1,1-Bis(3’-indolyl)-1-(p-substituted phenyl)methanes inhibit ovarian cancer cell growth through peroxisome proliferator–activated receptor–dependent and independent pathways

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Abstract
1,1-Bis(3’-indolyl)-1-(p-t-butylphenyl)methane (DIM-C-pPhtBu) is a peroxisome proliferator–activated receptor γ (PPARγ) agonist, and treatment of SKOV3 ovarian cancer cells with this compound (5 μmol/L) inhibits cell proliferation, whereas up to 15 μmol/L rosiglitazone had no effect on cell growth. DIM-C-pPhtBu also inhibits G0-G₁ to S phase cell cycle progression and this is linked, in part, to PPARγ-dependent induction of the cyclin-dependent kinase inhibitor p21. DIM-C-pPhtBu induces PPARγ-independent down-regulation of cyclin D1 and we therefore further investigated activation of receptor-independent pathways. DIM-C-pPhtBu also induced apoptosis in SKOV3 cells and this was related to induction of glucose-related protein 78, which is typically up-regulated as part of the unfolded protein response during endoplasmic reticulum (ER) stress. Activation of ER stress was also observed in other ovarian cancer cell lines treated with DIM-C-pPhtBu. In addition, DIM-C-pPhtBu induced CCAAT/enhancer binding protein homologous protein through both ER stress and c-Jun NH₂-terminal kinase–dependent pathways, and CCAAT/enhancer binding protein homologous protein activated death receptor 5 and the extrinsic pathway of apoptosis. These results show that DIM-C-pPhtBu inhibits growth and induces apoptosis in ovarian cancer cells through both PPARγ-dependent and PPARγ-independent pathways, and this complex mechanism of action will be advantageous for future clinical development of these compounds for treatment of ovarian cancer.

Introduction
Ovarian cancer is the major gynecologic cancer, and it has been estimated that in 2005 there will be >16,000 deaths from this disease. Patients with ovarian cancer generally receive a platinum-based drug plus a taxane-derived compound such as paclitaxel; however, the response to treatment is dependent on the stage of the tumor when first detected (1–5). Although overall survival times have been increasing, ovarian cancer is frequently not detected in its early stages. The overall 5-year survival rate is ~50% and this decreases to 20% to 25% in women with advanced-stage disease. Etiologic factors that contribute to development of ovarian cancer are complex. Family history of ovarian cancer is an important risk factor (6), and ~10% of all epithelial ovarian cancers have been linked to germ-line mutations in BRCA1 and BRCA2, which are also genetic risk factors for breast cancer (7–10). Similarly with breast cancer, early menarche, low parity, and late menopause are also associated with increased risk for ovarian cancer (11, 12). Other specific risks for ovarian cancer are not well defined but include chemical carcinogens and environmental factors (lifestyle and diet; refs. 13–17).

In addition to treatment with various taxane/platinum combinations, several new therapies are being developed for treatment of ovarian cancer and these include tyrosine kinase inhibitors, IFN, immunotherapy, and antiangiogenic agents. These approaches target immune surveillance and specific pathways involved in ovarian cancer growth and metastasis and are also commonly used for treatment of other cancers (1–3).

Peroxisome proliferator–activated receptors (PPAR) are ligand-activated transcription factors and are members of the nuclear receptor superfamily, which includes steroid hormone, thyroid hormone, vitamin D, and retinoic acid receptors and orphan receptors (18–20). PPARγ agonists have been developed for treatment of metabolic diseases, and thiazolidinediones are PPARγ agonists used by millions of patients in the United States for their antidiabetic properties and treatment of insulin-resistant type II diabetes (21–25). PPARγ expression was reported in 339 clinical samples from multiple tumor types and cancer cell lines that primarily expressed wild-type PPARγ (26). The growth inhibitory effects of endogenous and synthetic PPARγ agonists have been reported in cancer cells derived from multiple tumors (27–35), and growth inhibitory responses have been linked to decreased G0-G₁ to S phase progression associated with induction of p21 or p27, downregulation of cyclin D1, and induction of apoptosis. Research in this laboratory has identified a series of
PPARγ-active 1,1-bis(3′-indolyl)-l-(p-substituted phenyl)-methanes (C-DIM; refs. 36–41), and it was shown that growth inhibition of pancreatic and colon cancer cells was linked to PPARγ-dependent induction of p21 and caveolin-1, respectively (37, 38).

Recent studies in PPARγ+/− heterozygous transgenic mice showed that partial loss of PPARγ expression resulted in increased carcinogen-induced mammary and ovarian tumors compared with wild-type mice (42). These data complement human studies suggesting that PPARγ is a tumor suppressor gene. PPARγ is widely expressed in ovarian cancer cells and ovarian tumors (43), and the triterpenoid-derived PPARγ agonist 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid (CDDO) inhibited growth of several ovarian cancer cell lines with IC50 values in the low micromolar range (44). This study shows that PPARγ-active C-DIMs inhibit SKOV3 ovarian cancer cell growth by both receptor-dependent activation of p21 and receptor-independent down-regulation of cyclin D1 and activation of c-Jun NH2-terminal kinase (JNK) and endoplasmic reticulum (ER) stress pathways. The results show that PPARγ-active C-DIMs may have potential for clinical treatment of ovarian tumors through activation of PPARγ-dependent and PPARγ-independent pathways.

Materials and Methods
Reagents and Antibodies
DIM-C-pPhBu and PPARγ antagonist GW9662 were synthesized in this laboratory and confirmed by gas chromatography-mass spectrometry. Rosiglitazone was purchased from LKT Laboratories, Inc. (St. Paul, MN). MG132 was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for cyclin D1, poly(ADP-ribose) polymerase (PARP), p21, phospho-retinoblastoma (p-Rb), PPARγ, β-tubulin, CCAAT/enhancer binding protein homologous protein (CHOP), glucose-related protein 78 (GRP78), small inhibitory RNA (siRNA) duplexes for PPARγ (iPPARγ), c-Jun siRNA, control siRNA-A, and horseradish peroxidase substrate for Western blot analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-JNK, JNK, phospho-c-Jun(Ser63), and c-Jun were from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibody to death receptor 5 (DR5) was from Abcam (Cambridge, MA). Plasmid preparation kits were purchased from Qiagen, Inc. (Santa Clarita, CA) and Lipofectamine 2000 transfection kits were obtained from Invitrogen (Carlsbad, CA). Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). β-Galactosidase (β-gal) reagents were from Tropix (Bedford, MA). [γ32P]ATP (300 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, MA). Poly(deoxyinosinic-deoxycytidylic acid) and T4 polynucleotide kinase and the cell proliferation agent WST-1 were purchased from Roche Molecular Biochemicals (Indianapolis, IN). The pancaspase inhibitor N-carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) was obtained from BD Pharmingen (San Diego, CA).

Cell Culture and Compound Treatment
SKOV3 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM/Ham’s F-12 (Sigma) supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), and 10 mL/L...
of 100× antibiotic/antimycotic solutions (Sigma) in an atmosphere of 5% CO₂. Cells were trypsinized, suspended in medium, and the cell number was determined with Z1 DualCoulterParticleCounter (Beckman Coulter, Fullerton, CA). Equal numbers of cells were seeded into 96-well, 6-well, or 12-well plates and allowed to attach overnight. Then the medium was replaced with DMEM/Ham’s F-12 without phenol red supplemented with 2.5% charcoal-stripped fetal bovine serum containing either vehicle (DMSO) or various concentrations of compounds for the indicated time.

Plasmids
The Gal4 reporter containing 5× 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 from Dr. Jennifer L. Oberfield (Glaxo Wellcome Research and Development, Research Triangle Park, NC). p21 promoter reporter construct pWWP was provided by Dr. Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). PPRE3Luc luciferase reporter was constructed using pGL2 with a minimal TATA sequence and triple consensus PPARγ response elements (PPRE). The GRP78 promoter-luciferase construct was provided by Dr. K. Park (Center for Molecular Medicine, Sungkyunkwan University, Seoul, Korea). Human CHOP promoter constructs were provided by Dr. Pierre Fafournoux (Saint Genes Champarelle, France) and the DR5 constructs were from Dr. H.G. Wang (Moffitt Cancer Center, Tampa, FL).

WST-1 Cell Proliferation and Cell Survival Assays
SKOV3 cells were seeded in 96-well plates at a density of 2 × 10⁴ per well and then treated with DMSO or various compounds. The WST-1 assay was done according to the instructions of the manufacturer. The absorbance of each sample was analyzed with FLUOstar OPTIMA Elisa reader (Offenburg, Germany) at 450 nm with 620 nm as reference wavelength. All experiments were done in triplicate at least twice and results are expressed as means ± SD for each
treatment group. Cell survival assays used a similar procedure in six-well plates treated with trypan blue solution and counted with a hemocytometer. The rate of cell survival is the percentage of live cells in the treated samples divided by the controls (DMSO).

**Luciferase Assay**

SKOV3 cells were plated in six-well plates at 80% confluence and allowed to attach overnight. Various amounts of DNA [i.e.,Gal4Luc (0.4 μg), PPRE3Luc (0.4 μg), β-gal DNA (0.1 μg), PPARy (0.4 μg), gPPARy (0.4 μg), GRP78Luc (0.4 μg), CHOPLuc (0.4 μg)] or siRNA (different concentrations as indicated) were transfected with Lipofectamine (Invitrogen) according to the instructions of the manufacturer. Cells were treated with either DMSO or compounds at indicated concentrations for 24 hours. Cells were lysed with 200 μL of reporter lysis buffer and 30 μL of cell extract were subjected to luciferase and β-gal assays. Luciferase and β-gal activities were measured with a multifunctional microplate reader (FLUOstar OPTIMA) and luciferase activities were normalized to β-gal activities.

**Flow Cytometric Analysis**

SKOV3 cells were plated and synchronized in serum-free medium for 48 hours and then treated with either DMSO or compounds at indicated concentrations for 24 hours. Propidium iodide staining was done and samples were analyzed with a flow cytometer (Beckman-Coulter EPICS XL-MCL) and at least 10,000 cells were acquired for each sample. ModFit LT (Verity Software House, Topsham, ME) was used for the subsequent cell cycle analysis.

**Western Blot Analysis**

Whole-cell lysates were extracted with high-salt lysis buffer [50 mmol/L HEPES, 0.5 mol/L sodium chloride, 1.5 mmol/L magnesium chloride, 10% (v/v) glycerol, 1% Triton X-100, and 5 μL/mL of Protease Inhibitor Cocktail (Sigma)] and quantified with Bio-Rad Protein Assay (Hercules, CA). An equal amount of protein from each treatment group was separated on an

![Figure 3](image_url)

**Figure 3.** Effects of DIM-C-pPhtBu on cell cycle progression. A, G2–G1 to S phase arrest. SKOV3 cells were treated with DMSO, 10 or 15 μmol/L DIM-C-pPhtBu, or 15 μmol/L rosiglitazone for 24 h, and the percent distribution of cells in G2–G1, G2–M, and S phase was determined by fluorescence-activated cell sorting analysis as described in Materials and Methods. B, DIM-C-pPhtBu affects cell cycle genes. SKOV3 cells were treated with DMSO and 5 to 15 μmol/L DIM-C-pPhtBu for 24 h, and protein levels of p-Rb (CD1), cyclin D1 (CD1), p21, and β-tubulin (loading control) were determined by Western blot analysis as described in Materials and Methods. Effects of GW9662 on DIM-C-pPhtBu–dependent modulation of p21 (C) and p21-reporter (D) gene expression. SKOV3 cells were treated with DMSO or DIM-C-pPhtBu and levels of p21 or cyclin D1 protein or luciferase activity were determined as described in Materials and Methods. *, **, P < 0.05, significant induction by DIM-C-pPhtBu or inhibition after cotreatment with 5 μmol/L GW9662, respectively. E, inhibition of cyclin D1 degradation by MG132. SKOV3 cells were treated with DMSO or DIM-C-pPhtBu alone or in combination with MG132, and cyclin D1 protein was detected by Western blot analysis as described in Materials and Methods (β-tubulin served as a loading control).
SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA). The polyvinylidene difluoride membrane was then blocked with 5% milk in TBS-T (1.576 g/L Tris, 8.776 g/L NaCl, 0.5 mL/L Tween 20) and probed with primary antibodies, followed by incubation with horseradish peroxidase–conjugated secondary antibodies as indicated. For protein knockdown experiments, the siRNA was transfected for 36 to 48 hours before isolation of whole-cell lysates (37, 38). Western blots are representatives of at least three independent experiments.

**Apoptosis Assay**

SKOV3 cells were treated with DMSO or the indicated compounds for 24 hours, then trypsinized and collected through centrifugation at 200 × g for 5 minutes. Levels of apoptosis were detected with a cell death detection ELISA kit according to the instructions of the manufacturer (Roche Applied Science, Penzberg, Germany).

**Electrophoretic Mobility Shift Assay**

Nuclear extracts from SKOV3 cells were isolated as previously described (45) and aliquots were stored at −80°C until use. ER stress response elements (ERSE) were synthesized and annealed, and 5 pmol aliquots were 5'-end labeled using T4 kinase and [γ-32P]ATP. A 30-μL electrophoretic mobility shift assay reaction mixture contained ~100 mmol/L potassium chloride, 3 μg of crude nuclear protein, 1 μg of poly(deoxyinosinic-deoxycytidylic acid), with or without unlabeled competitor oligonucleotide, and 10 fmol radiolabeled probe. After incubation for 20 minutes on ice, antibodies against selected proteins were added and incubated for another 20 minutes on ice. Protein-DNA complexes were resolved by 5% PAGE as previously described (45). Specific DNA-protein and antibody-supershifted complexes were observed as retarded bands in the gel. ERSE sequence used in gel shift analysis is given below (the NF-Y/CBF and YY1 motifs are underlined).

Human GRP78-94 ERSE: GGGCCAATGAACGGCCTCAACGA

Human GADD153-103 ERSE: GGGGCCAATGCCGGCGTGCCACTTTCT

Human DR5-276 CHOP site: TTGCGGAGGATTGC-GTTGACGA.

**Statistical Analysis**

Statistical differences between different groups were determined by ANOVA and Scheffe’s test for significance. Data are presented as means ± SD for at least three separate determinations for each treatment.

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**Figure 4.** Induction of ER stress by DIM-C-pPhtBu. Induction of GRP78 in SKOV3 (A) and other ovarian cancer cells (B). Cells were treated with DMSO or 5 to 15 or 2 to 12.5 μmol/L DIM-C-pPhtBu for 24 h, and whole-cell lysates were analyzed by Western blot analysis for GRP78 and β-tubulin (loading control) proteins as described in Materials and Methods. C, activation of pGRP78 by DIM-C-pPhtBu. SKOV3 cells were transfected with pGRP78, treated with DMSO, DIM-C-pPhtBu, tunicamycin (Tm), or rosiglitazone, and luciferase activity was determined as described in Materials and Methods. *, P < 0.05, significant induction of activity. Columns, mean of three replicate determinations for each treatment group; bars, SE. D, electrophoretic mobility shift assay analysis. Nuclear extracts from SKOV3 cells treated with DMSO or various ER stress reagents were incubated with −94GRP78-ERSE(32P) and antibodies or oligonucleotides and analyzed in a gel mobility shift assay as described in Materials and Methods.
Results

DIM-C-pPhtBu Activates PPARγ and Inhibits SKOV3 Cell Proliferation

1,1-Bis(3-indolyl)-1-(p-t-butylphenyl)methane (DIM-C-pPhtBu) is a PPARγ agonist (36–40) and the comparative growth inhibitory effects of DIM-C-pPhtBu and the thiazolidinedione rosiglitazone were investigated in SKOV3 ovarian cancer cells. The results (Fig. 1) show that DIM-C-pPhtBu inhibits SKOV3 cell proliferation with an IC50 value of <5 μmol/L, whereas minimal growth

Figure 5. Activation of CHOP and DR5 by DIM-C-pPhtBu. A, induction of CHOP and DR5 proteins by DIM-C-pPhtBu. SKOV3 cells were treated with DMSO or 5 to 15 μmol/L DIM-C-pPhtBu for 24 h, and whole-cell lysates were analyzed for CHOP, DR5, and β-tubulin (loading control) by Western blot analysis as described in Materials and Methods. B, activation of pCHOP. SKOV3 cells were transfected with pCHOP, treated with DMSO, 5 to 15 μmol/L DIM-C-pPhtBu, 0.5 μg/mL tunicamycin, and 15 μmol/L rosiglitazone, and luciferase activity was determined as described in Materials and Methods. *, P < 0.05, significant induction. Columns, mean of three replicate determinations for each treatment group; bars, SE. C, activation of pDR5 constructs. SKOV3 cells were transfected with pDR5 constructs treated with DMSO or 15 μmol/L DIM-C-pPhtBu and luciferase activity was determined as described in Materials and Methods. *, P < 0.05, significant induction. D, electrophoretic mobility shift assay analysis. Nuclear extracts from SKOV3 cells treated with DMSO or various ER stress reagents were incubated with −276DR5[32P] and antibodies or oligonucleotides and analyzed in a gel mobility shift assay as described in Materials and Methods.
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inhibition was observed for rosiglitazone at concentrations as high as 30 μmol/L. DIM-C-pPhtBu decreased cell survival after treatment for 2 days as illustrated in Fig. 1C. The higher growth inhibitory activity of DIM-C-pPhtBu and related compounds compared with thiazolidinediones has also been observed in bladder, colon, and pancreatic cancer cells (37, 38, 41).

SKOV3 cells express PPARγ (Fig. 2A) and treatment with DIM-C-pPhtBu for 24 hours did not affect PPARγ protein levels. DIM-C-pPhtBu induced transactivation in SKOV3 cells transfected with GAL4-PPARγ/pGAL4, whereas an equivalent dose (15 μmol/L) of rosiglitazone was inactive (Fig. 2B). DIM-C-pPhtBu (15 μmol/L) also induced luciferase activity in cells transfected with a construct (pPPRE) containing three tandem PPREs linked to a luciferase reporter gene, and this response was significantly inhibited by the PPARγ antagonist GW9662 (Fig. 2C). Moreover, DIM-C-pPhtBu induced transactivation in cells transfected with iScr (nonspecific) RNA and pPPRE, and this response was inhibited in cells cotransfected with iPPARγ, confirming that DIM-C-pPhtBu induces PPARγ-dependent transactivation in SKOV3 cells (Fig. 2D). The results in Fig. 2E show that iPPARγ significantly decreased PPARγ protein expression in SKOV3 cells.

Figure 6. Induction of DR5 and apoptosis by DIM-C-pPhtBu. A, induction of DR5, procaspase, and PARP cleavage. SKOV3 cells were treated with DMSO or 5 to 15 μmol/L DIM-C-pPhtBu for 24 h, and whole-cell lysates were analyzed for PARP cleavage, procaspase-3, procaspase-8, DR5, and β-tubulin (loading control) by Western blot analysis as described in Materials and Methods. B, induction of apoptosis. SKOV3 cells were treated with DMSO, 15 μmol/L DIM-C-pPhtBu alone or in combination with Z-VAD-FMK, or Z-VAD-FMK alone, and apoptosis was determined with cell death detection ELISA kit as described in Materials and Methods. *, **, P < 0.05, significant induction of apoptosis or inhibition of this response, respectively. C, inhibition of PARP cleavage. SKOV3 cells were treated with DMSO, 15 μmol/L DIM-C-pPhtBu or Z-VAD-FMK (or combinations) for 24 h, and whole-cell lysates were analyzed for PARP cleavage and β-tubulin (loading control) by Western blot analysis as described in Materials and Methods. B, columns, mean of three replicated determinations for each treatment group; bars, SE.

Figure 7. Role of JNK pathway in activation of CHOP by DIM-C-pPhtBu. A, time-dependent (A) and concentration-dependent (B) activation of the JNK pathway by DIM-C-pPhtBu. SKOV3 cells were treated with DMSO and different concentrations of DIM-C-pPhtBu, and at different time points, cells were harvested and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods. C, effects of SP600125 on DIM-C-pPhtBu–induced activation of JNK, c-Jun, CHOP, and DR5. SKOV3 cells were treated with DMSO or pretreated with 10 or 30 μmol/L SP600125 for 2 h, then cotreated with 15 μmol/L DIM-C-pPhtBu for 24 h, and whole-cell lysates were analyzed for various proteins by Western blot analysis as described in Materials and Methods. D, effects of siRNA for c-jun on transactivation. SKOV3 cells were transfected with pCHOP, a nonspecific siRNA (control siRNA), or c-Jun siRNA, treated with 15 μmol/L DIM-C-pPhtBu, 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, significantly decreased activity. E, decreased c-jun and phospho-c-jun expression by RNA interference. SKOV3 cells were transfected with control siRNA or siRNA for c-jun and then treated with DMSO or 15 μmol/L DIM-C-pPhtBu, and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods.
in MCF-7 breast cancer cells (36). DIM-C-pPhtBu also induced transactivation in cells transfected with p21-luc, which contains the GC-rich PPAR-responsive proximal region of the p21 promoter, and this response was also inhibited by GW9662 (Fig. 3D). Thus, DIM-C-pPhtBu–induced p21 protein/reporter gene activity was inhibited by the PPAR antagonist and this was also observed in Panc28 cells treated with DIM-C-pPhtBu (38) where it was shown that up-regulation of p21 was due to PPARγ/Sp1–dependent interaction with this region of the p21 promoter. Thus, DIM-C-pPhtBu induced a PPARγ-dependent growth inhibitory pathway in SKOV3 cells. In contrast, DIM-C-pPhtBu–induced down-regulation of cyclin D1 was not affected by cotreatment with the PPARγ antagonist GW9662 (Fig. 3C); however, this response was reversed by 10 µmol/L concentration of the proteasome inhibitor MG132 (Fig. 3E) and this receptor-independent pathway was comparable to that observed in MCF-7 cells treated with PPARγ-active C-DIMs (36).
DIM-C-pPhtBu Activates ER Stress and Apoptosis in SKOV3 Cells

A recent report showed that DIM, 5,5’-dibromoDIM, and PPARγ-active C-DIMs induced cyclin D1 down-regulation and growth inhibition through receptor-independent activation of ER stress pathways (45). Results in Fig. 4A show that DIM-C-pPhtBu induces a concentration-dependent increase in GRP78 protein after treatment of SKOV3 cells for 24 hours. The induction of GRP78 by DIM-C-pPhtBu was also observed in OVCA-3, PE01, and ES2 ovarian cancer cell lines and the fold induction (~2-fold) was lowest in ES2 cells due to relatively high basal GRP78 expression in the untreated cells (Fig. 4B). Induction of this ER stress–induced protein was complemented by induction of transactivation by DIM-C-pPhtBu in SKOV3 cells transfected with pGRP78 that contains the ER-stress responsive region (−374 to +1) of the GRP78 promoter (Fig. 4C). Rosiglitazone (15 μmol/L) did not induce ER stress in SKOV3 cells, whereas tunicamycin, a prototypical inducer of ER stress, also activated the pGRP78 promoter. The effects of DIM-C-pPhtBu on binding of nuclear extracts from SKOV3 cells to the ERSE in the GRP78 promoter were investigated in gel mobility shift assays (Fig. 4D). Binding of nuclear extracts from solvent-treated cells (lane 1) to 32P-labeled −94GRP78-ERSE gave retarded bands corresponding to the ER stress factor (ERSF) and NF-Y and YY-1 complexes as previously reported (46). Extracts from SKOV3 cells treated with DIM-C-pPhtBu (lane 2) or tunicamycin (lane 3) gave a more intense ERSF-ERSE complex, which was not affected after cotreatment with nonspecific immunoglobulin G (lane 4) but was decreased after cotreatment with TFII-I antibodies (lane 5) or 100-fold excess of unlabeled −94GRP78-ERSE (lane 6). The enhanced ERSF retarded band is primarily due to induction of TFII-I by ER stressors (47). These results are consistent with induction of an ER stress response by DIM-C-pPhtBu, which is accompanied by induction of TFII-I and enhanced formation of the ERSF-ERSE complex. The supershifted band was not observed after coinubation with TFII-I antibodies (Fig. 4D, lane 5), however, immunodepletion of this protein resulted in a decreased retarded band intensity.

CHOP is another ER stress–induced protein and the results in Fig. 5A show that treatment of SKOV3 cells with 5 to 15 μmol/L DIM-C-pPhtBu for 24 hours induced CHOP protein, and this was also accompanied by induction of DR5. DIM-C-pPhtBu also induced transactivation in SKOV3 cells transfected with the pCHOP construct, which contains the −954 to +1 region of the CHOP promoter and two ERSEs at −103 and −75 and an activator protein-1 site at −245 (48). Results of transfection of pCHOP in SKOV3 cells (Fig. 5B) showed that 5 to 15 μmol/L DIM-C-pPhtBu induced luciferase activity and 0.5 μg/mL tunicamycin was also active in this assay. In contrast, 15 μmol/L rosiglitazone was inactive and this was consistent with the failure of this compound to activate pGRP78 (Fig. 4C). Recent studies show that activation of CHOP through ER stress also resulted in activation of DR5 and the extrinsic pathway of apoptosis (49–51). The role of CHOP induction by DIM-C-pPhtBu on activation of DR5 was initially investigated in SKOV3 cells transfected with constructs containing DR5 promoter inserts (Fig. 5C). The DR5 gene promoter contains both a CHOP and a GC-rich binding site at −276 and −198, which have been linked to activation of this gene by different compounds (49–52). DIM-C-pPhtBu induced transactivation in SKOV3 cells transfected with constructs pDR5a and pDR5b, which contain the CHOP element in the DR5 promoter; however, induction was not observed in cells transfected with pDR5c, which contains the GC-rich site but not the CHOP element (Fig. 5C). These results suggest that activation of ER stress and CHOP by DIM-C-pPhtBu is paralleled by activation of DR5 promoter constructs in which the CHOP response element is required for transactivation. We further investigated the induction of CHOP by DIM-C-pPhtBu and interaction with the DR5 promoter in an electrophoretic mobility shift assay using nuclear extracts from SKOV3 cells incubated with −276DR5[32P] containing the CHOP response element from the DR5 promoter (Fig. 5D). DIM-C-pPhtBu and tunicamycin induced a retarded band (lanes 3 and 4, respectively) compared with DMSO (lane 2). CHOP antibodies (lane 5) and 100-fold excess of unlabeled oligonucleotide (lane 7) decreased retarded band intensity, whereas immunoglobulin G had no effect (lane 6). These results are consistent with induction of CHOP by DIM-C-pPhtBu (Fig. 5A), which in turn activates the DR5 promoter through the CHOP response element (Fig. 5C).

Based on reports showing that ER stress and induction of DR5 resulted in activation of apoptosis (49–51), we investigated the effects of DIM-C-pPhtBu on induction of apoptosis in SKOV3 cells. Treatment of SKOV3 cells with 5 to 15 μmol/L DIM-C-pPhtBu also induced PARP cleavage at the high dose, and this was accompanied by a marked decrease in procaspase-8 and some decrease in procaspase-3 protein expression (Fig. 6A). Using an ELISA assay for apoptosis, DIM-C-pPhtBu significantly induced apoptosis, and this response was significantly blocked by the pancaspase inhibitor Z-VAD-FMK (Fig. 6B). These results are consistent with activation of the extrinsic pathway of apoptosis as previously reported for other compounds that activate ER stress and DR5 (49–52). DIM-C-pPhtBu–induced PARP cleavage was also inhibited by Z-VAD-FMK, confirming that this compound activated caspase-dependent apoptosis (Fig. 6C).

Although these results are consistent with an ER stress–dependent apoptotic pathway, other reports have linked chemical-induced up-regulation of DR5 to activation of JNK (53–55). Figure 7A summarizes the time-dependent induction of CHOP and phosphorylation of c-Jun and p54/p46-JNK by DIM-C-pPhtBu. The results show initial induction of JNK phosphorylation within 2 hours after treatment with 15 μmol/L DIM-C-pPhtBu, and there is a parallel increase in phospho-Jun and CHOP proteins after treatment for 4, 8, and 12 hours. The coordinate effects of JNK activation and induction of CHOP are paralleled by the concentration-dependent effects of 5, 10, and 15 μmol/L.
DIM-C-pPhtBu, where only the highest dose results in c-Jun/JNK phosphorylation (Fig. 7B) and induction of CHOP and DR5 (Fig. 5A). The role of DIM-C-pPhtBu–dependent activation of JNK in the induction of CHOP and DR5 was also investigated with the JNK inhibitor SP600125 (Fig. 7C). The results show that SP600125 inhibited phosphorylation of JNK and c-Jun (>75%) and this was accompanied by 40% to 50% inhibition of CHOP and DR5 protein expression. These results are consistent with the activation of CHOP not only through induction of activating transcription factor-6, TFII-1, and other factors that activate ERSEs (Fig. 4; ref. 45) but also through activation of c-jun–dependent transcription. Previous studies show that CHOP expression can be enhanced through activator protein-1 promoter sites (56), and results in Fig. 7D show that siRNA for c-jun decreases DIM-C-pPhtBu–induced transactivation in SKOV3 cells transfected with the pCHOP construct that contains the −954 to +1 region of the CHOP promoter. The results also show that transfection of the siRNA for c-jun decreases up-regulation c-jun and phospho-c-jun (Fig. 7E) as determined in Western blot analysis of whole-cell lysates. The data indicate that DIM-C-pPhtBu–dependent activation of CHOP is dependent on activation of transcription factors c-jun and ERSE binding factors, and that both the activator protein-1 and ERSE motifs are required for this response. These results, coupled with the PPARγ-dependent activation of p21 (Fig. 3), show that DIM-C-pPhtBu inhibits growth and induces apoptosis in ovarian cancer cells through activation of both PPARγ-dependent and PPARγ-independent pathways (Fig. 8).

Discussion

PPARγ is overexpressed in many tumor types and there is also evidence for expression of this receptor in ovarian cancer cells and tumors (26–35, 42–44). Different structural classes of PPARγ agonists, including thiazolidinediones and pentacyclic triterpenoids such as CDDO, inhibit growth of multiple tumors. However, the antitumorigenic activity of PPARγ agonists such as CDDO has been linked to both receptor-dependent and receptor-independent activation of different pathways associated with growth inhibition and cell death (53, 57–63). For example, CDDO and related compounds activate PPARγ-dependent transactivation and several responses are blocked in cells after cotreatment with PPARγ antagonists (57, 58). Mix et al. (63) recently reported that CDDO and prostaglandin J2 inhibit matrix metalloproteinases in chondrosarcoma cells and these responses were not inhibited by the PPARγ antagonist GW9662. Studies in this laboratory showed that CDDO and related methyl ester and imidazole derivatives inhibited colon cancer cell growth and induced apoptosis in colon cancer cells through both PPARγ-dependent and PPARγ-independent pathways (58). At lower concentrations, these compounds induced the tumor suppressor gene caveolin-1, and this response was inhibited by PPARγ antagonists. At higher doses, CDDO induced apoptosis, which was unaffected by PPARγ antagonists, and we have recently observed similar responses for PPARγ-active C-DIMs in the same cell line.3 This suggests that in some cell lines, PPARγ agonists may activate both PPARγ-dependent and PPARγ-independent pathways and their effects may be separable at different concentrations of these compounds. PPARγ is overexpressed in some ovarian cancer cells and tumors; however, Melichar et al. (44) showed that the growth inhibitory effects of CDDO in ovarian cancer cell lines were PPARγ independent. In this study, we used the PPARγ-active DIM-C-pPhtBu as a model and showed that in SKOV3 ovarian cancer cells, this compound inhibited cell proliferation (IC50 <5 μmol/L) and activated PPARγ-dependent transactivation (Figs. 1 and 2). Moreover, DIM-C-pPhtBu was more potent than rosiglitazone in both growth inhibition and transactivation assays, and these potency differences for one or both of these assays have been observed in other cancer cell lines (36–38).

PPARγ agonists typically inhibit G1–G0 to S phase progression in cancer cell lines, and this has been linked to modulation of one or more cell cycle genes such as p21, p27, or cyclin D1. Results in Fig. 3 show that DIM-C-pPhtBu also inhibits G1–G0 to S phase progression and significantly induces p21 (but not p27) protein expression, which is accompanied by decreased Rb phosphorylation and down-regulation of cyclin D1 protein. Interestingly, both up-regulation of p21 and down-regulation of cyclin D1 protein are observed at similar concentrations; however, the former is inhibited by the PPAR antagonist GW9662 whereas the latter is unaffected by GW9662. These results are consistent with previous

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studies on PPARγ-active C-DIMs, which also show PPARγ-dependent induction of p21 in pancreatic cancer cells and PPARγ-independent down-regulation of cyclin D1 in breast cancer cells (36, 38).

PPAR-independent down-regulation of cyclin D1 in pancreatic cancer cells was accompanied by ER stress (45), which can lead to cell death through multiple pathways including activation of DR5- and caspase-8-dependent apoptosis (49–52). Results in Fig. 4 show that 10 μmol/L DIM-C-pPhtBu induces the stress protein GRP78 in SKOV3 cells and this protein is also induced in OVCAR-3, PE01, and ES2 ovarian cancer cells. This suggests that activation of ER stress may be a general response of ovarian cancer cells to C-DIM compounds, although the magnitude of GRP78 induction in ES2 cells was relatively low (2-fold) due to high constitutive GRP78 expression. Both tunicamycin and thapsigargin are prototypical inducers of ER stress (49, 50, 64), and both tunicamycin and DIM-C-pPhtBu induced GRP78, activated a GRP78 promoter construct, and enhanced binding to an ERSE in an electrophoretic mobility shift assay using an ERSE from the GRP78 promoter (Fig. 4).

Previous studies with tunicamycin and thapsigargin show that both compounds activate apoptosis through induction of CHOP, which in turn activates DR5 and the extrinsic pathway of apoptosis, and similar results were reported for the proteasome inhibitor MG132 (49, 50). Our studies indicate that DIM-C-pPhtBu also activates expression of CHOP protein and CHOP promoter constructs and binding to a CHOP response element from the DR5 promoter in a gel mobility shift assay (Fig. 5). DIM-C-pPhtBu also induces apoptosis in SKOV3 cells and this is accompanied by induction of DR5 protein and cleavage of procaspase-8 (Fig. 6). Moreover, analysis of DR5 promoter constructs shows that DIM-C-pPhtBu induces transactivation only with constructs containing the CHOP response element (pDR5a and pDR5b), whereas pDR5c, which contains a GC-rich Sp1 binding site, is not activated by DIM-C-pPhtBu. These results are in contrast to the mechanism of DR5 induction by bile acids and sodium butyrate in human hepatocellular carcinoma and colon cancer cells, respectively, where the proximal GC-rich site in the DR5 promoter is required for activation (52, 54).

Our results show that induction of ER stress by DIM-C-pPhtBu results in activation of CHOP, which in turn up-regulates expression of DR5; however, there is also evidence that CHOP and/or DR5 can also be induced via the JNK pathway (53–55). Moreover, the synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-diene-28-oate, which activates PPARγ, also induces phosphorylation of c-Jun, which is critical for increased expression of DR5 and apoptosis in human lung cancer cells (53). The induction of CHOP expression and its role in up-regulation of DR5 in the lung cancer cells were not investigated (53). Our results clearly show that DIM-C-pPhtBu also increases phosphorylation of JNK and c-Jun and this is accompanied by induction of CHOP and DR5 (Fig. 7A and B). Moreover, inhibition of JNK activity or knockdown of c-Jun by RNA interference partially blocks DIM-C-pPhtBu–dependent up-regulation of CHOP and DR5 (Fig. 7C–E). A previous study showed that ER stress–dependent activation of DR5 and apoptosis in colon cancer cells was dependent on induction of CHOP (49) as observed in this study with the PPARγ-active DIM-C-pPhtBu. However, our data also show that CHOP up-regulation by DIM-C-pPhtBu in SKOV3 cells was due to simultaneous induction of ER stress and JNK pathways, which activate factors binding to ERSE and activator protein-1 motifs in the CHOP gene promoter.

DIM-C-pPhtBu is a PPARγ agonist and induces the cyclin-dependent kinase inhibitor p21 through activation of PPARγ. DIM-C-pPhtBu induces apoptosis in SKOV3 cells through a receptor-independent pathway involving parallel activation of ER stress and JNK, cooperatively increasing expression of CHOP, which in turn up-regulates DR5 (Fig. 8). These results show that PPARγ-active C-DIMs induce both receptor-dependent and receptor-independent pathways at similar concentrations that inhibit growth and induce apoptosis. Moreover, in vivo studies with C-DIM compounds indicate that they induce minimal toxic side effects (36, 41, 65, 66) This complex mechanism of action will be highly advantageous for development of these compounds and related analogues for treatment of ovarian cancer or for applications in combination therapy.

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Mol Cancer Ther 2006;5:2324-2336.

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