

1,1-Bis(3'-indolyl)-1-(*p*-substituted phenyl)methanes inhibit proliferation of estrogen receptor-negative breast cancer cells by activation of multiple pathways

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Abstract 1,1-Bis(3'-indolyl)-1-(*p*-substituted phenyl)methanes containing *para*-trifluoromethyl (DIM-C-pPhCF₃), *t*-butyl (DIM-C-pPh_tBu), and phenyl (DIM-C-pPhC₆H₅) groups are methylene-substituted diindolylmethanes (C-DIMs) that activate peroxisome proliferator-activated receptor γ (PPAR γ) in estrogen receptor α -negative MDA-MB-231 and MDA-MB-453 breast cancer cells. C-DIMs inhibit breast cancer cell proliferation; however, inhibition of G₀/G₁ to S phase progression and cyclin D1 downregulation was observed in MDA-MB-231 but not MDA-MB-453 cells. Nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1), a transforming growth factor β -like peptide, was also induced by these compounds, and the response was dependent on cell-context dependent activation of kinase pathways. However, inhibition of cell growth, induction of NAG-1 and activation of kinases by C-DIMs were not inhibited by PPAR γ antagonists. Despite the induction of NAG-1 and downregulation of the antiapoptotic protein survivin by

C-DIMs in both MDA-MB-231 and MDA-MB-453 cells, apoptotic cell death was not observed. Nevertheless, the cytotoxicity of C-DIMs *in vitro* was complemented by inhibition of tumor growth in athymic nude mice bearing MDA-MB-231 cells as xenografts and treated with DIM-C-pPhC₆H₅ (40 mg/kg/day). The growth inhibition of tumors derived from highly aggressive MDA-MB-231 cells suggests a potential role for the C-DIM compounds in the clinical treatment of ER-negative breast cancer.

Keywords Breast cancer · C-DIMs · ER negative · Growth inhibition · PPAR γ

Introduction

Peroxisome proliferator-activated receptor γ (PPAR γ) is a member of the nuclear receptor superfamily of transcription factors [1–3]. The PPAR subfamily includes α , β (δ) and γ sub-types that bind fatty acids and lipids, and the receptors play a role in lipid metabolism and metabolic diseases that include insulin-resistance, coronary artery disease and hyperlipidemia [3–6]. PPARs are differentially expressed in various tissues and exhibit some overlapping or interconnected activities such as in cholesterol transport, metabolism and function [5]. PPAR α regulates genes involved in fatty acid utilization during fasting and PPAR α agonists are drugs used for treating patients with hyperlipidemia. Thiazolidinediones (TZDs) such as rosiglitazone and pioglitazone are PPAR γ agonists which are insulin-sensitizing agents used extensively for treatment of type II diabetes [3–6].

PPAR γ is overexpressed in multiple tumor types and their derived cancer cell lines [7, 8] and is considered a potential target for development of anticancer drugs. Not

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surprisingly, several different structural classes of PPAR γ agonists have been identified and their anticancer activities have been investigated. These include TZDs, 15-deoxy- Δ^{12-14} -prostaglandin J2 (PGJ2), 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and related compounds, 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl)methanes [methylene-substituted diindolylmethanes (C-DIMs)], and several other chemical classes [1–3, 9–16]. All PPAR γ agonists typically induce fat cell differentiation and, using RNA interference and PPAR γ antagonists, several PPAR γ -dependent responses have been characterized in various cancer cell lines. For example, CDDO induces the tumor suppressor gene caveolin 1 in colon cancer cells and this response is inhibited by the PPAR γ antagonist GW9662. However, many other CDDO-induced responses are receptor-independent or the role of PPAR γ has not been investigated [17–23].

Research in this laboratory has identified 1,1-bis(3'-indolyl)-1-(*p*-trifluoromethylphenyl)methane (DIM-C-pPhCF₃), 1,1-bis(3'-indolyl)-1-(*p*-*t*-butylphenyl)methane (DIM-C-pPh_tBu), 1,1-bis(3'-indolyl)-1-(*p*-biphenyl)methane (DIM-C-pPhC₆H₅) as PPAR γ agonists in several different cancer cell lines [13, 24–32]. The PPAR γ -active C-DIMs induce PPAR γ -dependent transactivation in several different cancer cells transfected with receptor-responsive constructs and their induction of caveolin-1 and p21 is also PPAR γ -dependent but varied with cell context [25, 30]. C-DIM compounds also induce receptor-independent responses including endoplasmic reticulum (ER) stress, activation of multiple kinases, induction of non-steroidal anti-inflammatory drug activated gene-1 (NAG-1), and proteasome-dependent downregulation of cyclin D1. A previous study shows that PPAR γ -active C-DIMs inhibits growth of ER-positive MCF-7 breast cancer cells, induces apoptosis, and downregulates expression of estrogen receptor α and cyclin D1 proteins [13]. In this study, we show that DIM-C-pPhCF₃, DIM-C-pPh_tBu, DIM-C-pPhC₆H₅ inhibited growth of MDA-MB-231 and MDA-MB-453 cells and induced transactivation in cells transfected with a PPAR γ -responsive construct. The C-DIM compounds differentially affected cell cycle progression and modulation of p21, p27 and cyclin D1 expression in both cell lines and induced NAG-1 in MDA-MB-231 and MDA-MB-453 cells through activation of different kinases. These responses were not inhibited after cotreatment with the PPAR γ antagonist 2-chloro-5-nitro-*N*-phenylbenzamide (GW9662). In addition, C-DIMs also decreased expression of the anti-apoptotic protein survivin in these cells; however, this was not accompanied by apoptosis. In athymic nude mice bearing MDA-MB-231 cells as xenografts, treatment with 40 mg/kg/day of DIM-C-pPhC₆H₅ significantly inhibited tumor growth, confirming the anticarcinogenic activity of C-DIMs both in vivo and in vitro.

Materials and methods

Cells, chemicals and other materials

MG132 was obtained from Sigma Chemical Co. (St. Louis, MO). The human breast cancer cell lines MDA-MB-231 and MDA-MB-453 were obtained from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231 cells were maintained in DMEM:F-12 supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (FBS), and 2 ml/l antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO). MDA-MB-453 cells were maintained in RPMI supplemented with 0.15% sodium bicarbonate, 0.12% HEPES, 10% FBS, and 2 ml/l antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO). Cells were grown in 150 cm² culture plates in an air/CO₂ (95:5) atmosphere at 37°C and passaged approximately every 5 days. p21 (C-19), p27 (C-19), cyclin D1 (M-20), cyclin D3 (C-16), caveolin-1 (N-20), caveolin-2 (N-20), p-Erk (K-23), Erk (E-4), p-Akt (Ser473), Akt (H-136), p-c-Jun (KM-1), c-Jun (D), Grp78 (H-129), ATF-3 (C-19), survivin (FL-142) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The NAG-1 antibody was purchased from Upstate (Lake Placid, NY). Horseradish peroxidase substrate for Western blot analysis was purchased from NEN Life Science Products (Boston, MA). 2-Chloro-5-nitro-*N*-phenylbenzamide (GW9662) was prepared in this laboratory (>98% pure).

Cell proliferation assays

Cells were plated at a density of 3–5 × 10⁴/well in 12-well plates and media was replaced the next day with DMEM:Ham's F-12 media containing 2.5% charcoal-stripped FBS and either vehicle (DMSO) or the indicated ligand and concentration. Similar cytostatic responses were observed for stripped and non-stripped serum; however, in this study, experiments were carried out using stripped serum which would minimize the endogenous effects of ER β which is expressed in MDA-MB-231 cells. Fresh media and compounds were added every 48 h. Cells were counted at the indicated times using a Coulter Z1 cell counter. Each experiment was completed in triplicate and results are expressed as means \pm SE for each determination.

Transient transfection assays

Breast cancer cells were plated in 12-well plates at 1.5 × 10⁵ cells/well in DME-F12 media supplemented with 2.5% charcoal-stripped FBS. PPAR γ -Gal4 (0.2 μ g/well), Gal4-LUC (0.5 μ g/well) and pCDNA3.1-His-LacZ (0.04 μ g/well, Invitrogen, Carlsbad, CA) expression plasmid (for

normalization of transfection efficiency) were transiently cotransfected into MDA-MB-231 and MDA-MB-453 cells using Lipofectamine according to the manufacturer's protocol. After 5–6 h, cells were treated for 24 h with fresh 2.5% stripped bovine medium containing 1, 5, or 10 μM DIM-C-pPhCF₃, DIM-C-pPhtBu, DIM-C-pPhC₆H₅ dissolved in DMSO, or DMSO alone as a solvent control. After 24 h of treatment, cells were then lysed with 150 μl of 1 \times reporter lysis buffer from Promega (Madison, WI) and 30 μl of cell extract were used for luciferase and β -gal assays. Significance was determined by SuperANOVA ($P < 0.05$).

Fluorescence-activated cell-sorting assays (FACS)

MDA-MB-231 cells and MDA-MB-453 cells were treated with either the vehicle (DMSO) or the indicated compounds for 48 h. Cells were trypsinized, centrifuged and resuspended in staining solution containing 50 mg/ml propidium iodide, 4 mM sodium citrate, 30 units/ml RNase, and 0.1% Triton X-100. After incubation at 37°C for 10 min, sodium chloride was added to give a final concentration of 0.15 M. Cells were analyzed on a FACS Calibur flow cytometer using CellQuest acquisition software. PI fluorescence was collected through a 585/42 nm bandpass filter, and list mode data were acquired on a minimum of 20,000 single cells defined by a dot plot of PI width versus PI area. Data analysis was performed in ModFit LT using PI width versus PI to exclude cell aggregates.

Western blots assays

MDA-MB-231 and MDA-MB-453 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 indicated compounds. Whole cell lysates were obtained using high salt buffer [50 mM HEPES, 500 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol and 1% Triton X-100 pH 7.5 and 5 $\mu\text{l}/\text{ml}$ of Protease Inhibitor Cocktail]. Protein samples were incubated at 100°C for 2 min, separated on 10% SDS-PAGE at 120 V for 3–4 h in 1 \times running buffer [25 mM Tris-base, 192 mM glycine, and 0.1% SDS (pH 8.3)] and transferred to polyvinylidene difluoride membrane at 0.2 V for 16 h at 4°C in 1 \times transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 0.025% SDS). The PVDF membrane was blocked in 5% TBST-Blotto [10 mM Tris-HCl, 150 mM NaCl (pH 8.0), and 0.05% Triton X-100 and 5% non-fat dry milk] with gentle shaking for 30 min and incubated in fresh 5% TBST-Blotto at 1:200–1:1,000 with primary antibody overnight at 4°C with gentle shaking. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody (1:5,000) in 5% TBST-Blotto for 2 h.

The membrane was washed with TBST for 10 min and incubated with 10 ml of chemiluminescence substrate for 1.0 min and exposed to Kodak X-OMAT AR autoradiography film.

Anti-tumor efficacy studies

Female Balb/c athymic nude mice (Nu^{-/-}), 4–6 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a ventilated rack system. Irradiated chow and autoclaved water were provided ad libitum. The mice were acclimated for 7 days prior to beginning the experiments. Athymic nude mice (Nu^{-/-}) were injected i.p. with 75 μg of a rat anti-mouse asialo GM1 antibody (Wako Chemical Company, Richmond, VA) to reduce natural killer cells. Injections were done on days 4 and 2 prior to the injection of MDA-MB-231 cells. At day 0, mice were injected subcutaneously in the left flank with 10⁷ MDA-MB-231 cells in 100–200 μl serum-free medium. Three groups of mice (12 mice/group) were then treated i.p. with 40 mg/kg DIM-C-pPhC₆H₅ in 50 μl placebo, 50 μl placebo or 50 μl PBS, every day for 35 total injections starting at day 4 post-tumor inoculations. Tumor size was measured with calipers, based on the formula $L \times W^2$ where L is the length and W is the width of the tumor. Moribund mice and mice whose tumor burdens exceeded 20% of their body weight were euthanized following institutional regulation.

Results

DIM-C-pPhCF₃, DIM-C-pPhtBu and DIM-C-pPhC₆H₅ are PPAR γ agonists and inhibit growth of breast, bladder, colon, ovarian and pancreatic cancer cells [13, 24–32]. Results in Fig. 1A and B shows that these PPAR γ -active C-DIMs significantly induced transactivation in ER-negative MDA-MB-231 and MDA-MB-453 cells transfected with PPAR γ -GAL4/pGAL4. Rosiglitazone, a thiazolidinedione PPAR γ agonist, also induced transactivation in both cell lines. GW9662 is a PPAR γ antagonist, and the induction of transactivation by DIM-C-pPhCF₃, DIM-C-pPhtBu and DIM-C-pPhC₆H₅ in MDA-MB-231 (Fig. 1C) and MDA-MB-453 cells (Fig. 1D) transfected with PPAR γ -GAL4/pGAL4 was inhibited after cotreatment with GW9662. These results demonstrate that the PPAR γ -active C-DIMs activate PPAR γ in ER-negative breast cancer cell lines, and these results were similar to those previously reported for the same compounds in MCF-7 cells [13].

Results illustrated in Fig. 2 show that DIM-C-pPhCF₃, DIM-C-pPhtBu and DIM-C-pPhC₆H₅ inhibit proliferation of ER-negative MDA-MB-231 and MDA-MB-453 breast cancer cells and IC₅₀ values were generally between 1 and

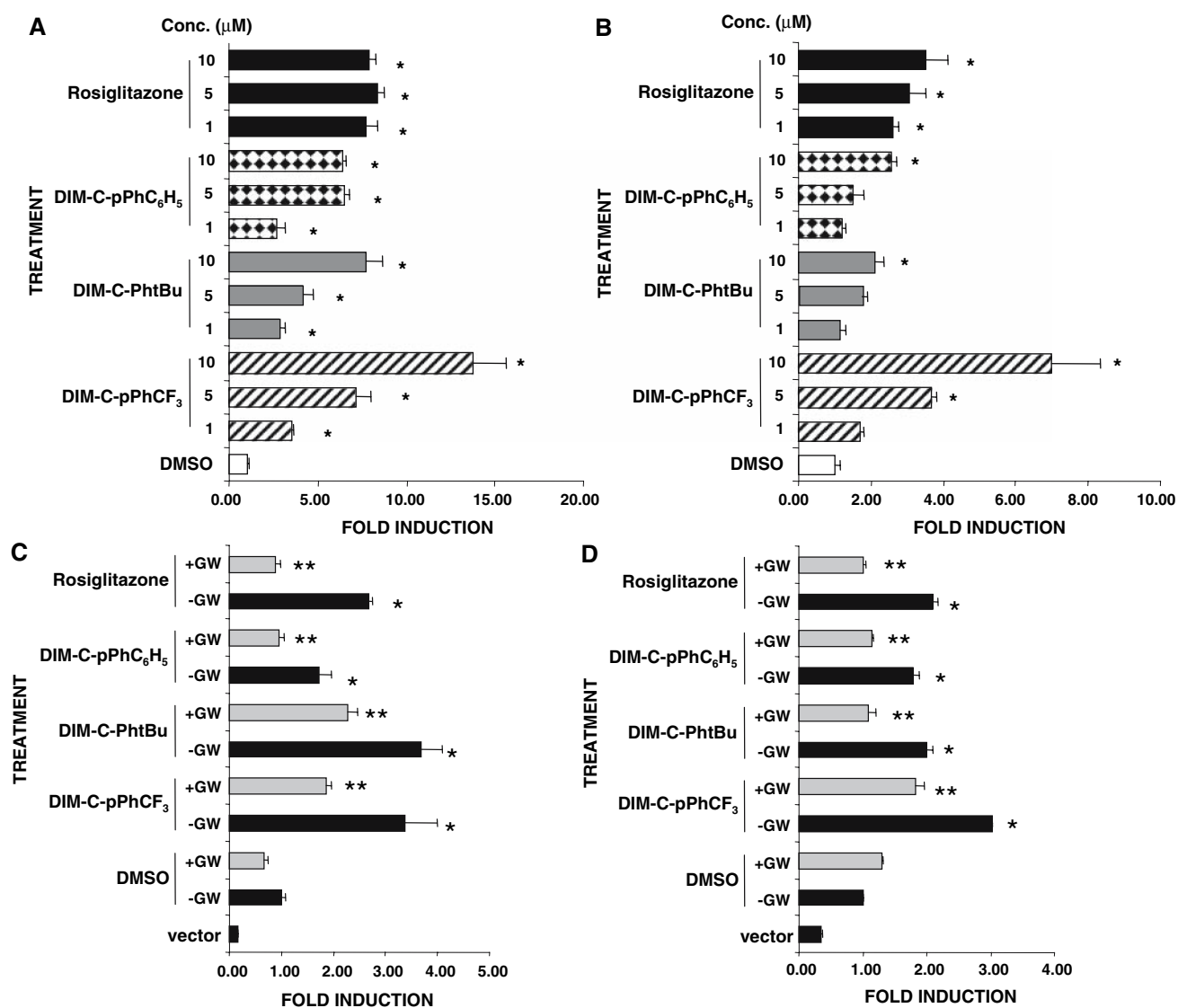


Fig. 1 Ligand-induced activation of PPAR γ and effects of PPAR γ antagonists. Ligand-dependent activation of PPAR γ -GAL4/pGAL4 in MDA-MB-231 (**A**) and MDA-MB-453 (**B**) cells. Cells were transfected with PPAR γ -GAL4/pGAL4, treated with 1, 5, or 10 μ M of DIM-C-pPhCF₃, DIM-C-pPhtBu, DIM-C-pPhC₆H₅ and rosiglitazone (positive control), and luciferase activity was determined as described in Materials and methods. Results of all transactivation studies in this figure are presented as means \pm SE for at least three separate determinations for each treatment group, and significant

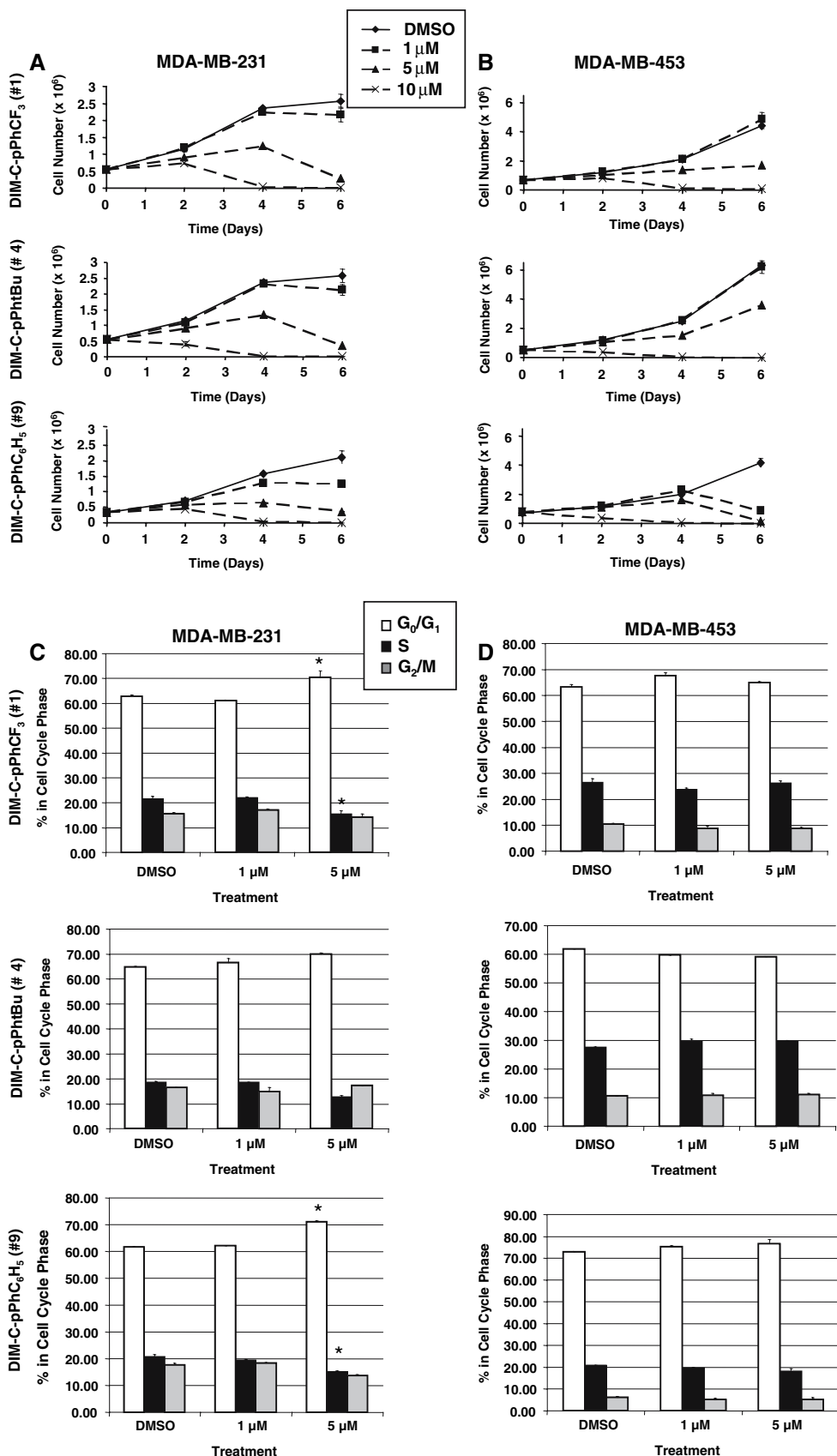
($P < 0.05$) induction compared with solvent (DMSO) control is indicated (*). Inhibition of transactivation in MDA-MB-231 (**C**) and MDA-MB-453 (**D**) cells by PPAR γ antagonist GW9662. Cells were transfected with PPAR γ -GAL4/pGAL4, treated with 10 μ M rosiglitazone or C-substituted DIMs alone or in combination with 7.5 μ M GW9662, and luciferase activities were determined as described in (**A**). Significant ($P < 0.05$) inhibition of induced transactivation by GW9662 is indicated (**)

5 μ M. The only exception was the decreased potency of DIM-C-pPhtBu in MDA-MB-453 cells where the IC₅₀ was slightly greater than 5 μ M. MDA-MB-231 and MDA-MB-453 cells were treated with C-DIMs alone for 24 or 48 h or in combination with 10 μ M GW9662 (PPAR γ antagonist). GW9662 did not significantly block growth inhibition by C-DIMs (data not shown). C-DIMs also affected the % distribution of MDA-MB-231 cells in G₀/G₁, S and G₂/M phase of the cell cycle (Fig. 2C). Even at concentrations as low as 1 or 5 μ M, the C-DIM compounds consistently increased the

% of cells in G₀/G₁ and decreased the % of cells in S phase of the cell cycle. In contrast, these same concentrations had minimal effects on the % distribution of MDA-MB-453 cells in G₀/G₁, S or G₂/M phase of the cell cycle (Fig. 2D), indicating some cell context-dependent differences in the effects of C-DIMs on cell cycle progression.

C-DIMs modulate p21, p27 (induction), and decrease cyclin D1 expression in breast, colon and pancreatic cancer cells [13, 25, 30]. Results in Fig. 3A show that after treating MDA-MB-231 cells for 12 h with 10 μ M C-DIMs,

Fig. 2 Growth inhibition studies and fluorescence-activated cell sorting (FACS) analysis. MDA-MB-231 (A) and MDA-MB-453 (B) breast cancer cells were treated with 1–10 μ M DIM-C-pPhCF₃, DIM-C-pPhBu, and DIM-C-pPhC₆H₅ for 6 days, and cell numbers were determined using a Coulter Counter as described in the Materials and methods. Results are expressed as means \pm SE for three separate determinations at each time point. MDA-MB-231 (C) and MDA-MB-453 (D) cells were treated for 48 h with 1–5 μ M DIM-C-pPhCF₃, DIM-C-pPhBu and DIM-C-pPhC₆H₅, and analyzed by FACS analysis as described in the Materials and methods. Experiments were carried out in 2.5% charcoal-stripped serum and DMSO served as the solvent control. Results are expressed as means \pm SE for three separate determinations for each treatment group and significance ($P < 0.05$) is indicated (*)



there was decreased expression of cyclin D1 and slightly increased levels of p21 or p27 proteins and similar results were observed after treatment for 24 h (data not shown). Cotreatment of both cell lines with C-DIMs plus GW9662 did not alter the effects of C-DIMs on p21, p27 or cyclin D1 expression (data not shown). In contrast, the PPAR γ -active C-DIMs did not affect cyclin D1, p21 or p27 levels in MDA-MB-453 cells after treatment for 12 h (Fig. 3B) or 24 h (data not shown). The C-DIM-induced downregulation of cyclin D1 in MDA-MB-231 cells was inhibited in cells cotreated with the proteasome inhibitor MG132 (Fig. 3C) and this PPAR γ -independent response was previously observed with these compounds in MCF-7 cells [13]. Thus, the major effects of the PPAR γ -active C-DIMs was induction of proteasome-dependent degradation of cyclin D1 (Fig. 3C) which correlates with the inhibition of G₀/G₁ to S phase progression in MDA-MB-231 cells (Fig. 2D). However, results of inhibition studies indicate that these responses were PPAR γ -independent.

C-DIMs induce several growth inhibitory factors or pathways in cancer cell lines including receptor-dependent induction of caveolin-1 and receptor-independent induction of ER stress and NAG-1 [24, 26, 28]. In contrast, C-DIM compounds did not affect expression of the stress protein GRP78 in MDA-MB-231 or MDA-MB-453 cells (data not shown). Figure 3D shows that caveolin 1 expression is not induced in MDA-MB-453 cells by the C-DIM compounds, whereas in MDA-MB-231 cells, concentration-independent effects on caveolin-1 expression were observed. In contrast, the same compounds induced caveolin 1 in colon cancer cells and cotreatment with PPAR γ antagonists blocked caveolin-1 induction [25].

NAG-1 is a proapoptotic and growth inhibitory protein induced in cancer cell lines by diverse agents including C-DIM compounds [26, 33–37], and results in Fig. 4A and B shows that NAG-1 is induced in MDA-MB-453 and MDA-MB-231 cells after treatment with 10 μ M DIM-C-pPhCF₃, DIM-C-pPhTbu and DIM-C-pPhC₆H₅. ATF3,

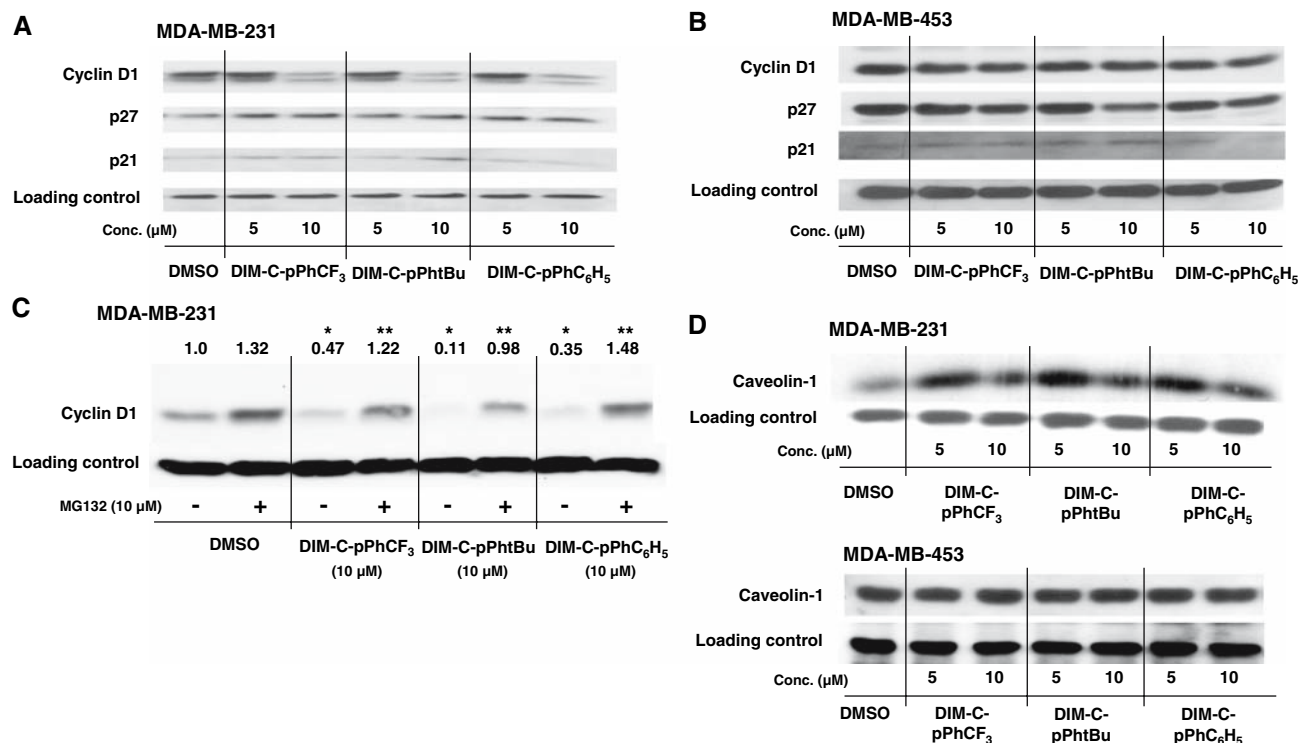


Fig. 3 Modulation of cell cycle, ER stress and caveolin proteins. MDA-MB-231 (A) or MDA-MB-453 (B) cells were treated with 5 or 10 μ M DIM-C-pPhCF₃ or DIM-C-pPhC₆H₅ for 12 h, and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. MDA-MB-231 (C) cells were pre-treated for 30 min with 10 μ M proteasome inhibitor MG132 or vehicle control and subsequently treated for 12 h with DMSO, 10 μ M DIM-C-pPhCF₃, or DIM-C-pPhC₆H₅ for 12 h, and whole cell lysates were analyzed by Western blot analysis as described in Materials and methods. Experiments were done in triplicate and results shown are typical of cyclin D1, p21, and p27 protein levels for treatment

replicates. (D) MDA-MB-231 and MDA-MB-453 cells were treated with DIM-C-pPhCF₃, DIM-C-pPhTbu and DIM-C-pPhC₆H₅ for 72 h, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and methods. Experiments were done in triplicate and results shown are typical of treatment replicates. These compounds did not induce GRP78, a marker of ER stress, whereas thapsigargin, an ER stress inducer, significantly increased GRP78 expression (data not shown). Significant ($P < 0.05$) induction or inhibition by C-DIMs is indicated (*) and the effects of MG132 on cyclin D1 downregulation are indicated (**)

which is often co-induced with NAG-1, was also elevated in the ER-negative breast cancer cells treated with PPAR γ -active C-DIMs. Using DIM-C-pPhC₆H₅ as a model, we showed that NAG-1 induction was not inhibited by the PPAR γ antagonist GW9662 (Fig. 4A, B). Similar results were observed for PPAR γ -active C-DIMs in colon cancer cells where induction of NAG-1 was also PI3-K dependent [26]. NAG-1 is induced through multiple pathways including activation of kinases in colon cancer cells where PPAR γ -active C-DIMs induced NAG-1 through PI3-K dependent activation of Egr-1 which binds the proximal region of the NAG-1 promoter [26]. The induction of NAG-1 by DIM-C-pPhC₆H₅ in the absence or presence of inhibitors of p38 MAPK (SB203580), JNK (SP600125), PKC (GF109203X), p44/42 MAPK (PD98059), and PI3-K (LY294002) is summarized in Fig. 4C. In MDA-MB-453 cells, inhibition of NAG-1 induction by DIM-C-pPhC₆H₅ was observed in cells cotreated with p44/42 MAPK, PI3-K and JNK inhibitors with the latter inhibitor being the most effective. In contrast, only LY294002 (PI3-K inhibitor) blocked induction of NAG-1 by DIM-C-pPhC₆H₅ in MDA-MB-231 cells, and the JNK inhibitor actually enhanced induction of NAG-1 by the C-DIM compound (Fig. 4C). Results in Fig. 4D show that DIM-C-pPhC₆H₅ induces PI3-K-dependent phosphorylation of Akt in MDA-MB-231 cells and phosphorylation of MAPK, Akt and c-Jun in MDA-MB-453 cells (Fig. 4E), and these induced kinases play a role in the induction of NAG-1 in the same cell lines. Both cell lines were also cotreated with DIM-C-pPhC₆H₅ plus the PPAR γ antagonist GW9662, and the latter compound did not inhibit activation of kinases by DIM-C-pPhC₆H₅ (data not shown).

Previous studies showed that DIM, ring-substituted DIMs, and C-DIMs induced caspase-dependent apoptosis in ER-positive MCF-7 cells, whereas apoptosis was not observed in MDA-MB-231 cells [13, 38]. The effects of the PPAR γ -active C-DIM compounds on induction of apoptosis were investigated in both MDA-MB-231 and MDA-MB-453 cells by determining caspase-dependent PARP cleavage, bax/bcl-2 expression (Fig. 5A, B), and effects on the antiapoptotic protein survivin (Fig. 5C, D). There was an increase in bax and a decrease in bcl-2 expression observed after treatment of MDA-MB-231 and MDA-MB-453 cells with C-DIMs; however, activation of the intrinsic pathway for apoptosis as evidenced by caspase-dependent PARP cleavage was not observed. The antiapoptotic protein survivin was expressed in MDA-MB-231 and MDA-MB-453 cells, and treatment with PPAR γ -active C-DIM compounds decreased survivin protein expression (Fig. 5C, D); however, this response was insufficient for activation of caspase-dependent apoptotic pathways.

Results in Fig. 6 show that treatment with DIM-C-pPhC₆H₅ (40 mg/kg/day) decreased tumor growth in

athymic nude mice bearing MDA-MB-231 cells as xenografts. These results, coupled with the cancer cell growth inhibition studies (Fig. 2), clearly show that PPAR γ -active C-DIMs effectively inhibited growth of ER-negative breast cancer cells, and this was comparable to inhibition of colon and bladder tumor growth in mouse xenograft models [28, 31].

Discussion

Several different structural classes of PPAR γ agonists have been identified and many of these, including PGJ2, thiazolidinediones and CDDO, inhibit proliferation of breast and other cancer cell lines through multiple receptor-dependent and -independent pathways [17–23, 39–46]. For example, in one study 10 μ M PGJ2 induced rapid morphological changes associated with apoptosis in MDA-MB-231 cells, whereas 100 μ M troglitazone induced minimal apoptosis after prolonged (50 h) treatment [39]. CDDO alone did not induce apoptosis in ER-positive T47D or ER-negative MDA-MB-453 cells [41]; however CDDO enhanced TRAIL-induced apoptosis in both cell lines. PPAR γ -active C-DIM compounds have been identified in this laboratory as a new class of PPAR γ agonists and several receptor-dependent and -independent responses have been characterized. Results of transactivation studies (Fig. 1) clearly demonstrate that the three C-DIMs induced PPAR γ -dependent activity in MDA-MB-231 and MDA-MB-453 cells transfected with PPAR γ -GAL4/pGAL4 constructs as previously reported in other cancer cell lines [13, 25–32].

PPAR γ -active C-DIMs inhibit growth of ER-negative MDA-MB-231 and MDA-MB-453 cells (Fig. 2) but induced only a modest increase in cells in G₀/G₁ and decrease in cells in S-phase in MDA-MB-231 but not in MDA-MB-453 cells (Fig. 2C, D). p21 and p27 were only slightly induced in MDA-MB-231 cells but not in MDA-MB-453 cells, and 5–10 μ M C-DIMs induced cyclin D1 downregulation in MDA-MB-231 but not MDA-MB-453 cells (Fig. 3A–C). This was most pronounced after treatment for 12 h and was reversed by the proteasome inhibitor MG132 (Fig. 3C), whereas the PPAR γ antagonist GW9662 did not affect either the growth inhibitory effects of C-DIMs or the decreased expression of cyclin D1 in MDA-MB-231 cells (data not shown). The results suggest that inhibition of MDA-MB-231 cell proliferation by C-DIMs is primarily due to decreased cyclin D1 expression, whereas other factors must be involved for inhibition of MDA-MB-453 cell proliferation and these effects were PPAR γ -independent.

C-DIMs induce receptor-dependent caveolin 1 expression and receptor-independent ER stress in pancreatic,

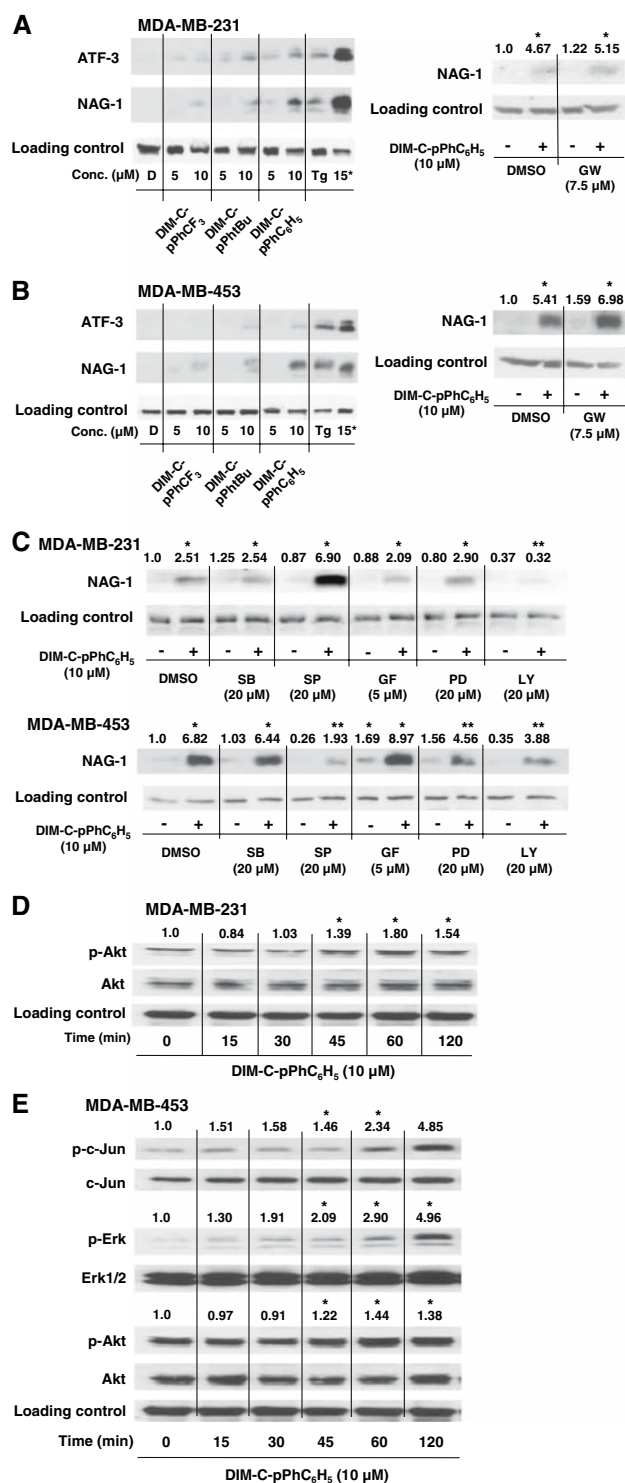


Fig. 4 Induction of NAG-1 proteins (**A**, left and **B**, left). MDA-MB-231 (**A**, left) and MDA-MB-453 (**B**, left) cells were treated with DMSO or 5–10 μM DIM-C-pPhC₆H₅, DIM-C-pPhC₆H₅ or 0.3 μM thapsigargin (Tg) for 24 h, and whole cell lysates were analyzed by Western blot analysis for NAG-1 and ATF3. 15* represents whole cell lysate sample isolated from SW480 cells and treated with 15 μM DIM-C-pPhC₆H₅ and, based on previous published data, served as a positive control for NAG-1 and ATF3 induction. PPAR γ -independent activation of NAG-1 (**A**, right and **B**, right). MDA-MB-231 (**A**, right) or MDA-MB-453 cells (**B**, right) were treated for 24 h with DMSO or 10 μM DIM-C-pPhC₆H₅, and with or without co-treatment with 10 μM PPAR γ inhibitor GW9662. Whole cell lysates were analyzed by Western blot analysis and probed for NAG-1. Role of kinases in NAG-1 induction (**C**). MDA-MB-231 and MDA-MB-453 cells were treated with DMSO or 10 μM DIM-C-pPhC₆H₅ and with or without co-treatment of the following kinase inhibitors: 20 μM p38 MAPK (SB203580), 20 μM JNK (SP600125), 5 μM PKC (GF109203X), 20 μM p44/42 MAPK (PD98059) and 20 μM PI3-K (LY294002) for 24 h. Whole cell lysates were probed for NAG-1. Activation of kinases in 0–120 min treatment with 10 μM DIM-C-pPhC₆H₅ (**D**, **E**). Whole cell lysates isolated from MDA-MB-231 (**D**) cells were analyzed by Western blot analysis and probed for p-Akt and Akt. MDA-MB-453 (**E**) cells were analyzed by Western blot analysis for p-Akt, Akt, p-Erk, Erk, p-c-Jun, c-Jun. Experiments were carried out in triplicate. Results shown are typical of treatment replicates, and values given in the figure demonstrate the magnitude of each treatment relative to that observed for DMSO (set at 1.0). Significant ($P < 0.05$) induction of NAG-1 or kinase activity (*) or inhibition of these responses by kinase inhibitors (**) are indicated

ATF3 (Fig. 4A, B) which is often co-regulated with NAG-1 [26, 36]. NAG-1 is induced by several proapoptotic and growth inhibitory agents including PPAR γ agonists such as the C-DIMs, troglitazone and PGJ2; however, the mechanisms of induction are variable. Baek and coworkers [39, 40] showed that induction of NAG-1 in HCT116 colon cancer cells by troglitazone and PGJ2 was PPAR γ -independent and -dependent, respectively, whereas induction of NAG-1 by C-DIMs was PPAR γ -independent [26]. Both troglitazone and C-DIMs induced NAG-1 through activation of PI3K. In ER-negative MDA-MB-453 cells activation of NAG-1 by DIM-C-pPhC₆H₅ was decreased in cells cotreated with the JNK inhibitor SP600125, the MAPK and PI3K inhibitors PD98059 and LY294002, respectively (Fig. 4C). These results correlated with DIM-C-pPhC₆H₅-dependent enhancement of all three kinase pathways in the same cell line (Fig. 4E) and these responses were receptor-independent (data not shown). However, in MDA-MB-231 cells, only the PI3-K inhibitor LY294002 blocked induction of NAG-1 by DIM-C-pPhC₆H₅ in MDA-MB-231 cells (Fig. 4C) and only this kinase was induced by the C-DIM compound in this cell line (Fig. 4D). Thus, induction of NAG-1 by DIM-C-pPhC₆H₅ in ER-negative breast cancer cells was due to cell context-dependent activation of kinases, and current studies in this laboratory are investigating upstream targets that are activated by C-DIMs and mediate selective activation of kinase pathways in different cell lines.

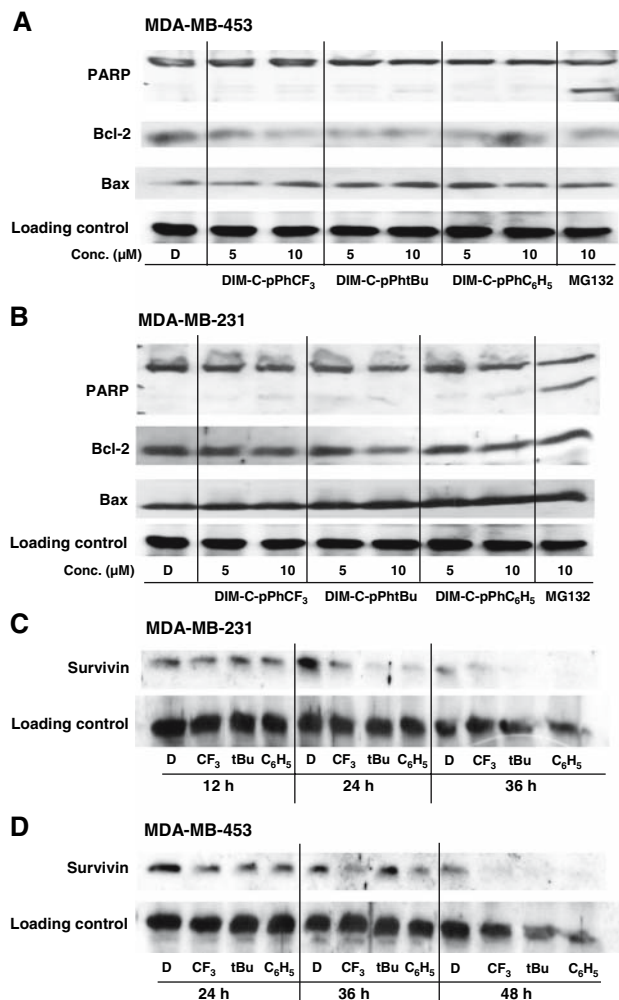


Fig. 5 Effects of PPAR γ agonists on apoptosis. MDA-MB-231 (A) and MDA-MB-453 cells (B) were treated with DMSO or 5–10 μ M DIM-C-pPhCF $_3$, DIM-C-pPhtBu and DIM-C-pPhC $_6$ H $_5$, or 10 μ M MG132 for 24 h. Whole cell lysates were analyzed by Western blot analysis for PARP112/85, bcl-2 and bax. Time-dependent activation of survivin. MDA-MB-231 (C) and MDA-MB-453 cells (D) were treated for 12–36 h or 24–48 h with DMSO or 10 μ M DIM-C-pPhCF $_3$, DIM-C-pPhtBu and DIM-C-pPhC $_6$ H $_5$. Whole cell lysates were analyzed by Western blot analysis and probed for survivin. Experiments were done in triplicate and results shown are typical of treatment replicates

Although NAG-1 is associated with apoptotic activity, we did not observe caspase-dependent PARP cleavage after treatment of MDA-MB-453 and MDA-MB-231 cells with PPAR γ -active C-DIM compounds, whereas the proteasome inhibitor MG132 (positive control) induced PARP cleavage (Fig. 5A, B). Bax/bcl-2 ratios were increased in MDA-MB-453 cells treated with 5 or 10 μ M PPAR γ -active C-DIMs. However, increased bax/bcl-2 ratios combined with C-DIM-dependent downregulation of the antiapoptotic protein survivin in both cell lines (Fig. 5C, D) was not accompanied by activation of caspase-mediated apoptosis.

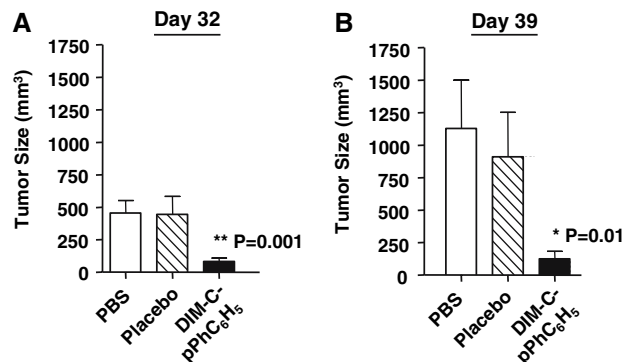


Fig. 6 In vivo antitumor activity of DIM-C-pPhC $_6$ H $_5$. MDA-MB-231 s.c. tumor size (mean \pm SE) in athymic mice treated i.p. daily starting at day 4 post-tumor inoculation for 35 total doses with PBS (50 μ l), placebo (50 μ l) and DIM-C-pPhC $_6$ H $_5$ (40 mg/kg in 50 μ l placebo). The tumor sizes were measured on day 35 and day 39. On day 32, the tumor sizes were 446.1 \pm 138.3 mm 3 (placebo treated), 454.3 \pm 97.5 mm 3 (PBS treated), 83.3 \pm 25.1 mm 3 (DIM-C-pPhC $_6$ H $_5$ treated) (*t*-test, *P* = 0.001 versus PBS treated group). On day 39, the tumor sizes were 909.9 \pm 342.6 mm 3 (placebo treated), 1129 \pm 371.9 mm 3 (PBS treated), 124.7 \pm 54.8 mm 3 (DIM-C-pPhC $_6$ H $_5$ treated) (*t*-test, *P* = 0.01 versus PBS treated group)

The failure to induce apoptosis was not unprecedented since other studies also show that ER-negative breast cancer cells are somewhat resistant to PPAR γ agonist-induced apoptosis [38, 39, 41]. Treatment of MDA-MB-231 cells with high concentrations of troglitazone (100 μ M) for 50 h induced apoptosis in only 30% of the cells [39], and CDDO did not cause caspase-dependent PARP cleavage in ER-negative MDA-MB-468 cells [41]. High concentrations of troglitazone (50 μ M) were cytotoxic to ER-negative MDA-MB-435 cells and decreased survivin expression [47], and similar results were observed for the C-DIM compounds (Figs. 2, 5C, D). Moreover, like the C-DIM compounds, troglitazone also did not induce apoptosis in MDA-MB-435 cells. However, the lack of induction of apoptosis by PPAR γ -active C-DIMs did not affect their in vitro cytotoxicity or their inhibition of tumor growth in athymic nude mice bearing MDA-MB-231 cells as xenografts (Fig. 6).

Although C-DIMs activate transfected PPAR γ -GAL4/pGAL4 in ER-negative breast cancer cells (Fig. 1), their effects as inhibitors of cell proliferation and cell cycle progression (Fig. 2) and activators of other growth inhibitory responses were receptor independent. Despite the potent antiproliferative effects of these compounds in vitro and in vivo, their downregulation of the antiapoptotic survivin protein, and induction of the proapoptotic NAG-1 protein in both cell lines, we did not observe caspase-dependent PARP cleavage (Fig. 5) or other indicators of apoptosis such as positive Annexin V staining (data not shown). In ongoing studies, we are investigating the potential role of other cell death pathways in breast cancer

cells and tumors in order to delineate critical markers associated with the anticarcinogenic activity of C-DIMs. The cytotoxicity of C-DIMs which is not accompanied by apoptosis is comparable to the effects reported for TZDs and CDDO in ER-negative cancer cell lines [41, 47], and similarities and differences in the effects of these compounds and C-DIMs are also being investigated.

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