 and EGR-1), and 1:5000 (for β-actin) primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the polyvinylidene difluoride membrane was incubated with secondary antibody (1:5000) in 5% TBST-BLOTTO for 90 min. The membrane was washed with TBST for 10 min and incubated with 10 ml of chemiluminescence substrate (PerkinElmer Life and Analytical Sciences) for 1.0 min

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and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

Quantitative Real-time PCR: Total RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 30 μL RNase-free water and stored at -80°C. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was prepared from the LNCaP cell line using a combination of oligodeoxythymidylic acid (Oligo-d(T)16), and dNTP mix (Applied Biosystems) and Superscript II (Invitrogen). Each PCR was carried out in triplicate in a 25 μL volume using Sybr Green Mastermix (Applied Biosystems) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The ABI Dissociation Curves software was used following a brief thermal protocol (95°C 15 s and 60°C 20 s, followed by a slow ramp to 95°C) to control for multiple species in each PCR amplification. The comparative CT method was used for relative quantitation of samples. Values for each gene were normalized to expression levels of TATA-binding protein. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of the primers used for reverse transcription-PCR were as follows: AR forward, 5'-GTA CCC TGG CGG CAT GGT-3', AR reverse, 5'-CCC ATT TCG CTT TTG ACA CA -3'; PSA forward, 5'-GCA TTG AAC CAG AGG AGT TCT TG -3', PSA reverse, 5'-TTG CGC ACA CAC GTC ATT G-3'; and TATA-binding protein forward, 5'-TGC ACA GGA GCC AAG AGT GAA-3', reverse, 5'-CAC ATC ACA GCT CCC CAC CA-3'.

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Statistical Analysis: Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean ± standard deviation for at least three separate determinations for each treatment.

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RESULTS

1. PPARγ-active C-DIMs induced transactivation and inhibit LNCaP cell growth

Previous studies have identified DIM-C-pPhCF$_3$, DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ as PPARγ agonists in breast, colon, pancreatic and bladder cancer cells (Chintharlapalli et al., 2004; Hong et al., 2004; Contractor et al., 2005; Chintharlapalli et al., 2005a; Abdelrahim et al., 2006; Kassouf et al., 2006). Results in Figure 1A show that all three compounds induce transactivation in LNCaP cells transfected with a GAL4-PPARγ chimera and a construct (pGAL4) containing five tandem GAL4 response elements linked to a luciferase reporter gene. The relative potency for these compounds in this assay was DIM-C-pPhCF$_3$ > DIM-C-pPhtBu ≈ DIM-C-pPhC$_6$H$_5$. Induction of luciferase activity by the PPARγ-active C-DIMs was also inhibited by cotreatment with the PPARγ antagonist GW9662 (10 μM). A parallel transactivation experiment was also carried out using a PPRE-luc construct which contains three tandem PPARγ response elements linked to a luciferase reporter gene (Fig. 1B). This assay relies on endogenous PPARγ and its heterodimeric partner RXR, and the results were similar to those observed using the GAL4-PPARγ/pGAL4 assay. All compounds induced transactivation which was inhibited by 10 μM GW9662, and DIM-C-pPhCF$_3$ was the most potent compound in both transactivation assays. In cell proliferation assays, ≥ 5 μM DIM-C-pPhCF$_3$ (Fig. 2A), DIM-C-pPhtBu (Fig. 2B) and DIM-C-pPhC$_6$H$_5$ (Fig. 2C) inhibited LNCaP cell proliferation, whereas 10 μM not only inhibited growth but the number of surviving cells was lower than the original number of seeded cells. This pattern was similar to that observed for these compounds in other cancer cell lines where there was a concentration-dependent inhibition of cell proliferation and induction

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of cell death (Chintharlapalli et al., 2004; Contractor et al., 2005; Chintharlapalli et al., 2005a; Kassouf et al., 2006). The growth inhibitory effects of rosiglitazone were also investigated and IC_{50} values were > 20 μM (data not shown), indicating the decreased potency of TZDs compared to C-DIMs as previously observed in other cancer cell lines (Qin et al., 2003; Chintharlapalli et al., 2004; Hong et al., 2004).

2. PPARγ-active C-DIMs modulate expression of cell cycle proteins and decrease AR and PSA expression

Different structural classes of PPARγ agonists inhibit prostate cancer cell growth and affect androgen-responsiveness through receptor-dependent and -independent pathways (Kubota et al., 1998; Mueller et al., 2000; Moretti et al., 2001; Segawa et al., 2002; Jiang et al., 2004; Jarvis et al., 2005; Yang et al., 2006). Results in Figure 3A illustrate the concentration-dependent effects of PPARγ-active C-DIMs on cyclin D1 and p27 protein expression. p27 is induced by all three compounds at lower concentrations (< 10 μM), whereas the induction response is lost at higher concentrations (10 - 15 μM). In contrast, cyclin D1 is downregulated at higher concentrations (10 - 15 μM) but unaffected by concentrations < 10 μM. Minimal effects were observed for induction of p21 protein (data not shown). A recent study reported that the PPARγ agonist troglitazone differentially decreased PSA and AR protein expression in LNCaP cells at low (IC_{50} < 10 μM) and high (IC_{50} = 40 μM) concentrations (Yang et al., 2006). Results in Figure 3A show that 7.5 - 15 μM concentrations of PPARγ-active C-DIMs significantly decrease both PSA and AR protein expression. PSA tends to be downregulated by C-DIMs at slightly lower concentrations than AR although, in contrast to the effects of

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troglitazone, both responses are observed within a narrow dose range. Troglitazone also decreased DHT-induced expression of PSA in LNCaP cells, and the results in Figure 3B show that DHT induced PSA protein expression was inhibited after treatment with C-DIMs for 24 hr, and levels of AR protein were also decreased. We also used lower concentrations of C-DIM compounds (10 μM) to examine the subcellular distribution of AR in LNCaP cells. Ten nM DHT enhances AR levels and redistributes most of the receptor in the nuclear fraction (Fig. 3C). Cotreatment with 10 μM DIM-C-pPhC_6H_5 or DIM-C-p$PhCF_3$ slightly decreased AR levels but did not affect DHT-induced nuclear uptake of AR. These results suggest that the C-DIM (10 μM)-induced downregulation of basal and DHT-induced PSA (Fig. 3B) is not due to the effects of these compounds on DHT-induced nuclear AR accumulation.

NAG-1 and ATF-3 are induced by NSAIDs (nonsteroidal anti-inflammatory drugs), PPARγ agonists, phorbol esters, and other apoptosis-inducing agents in different cancer cell lines (Baek et al., 2001; Baek et al., 2003; Baek et al., 2004; Kim et al., 2005; Jarvis et al., 2005; Shim and Eling, 2005; Chintharlapalli et al., 2005a; Chintharlapalli et al., 2005b; Kassouf et al., 2006; Chintharlapalli et al., 2006). Phorbol esters induce NAG-1 in LNCaP cells (Shim and Eling, 2005) and PPARγ-active C-DIMs induce NAG-1 in colon cancer cells (Chintharlapalli et al., 2005a; Chintharlapalli et al., 2006). Figure 4A shows that treatment of LNCaP cells with DIM-C-p$PhCF_3$, DIM-C-pPhtBu and DIM-C-pPhC_6H_5 for 24 hr significantly induces NAG-1 and ATF3 protein. ATF3 induction is observed at concentrations ≥ 7.5 μM, whereas NAG-1 is induced by ≥ 10 μM concentrations of PPARγ-active C-DIMs, and this is accompanied by PARP cleavage which is indicative of caspase-dependent apoptosis. Previous studies in colon cancer

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cells show that induction of NAG-1 after treatment with C-DIM compounds for ≥ 4 hr was dependent on prior induction of EGR-1 (2 hr) (Chintharlapalli et al., 2005a), whereas a comparable time-course study in LNCaP cells (Fig. 4B) shows that EGR-1 is induced 4, 8 - 12 hr after treatment and decreases to background levels after 24 hr. These results have been quantitated and are provided as Supplemental Material. Both NAG-1 and ATF3 proteins are also induced within 4 to 8 hr, and levels of both proteins exhibited a time-dependent increase during the 24 hr treatment period, suggesting that EGR-1 induction is not required for induction of NAG-1 or ATF3.

PPARγ-active C-DIMs enhance phosphatidylinositol-3-kinase (PI3K) activity in colon cancer cells, and this pathway was required for activation of Egr-1 which subsequently activated NAG-1 (Chintharlapalli et al., 2005a). Results in Figure 4C show the effects of various kinase inhibitors on DIM-C-pPhCF$_3$-induced NAG-1 and ATF3 expression and downregulation of AR protein. DIM-C-pPhCF$_3$-dependent induction of NAG-1 was not inhibited by the PKC (GF109203X), PI3K (LY294002), or JNK (SP600125) inhibitors, whereas inhibition of MAPK by PD98059 significantly decreased induction of NAG-1. These results contrast with induction of NAG-1 by TPA in LNCaP cells which is blocked by the PKC inhibitor GF109203X (Shim and Eling, 2005). The various kinase inhibitors do not affect induction of ATF3 or did not significantly reverse the C-DIM-mediated downregulation of AR or PSA protein, indicating a clear mechanistic distinction between the regulation of NAG-1 by DIM-C-pPhCF$_3$ versus the kinase-independent regulation of AR and ATF3. The JNK inhibitor alone also decreased AR protein levels, and the other kinase inhibitors alone either did not affect or induce AR and PSA protein expression. DIM-C-pPhCF$_3$ induces a time-

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dependent increase in MAPK phosphorylation in LNCaP cells (Fig. 4D) which correlates with induction of NAG-1 since the MAPK kinase inhibitor PD98059 inhibits this response (Fig. 4C). The p38 inhibitor SB203580 also exhibited some inhibition of DIM-C-pPhCF$_3$-induced NAG-1 expression; however, increased phosphorylation of p38 was not observed (data not shown).

3. Effects of C-DIM compounds on caveolin-1 and other responses after treatment for 96 hr

PPARγ agonists including C-DIMs induce caveolin-1 in some colon and bladder cancer cells (Chintharlapalli et al., 2004; Kassouf et al., 2006; Chintharlapalli et al., 2006), and the response was receptor-dependent and not observed until 48 - 96 hr after treatment. Results in Figure 5A show that after treatment of LNCaP cells for 96 hr with 2.5, 5.0 or 7.5 μM of the C-DIM compounds, there was a significant decrease in caveolin-1 protein, whereas 10 and 15 μM rosiglitazone significantly increased caveolin-1 expression. The lack of induction of caveolin-1 by C-DIMs is not unprecedented since previous studies in several colon cancer cell lines showed that caveolin-1 was induced in HT-29 and HCT-15 but not in HCT-116 colon cancer cells (Chintharlapalli et al., 2004; Chintharlapalli et al., 2005a; Chintharlapalli et al., 2006). In contrast, caveolin-1 expression was decreased by the C-DIM compounds in LNCaP cells (Fig. 5A), whereas DIM-C-pPhCF$_3$ induced caveolin-1 in HT-29 cells (Fig. 5B) as previously described (Chintharlapalli et al., 2004). We also examined induction of ATF3 and NAG-1, PARP cleavage and decreased AR and PSA expression after treatment of LNCaP cells for 96 hr with C-DIM compounds (Fig. 5C), and these responses were observed at lower

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concentrations (2.5 - 7.5 μM) than required for these same responses after treatment for only 24 hr (Figs. 3 and 4). Induction of NAG-1, ATF3 and PARP cleavage, and decreased PSA expression were observed at 2.5 - 5.0 μM DIM-C-pPhCF$_3$, whereas AR was decreased at the 7.5 μM concentration. DIM-C-pPhtBu and DIM-C-pPhC$_6$H$_5$ were less active than DIM-C-pPhCF$_3$ in this 96 hr experiment. Further confirmation of the proapoptotic effects of the C-DIM compounds is illustrated in Figure 5D which shows that after treatment for 24 hr DNA fragmentation is observed.

4. Effects of GW9662 (PPARγ agonists) and MG132 (proteasome inhibitor) on C-DIM-induced responses

PPARγ-active C-DIMs modulate expression of cell cycle genes, AR and androgen-regulated genes and NAG-1 in LNCaP cells (Figs. 3 and 4), and the role of PPARγ in mediating these responses was investigated using the PPARγ antagonist GW9662 (Fig. 6A). The antagonist alone did not affect cyclin D1, p27, AR or PSA protein expression and did not induce NAG-1 or PARP cleavage and in LNCaP cells, GW9662 (10 μM) did not affect induction of NAG-1 and PARP cleavage or downregulation of AR, cyclin D1 and PSA by the C-DIM compounds. These results suggest that the cell cycle, androgenic and NAG-1 responses are modulated by C-DIMs in LNCaP cells through receptor-independent pathways. LNCaP cells were also treated with different concentrations of 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl)-methane containing *p*-methyl (DIM-C-pPhOCH$_3$) or *p*-hydroxyl (DIM-C-pPhOH) substituents. These compounds are PPARγ-inactive (Qin et al., 2004), but also induce downregulation of AR and PSA proteins (Fig. 6B), further confirming that these effects

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are PPARγ-independent. Results presented in Figures 6A and 6B have been quantitated and are provided as Supplemental Material.

Previous studies showed that PPARγ-active C-DIMs induce downregulation of cyclin D1 in several cancer cell lines through activation of the proteasome pathways (Chintharlapalli et al., 2004; Hong et al., 2004; Chintharlapalli et al., 2005a; Kassouf et al., 2006; Chintharlapalli et al., 2006). Results in Figure 6C demonstrate that DIM-C-pPhCF_3- and DIM-C-pPhC_6H_5-induced downregulation of cyclin D1 in LNCaP cells was inhibited after cotreatment with 10 μM MG132 (proteasome inhibitor), whereas decreased expression of AR and PSA was not reversed by the proteasome inhibitor which slightly decreased AR protein levels. These results indicate that decreased expression of AR/PSA and cyclin D1 are mediated through different pathways. Caveolin-1 expression is decreased in LNCaP (Fig. 5A) and increased in HT-29 (Fig. 5B) cells, and the latter response is inhibited by the PPARγ antagonist GW9662 (Chintharlapalli et al., 2004). In contrast, GW9662 did not block C-DIM-induced downregulation of caveolin-1 in LNCaP cells (Fig. 6D), suggesting that this response was PPARγ-independent.

5. C-DIMs decrease PSA and AR gene expression

Since downregulation of AR and PSA in LNCaP cells treated with PPARγ-active C-DIMs was PPARγ-, proteasome- and kinase-independent, we further investigated the effects of these compounds on mRNA levels and reporter gene activity in transfected cells. All three compounds significantly decreased AR mRNA levels (Fig. 7A) and in LNCaP cells transfected with the pAR-luc construct containing the -5400 to +580 region

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of the AR promoter (linked to luciferase), the C-DIM compounds also decreased luciferase activity (Fig. 7B). We also examined the effects of the C-DIM compounds on PSA mRNA levels (Fig. 7C) and PSA-dependent transactivation in cells transfected with the construct pPSA-luc (Fig. 7D) containing the androgen-responsive (5.85 Kb) region of the PSA promoter linked to the luciferase gene. The results show that both mRNA levels and luciferase activity were decreased by these compounds, suggesting that decreased protein expression (Fig. 3B) is paralleled by decreased PSA gene expression. Interestingly, hormone-induced expression of luciferase activity in LNCaP cells transfected with pPSA-luc was also decreased after cotreatment with C-DIMs (Fig. 7E). Differences in the mechanism of action of the C-DIM compounds on downregulation of PSA and AR mRNA levels was demonstrated in experiments using the protein synthesis inhibitor cycloheximide. Cycloheximide did not affect C-DIM-induced downregulation of AR mRNA (Fig. 8A) but partially reversed the downregulation of PSA mRNA (Fig. 8B), indicating that both genes are differentially regulated by the C-DIM compounds and the latter response requires induction of an "inhibitory" factor. These data show that both PSA and AR gene expression and promoter gene activity are decreased by the C-DIM compounds through different pathways, and this represents a novel receptor-independent pathway that decreases androgen-responsiveness at concentrations that also inhibit LNCaP cell growth and induce apoptosis.

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DISCUSSION

PPARγ is an orphan nuclear receptor that binds fatty acids, prostaglandins, and other endogenous lipids, and its expression is highly tissue-specific. This receptor is a major drug target for treating type II diabetes, and the thiazolidinedione compounds rosiglitazone and pioglitazone are insulin-sensitizers widely used for treated this disease (Rosen and Spiegelman, 2001; Willson et al., 2001; Lee et al., 2003; Staels and Fruchart, 2005; Boden and Zhang, 2006). Since PPARγ is overexpressed in cancer cells and tumors (Ikezoe et al., 2001), there is considerable interest in developing new anticancer drugs that specifically target PPARγ and activate growth inhibitory and proapoptotic pathways (Grommes et al., 2004). CDDO and structurally-related triterpenoids are PPARγ agonists that are highly potent anticancer drugs against multiple tumor types; however, detailed mechanistic studies indicate that many of their effects are receptor-independent (Ikezoe et al., 2001; Melichar et al., 2004; Zou et al., 2004; Samudio et al., 2005; Chintharlapalli et al., 2005b).

Studies in this laboratory have characterized C-DIMs as a new class of PPARγ agonists with the most active analogs containing DIM-C-pPhCF_3, DIM-C-pPhtBu, and DIM-C-pPhC_6H_5 substituents (Chintharlapalli et al., 2004; Hong et al., 2004; Contractor et al., 2005; Kassouf et al., 2006; Chintharlapalli et al., 2006). These three compounds induce PPARγ-dependent transactivation and inhibit growth of breast, colon, pancreatic and bladder cancer cells, and similar results have been observed in LNCaP cells (Figs. 1 and 2). Mechanistic studies with PPARγ-active C-DIMs have identified both receptor-dependent and -independent responses that are observed only in some cell lines. For example, low dose growth inhibitory effects of these compounds in pancreatic and colon

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cancer cells are associated with receptor-dependent induction of p21 and caveolin-1, respectively (Chintharlapalli et al., 2004; Hong et al., 2004; Chintharlapalli et al., 2005a). In contrast, higher concentrations of C-DIMs that induce apoptosis are linked to receptor-independent pathways including activation of ER stress in pancreatic cancer cells (Abdelrahim et al., 2006), induction of NAG-1 in some colon cancer cells (Chintharlapalli et al., 2005a; Chintharlapalli et al., 2006), and proteasome-dependent degradation of cyclin D1 in most cancer cell lines.

In LNCaP cells, PPARγ-active C-DIMs primarily induce receptor-independent degradation of cyclin D1 (Fig. 6A) which is reversed by the proteasome inhibitor MG132; p21 levels are constitutively low and not induced (data not shown), whereas effects on p27 are biphasic with induction at lower but not higher concentrations of these compounds (Figs. 3A and 5A). NAG-1 is induced by multiple agents including C-DIM compounds and, in colon cancer cells, this response is PPARγ-independent and accompanied by induction of ATF3 and prior (rapid) induction of EGR-1 (Chintharlapalli et al., 2005a). C-DIM compounds induced a parallel increase of both NAG-1 and ATF3 (Figs. 4A and 4B); a similar induction pattern was observed for EGR-1 and this was in contrast to studies in colon cancer cells. NAG-1 and EGR-1 induction by C-DIMs in colon cancer cells was blocked by PI3K inhibitors, whereas the MAPK inhibitor PD98059, but not the PI3K inhibitor, blocked induction of NAG-1 in LNCaP cells (Fig. 4C). Phorbol esters also induced NAG-1 and apoptosis in LNCaP cells, and the former response was blocked by the PKC inhibitor GF109203 but not by PD98059 (MAPK inhibitor), SB203580 (p38 MAPK inhibitor), or SP600125 (JNK inhibitor) (Tanaka et al., 2003; Shim and Eling, 2005). These results clearly demonstrate a novel mechanism for

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induction of NAG-1 in LNCaP cells which is MAPK-dependent, and this differs from the PI3-K-dependent induction of NAG-1 by C-DIMs in colon cancer cells and the PKC-dependent activation of NAG-1 by TPA in LNCaP cells (Shim and Eling, 2005; Chintharlapalli et al., 2005a; Chintharlapalli et al., 2006). Currently, we are investigating the mechanisms associated with cell context-dependent activation of kinases by C-DIMs and delineating their critical downstream proapoptotic responses.

PPARγ-dependent induction of caveolin-1 in colon and bladder cancer cells is only observed after prolonged treatment with C-DIMs, CDDO compounds, or rosiglitazone (Chintharlapalli et al., 2004; Chintharlapalli et al., 2005a; Chintharlapalli et al., 2005b; Chintharlapalli et al., 2006), and this was also observed in HT-29 colon cancer cells used in this study (Fig. 5B). However, in LNCaP cells, the PPARγ-active C-DIMs decreased caveolin-1 expression, whereas rosiglitazone induced approximately a 2-fold increase in caveolin-1 (Fig. 5A). The reason for these differences in modulation of caveolin-1 are unknown; however, the downregulation of caveolin-1 by C-DIMs enhances their anticarcinogenic activity in prostate cancer since previous reports suggest that caveolin-1 may enhance prostate tumor development and growth (Yang et al., 1998; Williams et al., 2005).

Thiazolidinedione PPARγ agonists inhibit prostate cancer cell growth and tumors in mouse xenograft experiments. This was also accompanied (*in vitro*) by decreased expression of PSA (Kubota et al., 1998; Yang et al., 2006), and this response was PPARγ-independent (Yang et al., 2006). Thiazolidinediones also decreased AR expression; however, IC_{50} values for troglitazone were 40 μM, where ≤ 10 μM troglitazone was sufficient to significantly decrease PSA expression (Yang et al., 2006).

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It was concluded that these concentration-dependent differences indicate that troglitazone-induced downregulation of AR and PSA were mediated through different pathways. Like troglitazone, 5.0 - 10 μM C-DIM compounds also decrease constitutive or DHT-induced PSA protein expression in LNCaP cells, and these compounds did not affect DHT-induced intracellular location of AR (Figs. 3 and 4). Moreover, C-DIMs also decrease PSA mRNA levels (Fig. 7C) and both basal and DHT-induced transactivation in cells transfected with pPSA-luc (Figs. 7D and 7E), and these results were similar to those reported for troglitazone (Yang et al., 2006). The C-DIM compounds also decrease AR protein (Fig. 3A), AR mRNA (Fig. 7A), and reporter gene activity in LNCaP cells transfected with pAR-luc (Fig. 7B). In contrast to the large differences in the concentrations of troglitazone required for decreasing PSA and AR protein expression in LNCaP cells, C-DIM compounds induced both responses within a narrow range of concentrations (7.5 - 10 μM), where effects on PSA were generally observed at slightly lower concentrations (Fig. 3A). However, it was also apparent from cycloheximide experiments (Fig. 8) that downregulation of PSA but not AR mRNA levels by C-DIM compounds was affected by the protein synthesis inhibitor, suggesting that decreased expression of PSA and AR in LNCaP cells treated with C-DIMs occurs via two different pathways and these are currently being investigated.

A recent study using RNA interference showed that ablation of the AR in LNCaP cells decreased cell survival and enhanced apoptosis (Liao et al., 2005), suggesting that the C-DIM-induced degradation of AR in this study contributes to their proapoptotic effects (Figs. 4A, 5D and 6A). Lower concentrations of DIM-C-pPhCF_3 (2.5 - 5.0 μM) induced apoptosis and decreased caveolin-1 protein expression, whereas AR

degradation was observed at 7.5 μM, and this trend was similar for all PPARγ-active C-DIMs (Figs. 5A and 5C). Caveolin-1 expression and androgen sensitivity have been reported (Nasu et al., 1998), and antisense caveolin-1 in castrated animal models decreased tumor growth and increased apoptosis. This suggests that caveolin-1 downregulation by C-DIMs may also contribute to their apoptotic effects, particularly at lower concentrations.

In summary, results of this study demonstrate that C-DIMs activate multiple growth inhibitory/proapoptotic LNCaP cells (Fig. 8C) including induction of PPARγ-dependent transactivation; however, their induction of growth inhibitory and proapoptotic responses are primarily receptor-independent. These compounds decrease cyclin D1 expression, induce p27 (at some concentrations) and NAG-1, and downregulate caveolin-1, and all of these responses are associated with the observed growth inhibition and apoptosis.. In addition, C-DIMs induce degradation of AR and PSA protein, mRNA and reporter gene activity, and the effects on AR protein were proteasome-independent. There are critical differences in the effects of C-DIMs on AR and PSA expression compared to troglitazone (Yang et al., 2006), and transfection studies indicate that C-DIM-induced responses may be directly related to effects on both the AR and PSA promoters. These results demonstrate that C-DIMs induce multiple pathways that lead to growth inhibition, apoptosis, and AR ablation in prostate cancer cells. Current studies are further investigating the mechanisms of these responses and the pathways which are dominant in *in vivo* models.

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FOOTNOTES

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FIGURE CAPTIONS

Figure 1. C-DIMs activate PPARγ in LNCaP cells. Activation of PPARγ-GAL4 (A) and PPRE-luc (B) by C-DIMs. LNCaP cells were transfected with PPARγ-GAL4/pGAL4 or PPRE-luc, treated with DMSO or PPARγ-active C-DIMs alone or in combination with 10 μM GW9662, and luciferase was determined as described in the Materials and Methods. Results are expressed as means ± SE for three separate determinations for each treatment group, and significant ($p < 0.05$) induction by C-DIMs (*) or inhibition of this response by GW9662 (**) are indicated.

Figure 2. PPARγ-active C-DIMs inhibit LNCaP cell proliferation. LNCaP cells were treated with DIM-C-pPhCF_3 (A), DIM-C-pPhtBu (B), and DIM-C-pPhC_6H_5 for up to 6 days. Cell numbers were determined every second day as described in the Materials and Methods. Results are expressed as means ± SE for at least three replicate determinations for each time point.

Figure 3. PPARγ-active C-DIMs modulate cell cycle genes AR and PSA in LNCaP cells. LNCaP cells were treated with PPARγ-active C-DIMs alone (A) and in combination with DHT (B and C) for 24 hr. Whole cell lysates were analyzed for specific proteins by Western blot analysis as described in the Materials and Methods. Similar results were observed in replicate experiments. β-Actin served as a protein loading control for all treatment groups and Sp1 protein was used as a nuclear marker protein to confirm efficient separation of cytosolic (C) and nuclear (N) fractions in data summarized in Figure 3C. The results in Figures 3A - 3C have been quantitated (Fig. 3B is in the

Appendix) and are presented as means ± SE for three replicate determination. In Figure 3B, AR/β-actin and PSA/β-actin ratios were determined and values for DMSO were set at 1.0. Significantly decreased protein expression is indicated by an asterisk. AR/β-actin ratios in Figure 3C are given relative to that observed in the cytosolic (C) fraction in control (DMSO) cells. Relative AR levels in the C and nuclear (N) fractions were similar in treated and untreated groups.

Figure 4. Activation of EGR-1, NAG-1, ATF3, and PARP cleavage and the effects of kinase inhibitors. LNCaP cells were treated with different concentrations of PPARγ-active C-DIMs for 24 hr (A), 15 μM DIM-C-pPhCF$_3$ or DIM-C-pPhC$_6$H$_5$ for 2, 4, 8, 12 and 24 hr (B). Whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. These studies were replicated (3X), normalized to β-actin protein, and significant ($p < 0.05$) induction of NAG-1/ATF-3 (A) or NAG-1/ATF-3/EGR-1 (B) is indicated (*). The quantitative results obtained for Figure 4B are provided as Supplemental Material. (C) Cotreatment with kinase inhibitors. LNCaP cells were treated with 15 μM DIM-C-pPhCF$_3$ alone or in combination with 5 μM GF109203X, 20 μM LY294002, 20 μM SP600125, or 20 μM PD98059 for 24 hr. Whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. The effects of kinase inhibitors on NAG-1, ATF-3, AR and PSA were also quantitated as described in Figure 3A and compared to the control (DMSO) protein levels. Significant ($p < 0.05$) inhibition of a C-DIM-induced response by a kinase inhibitor in indicated (*). (D) Time-dependent activation of MAPK. LNCaP cells were treated with DIM-C-pPhCF$_3$

for different periods of time and levels of p-Erk and Erk proteins were determined by Western blot analysis as described in the Materials and Methods.

Figure 5. Effects of PPARγ-active C-DIMs on caveolin 1 expression and other responses after treatment for 96 hr. Induction of caveolin-1 in LNCaP (A) and HT-29 (B) cells. LNCaP or HT-29 cells were treated with different concentrations of PPARγ-active C-DIMs or rosiglitazone (10 or 15 μM) for 96 hr, and caveolin-1 protein expression was determined by Western blot analysis as described in the Materials and Methods. Decreased caveolin-1/β-actin protein ratios were determined in triplicate, and results are expressed as means ± SE relative to caveolin-1 levels in the DMSO (control) group. Significantly ($p < 0.05$) decreased caveolin-1 after treatment with C-DIM compounds is indicated (*), and significant induction by rosiglitzaone is also observed (**). (C) Effects of treatment of LNCaP cells with C-DIMs for 96 hr on other responses. Cells were treated with 2.5 - 7.5 μM C-DIMs for 96 hr and the expression of NAG-1, ATF3, AR and PSA proteins and PARP cleavage was determined by Western blot analysis as described in the Materials and Methods. (D) DNA fragmentation. The effects of C-DIM compounds on apoptosis was also determined in LNCaP cells treated with DMSO or PPARγ-active C-DIM compounds for 24 hr, followed by measuring DNA fragmentation as described in the Materials and Methods.

Figure 6. Effects of proteasome inhibitors and PPARγ antagonists on C-DIM-induced responses in LNCaP cells. (A) Effects of PPARγ antagonist GW9662. LNCaP cells were treated with 10 or 15 μM alone or in combination with 10 μM GW9662 for 24 hr,

and expression of various proteins in whole cell lysates were determined by Western blot analysis as described in the Materials and Methods. (B) Effects of PPARγ-inactive C-DIM compounds. LNCaP cells were treated with DMSO or different concentrations of PPARγ-inactive C-DIM compounds, and expression of AR, PSA, and β-actin was determined by Western blot analysis as described in the Materials and Methods. Quantitation of results in Figures 6A and 6B are provided as Supplemental Material. (C) Cotreatment with the proteasome inhibitor MG132. LNCaP cells were treated with 10 or 15 μM C-DIM compounds alone or in combination with 10 μM MG132 for 24 hr, and proteins in whole cell lysates were analyzed by Western blot analysis. (D) Effects of GW9662 on caveolin-1 expression. LNCaP cells were treated with 2.5 - 7.5 μM DIM-C-pPhCF_3 or DIM-C-pPhC_6H_5 alone or in combination with 7.5 μM GW9662 for 96 hr, and caveolin-1 levels were determined by Western blot analysis as described in the Materials and Methods.

Figure 7. PPARγ-active C-DIMs inhibit PSA and AR transcription. Inhibition of AR mRNA (A) or reporter gene activity (B) by C-DIMs. LNCaP cells were treated with C-DIMs for 6 or 12 hr. mRNA levels were determined, or cells were transfected with AR-luc and luciferase activity was determined as described in the Materials and Methods. Results are given as means ± SE for three replicate determinations for each treatment group. Significantly ($p < 0.05$) decreased responses are indicated (*). Inhibition of PSA mRNA (C) or reporter gene activity (D) by C-DIMs. LNCaP cells were treated with C-DIMs for 6 and 12 hr. mRNA levels were determined, or cells were transfected with PSA-luc and luciferase activity was determined as described in the Materials and

Methods. Results are given as means ± SE for three replicate determinations for each treatment, and significantly ($p < 0.05$) decreased responses are indicated (*). (E) Inhibition of DHT-induced pPSA-luc by C-DIMs. LNCaP cells were treated with C-DIMs alone or in combination with 10 nM DHT as described above in (D), and results are expressed as means for SE for three replicate determinations for each treatment group. Significantly ($p < 0.05$) induced activity by DHT (*) and inhibition of this response by C-DIMs (**) are indicated.

Figure 8. Effects of cycloheximide and mechanism of action of C-DIM compounds. LNCaP cells were pretreated with cycloheximide (15 μg/ml), treated with DMSO (O), 10 - 20 μM DIM-C-pPhCF$_3$ or DIM-C-pPhC$_6$H$_5$ for 12 hr. AR (A) and PSA (B) mRNA levels were determined as described in the Materials and Methods. Results are expressed as means ± SE for three replicate experiments. A significant ($p < 0.05$) decrease in AR or PSA mRNA levels is indicated (*) and significant inhibition of this response by cycloheximide is also indicated (**). (C) Proposed mechanisms of action of C-DIM compounds in LNCaP cells.

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Figure 1

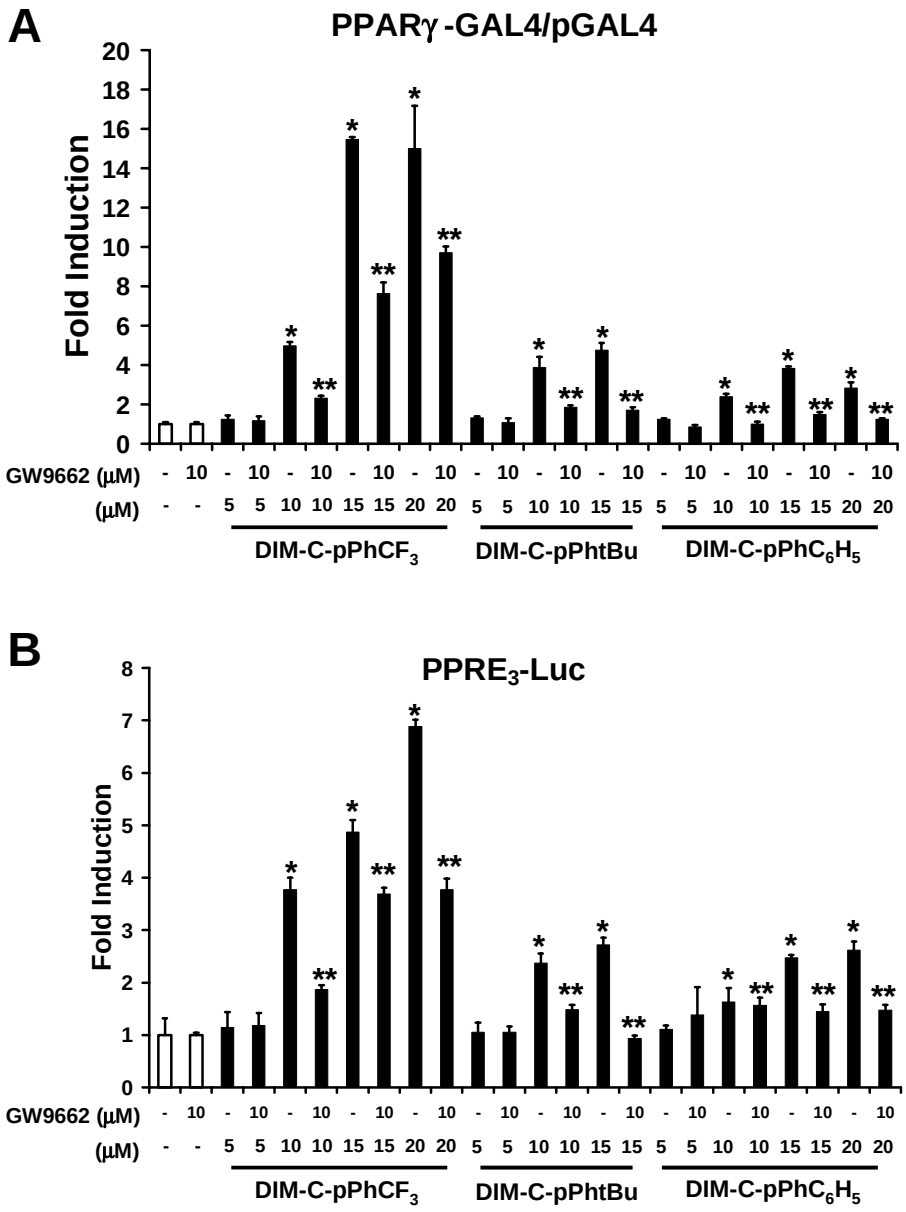

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Figure 2

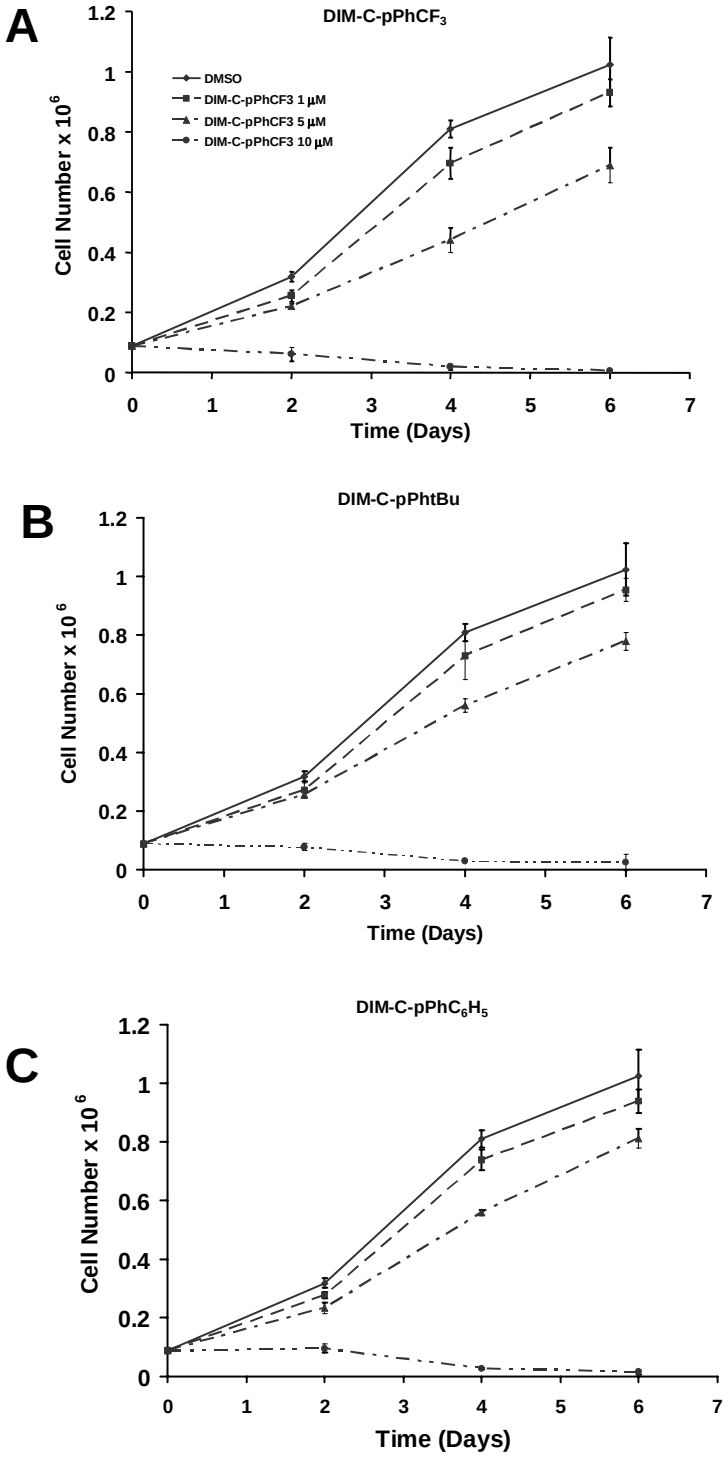

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Figure 3

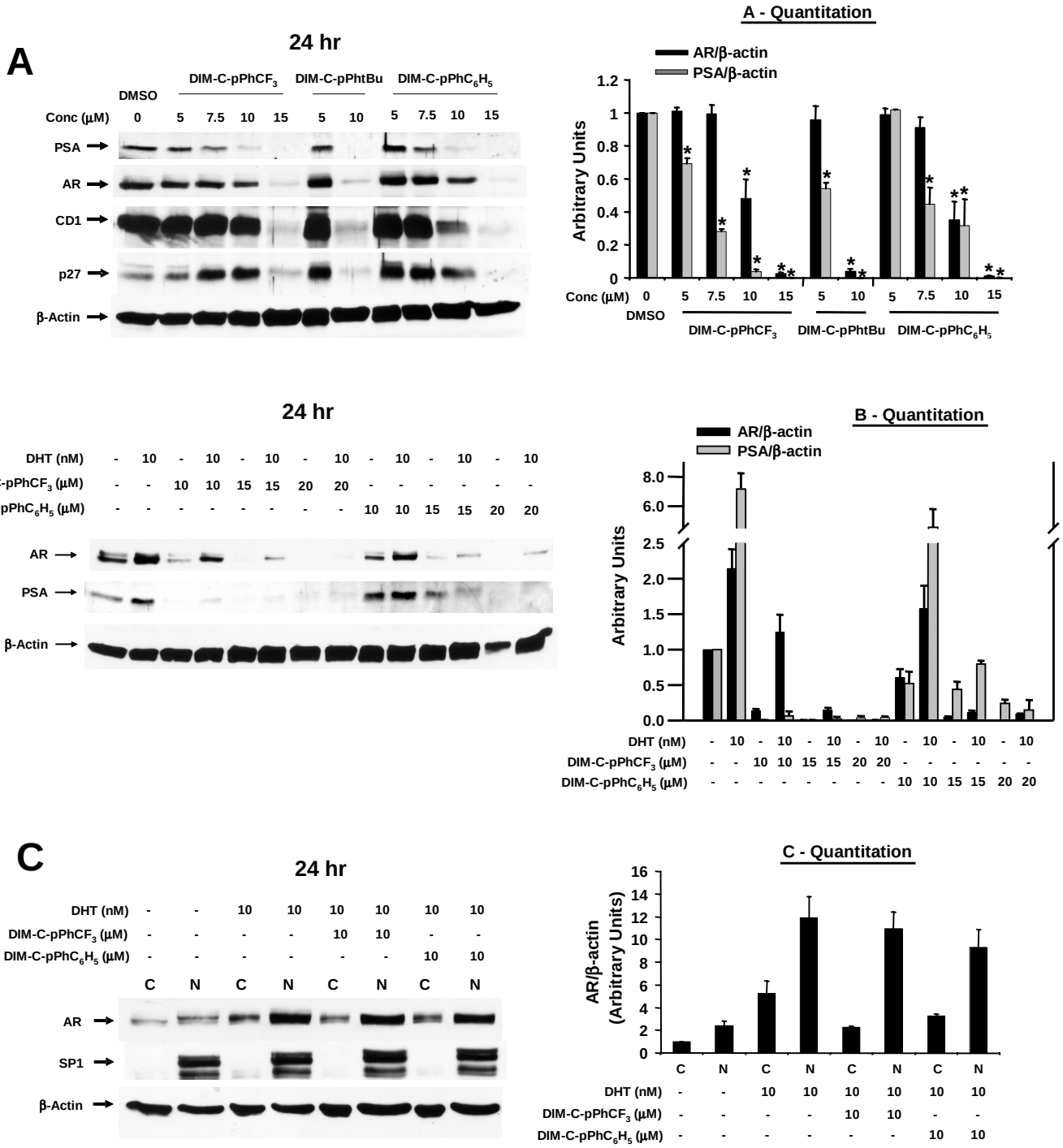

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Figure 4

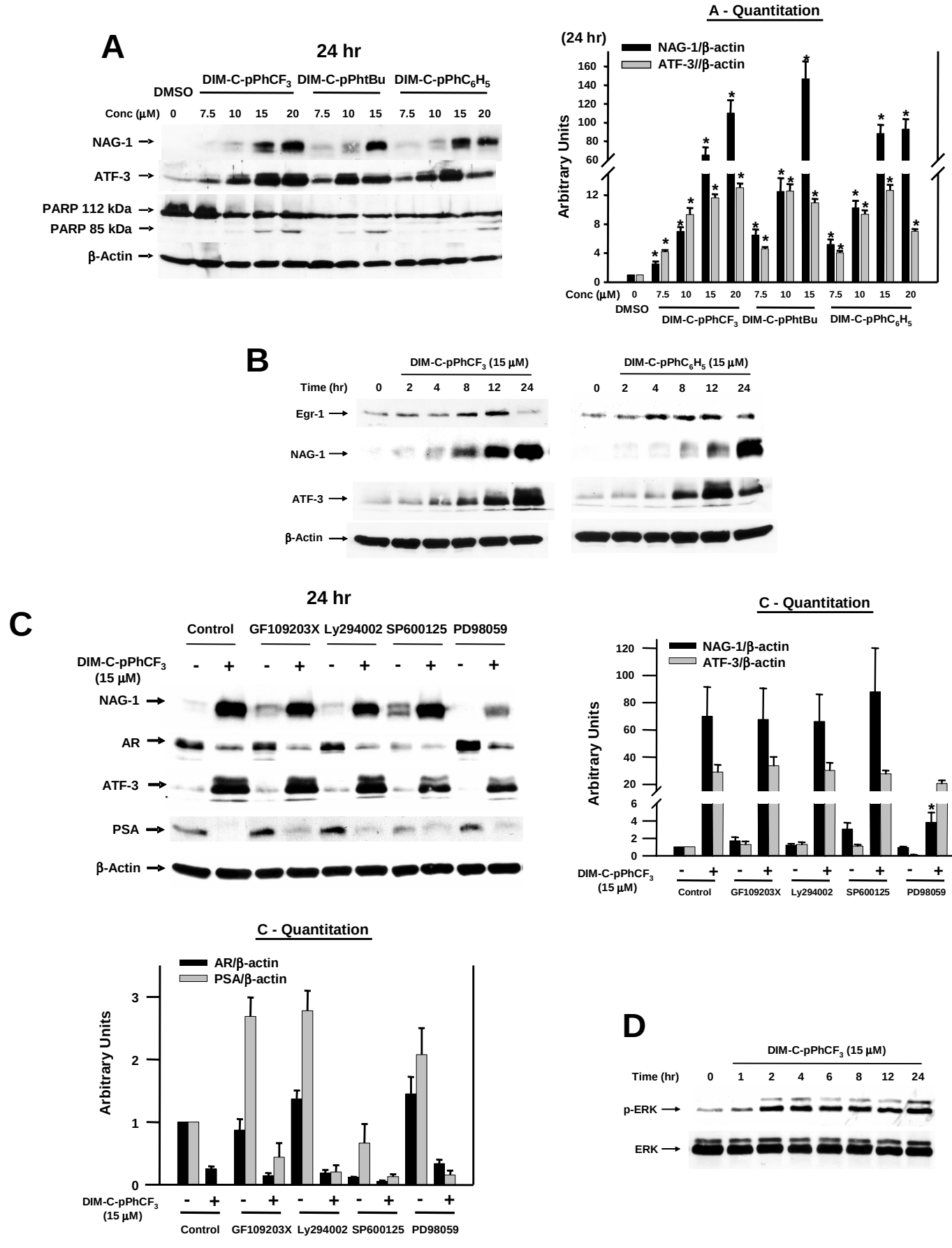

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Figure 5

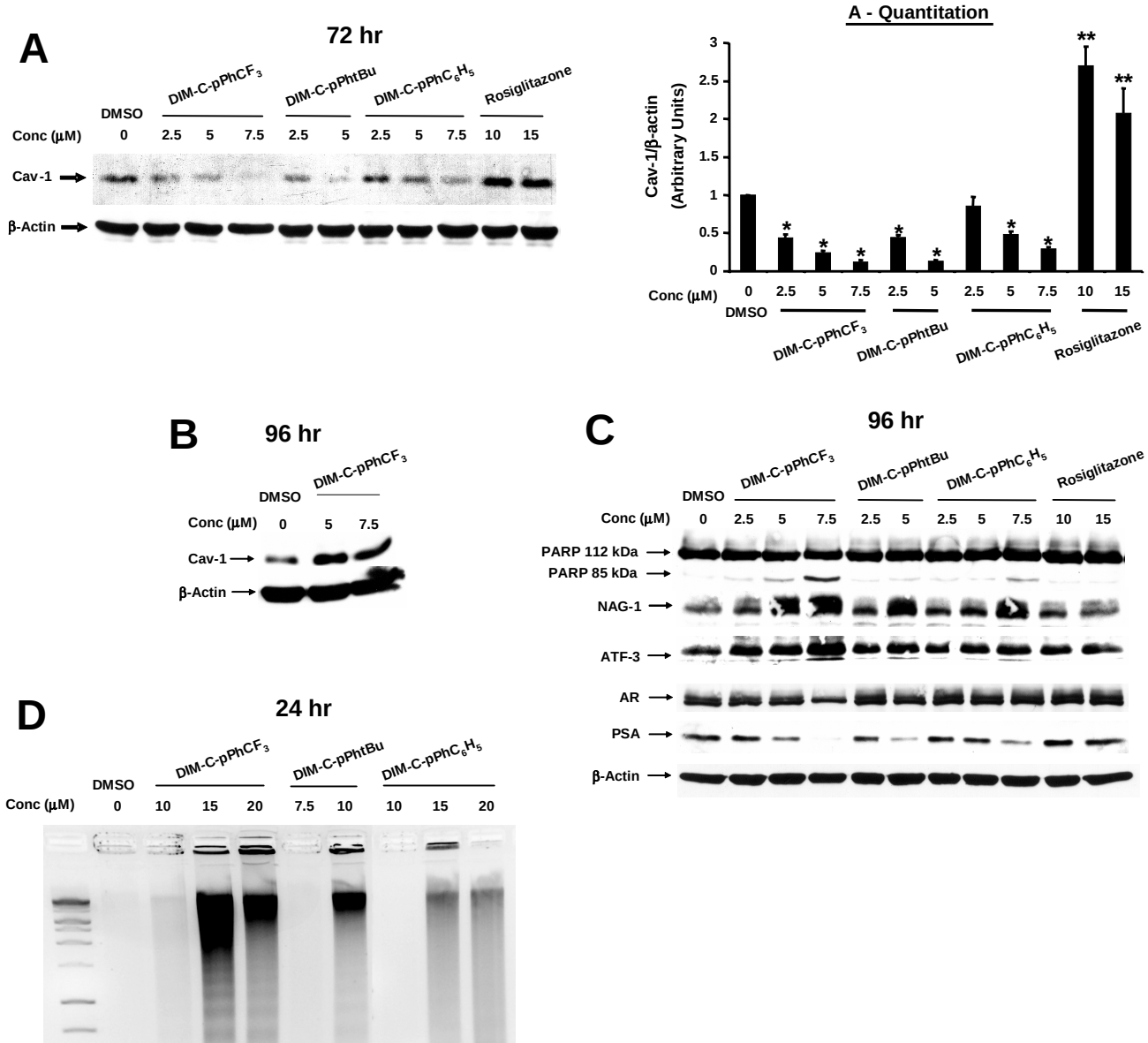

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Figure 6

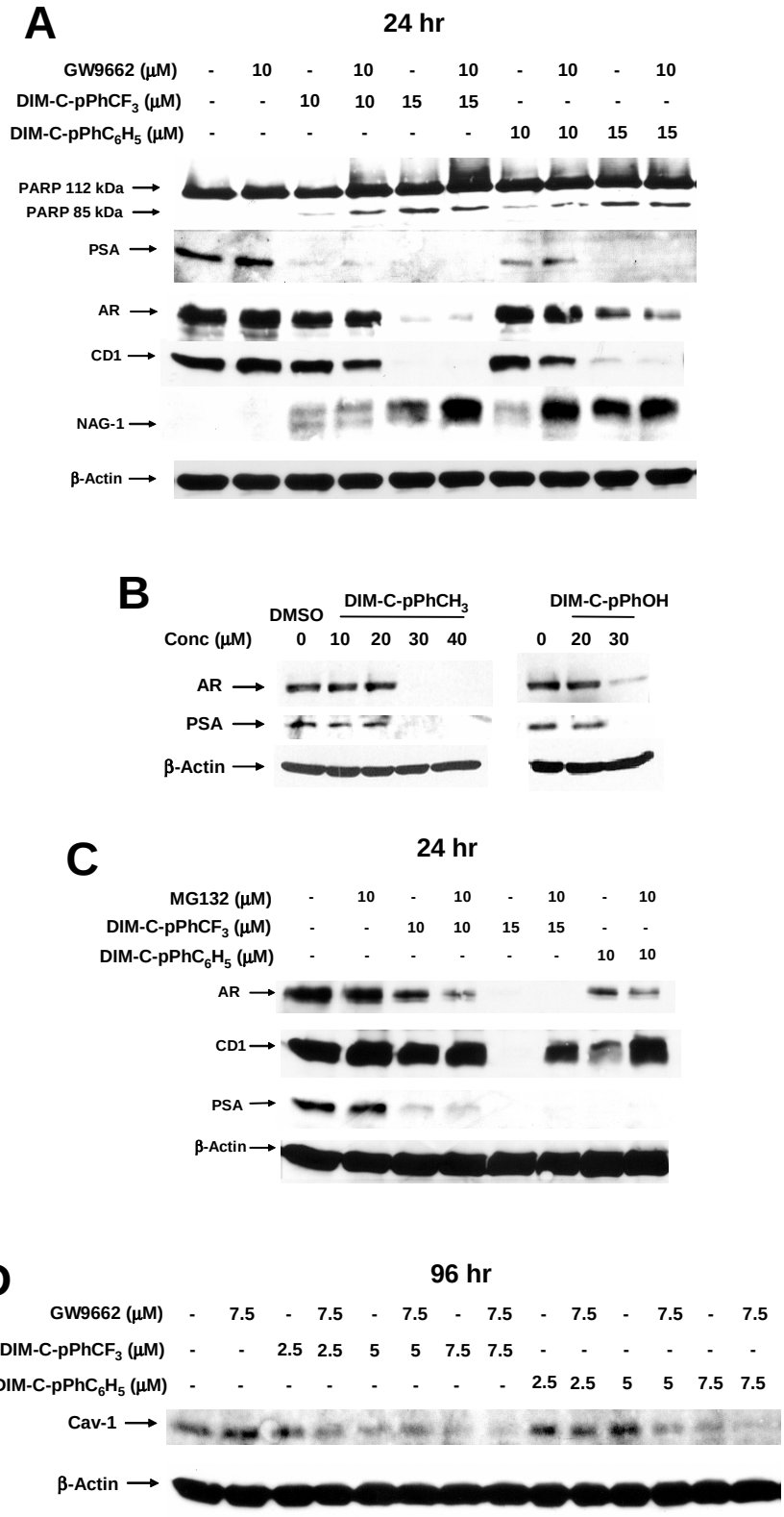

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Figure 7

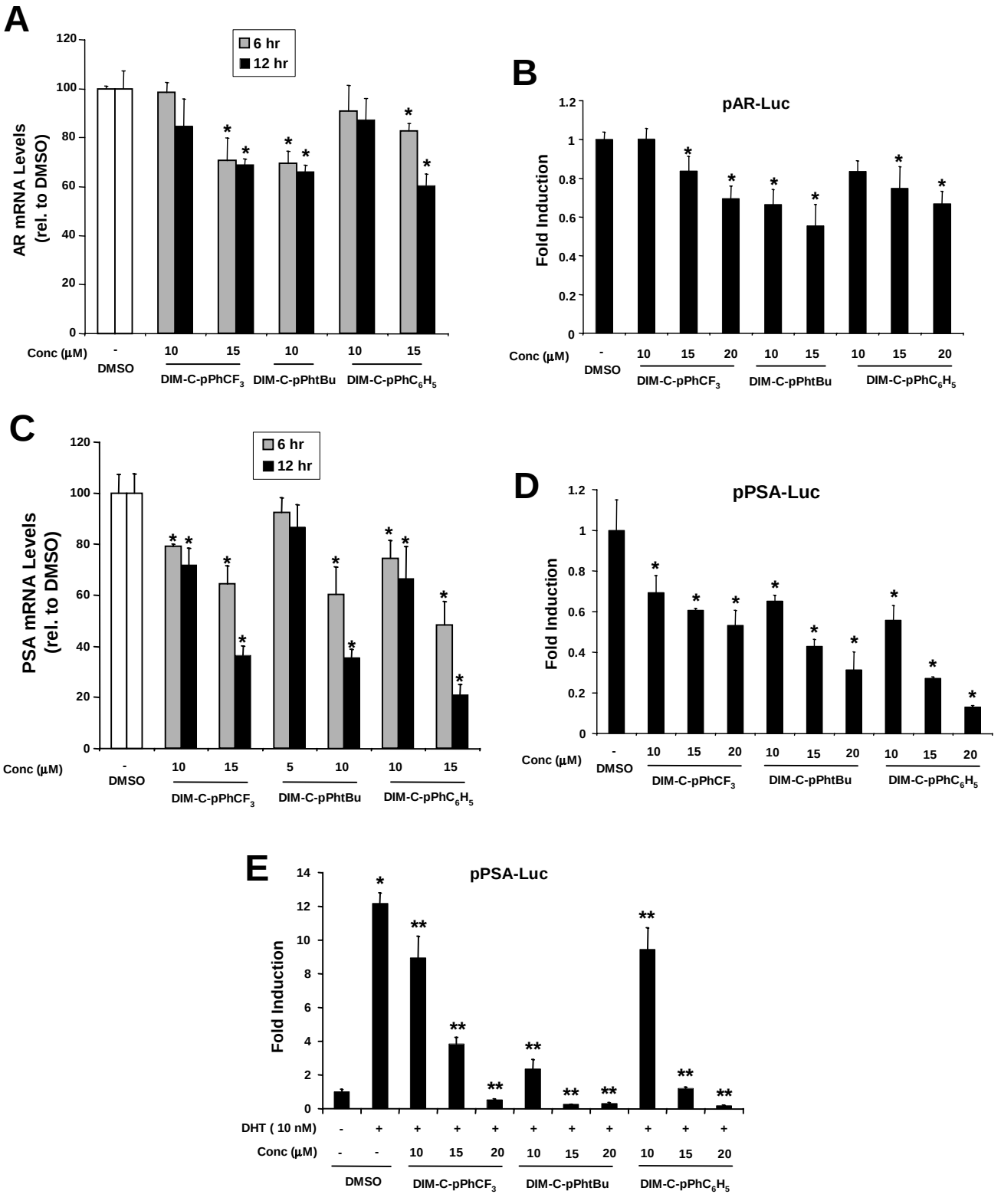

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Figure 8