1,1-BIS(3'-INDOLYL)-1-(*P*-SUBSTITUTEDPHENYL)METHANES ARE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ AGONISTS BUT DECREASE HCT-116 COLON CANCER CELL SURVIVAL THROUGH RECEPTOR-INDEPENDENT ACTIVATION OF EARLY GROWTH RESPONSE-1 AND NAG-1

Sudhakar Chintharlapalli, Sabitha Papineni, Seung Joon Baek, Shengxi Liu and Stephen Safe

Department of Biochemistry and Biophysics, College of Agriculture and Life Sciences, Texas A&M University, College Station, TX 77843 (S.C.)

Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843 (S.P., S.S.)

Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996 (S.J.B.)

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

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Running Title: Activation of NAG-1 in HCT-116 cells by C-DIMs

Address correspondence to:
Stephen Safe
Department of Veterinary Physiology and Pharmacology
Texas A&M University
College Station, TX 77843-4466
Tel: 979-845-5988 / Fax: 979-862-4929
Email: ssafe@cvm.tamu.edu

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Abbreviations:
C-DIMs, methylene-substituted diindolylmethanes; DIM, diindolylmethane; DIM-C-pPhtBu, 1,1-bis(3'-indolyl)-1-(*p*-*t*-butylphenyl)methane; DIM-C-pPhCF_3, 1,1-bis(3'-indolyl)-1-(*p*-trifluoromethylphenyl)methane; DIM-C-pPhC_6H_5, 1,1-bis(3'-indolyl)-1-(*p*-biphenyl)methane; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Egr-1, early growth response-1; MAPK, mitogen-activated protein kinase; NAG-1, NSAID-activated gene-1; NSAID, non-steroidal anti-inflammatory drug; PI3-K, phosphatidylinositol-3-kinase; PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; PPARγ, peroxisome proliferator-activated receptor γ; SRE, serum response element.

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ABSTRACT

1,1-Bis-(3'-indolyl)-1-(*p*-substitutedphenyl)methanes containing *p*-trifluoromethyl (DIM-C-pPhCF_3), *p*-*t*-butyl (DIM-C-pPhtBu), and phenyl (DIM-C-pPhC_6H_5) substituents decrease survival of HCT-116 colon cancer cells and activate peroxisome proliferator activated receptor γ (PPARγ) in this and other cancer cell lines. These PPARγ-active compounds had minimal effects on expression of cell cycle proteins and did not induce caveolin-1 in HCT-116 cells. However, these compounds induced NSAID-activated gene-1 (NAG-1) and apoptosis in HCT-116 cells and, in time-course studies, the PPARγ agonists maximally induced early growth response-1 (Egr-1) protein within 2 hr, whereas a longer time course was observed for induction of NAG-1 protein. These data, coupled with deletion and mutation analysis of both the Egr-1 and NAG-1 gene promoters, indicate that activation of NAG-1 by these compounds was dependent on prior induction of Egr-1, and induction of these responses were PPARγ-independent. Results of kinase inhibitor studies also demonstrated that activation of Egr-1/NAG-1 by methylene-substituted diindolylmethanes (C-DIMs) was phosphatidylinositol-3-kinase-dependent, and this represents a novel receptor-independent pathway for C-DIM-induced growth inhibition and apoptosis in colon cancer cells.

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INTRODUCTION

NSAID-activated gene-1 (NAG-1) is a transforming growth factor β (TGFβ)-like secreted protein and was initially characterized as a p53-regulated gene (Tan et al., 2000; Li et al., 2000). Overexpression of NAG-1 in breast cancer cells resulted in growth arrest and apoptosis, and similar results were also observed in colon cancer cells (Li et al., 2000; Baek et al., 2001b). Baek, Eling and their coworkers have extensively investigated the induction of NAG-1 by several different structural classes of drugs and chemoprotective phytochemicals in HCT-116 colon and other cancer cell lines (Baek et al., 2001a; Baek et al., 2001b; Kim et al., 2002; Bottone, Jr. et al., 2002; Baek et al., 2002; Wilson et al., 2003; Newman et al., 2003; Yamaguchi et al., 2004; Kim et al., 2004; Baek et al., 2004a; Baek et al., 2004b; Baek et al., 2005; Kim et al., 2005). Chemicals that induce NAG-1 expression in cancer cell lines generally inhibit growth and/or induce apoptosis in these cells, and these effects are due, in part, to induction of NAG-1 protein. In addition to NSAIDs, other agents that induce NAG-1 include phorbol esters, cyclooxygenase inhibitors, genistein, plant polyphenolics, diallyl disulfide, retinoids, indole-3-carbinol (I3C), diindolylmethane (DIM), and peroxisome proliferator-activated receptor γ (PPARγ) agonists (Baek et al., 2001a; Baek et al., 2001b; Kim et al., 2002; Bottone, Jr. et al., 2002; Baek et al., 2002; Wilson et al., 2003; Newman et al., 2003; Yamaguchi et al., 2004; Kim et al., 2004; Baek et al., 2004a; Baek et al., 2004b; Baek et al., 2005; Kim et al., 2005).

Several mechanisms of NAG-1 induction have been described and these are dependent not only on the structure or class of inducing agents but also on cell context. For example, diallyl disulfide and genistein are antitumorigenic components of garlic and soy, and their induction of NAG-1 in HCT-116 cells is p53-dependent (Bottone, Jr. et al., 2002; Wilson et al.,

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2003). In contrast, induction of NAG-1 by indole-3-carbinol and diindolylmethane (DIM), two anticarcinogenic components in cruciferous vegetables, is p53-independent in the same cell line (Lee et al., 2005). Two PPARγ agonists, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PGJ2) and troglitazone, also induce NAG-1 expression in HCT-116 cells, and the PPARγ antagonist 2-chloro-5-nitrobenzanilide (GW9662) inhibits the induction response by PGJ2 but not troglitazone (Baek et al., 2004b). It was also shown that the PPARγ-independent activation of NAG-1 by troglitazone is due to induction of early growth response gene (Egr-1) which in turn activates NAG-1 (Baek et al., 2003; Baek et al., 2004b).

Recent studies in this laboratory have identified 1,1-bis-(3'-indolyl)-1-(*p*-substituted-phenyl)methanes [methylene-substituted DIMs (C-DIMs)] as PPARγ agonists, and the most active compounds contain *p*-trifluoromethyl (DIM-C-pPhCF_3), *p*-*t*-butyl (DIM-C-pPhtBu), and phenyl (DIM-C-pPhC_6H_5) (Qin et al., 2004; Chintharlapalli et al., 2004; Hong et al., 2004; Contractor et al., 2005). These PPARγ agonists decrease survival and induce apoptosis in breast, leukemia, pancreatic and colon cancer cells. In the latter two cell lines, growth inhibition is associated with receptor-dependent activation of p21 (Hong et al., 2004) and the tumor suppressor gene caveolin-1 (Chintharlapalli et al., 2004). There is also evidence that decreased cancer cell survival induced by these compounds is also receptor-independent (Qin et al., 2004; Contractor et al., 2005). The PPARγ-active methylene-substituted diindolylmethanes (C-DIMs) also decrease cell survival and induce apoptosis in HCT-116 cells but do not induce caveolin-1, as previously reported in HT-29 and HCT-15 colon cancer cells (Chintharlapalli et al., 2004). In contrast, these compounds induce NAG-1 in HCT-116 cells and this response is not inhibited by PPARγ antagonists. Like troglitazone, the PPARγ-active C-DIMs also induce Egr-1 which in turn interacts with proximal (GC-rich) Egr-1 motifs in the NAG-1 gene promoter. However, in

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contrast to troglitazone, the C-DIM compounds induce Egr-1 through a phosphatidylinositol-3-kinase (PI3K)-dependent pathway which in turn activates serum response elements in the Egr-1 promoter. This represents a novel pathway for induction of Egr-1 and NAG-1, and these responses contribute to the induction of growth inhibition and apoptosis by the PPARγ-active C-DIMs in colon cancer cells. Moreover, these results also distinguish the mode of action of these C-DIM analogs from that of troglitazone and DIM and identify an important PPARγ-independent mode of action.

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MATERIALS AND METHODS

Cell Lines. HCT-116 (human colon carcinoma cell line) and LNCap (human prostate cancer cell line) were obtained from American Type Culture Collection (Manassas, VA). HCT-116 and LnCap cells were maintained in RPMI 1640 (Sigma-Aldrich, 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-Aldrich). Cells were maintained at 37°C in the presence of 5% CO_2.

Antibodies and Reagents. Antibodies for poly(ADP-ribose) polymerase (sc-8007), cyclin D1 (sc-718), p27 (sc-528), phospho-Akt (sc-7985R), Akt (sc-8312) p53 (sc-126) and caveolin 1 (sc-894) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), NAG-1 from Upstate Biotechnology (Lake Placid, NY), and Egr-1 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 supplied by Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA) and Lipofectamine reagent was purchased from Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagent was from Perkin-Elmer Life Sciences (Boston, MA). Rosiglitazone was purchased from LKT Laboratories, Inc. (St. Paul, MN). The C-substituted DIMs were prepared in this laboratory as previously described (Qin et al., 2004; Chintharlapalli et al., 2004) and the Egr-1 constructs were kindly provided by Mr. C.-C. Chen and Mrs. W.-R. Lee (Texas A&M University).

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 (GlaxoSmithKline Research

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and Development, Research Triangle Park, NC). PPRE-luc construct contains three tandem PPREs with a minimal TATA sequence in pGL2 (Qin et al., 2004; Chintharlapalli et al., 2004). pNAG-1A - pNAG-1D, pNAG-1Dm1, pNAG-1Dm2 and pNAG-1Dm3 were generated previously (Baek et al., 2001a; Baek et al., 2003). pEGR-1A - pEGR-1E constructs containing Egr-1 promoter inserts have also previously been described (Chen et al., 2004).

Transfection and Luciferase Assay. HCT-116 cells (1×10^5 cells/well) were plated in 12-well plates in DMEM:Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After 16 hr, various amounts of DNA [*i.e.* Gal4Luc (0.4 μg), β-gal (0.04 μg), PPRE-Luc (0.04 μg)] and 0.25 μg of the NAG-1/EGR-1 constructs were transfected by Lipofectamine (Invitrogen) according to the manufacturer's protocol. Five hours 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 were used for luciferase and β-gal assays. A Lumicount luminometer (PerkinElmer Life and Analytical Sciences) was used to quantitate luciferase and β-gal activities, and the luciferase activities were normalized to β-gal activity.

Cell Proliferation Assay. HCT-116 cells (2×10^4/well) were plated in 12-well plates. After cell attachment for 24 hr, the medium was changed to DMEM:Ham's F-12 media containing 2.5% charcoal-stripped FBS and either vehicle (DMSO) or the indicated compound. Fresh media and compounds were added every 48 hr, and the cells were then trypsinized and counted at the indicated times using a Coulter Z1 cell counter. Each experiment was done in triplicate, and results are expressed as means ± SE for each determination.

Western Blot Analysis. HCT-116 cells were seeded in DMEM:Ham's F-12 media containing 2.5% charcoal-stripped FBS for 24 h and then treated with either the vehicle (DMSO) or the

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compounds for different times as indicated. Cells were collected by scraping in 150 μL high salt lysis buffer (50 mM HEPES, 0.5 M NaCl, 1.5mM $MgCl_2$, 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 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 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 onto polyvinylidene membranes (PVDF; Bio-Rad, Hercules, CA) by semidry electroblotting in a buffer containing 25 mM Tris, 192 mM glycine and 20% methanol for 1.5 hr at 180 mA. 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% non-fat dry milk) and incubated in fresh 5% TBST-Blotto with 1:1000 (for caveolin 1, p27 and cyclin D1), 1:250 (for PARP), 1:500 (for NAG-1 and Egr-1), 1:5000 (for β-actin) 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 90 min. 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 Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

Chromatin immunoprecipitation (ChIP) assay. HCT-116 cells ($2x10^7$) were treated with Me_2SO (time 0), or DIM-C-pPhC_6H_5 (20 μM) for 1 or 2 hr. Cells were then fixed with 1.5% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to desired chromatin length (~500 bp). The chromatin was pre-cleared twice by addition of protein A-

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conjugated beads (PIERCE), and then incubated at 4°C for 1 hr with gentle agitation. The beads were pelleted, and the pre-cleared chromatin supernatants were immunoprecipitated with antibodies specific to IgG, Sp1, Sp3, Sp4 (Santa Cruz Biotechnology), and Egr-1 (Cell Signaling Technology) at 4°C overnight. Protein-antibody complexes were collected by addition of protein A-conjugated beads at room temperature for 1 hr. Beads were extensively washed; the protein-DNA crosslinks were eluted and reversed. DNA was purified by phenol extraction/ethanol precipitation followed by PCR amplification. The NAG-1 primers are: 5' - TAC TGA GGC CCA GAA ATG TG - 3' (forward), and 5' - GAG CTG GGA CTG ACC AGA TG - 3' (reverse). These primers amplify a 211-bp region of the human NAG-1 promoter containing two Sp1/Egr-1 binding sites. The positive control primers are: 5' - TAC TAG CGG TTT TAC GGG CG - 3' (forward), and 5' - TCG AAC AGG AGG AGC AGA GAG CGA - 3' (reverse), which amplify a 167-bp region of human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene. The negative control primers are: 5' - ATG GTT GCC ACT GGG GAT CT - 3' (forward), and 5' - TGC CAA AGC CTA GGG GAA GA - 3' (reverse), which amplify a 174-bp region of human CNAP1 exon. PCR products were resolved on a 2% agarose gel in the presence of 1:10 000 SYBR gold (Molecular Probes, Eugene, OR).

Statistical Analysis. Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. The results are expressed as means ± SE for at least three separate (replicate) experiments for each treatment.

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RESULTS

Studies in this laboratory have characterized selected C-DIMs as PPARγ agonists that inhibit growth of colon and other cancer cell lines through receptor-dependent and -independent pathways (Qin et al., 2004; Chintharlapalli et al., 2004; Hong et al., 2004; Contractor et al., 2005). Results illustrated in Figure 1 show that three PPARγ-active C-DIM compounds, namely DIM-C-pPhCF_3, DIM-C-pPhtBu and DIM-C-pPhC_6H_5, decreased HCT-116 colon cancer cell survival at concentrations of 1 - 10 μM after treatment for 48 or 96 hr. Treatment for 96 hr resulted in cell death using 10 μM concentrations (i.e. cell numbers were lower than the initial number of seeded cells). In contrast, 10 μM rosiglitazone decreased cell survival but did not induce cell death, which is observed only at higher concentrations of this compound (data not shown). Previous studies in colon cancer cells show that PPARγ-active C-DIMs induced transactivation in cells transfected with PPRE-luc or GAL4-PPRγ/pGAL4-luc constructs (Chintharlapalli et al., 2004). In HCT-116 cells, PPARγ-active C-DIMs induced a concentration-dependent increase in transactivation in cells transfected with PPARE-luc, and 10 μM rosiglitazone also increased activity but with lower fold-inducibility (Fig. 2A). The same compounds also induced transactivation in HCT-116 cells transfected with GAL4-PPARγ/GAL4-luc constructs and treated with 5 and 10 μM of the C-DIMs, and cotreatment with the PPARγ antagonist GW9662 significantly inhibited this response (Fig. 2B). These results confirm that PPARγ-active C-DIMs induce receptor-dependent transactivation in HCT-116 cells, and this complements results of recent studies that show similar response in HT-29 and HCT-15 colon cancer cell lines (Chintharlapalli et al., 2004).

Treatment of HCT-116 cells for 24 hr with 2.5, 5.0 and 7.5 μM DIM-C-pPhCF_3, DIM-C-pPhtBu or DIM-C-pPhC_6H_5 did not affect expression of cyclin D1 or p27 proteins (Fig. 3A), and

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p21 levels were barely detectable (data not shown). PPARγ agonists frequently affect expression of these proteins in other cancer cell lines (Clay et al., 1999; Motomura et al., 2000; Inoue et al., 2001; Palakurthi et al., 2001; Clay et al., 2002; Qin et al., 2003; Hong et al., 2004); however, the results (Fig. 3A) are comparable to those observed for the same compounds in HT-29 and HCT-15 cells (Chintharlapalli et al., 2004). The growth inhibitory effects of these compounds in HT-29 and HCT-15 cells are associated with receptor-dependent activation of the tumor suppressor gene caveolin-1 (Chintharlapalli et al., 2004) which inhibits colon cancer cell growth. However, in HCT-116 cells, treatment with 5, 10 or 15 μM DIM-C-pPhCF$_3$ or DIM-C-pPhC$_6$H$_5$ for 72 hr did not affect caveolin-1 protein expression (Fig. 3B). Moreover, unlike HT-29 and HCT-15 cells, relatively high levels of caveolin-1 were detected in solvent (DMSO)-treated HCT-116 cells. Other PPARγ agonists such as PGJ2 and troglitazone, induce the TGFβ-like protein NAG-1 in HCT-116 cells (Baek et al., 2003; Wilson et al., 2003), and therefore induction of NAG-1 protein and apoptosis by PPARγ-active C-DIMs was investigated in HCT-116 cells treated with relatively high concentrations (10 - 20 μM) over 24 hr. The results (Fig. 3C) show that NAG-1 protein expression was not detectable in solvent-treated cells; however, treatment with 10 - 20 μM DIM-C-pPhCF$_3$, DIM-C-pPhtBu or DIM-C-pPhC$_6$H$_5$ induced NAG-1 protein expression. We also observed induction of NAG-1 by C-DIMs in LNCaP cells as previously described for other NAG-1 inducers (Newman et al., 2003); however, NAG-1 was not induced in SW-480 colon cancer cells (data not shown). The induction of NAG-1 in HCT-116 cells was accompanied by activation of apoptosis as indicated by PARP cleavage. In contrast, p27 expression was unaffected by this treatment (data not shown) and cyclin D1 was downregulated only at the highest dose level. The coordinate induction of NAG-1 and apoptosis in HCT-116 cells by PPARγ-active C-DIMs after treatment for 24 hr is consistent with the effects of these

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compounds on decreased cell survival (Fig. 1). However, the induction of NAG-1 by PPARγ-active C-DIMs was not strictly a high dose effect since treatment of HCT-116 cells with 2.5, 5.0 and 7.5 μM DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ for 72 hr show that NAG-1 protein was induced at concentrations as low as 2.5 μM (Fig. 3D).

The concentration-dependent effects of DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ on induction of NAG-1 and other proteins that are often induced along with NAG-1 were determined in HCT-116 cells after treatment for 24 hr (Fig. 4A). NAG-1 protein levels were elevated at concentrations as low as 7.5 μM. After 24 hr, maximal induction was observed at concentrations ≤ 15 μM and this was accompanied by PARP cleavage. Induction of NAG-1 by some compounds is accompanied by increased expression of p53 protein (Bottone, Jr. et al., 2002; Wilson et al., 2003) or ATF3 (Baek et al., 2004a; Lee et al., 2005), and the PPARγ-active C-DIMs induced the latter protein but not p53. Although other PPARγ agonists induce NAG-1, the role of the receptor in mediating these responses is structure dependent since studies with PPARγ antagonists showed that induction of NAG-1 by PGJ2 and troglitazone was PPARγ-dependent and -independent, respectively (Baek et al., 2004b). Treatment of HCT-116 cells with DIM-C-pPhCF$_3$ (Fig. 4B) or DIM-C-pPhC$_6$H$_5$ (Fig. 4C) alone or in combination with the PPARγ antagonist GW9662 shows that induction of NAG-1 or apoptosis by the C-DIM compounds is not inhibited by GW9662. We have also repeated the same experiment with another PPARγ antagonist, 2-chloro-5-nitro-*N*-4-pyridinyl-benzamide (T007) and observed no inhibition of the C-DIM-mediated induction of NAG-1 or PARP cleavage (data not shown). In addition, GW9662 did not affect decreased HCT-116 cell survival after treatment with the C-DIM compounds for 96 hr (Fig. 4D) and similar results were observed after 48 hr (data not shown). Apoptosis (PARP cleavage) was induced by PPARγ-active C-DIMs at concentrations as low as

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10 μM (after 24 hr); this was more pronounced after 48 hr (data not shown) and correlated with the cell survival results (Fig. 1). Thus, like troglitazone, the PPARγ-active C-DIMs induce NAG-1 and apoptosis in HCT-116 cells via a PPARγ-independent pathway.

Previous reports have linked the anticarcinogenic activity of troglitazone to induction of both NAG-1 and Egr-1 and enhanced Egr-1 expression has been linked to upregulation of NAG-1 in HCT-116 cells (Baek et al., 2003; Baek et al., 2004b). The results in Figure 5A show that both DIM-C-pPhCF_3 and DIM-C-pPhC_6H_5 induce Egr-1 and NAG-1 proteins in HCT-116 cells; however, the temporal expression of both proteins is different. Egr-1 is maximally induced within 2 hr after treatment and levels then decline 4 to 24 hr after treatment. In contrast, induction of NAG-1 protein increases over the 24 hr treatment period and the highest levels are observed after 24 hr. The temporal sequence of C-DIM-induced NAG-1 and Egr-1 expression is comparable to that observed for troglitazone in HCT-116 cells except that the time-course for induction of both proteins is somewhat delayed (Baek et al., 2003).

We also investigated the effects of the PPARγ-active C-DIMs on transactivation in cells transfected with constructs containing -600 to +12 (pEGR-1A), -460 to +12 (pEGR-1B), and -164 to +12 (pEGR-1C) Egr-1 promoter inserts (Figs. 5B - 5D). All three compounds induced transactivation in cells transfected with pEGR-1A and pEGR-1B but not pEGR-1C, suggesting that SRE2-4 motifs within the -460 to -164 region of the promoter were required for activation of Egr-1. Further deletion of the 3' region of the promoter was investigated in cells transfected with pEGR-1D (-480 to -285) and pEGR-1E (-480 to -376). The C-DIM compound induced transactivation only in cells transfected with pEGR-1D and not pEGR-1E demonstrating that the minimal region of the Egr-1 promoter required for transactivation (-376 to -285) contained SRE3 and SRE2.

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The potential role of Egr-1 in mediating induction of NAG-1 was further investigated in HCT-116 cells transfected with a series of constructs containing the -3500 to +41 (pNAG-1A), -1086 to +41 (pNAG-1B), -474 to +41 (pNAG-1C), and -133 to +41 (pNAG1D) NAG-1 promoter inserts (Figs. 6A - 6D). The results show that the C-DIM compounds induce transactivation in HCT-116 cells transfected with all four constructs. Previous studies show that the -73 to -44 region of the NAG-1 gene promoter contains two GC-rich Sp1 binding sites that overlap two Egr-1 sites (Baek et al., 2001a; Baek et al., 2001b; Baek et al., 2004b). Therefore, cells were transfected with pNAG-1D or constructs containing a single Egr-1 site mutation (pNAG-1Dm1 and pNAG-1Dm2) or both sites mutated (pNAG-1Dm3). The results (Fig. 6E) show that C-DIM-induced transactivation was decreased in cells transfected with pNAG-1Dm1 and pNAG-1Dm2 and no significant induction was observed in cells transfected with pNAG-1Dm3. Thus, mutation of the Egr-1 sites resulted in loss of inducibility of the NAG-1 constructs. Moreover, in cells transfected with pNAG-1D, cotransfection with Egr-1 expression plasmid also induces transactivation (Fig. 7A). These results are consistent with a mechanism of NAG-1 induction by C-DIMs which involves initial activation of Egr-1 which in turn activates the NAG-1 promoter through proximal Egr-1 motifs. We also investigated interactions of Egr-1 with the NAG-1 promoter in a ChIP assay with PCR primers that target the proximal region of the NAG-1 promoter that contain the Egr-1 motifs. HCT-116 cells were treated with DMSO or 20 μM DIM-C-pPhC_6H_5 for 1 or 2 hr; cells were then crosslinked with formaldehyde and the ChIP assay procedure was used to determine interactions of Sp proteins and Egr-1 with the NAG-1 promoter (Fig. 7B). The results show binding of Sp1, Sp3 and Sp4 proteins to the NAG-1 promoter and the band intensities are increased after treatment with DIM-C-pPhC_6H_5. Similar results were observed for Egr-1 suggesting that induction of Egr-1 protein (Fig. 5) facilitates

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recruitment of Egr-1 and Sp proteins to the NAG-1 promoter. As a control experiment for the ChIP assay, the binding of TFIIB to the GAPDH promoter, but not exon 1 of the CNAP1 gene, is illustrated in Figure 7C as previously described (Hong et al., 2004).

Egr-1 is an immediate early gene that is activated by multiple factors in different cell lines, including UV light, ER stress, hormones, phorbol esters, and troglitazone (Sukhatme et al., 1987; Christy and Nathans, 1989; Sukhatme, 1990; Cicatiello et al., 1993; Muthukkumar et al., 1995; Dziema et al., 2003; Baek et al., 2003; Baek et al., 2004b; Baek et al., 2005), and many of the responses involve activation of kinases. Moreover, troglitazone activates Egr-1 and NAG-1 in HCT-116 cells through activation of the mitogen-activated protein kinase (MAPK) pathway (Baek et al., 2003; Baek et al., 2004b). The role of kinases in activation of NAG-1 by C-DIM compounds was therefore investigated in HCT-116 cells treated with 15 μM DIM-C-pPhC_6H_5 for 24 hr in the presence or absence of PD98059 or LY294002 which inhibit MAPK and PI3-K-dependent phosphorylation, respectively. The results showed that induction of NAG-1 protein by DIM-C-pPhC_6H_5 was inhibited by LY294002 but not PD98059 (Fig. 8A); similar inhibitory responses were also observed for induction of Egr-1 (Fig. 8B), and DIM-C-pPhCF_3-induced responses were also inhibited by LY294002 (data not shown). Interestingly, PD98059 alone also induced Egr-1 (Fig. 8B) but not NAG-1 (Fig 8A), suggesting that Egr-1 alone is not sufficient for activation of NAG-1. The inhibitory effects of LY294002 suggest that the C-DIM compounds induce PI3-K. Moreover, in cells transfected with pEGR-1D, induction of luciferase activity by the C-DIM compounds was inhibited by cotreatment with LY294002 (Fig. 8C). The results in Figure 8D show the time-dependent induction of Akt phosphorylation by DIM-C-pPhC_6H_5 and a similar induction response was observed for DIM-C-pPhCF_3 and DIM-C-pPhtBu. Activation of PI3-K was time-dependent since induction of Akt phosphorylation was not observed after

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treatment for 2 hr (data not shown). These results demonstrate that PPARγ-active C-DIMs coordinately induce Egr-1 and NAG-1 through a novel pathway which involves initial PI3-K-dependent activation of Egr-1 through SRE3 and SRE2 motifs on the Egr-1 promoter.

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DISCUSSION

PPARγ-active C-DIMs and other PPARγ agonists inhibit growth and induce apoptosis in several different cell lines, and these responses are both receptor-dependent and -independent (Sukhatme et al., 1987; Takahashi et al., 1999; Clay et al., 1999; Motomura et al., 2000; Palakurthi et al., 2001; Clay et al., 2002; Qin et al., 2003; Qin et al., 2004; Chintharlapalli et al., 2004; Hong et al., 2004; Contractor et al., 2005). For example, studies in this laboratory have demonstrated that induction of p21 in Panc-28 cells and upregulation of caveolin-1 in HT-29 and HCT-15 cells is PPARγ-dependent and inhibited by PPARγ antagonists or by PPARγ knockdown with small inhibitory RNAs (Chintharlapalli et al., 2004; Hong et al., 2004). In contrast, induction of apoptosis and downregulation of cyclin D1 in breast cancer cells was PPARγ-independent (Qin et al., 2004). The interplay between receptor-dependent and -independent pathways has also been reported for the potent triterpenoid-derived PPARγ agonist 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid and related compounds in HCT-15, HT-29 and SW-480 colon cancer cells (Chintharlapalli et al., 2005). At lower growth-inhibitory concentrations, CDDO induced caveolin-1, and this response was inhibited in cells cotreated with the PPARγ antagonist T007. In contrast, at higher concentrations, caveolin-1 expression was decreased and apoptosis was induced and this latter response was receptor-independent and not inhibited by T007. The type of differential concentration-dependent induction of PPARγ-dependent and -independent responses has also been observed for PPARγ-active C-DIM compounds in SW-480 cells (unpublished results).

In this study, we have investigated the effects of PPARγ-active C-DIMs in HCT-116 cells and, as previously reported in HCT-15 and HT-29 cells (Chintharlapalli et al., 2004), these compounds decrease HCT-116 cell survival (Fig. 1), activate PPARγ-dependent transactivation

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(Fig. 2), and p21, p27 and cyclin D1 levels were unchanged except for downregulation of cyclin D1 at high concentrations (Fig. 3). In contrast to observations in HT-29 and HCT-15 cells (Chintharlapalli et al., 2004), the PPARγ-active compounds did not induce caveolin-1 in HCT-116 cells (Fig. 3), and this may be due, in part, to the relatively high constitutive expression of caveolin-1 in this cell line. Thus, the growth inhibitory responses of C-DIMs in HCT-116 cells must be related to activation of other pathways. Previous studies in HCT-116 cells have reported that several different structural classes of growth inhibitory/antitumorigenic compounds, including the PPARγ agonists PGJ2 and troglitazone, and DIM induced NAG-1 expression and this protein exhibits both growth inhibitory and pro-apoptotic activity (Baek et al., 2001a; Baek et al., 2001b; Kim et al., 2002; Bottone, Jr. et al., 2002; Baek et al., 2002; Baek et al., 2003; Wilson et al., 2003; Newman et al., 2003; Yamaguchi et al., 2004; Kim et al., 2004; Baek et al., 2004a; Baek et al., 2004b; Lee et al., 2005; Baek et al., 2005; Kim et al., 2005). Our results show that PPARγ-active C-DIMs also induce NAG-1 protein expression in HCT-116 cells (Figs. 3 and 4), and this is consistent with their growth inhibitory (Fig. 1) and apoptotic (Figs. 3 and 4) effects in HCT-116 cells. It was also shown that induction of NAG-1 and apoptosis in HCT-116 cells by DIM-C-pPhCF_3 and DIM-C-pPhC_6H_5 was not inhibited by the PPARγ antagonists GW9662 or T007 and the PPARγ antagonists also did not affect C-DIM-induced growth inhibition (Fig. 4). Thus, induction of NAG-1 by PPARγ-active C-DIMs and troglitazone was receptor-independent, and this was in contrast to induction of NAG-1 by PGJ2 since this response was inhibited by a PPARγ antagonist (Baek et al., 2004b).

Induction of NAG-1 by troglitazone was linked to activation of the tumor suppressor gene Egr-1 which in turn directly activates NAG-1 promoter constructs. The C-DIM compounds also rapidly activate Egr-1 protein expression in HCT-116 cells and maximal induction was

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observed within 2 hr after treatment, whereas NAG-1 protein expression is increased with time over 24 hr (Fig. 5A). In contrast, DIM induced NAG-1 but did not affect Egr-1 expression in HCT-116 cells (Lee et al., 2005), and this clearly differentiated between diarylmethane (DIM) from the triarylmethane (C-DIM) compounds. Constructs containing Egr-1 promoter inserts (Figs. 5B - 5E) are also activated by the C-DIM compounds, and the minimal promoter region required for transactivation contains SRE3 and SRE2 which have previously been identified as critical *cis*-elements required for activation of Egr-1 (Chen et al., 2004). Moreover, these compounds also activate NAG-1 promoter constructs (Fig. 6) and deletion/mutation analysis of the NAG-1 promoter indicates that mutation of the proximal Egr-1 sites resulted in loss of C-DIM-induced transactivation (Fig. 6E). These results, coupled with the observed interactions of Egr-1 with the NAG-1 promoter in a ChIP assay (Fig. 7), clearly link the induction of NAG-1 by C-DIMs to the rapid induction of Egr-1 which subsequently activates NAG-1 expression in HCT-116 cells.

Thus, the induction of NAG-1 by PPARγ-active C-DIMs and troglitazone in HCT-116 cells is receptor-independent and also involves Egr-1 induction. However, induction of NAG-1 protein and reporter gene activity by troglitazone was inhibited by the MAPK inhibitor PD98059 suggesting that this response may be due, in part, to induction of Egr-1 through activation of kinases by troglitazone (Baek et al., 2004b). Kinase-dependent activation of NAG-1 by C-DIMs was further investigated by determining the effects of PI3-K and MAPK inhibitors on induction of NAG-1 and Egr-1 proteins and reporter gene activity (pEGR-1D) in HCT-116 cells (Figs. 8A - 8C). The results show that PI3-K, and not MAPK inhibitors, block induction of NAG-1 and Egr-1 proteins and induction of transactivation in cells transfected with pEGR-1D, and this was consistent with the identification of kinase-responsive SRE3 and SRE2 as critical *cis*-elements in

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the Egr-1 promoter that are required for C-DIM-dependent activation of the Egr-1 constructs (Fig. 5). Kinase-mediated activation of Egr-1 is highly variable and dependent on cell context (Sukhatme et al., 1987; Christy and Nathans, 1989; Sukhatme, 1990; Cicatiello et al., 1993; Muthukkumar et al., 1995; Dziema et al., 2003; Chen et al., 2004; Sarker and Lee, 2004); however, it is apparent from this study that activation of Egr-1 by C-DIMs is PI3-K-dependent, whereas troglitazone activation of Egr-1/NAG-1 was linked to a MAPK pathway (Baek et al., 2004b). In this study, 20 μM LY294002 alone did not affect NAG-1 or Egr-1 protein expression, but inhibited induction of these proteins by PPARγ-active C-DIMs (Fig. 8), and induction of Egr-1-derived promoter constructs by DIM-C-pPhtBu was also inhibited by LY294002 (data not shown). In contrast, a recent study reported that higher concentrations of LY294002 (50 μM) induced NAG-1 protein expression and this was linked to activation of GSK-3β through dephosphorylation of this protein (Baek et al., 2005). Thus, PI3-K plays a pivotal role in activation of Egr-1 (and NAG-1) by the C-DIM compounds, whereas inactivation of PI3-K after treatment with high concentrations of LY294002 results in GSK-3β-dependent activation of NAG-1 (Baek et al., 2005).

In summary, our results show that PPARγ-active C-DIMs induce both NAG-1 and Egr-1 in HCT-116 cells through receptor-independent pathways. NAG-1 upregulation is linked to prior PI3-K-dependent induction of Egr-1 which directly activates NAG-1 through interaction with Egr-1 *cis*-elements in the NAG-1 promoter. It is also possible that the decreased HCT-116 cell survival and apoptosis observed after treatment with the C-DIM compounds is due, in part, to activation of other Egr-1-dependent genes which mediate the apoptotic and growth inhibitory effects (Huang et al., 1995). The induction of PI3-K by the C-DIM compounds (Fig. 8D) is somewhat paradoxical since this kinase is linked to cell survival pathways. However, recent

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studies have demonstrated that activation of PI3-K can sensitize caveolin-1-expressing HeLa and 293 cells to the cytotoxicity of arsenite and hydrogen peroxide (Shack et al., 2003). Moreover, caveolin-1 and PI3-K also sensitize L929 cells to tumor necrosis factor-α-induced cell death, and it was postulated that this may be due to Akt-dependent inactivation of forkhead transcription factors (Ono et al., 2004). Although the C-DIM compounds do not induce caveolin-1 in HCT-116 cells, the high endogenous expression of caveolin-1 in these cells (Fig. 3B) coupled with the induction of PI3-K may also contribute to the decreased HCT-116 cell survival and apoptosis after treatment with the C-DIMs. Thus, like other PPARγ agonists, the C-DIM compounds activate receptor-independent responses that contribute to their effectiveness as a new class of drugs with potential clinical applications for cancer chemotherapy.

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FIGURE CAPTIONS

Figure 1. PPARγ-active C-DIMs decrease HCT-116 cancer cell survival. HCT-116 cells were treated for 48 [A] or 96 [B] hr with DMSO or different concentrations of C-DIMs, and cell numbers as a percentage of DMSO-treated cells were determined as described in the Materials and Methods. Results are expressed as means ± SE for three separate determinations for each treatment group and a significant ($p < 0.05$) decreased in cell survival is indicated with an asterisk.

Figure 2. Activation of PPARγ-dependent transactivation. HCT-116 cells were transfected with $PPRE_3$-luc [A] or PPARγ-GAL4/pGAL4 [B], treated with DMSO, different concentrations of C-DIMs or rosiglitazone alone or in combination with GW9662, and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means ± SE for replicate determinations for each treatment group, and significant ($p < 0.05$) induction (*) or inhibition after cotreatment with GW9662 (**) are indicated.

Figure 3. Modulation of cell cycle proteins, caveolin-1, PARP cleavage, and NAG-1 by PPARγ-active C-DIMs. HCT-116 cells were treated with DMSO or different concentrations of C-DIMs and analyzed for cyclin D1 (CD1)/p27 [A], caveolin-1 [B], PARP cleavage/NAG-1 and CD1 [C], and NAG-1 [D] by Western blot analysis as outlined in the Materials and Methods. Treatment times were varied and similar results were observed in duplicate experiments for [A] - [D].

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Figure 4. Induction of NAG-1 and related proteins by C-DIMs and the role of PPARγ. [A] Induction of NAG-1 and related proteins by C-DIMs. HCT-116 cells were treated with DMSO or 7.5 - 20 μM C-DIMs for 24 hr and proteins were analyzed by Western immunoblot analysis as described in the Materials and Methods. [B]/[C] Role of PPARγ in activation of NAG-1/PARP cleavage by C-DIMs. HCT-116 cells were treated for 24 hr with DMSO, 10 or 15 μM C-DIMs alone or in combination with 10 μM GW9662 and whole cell lysates were analyzed by Western immunoblot analysis as described in the Materials and Methods. [D] Role of PPARγ in mediating C-DIM-induced cell survival. Cell survival data (after 96 hr) were obtained as described in Figure1, and the effects of C-DIMs alone or in combination with GW9662 were determined. No significant ($p < 0.05$) effects on cell survival were observed in cells treated with D-DIMs plus GW9662. Experiments illustrated in [B] - [D] were also determined using the PPARγ antagonist T007 and similar results were obtained.

Figure 5. Induction of Egr-1 protein/reported gene activity by C-DIMs. [A] Time course induction of NAG-1 and Egr-1. HCT-116 cells were treated with DMSO or 15 μM C-DIMs for up to 24 hr, and whole cell lysates were analyzed for Egr-1, NAG-1 and β-actin (control) by Western immunoblot analysis as described in the Materials and Methods. Activation of EGR-1A [B], EGR-1B [C], EGR-1C [D], EGR-1D [E], and EGR-1E [F] by C-DIMs. HCT-116 cells were transfected with the various constructs treated with DMSO or C-DIMs 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 is indicated by an asterisk.

Figure 6. Activation of NAG-1 promoter constructs by C-DIMs and Egr-1. HCT-116 cells were transfected with pNAG-1A [A], pNAG-1B [B], pNAG-1C [C], pNAG-1D [D], or pNAG-1D mutants [E], treated with DMSO or C-DIMs, and luciferase activity determined as described in the Materials and Methods. Results in [A] - [E] are expressed as means ± SE for three replicate determinations for each treatment group, and significant ($p < 0.05$) induction is indicated by an asterisk.

Figure 7. Activation of NAG-1 by Egr-1. [A] Activation of NAG-1 promoter constructs by Egr-1 expression plasmid. HCT-116 cells were transfected with pNAG-1D and different amounts of Egr-1 expression plasmid (or empty vector to maintain a constant amount of transfected DNA), and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each group, and significant ($p < 0.05$) induction compared to empty vector is indicated by an asterisk. [B] ChIP assay. HCT-116 cells were treated with DMSO or 20 μM DIM-C-pPhC_6H_5, and interactions of various proteins with the NAG-1 promoter were determined in a ChIP assay as described in the Materials and Methods. [C] Control binding of TFIIB. The control ChIP assay illustrates binding of TFIIB to the GAPDH promoter but not to exon 1 of the CNAP1 gene (negative control).

Figure 8. C-DIM compounds activate PI3-K in HCT-116 cells. Effects of kinase inhibitors on induction of NAG-1 [A] or Egr-1 [B] by C-DIMs. HCT-116 cells were treated with DMSO, C-DIMs alone or in combination with 20 μM PD98059 or 20 μM LY294002 for 24 [A] or 2 [B] hr, and whole cell lysates were analyzed by Western immunoblot analysis as described in the

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Materials and Methods. Similar results were observed using DIM-C-pPhCF_3 or DIM-C-pPhtBu. [C] Inhibition of pEGR-1D activation by LY294002. HCT-116 cells were transfected with pEGR-1D, treated with 10 or 15 μM C-DIM compounds alone or in combination with 20 μM LY294002 and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SE (triplicate). Significant ($p < 0.05$) induction by C-DIMs and inhibition by LY294002 are indicated by (*) and (**), respectively. [D] Activation of Akt phosphorylation by C-DIMs. HCT-116 cells were treated with the C-DIM compounds for different periods of time, and whole cell lysates were analyzed by Western blot analysis for phospho-Akt and Akt as described in the Materials and Methods. Results shown in the bar graph are means of two duplicate determinations.

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Figure 1

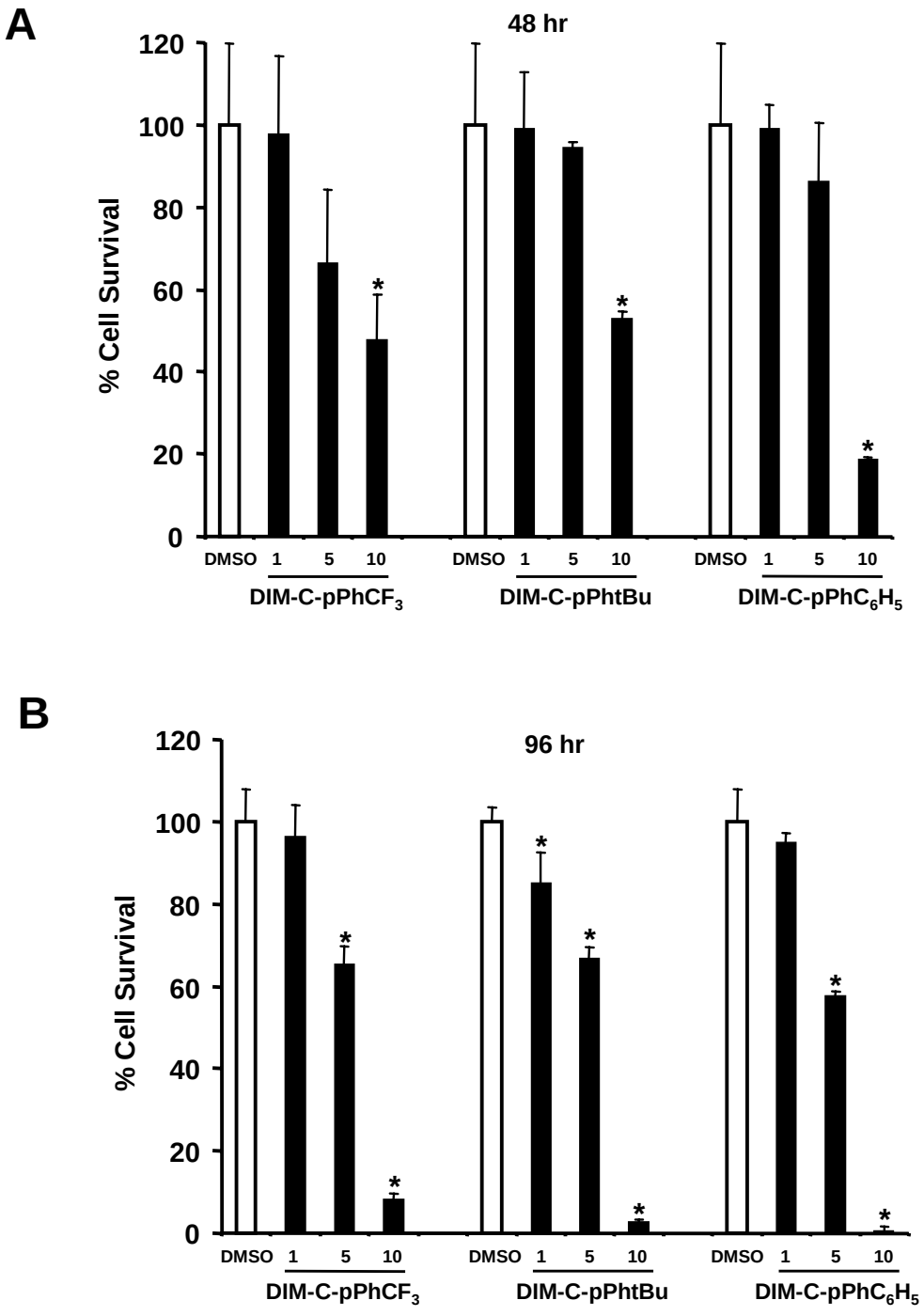

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Figure 2

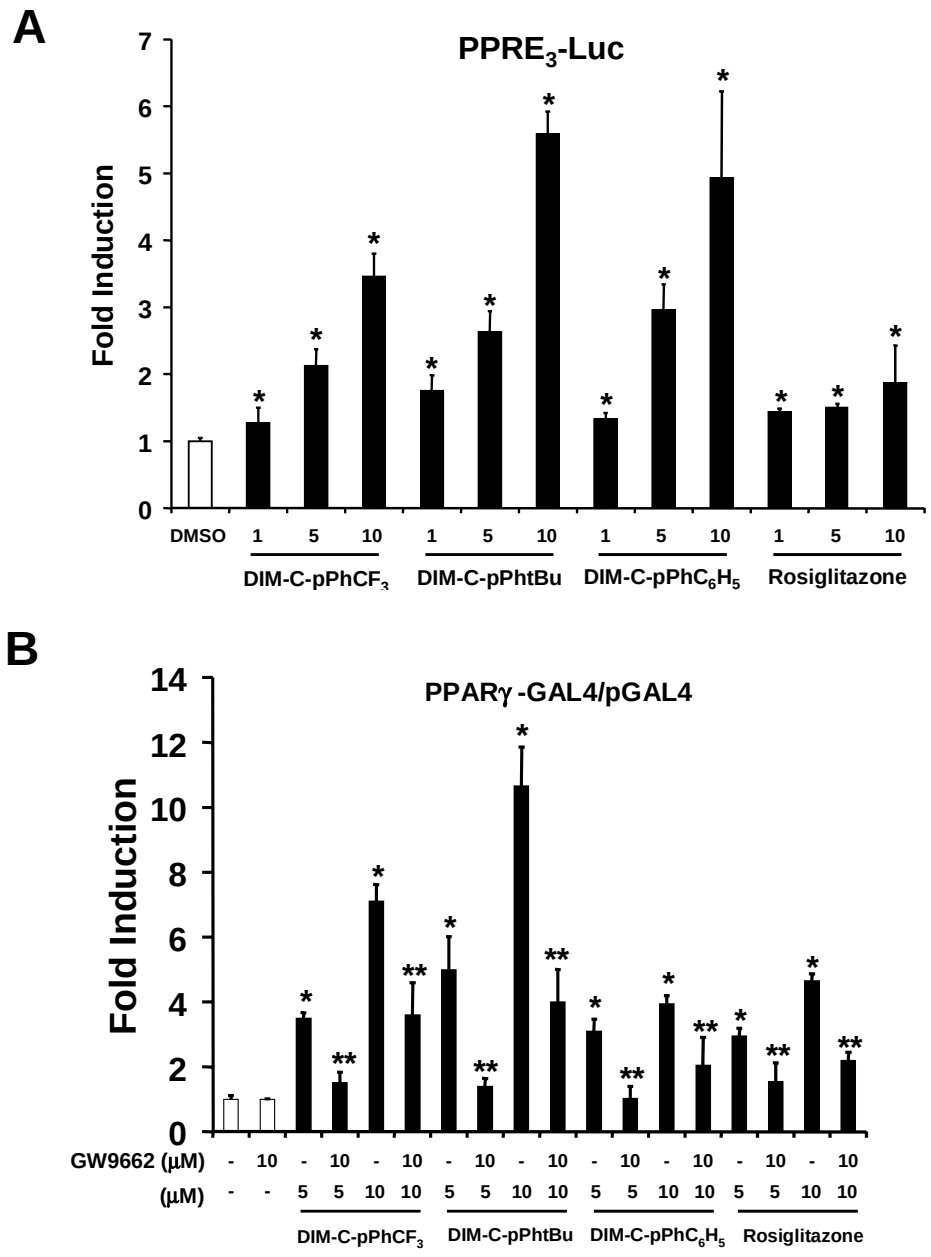

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Figure 3

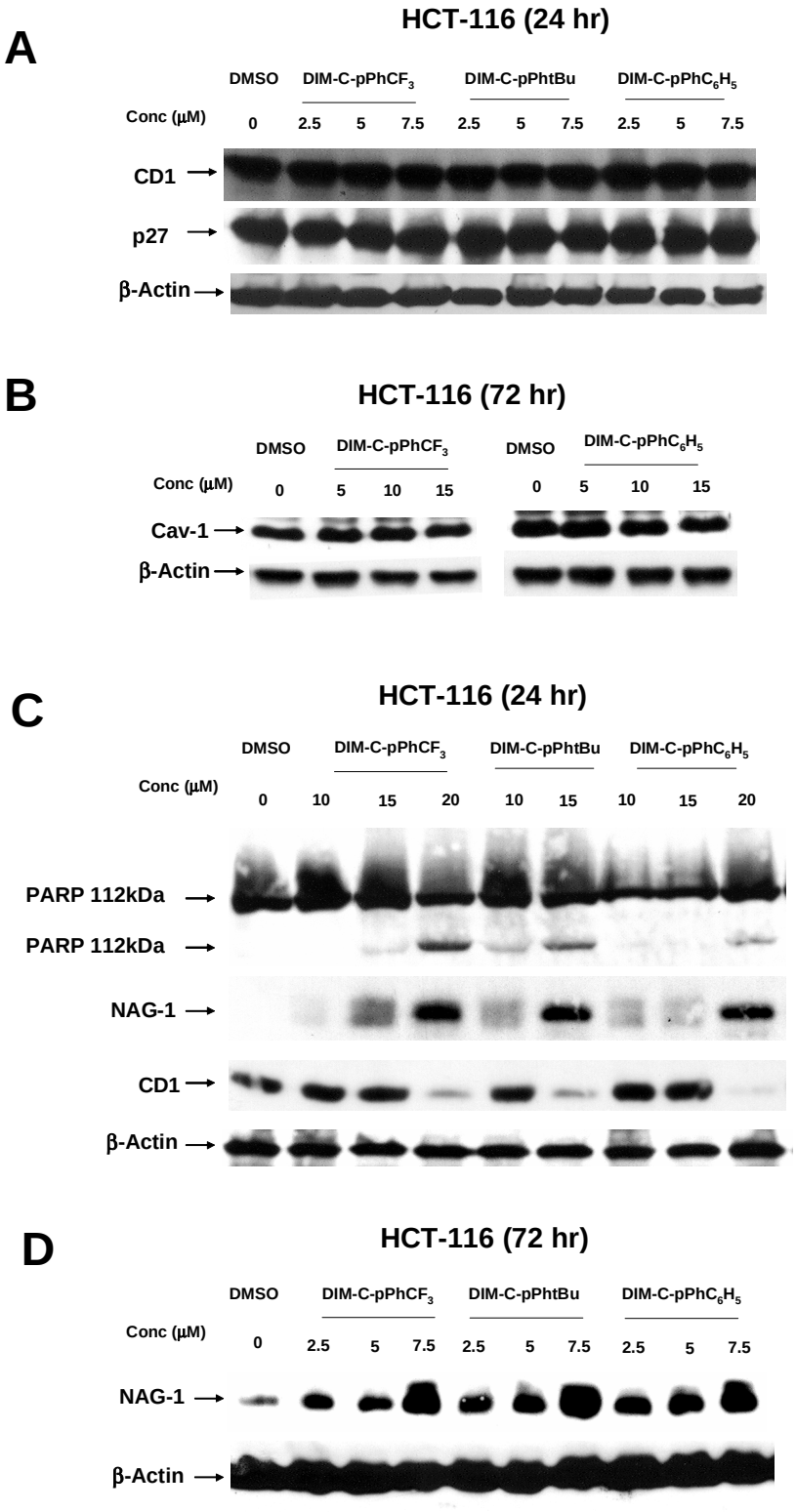

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Figure 4

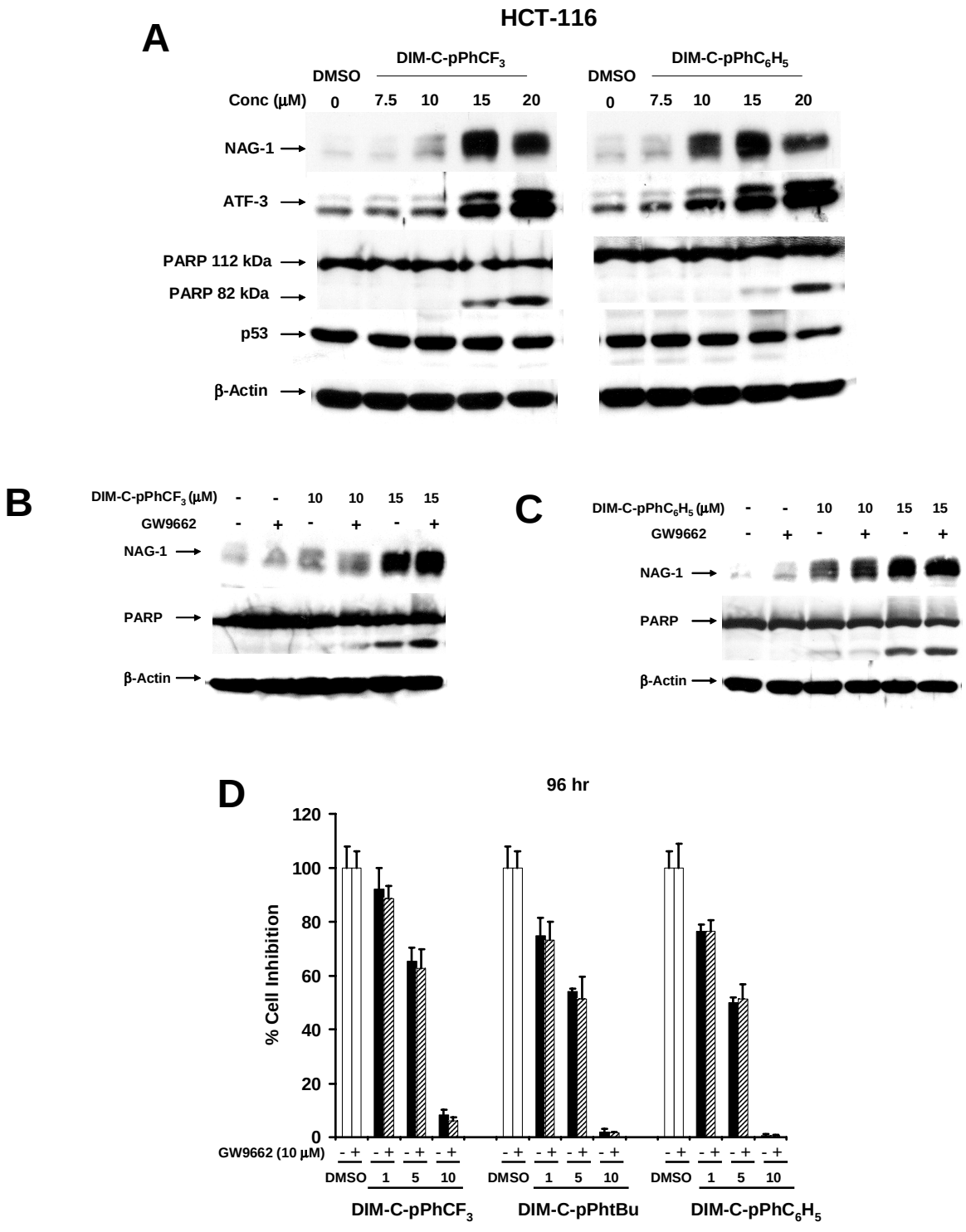

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Figure 5

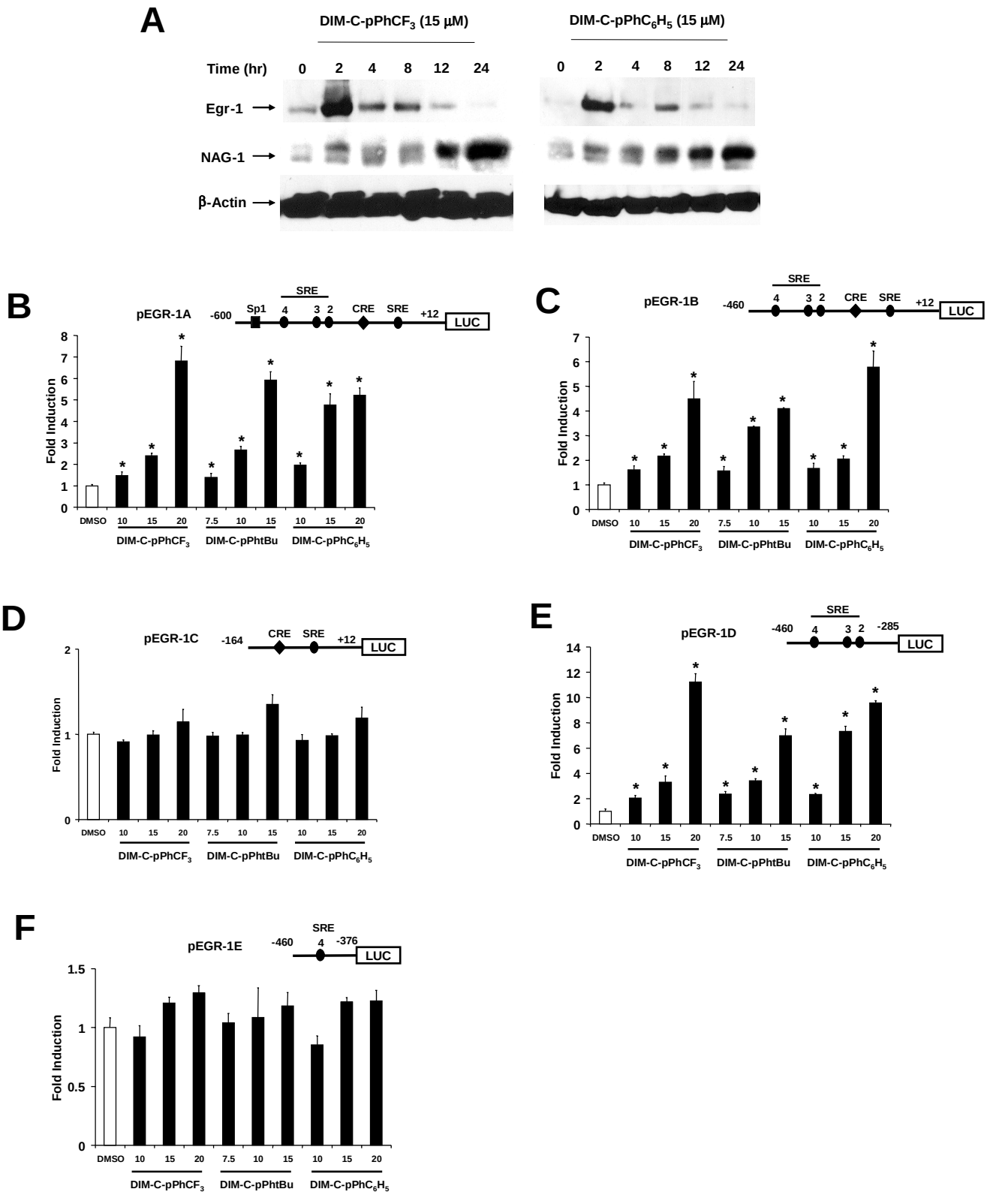

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Figure 6

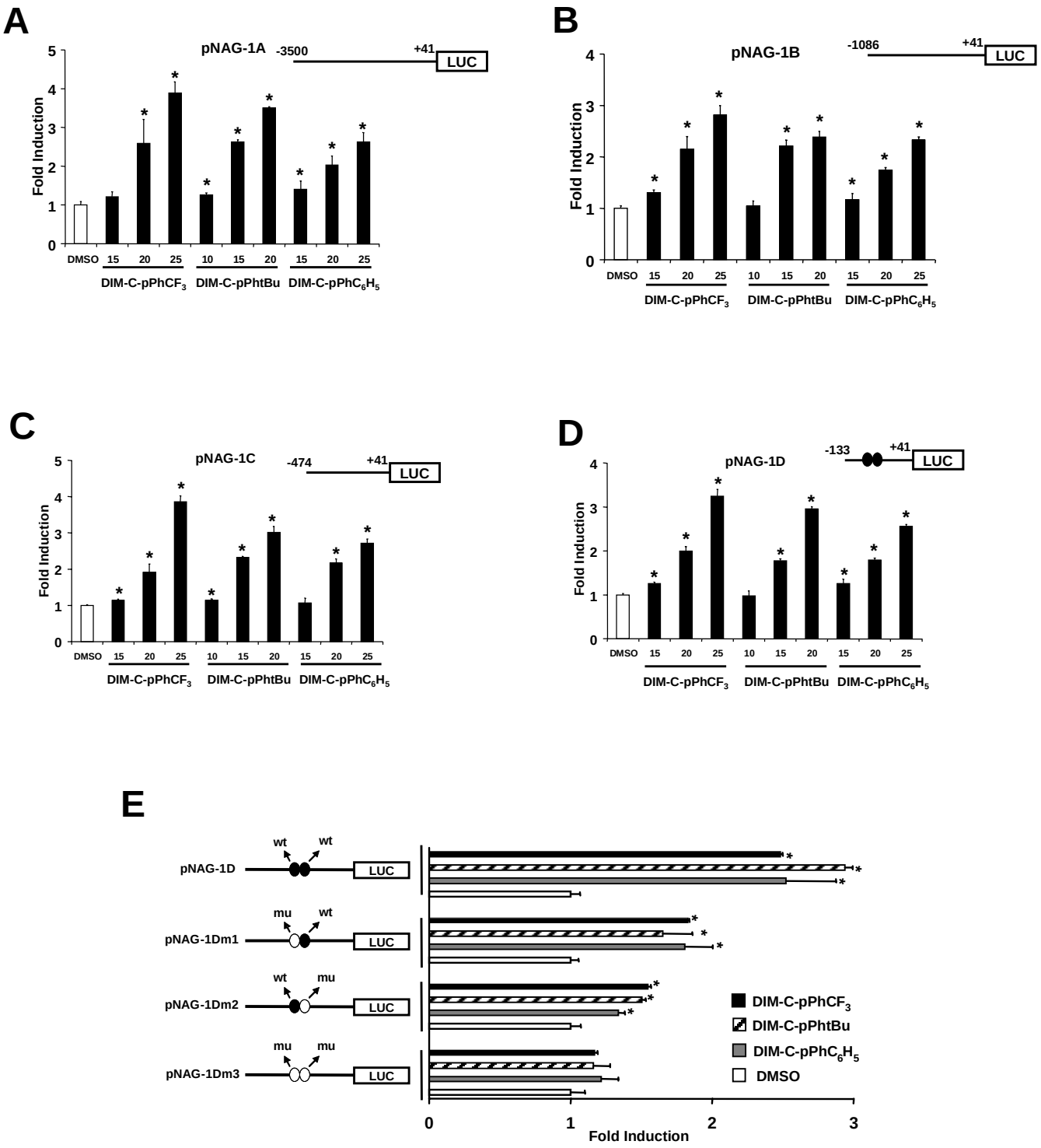

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Figure 7

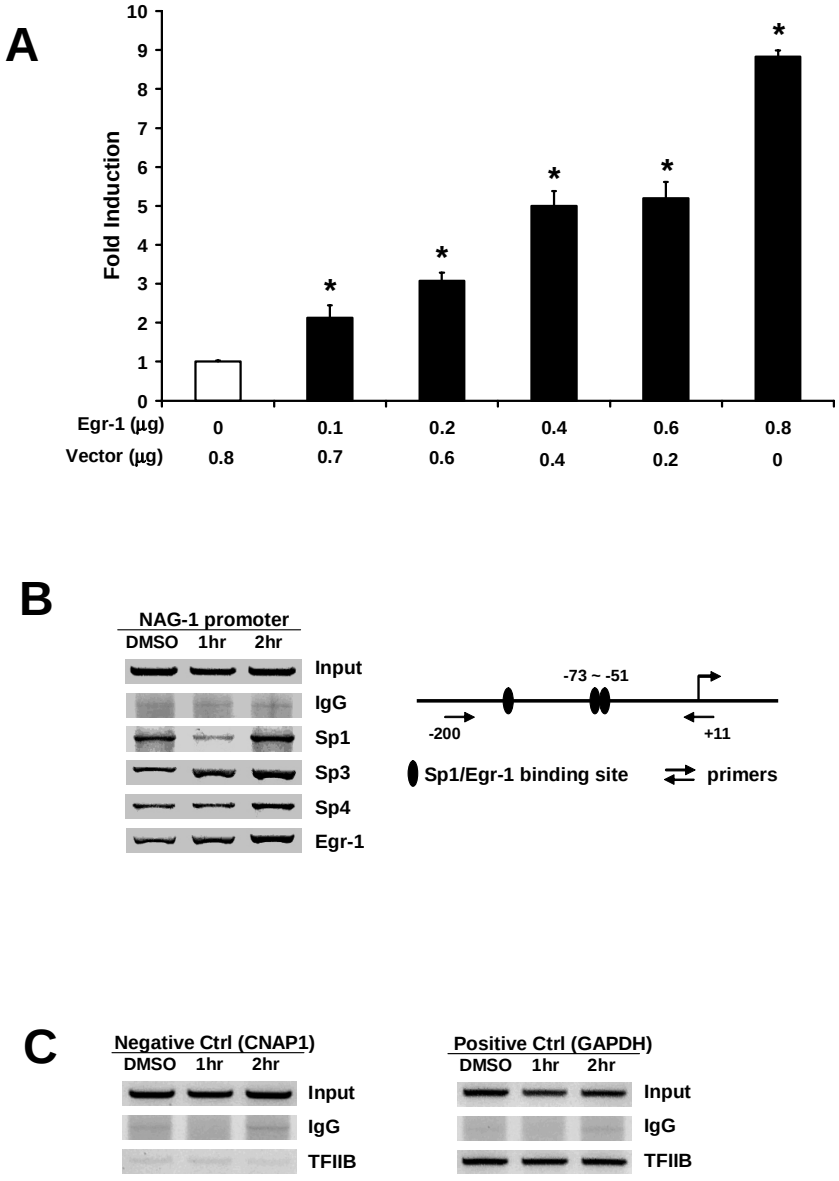

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Figure 8