

# Structure-dependent activity of glycyrrhetic acid derivatives as peroxisome proliferator-activated receptor $\gamma$ agonists in colon cancer cells

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## Abstract

Glycyrrhizin, a pentacyclic triterpene glycoside, is the major phytochemical in licorice. This compound and its hydrolysis product glycyrrhetic acid have been associated with the multiple therapeutic properties of licorice extracts. We have investigated the effects of 2-cyano substituted analogues of glycyrrhetic acid on their cytotoxicities and activity as selective peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) agonists. Methyl 2-cyano-3,11-dioxo-18 $\beta$ -olean-1,12-dien-30-oate ( $\beta$ -CDODA-Me) and methyl 2-cyano-3,11-dioxo-18 $\alpha$ -olean-1,12-dien-30-oate ( $\alpha$ -CDODA-Me) were more cytotoxic to colon cancer cells than their des-cyano analogues and introduction of the 2-cyano group into the pentacyclic ring system was necessary for the PPAR $\gamma$  agonist activity of  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers. However, in mammalian two-hybrid assays, both compounds differentially induced interactions of PPAR $\gamma$  with coactivators, suggesting that these isomers, which differ only in the stereochemistry at C18 which affects conformation of the E-ring, are selective receptor modulators. This selectivity in colon cancer cells was shown for the induction of two proapoptotic proteins, namely *caveolin-1* and the tumor-suppressor gene *Krüppel-like factor-4* (*KLF-4*).  $\beta$ -CDODA-Me but not  $\alpha$ -CDODA-Me induced *caveolin-1* in SW480 colon cancer cells, whereas

*caveolin-1* was induced by both compounds in HT-29 and HCT-15 colon cancer cells. The CDODA-Me isomers induced *KLF-4* mRNA levels in HT-29 and SW480 cells but had minimal effects on *KLF-4* expression in HCT-15 cells. These induced responses were inhibited by cotreatment with a PPAR $\gamma$  antagonist. This shows for the first time that PPAR $\gamma$  agonists derived from glycyrrhetic acid induced cell-dependent *caveolin-1* and *KLF-4* expression through receptor-dependent pathways. [Mol Cancer Ther 2007;6(5):1588–98]

## Introduction

Licorice root extracts have been extensively used for their therapeutic properties, which include the potentiation of cortisol action, inhibition of testosterone biosynthesis, reduction in body fat mass, and other endocrine effects (1–4). The activities of these extracts are linked to different classes of phytochemicals particularly the major water-soluble constituent glycyrrhizin and its hydrolysis product 18 $\beta$ -glycyrrhetic acid (Fig. 1). Glycyrrhizin is a pentacyclic triterpenoid glycoside, which is hydrolyzed in the gut to glycyrrhetic acid, and many of the properties of licorice root can be attributed to glycyrrhetic acid. For example, glycyrrhetic acid inhibits 11 $\beta$ -hydroxysteroid dehydrogenase activity, increasing corticosterone levels. This has been linked to apoptosis in murine thymocytes, splenocytes, and decreased body fat index in human studies (5–9). Glycyrrhetic acid also directly acts on mitochondria to induce apoptosis through increased mitochondrial swelling, loss of mitochondrial membrane potential, and release of cytochrome *c* (10, 11).

Glycyrrhetic acid has also been used as a template to synthesize bioactive drugs. For example carbenoxolone, the 3-hemisuccinate derivative of glycyrrhetic acid, has been used for the treatment of gastritis and ulcers (12). Some of the activity of carbenoxolone may be due to hydrolysis to glycyrrhetic acid; however, carbenoxolone itself induced oxidative stress in liver mitochondria and decreased mitochondrial membrane potential. Other carboxyl and hydroxyl derivatives of glycyrrhizic acid inhibit HIV and exhibit anti-inflammatory and immunomodulatory activities (13). In addition, glycyrrhetic acid derivatives containing a reduced carboxylic acid group at C-30 (CH<sub>2</sub>OH) and some additional functional changes exhibited strong antioxidant activity (14).

Structure-activity studies on the anti-inflammatory activities and cytotoxicity of several oleanolic and ursolic acid derivatives showed that addition of a 2-cyano substituent greatly enhanced their activity (15–19). Moreover, one of the 2-cyano analogues of oleanolic acid, namely 2-cyano-3,12-dioxo-17 $\alpha$ -olean-1,9(11)-diene-28-oic acid (CDDO) and its

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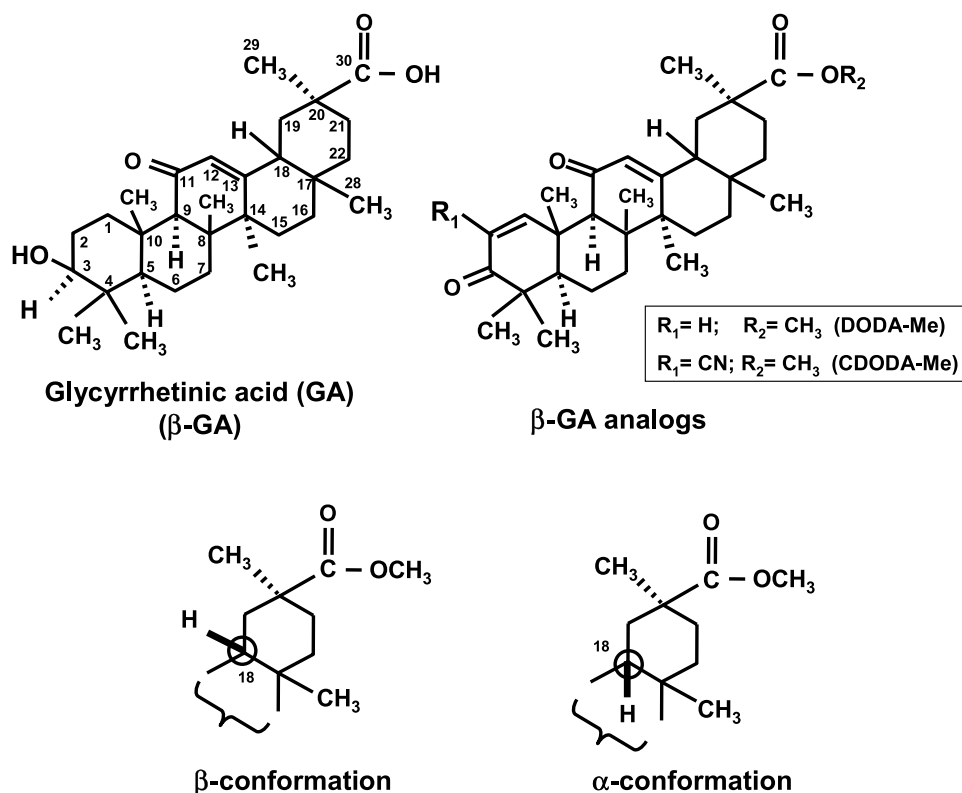
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**Figure 1.** Structures of glycyrrhetic acid (GA),  $\beta$ -glycyrrhetic acid derivatives, and differences in the stereochemistry at C-18 of the  $\alpha$ -glycyrrhetic acid- and  $\beta$ -glycyrrhetic acid-derived compounds.



methyl ester (CDDO-Me) exhibited peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) agonist activity (20–22). Although glycyrrhetic acid also has an oleanane triterpenoid backbone, there are major structural differences between glycyrrhetic acid and oleanolic acid and between CDDO-Me and the synthetic glycyrrhetic acid analogue methyl 2-cyano-3,11-dioxo-18 $\beta$ -olean-1,12-dien-30-oate ( $\beta$ -CDODA-Me). CDODA-Me ( $\beta$ -CDODA-Me) is isomeric with CDDO-Me ( $\alpha$ -CDDO-Me) but differs with respect to the carboxy substitution in the E ring, the stereochemistry at C-18 ( $\beta$  versus  $\alpha$ ) in the E/D ring junction, and the enone function in the C ring (Fig. 1). To more fully investigate the importance of the stereochemistry at C-18 in modulating cytotoxicity and PPAR $\gamma$  agonist activity of triterpenoid acids, we also synthesized methyl 2-cyano-3,11-dioxo-18 $\alpha$ -olean-1,12-dien-30-oate ( $\alpha$ -CDODA-Me). Our results show that introduction of the 2-cyano group into  $\alpha$ -glycyrrhetic acid or  $\beta$ -glycyrrhetic acid resulted in enhanced cytotoxicity, and both compounds induced PPAR $\gamma$ -dependent transactivation in colon cancer cells, including receptor- and cell context-dependent activation of *caveolin-1* and *Krüppel-like factor-4* (*KLF-4*), two genes associated with growth-inhibitory responses in colon cancer. However, it was also apparent that the different stereochemistries at C18 and the altered conformation of the E-ring resulted in different PPAR $\gamma$ -dependent effects in colon cancer cells, suggesting that the  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers are selective receptor modulators.

## Materials and Methods

### Cell Lines

Human colon carcinoma cell lines SW480, HCT-15, and HT29 were provided by Dr. Stan Hamilton (M.D. Anderson Cancer Center, Houston, TX); SW-480 and HT-29 cells were maintained in DMEM nutrient mixture with Ham's F-12 (DMEM/Ham's F-12; Sigma-Aldrich) with phenol red supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, and 5% fetal bovine serum and 10 mL/L 100 $\times$  antibiotic antimycotic solution (Sigma-Aldrich). HCT-15 cells were maintained in RPMI 1640 (Sigma) supplemented with 0.22% sodium bicarbonate, 0.11% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% fetal bovine serum, and 10 mL/L of 100 $\times$  antibiotic antimycotic solution. Cells were maintained at 37 $^{\circ}$ C in the presence of 5% CO $_2$ .

### Synthesis

**3,11-Dioxo-18 $\beta$ -Oleana-1,12-Dien-30-Oic Acid and 3,11-Dioxo-18 $\alpha$ -Oleana-1,12-Dien-30-Oic Acid.** A mixture of 18 $\beta$ -glycyrrhetic acid (157 mg, 0.3333 mmol; Sigma-Aldrich) and 2-iodoxybenzoic acid (373.4 mg, 1.333 mmol, 4 equiv) in 7 mL DMSO was stirred with heating at 85 $^{\circ}$ C for 21 h. After cooling, the solution was poured into water (100 mL) giving a white precipitate that was filtered and washed with methanol/methylene chloride (1:9). This material (381 mg) was triturated with ethyl acetate (5 mL), washed several times with this solvent, and the dissolved material was recovered by evaporation and purified by preparative scale TLC using methanol/CH $_2$ Cl $_2$  (1:19) as

eluant. The main band gave 3,11-dioxo-18 $\beta$ -oleana-1,12-dien-30-oic acid ( $\beta$ -DODA) as a white solid (133 mg, 85.5%), which was crystallized from methanol (104 mg), mp 270°C to 275°C.  $^1\text{H}$  nuclear magnetic resonance (NMR)  $\delta$  7.746 (1H, d,  $J$  = 10.4 Hz, C1-H), 5.816 (1H, d,  $J$  = 10.4 Hz, C2-H), 5.817 (1H, s, C12-H), 2.691 (1H, s, C9-H), 1.422, 1.401, 1.245, 1.191, 1.169, 1.118, 0.872 (all 3H, s, CMe). A similar procedure was used for the synthesis of 3,11-dioxo-18 $\alpha$ -oleana-1,12-dien-30-oic acid ( $\alpha$ -DODA) from 18 $\alpha$ -glycyrrhetic acid (Sigma-Aldrich).

**$\beta$ -DODA-Me and  $\alpha$ -DODA-Me.** Methyl 18 $\beta$ -glycyrrhetinate was prepared by diazomethylation of 18 $\beta$ -glycyrrhetic acid and a sample (161 mg, 0.3333 mmol) reacted with the 2-iodoxybenzoic acid reagent (373 mg, 1.333 mmol, 4 equiv) as above for the parent acid. After a similar work-up, the recovered product was purified by preparative TLC (methanol/ $\text{CH}_2\text{Cl}_2$ ; 1:19) to give a colorless solid (155.3 mg, 96.9%), which, on crystallization, gave needles (140 mg), mp 192°C to 194°C.  $^1\text{H}$  NMR  $\delta$  7.745 (1H, d,  $J$  = 10.0 Hz, C1-H), 5.812 (1H, d,  $J$  = 10.0 Hz, C2-H), 5.770 (1H, s, C12-H), 3.078 (3H, s, OMe), 2.681 (1H, s, C9-H), 1.419, 1.390, 1.184, 1.166, 1.159, 1.118, 0.833 (all 3H, s, CMe). A similar procedure was used for the synthesis of  $\alpha$ -DODA-Me from  $\alpha$ -DODA.

**$\beta$ -CDODA and  $\alpha$ -CDODA.** The two cyano derivative of 18 $\beta$ -glycyrrhetic acid was synthesized as previously described (23), and a sample (422 mg, 0.8961 mmol) of this compound and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (247 mg, 1.088 mmol) in dry benzene (55 mL) was heated to reflux, with stirring, for 6 h. The reaction mixture was filtered and washed with benzene, and the filtrate plus washings were combined, evaporated, and purified by preparative TLC (ethyl acetate/hexane, 1:1) to give  $\beta$ -CDODA (149 mg, 33.7%). This material was crystallized twice from ethyl acetate/hexane to give a yellow solid (55.5 mg), mp 195°C to 197°C.  $^1\text{H}$  NMR  $\delta$  8.550 (1H, s, C1-H), 5.846 (s, C12-H), 2.2715 (1H, s, C9-H), 1.455, 1.404, 1.255, 1.225, 1.200, 1.162, 0.876 (all 3H, s, CMe). A similar procedure was used for the synthesis of  $\alpha$ -CDODA from 18 $\alpha$ -glycyrrhetic acid.

**$\beta$ -CDODA-Me and  $\alpha$ -CDODA-Me.** The nitrile was also prepared from methyl 18 $\beta$ -glycyrrhetinate, and the resulting ester (246 mg, 0.4863 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (134 mg, 0.5905 mmol) in dry benzene (20 mL) was refluxed for 5 h to give  $\beta$ -CDODA-Me. The compound was purified by TLC (ethyl acetate/hexane; 1:3) to give  $\beta$ -CDODA-Me and crystallized from ethyl acetate/hexane (138 mg), mp 243°C to 245°C.  $^1\text{H}$  NMR  $\delta$  8.553 (1H, s, C1-H), 5.805 (s, C12-H), 3.716 (3H, s, OMe), 2.706 (1H, s, C9-H), 1.454, 1.393, 1.223, 1.194, 1.168, 1.161, 0.834 (all 3H, s, CMe). A similar procedure was used for the synthesis of  $\alpha$ -CDODA-Me from  $\alpha$ -DODA.

#### Antibodies and Reagents

Caveolin-1 antibody was purchased from Santa Cruz Biotechnology, Inc. Monoclonal  $\beta$ -actin antibody and 18 $\alpha$ -glycyrrhetic acid and 18 $\beta$ -glycyrrhetic acid were purchased from Sigma-Aldrich. Reporter lysis buffer and luciferase reagent for luciferase studies were supplied by

Promega.  $\beta$ -Galactosidase ( $\beta$ -Gal) reagent was obtained from Tropix, and LipofectAMINE reagent was purchased from Invitrogen. Western Lightning chemiluminescence reagent was obtained from Perkin-Elmer Life and Analytical Sciences. The PPAR $\gamma$  antagonists 2-chloro-5-nitro-*N*-phenylbenzamide (GW9662) and *N*-(4'-aminopyridyl)-2-chloro-5-nitrobenzamide (T007) were synthesized in this laboratory, and their identities and purity (>98%) were confirmed by gas chromatography-mass spectrometry. Melting points were determined with a Kofler hot-stage apparatus.  $^1\text{H}$  NMR spectra were run in  $\text{CDCl}_3$  on a Bruker Avance-400 spectrometer using  $\text{Me}_4\text{Si}$  as an internal standard. For analytic and preparative use, TLC plates were spread with Silica Gel 60 GF (Merck). Elemental microanalyses were carried out by Guelph Chemical Laboratories, Ltd.

#### Plasmids

The Gal4 reporter containing 5 $\times$  Gal4 response elements (pGal4) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4DBD-PPAR $\gamma$  construct was a gift of Dr. Jennifer L. Oberfield (GlaxoSmithKline Research and Development). PPRE $_3$ -luc construct contains three tandem PPREs with a minimal TATA sequence in pGL2. The GAL4 coactivator (PM coactivator) and VP-PPAR $\gamma$  chimeras were provided by Dr. S. Kato, University of Tokyo (Tokyo, Japan; ref. 24).

#### Transfection and Luciferase Assay

Colon cancer cell lines SW480 and HT29 ( $1 \times 10^5$  cells per well) were plated in 12-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum. After 16 h, various amounts of DNA [i.e., Gal4Luc (0.4  $\mu\text{g}$ ),  $\beta$ -galactosidase (0.04  $\mu\text{g}$ ), and Gal4PPAR $\gamma$  and PPRE-Luc (0.04  $\mu\text{g}$ )] were transfected using LipofectAMINE reagent (Invitrogen) following the manufacturer's protocol. Five hours after transfection, the transfection mix was replaced with complete medium containing either vehicle (DMSO) or the indicated ligand for 20 to 22 h. Cells were then lysed with 100  $\mu\text{L}$  of 1 $\times$  reporter lysis buffer, and 30  $\mu\text{L}$  of cell extract was used for luciferase and  $\beta$ -galactosidase assays. A LumiCount luminometer (Perkin-Elmer Life and Analytical Sciences) was used to quantitate luciferase and  $\beta$ -galactosidase activities, and the luciferase activities were normalized to  $\beta$ -galactosidase activity. Results are expressed as means  $\pm$  SE for at least three replicate determinations for each treatment group.

#### Mammalian Two-Hybrid Assay

SW480 cells were plated in 12-well plates at  $1 \times 10^5$  per well in DMEM/F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum. After growth for 16 h, various amounts of DNA [i.e., Gal4Luc (0.4  $\mu\text{g}$ ),  $\beta$ -gal (0.04  $\mu\text{g}$ ), VP-PPAR $\gamma$  (0.04  $\mu\text{g}$ ), pMSRC1 (0.04  $\mu\text{g}$ ), pMSRC2 (0.04  $\mu\text{g}$ ), pMSRC3 (0.04  $\mu\text{g}$ ), pMPGC-1 (0.04  $\mu\text{g}$ ), pMDRIP205 (0.04  $\mu\text{g}$ ), and pMCARM-1 (0.04  $\mu\text{g}$ )] were transfected by LipofectAMINE (Invitrogen) according to the manufacturer's protocol. After 5 h, the transfection mix was replaced with complete medium containing either vehicle (DMSO) or the indicated ligand for 20 to 22 h. Cells

were then lysed with 100 mL of  $1\times$  reporter lysis buffer, and 30  $\mu\text{L}$  of cell extract was used for luciferase and  $\beta$ -galactosidase assays. LumiCount was used to quantitate luciferase and  $\beta$ -galactosidase activities, and the luciferase activities were normalized to  $\beta$ -galactosidase activity.

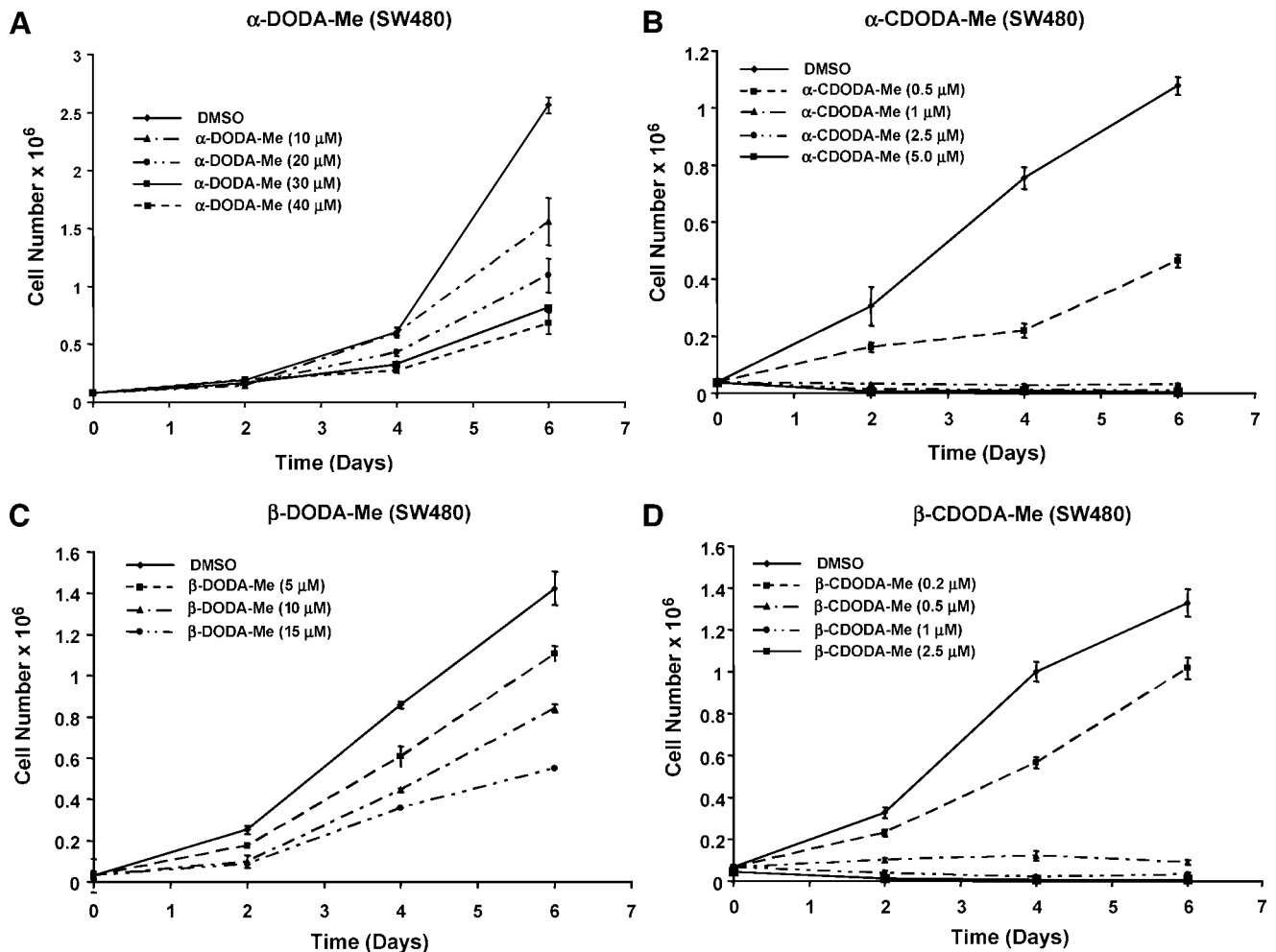
#### Cell Proliferation Assay

SW480, HCT-15, and HT 29 cells ( $2 \times 10^4$ ) were plated in 12-well plates, and the medium was replaced the next day with DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped fetal bovine serum and either vehicle (DMSO) or the indicated ligand and dissolved in DMSO. Fresh medium and compounds were added every 48 h. Cells were counted at the indicated times using a Coulter Z1 cell counter. Each experiment was done in triplicate, and results are expressed as means  $\pm$  SE for each determination.

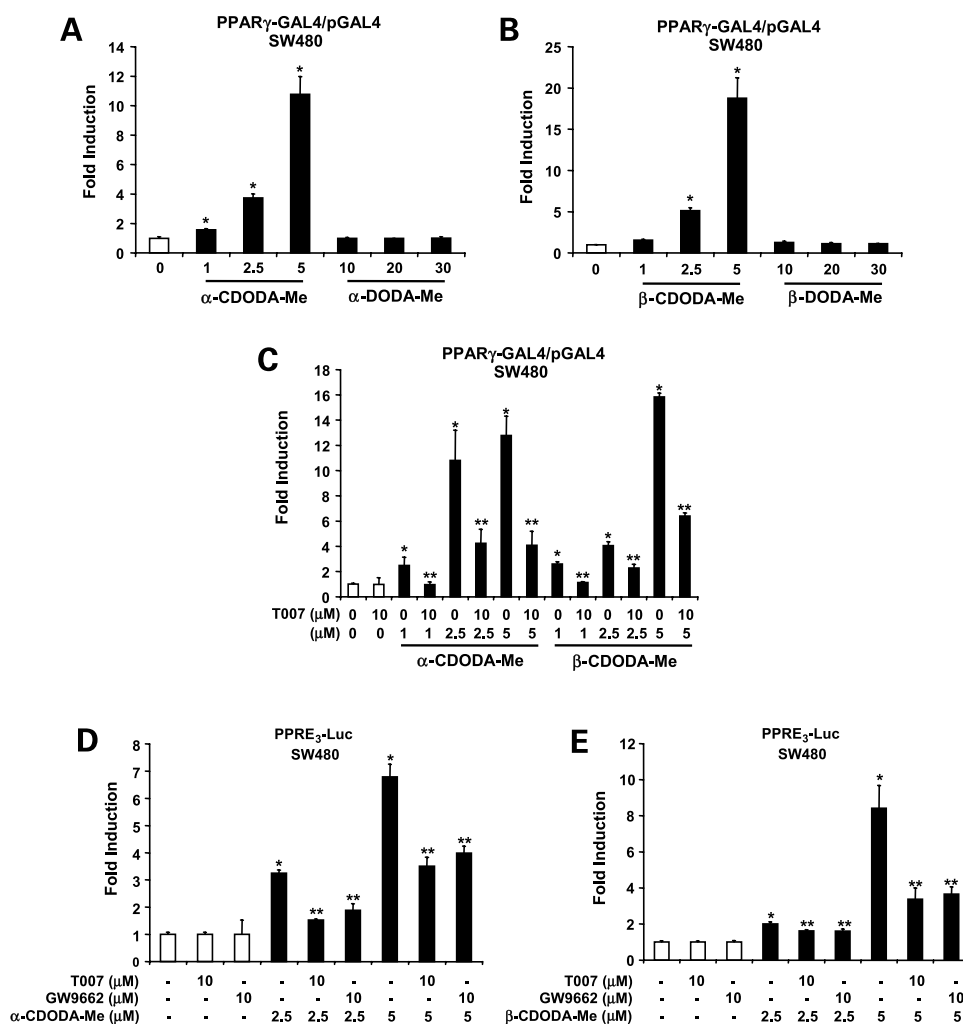
#### Western Blot Analysis

SW-480, HCT-15, and HT-29 ( $3 \times 10^5$ ) cells were seeded in six-well plates in DMEM/Ham's F-12 medium containing

2.5% charcoal-stripped fetal bovine serum 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 mmol/L HEPES, 500 mmol/L NaCl, 1.5 mmol/L  $\text{MgCl}_2$ , 1 mmol/L EGTA, 10% glycerol, and 1% Triton X-100 (pH 7.5), and 5  $\mu\text{L}/\text{mL}$  protease inhibitor cocktail (Sigma-Aldrich)]. Protein samples were incubated at  $100^\circ\text{C}$  for 2 min, separated on 10% SDS-PAGE at 120 V for 3 to 4 h in  $1\times$  running buffer [25 mmol/L Tris-base, 192 mmol/L glycine, and 0.1% SDS (pH 8.3)], and transferred to polyvinylidene difluoride membrane (Bio-Rad) at 0.1 V for 16 h at  $4^\circ\text{C}$  in  $1\times$  transfer buffer (48 mmol/L Tris-HCl, 39 mmol/L glycine, and 0.025% SDS). The polyvinylidene difluoride membrane was blocked in 5% TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, and 5% nonfat dry milk] with gentle shaking for 30 min and was incubated in fresh 5% TBST-Blotto with 1:1,000 (for *caveolin-1*), and 1:5,000 (for  $\beta$ -actin) primary



**Figure 2.** Growth inhibition studies. SW480 colon cancer cells were treated with different concentrations of  $\alpha$ -DODA-Me (**A**),  $\alpha$ -CDODA-Me (**B**),  $\beta$ -DODA-Me (**C**), and  $\beta$ -CDODA-Me (**D**) for 6 d, and cell numbers were determined using a Coulter counter as described in Materials and Methods. Points, mean for three separate determinations; bars, SE. Significant ( $P < 0.05$ ) inhibition of cell growth was observed for  $\beta$ -DODA-Me ( $\leq 5 \mu\text{mol/L}$ ),  $\beta$ -CDODA-Me ( $\leq 0.2 \mu\text{mol/L}$ ),  $\alpha$ -DODA-Me ( $\leq 10 \mu\text{mol/L}$ ), and  $\alpha$ -CDODA-Me ( $\leq 0.5 \mu\text{mol/L}$ ).



**Figure 3.** Ligand-induced activation of PPAR $\gamma$  and effects of PPAR $\gamma$  antagonists. Activation of PPAR $\gamma$ -GAL4/pGAL4 in SW480 cells treated with  $\alpha$ -DODA-Me/ $\alpha$ -CDODA-Me (A),  $\beta$ -DODA-Me/ $\beta$ -CDODA-Me (B), and both isomers plus T007 (C). Cells were transfected with PPAR $\gamma$ -GAL4/pGAL4, treated with different concentrations of the triterpenoids alone or in combination with T007, and luciferase activity was determined as described in Materials and Methods. Columns, means for at least three separate determinations for each treatment group; bars, SE. Significant ( $P < 0.05$ ) induction compared with solvent (DMSO) control (\*) and inhibition by cotreatment with T007 (\*\*). Activation of PPRE-luc in SW480 cells treated with  $\alpha$ -CDODA-Me (D) or  $\beta$ -CDODA-Me (E) alone or in combination with PPAR $\gamma$  antagonists. SW480 cells were transfected with PPRE-Luc, treated with different concentrations of CDODA-Me isomers alone or in combination with 10  $\mu$ mol/L GW9662 and/or T007, and luciferase activities were determined as described in A. Significant ( $P < 0.05$ ) induction of luciferase activity (\*) and inhibition of induced transactivation by GW9662 or T007 (\*\*).

antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the polyvinylidene difluoride membrane was incubated with secondary antibody (1:5,000) in 5% TBST-Blotto for 90 min. The membrane was washed with TBST for 10 min, incubated with 10 mL of chemiluminescence substrate (Perkin-Elmer) for 1.0 min, and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak).

#### Semiquantitative Reverse Transcription-PCR Analysis

SW480, HT-29, and HCT-15 cells were treated with either DMSO (control) or with the indicated concentration of the compound for 12 h. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Inc.), and 1  $\mu$ m of RNA was used to synthesize cDNA using Reverse Transcription System (Promega). The PCR conditions were as follows: initial denaturation at 94°C (1 min) followed by 28 cycles of denaturation for 30 s at 94°C, annealing for 60 s at 55°C and extension at 72°C for 60 s, and a final extension step at 72°C for 5 min. The mRNA levels were normalized using *GAPDH* as an internal housekeeping gene. Primers were obtained from IDT and used for amplification were as follows: *KLF4* (sense 5'-CTATGGCAGGGAGTCCGCTCC-3'; antisense

5'-ATGACCGACGGGCTGCCGTAC-3') and *GAPDH* (sense 5'-ACGGATTGGTCGTATTGGGCG-3'; antisense 5'-CTCCTGGAAGATGGTATGG-3'). PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and visualized under UV transillumination.

#### Statistical Analysis

Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean  $\pm$  SD for at least three separate determinations for each treatment.

## Results

### Growth-Inhibitory Effects of Isomeric Glycyrrhetic Acid Derivatives

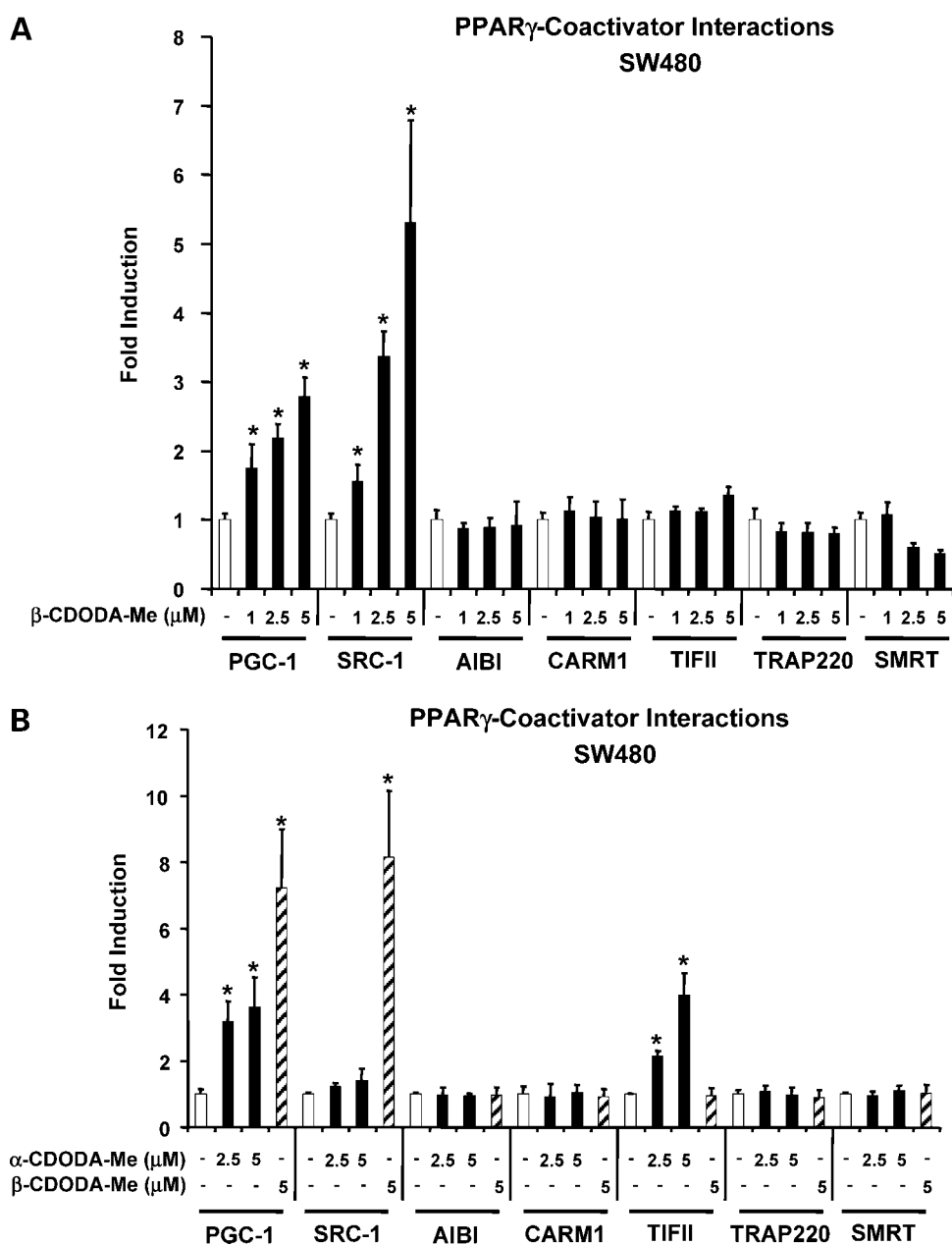
This study compares the cytotoxicity of 18 $\beta$ -glycyrrhetic acid and 18 $\alpha$ -glycyrrhetic acid derivatives and results in Fig. 2 summarize the cytotoxicity of  $\beta$ -DODA-Me,  $\beta$ -CDODA-Me, and the corresponding 18 $\alpha$  isomers. Initial studies showed that glycyrrhetic acid and its methyl esters exhibit minimal cytotoxicity and the methyl esters were more potent than the corresponding free triterpenoid acids (data not shown). The  $\alpha$ -DODA and

$\beta$ -DODA methyl esters exhibited growth-inhibitory  $IC_{50}$  values of 10 to 20  $\mu\text{mol/L}$  and 10 to 15  $\mu\text{mol/L}$ , respectively, whereas introduction of the 2-cyano substituents into the  $\alpha$  and  $\beta$  isomers greatly enhanced cytotoxicity. The  $IC_{50}$  values for  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me were 0.5  $\mu\text{mol/L}$  and 0.2 to 0.5  $\mu\text{mol/L}$ , respectively, demonstrating the greatly enhanced cytotoxicity of the glycyrrhetic acid derivatives containing the 2-cyano substituents. Similar results were observed in HT-29 and HCT-15 colon cancer cells (data not shown).

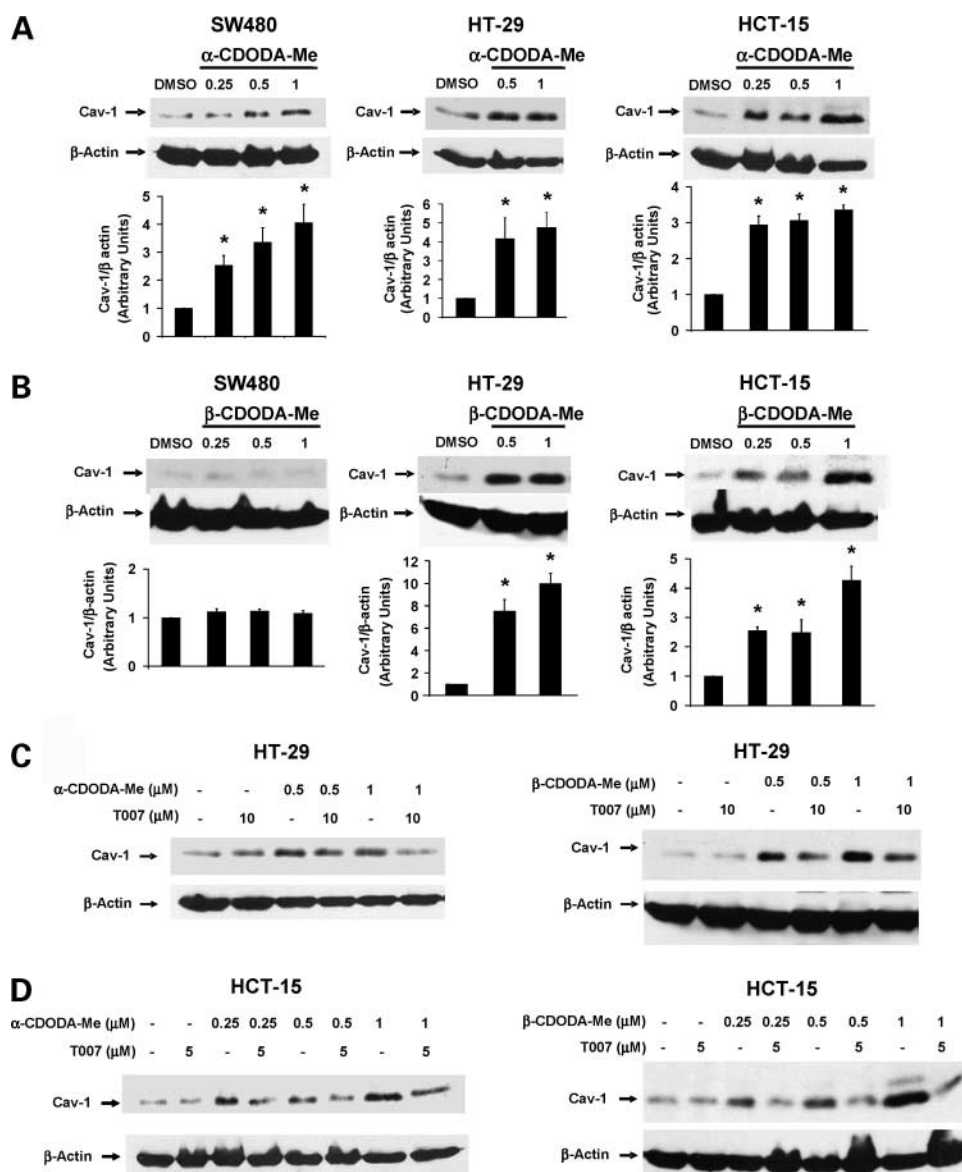
#### $\alpha$ -CDODA-Me and $\beta$ -CDODA-Me Activate PPAR $\gamma$

Previous studies have shown that introduction of 2-cyano substituents into oleanolic acid and ursolic acid derivatives enhances cytotoxicity of these triterpenoid acids (15, 16, 19)

as observed in this study for the  $\alpha$ -glycyrrhetic acid and  $\beta$ -glycyrrhetic acid derivatives (Fig. 2). 2-Cyano derivatives of oleanolic acid also exhibit PPAR $\gamma$  agonist activity (22) and, in this study, we have investigated the PPAR $\gamma$  agonist activity of  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers, which exhibit major structural differences in the E-ring of glycyrrhetic acid. Results in Fig. 3A compare activation of PPAR $\gamma$  by  $\alpha$ -CDODA-Me and  $\alpha$ -DODA-Me in SW480 cells transfected with PPAR $\gamma$ -GAL4/pGAL4; 5  $\mu\text{mol/L}$   $\alpha$ -CDODA-Me induces a >10-fold increase in activity, whereas  $\alpha$ -DODA-Me was inactive at concentrations as high as 20  $\mu\text{mol/L}$ . In a separate experiment, similar results were obtained for  $\beta$ -CDODA-Me and  $\beta$ -DODA-Me. The former compound (5  $\mu\text{mol/L}$ ) induced a



**Figure 4.** Ligand-induced PPAR $\gamma$ -coactivator interactions. SW480 cells were transfected with VP-PPAR $\gamma$ , coactivator-GAL4/pGAL4, treated with different concentrations of  $\alpha$ -CDODA-Me (A) or both  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me (B), and luciferase activity was determined as described in Materials and Methods. Columns, mean for three replicate determinations for each treatment group; bars, SE. \*,  $P < 0.05$ , significant induction.



**Figure 5.** Induction of *caveolin-1* in colon cancer cells. SW480, HT-29, or HCT-15 colon cancer cells were treated with  $\alpha$ -CDODA-Me (**A**) or  $\beta$ -CDODA-Me (**B**) for 72 h as previously described (20), and whole-cell lysates were analyzed by Western immunoblot analysis as described in Materials and Methods. Inhibition of *caveolin-1* induction by  $\alpha$ -CDODA-Me or  $\beta$ -CDODA-Me by T007 in HT-29 (**C**) or HCT-15 (**D**) cells. Cells were treated with 5  $\mu$ mol/L T007, 0.5 to 1.0  $\mu$ mol/L  $\alpha$ -CDODA-Me or  $\beta$ -CDODA-Me, or combinations (as indicated) for 72 h, and whole-cell lysates were analyzed by Western immunoblot analysis as described in Materials and Methods. Similar results were observed for  $\alpha$ -CDODA-Me in SW480 cells (data not shown). Caveolin-1 protein expression (relative to  $\beta$ -actin) in the DMSO-treated cells (**A** and **B**) was set at 1.0. \*,  $P < 0.05$ , significant induction. Columns, mean for three replicate determinations for each treatment group; bars, SE.

>18-fold increase in luciferase activity, whereas the latter compound (30  $\mu$ mol/L) was inactive (Fig. 3B). A direct comparison of both  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me is shown in Fig. 3C, where 5  $\mu$ mol/L of both compounds induced a 12- to 16-fold increase in luciferase activity in SW480 cells transfected with PPAR $\gamma$ -GAL4/pGAL4. Cotreatment with 10  $\mu$ mol/L of the PPAR $\gamma$  antagonist T007 significantly decreased  $\alpha$ -CDODA-Me/ $\beta$ -CDODA-Me-induced transactivation. Both  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me also induced transactivation (Figs. 3D and E) in SW480 cells transfected with PPRE3-luc, a construct that contains three tandem PPAR $\gamma$  response elements linked to luciferase and that relies on activation of the endogenous PPAR $\gamma$ -retinoid X receptor complex expressed in this cell line (20). In addition, both PPAR $\gamma$  antagonists T007 and GW9662 inhibited  $\alpha$ -CDODA-Me/ $\beta$ -CDODA-Me-induced

transactivation. These results show for the first time that introduction of a 2-cyano group into the glycyrrhetic acid triterpenoid acid structure is sufficient for conferring PPAR $\gamma$  agonist activity on the resulting compound. Moreover, both the  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers exhibit similar potencies as PPAR $\gamma$  agonists ( $\beta$ -CDODA-Me  $\geq$   $\alpha$ -CDODA-Me), suggesting that the conformational differences in the E-ring, which are observed for the 18 $\alpha$  and 18 $\beta$  isomers do not affect their PPAR $\gamma$  agonist activities in the PPAR $\gamma$ -GAL4/pGAL4 and PPRE-luc transactivation assays.

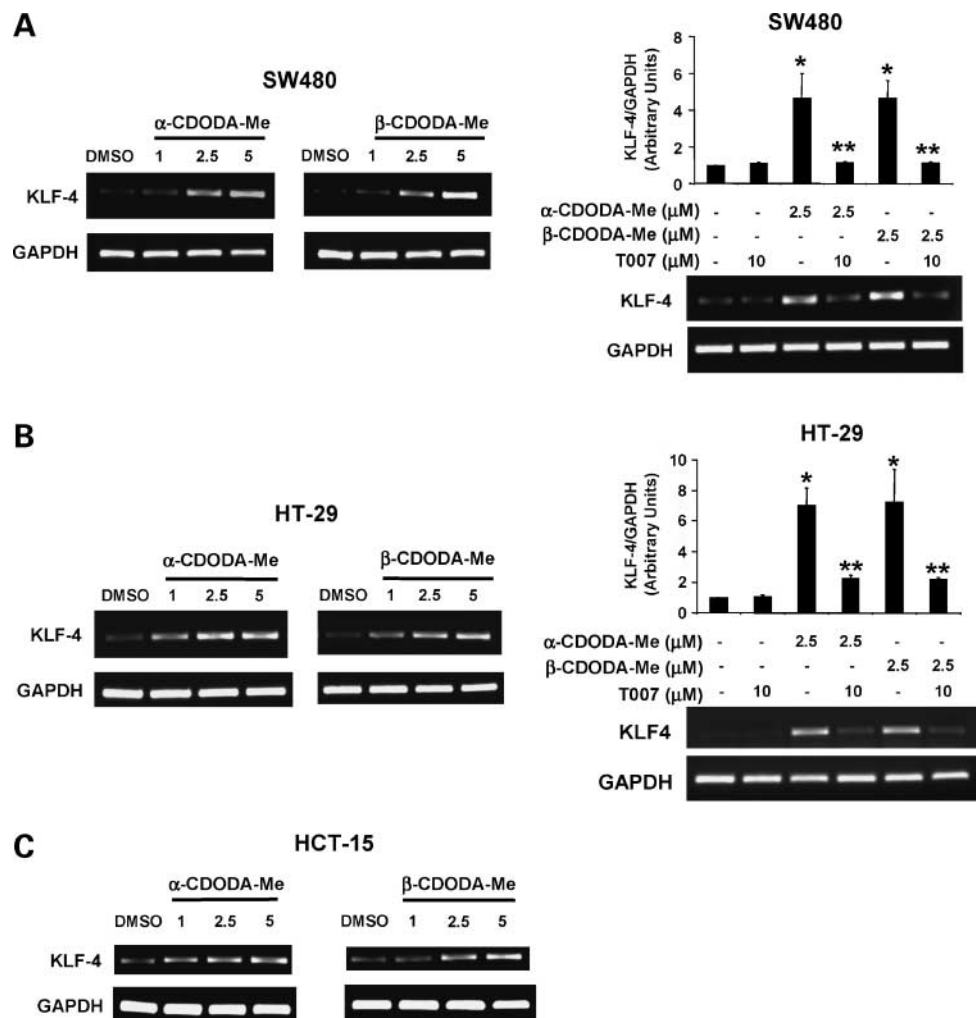
#### $\alpha$ -CDODA-Me and $\beta$ -CDODA-Me as Selective Receptor Modulators

PPAR $\gamma$  agonists are structurally diverse (24–27) and there is evidence that many of these compounds are selective PPAR $\gamma$  modulators that exhibit tissue-specific

differences in their activation of receptor-dependent genes/protein. The selectivity of various structural classes of PPAR $\gamma$  ligands is due, in part, to differential interactions within the ligand binding domain of PPAR $\gamma$ , which can lead to different conformations of the receptor. This can result in differential interactions of the ligand-bound PPAR $\gamma$  with nuclear receptor coactivators (24), and results in Fig. 4A summarize  $\beta$ -CDODA-Me-induced transactivation in SW480 cells transfected with GAL4-coactivator and VP-PPAR $\gamma$  (ligand binding domain) chimeras and a pGAL4 reporter gene. In this mammalian two-hybrid assay,  $\beta$ -CDODA-Me induced PPAR $\gamma$  interactions only with PGC-1 and SRC-1 but not with AIB1 (SRC-3), TIFII (SRC-2), CARM1, TRAP220, and the corepressor SMRT.  $\alpha$ -CDODA-Me also induced PPAR $\gamma$ -PGC-1 interactions (Fig. 4B); however, in contrast with the  $\beta$  isomer,  $\alpha$ -CDODA-Me induced PPAR $\gamma$  interactions with TIFII but not with SRC-1, AIB1, CARM1, TRAP220, or SMRT. These results suggest that the two  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers, which differ only in the conformations of their E-rings, are selective receptor modulators and induce different patterns of coactivator-receptor interactions in a

mammalian two-hybrid assay. These results also suggest that the  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers should exhibit some tissue/cell or response-specific differences in their activation of receptor-dependent genes.

PPAR $\gamma$  agonists induce *caveolin-1* in colon cancer cells through a receptor-dependent mechanism (28, 29). The effects of  $\alpha$ -CDODA-Me on *caveolin-1* expression in HT-29, HCT-15, and SW480 cells is summarized in Fig. 5A, and induction was observed in all three cell lines. In contrast,  $\beta$ -CDODA-Me induced *caveolin-1* in HT-29 and HCT-15 but not SW480 colon cancer cells (Fig. 5B), and the failure to observe induction of *caveolin-1* in SW480 cells was noted in several replicate experiments. Figure 5 (C and D) shows that induction of *caveolin-1* by  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers was inhibited in HT-29 and HCT-15 cells cotreated with the PPAR $\gamma$  antagonist T007, and similar results were observed for  $\alpha$ -CDODA-Me in SW480 cells (data not shown). These results show the tissue-selective induction of *caveolin-1* expression by  $\beta$ -CDODA-Me and this is consistent with the activity of  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers as selective receptor modulators.



**Figure 6.** Induction of *KLF-4* gene expression by  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me. Induction of *KLF-4* in SW480 (A), HT-29 (B), and HCT-15 (C) cells. Cells were treated with different concentrations of CDODA isomers or T007 alone or in combination and *KLF-4* mRNA levels were determined by RT-PCR as described in Materials and Methods. Columns (A and B), mean from three replicate experiments; bars, SE. Significant ( $P < 0.05$ ) induction or inhibition of *KLF-4* mRNA levels (\*) and inhibition of these responses after cotreatment with T007 (\*\*). Induction of *KLF-4* in HCT-15 cells was highly variable (<2-fold) and was not further quantitated.



Based on results of preliminary studies on growth-inhibitory/proapoptotic genes induced by CDODA-Me isomers, we investigated the induction of the tumor-suppressor gene *KLF-4* in colon cancer cells. Results in Fig. 6A show that 1 to 5  $\mu\text{mol/L}$  concentrations of both  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers induced *KLF-4* mRNA levels in SW480 cells. The PPAR $\gamma$  antagonist T007 (10  $\mu\text{mol/L}$ ) alone did not induce *KLF-4*. In SW480 cells cotreated with T007 and the CDODA-Me isomers, there was a significant inhibition of the induced response. A similar experiment was carried out in HT-29 cells (Fig. 6B), and both CDODA-Me isomers induced *KLF-4* mRNA levels, which were inhibited after cotreatment with T007. In contrast,  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me isomers did not consistently alter expression of *KLF-4* mRNA levels in HCT-15 cells (<2-fold and variable; Fig. 6C). These results show that CDODA-Me isomers exhibited similar activities as PPAR $\gamma$  agonists in HT-29 and SW480 colon cancer cells; however, induction of *KLF-4* mRNA was cell context-dependent and, over several experiments, we did not observe significant induction of *KLF-4* in HCT-15 cells. These results on the receptor-dependent induction of *KLF-4* gene expression by CDODA-Me isomers contrasts to the reported receptor-independent induction of *KLF-4* gene expression by the PPAR $\gamma$  agonist 15-deoxy- $\Delta$ 12,14-prostaglandin J<sub>2</sub> (PGJ<sub>2</sub>) in HT-29 cells (30).

## Discussion

PPAR $\gamma$  and other members of the nuclear receptor superfamily are characterized by their modular structure, which contains several regions and domains that are required for critical receptor-protein and receptor-DNA interactions (25–27). Nuclear receptors typically contain NH<sub>2</sub>- and COOH-terminal activation functions (AF1 and AF2, respectively), a DNA-binding domain, and a flexible hinge region. The addition of receptor ligand usually results in formation of a transcriptionally active nuclear receptor complex that binds cognate response elements in promoter regions of target genes and activates transcription. However, receptor-mediated transactivation is dependent on several factors including cell context-specific expression of coregulatory proteins (e.g., coactivators), gene promoter accessibility, and ligand structure (31). The complex pharmacology of receptor ligands is due, in part, to the ligand structure-dependent conformational changes in the bound receptor complex that may differentially interact with coregulatory factors and exhibit tissue-specific agonist and/or antagonist activity (31, 32). This has led to development of selective receptor modulators for several nuclear receptors that selectively activate or block specific receptor-mediated responses in different tissues/cells.

There is evidence that different structural classes of PPAR $\gamma$  agonists are also selective receptor modulators and induce tissue-specific receptor-dependent and receptor-independent responses. For example, induction of *NAG-1* in HCT116 colon cancer cells by PGJ<sub>2</sub> was PPAR $\gamma$  dependent, whereas both troglitazone and PPAR $\gamma$ -active 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl) methanes also

enhanced *NAG-1* expression through receptor-independent pathways in the same cell line (33–35). Evidence that different structural classes of PPAR $\gamma$  agonists are selective receptor modulators has been reported in mammalian two-hybrid assays in which cells have been transfected with VP-PPAR $\gamma$  and GAL4-coactivator constructs. For example, PGJ<sub>2</sub> and rosiglitazone differentially induced coactivator-PPAR $\gamma$  interactions in COS-1 cells (24), and differences in ligand-dependent coactivator-receptor interactions were also observed for rosiglitazone and PPAR $\gamma$ -active 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl) methanes in colon cancer cells (29).

Previous studies showed that introduction of a cyano group at C-2 of oleanolic acid or ursolic acid enhanced the cytotoxicity of the resulting synthetic analogues (15). Moreover, the oleanolic acid derivatives CDDO and CDDO-Me exhibited PPAR $\gamma$  agonist activities (20–22). Results of this study also showed that 2-cyano analogues of the  $\alpha$ -glycyrrhetic acid and  $\beta$ -glycyrrhetic acid methyl esters also exhibited increased cytotoxicity (Fig. 2) and PPAR $\gamma$  agonist activity. Similar results were observed for the corresponding acid derivatives that were less active than  $\alpha$ -CDODA-Me or  $\beta$ -CDODA-Me (data not shown). Thus, introduction of the 2-cyano group into the oleanolic acid and glycyrrhetic acid backbone is necessary for their PPAR $\gamma$  agonist activities and differences in their substitution in the C-ring and the position of carboxymethyl groups at C-30 (in glycyrrhetic acid) or C-28 (in oleanolic acid) did not affect PPAR $\gamma$  agonist activity. Glycyrrhetic acid and oleanolic acid are 18 $\beta$  and 18 $\alpha$  isomers, respectively (e.g., Fig. 1), and their different stereochemistries at C-18 results in conformational differences in the E-ring of these triterpenoids. Therefore, to directly compare the effects of different E-ring conformations on cytotoxicity and PPAR $\gamma$  agonist activity, we investigated the comparative effects of  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me. Both isomers exhibited similar cytotoxicities and PPAR $\gamma$  agonist activities, suggesting that the stereochemical differences at C-18 do not affect PPAR $\gamma$ -dependent transactivation in reporter gene assays (Fig. 3), indicating that the PPAR $\gamma$  agonist activity in this assay was primarily governed by the 2-cyano substituents.

However, results of the mammalian two-hybrid assay (Fig. 4) show that  $\alpha$ -CDODA-Me induces interactions between PGC-1 and TIFII (SRC-2), whereas  $\beta$ -CDODA-Me induces interactions between PGC-1 and SRC-1. These differences must be due to the unique conformations of the E-ring of these isomeric triterpenoids, which is dependent on the different stereochemistries ( $\alpha$  and  $\beta$ ) at C-18 located at the E/D ring junction (Fig. 1). The mammalian two-hybrid assay uses the GAL4-coactivator chimeras as probes for investigating differences in ligand-dependent conformational changes in PPAR $\gamma$ . These results do not necessarily identify which coactivators are important for activation of PPAR $\gamma$  because this will also depend on tissue-specific expression of coactivators and other important coregulatory proteins. However, data from the two-hybrid assay suggest that, like other structural classes of

PPAR $\gamma$  agonists,  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me are selective receptor modulators and this selectivity was further investigated using induction of *caveolin-1* and *KLF-4* as end points. Both *caveolin-1* and *KLF-4* were selected as potential PPAR $\gamma$ -dependent responses based on results of previous studies showing that both genes are induced by one or more structural classes of PPAR $\gamma$  agonists (20, 28, 30, 36). *Caveolin-1* expression in colon cancer and some other cancer cell lines is associated with reduced rates of cancer cell proliferation and anchorage-independent growth (28, 37–39). *KLF-4* is a member of the Sp/KLF family of zinc finger transcription factors (40, 41), and *KLF-4* expression is also correlated with tumor/cancer cell growth inhibition in gastric and colon cancer, suggesting that *KLF-4* acts as a tumor-suppressor gene (42–45). Previous studies have shown that *caveolin-1* is induced by thiazolidinediones, CDDO/CDDO-Me, and 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes in HT-29 and other colon cancer cell lines. However, PPAR $\gamma$ -active 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes, but not rosiglitazone, induced *caveolin-1* in HCT-15 cells (20, 29) and this was related, in part, to a mutation in PPAR $\gamma$  expressed in the HCT-15 cell line. The differences in *caveolin-1* induction between the two structurally unrelated PPAR $\gamma$  agonists in HCT-15 cells is an example of the selective receptor modulator activity of different structural classes of PPAR $\gamma$  agonists. We also observed cell-specificity differences between  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me with respect to their induction of *caveolin-1* in colon cancer cells (Fig. 5). Although  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me induced *caveolin-1* in HT-29 and HCT-15 cells, only the former isomer induced this response in SW480 cells and this was observed in replicate experiments. Because  $\alpha$ -CDODA-Me and CDDO-Me contain the 18 $\alpha$  configuration and both compounds also induce *caveolin-1* (Fig. 5; ref. 20), this suggests that differences in *caveolin-1* induction by  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me are due to their different E-ring conformations (Fig. 1), which also affects ligand-induced PPAR $\gamma$ -coactivator interactions (Fig. 4).

A previous report (30) showed that *KLF-4* induction by PGJ2 was PPAR $\gamma$ -independent and this response was used as a model to investigate mechanistic differences in *KLF-4* induction by  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me and PGJ2.  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me induce *KLF-4* mRNA levels in HT-29 and SW480 cells (Fig. 6A and B), and cotreatment of these cells with the PPAR $\gamma$  antagonist T007 inhibits induction of *KLF-4*. Similar results were observed for induction of *KLF-4* protein (data not shown) demonstrating receptor-dependent ( $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me) and receptor-independent (PGJ2) induction of *KLF-4* in HT-29 cells and that the two different structural classes of PPAR $\gamma$  agonists exhibit selective receptor modulator-like activity.

Results of this study show for the first time that introduction of 2-cyano substituents into the A ring of  $\alpha$ -glycyrrhetic acid and  $\beta$ -glycyrrhetic acid significantly enhances their cytotoxicity and is necessary for their activity as PPAR $\gamma$  agonists. This represents an important

extension of the potential therapeutic applications of synthetic analogues of glycyrrhetic acid, a major component of licorice extracts. In addition, we also show that both  $\alpha$ -CDODA-Me and  $\beta$ -CDODA-Me are selective receptor modulators based on their tissue-selective induction of *caveolin-1* and *KLF-4* in colon cancer cells. These differences in activity are consistent with their structure-dependent induction of PPAR $\gamma$  interactions with different coactivators in SW480 cells. Thus, synthetic analogues of glycyrrhetic acid exhibit potent anticancer activity in colon cancer cells and mechanisms of their induction of *KLF-4* and other receptor-dependent and receptor-independent responses and *in vivo* applications of these compounds as a new class of anticancer drugs are currently being investigated.

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