1,1-Bis(3′-indolyl)-1-(*p*-substituted phenyl)methanes inhibit colon cancer cell and tumor growth through PPARγ-dependent and PPARγ-independent pathways

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Abstract

1,1-Bis(3′-indolyl)-1-(*p*-substituted phenyl)methanes containing *p*-trifluoromethyl, *t*-butyl, and phenyl [1,1-bis(3′-indolyl)-1-(*p*-phenyl)methane (DIM-C-pPhC$_6$H$_5$)] substituents induce peroxisome proliferator-activated receptor γ (PPARγ)–mediated transactivation in SW480 colon cancer cells. These PPARγ-active compounds also inhibit cell proliferation and modulate some cell cycle proteins. At concentrations from 2.5 to 7.5 μmol/L, the PPARγ agonists induce caveolin-1 and phosphorylation of Akt and cotreatment with the PPARγ antagonist GW9662 inhibited the induction response. In contrast, higher concentrations (10 μmol/L) of 1,1-bis(3′-indolyl)-1-(*p*-substituted phenyl)methanes containing 1,1-bis(3′-indolyl)-1-(*p*-trifluoromethyl)methane and DIM-C-pPhC$_6$H$_5$ induce apoptosis, which is PPARγ independent. This was accompanied by loss of caveolin-1 induction but induction of proapoptotic nonsteroidal anti-inflammatory drug activated gene-1. In athymic nude mice bearing SW480 cell xenografts, DIM-C-pPhC$_6$H$_5$ inhibits tumor growth at doses of 20 and 40 mg/kg/d and immunohistochemical staining of the tumors showed induction of apoptosis and nonsteroidal anti-inflammatory drug activated gene-1 expression. Thus, the indole-derived PPARγ-active compounds induce both receptor-dependent and receptor-independent responses in SW480 cells, which are separable over a narrow range of concentrations. This dual mechanism of action enhances their antiproliferative and anticancer activities. [Mol Cancer Ther 2006;5(5):1362–70]

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Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors (1–6). Although the endogenous ligand for PPARγ has not been determined, several endogenous molecules, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$, other prostaglandin-derived compounds, fatty acids, and flavonoids, activate PPARγ. It has recently been reported that nitrolinoleic acid, a stress-induced fatty acid oxidation product, may be an endogenous PPARγ agonist (7). Several different structural classes of PPARγ agonists have been synthesized and these include thiazolidinediones, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and related triterpenoids, 3-benzyl and acyl-substituted indoles, substituted chromane carboxylic acids, and phosphonophosphates (8–13). Among the synthetic PPARγ agonists, the thiazolidinediones are now being extensively used as insulin-sensitizing agents for treatment of type II diabetes (1, 3). PPARγ is highly expressed in many tumor samples and cancer cell lines derived from hematopoietic and nonhematopoietic tumors (14), and several studies show that PPARγ agonists inhibit growth and/or induce apoptosis in multiple cancer cell lines and in *in vivo* tumor models (15–33).

3,3′-Diindolylmethane is a metabolite of the phytochemical indole-3-carbinol and both compounds exhibit a broad spectrum of anticancer activities (34, 35). Methylene-substituted 3,3′-diindolylmethanes (C-DIM) are synthetic analogues of 3,3′-diindolylmethane, which also exhibit anticancer activity and bind to orphan receptors, such as PPARγ and Nur77 (36–40). The 1,1-bis(3′-indolyl)-1-(*p*-substituted phenyl)methanes containing *p*-trifluoromethyl [1,1-bis(3′-indolyl)-1-(*p*-trifluoromethyl)methane (DIM-C-pPhCF$_3$)], *t*-butyl [1,1-bis(3′-indolyl)-1-(*p*-*t*-butyl)methane (DIM-C-pPhtBu)], and phenyl [1,1-bis(3′-indolyl)-1-(*p*-phenyl)methane (DIM-C-pPhC$_6$H$_5$)] substituents activate PPARγ in several cancer cell lines, including colon cancer cells (36–39). The effects of these compounds depend on cell context and this is commonly observed for other PPARγ agonists. The PPARγ-active C-DIMs inhibit growth and/or induce apoptosis in cancer cells; in HT-29 and HCT-15 colon cancer cells, these compounds induce caveolin-1, whereas p21 is induced in Panc28 pancreatic cancer cells and both responses are inhibited by PPARγ antagonists (37, 38). In contrast, induction of cyclin D1 down-regulation and apoptosis in breast cancer cells (36) and induction of nonsteroidal anti-inflammatory drug activated gene-1 (NAG-1) are PPARγ independent (41, 42).

In this study, we show that PPARγ-active C-DIMs inhibit growth of SW480 colon cancer cells and this is associated with PPARγ-dependent induction of caveolin-1 and

receptor-independent activation of both NAG-1 and poly (ADP-ribose) polymerase (PARP) cleavage. Moreover, in athymic nude mice bearing SW480 cell xenografts, DIM-C-pPhC_6H_5 (20 and 40 mg/kg/d) inhibits tumor growth and immunostaining of tumors shows both overexpression of caspase-3 and NAG-1 in the treated groups. These data show that PPARγ-active C-DIMs inhibit colon cancer cell/tumor growth and confirm that PPARγ agonists are an important class of compounds for potential applications in the treatment of colon cancer.

Materials and Methods

Cell Lines, Plasmids, Chemicals, and Reagents

The SW480 human colon cancer cell line was provided by Dr. S. Hamilton (M. D. Anderson Cancer Center, Houston, TX). SW480 cells were maintained in DMEM/Ham's F-12 (Sigma, St. Louis, MO) with phenol red supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, and 5% fetal bovine serum (FBS) and 10 ml/L of 100× antibiotic-antimycotic solution (Sigma). The Gal4 reporter containing 5× Gal4DBD (Gal4Luc) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4DBD-PPARγ construct was a gift of Dr. Jennifer L. Oberfield (Glaxo Wellcome Research and Development, Research Triangle Park, NC) and chimeric pM-PPARγ coactivator-1 (PGC-1) was a gift of Dr. Bruce M. Spiegelman (Harvard University, Boston, MA). The PPARγ2-VP16 fusion plasmid (VP-PPARγ) contained the DEF region of PPARγ (amino acids 183–505) fused to the pVP16 expression vector and the Gal4-coactivator fusion plasmids pM-SRC-1, pM-AIBI, pM-TIFII, pM-DRIP205, pM-TRAP220, and pM-CARM-1 were kindly provided by Dr. Shigeaki Kato (University of Tokyo, Tokyo, Japan). Rosiglitazone was purchased from LKT Laboratories, Inc. (St. Paul, MN). Horseradish peroxidase substrate for Western blot analysis was purchased from NEN Life Science Products (Boston, MA). Cell lysis buffer and luciferase reagent were purchased from Promega (Madison, WI), and β-galactosidase reagent was from Tropix (Bedford, MA). Antibodies for cyclin D1 (sc-718), p27 (sc-528), phosphorylated Akt (sc-7985R), Akt (sc-8312), Bcl-2 (sc-7382), Bax (sc-20067), caveolin-1 (sc-894), and PARP (sc-8007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). NAG-1 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY); monoclonal anti-β-actin was purchased from Sigma.

Cell Proliferation Assay

Cells were plated at a density of 2×10^4 per well in 12-well plates in DMEM/Ham's F-12 and 5% FBS, and after 24 hours, this was replaced with DMEM/Ham's F-12 containing 2.5% charcoal-stripped FBS. Cells were then treated with either vehicle (DMSO) or the indicated ligand in DMSO. Fresh medium and compounds were added every 48 hours. Cells were counted at the indicated times using a Coulter Z1 cell counter (Beckman-Coulter, Fullerton, CA). Each experiment was carried out in triplicate and results are expressed as mean ±SE for each determination.

Transfection and Luciferase Assay

SW480 cells were plated in 12-well plates at 1×10^5 per well in DMEM/Ham's F-12 supplemented with 2.5% charcoal-stripped FBS. After growth for 16 hours, various amounts of DNA [i.e., Gal4Luc (0.4 μg), β-galactosidase (0.04 μg), VP-PPARγ (0.04 μg), pM-SRC-1 (0.04 μg), pM-TIFII (0.04 μg), pM-AIBI (0.04 μg), pM-DRIP205 (0.04 μg), and pM-CARM-1 (0.04 μg)] were transfected by LipofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 2 μL LipofectAMINE was mixed with 50 μL serum-free medium. Appropriate concentrations of plasmid DNA were mixed with 50 μL serum-free medium. The LipofectAMINE solution was then mixed with the DNA solution and the mixture was allowed to incubate at room temperature for 45 minutes to form the DNA/LipofectAMINE complex. In the mean time, cells grown in the presence DMEM/Ham's F-12 supplemented with 2.5% charcoal-stripped FBS were washed with serum-free DMEM/Ham's F-12, and 400 μL serum-free medium was added. Following 45-minute incubation, the LipofectAMINE/DNA complex was carefully dropped over the cells and incubated for 5 to 6 hours at 37°C. Five hours after transfection, the transfection mix was replaced with complete medium containing either vehicle (DMSO) or the indicated ligand for 20 to 22 hours. Cells were then lysed with 100 μL of 1× reporter lysis buffer, and 30 μL cell extracts were used for luciferase and β-galactosidase assays. A Lumicount luminometer (Perkin-Elmer Life Sciences, Boston, MA) was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

Western Blot Analysis

SW480 cells were seeded in DMEM/Ham's F-12 containing 2.5% charcoal-stripped FBS for 24 hours and then treated with either the vehicle (DMSO) or the indicated compounds. Whole-cell lysates were obtained using high-salt buffer [50 mmol/L HEPES, 500 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100 (pH 7.5), 5 μL/mL protease inhibitor cocktail (Sigma)].

Protein samples were incubated at 100°C for 2 to 3 minutes, separated on 10% SDS-PAGE at 120 V for 3 to 4 hours in 1× running buffer [25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS (pH 8.3)], and transferred to polyvinylidene difluoride (Bio-Rad, Hercules, CA) at 0.1 V for 16 hours at 4°C in 1× transfer buffer (48 mmol/L Tris-HCl, 39 mmol/L glycine, 0.025% SDS). The polyvinylidene difluoride membrane was blocked in 5% TBS-Tween 20-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, 5% nonfat dry milk] with gentle shaking for 30 minutes and incubated in fresh 5% TBS-Tween 20-Blotto with 1:1,000 (for CD1, p27, Bcl-2, Bax, and caveolin-1), 1:500 (for PARP and NAG-1), and 1:5,000 (for β-actin) primary antibody overnight with gentle shaking at 4°C. After washing with TBS-Tween 20 for 10 minutes, the polyvinylidene difluoride membrane was incubated with secondary antibody (1:5,000) in 5% TBS-Tween 20-Blotto for 90 minutes. The membrane was washed with TBS-Tween

20 for 10 minutes and incubated with 10 mL chemiluminescence substrate (Perkin-Elmer Life Sciences) for 1 minute and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

Xenograft Experiment

Male athymic BALB/c nude mice (age 4–6 weeks) were purchased from Harlan (Indianapolis, IN). Mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions. SW480 cells were concentrated to 2×10^6 per 200 μL and injected s.c. into the left flank of each mouse using a 30-gauge needle. Six days after cell inoculation, animals were divided into three equal groups of 10 mice each. The first group received 70 μL vehicle (corn oil) by oral gavage and the second and third groups of animals received 20 and 40 mg/kg/d doses of DIM-C-pPhC_6H_5 in vehicle every second day for 20 days (10 doses). The mice were weighed, and tumor areas were measured throughout the study. After 21 days, the animals were sacrificed, final body and tumor weights were determined, and selected tissues were further examined by routine H&E staining and immunohistochemical analysis.

Immunohistochemistry

Tissue sections (4–5 μm thick) mounted on poly-L-lysine-coated slide were deparaffinized by standard methods. Endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 10 minutes. Antigen retrieval for NAG-1 staining was done for 7.5 minutes in 10 mmol/L sodium citrate buffer (pH 6) heated at 95°C in a steamer followed by cooling for 15 minutes. The slides were washed with PBS and incubated for 30 minutes at room temperature with a protein blocking solution (Biostain Rabbit IgG System, Biomeda, Foster City, CA). Excess blocking solution was drained, and the samples were incubated overnight at 4°C with one of the following: a 1:100 dilution of activated caspase-3 antibody or a 1:100 dilution of NAG-1 antibody.

Sections were then incubated with biotinylated secondary antibody followed by streptavidin (Biostain Rabbit IgG System). The color was developed by exposing the peroxidase to diaminobenzidine reagent (Vector Laboratories, Burlingame, CA), which forms a brown reaction product. The sections were then counterstained with Gill's hematoxylin. Activated caspase-3 and NAG-1 expression was identified by the brown cytoplasmic staining.

Assessment of Apoptotic Cells

Four slides per group were stained and apoptotic cells were identified by the dark brown cytoplasmic staining. Ten microscopic fields in each slide were counted for cytoplasmic staining and averaged.

Results

Inhibition of Cell Proliferation and Activation of PPARγ

Previous studies in colon and pancreatic cancer cells showed that PPARγ-active C-DIMs inhibit cell growth (38, 39), and results in Fig. 1 also show the DIM-C-pPhCF_3, DIM-C-pPhtBu, and DIM-C-pPhC_6H_5 inhibit SW480 cancer cell proliferation. IC_{50}s varied between 1 and 10 μmol/L and the relative order of potency was DIM-C-pPhCF_3 > DIM-C-pPhtBu > DIM-C-pPhC_6H_5. In contrast, 1 to 10 μmol/L rosiglitazone did not significantly affect growth of SW480 cells.

Activation of PPARγ was also investigated in SW480 cells transfected with chimeric PPARγ-Gal4 and a Gal4 response

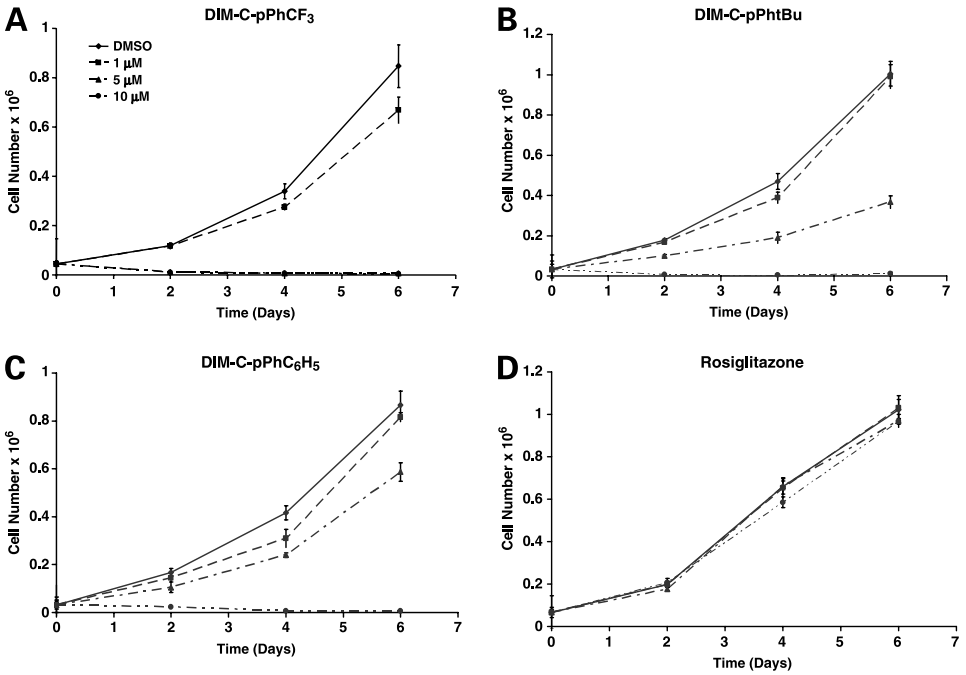

Figure 1. Cell proliferation assays. SW480 cells were treated with DIM-C-pPhCF_3 (**A**), DIM-C-pPhtBu (**B**), DIM-C-pPhC_6H_5 (**C**), or rosiglitazone (**D**), and cell numbers were determined after 2, 4, and 6 d as described in Materials and Methods. Significant ($P < 0.05$) inhibition of cell proliferation was observed for the C-DIMs (≥5 μmol/L). *Points,* mean of three replicate determinations for each treatment group; *bars,* SE.

element reporter plasmid containing five-tandem Gal4 response elements linked to a luciferase reporter gene. The results showed that DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$ significantly induced transactivation with a maximal 17-fold increase observed for 10 μmol/L DIM-C-pPhCF$_3$, whereas <4-fold induction of luciferase activity was observed for rosiglitazone (Fig. 2A). We also investigated ligand-induced interactions of PPARγ with a panel of coactivators in a mammalian two-hybrid assay in SW480 cells (Fig. 2B). The results show that the PPARγ-active C-DIMs induce interactions between PPARγ and PGC-1 and similar results were obtained for the compounds in previous studies in HCT-15 cells, whereas in HT-29 cells PGC-1-PPARγ interactions were induced by DIM-C-pPhCF$_3$ and DIM-C-pPhtBu but not DIM-C-pPhC$_6$H$_5$ (37). In contrast to results obtained in HT-29/HCT-15 cells, the PPARγ-active C-DIMs also significantly induced interactions of PPARγ with SRC-1, SRC-2 (TIFII), SRC-3 (AIBI), and the corepressor SMRT, and this pattern of ligand-induced PPARγ-coactivator/corepressor interactions in SW480 cells was different from that observed in HCT-15 and HT-29 cells. These cell context–dependent differences suggest that these compounds are selective PPARγ modulators. In addition, rosiglitazone was inactive in the mammalian two-hybrid assay in SW480 cells and this assay distinguished between the activities of this compound and the PPARγ-active C-DIMs. The selectivity of these ligand-induced PPARγ-coactivator interactions indicates the potential for cell context–dependent differences in the activation of PPARγ-dependent responses by the C-DIMs.

Effects of PPARγ-Active C-DIMs on Expression of Proteins Associated with Cell Proliferation and Cell Death

The effects of two prototypical PPARγ-active C-DIMs on expression of various cell cycle proteins and apoptosis (PARP cleavage) was investigated in SW480 cells treated with 5 to 10 μmol/L DIM-C-pPhCF$_3$, DIM-C-pPhC$_6$H$_5$, or rosiglitazone for 24 hours (Fig. 3A). The PPARγ-active C-DIMs enhanced p27 expression and down-regulated cyclin D1 protein only at the highest concentration and this was also accompanied by PARP cleavage. Previous studies with different structural classes of PPARγ agonists have reported induction of p27 in colon and other cancer cell lines, and we have also observed cyclin D1 down-regulation and PARP cleavage in other cancer cell lines treated with C-DIM compounds (36–39, 41, 42). There were minimal compound-induced changes in Bax levels and decreased expression of Bcl-2 at the 10 μmol/L concentrations of DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$, whereas up to 10 μmol/L rosiglitazone did not affect expression of apoptotic or cell cycle proteins. The growth inhibitory effects of PPARγ-active C-DIMs in HT-29 and HCT-15 cells have been linked, in part, to induction of caveolin-1 (37) and the results in Fig. 3B show that at doses as low as 2.5 μmol/L there was induction of caveolin-1 in SW480 cells. This is a response that was not observed until 48 hours after treatment and only minimal apoptosis was induced in the

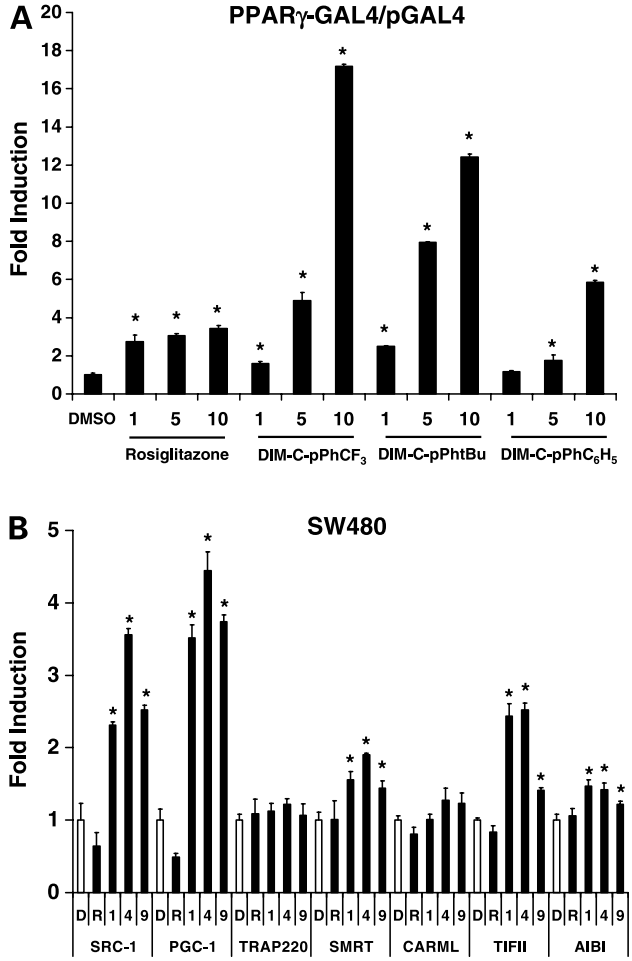

Figure 2. C-DIMs activate PPARγ. **A,** transactivation. SW480 cells were transfected with PPARγ-Gal4/pGal4 and treated with DMSO or different concentrations of compounds, and luciferase activity was determined as described in Materials and Methods. **B,** mammalian two-hybrid assay. SW480 cells were transfected with Gal4 chimeras, VP-PPARγ (LBD) and pGal4Luc, and treated with various compounds [rosiglitazone (*R*), DIM-C-pPhCF$_3$ (*1*), DIM-C-pPhtBu (*4*), and DIM-C-pPhC$_6$H$_5$ (*9*)], and luciferase activity was determined as described in Materials and Methods. *Columns,* mean of three replicate determinations for each treatment group; *bars,* SE. *, $P < 0.05$, significant induction.

7.5 μmol/L DIM-C-pPhCF$_3$ treatment group (data not shown), suggesting that caveolin-1 was primarily associated with growth inhibitory responses observed at lower concentrations (≤7.5 μmol/L). Induction of caveolin-1 by 5 μmol/L DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ was inhibited after cotreatment with the PPARγ antagonist GW9662 (Fig. 3C), showing that this response was PPARγ dependent, and this was observed previously in HT-29 and HCT-15 cells (37). We also recently reported that PPARγ-dependent induction of caveolin-1 was observed in SW480 cells treated with CDDO and related compounds (43) and this was accompanied by induction of phosphatidylinositol 3-kinase–dependent phosphorylation of Akt. The results in Fig. 3D also show that 5 μmol/L DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ also induce Akt phosphorylation, and in

SW480 cells cotreated with GW9662, there was a dose-dependent decrease in Akt phosphorylation. Thus, the PPARγ-active C-DIMs coordinately induced caveolin-1 and phosphorylated Akt in SW480 cells and it has been reported that combined activation of caveolin-1 and phosphatidylinositol 3-kinase enhances the effects of agents that decrease cancer cell survival (44, 45).

The role of PPARγ in mediating DIM-C-pPhCF$_3$- and DIM-C-pPhC$_6$H$_5$-induced apoptosis was investigated in SW480 cells cotreated with C-DIMs and the PPARγ antagonist GW9662 (Fig. 4A). The results show that GW9662 did not affect induced PARP cleavage, suggesting that this response was PPARγ independent and similar results were observed for cyclin D1 down-regulation (data not shown). Interestingly, we also observed that at higher concentrations (>7.5 μmol/L) of C-DIMs that induce apoptosis in SW480 cells (Fig. 4A) there is a loss of caveolin-1 induction by these same compounds (Fig. 4B). The reason for this concentration-dependent switch between PPARγ-dependent and PPARγ-independent responses is unknown; however, we have also observed similar responses for CDDO and related compounds (data not shown). Recent studies in this laboratory show that PPARγ-active C-DIMs induced cell death through activation of endoplasmic reticulum stress in pancreatic cancer cells (42) or through induction of NAG-1 in HCT-116 colon cancer cells (41). Results in Fig. 4C show that induction of apoptosis in SW480 cells treated with DIM-C-pPhCF$_3$ or DIM-C-pPhC$_6$H$_5$ for 24 hours is not accompanied by induction of the stress protein GRP78.

NAG-1 is a transforming growth factor-β–like peptide, which is induced by C-DIMs and many other growth inhibitory agents, including rosiglitazone, in selected cell lines, including HCT-116 colon cancer cells. Preliminary studies did not detect induction of NAG-1 by C-DIMs in SW480 cells (42); however, repetition of this experiment with a different antibody showed that within 24 hours after treatment with DIM-C-pPhCF$_3$ or DIM-C-pPhC$_6$H$_5$ there

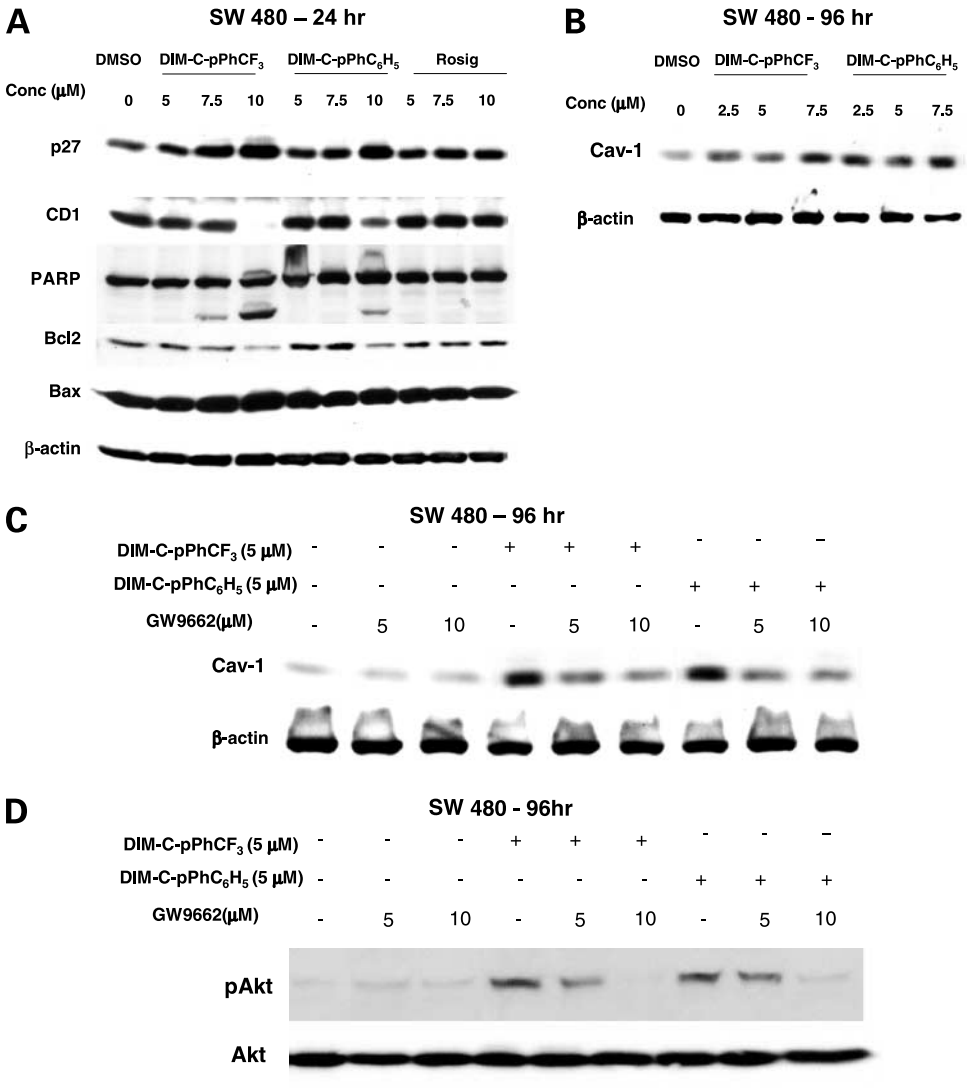

Figure 3. Cell cycle proteins, caveolin-1 expression, and Akt phosphorylation. **A,** cell cycle proteins. SW480 cells were treated with different concentrations of compounds for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods. Induction of caveolin-1 (**B, C**), Akt phosphorylation (**D**), and inhibition by GW9662 (**C, D**). SW480 cells were treated with different concentrations of compounds alone or in combination, and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods. β-Actin was used as a loading control for these experiments.

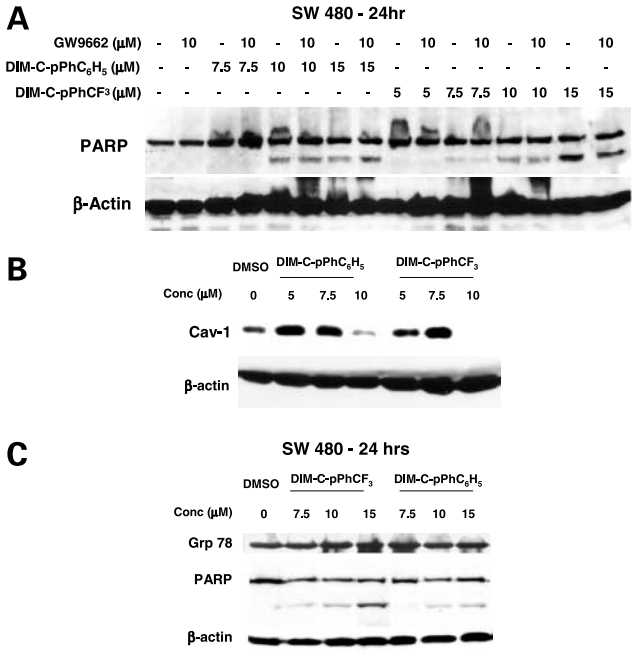

Figure 4. Effects of higher concentrations of C-DIMs on PARP cleavage (**A**) and caveolin-1 (**B**) and GRP78 (**C**) expression. SW480 cells were treated with different compounds (alone or combined) for 24 h (**A** and **C**) or 72 h (**B**), and whole-cell lysates were assayed by Western blot analysis as described in Materials and Methods. β-Actin served as a loading control for all experiments.

was induction of NAG-1 protein (Fig. 5A). Moreover, in SW480 cells cotreated with PPARγ-active C-DIMs (5–10 μmol/L) plus the PPARγ agonist GW9662 (10 μmol/L), the antagonist did not block induction of NAG-1 (Fig. 5B), suggesting that this response was PPARγ independent as reported previously in HCT-116 cells (42). We also observed induction of NAG-1 after treatment with C-DIMs for 96 hours (Fig. 5C), and at both 24- and 96-hour time points, effective doses were in the 7.5 to 10 μmol/L range.

The *in vivo* antitumorigenic activity of PPARγ-active C-DIMs was further investigated in male athymic nude mice bearing SW480 cell xenografts. After palpable tumors were first observed (<15 mm^2), animals were treated with corn oil (70 μL/mouse) or DIM-C-pPhC$_6$H$_5$ (20 or 40 mg/kg) in corn oil by oral gavage every second day for 21 days and tumor areas were determined. The results (Fig. 6A) show that tumor areas were significantly decreased after treatment with DIM-C-pPhC$_6$H$_5$ compared with the corn oil (control) group, and after sacrifice, tumor weights were also decreased in both treatment groups (Fig. 6B). We also investigated the effects of DIM-C-pPhC$_6$H$_5$ (40 mg/kg/d) on expression of the apoptotic gene caspase-3 (Fig. 6C and D) and NAG-1 in tumors by immunohistochemical analysis. DIM-C-pPhC$_6$H$_5$ significantly induced caspase-3 expression compared with control (corn oil) tumors (Fig. 6C). NAG-1 was not detected in tumors from control animals, whereas massive expression (brown staining) of NAG-1 was detected in tumors from DIM-C-pPhC$_6$H$_5$-treated mice (Fig. 6D), suggesting an important role for this protein in the antitumorigenic activity of this C-DIM compound. In contrast, minimal NAG-1 expression was observed at the 20 mg/kg dose and the available caveolin-1 antibodies did not give consistent staining. These data complement the *in vitro* studies and show that PPARγ-active C-DIMs inhibit colon cancer cell/tumor growth and these compounds are being developed for future clinical applications.

Discussion

Several studies report that PPARγ agonists inhibit growth of colon cancer cells; however, their induced responses and mechanisms of action are dependent on multiple factors, including ligand structure, cell context, and wild-type or variant PPARγ expression. For example, troglitazone (a thiazolidinedione), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, and PPARγ-active C-DIMs induce the transforming growth factor-β–like peptide NAG-1 in HCT-116 cells and GW9662 inhibits the effects of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 but had no effect on troglitazone or the PPARγ-active C-DIMs (27, 41). Although troglitazone, DIM-C-pPhCF$_3$, DIM-C-pPhtBu, and DIM-C-pPhC$_6$H$_5$ induce NAG-1 through prior activation of early growth response gene (27, 41), the former response is dependent on activation of mitogen-activated protein kinase, whereas the C-DIMs activate EGR-1 and NAG-1 through induction of phosphatidylinositol 3-kinase. Dubois and coworkers (20, 21, 23) have reported that the effects of rosiglitazone on cell proliferation and differentiation in several colon cancer cell lines

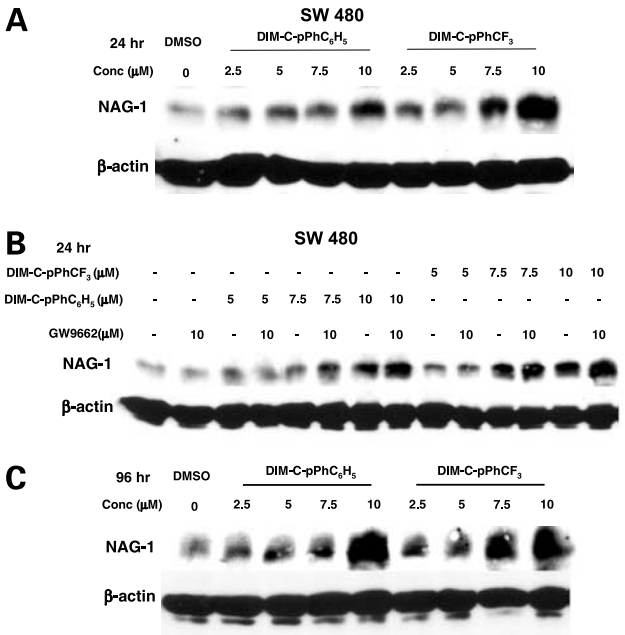

Figure 5. Induction of NAG-1 by C-DIM compounds. SW480 cells were treated for 24 h with DIM-C-pPhCF$_3$ or DIM-C-pPhC$_6$H$_5$ alone (**A**) or in combination with GW9662 (**B**) or for 96 h (**C**). Whole-cell lysates were analyzed for NAG-1 expression by Western blot analysis as described in Materials and Methods. β-Actin was used as a loading control for these experiments.

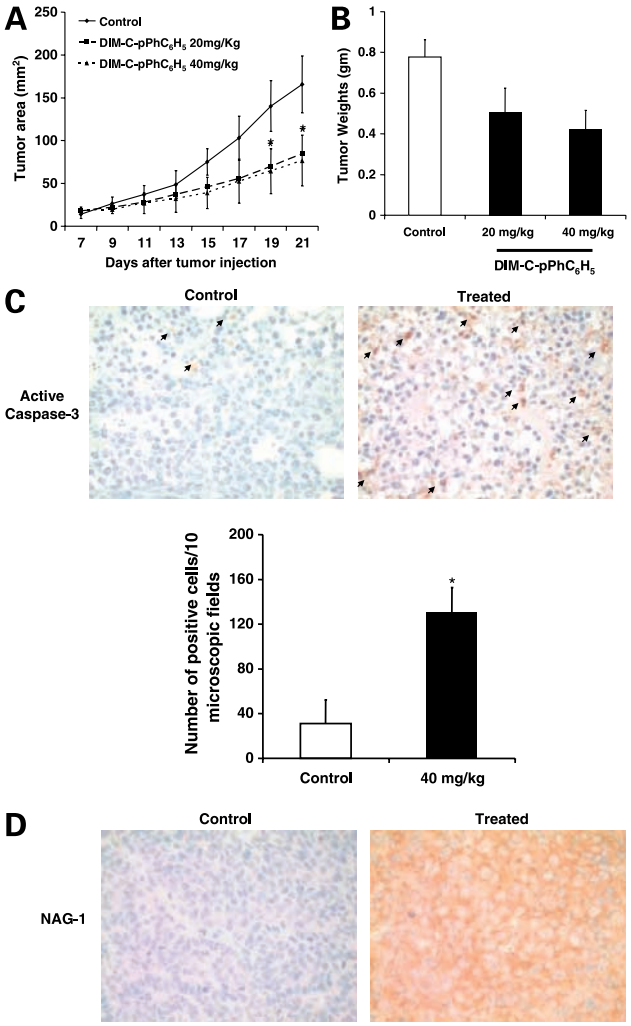

Figure 6. *In vivo* antitumorigenic activity of DIM-C-pPhC$_6$H$_5$. Tumor areas (**A**) and weights (**B**). When palpable tumors were first observed (15 mm^2), athymic nude mice bearing SW480 colon cancer cell xenografts were given corn oil (control) or DIM-C-pPhC$_6$H$_5$ (20 or 40 mg/kg/d) in corn oil by oral gavage, and tumor areas and weights were determined as described in Materials and Methods. *Points* (**A**) and *columns* (**B**), mean for 10 animals for each treatment group; *bars,* SE. *, $P < 0.05$, significant decrease in tumor areas and weights in the treatment groups compared with the corn oil (control) group. **C,** caspase-3 expression was quantitated by immunohistochemical analysis of caspase-3 in tumor sections from control and treated (DIM-C-pPhC$_6$H$_5$, 40 mg/kg/d) mice as described in Materials and Methods. *Arrows,* positive caspase-3 staining. **D,** immunohistochemical analysis of NAG-1. Tumors from control or DIM-C-pPhC$_6$H$_5$-treated mice were analyzed for NAG-1 expression by immunohistochemistry as described in Materials and Methods. There were no significant differences in animal weight gain, organ weights, or histopathology in any of the treatment groups.

were dependent on their expression of wild-type or mutant K422Q PPARγ where rosiglitazone responsiveness was observed only in cells (e.g., HT-29) expressing wild-type receptor (21).

PPARγ-active C-DIMs inhibit growth of HT-29 and HCT-15 colon cancer cell lines expressing wild-type and mutant PPARγ, respectively, and these compounds also induce caveolin-1 in both cell lines (37). The results in Figs. 1 and 2 show that the C-DIM compounds but not rosiglitazone inhibit SW480 cancer cell growth and induced PPARγ-dependent transactivation. These data are similar to those previously reported in rosiglitazone-nonresponsive HCT-15 cells, suggesting that SW480 cells may also express mutant PPARγ, which is not responsive to the growth inhibitory effects of rosiglitazone.

The short-term effects of DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ on cell cycle protein expression and apoptosis in SW480 (Fig. 4) versus HT-29/HCT-15 cells (37) were different. Growth inhibitory concentrations (5.0 and 7.5 μmol/L) did not affect p21 (data not shown) or cyclin D1 expression in SW480, HT-29, or HCT-15 cells, whereas p27 protein levels were slightly elevated in SW480 cells after treatment with 7.5 μmol/L DIM-C-pPhCF$_3$ and 10 μmol/L DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$. Another major difference observed in this study with SW480 cells was the induction of apoptosis and down-regulation of cyclin D1 after treatment with 10 μmol/L DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ for 24 hours, whereas these responses were not observed in HT-29 or HCT-15 cells (37).

Despite the cell context–dependent differences in the effects of PPARγ-active C-DIMs in colon cancer cells, the results in Fig. 3 show that PPARγ-active C-DIMs induce caveolin-1 expression in SW480 cells after prolonged treatment (3 days) with concentrations as low as 2.5 μmol/L. This response in SW480 cells was inhibited by GW9662 and similar results were observed for PPARγ-active C-DIMs and for CDDO and related compounds in colon cancer cells (37, 43). In this study, we also observed PPARγ-dependent up-regulation of phosphorylated Akt in SW480 cells and similar results have been observed for CDDO and related compounds in the same cell line (43). The induction of caveolin-1 at the lower doses (<7.5 μmol/L) of DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ is consistent with the growth inhibitory effects of these compounds because caveolin-1 expression has been linked to inhibition of colon cancer cell/tumor proliferation in both *in vitro* and *in vivo* models (44, 45). The observation that DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ induce phosphatidylinositol 3-kinase–dependent phosphorylation of Akt, which is normally a cell survival pathway, was also consistent with the observed growth inhibitory effects of these compounds. Phosphatidylinositol 3-kinase and caveolin-1 coexpression sensitizes HeLa and 293 cells to the cytotoxicity of arsenite and hydrogen peroxide (46) and sensitizes L929 cells to tumor necrosis factor α–induced cell death (47).

Several studies show that PPARγ agonists induce both receptor-dependent and receptor-independent responses (26, 27, 29, 36–39, 41–43), and in this study, these pathways are separable at different concentrations of C-DIMs. At low concentrations (≤7.5 μmol/L), DIM-C-pPhCF$_3$ and DIM-C-pPhC$_6$H$_5$ induce receptor-dependent up-regulation of caveolin-1 (Fig. 3), whereas at higher concentrations (>7.5 μmol/L) these same compounds induce apoptosis, which was not inhibited by GW9662, a PPARγ antagonist (Fig. 4A). Similar results were observed for CDDO and

related compounds in SW480 cells (43). Interestingly, there seems to be a narrow concentration range for the switch between receptor-dependent and receptor-independent responses. Moreover, induction of PPARγ-dependent caveolin-1 expression by ≥7.5 μmol/L DIM-C-pPhCF$_3$ or DIM-C-pPhC$_6$H$_5$ is lost in cells treated with 10 μmol/L concentrations of these same compounds (Fig. 4B) where extensive apoptosis is observed. In other cell lines, we have recently shown that C-DIMs induce endoplasmic reticulum stress, which leads to activation of DR5 and the extrinsic pathway for apoptosis (41); however, this response was not observed in SW480 cells (Fig. 4C). In contrast, both DIM-C-pPhC$_6$H$_5$ and DIM-C-pPhCF$_3$ induced NAG-1 expression at concentrations (≥7.5 μmol/L) similar to those required for induction of apoptosis (PARP cleavage) and both responses were PPARγ independent. NAG-1 is up-regulated by different classes of compounds that inhibit growth and induce apoptosis in colon and other cancer cell lines (29, 41, 48–51), and a recent study showed that inhibition of NAG-1 induction by sulindac sulfide using RNA interference also reversed the growth inhibitory effects of this compound in SKOV3 cells (50). Moreover, overexpression of NAG-1 in breast cancer cells significantly decreased cell viability (52), and purified NAG-1 induced apoptosis in prostate cancer cells (53). These results suggest that PPARγ-independent induction of apoptosis in SW480 cells by C-DIM compounds is due to up-regulation of NAG-1 expression. These *in vitro* responses are also paralleled in the athymic nude mouse xenograft studies where DIM-C-pPhC$_6$H$_5$ not only inhibits tumor growth but also induces caspase-3 and NAG-1 expression in the tumors (Fig. 6).

In summary, our results show that PPARγ-active C-DIMs inhibit SW480 cell proliferation and inhibit colon tumor growth in an athymic nude mouse xenograft model (Fig. 6). The antitumorigenic activity of these compounds may be due to activation of both PPARγ-dependent (caveolin-1) or PPARγ-independent (NAG-1) responses; however, the relative contributions of these pathways to the antitumorigenic action of the C-DIMs is unknown. Ongoing studies using RNA interference (*in vitro*) and transgenic mouse models (*in vivo*) will provide critical insights on the relative importance of the receptor-dependent and receptor-independent responses to the antitumorigenic activity of C-DIMs and guide development of more effective C-DIM analogues for applications in colon cancer chemotherapy.

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