

2-Cyano-3,12-dioxolean-1,9-dien-28-oic Acid (CDDO) and Related Compounds Inhibit Growth of Colon Cancer Cells Through Peroxisome Proliferator-Activated Receptor γ -Dependent and -Independent Pathways

Sudhakar Chintharlapalli, Sabitha Papineni, Marina Konopleva, Michael Andreef,

Ismael Samudio and Stephen Safe

Department of Biochemistry and Biophysics (S.C., S.P., S.S.) and Department of Veterinary Physiology and Pharmacology (S.S.), Texas A&M University, College Station, TX 77843; Department of Blood and Marrow Transplantation, Section of Molecular Hematology and Therapy, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030 (M.K., M.A., I.S.); Institute of Biosciences and Technology, Texas A&M University System Health Science Center, 2121 W. Holcombe Blvd., Houston, TX 77030 (S.S.)

Running Title: PPAR γ -dependent and -independent growth inhibition of colon cancer cells

Correspondence should be sent to:

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

Manuscript information:

Text pages: 29
Figures: 5
References: 38
Abstract: 197 words
Introduction: 624 words
Discussion: 1030 words

Abstract

2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and the corresponding methyl (CDDO-Me) and imidazole (CDDO-Im) esters induce peroxisome proliferator-activated receptor gamma (PPAR γ)-dependent transactivation in SW-480 colon cancer cells, and these responses were inhibited by small inhibitory RNA for PPAR γ (iPPAR γ). Moreover, in a mammalian two-hybrid assay using VP-PPAR γ and GAL4-coactivator/corepressor chimeras and a construct (pGAL4) containing five tandem GAL4 response elements, CDDO, CDDO-Me and CDDO-IM induce transactivation and PPAR γ interaction with multiple coactivators. A major difference among the three PPAR γ agonists was the higher activity of CDDO-Im to induce PPAR γ interactions with the corepressor SMRT. CDDO, CDDO-Me and CDDO-Im inhibited SW-480, HCT-116 and HT-29 colon cancer cell proliferation at low concentrations and induced cell death at higher concentrations. Growth inhibition at lower concentrations correlated with induction of the tumor suppressor gene caveolin-1 which is known to inhibit colon cancer cell growth. Induction of caveolin-1 by CDDO, CDDO-Me and CDDO-Im was inhibited by the PPAR γ antagonist N-(4'-aminopyridyl-2-chloro-5-nitrobenzamide (T007), whereas higher doses induced apoptosis (PARP cleavage) which was not inhibited by T007. These results illustrate that CDDO-, CDDO-Me and CDDO-Im induce both PPAR γ -dependent and -independent responses in colon cancer cells and activation of these pathways are separable and concentration-dependent for all three compounds.

INTRODUCTION

2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) is a synthetic triterpenoid-derived compound structurally-related to the pentacyclic triterpenoids oleanolic and ursolic acids which exhibit anti-inflammatory and anticarcinogenic activities (Nishino et al., 1988; Huang et al., 1994; Suh et al., 1998). CDDO and related compounds inhibit growth of multiple cancer cell lines, induced differentiation and inhibited inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 activities (Nishino et al., 1988; Honda et al., 1997; Honda et al., 1998; Suh et al., 1998; Suh et al., 1999; Honda et al., 2000; Place et al., 2003). CDDO and the methyl ester derivative (CDDO-Me) bound peroxisome-proliferator-activated receptor γ (PPAR γ) and CDDO induced transactivation in CV-1 cells transfected with PPAR γ -responsive GAL4-PPAR γ or PPRE (promoter) constructs (Wang et al., 2000). CDDO alone or in combination with the RXR agonist LG100268 induced 3T3-L1 differentiation, whereas CDDO-Me was inactive in this assay. It was suggested that CDDO was a PPAR γ agonist, whereas CDDO-Me exhibited partial antagonist activity (Wang et al., 2000).

CDDO, CDDO-Me and an imidazole ester (CDDO-Im) decrease cancer cell survival, and their cell context-dependent responses and mechanisms of action have been investigated (Ito et al., 2000; Stadheim et al., 2002; Pedersen et al., 2002; Konopleva et al., 2002; Suh et al., 2003; Ikeda et al., 2003; Konopleva et al., 2004a; Konopleva et al., 2004b). These triterpenoid compounds are potent inducers of differentiation and apoptosis in leukemia cells; however, their pro-apoptotic effects were somewhat variable among different cell lines. CDDO-Me induced intrinsic apoptotic pathways in HL-60 cells and enhanced responses were observed after cotreatment with RXR ligands (Konopleva et al., 2002). CDDO-induced apoptosis in HL-60 and U937 cells were inhibited by dominant negative PPAR γ expression and the PPAR γ antagonist N-

(4'-aminopyridyl-2-chloro-5-nitrobenzamide (T007) (Konopleva et al., 2004a). CDDO and related compounds also induced caspase 8-dependent pathways in leukemia cells (Stadheim et al., 2002; Pedersen et al., 2002; Suh et al., 2003; Ikeda et al., 2003; Konopleva et al., 2004a), and this may be due, in part, to downregulation of FLIP, an endogenous inhibitor of caspase-8 activation. CDDO also induced apoptosis in leukemia cells through enhanced oxidative stress (Ikeda et al., 2003) and loss of mitochondrial membrane potential (Konopleva et al., 2004b). In breast cancer cells, CDDO inhibits growth of ER-positive and ER-negative cells and tumor growth in athymic nude mouse models and this correlated with modulation of genes associated with cell cycle progression, apoptosis and ER stress (Lapillonne et al., 2003). In COLO 16 human skin cancer cells, CDDO induced apoptosis and this was due, in part, to ER stress and direct mitochondrial effects that disrupted calcium homeostasis (Hail, Jr. et al., 2004). CDDO-Me induced both the intrinsic and extrinsic apoptosis pathway in lung cancer cell lines (Kim et al., 2002; Zou et al., 2004), and a caspase 8-dependent apoptotic pathway was activated by CDDO in human osteosarcoma cells (Ito et al., 2001). CDDO also inhibited growth of several ovarian cancer cell lines that express PPAR γ and, since cotreatment with the PPAR γ antagonist T007 did not block the effects of CDDO, it was concluded that this response was PPAR γ -independent (Kodera et al., 2000; Melichar et al., 2004).

This study reports the effects of CDDO compound on SW-480 and other colon cancer cell lines and investigates the concentration-dependent induction of PPAR γ -dependent and -independent responses. Growth inhibitory IC₅₀ values for CDDO-Me and CDDO-Im were $\leq 0.2 \mu\text{M}$ in SW-480, HCT-116 and HT-29 colon cancer cells, whereas IC₅₀ values for CDDO were $\leq 0.5 \mu\text{M}$ after 6 days of growth. CDDO, CDDO-Im and CDDO-Me also induced PPAR γ -dependent transactivation and coactivator-PPAR γ interactions in mammalian two-hybrid assays

in SW-480 cells. In the same cell lines, CDDO and related compounds induce PPAR γ -dependent upregulation of the tumor suppressor gene caveolin-1 and PPAR γ -independent apoptosis and these responses were activated over distinct and separable concentrations in SW-480 cells.

MATERIALS AND METHODS

Cell culture

Human colon cancer cell lines SW480 and HT-29 were provided by M.D. Anderson Cancer Center; HCT-116 cells were obtained from American Type Culture Collection (Manassas, VA). SW480 and HT-29 cells were maintained in Dulbecco's Modified Eagle's Medium nutrient mixture F-12 Ham (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) (Intergen, Purchase, NY) and 10 ml/L of 100X antibiotic antimycotic solution (Sigma). HCT-116 cells were maintained in RPMI-1640 medium (Sigma) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS and 10 ml/L of 100X antibiotic antimycotic solution (Sigma).

Chemicals, reagents, plasmids and antibodies

The PPAR γ antagonist *N*-(4'-aminopyridyl)-2-chloro-5-nitrobenzamide (T007) was synthesized in this laboratory and confirmed by gas chromatography-mass spectrometry. CCDO compounds were provided by Dr. Edward Sausville (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) through the Rapid Access to Intervention Developmental Program. 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 (β -gal) reagent was from Tropix (Bedford, MA). Small inhibitory RNA (siRNA) duplexes were prepared by Dharmacon Research (Lafayette, CO). Previous studies in this laboratory have reported oligonucleotide sequences for PPAR γ and lamin A/C siRNA (Qin et al., 2004). The Gal4 reporter

containing 5X Gal4 DBD (Gal4Luc) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4DBD-PPAR γ construct (gPPAR γ) was a gift of Dr. Jennifer L. Oberfield (Glaxo Wellcome Research and Development, Research Triangle Park, NC) and PPAR γ expression plasmid and pM-PPAR γ coactivator-1 (PGC1) were gifts of Dr. Bruce M. Spiegelman (Harvard University, Boston, MA). The PPRE-luc construct contains three tandem PPAR γ response elements (PPREs) with a minimal TATA sequence in pGL2. The PPAR γ_2 -VP16 fusion plasmid (VP-PPAR γ) contained the DEF region of PPAR γ (amino acids 183–505) fused to the pVP16 expression vector and the GAL4-coactivator fusion plasmids pM-SRC1, pMSRC2, pMSRC3, pM-DRIP205 and pM-CARM-1 were kindly provided by Dr. Shigeaki Kato (University of Tokyo, Tokyo, Japan). Antibodies for caveolin-1 (sc-894), PARP (sc-8007), Akt and phospho-Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti- β -actin was purchased from Sigma (St. Louis, MO).

Cell Proliferation Assay

Cells (2×10^4) were plated 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 dissolved in DMSO. Fresh media and compounds were added every 48 hr. Cells were counted at the indicated times using a Coulter Z1 cell counter. The proliferation of SW480 cells was also carried out as described above using the colorimetric WST1 assay according to the manufacturer's instructions (Roche, Mannheim, Germany). Cells (4000/well) were seeded in 96 well plates and assayed after 48 hr. Each experiment was carried out at least three times and results are expressed as means \pm SE for each determination.

Transfection and Luciferase Assay

Colon cancer cells (1×10^5) were seeded onto 24-well plates in DME-F12 media supplemented with 2.5% charcoal-stripped FBS and grown overnight. Transient transfections were performed using LipofectAmine reagent (Invitrogen) according to the protocol provided by the manufacturer. For transfections with siRNA for PPAR γ , the RNA concentration was 75 nM and Oligofectamine transfection reagent (Invitrogen) was used. Cotransfections were performed using Gal4Luc (0.4 μ g), β -gal 0.04 μ g), Gal4DBD-PPAR γ (0.04 μ g), VP-PPAR γ (0.04 μ g), pM SRC1 (0.04 μ g), pMSRC2 (0.04 μ g), pMSRC3 (0.04 μ g), pMPGC-1 (0.04 μ g), pMDRIP205 (0.04 μ g) and pMCARM-1 (0.04 μ g). After 5 hr of transfection, the transfection mix was replaced with complete media containing either vehicle (DMSO) or the indicated ligand for 20-22 hr. Cells were then lysed with 100 μ l of 1X reporter lysis buffer and 30 μ l of cell extract were used for luciferase and β -galactosidase assays. A Packard Lumicount luminator (Packard Instruments Co., Downers Grove, IL) was used to quantitate luciferase and β -galactosidase activities, and the luciferase activities were normalized to β -galactosidase activity.

Western Blot Analysis

SW480, HT-29 and HCT-116 (3×10^5) cells were seeded in six-well plates in DMEM:Ham's F-12 media containing 2.5% charcoal-stripped FBS for 24 hr and then treated with either the vehicle (DMSO) or the indicated compounds. 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 μ l/ml of Protease Inhibitor Cocktail (Sigma)]. Protein samples were incubated at 100°C for 2 min, separated on 10% SDS-PAGE at 120 V for 3-4 hr in 1 X running buffer [25 mM Tris-base, 192 mM glycine, and 0.1% SDS (pH 8.3)], and

transferred to polyvinylidene difluoride membrane (PVDF, Bio-Rad, Hercules, CA) at 0.1 V for 16 hr at 4°C in 1 X 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), 0.05% Triton X-100 and 5% non-fat dry milk] with gentle shaking for 30 min and incubated in fresh 5% TBST-Blotto with 1:1000 (for caveolin-1), 1:250 (for PARP), 1:5000 (for β -actin) primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody (1:5000) in 5% TBST-Blotto for 90 min. The membrane was washed with TBST for 10 min and incubated with 10 ml of chemiluminescence substrate (PerkinElmer Life Sciences) for 1.0 min and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY). Band intensities were evaluated by scanning laser densitometry (Sharp Electronics Corporation, Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corporation, Billerica, MA).

RESULTS

Initial studies investigated the effects of CDDO, CDDO-Me and CDDO-Im on growth of SW-480, HT-29 and HCT-116 colon cancer cells. Cells were treated with different concentrations of the test compounds on days 0, 2 and 4 (media and compounds were changed every 2 days) and cell numbers were determined 2, 4 and 6 days after treatment. Significant inhibition of cell proliferation was observed for all compounds after treatment for 2 and 4 days, and IC_{50} values were $\leq 0.2 \mu\text{M}$ for CDDO-Me and CDDO-Im and $\leq 0.5 \mu\text{M}$ for CDDO in all three cell lines (data not shown). Cell survival results after treatment with the CDDO compounds (Fig. 1) were derived from the growth inhibition curves, and the percent cell survival was the ratio of the number of cells in the treated/DMSO (solvent) groups at each time point. Higher concentrations of these compounds decreased cell numbers below the initial number of attached cells (i.e. cell death) and after 96 hr, the concentrations of CDDO, CDDO-Me and CDDO-Im that induced cell death in all three cell lines were 1.0-2.5, 0.2 and 0.2 μM , respectively. CDDO decreased proliferation of SW480 cells using the WST-1 colorimetric assay (Fig. 1D), and cotreatment of CDDO plus 5.0 μM T007 (PPAR γ antagonist) significantly reversed the growth inhibitory effects of CDDO. The results demonstrate a role for PPAR γ in mediating the effects of CDDO in this assay.

PPAR γ -Dependent Transactivation Assays

Previous studies showed that CDDO and related compounds activate PPAR γ (Wang et al., 2000) and this was further investigated in SW-480 cells transfected a GAL4-PPAR γ chimeric expression plasmid and a reporter construct (pGAL4) containing 5 tandem GAL4 response elements linked to a luciferase reporter gene. The results (Fig. 2A) show that CDDO, CDDO-

Me and CDDO-Im induced a concentration-dependent increase in luciferase activity and significant induction was observed at concentrations as low as 0.2, 0.025 and 0.025 μM , respectively. Both CDDO and CDDO-Me induced a maximal 35- to 45-fold increase in luciferase activity; the maximal induced value for CDDO-Im was lower (< 30 -fold); however, this may be due to toxicity of this compound at the higher concentrations or differential recruitment of coactivators or corepressors. Ligand-induced transactivation in SW-480 cells transfected with GAL4-PPAR γ /pGAL4 was also inhibited by the PPAR γ antagonist T007 (Fig. 2B). Maximal induction of luciferase activity by CDDO (2.5 μM), CDDO-Me (0.5 μM) and CDDO-Im (0.5 μM) was inhibited in cells cotreated with 5 μM T007. Similar results were observed at lower concentrations of the CDDO compounds and also with the PPAR γ antagonist GW966 (data not shown). The CDDO compounds also induced transactivation in SW-480 cells transfected with a construct (PPRE $_3$ -luc) containing three tandem PPAR γ response elements linked to firefly luciferase. The results (Fig. 2C) show that CDDO, CDDO-Me and CDDO-Im significantly induce transactivation at concentrations of 1.0, 0.2 and 0.2 μM , respectively, and maximal induction (> 30 -fold) was observed for CDDO-Me. Transactivation induced by CDDO compounds in SW-480 cells transfected with PPRE-luc was inhibited after cotransfection with iPPAR γ (Fig. 2D), and this correlates with comparable inhibition of PPAR γ -dependent gene expression induced by other PPAR γ agonists using iPPAR γ (Chintharlapalli et al., 2004). These results confirm that CDDO, CDDO-Me and CDDO-Im activate PPAR γ -dependent transactivation in SW-480 colon cancer cells.

Induction of PPAR γ -Coactivator Interactions by CDDO and Related Compounds

Previous reports show that different classes of PPAR γ agonist induce structure-dependent

interactions of PPAR γ with coactivators in mammalian two hybrid experiments (Kodera et al., 2000; Chintharlapalli et al., 2004) and therefore the effects of CDDO compounds were investigated in a mammalian two hybrid assay in SW-480 cells transfected with pGAL4, VP-PPAR γ and GAL4-coactivators/GAL4-SMRT (corepressor). The results show that CDDO, CDDO-Me and CDDO-Im induce VP-PPAR γ - pM coactivator interactions in SW-480 cells transfected with SRC-1, SRC-2, TRAP 220, CARM-1 and PGC-1. In contrast, CDDO and CDDO-Me but not CDDO-Im enhanced PPAR γ -SRC-3 interaction; however, it is possible that higher concentrations of CDDO-Im would induce transactivation. We also investigated interactions with the corepressor SMRT and the results indicate that all three compound increased transactivation but CDDO-Im was significantly more active than CDDO or CDDO-Im. These results confirm that CDDO and related esters induce interactions between VP-PPAR γ with pM-coactivators in SW-480 cells and differences between compounds were primarily concentration-dependent.

CDDO Compounds Induce Caveolin-1 in Colon Cancer Cells

Caveolin-1 is a tumor suppressor gene in colon cancer cells and previous reports show that PPAR γ agonists induce this response in some colon cancer cell lines (Burgermeister et al., 2003; Chintharlapalli et al., 2004). The results illustrated in Figure 4A show that after treatment of SW-480 cells with CDDO (0.25 - 0.5 μ M), CDDO-Me (0.025 - 0.1 μ M) and CDDO-Im (0.5 - 0.1 μ M) for 3 days, caveolin-1 was induced by all three compounds. In contrast, induction of PARP cleavage, a marker of apoptosis was not observed at these concentrations. A slight increase in p27 was observed, and cyclin D1 was unchanged by these treatments (data not shown). In a separate experiment, SW-480 cells were treated with the CDDO compounds or

T007 alone and in combination for 3 days and caveolin-1 protein expression was determined (Figs. 4B - 4D). The results show that 5 μ M T007 alone did not affect levels of caveolin-1 protein; however, in cells cotreated with CDDO, CDDO-Me or CDDO-Im plus T007, there was a decrease in caveolin-1 protein expression compared to cells treated with the CDDO compounds alone. These results complement the transactivation studies and demonstrate that induction of caveolin-1 by the CDDO compounds was inhibited by the PPAR γ antagonist T007. Induction of caveolin-1 by CDDO, CDDO-Me and CDDO-Im was also determined in HT-29 (Fig. 5A) and HCT-116 (Fig. 5B) cells, and induction was observed in both colon cancer cell lines. The effects of caveolin-1 induction by CDDO compounds on levels of phospho-Erk in SW480 cells were minimal (data not shown). However, these compounds induced phosphatidylinositol-3-kinase (PI3-K)-dependent phosphorylation of Akt (Fig. 4E), and this was consistent with results of a recent study in human 293 and HeLa cells overexpressing caveolin-1 (Shack et al., 2003). PARP cleavage was minimal or not observed at the concentrations of CDDO compounds used in this experiment.

Cell survival studies (Fig. 1) indicated that higher concentrations of CDDO and related esters induced cell death and therefore we further investigated induction of PARP cleavage in SW-480 cells treated with 0.5 - 2.5 μ M CDDO and 0.2 - 1.0 μ M CDDO-Me and CDDO-Im for 1 day (Fig. 5C). Longer treatment (i.e. 3 days) could not be determined due to extensive cell death and minimal cell survival. These results demonstrate that the higher concentrations of CDDO and related esters induced PARP cleavage and this was consistent with the cell survival studies. In contrast, the PPAR γ antagonist T007 did not inhibit induced PARP cleavage, suggesting that this response was PPAR γ -independent. Moreover, at these higher concentrations, there was also a significant downregulation of cyclin D1, and this response was also not affected by T007 (data

not shown). These results demonstrate that CDDO and related esters induce PPAR γ -dependent and -independent responses associated with growth inhibition/cell death in colon cancer cells, and these mechanistic differences are concentration-dependent.

DISCUSSION

PPAR γ is expressed in tumors from multiple tissues and cancer cell lines (Ikezoe et al., 2001), and chemicals that activate PPAR γ have been extensively investigated for their efficacy in cancer chemotherapy [reviewed in (Desvergne and Wahli, 1999; Murphy and Holder, 2000; Escher and Wahli, 2000; Fajas et al., 2001; Willson et al., 2001; Grommes et al., 2004)]. PPAR γ agonists typically decrease cancer cell survival due to induction of differentiation genes and apoptosis and modulation of genes/proteins linked to G1 \rightarrow S phase progression. It is somewhat paradoxical that induction of these responses by PPAR γ agonists, such as the thiazolidinediones (TZDs), 15-deoxy- Δ 12,14-prostaglandin J2 (PGJ2), and CDDO compounds, appears to be both PPAR γ -dependent and -independent and the relative contributions of these pathways are often not well defined. For example, troglitazone inhibits growth and induces apoptosis in colon cancer cell lines and induces early growth response-1 (Egr-1) gene expression which in turn activates downstream growth inhibitory effects (Baek et al., 2003). This response was unique to troglitazone among PPAR γ agonists and was induced via PPAR γ -independent pathways.

CDDO, CDDO-Me and CDDO-Im activate PPAR γ in transactivation assays and CDDO-induced apoptosis was inhibited by dominant negative PPAR γ in myeloid HL-60 cells and by T007 in myeloid U937 cells (Konopleva et al., 2004a). In another study, CDDO-Im induced differentiation in leukemia cells that was not inhibited by the PPAR γ antagonist GW9662 (Ito et al., 2001) and T007 did not affect inhibition of SKOV3 ovarian cancer cell growth by CDDO (Melichar et al., 2004). Thus, it is clear that CDDO and related compounds activate PPAR γ -dependent and -independent pathways that inhibit cancer cell growth and this dual action may be beneficial for cancer chemotherapy. Results of this study demonstrate that CDDO, CDDO-Me and CDDO-Im also inhibit growth and survival of colon cancer cells (Fig. 1) and we have

primarily used SW-480 cells as a model for determining activation of PPAR γ -dependent and -independent pathways by CDDO and related esters.

CDDO, CDDO-Me and CDDO-Im activate PPAR γ -dependent transactivation (Fig. 2) and coactivator interactions (Fig. 3) and differences between compounds were primarily associated with the fold-induction response where induction was lower for CDDO-Im due to the higher toxicity of this compound. Previous studies indicate that induced transactivation in the mammalian two hybrid assay using VP-PPAR γ and GAL4-coactivators was both structure and coactivator-dependent (Burgermeister et al., 2003; Chintharlapalli et al., 2004). For example, a series of 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes [methylene-substituted 1,1-bis(3'-indolyl)methanes (C-DIMs)], that activate PPAR γ , primarily induced interactions of PPAR γ primarily with PGC-1 in colon cancer cell lines (Chintharlapalli et al., 2004), whereas CDDO, CDDO-Me and CDDO-Im induced interactions with several coactivators including SRC-1, SRC-2, CARM-1, TRAP 220 and PGC-1. The main distinguishing feature among the CDDO compounds was the failure of CDDO-Im to induce PPAR γ -SRC-3 interactions with VP-PPAR γ and the relatively high activity of CDDO-Im to induce PPAR γ interactions with the co-repressor SMRT. These differences among CDDO compounds in PPAR γ -dependent interactions with coactivators might result in some tissue/cell selectivity in their PPAR γ dependent responses.

PPAR γ agonists such as TZDs, PGJ2 and C-DIMs typically inhibit genes/proteins associated with cell cycle progression and differentiation in cancer cell lines and these responses may be receptor dependent or independent. Previous studies in colon cancer cell lines with C-DIMs showed that these compounds had minimal effects on critical cell cycle proteins cyclin D1, p21 and p27 (Chintharlapalli et al., 2004); and similar results were observed in this study at the lower concentrations of CDDO compounds (data not shown). The induction of caveolin-1 by

PGJ2, C-DIMs and TZDs has been observed in colon cancer cell lines (Burgermeister et al., 2003; Chintharlapalli et al., 2004) and overexpression of caveolin-1 inhibits colon cancer cell growth *in vitro* and tumor growth in athymic nude mice (Bender et al., 2000; Burgermeister et al., 2003; Chintharlapalli et al., 2004). Results in Figures 4 and 5 show that CDDO, CDDO-Me and CDDO-Im significantly induce caveolin-1 in colon cancer cells at concentrations that also inhibit cell growth (Figs. 1). Moreover, in SW-480 cells, the induced caveolin-1 expression is inhibited by the PPAR γ antagonist T007. The relatively low concentrations of CDDO, CDDO-Me and CDDO-Im that inhibit SW-480 cell proliferation induce minimal to non-detectable apoptosis as indicated by PARP cleavage (Figs. 4, 5A and 5B). We also observed that CDDO compounds induced PI3-K-dependent Akt phosphorylation which is normally associated with increased cell survival. However, a recent report (Shack et al., 2003) showed that overexpression of caveolin-1 in human 293 and HeLa cells also resulted in upregulation of the PI3-K/Akt pathway which sensitized the cells to stress-induced cytotoxicity.

In contrast, higher concentrations of CDDO compounds induce apoptosis and PARP cleavage; however, this response was PPAR γ -independent and not inhibited by T007 (Fig. 5C). Moreover, at the higher concentrations of CDDO, CDDO-Me and CDDO-Im, there was also a marked decrease in cyclin D1 protein expression which was also not inhibited by T007 (data not shown). Similar results have also been observed in this laboratory after treatment of breast cancer cells by PGJ2, TZDs and C-DIMs (Qin et al., 2003; Qin et al., 2004). These data demonstrate that CDDO and related esters inhibit colon cancer cell growth through both PPAR γ -dependent and -independent pathways which are observed at different concentrations and are linked to growth inhibition and cell death, respectively.

Previous reports of mechanistic differences regarding the role of PPAR γ agonists in

mediating growth inhibitory responses in different cancer cell lines may be explained, in part, by results of this study. CDDO and related esters are a unique example of a specific structural class of PPAR γ agonists that induce both PPAR γ -dependent (caveolin induction) and -independent (apoptosis) responses over a relatively narrow dose range in colon cancer cells. It is possible that the antimitogenic and anticarcinogenic activities of PPAR γ agonists may be enhanced when the dose ranges for their PPAR γ -dependent and -independent responses are similar, and this may contribute to the high potencies observed for CDDO, CDDO-Me and CDDO-Im in colon cancer cell lines. Currently, we are investigating the dose-dependent effects of thiazolidinediones, CDDO and related esters and C-DIMs in different cancer cell lines to determine their relative potencies in activating PPAR γ -dependent and -independent pathways. This approach will provide important mechanistic insights into the activities of these compounds and their potential clinical applications for cancer chemotherapy.

REFERENCES

- Baek SJ, Wilson LC, Hsi LC and Eling TE (2003) Troglitazone, a peroxisome proliferator-activated receptor γ (PPAR γ) ligand, selectively induces the early growth response-1 gene independently of PPAR γ . A novel mechanism for its anti-tumorigenic activity. *J Biol Chem* **278**:5845-5853.
- Bender FC, Reymond MA, Bron C and Quest AF (2000) Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. *Cancer Res* **60**:5870-5878.
- Burgermeister E, Tencer L and Liscovitch M (2003) Peroxisome proliferator-activated receptor- γ upregulates caveolin-1 and caveolin-2 expression in human carcinoma cells. *Oncogene* **22**:3888-3900.
- Chintharlapalli S, Smith III R, Samudio I, Zhang W and Safe S (2004) 1,1-Bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes induce peroxisome proliferator-activated receptor γ -mediated growth inhibition, transactivation and differentiation markers in colon cancer cells. *Cancer Res* **64**:5994-6001.
- Desvergne B and Wahli W (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* **20**:649-688.
- Escher P and Wahli W (2000) Peroxisome proliferator-activated receptors: insight into multiple cellular functions. *Mutat Res* **448**:121-138.
- Fajas L, Debril MB and Auwerx J (2001) Peroxisome proliferator-activated receptor- γ : from adipogenesis to carcinogenesis. *J Mol Endocrinol* **27**:1-9.
- Grommes C, Landreth GE and Heneka MT (2004) Antineoplastic effects of peroxisome proliferator-activated receptor γ agonists. *Lancet Oncol* **5**:419-429.

- Hail N, Jr., Konopleva M, Sporn M, Lotan R and Andreeff M (2004) Evidence supporting a role for calcium in apoptosis induction by the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO). *J Biol Chem* **279**:11179-11187.
- Honda T, Finlay HJ, Gribble GW, Suh N and Sporn MB (1997) New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. *Bioorg Med Chem Lett* **7**:1623-1628.
- Honda T, Rounds BV, Bore L, Finlay HJ, Favalaro FG, Jr., Suh N, Wang Y, Sporn MB and Gribble GW (2000) Synthetic oleanane and ursane triterpenoids with modified rings A and C: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *J Med Chem* **43**:4233-4246.
- Honda T, Rounds BV, Gribble GW, Suh N, Wang Y and Sporn MB (1998) Design and synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages. *Bioorg Med Chem Lett* **8**:2711-2714.
- Huang MT, Ho CT, Wang ZY, Ferraro T, Lou YR, Stauber K, Ma W, Georgiadis C, Laskin JD and Conney AH (1994) Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer Res* **54**:701-708.
- Ikeda T, Sporn M, Honda T, Gribble GW and Kufe D (2003) The novel triterpenoid CDDO and its derivatives induce apoptosis by disruption of intracellular redox balance. *Cancer Res* **63**:5551-5558.
- Ikezoe T, Miller CW, Kawano S, Heaney A, Williamson EA, Hisatake J, Green E, Hofmann W, Taguchi H and Koeffler HP (2001) Mutational analysis of the peroxisome proliferator-activated receptor γ gene in human malignancies. *Cancer Res* **61**:5307-5310.

- Ito Y, Pandey P, Place A, Sporn MB, Gribble GW, Honda T, Kharbanda S and Kufe D (2000) The novel triterpenoid 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid induces apoptosis of human myeloid leukemia cells by a caspase-8-dependent mechanism. *Cell Growth Differ* **11**:261-267.
- Ito Y, Pandey P, Sporn MB, Datta R, Kharbanda S and Kufe D (2001) The novel triterpenoid CDDO induces apoptosis and differentiation of human osteosarcoma cells by a caspase-8 dependent mechanism. *Mol Pharmacol* **59**:1094-1099.
- Kim KB, Lotan R, Yue P, Sporn MB, Suh N, Gribble GW, Honda T, Wu GS, Hong WK and Sun SY (2002) Identification of a novel synthetic triterpenoid, methyl-2-cyano-3,12-dioxoleana-1,9-dien-28-oate, that potently induces caspase-mediated apoptosis in human lung cancer cells. *Mol Cancer Ther* **1**:177-184.
- Kodera Y, Takeyama K, Murayama A, Suzawa M, Masuhiro Y and Kato S (2000) Ligand type-specific interactions of peroxisome proliferator-activated receptor γ with transcriptional coactivators. *J Biol Chem* **275**:33201-33204.
- Konopleva M, Elstner E, McQueen TJ, Tsao T, Sudarikov A, Hu W, Schober WD, Wang RY, Chism D, Kornblau SM, Younes A, Collins SJ, Koeffler HP and Andreeff M (2004a) Peroxisome proliferator-activated receptor γ and retinoid X receptor ligands are potent inducers of differentiation and apoptosis in leukemias. *Mol Cancer Ther* **3**:1249-1262.
- Konopleva M, Tsao T, Estrov Z, Lee RM, Wang RY, Jackson CE, McQueen T, Monaco G, Munsell M, Belmont J, Kantarjian H, Sporn MB and Andreeff M (2004b) The synthetic triterpenoid 2-cyano-3,12-dioxoleana-1,9-dien-28-oic acid induces caspase-dependent and -independent apoptosis in acute myelogenous leukemia. *Cancer Res* **64**:7927-7935.
- Konopleva M, Tsao T, Ruvolo P, Stiouf I, Estrov Z, Leysath CE, Zhao S, Harris D, Chang S,

- Jackson CE, Munsell M, Suh N, Gribble G, Honda T, May WS, Sporn MB and Andreeff M (2002) Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. *Blood* **99**:326-335.
- Lapillonne H, Konopleva M, Tsao T, Gold D, McQueen T, Sutherland RL, Madden T and Andreeff M (2003) Activation of peroxisome proliferator-activated receptor γ by a novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest and apoptosis in breast cancer cells. *Cancer Res* **63**:5926-5939.
- Melichar B, Konopleva M, Hu W, Melicharova K, Andreeff M and Freedman RS (2004) Growth-inhibitory effect of a novel synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, on ovarian carcinoma cell lines not dependent on peroxisome proliferator-activated receptor- γ expression. *Gynecol Oncol* **93**:149-154.
- Murphy GJ and Holder JC (2000) PPAR- γ agonists: therapeutic role in diabetes, inflammation and cancer. *Trends Pharmacol Sci* **21**:469-474.
- Nishino H, Nishino A, Takayasu J, Hasegawa T, Iwashima A, Hirabayashi K, Iwata S and Shibata S (1988) Inhibition of the tumor-promoting action of 12-O-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoid compounds. *Cancer Res* **48**:5210-5215.
- Pedersen IM, Kitada S, Schimmer A, Kim Y, Zapata JM, Charboneau L, Rassenti L, Andreeff M, Bennett F, Sporn MB, Liotta LD, Kipps TJ and Reed JC (2002) The triterpenoid CDDO induces apoptosis in refractory CLL B cells. *Blood* **100**:2965-2972.
- Place AE, Suh N, Williams CR, Risingsong R, Honda T, Honda Y, Gribble GW, Leesnitzer LM, Stimmel JB, Willson TM, Rosen E and Sporn MB (2003) The novel synthetic triterpenoid, CDDO-imidazolide, inhibits inflammatory response and tumor growth in

- vivo. *Clin Cancer Res* **9**:2798-2806.
- Qin C, Burghardt R, Smith R, Wormke M, Stewart J and Safe S (2003) Peroxisome proliferator-activated receptor γ (PPAR γ) agonists induce proteasome-dependent degradation of cyclin D1 and estrogen receptor α in MCF-7 breast cancer cells. *Cancer Res* **63**:958-964.
- Qin C, Morrow D, Stewart J, Spencer K, Porter W, Smith III R, Phillips T, Abdelrahim M, Samudio I and Safe S (2004) A new class of peroxisome proliferator-activated receptor γ (PPAR γ) agonists that inhibit growth of breast cancer cells: 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes. *Mol Cancer Therap* **3**:247-259.
- Shack S, Wang XT, Kokkonen GC, Gorospe M, Longo DL and Holbrook NJ (2003) Caveolin-induced activation of the phosphatidylinositol 3-kinase/Akt pathway increases arsenite cytotoxicity. *Mol Cell Biol* **23**:2407-2414.
- Stadheim TA, Suh N, Ganju N, Sporn MB and Eastman A (2002) The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) potently enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *J Biol Chem* **277**:16448-16455.
- Suh N, Honda T, Finlay HJ, Barchowsky A, Williams C, Benoit NE, Xie QW, Nathan C, Gribble GW and Sporn MB (1998) Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. *Cancer Res* **58**:717-723.
- Suh N, Wang Y, Honda T, Gribble GW, Dmitrovsky E, Hickey WF, Maue RA, Place AE, Porter DM, Spinella MJ, Williams CR, Wu G, Dannenberg AJ, Flanders KC, Letterio JJ, Mangelsdorf DJ, Nathan CF, Nguyen L, Porter WW, Ren RF, Roberts AB, Roche NS,

- Subbaramaiah K and Sporn MB (1999) A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. *Cancer Res* **59**:336-341.
- Suh WS, Kim YS, Schimmer AD, Kitada S, Minden M, Andreeff M, Suh N, Sporn M and Reed JC (2003) Synthetic triterpenoids activate a pathway for apoptosis in AML cells involving downregulation of FLIP and sensitization to TRAIL. *Leukemia* **17**:2122-2129.
- Wang Y, Porter WW, Suh N, Honda T, Gribble GW, Leesnitzer LM, Plunket KD, Mangelsdorf DJ, Blanchard SG, Willson TM and Sporn MB (2000) A synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor γ . *Mol Endocrinol* **14**:1550-1556.
- Willson RM, Lambert MH and Kliewer SA (2001) Peroxisome proliferator-activated receptor γ and metabolic disease. *Annu Rev Biochem* **70**:341-367.
- Zou W, Liu X, Yue P, Zhou Z, Sporn MB, Lotan R, Khuri FR and Sun SY (2004) c-Jun NH₂-terminal kinase-mediated up-regulation of death receptor 5 contributes to induction of apoptosis by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate in human lung cancer cells. *Cancer Res* **64**:7570-7578.

Footnotes

This work was financially supported by the National Institutes of Health (ES09106 and CA112337), the M.D. Anderson Cancer Center Pancreatic Cancer Spore (P20 CA10193), and the Texas Agricultural Experiment Station.

FIGURE CAPTIONS

Figure 1. Effects of CDDO and related esters on colon cancer cell survival and proliferation. SW-480 (A), HT-29 (B) and HCT-116 (C) cells were treated with different concentrations of CDDO, CDDO-Me or CDDO-Im for 48 or 96 hr, and this percent cell survival was determined as described in the Materials and Methods. Solvent (DMSO)-treated cells survival was set at 100% and significantly ($p < 0.05$) decreased cell numbers after treatment are indicated by an asterisk. The percent cell survival is derived from the treated/control (DMSO) cell number ratios. (D) Effects of T007 on CDDO-induced cell proliferation. SW480 cells were treated with different concentrations of CDDO and 5 μM T007 for 48 hr, and cell proliferation was determined colorimetrically using the cell proliferation reagent WST-1 as described in the Materials and Methods. Significant ($p < 0.05$) inhibition of cell proliferation by CDDO is indicated by an asterisk (*) and reversal of these effects by 5 μM T007 is indicated (**).

Figure 2. PPAR γ -dependent transactivation by CDDO, CDDO-Me and CDDO-Im in SW-480 cells. Activation of PPAR γ -GAL4/pGAL4 (A) and inhibition by T007 (B). SW-480 cells were transfected with PPAR γ -GAL4/pGAL4, treated with different concentrations of CDDO compounds and luciferase activity determined as described in the Materials and Methods. In a separate experiment, cells were treated with CDDO (2.5 μM), CDDO-Me (0.5 and 1.0 μM), or CDDO-Im (0.2 and 0.5 μM) alone or in combination with 5 μM T007. Significant ($p < 0.05$) induction by CDDO, CDDO-Me and CDDO-Im is indicated by an asterisk and inhibition by T007 is also indicated (**). (C) Activation of PPRE₃-luc. SW-480 cells were transfected with

PPRE₃-luc, treated with CDDO compounds and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) induction is indicated by an asterisk. (D) RNA interference assay. Cells were transfected with PPRE-luc, treated with the different CDDO compounds, and small inhibitory RNA for lamin (non-specific control) or PPAR γ and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by PPAR γ agonist (*) and inhibition after cotransfection with iPPAR γ (**) is indicated. All experiments (A - D) are expressed as means \pm SE for at least three separate determinations for each treatment group.

Figure 3. CDDO-, CDDO-Me- and CDDO-Im-induced PPAR γ -coactivator interactions in SW-480 cells. Ligand-induced interactions with SRC-1 (A), SRC-2 (B), SRC-3 (C), TRAP 220 (D), PGC-1(E), CARM-1 (F), and SMRT (G). Cells were transfected with VP-PPAR γ and GAL4-coactivator chimeric expression plasmids, treated with the CDDO compounds, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for at least three separate determinations for each treatment group and significant ($p < 0.05$) induction as indicated by an asterisk.

Figure 4. Induction of caveolin-1, Akt phosphorylation, and PARP cleavage by CDDO and related esters in SW-480 cells. (A) Induction of caveolin-1 and PARP cleavage. SW-480 cells were treated with CDDO, CDDO-Me and CDDO-Im for 3 days and caveolin-1 and PARP cleavage proteins were determined by Western blot analysis of whole cell lysates as described in the Materials and Methods. T007 inhibition of caveolin-1 induction by CDDO (B), CDDO-Me (C), and CDDO-Im (D). Cells were

treated with the compounds alone or in combination with 5 μ M T007 as described in (A), and whole cell lysates were analyzed by Western blot analysis. β -Actin served as a loading control for these experiments. (E) CDDO compounds induce Akt phosphorylation. Cells were treated with the CDDO compounds as described in (A), and Akt/phospho-Akt were determined by Western blot analysis as described in the Materials and Methods.

Figure 5. Induction of caveolin-1 and PARP cleavage by CDDO compounds in colon cancer cell lines. Induction by CDDO, CDDO-Me and CDDO-Im in HT-29 (A) and HCT-116 (B) cells. Cells were treated with different concentrations of CDDO and related esters for 3 days, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. (C) Higher concentrations of CDDO, CDDO-Me and CDDO-Im induce PPAR γ -independent PARP cleavage in SW-480 cells. SW-480 cells were treated for 1 day with the indicated doses of CDDO compounds alone or in combination with 5.0 μ M T007 and PARP cleavage was determined by Western blot analysis of whole cell lysates as described in the Materials and Methods. β -Actin served as a loading control.

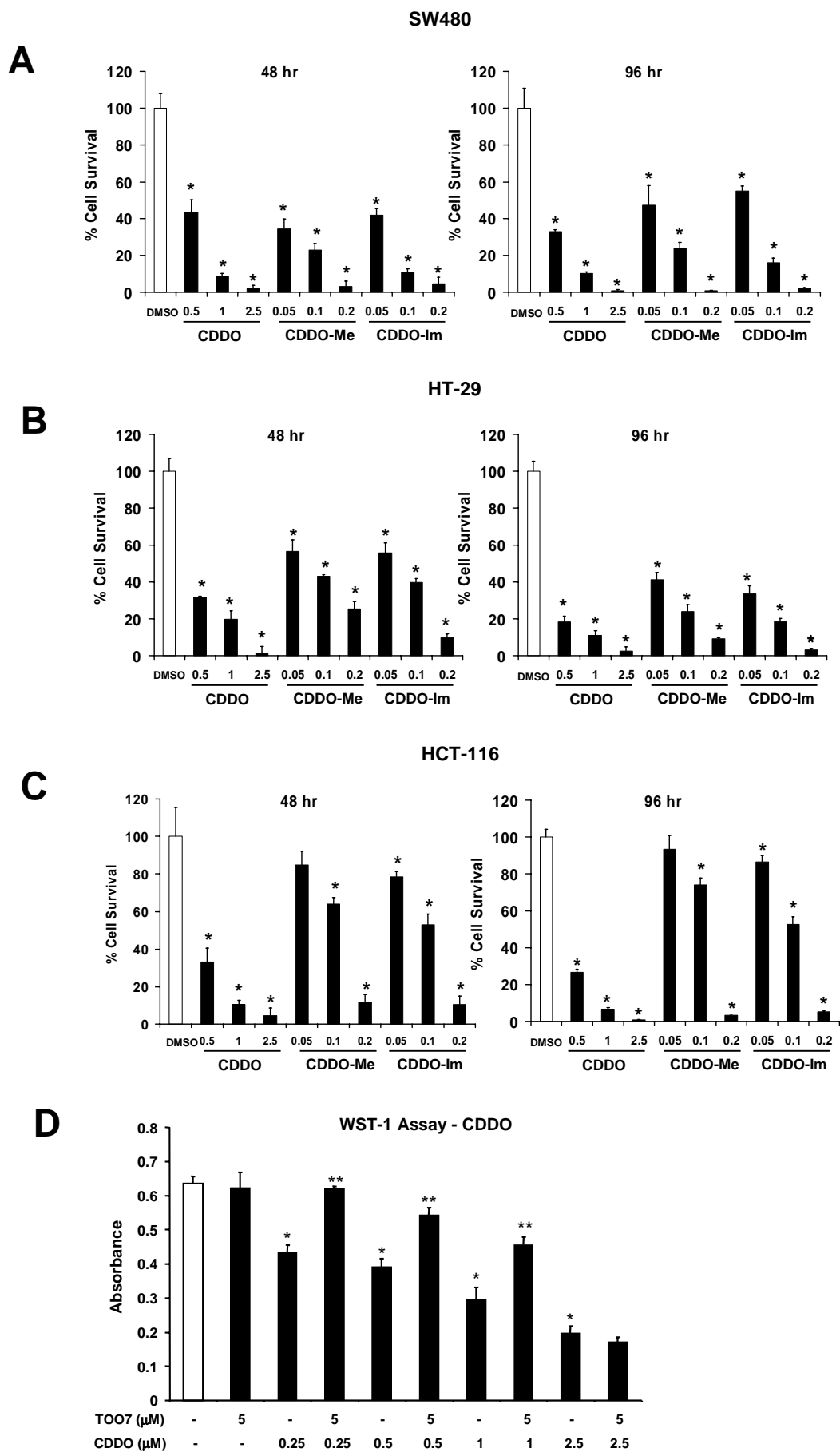


Figure 2

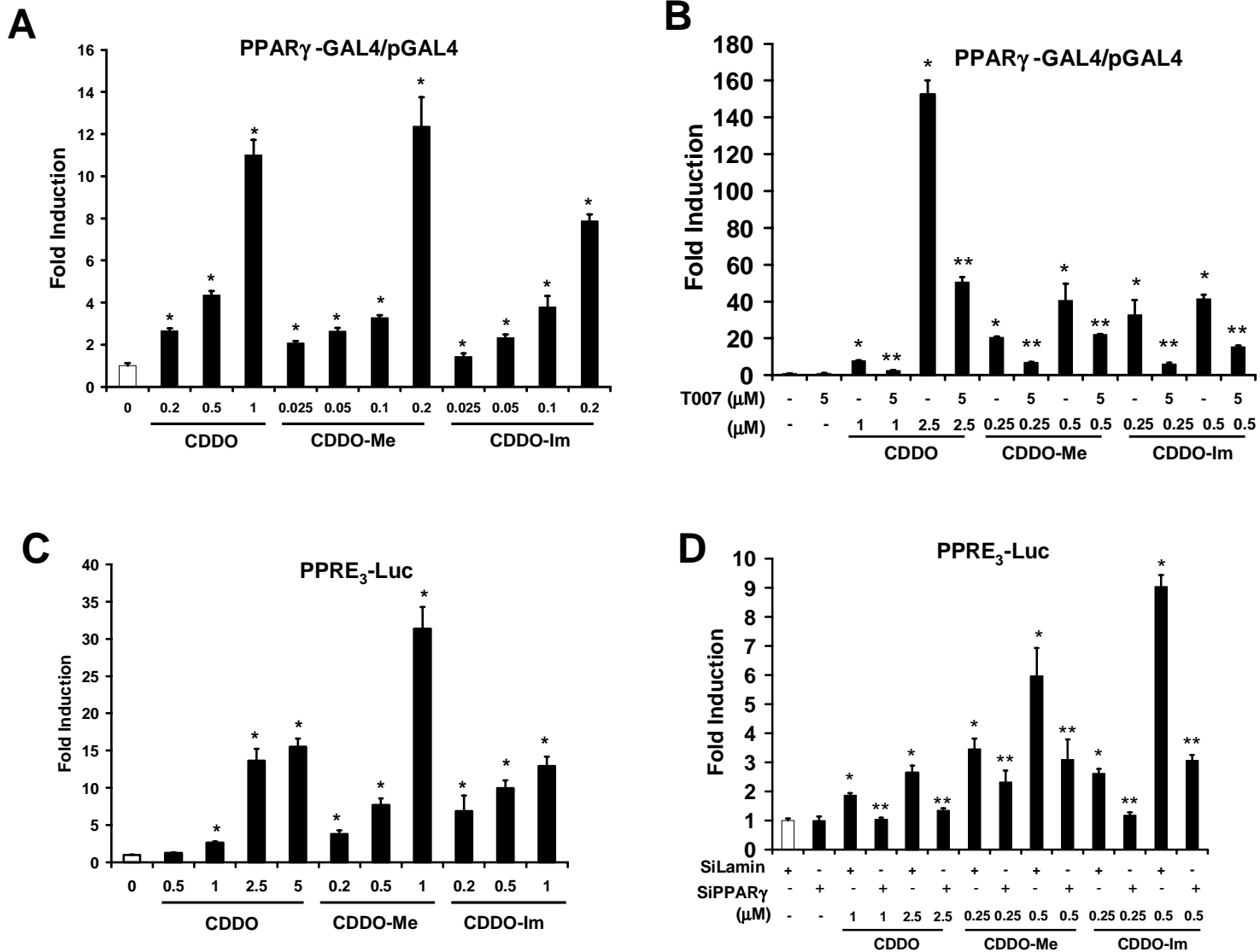


Figure 3

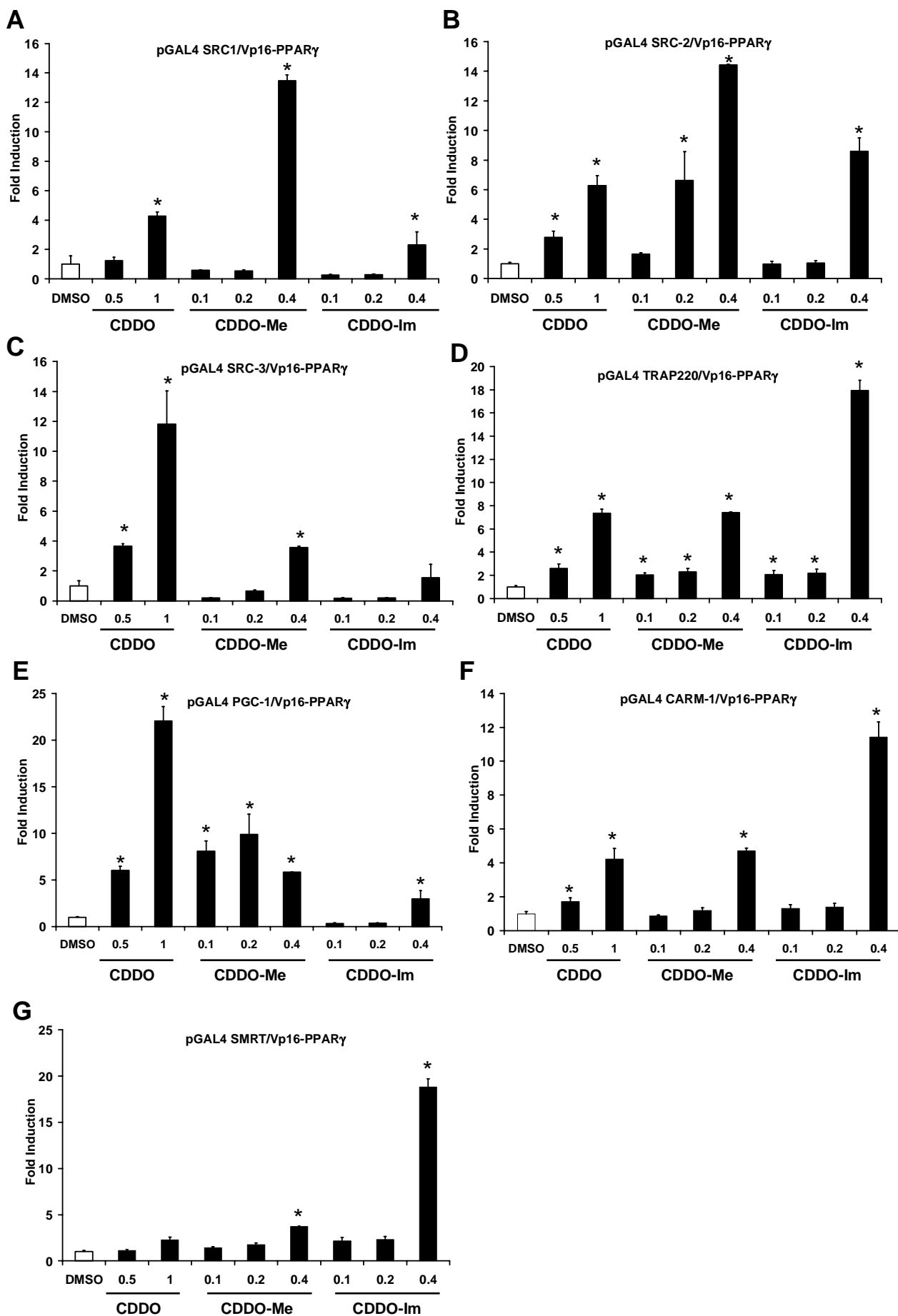


Figure 4

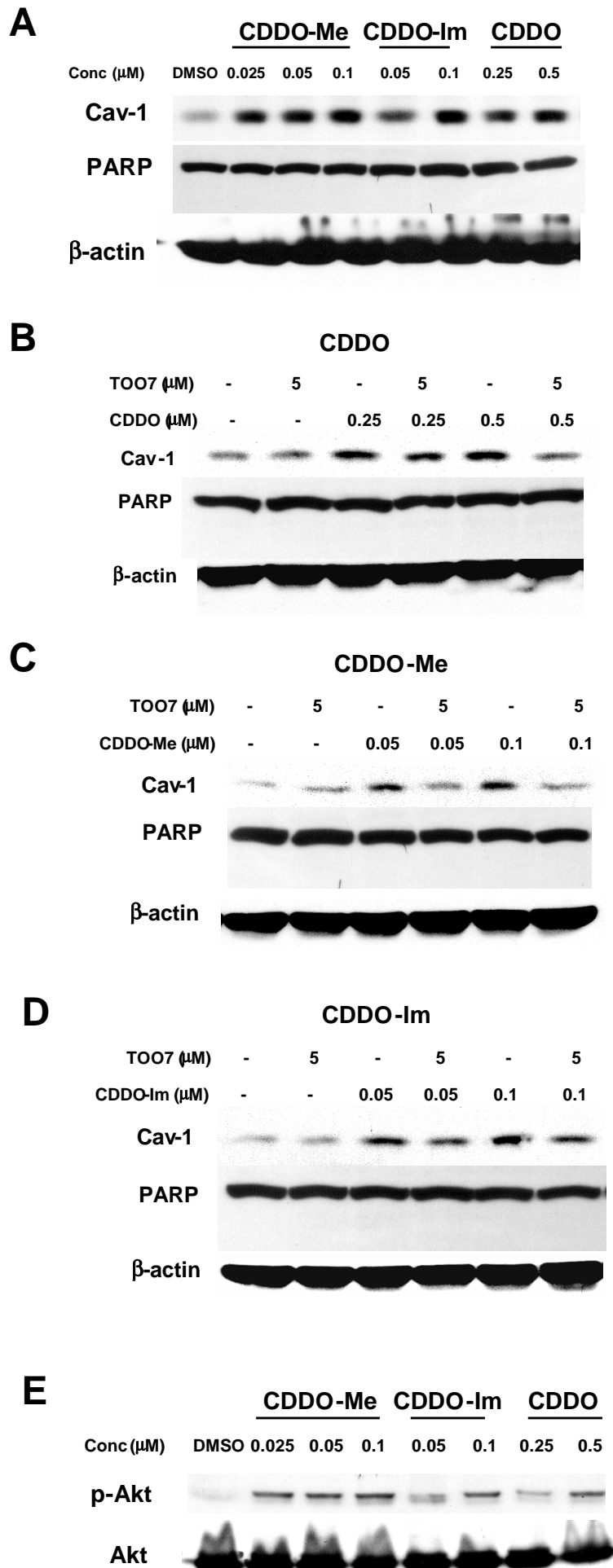


Figure 5

