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METHYL 2-CYANO-3,11-DIOXO-18β-OLEAN-1,12-DIEN-30-OATE IS A PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ AGONIST THAT INDUCES RECEPTOR-INDEPENDENT APOPTOSIS IN LNCaP PROSTATE CANCER CELLS

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Running Title:
β-CDODA-Me blocks cell growth and androgen responsiveness

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Abbreviations:
PPARγ, peroxisome proliferator-activated receptor γ; PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; TZDs, thiazolidinediones; CDDO, 2-cyano-3,12-dioxo-17α-olean-1,9-dien-28-oic acid; C-DIMs, 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methanes; GA, glycyrrhetinic acid; β-CDODA-Me, methyl 2-cyano-3,11-dioxo-18β-olean-1,12-diene-30-oate; KLF4, Krüppel-like Factor-4; NAG-1, nonsteroidal anti-inflammatory drug-activated gene 1; AR, androgen receptor; PSA, prostate specific antigen; β-Gal, β-Galactosidase; T007, N-(4'-aminopyridyl)-2-chloro-5-nitrobenzamide; PVDF, polyvinylidene difluoride; TRAMP, transgenic adenocarcinoma mouse prostate; DIM, 3,3'-diindolylmethane

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ABSTRACT

Methyl 2-cyano-3,11-dioxo-18β-olean-1,12-diene-30-oate (β-CDODA-Me) is a synthetic analog of the naturally-occurring triterpenoid glycyrrhetinic acid which contains a 2-cyano substituent in the A-ring. β-CDODA-Me was a potent inhibitor of LNCaP prostate cancer cell growth (IC_{50} ~ 1 μM) and activated peroxisome proliferator-activated receptor γ (PPARγ), whereas analogs without the cyano group were weakly cytotoxic and did not activate PPARγ. β-CDODA-Me induced p21 and p27 and downregulated cyclin D1 protein expression and also induced two other proapoptotic proteins, namely nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) and activating transcription factor-3 (ATF-3). However, induction of these responses by β-CDODA-Me was PPARγ-independent and due to activation of phosphatidylinositol-3-kinase (PI3K), mitogen activated protein kinase (MAPK), and jun N-terminal kinase (JNK) pathways by this compound. In contrast, β-CDODA-Me also decreased androgen receptor (AR) and prostate specific antigen (PSA) mRNA and protein levels through kinase-independent pathways. β-CDODA-Me repressed AR mRNA transcription, whereas decreased PSA mRNA levels were dependent on protein synthesis and was reversed by cycloheximide. Thus, potent inhibition of LNCaP cell survival by β-CDODA-Me is due to PPARγ-independent activation of multiple pathways that selectively activate growth inhibitory and proapoptotic responses.

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INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are a sub-family of the nuclear receptor superfamily of ligand-activated receptors (Mangelsdorf et al., 1995). The three members of this family, PPARα, PPARγ and PPARβ/σ are lipid sensors and play a key role in regulating tissue-specific lipid homeostasis and metabolism (Lee et al., 2003). PPARs also play an important role in many diseases particularly those related to obesity, metabolic disorders, cancer and atherogenesis (Escher and Wahli, 2000; Lee et al., 2003). Endogenous ligands for PPARs include fatty acid derived compounds and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PGJ2) which exhibits high affinity for PPARγ; however, PGJ2 may not be the endogenous ligand for this receptor due to the low cellular expression of this metabolite. Synthetic PPARγ agonists, such as the thiazolidinediones (TZDs) rosiglitazone and pioglitazone, are insulin-sensitizing drugs that are widely used for clinical treatment of type II diabetes.

Several different structural classes of PPARγ agonists have been characterized and these include flavones, various indole derivatives, and triterpenoids such as 2-cyano-3,12-dioxo-17α-olean-1,9-dien-28-oic acid (CDDO) and related compounds (Honda et al., 1998; Rieusset et al., 2002; Berger et al., 2003; Qin et al., 2004; Schopfer et al., 2005). PPARγ is overexpressed in many tumor types and cancer cell lines (Ikezoe et al., 2001) and PPARγ agonists show promise for the clinical treatment of various types of tumors (Kubota et al., 1998; Chang and Szabo, 2000; Gupta et al., 2003; Qin et al., 2004). Ligands for this receptor typically inhibit G_0/G_1 to S phase progression and this is accompanied by downregulation of cyclin D1 expression and induction of the cyclin-dependent kinase inhibitors p27 or p21. Research from our laboratory has identified a series of 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methanes [methylene-substituted diindolyl-methanes (C-DIMs)] which inhibit cancer cell and tumor growth (Qin et al.,

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2004; Chintharlapalli et al., 2004; Hong et al., 2004; Chintharlapalli et al., 2005a; Abdelrahim et al., 2006; Chintharlapalli et al., 2007b) through both receptor-dependent and independent pathways and similar observations have been reported for other PPARγ agonists (Clay et al., 2002; Konopleva et al., 2004; Samudio et al., 2005; Yang et al., 2006).

A new class of synthetic PPARγ agonists has been derived from glycyrrhetinic acid (GA), a major triterpenoid acid found in licorice extracts. Methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (β-CDODA-Me) is a 2-cyano derivative of GA and has the same oleanolic acid pentacyclic triterpene backbone structure as CDDO which is also a 2-cyano derivative of oleanolic acid (Honda et al., 1998). However, there are major structural differences between CDODA and CDDO with respect to the position of the carboxylic acid group in the E ring, the position of the double bonds and keto group in the C-ring. We recently reported that the 18α and 18β isomers of CDODA-Me activate PPARγ in colon cancer cells and induced both caveolin-1 and Krüppel-like Factor-4 (KLF4) through receptor-dependent pathways (Chintharlapalli et al., 2007a). In this study, we have investigated the effects of β-CDODA-Me on the proliferation of LNCaP prostate cancer cells and the IC_{50} value for growth inhibition was approximately 1 μM. In contrast to studies in colon cancer cells, β-CDODA-Me had minimal effects on caveolin-1 or KLF4 expression in LNCaP cells. The proapototic and growth inhibitory effects of β-CDODA-Me in LNCaP cells were associated with induction of p21 and p27 expression, downregulation of cyclin D1, and induction of nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1). β-CDODA-Me also decreased androgen receptor (AR) and prostate specific antigen (PSA) protein and RNA expression and all of these responses were PPARγ-independent. Thus, β-CDODA-Me, a PPARγ agonist, inhibited growth and induced

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apoptosis in LNCaP cells through activation of multiple receptor-independent pathways including ablation of AR gene expression.

MATERIALS AND METHODS

Cell lines- LNCaP, PC3 and DU145 human prostate carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). Fetal bovine serum was obtained from JRH Biosciences, Lenexa, KS. LNCaP, PC3 and DU145 cells were maintained in RPMI 1640 (Sigma Chemical, St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 mL/L of 100X antibiotic/antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO_2.

Antibodies and reagents- Antibodies for poly(ADP-ribose) polymerase, cyclin D1, p27, FKBP51, AR, ATF3, Akt and caveolin-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PSA was obtained from Dako Denmark A/S (Glostrup, Denmark); NAG-1 was purchased from Upstate Biotechnology (Charlottesville, VA); and EGR-1, pAKT, pERK, ERK, pJNK, JNK were obtained from Cell Signaling Technology Inc. (Danvers, MA). Monoclonal β-actin antibody and dihydrotesterone were 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), and lipofectamine reagents were supplied by Invitrogen (Carlsbad, CA). Western blotting chemiluminescence reagents were from Perkin-Elmer Life Sciences (Boston, MA). The PPARγ antagonist N-(4'-aminopyridyl)-2-chloro-5-nitrobenzamide (T007) was prepared in this

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laboratory and the synthesis of the GA derivatives has been previously described (Chintharlapalli et al., 2007a).

Cell proliferation and DNA fragmentation assays- LNCaP prostate cancer cells (2 x 10^4 per well) were added to 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM/Ham's F-12 media containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or the indicated compounds were added. Fresh medium and indicated compounds were added every 48 hr, and cells were then trypsinized and counted after 2, 4, and 6 days using a Coulter Z1 cell counter (Beckman Coulter, Fullerton, CA). Each experiment was done in triplicate, and results are expressed as means ± S.E. for each set of three experiments. The DNA fragmentation assay was performed using a BioVision Apoptotic DNA ladder extraction kit (BioVision, Mountain View, CA) according to the manufacturer's protocol.

Transfections- The Gal4 reporter construct containing 5X Gal4 response elements (pGal4) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). The Gal4DBD-PPARγ construct was a gift of Dr. Jennifer L. Oberfield (Glaxo Wellcome Research and Development, Research Triangle Park, NC). The PPRE-luc construct contains three tandem PPREs with a minimal TATA sequence linked to the luciferase gene 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-kilobase region of the PSA promoter was provided by Dr. Hong-Wu Cheng (University of California, Davis, CA). LNCaP cells (1 x 10^5) were seeded in 12-well plates in DMEM/Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS and grown overnight. Transient transfections were performed using Lipofectamine reagent (Invitrogen) according to the protocol provided by the manufacturer. Transfection studies were performed using 0.4 μg of

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Gal4Luc, 0.04 μg of β-galactosidase, 0.04 μg of Gal4DBD-PPARγ, 0.4 μg of AR-luc, and 0.3 μg of PSA-luc. 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 1 x 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.

Fluorescence-Activated Cell Sorting Analysis- LNCaP cells were treated with either the vehicle (DMSO) or the compound for 24 hr. Cells were trypsinized, centrifuged, and resuspended in staining solution containing 50 μg/ml propidium iodide, 4 mmol/L sodium citrate and 30 units/ml RNase. After incubation at room temperature for 1 hr, cells were analyzed on a FACS Vantage SE DiVa made by Becton Dickinson (BD), using BD FACSDiva Software V4.1.1. Propidium iodide (PI) fluorescence was collected through a 610SP bandpass filter, and list mode data were acquired on a minimum of 50,000 single cells defined by a dot plot of PI width *versus* PI area. Data analysis was performed in BD FACSDiva Software V4.1.1 using PI width *versus* PI area to exclude cell aggregates.

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 of RNasefree water and stored at -80°C. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA was prepared from the LNCaP cell line using a combination of oligodeoxythymidylic acid and dNTP mix (Applied Biosystems, Foster City, CA) and Superscript II (Invitrogen). Each PCR was carried out in triplicate in a 25-μl volume using SYBR Green Master mix (Applied Biosystems) for 15 min at

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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 (Applied Biosystems). The ABI Dissociation Curves software was used after 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' and AR reverse, 5'-CCC ATT TCG CTT TTG ACA CA-3'; PSA forward, 5'-GCA TTG AAC CAG AGG AGT TCT TG-3' and 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' and reverse, 5'-CAC ATC ACA GCT CCC CAC CA-3'.

Western blot analysis- 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 indicated compounds. Cells were collected by scraping in 150 µl high salt lysis buffer (50 mM HEPES, 0.5 M NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton-X-100 and 5 µL/ml of Protease Inhibitor Cocktail (Sigma). The lysates were incubated on ice for 1 hr with intermittent vortexing followed by centrifugation at 20,000 *g* for 10 min at 4°C. Before electrophoresis, samples were boiled for 3 min at 100°C; the amount of protein was determined and 60 µg protein applied per lane. Samples were subjected to SDS-PAGE on 10% gel at 120 V for 3 to 4 hr. Proteins were transferred on to polyvinylidene difluoride membrane (PVDF; Bio-Rad, Hercules, CA) at 0.9 amp for 90 min at 4°C in 1x transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 0.025% SDS). The membranes were blocked for 30 min with 5% TBST-Blotto (10

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mM Tris-HCl, 150 mM NaCl (pH 8.0), 0.05% Triton X-100 and 5% non-fat dry milk) and incubated in fresh 5% TBST-Blotto with primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody (1:5000) in 5% TBST-Blotto for 2-3 hr. The membrane was washed with TBST for 10 min and incubated with 10 ml of chemiluminiscence substrate (PerkinElmer Life Sciences) for 1.0 min and exposed to ImageTeK-H medical imaging film (Eastman American X-ray Supply, Inc.).

Statistical analysis- Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean ± S.E. for at least three separate determinations for each treatment group.

RESULTS

Cytotoxicity of β-CDODA-Me and activation of PPARγ. β-DODA or 1, 2-dehydro-GA exhibited minimal inhibition of LNCaP cell growth with a IC_{50} value > 15 μM whereas the IC_{50} for the corresponding methyl ester derivative was between 10-15 μM (Fig. 1A). Introduction of a 2-cyano group to give β-CDODA-Me increased the cytotoxicity by at least an order of magnitude and the IC_{50} was approximately 1 μM in LNCaP cells (Fig. 1A). These results were similar to those observed in colon cancer cells (Chintharlapalli et al., 2007a) and demonstrate the importance of 2-cyano substituents in mediating the cytotoxicity of GA derivatives. Figure 1B shows that decreased LNCaP cell survival induced by β-CDODA-Me was not affected after cotreatment with the PPARγ antagonist T007, suggesting that the cytotoxicity of this compound was receptor-independent. β-CDODA-Me was also cytotoxic to androgen-insensitive PC3 and DU145 prostate cancer cells (Fig. 1C). The induction of PPARγ-dependent transactivation by β-

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CDODA-Me was also investigated in LNCaP cells transfected with PPARγ-GAL4/GAL4-Luc or $PPRE_3$-Luc constructs and treated with 1-5 μM concentrations. β-CDODA-Me significantly induced luciferase activity (Fig. 1D) and in cells cotreated with β-CDODA-Me plus 10 μM T007 (a PPARγ antagonist), there was significant inhibition of induced transactivation. In contrast, β-DODA-Me did not activate PPARγ (data not shown) demonstrating the requirement for the 2-cyano substituent to confer PPARγ agonist activity on the GA derivative.

PPARγ agonists typically modulate expression of one or more of the cell cycle proteins p27, p21 and cyclin D1, and Figure 2A illustrates the effects of 1-5 μM β-CDODA-Me on expression of these proteins in LNCaP cells. There was a concentration-dependent induction of p27 and p21 and a decrease in cyclin D1 proteins and Rb phosphorylation in cells treated with β-CDODA-Me alone, and similar results were observed in cells cotreated with the PPARγ antagonist T007 and β-CDODA-Me (Fig. 2B) suggesting that these responses were PPARγ-independent. FACS analysis of LNCaP cells treated with different concentrations of β-CDODA-Me indicated that 1 μM β-CDODA-Me did not affect the percentage of cells in G_0/G_1, S or G_2/M, whereas at higher concentrations, the percentage of cells in these phases of the cell cycle decreased (Fig. 2C). β-CDODA-Me induced a concentration-dependent increase in the percentage of apoptotic subG1 LNCaP cells, and the proapoptotic effects of this compound were also observed in PC3 and DU145 cells (Fig. 2D) where β-CDODA-Me induced caspase-dependent PARP cleavage. Thus, the overall cytotoxicity of β-CDODA-Me in prostate cancer cells was associated with both inhibition of growth and induction of apoptosis.

Induction of proapoptotic responses by β-CDODA-Me. NAG-1 and ATF-3 are proapoptotic proteins induced by PPARγ agonists and results in Figure 3A show that 1-5 μM β-

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CDODA-Me induced NAG-1 and ATF-3 which are often co-induced and this was accompanied by caspase-dependent PARP cleavage, DNA fragmentation, and decreased bcl2 expression in LNCaP cells. In addition, the DNA laddering response was not affected after cotreatment with the PPARγ antagonist T007 (Fig. 3B). In LNCaP cells cotreated with β-CDODA-Me plus T007 (Fig. 3C), the induced responses were not inhibited by the PPARγ antagonist indicating that induction of these proapototic responses was receptor-independent. Previous studies show that different structural classes of PPARγ agonists downregulate AR expression in LNCaP cells and this response can also result in activation of apoptosis (Yang et al., 2006; Chintharlapalli et al., 2007b). Figure 3D summarizes the effects of β-CDODA-Me on AR expression in the presence or absence of 10 nM DHT and also on the expression of FKBP51 and PSA, two androgen-responsive genes in LNCaP cells. DHT increases expression of AR due to stabilization of the receptor and also induces both androgen-responsive FKBP51 and PSA genes and, in cells treated with 1-5 μM β-CDODA-Me, there was a concentration-dependent decrease in AR, PSA and FKBP51 expression in the presence or absence of DHT. In addition, downregulation of AR, PSA and FKBP51 proteins in LNCaP cells treated with β-CDODA-Me was not affected by cotreatment with the PPARγ antagonist T007 or the proteasome inhibitor MG132 (Fig. 3E). In contrast, β-CDODA-Me-dependent degradation of cyclin D1 was inhibited after cotreatment with MG132 and these observations are similar to those reported for other PPARγ agonists that induce proteasome-dependent degradation of cyclin D1 (Chintharlapalli et al., 2004; Chintharlapalli et al., 2005a; Chintharlapalli et al., 2005b). These results clearly show that β-CDODA-Me decreases expression of androgen-responsive genes and AR through PPARγ-independent pathways. The downregulation of AR in cells treated with β-CDODA-Me is

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consistent with the induction of apoptosis by this compound since decreased AR expression by small inhibitory RNAs in LNCaP cells also induces apoptosis (Liao et al., 2005).

β-CDODA-Me induces kinase-dependent activation of proapoptotic/growth inhibitory pathways. Previous studies show that NAG-1 is induced by some PPARγ agonists and other cytotoxic compounds in colon cancer cells (Baek et al., 2003; Baek et al., 2005; Chintharlapalli et al., 2005a) through PI3K-dependent activation of EGR-1 which acts as a *trans*-acting factor to induce NAG-1 expression. Figure 4A summarizes the time-dependent induction of EGR-1, ATF-3 and NAG-1 by 2.5 μM β-CDODA-Me and the induction responses followed a similar time course, whereas EGR-1 dependent induction of NAG-1 in colon cancer cells is associated with the increased expression of EGR-1 prior to induction of NAG-1 (Baek et al., 2005; Chintharlapalli et al., 2005a). Previous studies show that NAG-1 induction is kinase-dependent (Baek et al., 2005; Chintharlapalli et al., 2005a), and results in Figure 4B show that 2.5 μM β-CDODA-Me induces activation of the JNK (p-JNK), PI3K (p-Akt) and MAPK (p-Erk) pathways. Maximal activation of JNK and PI3K was observed after 8 and 8-12 hr, respectively, whereas p-Erk activation remained elevated for 24 hr. The effects of inhibitors of MAPK (PD98059), PI3K (LY294002), protein kinase C (GF109203X) and JNK (SP600125) on induction of NAG-1 and ATF3 and decreased expression of AR, PSA and FKBP51 was also investigated in LNCaP cells treated with 2.5 μM β-CDODA-Me (Fig. 4C). Both PD98059 and LY294002 inhibited induction of NAG-1 by β-CDODA-Me. However, the JNK inhibitor SP600125 was the most potent inhibitor of ATF-3 induction (Figs. 4C and 4D). In contrast, decreased expression of AR, PSA and FKBP51 in LNCaP cells treated with β-CDODA-Me was unaffected by kinase inhibitors. CDDO and related compounds also induce activation of JNK in leukemia cells through increased reactive of oxygen species (ROS) (Ikeda et al., 2003), and

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Figure 4E illustrates the effects of β-CDODA-Me on activation of JNK and ATF3 after cotreatment with the antioxidant NAC. Induction of JNK phosphorylation and ATF3 by β-CDODA-Me was inhibited after cotreatment with NAC demonstrating that like CDDO (Ikeda et al., 2003), β-CDODA-Me activates JNK through increased oxidative stress.

These results suggest that the underlying pathways associated with the growth inhibitory/proapoptotic pathways induced by β-CDODA-Me in LNCaP cells are due in part to activation of kinases. Therefore, the effects of kinase inhibitors on modulation of cell cycle proteins by β-CDODA-Me were also investigated and the downregulation of cyclin D1 and induction of p21 were partially blocked in cells cotreated with the MAPK inhibitor PD98059 (Fig. 5A), and MAPK-dependent activation of p21 has previously been observed (De Siervi et al., 2004). Results in Figure 5B show that the 1-5 μM β-CDODA-Me also induces luciferase activity in LNCaP cells transfected with constructs containing -2325 to +8 [p21-Luc (Fl)], -124 to +8 [p21-Luc (-124)], -101 to +8 [p21-Luc (-101)], and -60 to +8 [p21-Luc (-60)] p21 promoter inserts. The latter 3 constructs contain the 6 proximal GC rich site (VI - I) and the results of the transfection studies suggest that these GC-rich sites are necessary for β-CDODA-Me-induced transactivation. Deletion analysis of the p21 promoter indicates that loss of inducibility [i.e. p21-luc(60)] is associated with loss of GC-rich sites IV and III which are essential for MAPK-dependent activation of p21 by β-CDODA-Me. The role of MAPK in activation of the p21 promoter was confirmed in LNCaP cells transfected with p21-luc(101); β-CDODA-Me induced luciferase activity and cotreatment with the MAPK inhibitor PD98059 inhibited this response (Fig. 5C). These results show that the induction of p21 and the proapototic NAG-1 protein by β-CDODA-Me were related to the activation of MAPK and PI3K but were independent of PPARγ (Figs. 2B and 3C).

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β-CDODA-Me differentially decreases AR and PSA gene expression in LNCaP cells. β-CDODA-Me decreases expression of AR, PSA and FKBP51 protein levels through proteasome and PPARγ-independent pathways (Figs. 3D and 3E) and these responses are also not modulated by kinase inhibitors (Fig. 4C). The results in Figure 6A show that β-CDODA-Me also decreases AR mRNA levels after treatment for 18 hr, and cotreatment with the PPARγ antagonist T007 did not affect mRNA levels confirming the β-CDODA-Me-induced downregulation of AR mRNA levels was also PPARγ-independent. Similar results were obtained in LNCaP cells treated with β-CDODA-Me alone or in the presence of the protein synthesis inhibitor cycloheximide (10 μg/ml) (Fig. 6B); cycloheximide did not modulate the effects of β-CDODA-Me, suggesting that an induced inhibitory protein(s) does not mediate the effects of β-CDODA-Me on AR mRNA expression. β-CDODA-Me also decreased luciferase activity in LNCaP cells transfected with the AR-Luc construct that contains the -5400 to +580 region of the AR promoter linked to the luciferase genes (Fig. 6C). The results indicate that β-CDODA-Me inhibits AR transcription without the parallel induction of inhibitory *trans*-acting factors. Recent studies suggest that AR downregulation of a PPARγ-inactive thiazolidinedione analog was due to downregulation of Sp protein (Yang et al., 2007). Results in Figure 6D show that β-CDODA-Me induces a time-dependent induction of PARP cleavage and a decrease of both AR and Sp1.

PSA protein expression is also decreased in LNCaP cells treated with β-CDODA-Me (Fig. 3D) and similar effects were observed for PSA mRNA levels after treatment for 18 hr, and these responses were not inhibited after cotreatment with the PPARγ antagonist T007 (Fig. 7A). However, β-CDODA-Me-induced downregulation of PSA mRNA levels after treatment for 18 hr was significantly inhibited after cotreatment with cycloheximide (Fig. 7B). In addition, β-

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CDODA-Me inhibited transactivation in LNCaP cells transfected with the PSA-Luc construct (contains 5.85 kb of the PSA promoter insert) (Fig. 7C) and similar results were obtained for DHT-induced luciferase activity (Fig. 7D). Thus, in contrast to results obtained for AR, β-CDODA-Me inhibits PSA expression through induction of inhibitory *trans*-acting factors and the mechanisms associated with the decreased PSA expression and the *cis*-elements important for this response are currently being investigated.

DISCUSSION

PPARγ agonists have been extensively investigated in both *in vitro* and *in vivo* cancer models for their potential applications in cancer chemotherapy (Honda et al., 1998; Escher and Wahli, 2000; Berger et al., 2003; Qin et al., 2004). PPARγ agonists inhibit prostate cancer cell and tumor growth (Kubota et al., 1998; Moretti et al., 2001; Segawa et al., 2002; Chintharlapalli et al., 2007b) and the fact that approximately 40% of prostate cancer patients carry hemizygous deletions of PPARγ (Mueller et al., 2000) suggests that this receptor may serve as a tumor suppressor gene for prostate cancer. However, in animal studies using the transgenic adenocarcinoma mouse prostate (TRAMP) model with hemizygous deletion in PPARγ, it was shown that the loss of receptor expression did not enhance or inhibit prostate tumor development in these animals (Saez et al., 2003). Thus, at least in the TRAMP mouse model, PPARγ does not appear to act as a tumor suppressor gene.

One of the perplexing problems with PPARγ agonists is that although these compounds inhibit cancer cell and tumor growth, their mechanisms of action are both receptor-dependent and -independent in different cancer cell lines. For example, PGJ2, troglitazone and PPARγ-active C-DIMS induce NAG-1 in HCT-116 colon cancer cells; however, only induction by PGJ2

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is inhibited by a PPARγ antagonist (Baek et al., 2003; Chintharlapalli et al., 2005a). Caveolin-1 is induced by C-DIM compounds and CDDO in colon cancer cell lines, and this response is inhibited after cotreatment with PPARγ antagonists (Chintharlapalli et al., 2004; Chintharlapalli et al., 2005b). In contrast, C-DIMs decreased caveolin-1 expression in LNCaP cells and this response was PPARγ-independent (Chintharlapalli et al., 2007b).

β-CDODA-Me is a triterpenoid acid that contains an oleanolic acid backbone structure similar to that of CDDO and CDDO-Me (methyl ester) (Honda et al., 1998), but there are important structural differences in the C, D and E rings that differentiate between these compounds; however, for both compounds the 2-cyano group was necessary for activation of PPARγ.

In this study, we investigated the growth inhibitory and proapoptotic effects of β-CDODA-Me in LNCaP cells and the role of PPARγ in mediating these responses. β-CDODA-Me was a more potent inhibitor of LNCaP cell growth than analogs (β-DODA and β-DODA-Me) that did not contain a 2-cyano substituent (Fig. 1A). Moreover, β-CDODA-Me decreased survival of androgen-insensitive PC3 and DU145 cells (Fig. 1C) and also induced apoptosis in these cells (Fig. 2D). β-CDODA-Me activated PPARγ-dependent transactivation in transient transfection studies in LNCaP cells (Fig. 1D), and compounds without the CN-group were inactive (data not shown) as previously reported for these analogs in colon cancer cells (Chintharlapalli et al., 2007a). β-CDODA-Me induced p27 expression and downregulated levels of cyclin D1 protein (Figs. 2A and 2B). Similar effects were previously reported for C-DIMs in LNCaP cells (Chintharlapalli et al., 2007b) and the effects of both compounds were receptor-independent; however, β-CDODA-Me-induced responses were observed at lower concentrations (1 - 2.5 μM) than the C-DIM compounds (7.5 - 10 μM). C-DIMs did not induce p21 in LNCaP

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cells (Chintharlapalli et al., 2007b), whereas β-CDODA-Me induced p21 protein and this response was not inhibited after cotreatment with PPARγ antagonist T007 (Fig. 2B). Differences between PPARγ-active C-DIMs and β-CDODA-Me in their induction of p21 in LNCaP cells was due to activation of MAPK signaling by the latter compound (Fig. 4B) which was required for induction of p21 protein (Fig. 5A) and activation of the p21 promoter (Fig. 5C). This is a novel pathway for induction of p21 in LNCaP cells; however, previous studies in other cell lines have demonstrated MAPK-dependent induction of p21 expression (De Siervi et al., 2004).

NAG-1 and ATF3 are growth inhibitory and proapoptotic proteins (Hartman et al., 2004; Jang et al., 2006), and previous studies with PPARγ agonists report both receptor-dependent and -independent induction of NAG-1 (Baek et al., 2003; Chintharlapalli et al., 2005a; Chintharlapalli et al., 2005b). Induction of NAG-1 and ATF3 by β-CDODA-Me in LNCaP cells was also PPARγ-independent. Both PI3K and MAPK inhibitors blocked induction of NAG-1; however, the JNK inhibitor SP600125 was the most potent inhibitor of ATF-3 (but not NAG-1) induction. The inhibitory effects of SP600125 are consistent with previous studies showing that homocysteine also induces ATF3 in vascular cells through activation of JNK which activates c-*jun* and ATF-3 through an AP-1 site in the ATF-3 promoter (Cheng et al., 2006). The structurally-related triterpenoid CDDO induces oxidative stress in leukemia and pancreatic cancer cells (Ikeda et al., 2003; Samudio et al., 2005), and this is correlated with pro-oxidant-dependent induction of JNK phosphorylation and other responses which are inhibited by antioxidants such as NAC (Ikeda et al., 2003). Similar results were observed in this study where NAC also inhibits β-CDODA-Me-dependent activation of JNK and induction of ATF3 (Fig. 4E). The kinase-dependent induction of NAG-1 has previously been reported and these effects are both structure and cell context-dependent. For example, troglitazone and PPARγ-active C-DIMs

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induce NAG-1 in HCT116 colon cancer cells through rapid activation of Egr-1 which subsequently activates NAG-1 through direct interaction with the proximal region of the NAG-1 promoter (Chintharlapalli et al., 2005a). However, this induction response is MAPK-dependent for troglitazone and PI3-K-dependent for the C-DIM compound. In this study, the time-dependent induction of both EGR-1 and NAG-1 are similar in LNCaP cells (Fig. 4A), and inhibition of NAG-1 expression is observed with both PI3K and MAPK inhibitors (Fig. 4B). This may involve cooperative interactions of both kinase pathways for induction of NAG-1 by β-CDODA-Me in LNCaP cells, and mechanisms for these responses are currently being investigated. Interestingly, induction of NAG-1 by PPARγ-active C-DIMs in LNCaP cells was inhibited only by the MAPK inhibitor PD98059 (Chintharlapalli et al., 2007b), suggesting differences between β-CDODA-Me and C-DIMs in the same cell line. Thus, induction of both NAG-1 and ATF3 in LNCaP cells is differentially induced by two PPARγ agonists, C-DIMs and β-CDODA-Me through receptor-independent activation of different kinase pathways.

Recent reports show that in LNCaP cells AR knockdown by RNA interference results in apoptosis (Liao et al., 2005) and stable knockdown using short hairpin RNAs for AR results in decreased AR and PSA expression and inhibition of tumor growth *in vivo* (Cai et al., 2000). Like β-CDODA-Me, AR and PSA expressions are also decreased by C-DIMs and troglitazone in LNCaP cells, and 3,3'-diindolylmethane (DIM) also decreases expression of both genes and proteins (Bhuiyan et al., 2006; Li et al., 2007). Troglitazone differentially decreases PSA and AR expression at relatively low ($IC_{50} \leq 10$ μM) and high ($IC_{50} \sim 40$ μM) concentrations, respectively (Yang et al., 2006). In contrast, C-DIMs decreased AR and PSA mRNA, protein and reporter gene activity in cells transfected with PSA-Luc and AR-Luc constructs over a narrow range of concentrations (7.5 - 10 μM) (Chintharlapalli et al., 2007b) and similar results

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were observed for β-CDODA-Me (1 - 2.5 μM) in this study (Figs. 6 and 7). Moreover, cycloheximide reversed the β-CDODA-Me- and C-DIM-dependent downregulation of PSA but not AR mRNA levels, suggesting a similar mechanism of action for both compounds. One study reported that DIM inhibited nuclear uptake of AR in LNCaP cells (Le et al., 2003), and like β-CDODA-Me, DIM also decreased AR and PSA expression in LNCaP and androgen-insensitive C4-2B cells (Bhuiyan et al., 2006; Li et al., 2007). However, there are several differences between the pathways associated with downregulation of these genes by β-CDODA-Me and DIM and this includes the pivotal role for DIM as an inhibitor of phospho-Akt (Bhuiyan et al., 2006; Li et al., 2007), whereas β-CDODA-Me induces phospho-Akt (Fig. 4B) and the PI3K inhibitor LY294002 does not affect β-CDODA-dependent downregulation of AR, PSA or FKBP51 (Fig. 4C) or induction of p21 or p27 (Fig. 5A).

A recent report indicated that decreased AR expression in LNCaP cells treated with a PPARγ-inactive thiazolidinedione derivative was due to proteasome-dependent degradation of Sp1 (Yang et al., 2007). Our results also show a parallel decrease of AR and Sp1 in LNCaP cells treated with β-CDODA-Me (Fig. 6D); however, downregulation of AR was not reversed by a proteasome inhibitor (Fig. 3E), and the mechanism of this response is currently being investigated. Loss of AR by RNA interference results in the induction of apoptosis in LNCaP cells (Liao et al., 2005). In contrast, 2.5 μM β-CDODA-Me rapidly induces PARP cleavage and apoptosis in LNCaP cells prior to decreased AR expression (Fig. 6D) demonstrating that apoptotic pathways activated by β-CDODA-Me in LNCaP cells are not associated with loss of AR, and the proapoptotic mechanisms are currently being investigated.

Results of this study demonstrate that β-CDODA-Me is a potent inhibitor of LNCaP cell growth and induces proapoptotic responses through activation of kinases which differentially

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activate ATF3, NAG-1 and p21. In contrast, decreased expression of AR and PSA are kinase independent and occur through different pathways (Fig. 8). β-CDODA-Me, C-DIMs, DIM and troglitazone exhibited both differences and similarities in their modes of action in LNCaP cells, although all of these compounds decreased expression of AR and PSA. The growth inhibitory and proapoptotic effects of β-CDODA-Me were primarily PPARγ-independent in LNCaP cells (Figs. 1B and 3). β-CDODA-Me also decreased survival and induced apoptosis in androgen-insensitive PC3 and DU145 prostate cancer cells (Figs. 1C and 2D), and similar results have been observed for other PPARγ agonists. It is apparent that β-CDODA-Me decreases survival and induces apoptosis through multiple pathways including its potent antiandrogenic activity in LNCaP cells. The successful applications of β-CDODA-Me and other such compounds for single or combined prostate cancer chemotherapies will require insights on their mechanisms of action and prostate cancer cell context-dependent similarities and differences in activating critical pathways such as those illustrated in Figure 8.

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FOOTNOTES

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FIGURE CAPTIONS

Fig. 1. Effects of β-CDODA-Me and related compounds on LNCaP cell survival and activation of PPARγ. Cell survival of LNCaP (A and B), PC3 and DU145 cells (C) cells. Prostate cancer cells were treated with different concentrations of β-DODA, β-DODA-Me or β-CDODA-Me alone or in combination with 5 μM T007 (C) for 96 hr, and the % cell survival relative to DMSO (solvent control set at 100%) was determined as described in the Materials and Methods. Results are expressed as means ± SE for three separate determinations for each treatment group, and significantly ($p < 0.05$) decreased survival is indicated (*). (D) β-CDODA-Me activates PPARγ. LNCaP cells were treated with β-CDODA, T007 or their combination, transfected with PPARγ-GAL4/pGAL4 or PPRE-luc, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group, and significant ($p < 0.05$) induction by β-CDODA-Me (*) and inhibition after cotreatment with T007 (**) are indicated.

Fig. 2. β-CDODA-Me modulates the cell cycle and cell cycle genes and induces apoptosis in prostate cancer cells. Modulation of cell cycle genes by β-CDODA-Me alone (A) and in combination with T007 (B). Cells were treated as indicated for 24 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. (C) Cell cycle progression. LNCaP cells were treated with DMSO and different concentrations of β-CDODA-Me for 24 hr and analyzed for percent distribution of cells in different phases of the cell cycle by FACS analysis as described in the Materials and Methods. Results are expressed at means ± SE for three replicate determinations, and significant ($p < 0.05$) changes (compared to DMSO group) are

indicated by an asterisk. (D) Induction of PARP cleavage. PC3 and DU145 cells were treated for 24 hr with different concentrations of β-CDODA-Me, and whole cell lysates were analyzed by Western blots as described in the Materials and Methods.

Fig. 3. β-CDODA induces apoptotic pathways and decreases androgen-responsiveness in LNCaP cells. β-CDODA-Me alone (A) and in combination with T007 (B and C) induces proapoptotic pathways. LNCaP cells were treated as indicated for 24 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. β-CDODA-Me-induced DNA fragmentation (A and B) was also determined as described. Effects of β-CDODA-Me alone and in combination with DHT or T007 (D) or MG132 (E) on AR and androgen-responsive proteins. LNCaP cells were treated with DMSO or the various compounds for 24 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods.

Fig. 4. β-CDODA-Me induces proapoptotic proteins and kinases. Induction of NAG-1, ATF-3 and Egr-1 (A) and kinases (B) by β-CDODA-Me. LNCaP cells were treated with 2.5 μM β-CDODA-Me, and whole cell lysates isolated at different times after treatment were analyzed by Western blot analysis as described in the Materials and Methods. Effects of kinase inhibitors on proapoptotic responses (C) and quantitation of NAG-1 and ATF-3 expression (D). LNCaP cells were treated with 2.5 μM β-CDODA alone or in combination with various kinase inhibitors and after 24 hr, whole cell lysates were analyzed by Western blot analysis. Levels of NAG-1 and ATF-3 proteins (normalized to β-actin) (D) are means ± SE for three separate determinations for each treatment group and significantly ($p < 0.05$) decreased levels after cotreatment with a kinase inhibitor are indicated (**).

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Fig. 5. β-CDODA-Me induction of p21 is MAPK-dependent. (A) Effects of kinase inhibitors on induction of p21. LNCaP cells were treated with DMSO, 2.5 μM β-CDODA-Me alone or in combination with kinase inhibitors for 24 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. (B) β-CDODA-Me activates p21 promoter constructs. LNCaP cells were transfected with p21 promoter constructs, treated with DMSO or different concentrations of β-CDODA-Me, and luciferase activity was determined as described in the Materials and Methods. Results are means ± SE for three separate determinations for each treatment group, and significant ($p < 0.05$) induction of activity is indicated (*). (C) Inhibition by PD98059. Cells were transfected with p21-luc(101), treated with DMSO, β-CDODA-Me alone or in combination with 10 μM PD98059. Results are expressed as means ± SE for three separate determinations for each treatment group, and significant ($p < 0.05$) induction by β-CDODA-Me (*) and inhibition after cotreatment with PD98059 (**) are indicated.

Fig. 6. β-CDODA-Me decreases AR gene expression. Effects of T007 (A) and cycloheximide (B) on β-CDODA-Me-dependent effects on AR gene expression. LNCaP cells were treated with β-CDODA-Me alone or in combination with T007 or cycloheximide for 18 hr, and AR mRNA levels were determined by real time PCR as described in the Materials and Methods. Similar results were observed after treatment for 12 hr (data not shown). (C) β-CDODA-Me decreases AR promoter activity. LNCaP cells were transfected with AR-luc, treated with DMSO or β-CDODA-Me, and luciferase activity determined as described in the Materials and Methods. Results are means ± SE for three separate experiments for each treatment group and a significant ($p < 0.05$) decrease in activity is indicated (*). (D) Time-dependent effects of β-CDODA-Me on AR, Sp1 and PARP

(cleaved). LNCaP cells were treated with DMSO or β-CDODA-Me for up to 24 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods.

Fig. 7. β-CDODA-Me decreases PSA expression. Effects of T007 (A) and cycloheximide (B) on β-CDODA-Me-dependent effects on PSA gene expression. LNCaP cells were treated with β-CDODA-Me alone or in combination with T007 or cycloheximide for 18 hr, and PSA mRNA levels were determined by real time PCR as described in the Materials and Methods. Similar results were observed after treatment for 12 hr (data not shown). β-CDODA-Me decreases PSA promoter (C) and DHT-induced (D) PSA promoter activity. LNCaP cells were transfected with PSA-luc, treated with DMSO, β-CDODA-Me, DHT and β-CDODA-Me plus DHT (combined), and luciferase activity determined as described in the Materials and Methods. Results are means ± SE for three replicate determinations for each treatment group, and significantly ($p < 0.05$) decreased basal or DHT-induced luciferase activity by β-CDODA-Me is indicated (*).

Fig. 8. β-CDODA-Me-dependent activation of kinases and kinase-dependent genes and repression of AR and PSA.

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Figure 1

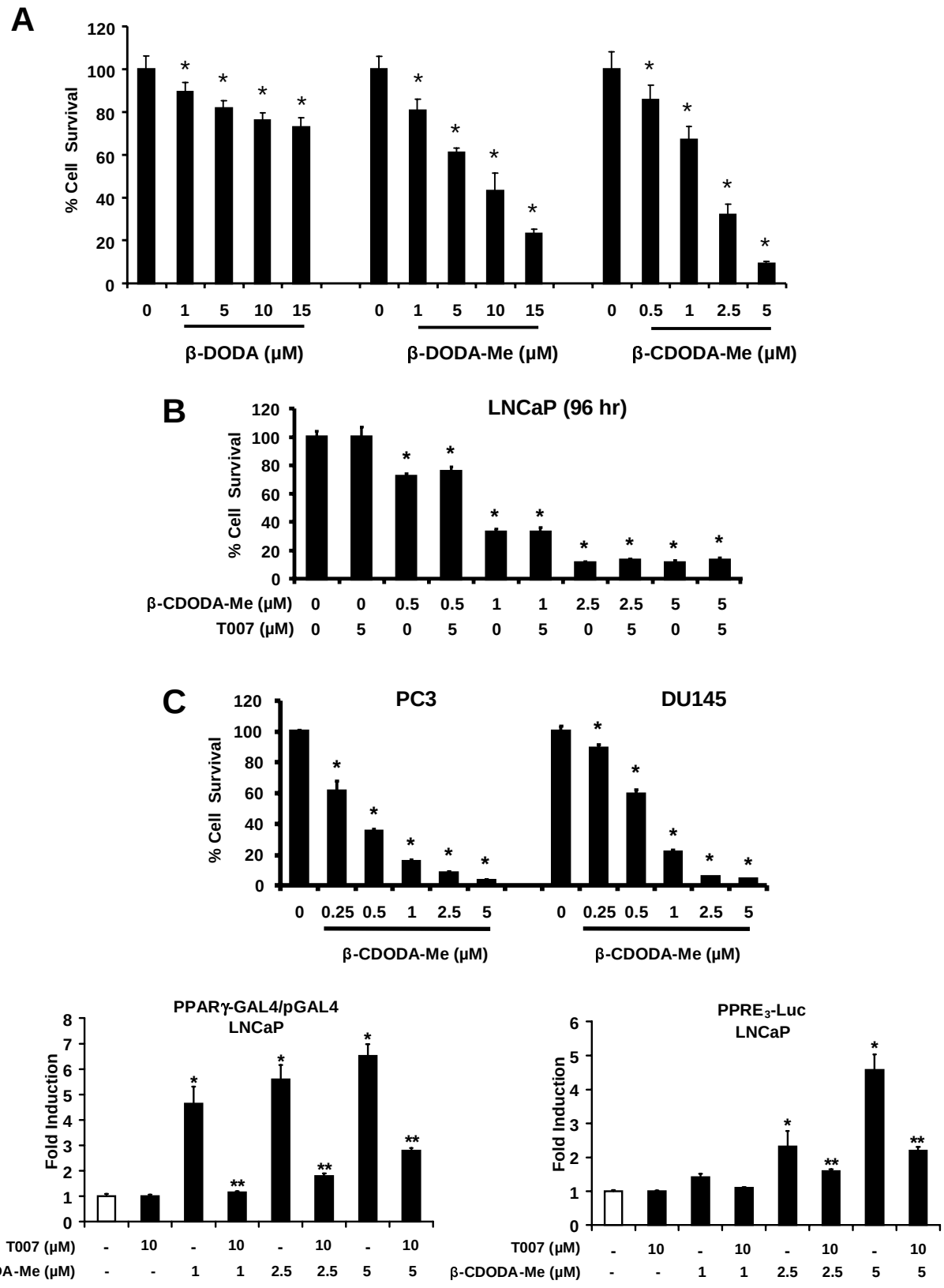

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Figure 2

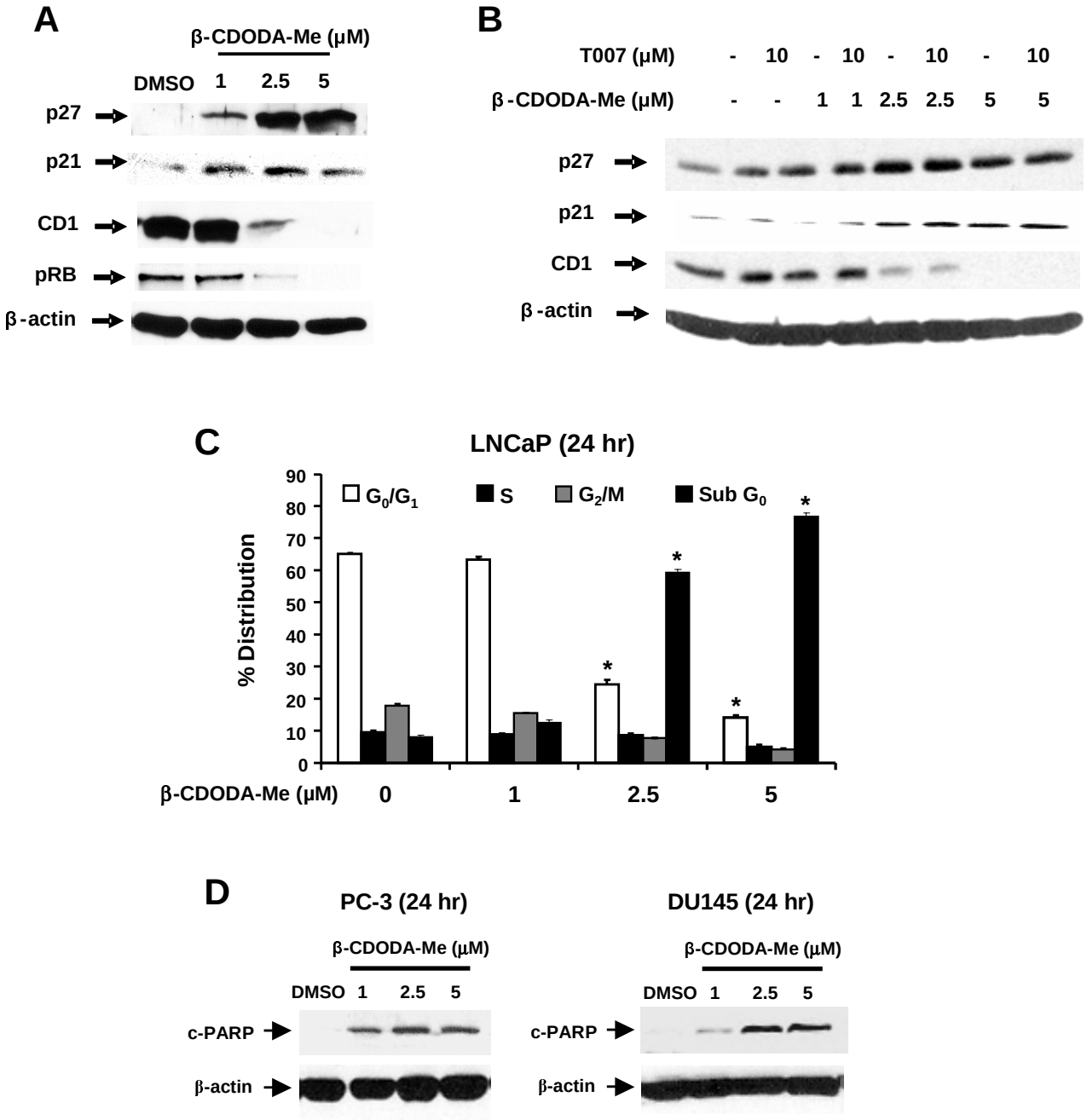

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Figure 3

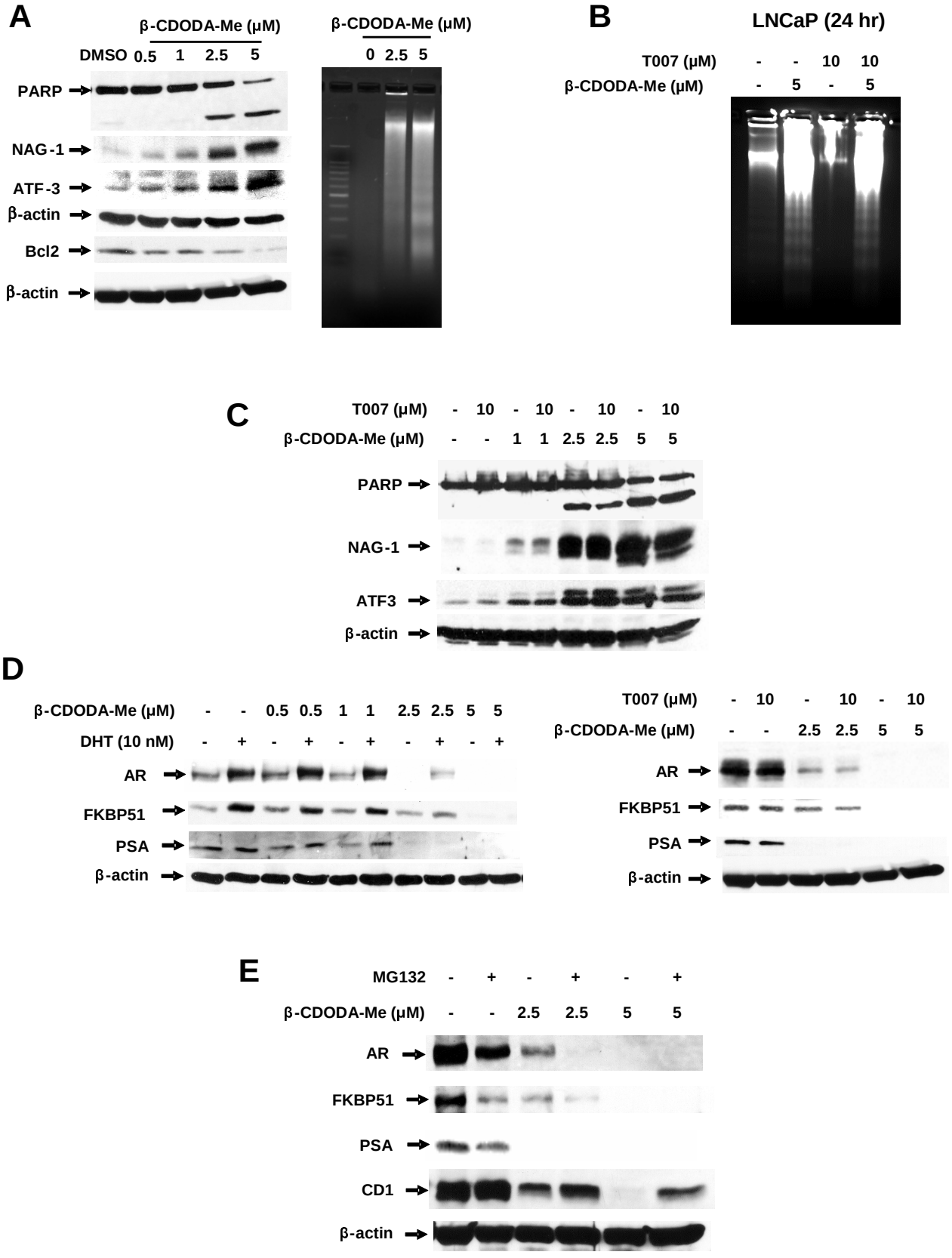

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Figure 4

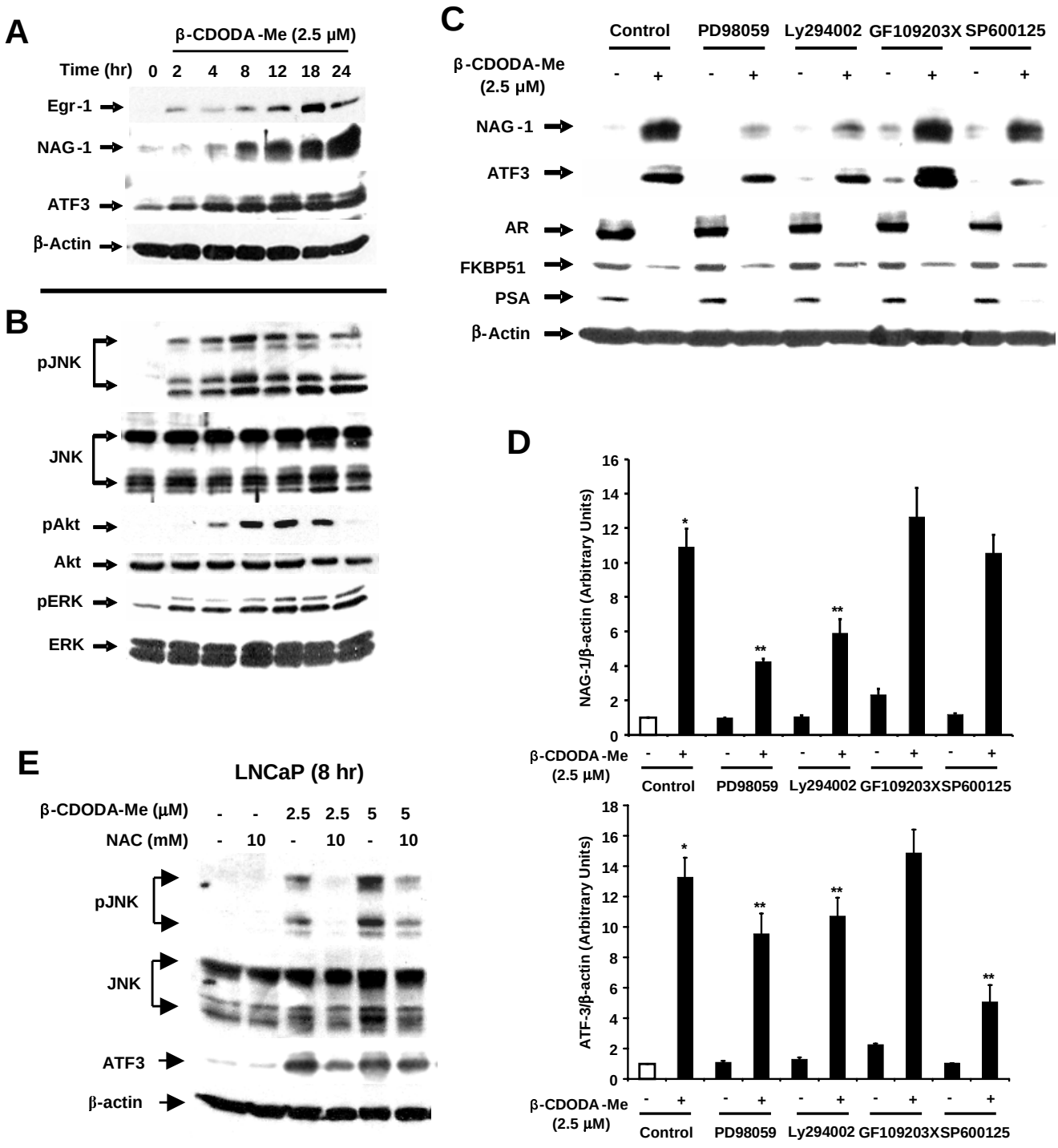

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Figure 5

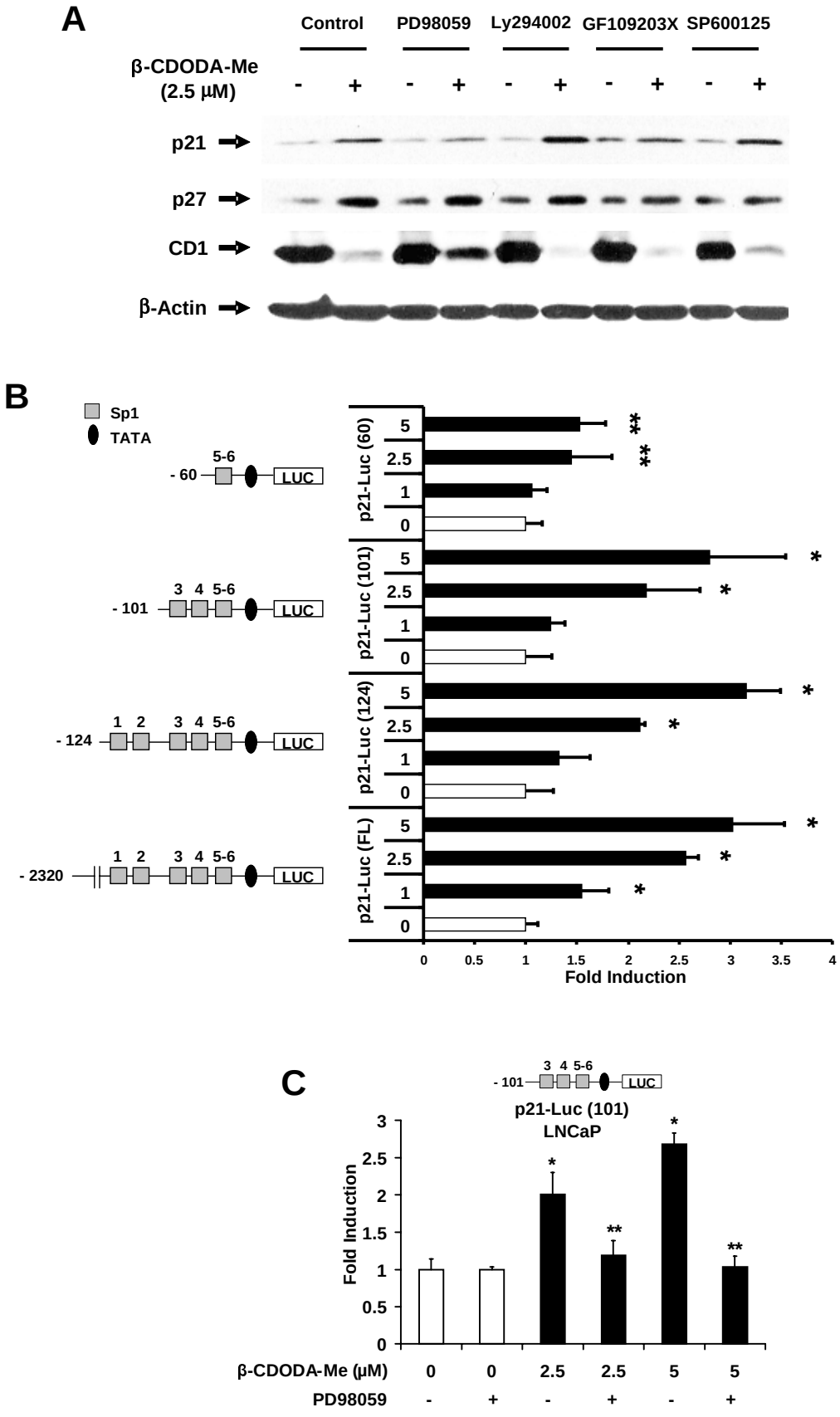

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Figure 6

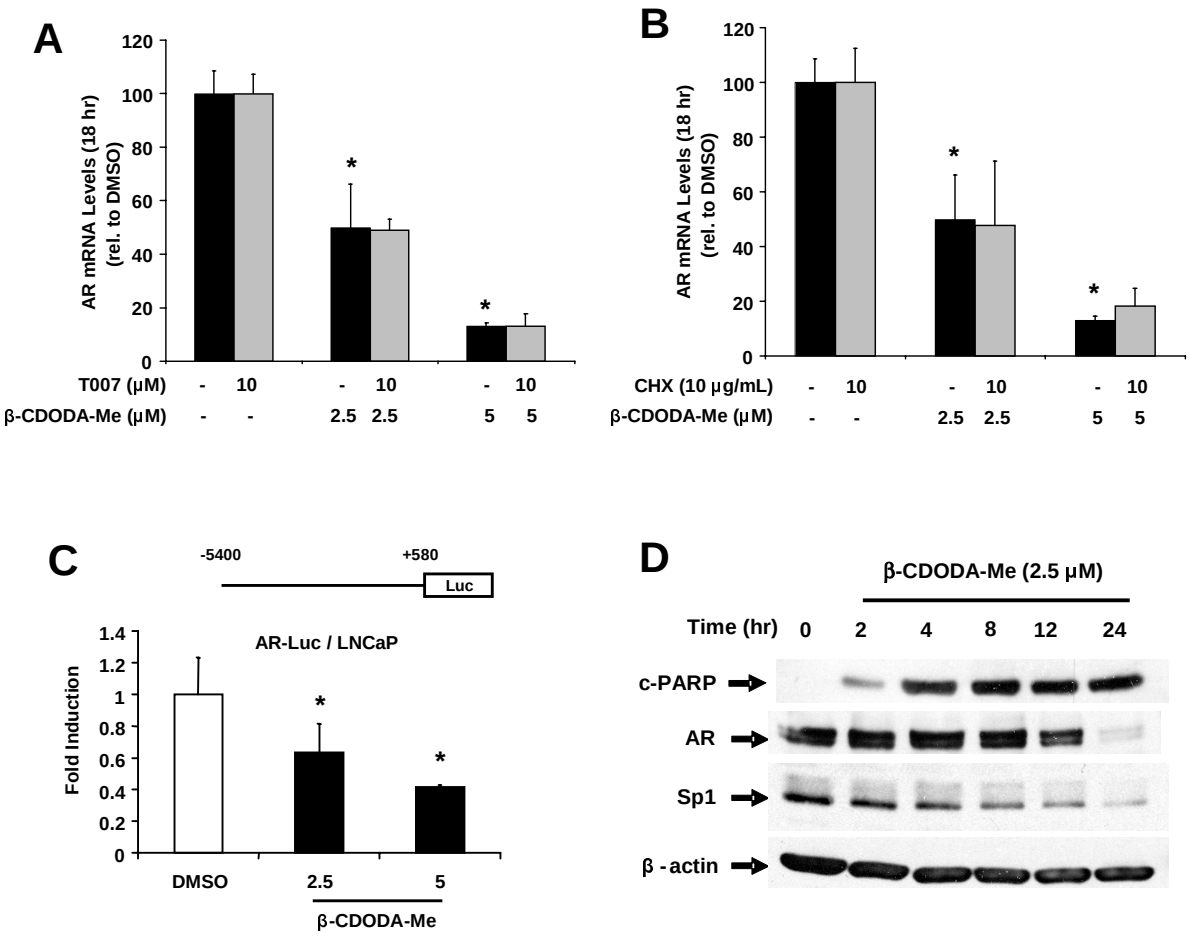

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Figure 7

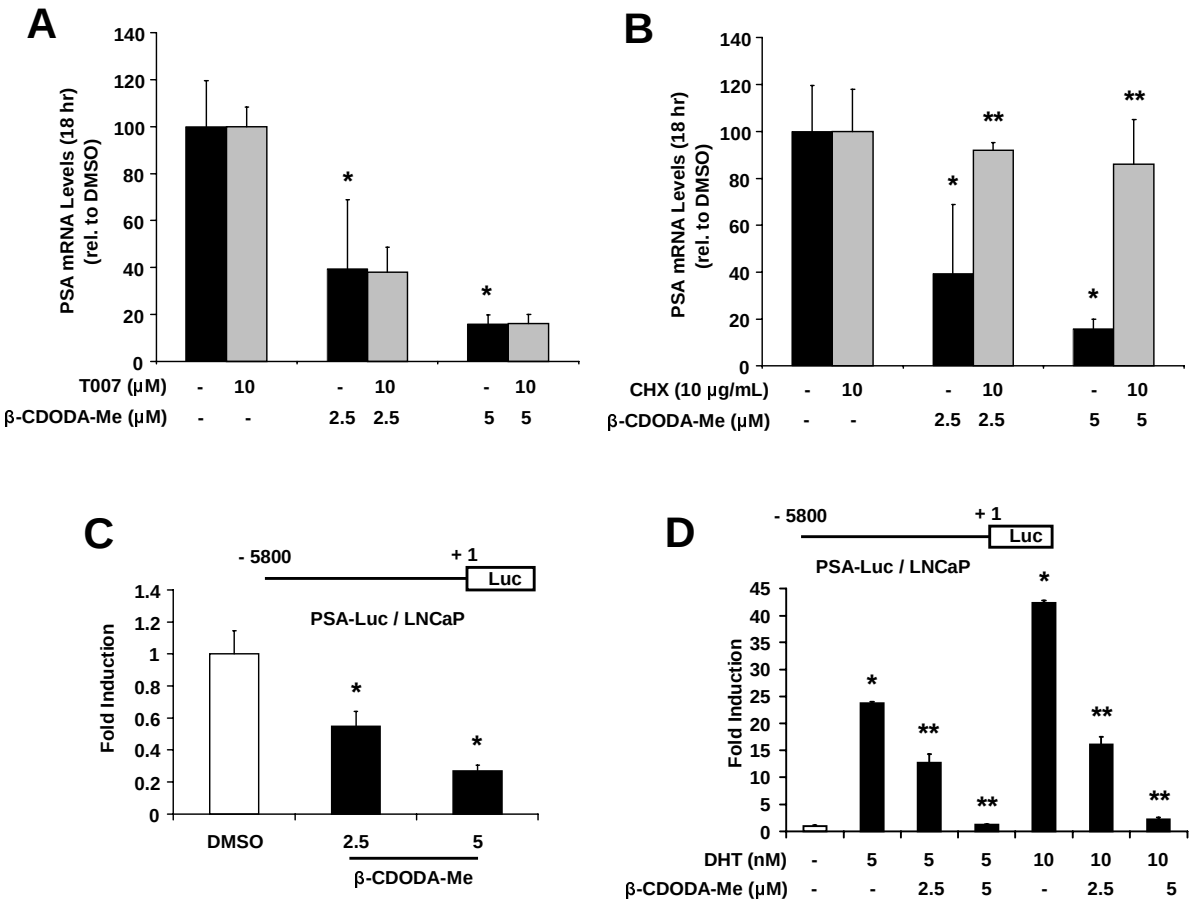

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Figure 8