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Induction of Apoptosis and Nonsteroidal Antiinflammatory Drug-Activated Gene 1 in Pancreatic Cancer Cells By A Glycyrrhetic Acid Derivative

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Abstract

Methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oate (CDODA-Me) is a synthetic triterpenoid derived from glycyrrhetic acid, a bioactive phytochemical in licorice. CDODA-Me inhibits growth of Panc1 and Panc28 pancreatic cancer cell lines and activates peroxisome proliferator-activated receptor γ (PPAR γ)-dependent transactivation in these cells. CDODA-Me also induced p21 and p27 protein expression and downregulates cyclin D1; however, these responses were receptor-independent. CDODA-Me induced apoptosis in Panc1 and Panc28 cells, and this was accompanied by receptor-independent induction of the proapoptotic proteins early growth response-1 (Egr-1), nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1), and activating transcription factor-3 (ATF3). Induction of NAG-1 and Egr-1 by CDODA-Me was dependent on activation of phosphatidylinositol-3-kinase (PI3-K) and/or p42 and p38 mitogen-activated protein kinase (MAPK) pathways but there were differences between Panc28 and Panc1 cells. Induction of NAG-1 in Panc28 cells was p38-MAPK- and PI3-K-dependent but Egr-1-independent, whereas induction in Panc1 cells was associated with activation of p38-MAPK, PI3-K and p42-MAPK and was only partially Egr-1-dependent. This is the first report of the induction of the proapoptotic protein NAG-1 in pancreatic cancer cells.

Keywords

CDODA-Me; pancreatic cancer; apoptosis

1. Introduction

Glycyrrhetic acid (GA) is a pentacyclic triterpenoid acid that is found as a conjugate (glycyrrhizin) in licorice extracts [1,2]. GA is one of the medicinally active compounds of licorice and exhibits multiple activities which include the enhancement of corticosterone levels which contributes to decreased body fat index in human studies with GA [3,4]. In addition, several derivatives of GA are also biologically active, and carbenoxolone, a 3-hemisuccinate of GA, has been used for the treatment of ulcers and arthritis [1,2,5]. Previous

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Conflict of Interest: None

studies with closely related triterpenoid acids, ursolic acid and oleanolic acid, have demonstrated that introduction of a 2-cyano-1-en-3-one function in their A ring greatly enhances their anti-inflammatory activity in a mouse macrophage model [6-8], and one of these compounds, 2-cyano-3,12-dioxo-18 β -olean-1,9(11)-dien-28-oic acid (CDDO), its methyl ester (CDDO-Me), and imidazole derivatives exhibit antitumorigenic activity [9,10]. We have synthesized 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oic acid (CDODA) and its methyl ester (CDODA-Me) from GA and have demonstrated that these compounds are highly cytotoxic in colon, prostate, bladder and pancreatic cancer cells [11-13]. The most active member of these GA derivatives is CDODA-Me (18 β isomer) which activates peroxisome proliferator-activator receptor γ (PPAR γ) and induces both receptor-dependent and -independent responses in colon and prostate cancer cells. For example, in colon cancer cells, β -CDODA-Me induced receptor-dependent caveolin-1 expression in HT-29 and HCT-15 but not SW480 colon cancer cells, whereas α -CDODA-Me induced receptor-mediated caveolin-1 protein levels in all three cell lines [11]. In contrast, the pattern of receptor-dependent induction of Krüppel-like factor-4 (KLF-4) in HT-29, HCT-15 and SW480 colon cancer cells was similar for both β - and α -CDODA-Me. β -CDODA-Me induced apoptosis and several proapoptotic proteins in LNCaP prostate cancer cells and these included nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) and activating transcription factor 3 (ATF3), and activation of these pathways was not inhibited by PPAR γ antagonists [12].

In this study, we demonstrate that β -CDODA-Me induced PPAR γ -dependent transactivation in Panc28 and Panc1 pancreatic cancer cells and β -CDODA-Me induced the characteristic PPAR γ -dependent differentiation of 3T3-L preadipocytes. β -CDODA-Me induced expression of several growth inhibitory and proapoptotic proteins including p21, p27, NAG-1 and ATF3 and downregulated cyclin D1 proteins, and effects on these growth inhibitory responses were receptor-independent. β -CDODA-Me also activated multiple kinases in pancreatic cancer cells including p38 and p42 mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3-K), and c-jun N-terminal kinase (JNK) pathways, and the role of these kinases in the induction of NAG-1 and apoptosis was cell context-dependent.

2. Materials and methods

2.1 Cell lines

The Panc28 cell line was a generous gift from Paul Chiao (University of Texas M.D. Anderson Cancer Center, Houston, TX) and Panc1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

2.2 Antibodies and reagents

Both pancreatic cancer cell lines were maintained in DMEM-F12 supplemented with 5% FBS, 0.22% sodium bicarbonate, and 10 ml/L of 100X antibiotic/antimycotic cocktail solution (Sigma Aldrich Co., St. Louis, MO). Cells were grown in 150 cm² culture plates in an air/CO₂ (95:5) atmosphere at 37°C. Cyclin D1, p21, p27, ATF3, p-c-jun, c-jun, p-Akt 1/2/3, Akt 1/2, p-Erk, Erk and p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP, Egr-1 and p-p38 antibody were purchased from Cell Signaling Technology (Danvers, MA) and NAG-1 antibody was purchased from Upstate USA, Inc. (Lake Placid, NY). Monoclonal β -actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase substrate for western blot analysis was obtained from NEN Life Science Products (Boston, MA). Proteinase K were obtained from Sigma Aldrich. Lipofectamine was purchased from Invitrogen (Carlsbad, CA). β -Galactosidase

reagent was obtained from Tropix (Bedford, MA). LY294002, PD98059 and SB203580 were purchased from EMD Chemicals, Inc (Gibbstown, NJ).

2.3 Cell proliferation assay

Pancreatic cancer cells (3×10^4 per well) were plated in 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or CDODA-Me were added. Fresh medium and test compounds were added every 48 hr, and cells were then trypsinized and counted at the indicated times using a Coulter Z1 particle counter. Each experiment was done in triplicate and results are expressed as means $\pm$ SE for each treatment group.

2.4 Transfection and luciferase assay

The pancreatic cancer cells (1×10^5 per well) were plated in 12-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 24 hr, various amounts of DNA (i.e., 0.4 μ g pGal4, 0.04 μ g β -galactosidase, and 0.04 μ g PPAR γ -GAL4 or 0.4 μ g of PPRE3-Luc) were transfected using Lipofectamine reagent according to the manufacturer's protocol. Five hours post-transfection, the transfection mix was replaced with complete medium containing either vehicle (DMSO) or the indicated compound in DMSO. After 22 hr, cells were then lysed with 100 μ L of 1 $\times$ reporter lysis buffer, and cell extracts (30 μ L) were used for luciferase and β -galactosidase assays. A Lumicount luminometer was used to quantitate luciferase and β -galactosidase activities, and the luciferase activities were normalized to β -galactosidase activity.

2.5 Western blots

Pancreatic cancer cells were initially seeded in DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS and, after 24 hr, cells were treated with either vehicle (DMSO) or the indicated compounds. Cells were collected using high-salt buffer [50 mmol/L HEPES, 0.5 mol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10% glycerol, and 1% Triton-X-100] and 10 μ L/mL of Protease Inhibitor Cocktail. Protein lysates were incubated for 3 min at 100°C before electrophoresis, and then separated on 10% SDS-PAGE 120 V for 3 to 4 hr. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by wet electroblotting in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol for 1.5 hr at 180 mA. Membranes were blocked for 30 min with 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] and incubated in fresh 5% TBST-Blotto with 1:500 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 2 hr by gentle shaking. The membrane was washed with TBST for 10 min, incubated with 6 mL of chemiluminescence substrate for 1 min, and exposed to Kodak X-OMAT AR autoradiography film.

2.6 DNA fragmentation

The isolation of DNA was performed according to the protocol 6.2 "Rapid Isolation of Mammalian DNA". Extracted DNA was run on 0.9% agarose gel and stained with 0.5 μ g/mL ethidium bromide and the fragmented DNA was visualized using a Transilluminator on an ultraviolet light.

2.7 Fluorescence-activated cell-sorting assays (FACS)

Both Panc1 and Panc28 pancreatic cancer cells were treated with either the vehicle (DMSO) or the indicated compounds for 48 hr. Cells were trypsinized, centrifuged and re-suspended in staining solution containing 50 mg/ml propidium iodide, 4 mM sodium citrate, 30 units/

ml RNase and 0.1% Triton X-100. After incubation at 37°C for 10 min, sodium chloride was added to give a final concentration of 0.15 M. Cells were analysed on a FACS Calibur flow cytometer using CellQuest acquisition software (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ). PI fluorescence was collected through a 585/42 nm band pass filter, and list mode data were acquired on a minimum of 20,000 single cells defined by a dot plot of PI width versus PI area. Data analysis was performed in Modfit LT using PI width versus PI to exclude cell aggregates.

2.8 Differentiation and oil red O staining

3T3-L1 preadipocytes were cultured on Lab-Tek Chamber 4-well Slide with DMEM-F12 and 10% FBS at 5% CO₂. At 2 days postconfluence, fresh media supplemented with 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (1 mM), insulin (1.7 mM), and DMSO or CDODA-Me (0.25 μM) was added. After 48 hr, fresh media was added and cells were treated with DMSO and CDODA-Me for 5 days. Cells without any treatment for the entire 7 days were used as controls. Cells were then fixed with 10% formalin, washed with 60% isopropanol and stained with filtered 60% Oil Red O in deionized water. After staining, cells were washed with water and photographed to visualize the staining.

3. Results

3.1 Effects on cell proliferation and apoptosis

Previous studies in colon and prostate cancer cells show that the cytotoxicity of a series of glycyrrhetic acid derivatives is dependent on the introduction of a 2-cyano group and a 1-en-3-one functionality in the A ring [11-13]. Figure 1 compares the cytotoxicities of DODA-Me, which contain the A-ring 1-en-3-one function, to CDODA-Me, which contains both the 1-en-3-one and 2-cyano substituents. At concentrations of 1 - 15 μM, DODA-Me had minimal effects on Panc1 and Panc28 pancreatic cancer cell growth (Figs. 1A and 1B) with growth inhibitory IC₅₀ values > 15 μM. In contrast, CDODA-Me was a potent inhibitor of pancreatic cancer cell growth. The IC₅₀ values for CDODA-Me in Panc1 and Panc28 cells were 1.21 and 1.79 μM, respectively. We also investigated the cytotoxicities of the free acids (DODA and CDODA); IC₅₀ values for DODA were > 15 μM and the values for CDODA were 7.31 and 3.8 μM in Panc28 and Panc1 cells, respectively (data not shown). Thus, the methyl ester was the most cytotoxic of the 2-cyano derivatives and was used as a model compound for subsequent studies in pancreatic cancer cells.

Figures 2A and 2B summarize the concentration-dependent effects of CDODA-Me on percent distribution of Panc28 and Panc1 cells in G₀/G₁, S and G₂/M phases of the cell cycle after treatment for 48 hr. In Panc28 cells, CDODA-Me decreased the percentage of cells in G₀/G₁ and increased the percentage in S and G₂/M phases, whereas in Panc1 cells, CDODA-Me induced cell cycle arrest in G₀/G₁ and inhibited G₀/G₁ to S phase progression. Thus, the effects of CDODA-Me on the cell cycle are cell context-dependent in the two pancreatic cancer cell lines. Treatment of Panc28 and Panc1 cells with CDODA-Me (5.0 and 7.5 μM) for 24 hr induced a DNA ladder indicative of apoptosis, and similar effects were observed in previous studies with this compound in prostate cancer cells [12].

3.2 Activation of PPARγ by CDODA-Me

CDODA-Me activates the nuclear receptor PPARγ in colon and prostate cancer cells [11,12]. Results in Figure 3A show that CDODA-Me induced transactivation in Panc28 and Panc1 cells transfected with a GAL4-PPARγ chimera and pGAL4₅ which contains 5 tandem GAL4 response elements linked to the luciferase gene. Similar results were obtained in the pancreatic cancer cells transfected with a PPRE₃-luc construct which relies on activation of endogenous PPARγ and RXR (Fig. 3B). Using this same construct, we also showed that

induction of luciferase activity in Panc28 and Panc1 (Fig. 3C) cells by CDODA-Me was inhibited after cotreatment with the PPAR γ antagonist T007. These results confirm that CDODA-Me exhibits PPAR γ agonist activity in pancreatic cancer cells, and the PPAR γ activity of CDODA-Me was confirmed using 3T3-L preadipocytes in which treatment with concentrations as low as 0.25 μ M induced differentiation of these cells and formation of highly characteristic oil-Red-O droplets (Fig. 3D). In contrast, these droplets were not observed in cells treated with solvent control (DMSO); however, these cells were also weakly stained due to endogenous triglycerides.

3.3 Modulation of cell cycle proteins

Several studies report the growth inhibitory and proapoptotic effects of PPAR γ agonists in pancreatic cancer cells [14-24]. The effects of CDODA-Me on expression of the key cell cycle genes p21, p27 and cyclin D1 that are often affected by different classes of PPAR γ agonists that inhibit growth of pancreatic cancer cells were also investigated in the Panc28 and Panc1 cell lines. In Panc28 cells, treatment with 0.5 - 7.5 μ M CDODA-Me induced expression of both p21 and p27 but downregulated cyclin D1 protein (Fig. 4A). p21 and cyclin D1 were induced and repressed, respectively, in Panc1 cells treated with CDODA-Me, whereas p27 protein was unchanged. This may be due, in part, to the high constitutive levels of p27 in Panc1 cells. There were also response-specific differences in the sensitivity of Panc1 and Panc28 cells to CDODA-Me. Induction of p21 in Panc1 and Panc28 cells was observed after treatment with 2.5 and 5.0 μ M CDODA-Me, respectively, whereas 7.5 and 1.0 μ M CDODA-Me were required for cyclin D1 downregulation, respectively. Thus, CDODA-Me differentially modulated expression of cell cycle genes in Panc1 and Panc28 cells. These effects correlated with the G₀/G₁ arrest in Panc1 but not in Panc28 cells (Figs. 2A and 2B) and the reason for these cell context-dependent differences are currently being investigated. Previous studies on PPAR γ -active 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl)methane (C-DIM) compounds show that induction of p21 is receptor-dependent in Panc28 cells and inhibited by the PPAR γ antagonist GW9662 [14]. Therefore, we examined the effects of the PPAR γ antagonist GW9662 on CDODA-Me-induced p21, p27 and cyclin D1 in Panc28 (Fig. 4C) and Panc1 (Fig. 4D) cells. The results show that cotreatment of these cells with GW9662 plus CDODA-Me did not modulate the activity of the latter compound and similar results were obtained for other PPAR γ antagonists (data not shown) and GW9662 alone did not affect cyclin D1, p27 or p21 protein expression in these cells. Previous studies showed that CDODA-Me induces receptor-dependent activation of caveolin-1 and KLF-4 in colon cancer cells [11]; however, in Panc1 and Panc28 cells, CDODA-Me did not induce expression of either protein (data not shown) demonstrating that CDODA-Me is a selective PPAR γ modulator in pancreatic cancer cells.

3.4 Induction of NAG-1 and other responses

CDODA-Me induced DNA fragmentation in pancreatic cancer cells (Figs. 2C and 2D) and, in prostate cancer cells, CDODA-Me also induced apoptosis and the proapoptotic proteins NAG-1, ATF3 and early growth response-1 (Egr-1) [12] and the effects of CDODA-Me on these proapoptotic responses was also investigated in pancreatic cancer cells. Figures 5A and 5B show that CDODA-Me induced NAG-1 and ATF3 in Panc28 and Panc1 cells, respectively. Significant induction of these proteins was observed at 2.5 or 5.0 μ M concentrations of CDODA-Me, and Panc28 cells were more sensitive than Panc1 cells for induction of these proteins. NAG-1 is induced by a variety of anticancer agents in colon and other cancer cell lines [25-36], and this includes induction by PPAR γ agonists such as PGJ2, troglitazone and PPAR γ -active C-DIMs [34-36]; however, among these compounds, PPAR γ -dependent induction is only observed for PGJ2 in colon cancer cells [34]. Results in Figures 5C and 5D show that different concentrations of CDODA-Me induced both NAG-1 and caspase-dependent PARP cleavage and cotreatment with the PPAR γ antagonist

GW9662 did not affect the magnitude of these responses, suggesting induction of these responses was also PPAR γ -independent. Similar results were observed for ATF3 (data not shown).

NAG-1 induction is complex and dependent on the chemical agent and cell context [12,25-36], and the effects of CDODA-Me on induction of the protein in Panc1 and Panc28 cells was further investigated. In a time course study (Fig. 6A), NAG-1 protein levels were increased in Panc28 cells after treatment for 8, 16 and 24 hr, whereas induction in Panc1 cells was observed at later time points (18 - 24 hr). ATF3 was induced 4 - 6 hr after treatment in both cell lines. Previous studies have shown that prior induction of Egr-1 is involved in enhanced expression of NAG-1 in some cell lines [12,26,27,33-36], and Figure 6A illustrates that Egr-1 protein was increased in Panc28 cells within 2 hr after treatment with CDODA-Me and was rapidly induced in Panc1 cells within 1 hr and declined thereafter. The temporal pattern of Egr-1 induction prior to NAG-1 induction in both cell lines is similar to that previously reported for other NAG-1 inducers including CDODA-Me in prostate cancer cells [12] in which CDODA-Me-dependent activation of PI3-K- and/or MAPK-dependent pathways was important for induction of Egr-1 and NAG-1. Results in Figure 6B were determined as part of the time course study shown in Figure 6A and show that CDODA-Me induced phosphorylation of Akt, c-jun and p38- and p42-MAPK after treatment of Panc28 and Panc1 cells for 1 hr, whereas levels of Akt, c-jun and MAPK proteins were unchanged. The temporal patterns of increased kinase phosphorylation were cell context-dependent (Fig. 6B); in Panc28 cells, increased phosphorylation of Akt and p42-MAPK was observed 1 - 8 and 1 - 2 hr, respectively, after treatment with CDODA-Me, whereas in Panc1 cells, enhanced phosphorylation persisted for at least 24 hr. In contrast, p38-MAPK phosphorylation was variable but maximally induced after treatment for 24 hr.

Results of kinase inhibitor studies show that induction of Egr-1 by CDODA-Me was inhibited by PD98059 but not by LY294002 or SB203580, suggesting that induction of Egr-1 was p42-MAPK-dependent in both cell lines (Fig. 6C). PD98059 also inhibited induction by NAG-1 by CDODA-Me in Panc1 cells, suggesting that this response may be, in part, Egr-1-dependent (Fig. 6D). However, even in Panc1 cells, NAG-1 induction was also inhibited by LY294002 and SB203580 and since these compounds did not affect Egr-1 expression, the induction of NAG-1 in Panc1 cells was Egr-1-independent. Moreover, in Panc28 cells NAG-1 induction was inhibited by LY294002 and SB203580 but not PD98059 (Fig. 6D), confirming an Egr-1-independent pathway in this cell line.

4. Discussion

PPAR γ is an orphan nuclear receptor that is overexpressed in multiple tumor types and cancer cell lines, and this receptor is a potential target for cancer chemotherapy [37,38]. Different structural classes of PPAR γ agonists, including the thiazolidinediones (TZDs), 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 (PGJ2), CDDO-Me and PPAR γ -active 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl)methanes (C-DIMs), activate overlapping and compound-specific growth inhibitory and proapoptotic responses in pancreatic cancer cells [17-24]. Many of these compounds induce p21 and/or p27 expression, downregulate cyclin D1 protein, and cause G₀/G₁ to S phase arrest and these effects are both cell context- and structure-dependent. For example, troglitazone, a TZD, induces p27 and not p21 protein expression in several pancreatic cancer cell lines, whereas in another study TZD induces p21 and differentiation markers in a number of pancreatic cancer cells [14-17]. PPAR γ -active C-DIMs also induce p21 but not p27 in Panc28 cancer cell lines and this is accompanied by a significantly higher percentage of cells in G₀/G₁ (28%) and a decreased percentage in S phase (21%) after treatment for 24 hr [17]. The induction of p21 in Panc28 cells by PPAR γ -

active C-DIMs is inhibited by PPAR γ agonists; however, most other studies in pancreatic cancer cells have not investigated the role of this receptor in mediating these responses.

In this study, CDODA-Me inhibited Panc1 and Panc28 cell proliferation (Fig. 1), and comparative studies with DODA-Me demonstrated the importance of the 2-cyano group which markedly enhanced antiproliferative activity. The 2-cyano group was necessary for the PPAR γ agonist activities of the glycyrrhetic acid derivatives [11,12] and structurally related olean and lupane derivatives [7,39]. CDODA-Me induced PPAR γ -dependent transactivation and activity (Fig. 3), whereas DODA-Me which does not contain a 2-cyano substituent exhibited decreased antiproliferative activity (Fig. 1) and did not exhibit PPAR γ agonist activity (data not shown). In addition, CDODA-Me induced differentiation of 3T3-L pre-adipocytes and this is a highly prototypical PPAR γ -dependent response (Fig. 3D). These results on activation of PPAR γ by DODA-Me and CDODA-Me in Panc1 and Panc28 cells are similar to those observed in prostate and colon cancer cells [11,12].

Like other PPAR γ agonists, CDODA-Me induced p21 and p27 and decreased cyclin D1 expression in Panc1 and Panc28 cells (Fig. 4) and, in Panc1 cells (Fig. 2B), this was accompanied by a G₀/G₁ to S phase arrest. However, studies with the PPAR γ antagonist GW9662 indicated that these responses were receptor-independent, and this contrasted to the receptor-dependent induction of p21 in Panc28 cells by PPAR γ -active C-DIMs and the 2-cyano derivative of betulinic acid which, like CDODA-Me, contains a 1-en-3-one function in the A-ring [17,38]. Thus, among the three PPAR γ agonists, there was a structure-dependent induction of p21 and similar results have been observed for induction of KLF4, suggesting that CDODA-Me, PPAR γ -active C-DIMs, and the cyano derivative of betulinic acid are selective PPAR γ modulators.

CDODA-Me also induces apoptosis (DNA laddering) in Panc1 and Panc28 cells (Fig. 2C) and previous studies indicate that CDODA-Me, other PPAR γ agonists, and anticancer drugs induce the proapoptotic proteins NAG-1 and ATF3 in colon, prostate and other cancer cells [25-36]; however, induction of these proteins has not previously been investigated in pancreatic cancer cells. Results in Figures 5A and 5B demonstrate that CDODA-Me induced NAG-1 and ATF3 proteins in Panc28 and Panc1 cells and cotreatment with the PPAR γ antagonist GW9662 did not affect the induction responses or activation of caspase-dependent PARP cleavage (Figs. 5C and 5D). Previously, we also observed receptor-independent induction of the proapoptotic proteins NAG-1 and ATF3 by CDODA-Me in LNCaP prostate cancer cells [12], and these responses were kinase-dependent and the prior induction of Egr-1 was associated with induction of NAG-1. In prostate cancer cells, induction of ATF3 by CDODA-Me was JNK-dependent [12]; however, in pancreatic cancer cells, JNK and other kinase inhibitors had no effect on ATF3 induction (data not shown) which was not further investigated.

Egr-1 was induced in Panc1 and Panc28 cells within 1 - 2 hr after treatment with CDODA-Me, whereas NAG-1 was induced at later time points in Panc28 (6 - 24 hr) and Panc 1 (24 hr) cells (Fig. 6A). This temporal pattern of NAG-1 and Egr-1 induction is similar to that observed in other studies where Egr-1 activates NAG-1 through interactions with the GC-rich proximal region of the NAG-1 promoter [26,29,33-36]. CDODA-Me induces phosphorylation of several kinases (PI3K, p38/p42MAPK and JNK) in both Panc28 and Panc1 cells (Fig. 6B) as previously observed in prostate cancer cells [12]; however, induction of Egr-1 was p42MAPK-dependent (Fig. 6C). In Panc1 cells, the p42MAPK inhibitor PD98059 also inhibited induction of NAG-1 which is consistent with a role for Egr-1 in mediating the induction of NAG-1 by CDODA-Me. However, the PI3-K and p38 MAPK inhibitors LY294002 and SB203580, respectively, also decreased induction of NAG-1, demonstrating the contributions of Egr-1-independent pathways in Panc1 cells.

Kinase inhibitor studies in Panc28 showed that induction of NAG-1 by CDODA-Me was primarily Egr-1-independent and was inhibited by LY294002 and SB20358 which had no effect on induction of Egr-1 in this cell line. This is one of the first examples of drug-dependent activation of both Egr-1 and NAG-1 in which induction of the latter gene is Egr-1-independent in one cell line (Panc28) and partially Egr-1-independent in another (Panc1 cells).

In summary, results of this study demonstrate for the first time that CDODA-Me inhibits growth and induces apoptosis in pancreatic cancer cells. Although CDODA-Me activates PPAR γ in Panc28 and Panc1 cells, induction of growth inhibitory and proapoptotic proteins and activation of multiple kinase activities is receptor-independent. This is the first report of the induction of the proapoptotic protein NAG-1 in pancreatic cancer cells; however, it was evident from studies with kinase inhibitors that the mechanisms of NAG-1 induction and the role of Egr-1 is cell context-dependent in Panc28 and Panc1 cells and differs from results of previous studies on NAG-1 induction [26,29,33-36]. Current studies are investigating the interplay between kinase activation, induction of proapoptotic proteins, and apoptosis by CDODA-Me in pancreatic cancer cells and the contributions of other pathways in mediating the proapoptotic effects of CDODA-Me in pancreatic cancer cells and tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Cytotoxicity of glycyrrhetic acid derivatives in pancreatic cancer cells. Effects of DODA-Me on Panc28 (A) and Panc1 (B) cell proliferation. Cells were treated with different concentrations of DODA-Me for up to 6 d and cell numbers were determined as described in the Materials and Methods. Growth inhibitory IC_{50} values were $> 15 \mu$ M in both cell lines. Effects of CDODA-Me on Panc28 (C) and Panc1 (D) cell proliferation. Cells were treated with different concentrations of CDODA-Me for up to 6 d and cell numbers were determined as described in the Materials and Methods. Growth inhibitory IC_{50} values were > 1.79 and 1.21μ M in Panc28 and Panc1 cell lines, respectively. Results are expressed as means $\pm$ SE for 3 replicate determinations for each treatment group. Similar studies were carried out for the corresponding free acids (DODA and CDODA) (data not shown) which exhibited lower cytotoxicity in both cell lines as previously described for these compounds in colon and pancreatic cancer cells [11,12].

Fig. 2.

Effects of CDODA-Me on cell cycle progression and apoptosis. Cell cycle progression in Panc28 (A) and Panc1 (B) cells. Cells were treated with DMSO or different concentrations of CDODA-Me for 48 hr, and % distribution of cells in G₀/G₁, S and G₂/M phases were determined by FACS analysis as described in the Materials and Methods. Results are expressed as means $\pm$ SE for 3 replicate determinations for each treatment group, and significant ($p < 0.05$) CDODA-Me-induced increases (*) or decreases (**) in percentages of a phase compared to the solvent (DMSO) control are indicated. Induction of DNA laddering in Panc28 (C) and Panc1 (D) cells. Cells were treated with DMSO or CDODA-Me for 24 hr and DNA laddering was determined as described in the Materials and Methods. The standard (S) of 1 kb was used as a marker for DNA laddering.

Fig. 3.

Activation of PPAR γ by CDODA-Me. Transfection with PPAR γ -GAL4/pGAL4 (A) and PPRE-luc (B). Panc1 or Panc28 cells were transfected with PPAR γ -GAL4/pGAL4 or PPRE-luc constructs, treated with DMSO or different concentrations of CDODA-Me, and luciferase activity was determined as described in the Materials and Methods. Inhibition of transactivation in Panc28 or Panc1 cells (C) by T007. Cells were transfected with PPAR γ -GAL4/pGAL4, treated with DMSO, CDODA-Me, T007 or their combination, and luciferase activity was determined as described in the Materials and Methods. Results in (A) - (C) are expressed as means $\pm$ SE for 3 replicate determinations for each treatment group, and significant ($p < 0.05$) induction by CDODA-Me (*) or inhibition after cotreatment with T007 (**) is indicated. (D) CDODA-Me induces differentiation. 3T3-L1 preadipocytes were treated with DMSO and CDODA-Me, and differentiation was detected by oil-red-O staining as described in the Materials and Methods.

Fig. 4.

Effects of CDODA-Me on cell cycle proteins. Induction of p21, p27, cyclin D1 in Panc28 (A) or Panc1 (B) cells by CDODA-Me, and the effects of PPAR γ antagonists on induction of these proteins in Panc28 (C) and Panc1 (D) cells. Cells were treated for 24 hr with DMSO, different concentrations of CDODA-Me alone, or in combination with GW9662, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. β -Actin served as a protein loading control.

Fig. 5.

Induction of apoptosis and proapoptotic NAG-1 and ATF3 proteins. Concentration-dependent induction of NAG-1/PARP cleavage (A) and ATF3 (B) by CDODA-Me. Panc28 and Panc1 cells were treated with DMSO and CDODA-Me for 24 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. Band intensities in (A) and (B) were quantitated relative to β -actin from 3 replicate determinations, and DMSO values were set at 1.0. Significant ($p < 0.05$) induction of NAG-1, PARP cleavage and ATF3 in Panc28 cells were observed at concentrations of CDODA-Me ≥ 2.5 , 2.5 and 1.0 μ M, respectively. The induction responses in Panc1 cells were observed at concentrations of ≥ 5.0 , 2.5 and 2.5 μ M, respectively. Effects of GW9662 on induction of NAG-1/PARP cleavage in Panc28 (C) and Panc1 (D) cells. Cells were treated and analyzed as described above in (A) and (B). β -Actin served as a protein loading control.

Fig. 6.

Kinase-dependent activation of NAG-1, Egr-1, PARP cleavage and ATF3 proteins induced by CDODA-Me. Time-dependent induction of NAG-1, Egr-1 and ATF3 (A) and kinases (B) by CDODA-Me in Panc1 and Panc28 cells. Cells were treated with DMSO or 5 μ M CDODA-Me for 1, 2, 4, 6, 8, 16 or 24 hr, and whole cell lysates from each treatment group were analyzed by western blots as described in the Materials and Methods. Effects of kinase inhibitors on induction of Egr-1 (C) and NAG-1 (D) in Panc1 and Panc28 cells. Cells were treated with DMSO, CDODA-Me alone or in the presence of kinase inhibitors, and whole cell lysates from each treatment group were analyzed by western blots as described in the Materials and Methods. β -Actin served as a protein loading control. The concentrations of

kinase inhibitors were 20 μ M LY294002 (LY), 20 μ M PD98059 (PD), and 20 μ M SB203580 and similar results we observed in duplicate studies. LY294002 and SB203580 inhibited Akt and p38 phosphorylation (positive control, data not shown). Band intensities in (C) and (D) were quantitated relative to β -actin from 3 replicate determinations and mean values are given relative to DMSO (set at 1.0). Significant ($p < 0.05$) inhibition of Egr-1 induction was observed for PD98059 in both cell lines. LY294002 and SB20358 significantly ($p < 0.05$) inhibited induction of NAG-1 in Panc1 and Panc28 cells, and PD98059 significantly ($p < 0.05$) inhibited induction of NAG-1 only in Panc1 cells.