Structure-dependent activity of glycyrrhetinic acid derivatives as peroxisome proliferator–activated receptor γ 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 glycyrrhetinic acid have been associated with the multiple therapeutic properties of licorice extracts. We have investigated the effects of 2-cyano substituted analogues of glycyrrhetinic acid on their cytotoxicities and activity as selective peroxisome proliferator–activated receptor γ (PPARγ) agonists. Methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (β-CDODA-Me) and methyl 2-cyano-3,11-dioxo-18α-olean-1,12-dien-30-oate (α-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 PPARγ agonist activity of α-CDODA-Me and β-CDODA-Me isomers. However, in mammalian two-hybrid assays, both compounds differentially induced interactions of PPARγ 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*). β-CDODA-Me but not α-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 PPARγ antagonist. This shows for the first time that PPARγ agonists derived from glycyrrhetinic 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 18β-glycyrrhetinic acid (Fig. 1). Glycyrrhizin is a pentacyclic triterpenoid glycoside, which is hydrolyzed in the gut to glycyrrhetinic acid, and many of the properties of licorice root can be attributed to glycyrrhetinic acid. For example, glycyrrhetinic acid inhibits 11β-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). Glycyrrhetinic 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).

Glycyrrhetinic acid has also been used as a template to synthesize bioactive drugs. For example carbenoxolone, the 3-hemisuccinate derivative of glycyrrhetinic acid, has been used for the treatment of gastritis and ulcers (12). Some of the activity of carbenoxolone may be due to hydrolysis to glycyrrhetinic 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, glycyrrhetinic acid derivatives containing a reduced carboxylic acid group at C-30 (CH_2OH) 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-17α-olean-1,9(11)-diene-28-oic acid (CDDO) and its

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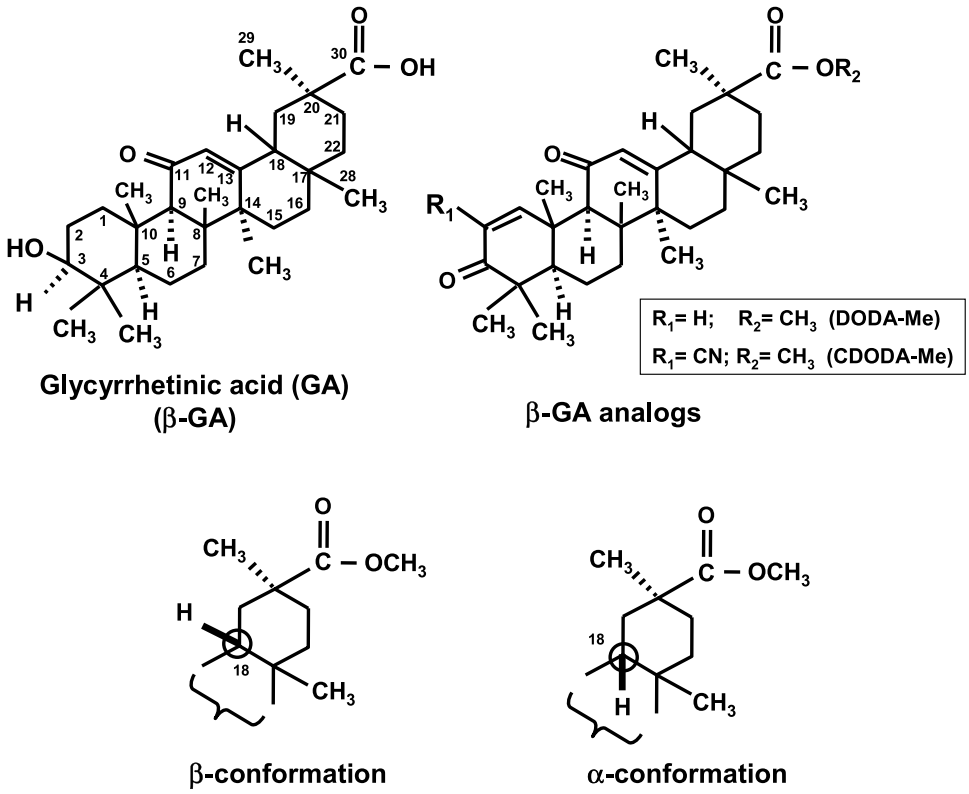

Figure 1. Structures of glycyrrhetinic acid (*GA*), β-glycyrrhetinic acid derivatives, and differences in the stereochemistry at C-18 of the α-glycyrrhetinic acid – and β-glycyrrhetinic acid – derived compounds.

methyl ester (CDDO-Me) exhibited peroxisome proliferator–activated receptor γ (PPARγ) agonist activity (20–22). Although glycyrrhetinic acid also has an oleanolane triterpenoid backbone, there are major structural differences between glycyrrhetinic acid and oleanolic acid and between CDDO-Me and the synthetic glycyrrhetinic acid analogue methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (β-CDODA-Me). CDODA-Me (β-CDODA-Me) is isomeric with CDDO-Me (α-CDDO-Me) but differs with respect to the carboxy substitution in the E ring, the stereochemistry at C-18 (β versus α) 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 PPARγ agonist activity of triterpenoid acids, we also synthesized methyl 2-cyano-3,11-dioxo-18α-olean-1,12-dien-30-oate (α-CDODA-Me). Our results show that introduction of the 2-cyano group into α-glycyrrhetinic acid or β-glycyrrhetinic acid resulted in enhanced cytotoxicity, and both compounds induced PPARγ-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 confirmation of the E-ring resulted in different PPARγ-dependent effects in colon cancer cells, suggesting that the α-CDODA-Me and β-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× 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× antibiotic antimycotic solution. Cells were maintained at 37°C in the presence of 5% CO_2.

Synthesis

3,11-Dioxo-18β-Oleana-1,12-Dien-30-Oic Acid and 3,11-Dioxo-18α-Oleana-1,12-Dien-30-Oic Acid. A mixture of 18β-glycyrrhetinic 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°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_2Cl_2 (1:19) as

eluant. The main band gave 3,11-dioxo-18β-oleana-1,12-dien-30-oic acid (β-DODA) as a white solid (133 mg, 85.5%), which was crystallized from methanol (104 mg), mp 270°C to 275°C. ^{1}H nuclear magnetic resonance (NMR) δ 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-18α-oleana-1,12-dien-30-oic acid (α-DODA) from 18α-glycyrrhetinic acid (Sigma-Aldrich).

β-DODA-Me and α-DODA-Me. Methyl 18β-glycyrrhetinate was prepared by diazomethylation of 18β-glycyrrhetinic 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/CH_2Cl_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}H NMR δ 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 α-DODA-Me from α-DODA.

β-CDODA and α-CDODA. The two cyano derivative of 18β-glycyrrhetinic 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 β-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}H NMR δ 8.550 (^{1}H, s, C1-H), 5.846 (s, C12-H), 2.2.715 (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 α-CDODA from 18α-glycyrrhetinic acid.

β-CDODA-Me and α-CDODA-Me. The nitrile was also prepared from methyl 18β-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 β-CDODA-Me. The compound was purified by TLC (ethyl acetate/hexane; 1:3) to give β-CDODA-Me and crystallized from ethyl acetate/hexane (138 mg), mp 243°C to 245°C. ^{1}H NMR δ 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 α-CDODA-Me from α-DODA.

Antibodies and Reagents

Caveolin-1 antibody was purchased from Santa Cruz Biotechnology, Inc. Monoclonal β-actin antibody and 18α-glycyrretinic acid and 18β-glycyrretinic acid were purchased from Sigma-Aldrich. Reporter lysis buffer and luciferase reagent for luciferase studies were supplied by Promega. β-Galactosidase (β-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 PPARγ 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}H NMR spectra were run in $CDCl_3$ on a Bruker Avance-400 spectrometer using Me_4Si 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× Gal4 response elements (pGal4) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4DBD-PPARγ construct was a gift of Dr. Jennifer L. Oberfield (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-PPARγ 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 (1×10^5 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 μg), β-galactosidase (0.04 μg), and Gal4PPARγ and PPRE-Luc (0.04 μg)] 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 μL of 1× reporter lysis buffer, and 30 μL of cell extract was used for luciferase and β-galactosidase assays. A LumiCount luminometer (Perkin-Elmer Life and Analytical Sciences) was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity. Results are expressed as means ± SE for at least three replicate determinations for each treatment group.

Mammalian Two-Hybrid Assay

SW480 cells were plated in 12-well plates at 1×10^5 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 μg), β-gal (0.04 μg), VP-PPARγ (0.04 μg), pMSRC1 (0.04 μg), pMSRC2 (0.04 μg), pMSRC3 (0.04 μg), pMPGC-1 (0.04 μg), pMDRIP205 (0.04 μg), and pMCARM-1 (0.04 μg)] 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× reporter lysis buffer, and 30 μL of cell extract was used for luciferase and β-galactosidase assays. LumiCount was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

Cell Proliferation Assay

SW480, HCT-15, and HT 29 cells (2×10^4) 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 ± SE for each determination.

Western Blot Analysis

SW-480, HCT-15, and HT-29 (3×10^5) 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 $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, and 1% Triton X-100 (pH 7.5), and 5 μL/mL protease inhibitor cocktail (Sigma-Aldrich)]. Protein samples were incubated at 100°C for 2 min, separated on 10% SDS-PAGE at 120 V for 3 to 4 h in 1× 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°C in 1× 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 β-actin) primary

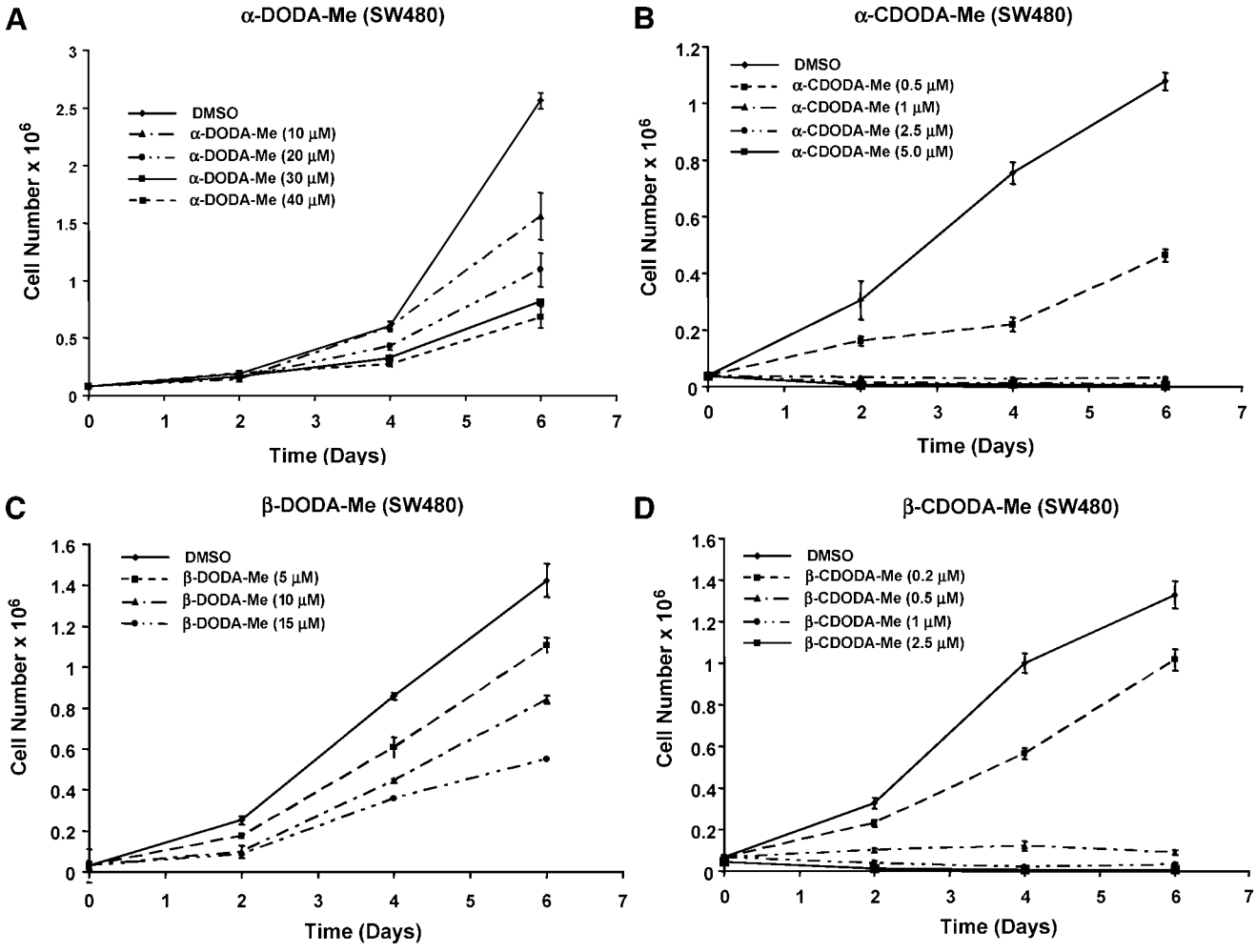

Figure 2. Growth inhibition studies. SW480 colon cancer cells were treated with different concentrations of α-DODA-Me (**A**), α-CDODA-Me (**B**), β-DODA-Me (**C**), and β-CDODA-Me (**D**) for 6 d, and cell numbers were determined using a Coulter counter as described in Materials and Methods. *Points,*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

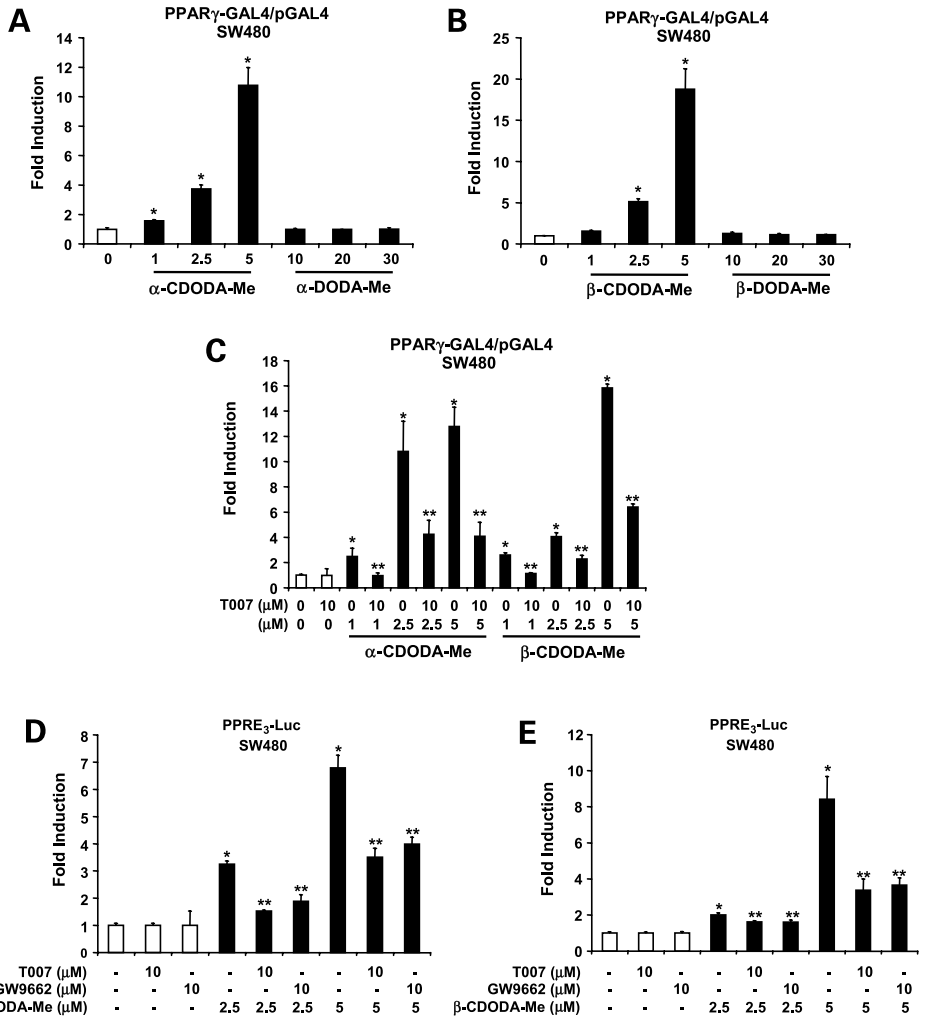

Figure 3. Ligand-induced activation of PPARγ and effects of PPARγ antagonists. Activation of PPARγ-GAL4/pGAL4 in SW480 cells treated with α-DODA-Me/α-CDODA-Me (**A**), β-DODA-Me/β-CDODA-Me (**B**), and both isomers plus T007 (**C**). Cells were transfected with PPARγ-GAL4/pGAL4, treated with different 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 α-CDODA-Me (**D**) or β-CDODA-Me (**E**) alone or in combination with PPARγ antagonists. SW480 cells were transfected with PPRE-Luc, treated with different concentrations of CDODA-Me isomers alone or in combination with 10 μmol/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 μm 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′-ACGGATTTGGTCGTATTGGGCG-3′; antisense 5′-CTCCTGGAAGATGGTGATGG-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 ± SD for at least three separate determinations for each treatment.

Results

Growth-Inhibitory Effects of Isomeric Glycyrrhetinic Acid Derivatives

This study compares the cytotoxicity of 18β-glycyrrhetinic acid and 18α-glycyrrhetinic acid derivatives and results in Fig. 2 summarize the cytotoxicity of β-DODA-Me, β-CDODA-Me, and the corresponding 18α isomers. Initial studies showed that glycyrrhetinic 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 α-DODA and

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

α-CDODA-Me and β-CDODA-Me Activate PPARγ

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

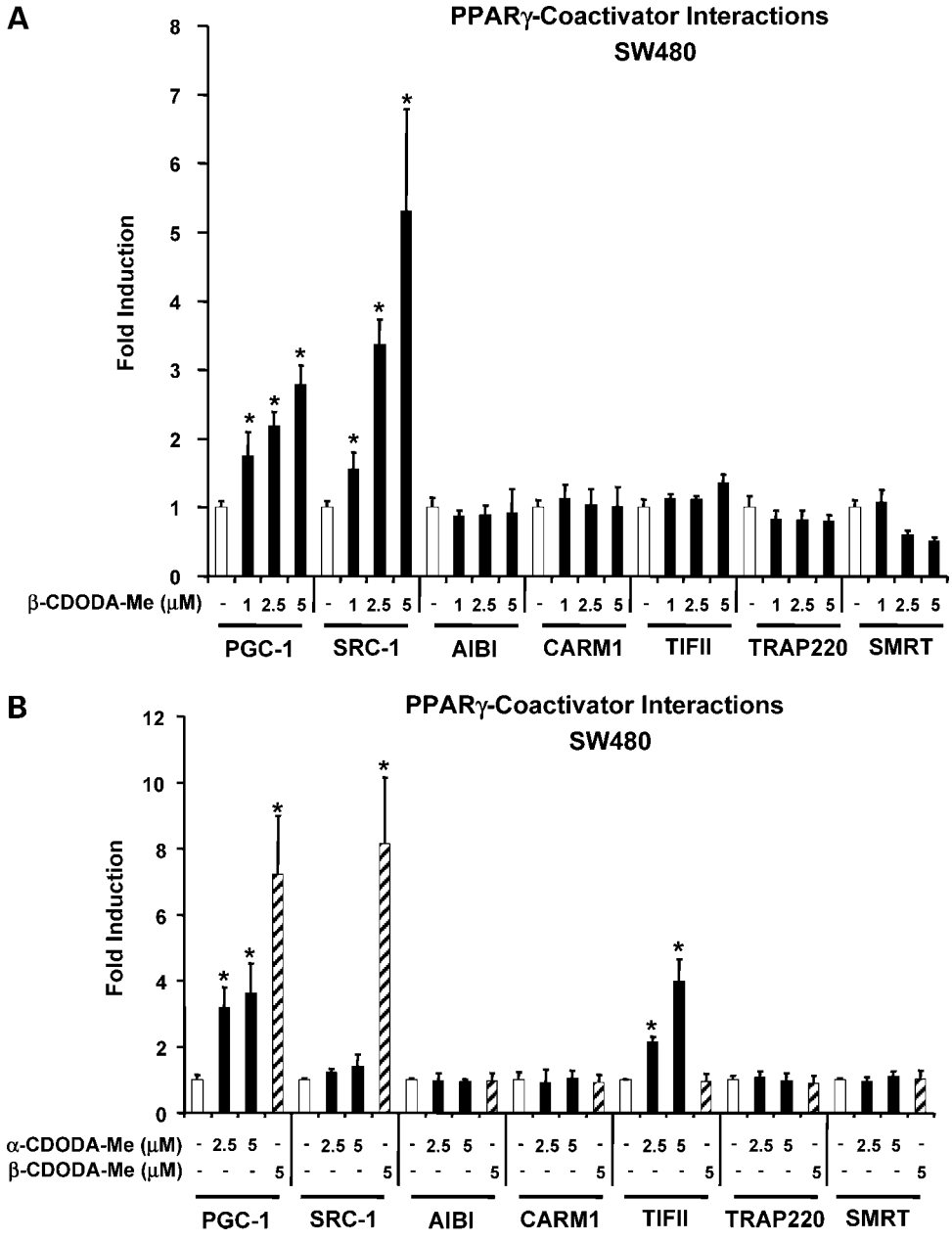

Figure 4. Ligand-induced PPARγ-coactivator interactions. SW480 cells were transfected with VP-PPARγ, coactivator-GAL4/pGAL4, treated with different concentrations of α-CDODA-Me (**A**) or both α-CDODA-Me and β-CDODA-Me (**B**), and luciferase activity was determined as described in Materials and Methods.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

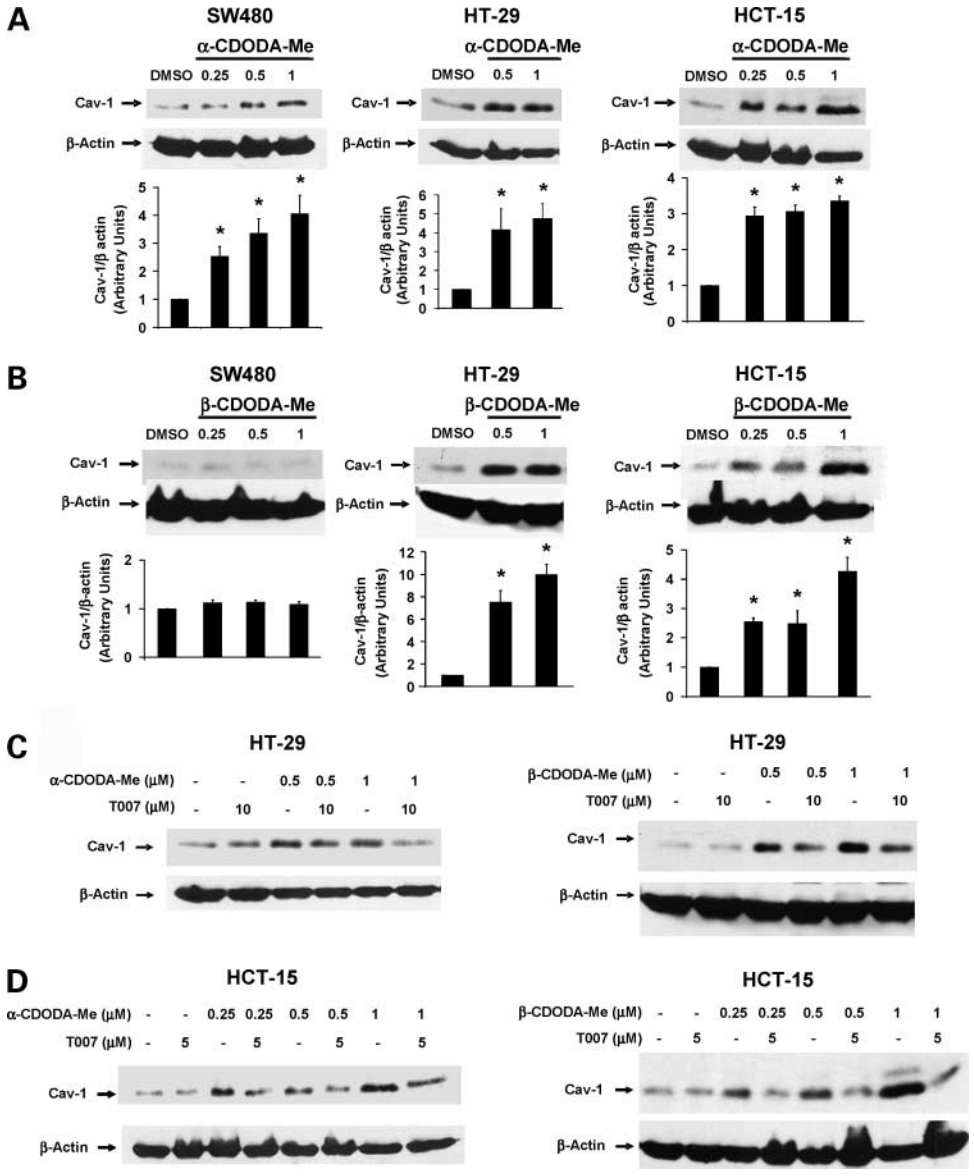

Figure 5. Induction of *caveolin-1* in colon cancer cells. SW480, HT-29, or HCT-15 colon cancer cells were treated with α-CDODA-Me (**A**) or β-CDODA-Me (**B**) for 72 h as previously described (20), and whole-cell lysates were analyzed by Western immunoblot analysis as described in Materials and Methods. Inhibition of *caveolin-1* induction by α-CDODA-Me or β-CDODA-Me by T007 in HT-29 (**C**) or HCT-15 (**D**) cells. Cells were treated with 5 μmol/L T007, 0.5 to 1.0 μmol/L α-CDODA-Me or β-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 α-CDODA-Me in SW480 cells (data not shown). Caveolin-1 protein expression (relative to β-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 μmol/L) was inactive (Fig. 3B). A direct comparison of both α-CDODA-Me and β-CDODA-Me is shown in Fig. 3C, where 5 μmol/L of both compounds induced a 12- to 16-fold increase in luciferase activity in SW480 cells transfected with PPARγ-GAL4/pGAL4. Cotreatment with 10 μmol/L of the PPARγ antagonist T007 significantly decreased α-CDODA-Me/β-CDODA-Me–induced transactivation. Both α-CDODA-Me and β-CDODA-Me also induced transactivation (Figs. 3D and E) in SW480 cells transfected with PPRE3-luc, a construct that contains three tandem PPARγ response elements linked to luciferase and that relies on activation of the endogenous PPARγ-retinoid X receptor complex expressed in this cell line (20). In addition, both PPARγ antagonists T007 and GW9662 inhibited α-CDODA-Me/β-CDODA-Me–induced transactivation. These results show for the first time that introduction of a 2-cyano group into the glycyrrhetinic acid triterpenoid acid structure is sufficient for conferring PPARγ agonist activity on the resulting compound. Moreover, both the α-CDODA-Me and β-CDODA-Me isomers exhibit similar potencies as PPARγ agonists (β-CDODA-Me ≥ α-CDODA-Me), suggesting that the conformational differences in the E-ring, which are observed for the 18α and 18β isomers do not affect their PPARγ agonist activities in the PPARγ-GAL4/pGAL4 and PPRE-luc transactivation assays.

α-CDODA-Me and β-CDODA-Me as Selective Receptor Modulators

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

differences in their activation of receptor-dependent genes/protein. The selectivity of various structural classes of PPARγ ligands is due, in part, to differential interactions within the ligand binding domain of PPARγ, which can lead to different conformations of the receptor. This can result in differential interactions of the ligand-bound PPARγ with nuclear receptor coactivators (24), and results in Fig. 4A summarize β-CDODA-Me–induced transactivation in SW480 cells transfected with GAL4-coactivator and VP-PPARγ (ligand binding domain) chimeras and a pGAL4 reporter gene. In this mammalian two-hybrid assay, β-CDODA-Me induced PPARγ interactions only with PGC-1 and SRC-1 but not with AIB1 (SRC-3), TIFII (SRC-2), CARM1, TRAP220, and the corepressor SMRT. α-CDODA-Me also induced PPARγ-PGC-1 interactions (Fig. 4B); however, in contrast with the β isomer, α-CDODA-Me induced PPARγ interactions with TIFII but not with SRC-1, AIB1, CARM1, TRAP220, or SMRT. These results suggest that the two α-CDODA-Me and β-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 α-CDODA-Me and β-CDODA-Me isomers should exhibit some tissue/cell or response-specific differences in their activation of receptor-dependent genes.

PPARγ agonists induce *caveolin-1* in colon cancer cells through a receptor-dependent mechanism (28, 29). The effects of α-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, β-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 α-CDODA-Me and β-CDODA-Me isomers was inhibited in HT-29 and HCT-15 cells cotreated with the PPARγ antagonist T007, and similar results were observed for α-CDODA-Me in SW480 cells (data not shown). These results show the tissue-selective induction of *caveolin-1* expression by β-CDODA-Me and this is consistent with the activity of α-CDODA-Me and β-CDODA-Me isomers as selective receptor modulators.

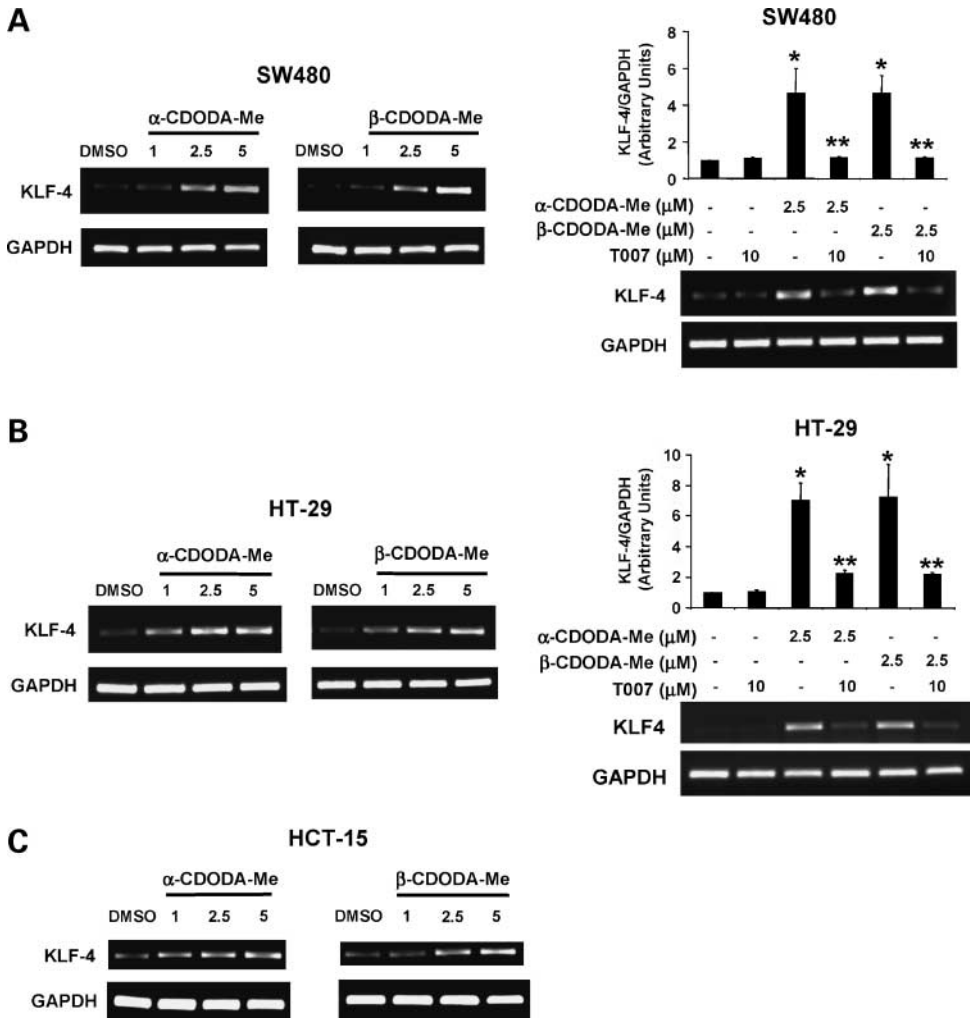

Figure 6. Induction of *KLF-4* gene expression by α-CDODA-Me and β-CDODA-Me. Induction of *KLF-4* in SW480 (**A**), HT-29 (**B**), and HCT-15 (**C**) cells. Cells were treated with different concentrations of CDODA isomers or T007 alone or in combination and *KLF-4* mRNA levels were determined by RT-PCR 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 μmol/L concentrations of both α-CDODA-Me and β-CDODA-Me isomers induced *KLF-4* mRNA levels in SW480 cells. The PPARγ antagonist T007 (10 μmol/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, α-CDODA-Me and β-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 PPARγ 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 PPARγ agonist 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2) in HT-29 cells (30).

Discussion

PPARγ 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_2- 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 PPARγ 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 PGJ2 was PPARγ dependent, whereas both troglitazone and PPARγ-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 PPARγ agonists are selective receptor modulators has been reported in mammalian two-hybrid assays in which cells have been transfected with VP-PPARγ and GAL4-coactivator constructs. For example, PGJ2 and rosiglitazone differentially induced coactivator-PPARγ interactions in COS-1 cells (24), and differences in ligand-dependent coactivator-receptor interactions were also observed for rosiglitazone and PPARγ-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 PPARγ agonist activities (20–22). Results of this study also showed that 2-cyano analogues of the α-glycyrrhetinic acid and β-glycyrrhetinic acid methyl esters also exhibited increased cytotoxicity (Fig. 2) and PPARγ agonist activity. Similar results were observed for the corresponding acid derivatives that were less active than α-CDODA-Me or β-CDODA-Me (data not shown). Thus, introduction of the 2-cyano group into the oleanolic acid and glycyrrhetinic acid backbone is necessary for their PPARγ agonist activities and differences in their substitution in the C-ring and the position of carboxymethyl groups at C-30 (in glycyrrhetinic acid) or C-28 (in oleanolic acid) did not affect PPARγ agonist activity. Glycyrrhetinic acid and oleanolic acid are 18β and 18α 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 PPARγ agonist activity, we investigated the comparative effects of α-CDODA-Me and β-CDODA-Me. Both isomers exhibited similar cytotoxicities and PPARγ agonist activities, suggesting that the stereochemical differences at C-18 do not affect PPARγ-dependent transactivation in reporter gene assays (Fig. 3), indicating that the PPARγ 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 α-CDODA-Me induces interactions between PGC-1 and TIFII (SRC-2), whereas β-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 (α and β) 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 PPARγ. These results do not necessarily identify which coactivators are important for activation of PPARγ 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

PPARγ agonists, α-CDODA-Me and β-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 PPARγ-dependent responses based on results of previous studies showing that both genes are induced by one or more structural classes of PPARγ 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, PPARγ-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 PPARγ expressed in the HCT-15 cell line. The differences in *caveolin-1* induction between the two structurally unrelated PPARγ agonists in HCT-15 cells is an example of the selective receptor modulator activity of different structural classes of PPARγ agonists. We also observed cell-specificity differences between α-CDODA-Me and β-CDODA-Me with respect to their induction of *caveolin-1* in colon cancer cells (Fig. 5). Although α-CDODA-Me and β-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 α-CDODA-Me and CDDO-Me contain the 18α configuration and both compounds also induce *caveolin-1* (Fig. 5; ref. 20), this suggests that differences in *caveolin-1* induction by α-CDODA-Me and β-CDODA-Me are due to their different E-ring conformations (Fig. 1), which also affects ligand-induced PPARγ-coactivator interactions (Fig. 4).

A previous report (30) showed that *KLF-4* induction by PGJ2 was PPARγ-independent and this response was used as a model to investigate mechanistic differences in *KLF-4* induction by α-CDODA-Me and β-CDODA-Me and PGJ2. α-CDODA-Me and β-CDODA-Me induce *KLF-4* mRNA levels in HT-29 and SW480 cells (Fig. 6A and B), and cotreatment of these cells with the PPARγ antagonist T007 inhibits induction of *KLF-4*. Similar results were observed for induction of KLF-4 protein (data not shown) demonstrating receptor-dependent (α-CDODA-Me and β-CDODA-Me) and receptor-independent (PGJ2) induction of *KLF-4* in HT-29 cells and that the two different structural classes of PPARγ 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 α-glycyrrhetinic acid and β-glycyrrhetinic acid significantly enhances their cytotoxicity and is necessary for their activity as PPARγ agonists. This represents an important extension of the potential therapeutic applications of synthetic analogues of glycyrrhetinic acid, a major component of licorice extracts. In addition, we also show that both α-CDODA-Me and β-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 PPARγ interactions with different coactivators in SW480 cells. Thus, synthetic analogues of glycyrrhetinic 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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