Methyl 2-Cyano-3,12-dioxooleana-1,9-dien-28-oate Decreases Specificity Protein Transcription Factors and Inhibits Pancreatic Tumor Growth: Role of MicroRNA-27a

Indira Jutooru, Gayathri Chadalapaka, Maen Abdelrahim, Md Riyaz Bashar, Ismael Samudio, Marina Konopleva, Michael Andreeff, and Stephen Safe

Department of Veterinary Physiology and Pharmacology (I.J., G.C., S.S.) and Institute of Biosciences and Technology (S.S.), Texas A&M University, College Station, Texas; Cancer Research Institute, M.D. Anderson Cancer Center, Orlando, Florida (M.A., M.R.B.); and Section of Molecular Hematology and Therapy, Department of Stem Cell Transplantation and Cellular Therapy, M.D. Anderson Cancer Center, Houston, Texas (I.S., M.K., M.A.)

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ABSTRACT

The anticancer agent 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me) typically induce a broad spectrum of growth-inhibitory, proapoptotic, and antiangiogenic responses. Treatment of Panc1, Panc28, and L3.6pL pancreatic cancer cells with low micromolar concentrations of CDDO or CDDO-Me resulted in growth inhibition, induction of apoptosis, and down-regulation of cyclin D1, survivin, vascular endothelial growth factor (VEGF), and its receptor (VEGFR2). RNA interference studies indicate that these repressed genes are regulated by specificity protein (Sp) transcription factors Sp1, Sp3, and Sp4, and Western blot analysis of lysates from pancreatic cancer cells treated with CDDO and CDDO-Me shows for the first time that both compounds decreased the expression of Sp1, Sp3, and Sp4. Moreover, CDDO-Me (7.5 mg/kg/day) also inhibited pancreatic human L3.6pL tumor growth and down-regulated Sp1, Sp3, and Sp4 in tumors using an orthotopic pancreatic cancer model. CDDO-Me also induced reactive oxygen species (ROS) and decreased mitochondrial membrane potential (MMP) in Panc1 and L3.6pL cells, and cotreatment with antioxidants (glutathione and dithiothreitol) blocked the formation of ROS, reversed the loss of MMP, and inhibited down-regulation of Sp1, Sp3, and Sp4. Repression of Sp and Sp-dependent genes by CDDO-Me was due to the down-regulation of microRNA-27a and induction of zinc finger and BTB domain containing 10 (ZBTB10), an Sp repressor, and these responses were also reversed by antioxidants. Thus, the anticancer activity of CDDO-Me is due, in part, to activation of ROS, which in turn targets the microRNA-27a:ZBTB10-Sp transcription factor axis. This results in decreased expression of Sp-regulated genes, growth inhibition, induction of apoptosis, and antiangiogenic responses.

Extracts of plants and microorganisms and individual natural products have been extensively used as traditional medicines for the treatment of several diseases, including cancer.

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Individual natural products including aspirin, morphine, quinine, statins, penicillins, taxanes, and many other compounds are widely used pharmaceutical agents and serve as templates for the synthesis of more potent analogs (Koehn and Carter, 2005). Triterpenoids are derived from cyclization of oxidosqualene, and different cyclization pathways coupled with postcyclization modifications can give several thousand possible analogs, including oleanolic acid, which contains a pentacyclic oleanane skeleton and a C28 carboxyl group. Oleanolic acid has been used by Sporn, Honda, and their collaborators as a template for extensive structure-activity

ABBREVIATIONS: CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; CDDO-IM, imidazole ester of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; CDDO-Me, methyl ester of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; CDDOA-Me, methyl ester of 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oic acid; GSH, glutathione; MMP, mitochondrial membrane potential; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; Sp, specificity protein; VEGF, vascular endothelial growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PARP, poly(ADP-ribose) polymerase; TBP, TATA binding protein; ZBTB10, zinc finger and BTB domain containing 10; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; TBST, Tris-buffered saline/Tween 20; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; DTT, dithiothreitol; as-miR-27a, antisense microRNA-27a; CM-H2DCFDA, 5-(and-6)-chlorodihydrofluorescein diacetate acetyl ester.
studies to identify anti-inflammatory drugs, and the most active compounds identified were 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its corresponding methyl ester (CDDO-Me) and imidazole (CDDO-Im) esters (Honda et al., 1998, 2000; Liby et al., 2007).

The anticancer activities of CDDO and related compounds have been extensively investigated in several different cancer cell lines and in vivo, and their remarkable potency is due to modulation of several important pathways (Liby et al., 2007). Initial studies showed that CDDO was a peroxisome proliferator-activated receptor γ (PPARγ) agonist (Wang et al., 2000); however, most subsequent studies indicate that the anticancer activities of CDDO and related compounds were PPARγ-independent (Melichar et al., 2004; Ray et al., 2006). The effects of CDDO, CDDO-Me, and CDDO-Im vary among different cell lines and are dependent on the specific parameters measured; however, treatment with these compounds invariably resulted in growth inhibition, antiangiogenic activity, and induction of apoptosis (Liby et al., 2007). Induction of these responses is associated with the modulation of several pathways and genes, including activation of endoplasmic reticulum stress, microtubule disruption, direct binding to specific thiol groups in proteins, inhibition of nuclear factor-κB signaling, and mitochonodriotoxic, resulting in decreased mitochondrial membrane potential (MMP) (Ikedo et al., 2003, 2004; Samudio et al., 2005, 2006; Yore et al., 2006; Yue et al., 2006; Liby et al., 2007). For example, in pancreatic cancer cells, CDDO-Im inhibits cell growth and induces apoptosis, and this is associated with decreased MMP and mitochondrial glutathione (GSH) and induction of reactive oxygen species (ROS) (Samudio et al., 2005).

Studies in this laboratory have characterized the anticancer activity of 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oic acid (CDDOA) and its methyl ester (CDDO-Me), which are structurally similar to CDDO and CDDO-Me but are derived from the triterpenoid glycyrrhetinic acid, a bioactive component of licorice (Chintharlapalli et al., 2007a; Chintharlapaka et al., 2008b; Papineni et al., 2008; Jutooru et al., 2009). A recent study reported that one of the underlying mechanisms of action of CDDO-Me in colon cancer cells was due to the down-regulation of specificity protein (Sp) transcription factors Sp1, Sp3, and Sp4 and Sp-dependent genes (Chintharlapalli et al., 2009). In this study, we demonstrate for the first time that CDDO and CDDO-Me also decrease the expression of Sp1, Sp3, and Sp4 and Sp-dependent gene products (VEGF, cyclin D1, survivin, and VEGF2R) in pancreatic cancer cells and tumors in an orthotopic mouse model. The mitochondriotoxicity of CDDO-Me results in decreased MMP, induction of ROS, ROS-dependent down-regulation of microRNA-27a, and induction of ZBTB10 (an Sp repressor protein), which in turn down-regulates Sp transcription factors and Sp-dependent genes. Thus, CDDO-Me-dependent repression of Sp1, Sp3, and Sp4 contributes to the potent anticancer activity of CDDO and related compounds.

**Materials and Methods**

**Cell Lines.** Panc28 cell line was a generous gift from Dr. Paul Chiao, and L3.6P-L cells were kindly provided by Dr. Isaiah Fidler (University of Texas M. D. Anderson Cancer Center, Houston, TX), and Panc1 cells were obtained from the American Type Culture Collection (Manassas, VA). The HPDE immortalized pancreatic cell line was kindly provided by Dr. M-T. Tsao (Ontario Cancer Institute, Toronto, ON, Canada).

**Antibodies and Reagents.** All three pancreatic cancer cell lines were maintained in DMEM/Ham’s F-12 medium supplemented with 5% FBS, 0.22% sodium bicarbonate, and 10 μl/ml of 100× antibiotic/antimycotic cocktail solution (Sigma-Aldrich, St. Louis, MO). Cells were grown in 150-cm² culture plates in air/CO₂ (95:5) atmosphere at 37°C. Cyclic D1, Sp3, Sp4, VEGF, and VEGFR2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP antibody was purchased from Cell Signaling Technology (Danvers, MA), and Sp1 antibody was purchased from Millipore (Billerica, MA). Survivin antibody was purchased from R&D systems (Minneapolis, MN). Monoclonal β-actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase substrate for Western blot analysis was obtained from Millipore. Dithiothreitol and glutathione (GSH) were obtained from Sigma-Aldrich. Superscript II, LipofectAMINE, and LipofectAMINE 2000 were purchased from Invitrogen (Carlsbad, CA). Reporter lysis buffer and luciferase reagent were purchased from Promega (Madison, WI). β-Galactosidase reagent was obtained from Tropix (Bedford, MA). Primers for TBP and ZBTB10 were purchased from Integrated DNA Technologies (Corvalle, IA). Primers for Sp3 and Sp4 were obtained from QIAGEN (Valencia, CA); ZBTB10 expression vector and empty vector (pCMV6-XL4) were from Origene (Rockville, MD). miRNA mirvation RNA extraction kits and the reverse transcription and real-time polymerase chain reaction (PCR) amplification kits were purchased from Applied Biosystems (Foster City, CA). The VEGF and survivin promoter constructs were provided by Drs. Gerhard Siemeister and Gunter Finkenzeller (Institute of Molecular Medicine, Tumor Biology Center, Freiburg, Germany) and Dr. M. Zhou (Emory University, Atlanta, GA). Sp1 and Sp3 promoter constructs were kindly provided by Drs. Carlos Cuidad and Veronique Noel (University of Barcelona, Barcelona, Spain).

**Cell Proliferation Assay.** Pancreatic cancer cells (3 × 10⁵/well) were plated in 12-well plates and allowed to attach for 24 h. The medium was then changed to DMEM/Ham’s F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or different doses of CDDO or CDDO-Me were added. Cells were trypsinized and counted every 48 h using a Coulter Z1 particle counter for 6 days (Beckman Coulter, Fullerton, CA). Fresh medium and test compounds were added every 48 h. Each experiment was done in triplicate, and results are expressed as means ± S.E. for each treatment group.

**Transfection and Luciferase Assay.** Pancreatic cancer cells (10⁴/well) were plated in 12-well plates in DMEM/Ham’s F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 24 h, various amounts of DNA [i.e., 0.4 μg of PGL2-Luc, 0.4 μg of PGL3-Luc, 0.04 μg of β-galactosidase, and 0.4 μg of pEGFP (269)-Luc] were transfected using Lipofectamine reagent according to the manufacturer’s protocol. Five hours after transfection, the transfection mix was replaced with complete medium containing either vehicle (DMSO) or the indicated compound in DMSO. After 22 h, cells were then lysed with 100 μl of 1X reporter lysis buffer, and cell extracts (30 ml) were used for luciferase and β-galactosidase assays. A Lumicoat luminometer was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

**Transfection With Antisense microRNA-27a, Small Inhibitory RNAs against Sp1, Sp3, and Sp4, and ZBTB10 Expression Vector.** Pancreatic cancer cells (10⁴/well) were plated in 12-well plates in DMEM/Ham’s F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 24 h, cells were transfected with empty vector (pCMV6-XL4) or 4 μg/well ZBTB10 expression plasmid pCMV6-XL4 vector using Lipofectamine 2000 reagent according to the manufacturer’s protocol. After transfection for 5 h, the transfection mix was replaced with complete medium and incubated for 48 h. A similar approach was used in studies with antisense microRNA-27a (as-miR-27a) to determine its effect on cell proliferation and apoptosis. Small inhibitory RNAs for Sp1 (siSp1), Sp3 (siSp3), Sp4
plates were loaded with 10 gen. After treatment for 20 to 24 h, cells plated on 96-well cell culture were determined with the plate reader set to an absorbance of 610 excitation and emission wavelengths, respectively. After reading of BioTek Synergy 4 plate reader (Winooski, VT) set at 480 and 525 nm once with serum-free medium, and analyzed for ROS levels using the SNAP-frozen in liquid nitrogen and stored at measured, and their weights were recorded. The tumor tissues were irradiated to confirm the initiation of tumor growth.

Animals and Orthotopic Implantation of Tumor Cells. Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). Mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations for growth inhibition by CDDO-Me and CDDO after treatment for 48 h were 5.2 and 0.37 (Panc1), 5.3 and 0.37 (Panc28), and 3.0 and 0.4 \( \mu \)M (L3.6pL), respectively. CDDO-Me is more active than CDDO in all three pancreatic cancer cell lines. After prolonged treatment (144 h) with CDDO-Me and CDDO-Im are cytotoxic to several different cancer cell lines, and the latter derivative was a potent inhibitor of pancreatic cancer cell formation HPDE pancreatic cells; however, this cell line was more resistant to the effects of CDDO-Me than pancreatic cancer cell lines, and there were minimal differences between cell lines to show that CDDO-Me also inhibits the growth of nontransfected HPDE pancreatic cells; however, this cell line was more resistant to the effects of CDDO-Me than pancreatic cancer cell lines.

Results

The synthetic oleandric acid derivatives CDDO, CDDO-Me, and CDDO-Im are cytotoxic to several different cancer cell lines, and the latter derivative was a potent inhibitor of pancreatic cancer cell proliferation (Samudio et al., 2005). In this study, we initially compared the effects of CDDO and CDDO-Me on the proliferation of Panc1, Panc28, and L3.6pL pancreatic cancer cell lines for up to 144 h (Fig. 1, A and B). IC_{50} values for growth inhibition by CDDO-Me and CDDO after treatment for 48 h were 5.2 and 0.37 (Panc1), 5.3 and 0.37 (Panc28), and 3.0 and 0.4 \( \mu \)M (L3.6pL), respectively. CDDO-Me was more active than CDDO in all three pancreatic cancer cell lines, and there were minimal differences between cell lines to the cytotoxic effects of both compounds. Supplemental Fig. 1 shows that CDDO-Me also inhibits the growth of nontransformed HPDE pancreatic cells; however, this cell line was more resistant to the effects of CDDO-Me than pancreatic cancer cell lines. After prolonged treatment (144 h) with CDDO-Me and CDDO, IC_{50} values were 0.25 and 1.8 (Panc1), 0.30 and 2.3 (Panc28), and 0.28 and 1.4 \( \mu \)M (L3.6pL), respectively, and the relative potencies of both compounds and cellular responsiveness were similar to that observed after 48 h.
Panc1, Panc28, and L3.6pL cell growth by CDDO and CDDO-Me was also accompanied by apoptosis, and Fig. 1, C and D, shows that both compounds induced caspase-dependent PARP cleavage in the pancreatic cancer cell lines.

CDDO and related compounds inhibit growth, induce apoptosis, and exhibit antiangiogenic activity in cancer cells derived from multiple tumor types (Liby et al., 2007), and the effects of CDDO-Me and CDDO on the expression of prototypical gene products representing these activities were investigated in the three pancreatic cancer cell lines. Results in Fig. 2A show that 0 to 1.25 μM CDDO-Me and 0 to 5 μM CDDO decrease the expression of cyclin D1, survivin, VEGF, and VEGFR2 proteins in Panc1 cells. Moreover, similar results were observed in Panc28 (Fig. 2B) and L3.6pL (Fig. 2C) cells, and results in Figs. 1 and 2 are consistent with previous studies on CDDO and related compounds in cancer cells (Honda et al., 1998, 2000; Wang et al., 2000; Ikeda et al., 2003, 2004; Melichar et al., 2004; Samudio et al., 2005, 2006; Yore et al., 2006; Yor et al., 2006; Liby et al., 2007).

Knockdown of Sp1, Sp3, and Sp4 transcription factors by RNA interference in pancreatic cancer cells showed that VEGF, VEGF-R1, and VEGF-R2 expression were regulated by Sp1, Sp3, and Sp4 (Abdelrahim et al., 2004, 2006; Higgins et al., 2006), suggesting that an underlying mechanism of action of CDDO and CDDO-Me may involve the down-regulation of Sp proteins as described previously for other bioactive triterpenoids in cancer cells (Chintharlapalli et al., 2007b, 2009). Results in Fig. 3A demonstrate that 0 to 1.25 μM CDDO-Me and 0 to 5.0 μM CDDO decreased the expression of Sp1, Sp3, and Sp4 proteins in Panc1 cells, and similar results were observed in Panc28 (Fig. 3B) and L3.6pL (Fig. 3C) cells. The observed CDDO-/CDDO-Me-dependent down-regulation of Sp transcription factors (Fig. 3, A–C) is consistent with their parallel decrease in Sp-dependent genes, as illustrated in Fig. 2. Previous studies show that the nonsteroidal anti-inflammatory drug tolfenamic acid induced proteasome-dependent down-regulation of Sp1, Sp3, and Sp4 in Panc1 cells that was blocked by the proteasome inhibitor N-benzoylloxycarbonyl (Z)-Leu-Leu-leucinal (MG132) (Abdelrahim et al., 2006); however, results in Fig. 3D using CDDO-Me indicate that down-regulation of Sp1, Sp3, and Sp4 by this compound in pancreatic cancer cells was not blocked by the proteasome inhibitor MG132, indicating a proteasome-independent pathway. The proximal regions of...
the Sp1, Sp3, VEGF, and survivin promoters are GC-rich, and the effects of CDDO-Me and CDDO on promoter activity were investigated in Panc1 cells transfected with pSp1For4, pSp3For5, pVEGF, and pSurvivn constructs, which contain −751 to −20 (Sp1), −417 to −38 (Sp3), −2018 to +50 (VEGF), and −269 to +49 (survivin) promoter inserts, respectively. CDDO and CDDO-Me significantly decreased luciferase (reporter gene) activity in Panc1 cells transfected with these constructs (Supplemental Fig. 2), indicating that CDDO and CDDO-Me act at the transcriptional level, and this is consistent with the observed proteasome-independent down-regulation results shown in Fig. 3D. We also observed that CDDO-Me decreased Sp1 mRNA levels (Supplemental Fig. 3), which is consistent with the inhibitory effects of this compound on transcription.

Previous reports showed that the imidazole derivative of CDDO was mitochondriotoxic in pancreatic cancer cells, and this was characterized by decreased MMP and accompanied ROS levels as determined by CM-H2DCFDA fluorescent dye, and this response was also attenuated after cotreatment with the antioxidant glutathione (Fig. 4A). Panc1 and L3.6pL cells were incubated with the fluorescent dye JC-1, and after incubation with the fluorescent dye JC-1, and after treatment with DMSO or GSH, the typical orange/red fluorescent staining of mitochondria was observed (Figs. 4A). However, in Panc1 or L3.6pL cells treated with 1.25 or 1.0 μM CDDO-Me, respectively, there was a significant decrease in orange/red staining and an increase in green fluorescence indicative of decreased MMP, and this response was attenuated after cotreatment with GSH in both cell lines (Fig. 4B). These results demonstrate that CDDO-Me was mitochondriotoxic in pancreatic cancer cells, and we therefore investigated the possible connection between CDDO-Me-induced ROS and mitochondrial effects and CDDO-Me-dependent down-regulation of Sp1, Sp3, and Sp4 proteins. Results in Fig. 4C demonstrate that Sp1, Sp3, and Sp4 are down-regulated in Panc1 and L3.6pL cells after treatment with CDDO-Me, and decreased expression of Sp1, Sp3, and Sp4 proteins was inhibited after cotreatment with thiol antioxidants (DTT and/or GSH) and similar results were observed in Panc28 cells (data not shown). Hydrogen peroxide or t-butyl hydroperoxide was used as prototypical model ROS, and both compounds decreased the expression of Sp1, Sp3, and Sp4 proteins (data not shown), confirming that induction of ROS in pancreatic cancer cells is a critical upstream response in targeting the repression of Sp transcription factors, and comparable results have been observed in other cancer cell lines.

CDDOA-Me, a triterpenoid structurally related to CDDO-Me, decreased Sp proteins in colon cancer cells through down-regu-

Fig. 2. CDDO and CDDO-Me decrease the expression of VEGF, VEGFR2, cyclin D1 (CD1), and survivin proteins in Panc1 (A), Panc28 (B) and L3.6pL (C) pancreatic cancer cell lines. Cells were treated with DMSO, CDDO (1.0, 2.5, or 5.0 μM), or CDDO-Me (0.5, 1.0, or 1.25 μM) for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described under Materials and Methods. β-Actin served as a loading control. The gels were typical of results of at least two replicate determinations per treatment group.
CDDO-Me down-regulates Sp proteins via ROS-initiated pathway.

We also investigated the comparative effects of as-miR-27a, ZBTB10 overexpression, and knockdown of Sp1, Sp3, and Sp4 alone or in combination as described previously (Chadalapaka et al., 2008a, 2010; Chintharlapalli et al., 2009) on growth inhibition and induction of PARP cleavage in Panc1 and L3.6pL cells (Fig. 6). Transfection of cells with as-miR-27a inhibited the growth of Panc1 and L3.6pL cell growth (Fig. 6B), and the results were similar to that observed for as-miR-27a; however, overexpression of ZBTB10 was more potent than as-miR-27a as an inducer of PARP cleavage. Because both as-miR-27a and ZBTB10 are involved in the down-regulation of Sp1, Sp3, and Sp4 (Fig. 5C) (Mertens-Talcott et al., 2007), the effects of individual Sp knockdown (iSp1, iSp3, and iSp4) or combined knockdown (iSp1/3/4) by RNA interference on cell growth and PARP cleavage were investigated (Fig. 6, C and D). Panc1 and L3.6pL cell growth was decreased in cells transfected with iSp1/3/4, and the effects of individual Sp knockdown were variable and cell context-dependent; however, loss of Sp1 resulted in the most pronounced growth inhibition in both cell lines. In Panc1 cells, iSp1, iSp3, iSp4, and iSp1/3/4 induced PARP cleavage; however, iSp3 was the most effective (Fig. 6C), whereas iSp4 and iSp1/3/4 induced PARP cleavage in L3.6pL cells (Fig. 6D). These results demonstrate that the
growth inhibitory and proapoptotic effects of CDDO-Me are also observed in pancreatic cancer cells transfected with as-
miR-27a or ZBTB10 overexpression or after knockdown of Sp transcription factors.

The in vivo anticancer activity of CDDO-Me was also investigated in an orthotopic model of pancreatic cancer in
which L3.6pL cells were injected directly into the pancreas of 8- to 12-week-old male thymic nude mice (Baker et al., 2002; Abdelrahim et al., 2006). Treatment with CDDO-Me (7.5 mg/kg daily by oral administration) was initiated 7 days after injection of the cells and continued for an additional 28 days. Treatment with CDDO-Me significantly decreased pancreatic tumor volume and weight (Fig. 7D) compared with the vehicle control group. In addition, lysate from tumors treated with the vehicle or CDDO-Me (from three different animals per group) were also analyzed by Western
blots, and there was a marked decrease in expression of Sp1, Sp3, and Sp4 proteins in tumors from mice treated with CDDO-Me compared with the control group (Fig. 7C). Moreover, we also observed decreased expression of VEGF, cyclin
D1, and survivin in tumors from CDDO-Me-treated mice compared with animals receiving vehicle control (Fig. 7D).
Body and organ weight changes and evidence for toxicity were not observed (data not shown), and this is consistent with the applications of CDDO compounds in chemoprevention
studies (Liby et al., 2007). These in vivo results are consistent with cell culture studies (Figs. 2 and 3) demonstrating for the first time that CDDO-Me represses the expression of Sp1, Sp3, and Sp4 transcription factors and Sp-dependent gene products, suggesting that this hitherto unrecognized pathway also contributes to the anticancer activity of CDDO-Me and related compounds.

Discussion
Sp1 was the first transcription factor identified and is a member of the Sp/Krüppel-like family of zinc finger trans-
scription factors that exhibit a broad range of tissue-specific and overlapping functions (Bouwman and Philip-
sen, 2002; Safe and Abdelrahim, 2005). Although Sp
transcription factors are important in humans and laboratory animals of a marked decrease in Sp1 with aging (Ammendola et al., 1992; Oh et al., 2007), and studies in this laboratory show that Sp1, Sp3, and Sp4 levels in nontumor tissue of mice are minimal to nondetectable (Abdelrahim et al., 2006, 2007; Chintharlapalli et al., 2007b). Several reports show that Sp1 protein is overexpressed in different human tumor types, including gastric, colorectal, pancreatic, epidermal, thyroid, and breast cancers (Zannetti et al., 2000; Shi et al., 2001; Chiefari et al., 2002; Wang et al., 2003; Hosoi et al., 2004; Yao et al., 2004; Mertens-Talcott et al., 2007, 2008). In patients with gastric cancer, there was an association between Sp1 expression with advanced stage of the tumor, VEGF expression, and poor survival (Yao et al., 2004). Although the mechanism of Sp overexpression has not been determined, Lou et al. (2005) have shown that malignant transformation of human fibroblasts resulted in an 8- to 18-fold increase in Sp1 expression and the transformed cells formed tumors in athymic nude mouse xenografts. In contrast, these transformed cells were not tumorigenic after knockdown of Sp1. Sp1 is overexpressed in pancreatic cancer cells (Shi et al., 2001), and there is a correlation between the expression of Sp1 and the angiogenic factor VEGF. Moreover, it was recently reported that Sp1 was a biomarker that identifies patients with a highly aggressive subtype of pancreatic ductal adenocarcinomas (Jiang et al., 2008). Studies in this laboratory show that Sp1, Sp3, and Sp4 are overexpressed in pancreatic and other cancer lines and knockdown of Sp1, Sp3, and Sp4 by RNA interference indicates that expression of several growth-promoting, prosurvival, and angiogenic genes/responses are regulated by these transcription factors (Abdelrahim et al., 2004, 2006; Chintharlapalli et al., 2007b; Mertens-Talcott et al., 2007, 2008a).

The high expression of Sp1, Sp3, and Sp4 proteins in cancer cells and tumors coupled with their regulation of several critical pro-oncogenic genes suggests that these transcription factors are potentially important drug targets. The nonsteroidal anti-inflammatory drug tolenamic acid, the triterpenoid betulinic acid, curcumin, and CDODA-Me have been characterized previously as agents that inhibit cancer cell proliferation and tumor growth in rodent models and induce down-regulation of Sp1, Sp3, and Sp4 through proteasome-dependent and -independent pathways (Abdelrahim et al., 2006; Chintharlapalli et al., 2007b, 2009; Chadalapaka et al., 2008a). CDDO/CDDO-Me and CDODA/CDODA-Me activate PPARγ and exhibit some common and different activities in colon and pancreatic cancer cells (Wang et al., 2000;
Chintharlapalli et al., 2007a). A recent study in colon cancer cells showed that CDODA-Me down-regulated the expression of Sp1, Sp3, and Sp4 and Sp-regulated gene products (Chintharlapalli et al., 2009). Results in Figs. 2 and 3 show that CDDO and CDDO-Me also decreased the expression of Sp1, Sp3, and Sp4 and Sp-regulated genes such as cyclin D1, VEGF, VEGFR2, and survivin in Panc1, Panc28, and L3.6pL pancreatic cancer cells. Moreover, repression of these gene products by CDDO and CDDO-Me were observed at concentrations that were comparable with those required for inhibition of Panc1, Panc28, and L3.6pL cell growth and induction of apoptosis (Fig. 1). In contrast to previous reports with tolfenamic acid in pancreatic cancer cells (Abdelrahim et al., 2006, 2007), CDDO-Me induced proteasome-independent down-regulation of Sp1, Sp3, and Sp4 in pancreatic cancer cells (Fig. 3D). CDDO-Me also decreased luciferase activity in cells transfected with constructs containing Sp1, Sp4, VEGF, and survivin gene promoter inserts and decreased Sp1 mRNA expression (Supplemental Figs. 2 and 3), which is consistent with the effects of CDDO-Me on transcription. These in vitro cell culture studies confirm the potency of CDDO-Me as an inhibitor of pancreatic cancer cell growth and demonstrate for the first time that the anticancer activity is due, in part, to targeting of Sp transcription factors and Sp-regulated genes. Further evidence for the role of this pathway in the activity of CDDO-Me was observed in an

**Fig. 6.** Growth inhibition and induction of apoptosis by as-miR-27a, ZBTB10, and Sp knockdown. Panc1 and L3.6pL cells were transfected with as-miR-27a (A), ZBTB10 expression plasmid (B), and small inhibitory RNAs against Sp transcription factors (C and D), and their effects on cell proliferation and induction of PARP cleavage were determined as described under Materials and Methods. Western blots are typical of at least two replicate determinations. Cell numbers are given as means ± S.E. of three replicate determinations. Significant (*P < 0.05) growth inhibition is indicated (*). RNA interference decreased expression of individual Sp proteins approximately 60 to 80% as described previously (Chadalapaka et al., 2008a, 2010).
orthotopic model for pancreatic cancer, in which CDDO-Me (7.5 mg/kg/day) not only inhibited tumor growth but also decreased the expression of Sp1, Sp3, and Sp4 proteins in pancreatic tumors (Fig. 7). These results suggest that CDDO-Me activates comparable pathways both in vitro and in vivo, and this has been observed previously for other agents that target Sp transcription factors (Abdelrahim et al., 2006, 2007; Chadalapaka et al., 2008a; Chintharlapalli et al., 2009).

CDDO-Im-dependent anticarcinogenic activity in pancreatic cancer cells has been linked to its mitochondrial activity (Samudio et al., 2005), which was characterized by decreased MMP and GSH and induction of ROS, and these responses were blocked in cells cotreated with thiol antioxidants. Not surprisingly, CDDO-Me also induced a similar pattern of mitochondrial responses in pancreatic cancer cells, which were inhibited after cotreatment with thiol antioxidants (Fig. 4). A recent study in prostate cancer cells also linked the proapoptotic activity of CDDO-Me to the induction of ROS (Deeb et al., 2010). Because induction of ROS and ROS-dependent responses play an important role in the anticancer activity of mitochondrial anticancer drugs such as arsenic trioxide (Miller et al., 2002), we hypothesized that repression of Sp transcription factors may be an ROS-dependent response. Results in Fig. 4D show that CDDO-Me-dependent down-regulation of Sp1, Sp3, and Sp4 proteins is significantly inhibited in Panc1 and L3.6P cells after cotreatment with GSH and DTT. Moreover, using hydrogen peroxide and tert-butyl hydroperoxide as prototypical ROS, both oxidants also decreased the expression of Sp1, Sp3, and Sp proteins in pancreatic cancer cells (data not shown). These results demonstrate that the effects of CDDO-Me on mitochondria are linked to ROS-dependent down-regulation of Sp1, Sp3, and Sp4, and we have also observed similar effects with arsenic trioxide in pancreatic cancer cells (Miller et al., 2002).

The role of miR-27a and its suppression of the zinc finger protein ZBTB10 in regulating the expression of Sp1, Sp3, and Sp4 has been characterized previously in breast and colon cancer cells (Mertens-Talcott et al., 2007; Chintharlapalli et al., 2009). Both antisense miR-27a and ZBTB10 overexpression decrease Sp and Sp-dependent genes in these cell lines (Abdelrahim et al., 2006; Mertens-Talcott et al., 2007), and this is related to the effects of ZBTB10, which binds GC-rich sites and acts as a transcriptional repressor (Tillotson, 1999). Treatment of Panc1 and L3.6P cells with CDDO-Me decreased the expression of miR-27a (Fig. 5A), and this was accompanied by the induction of ZBTB10 (Fig. 5B). These results are comparable with those observed for the structurally related triterpenoid CDDOA-Me in colon cancer cells (Mertens-Talcott et al., 2007; Chintharlapalli et al., 2009). We also confirmed that ZBTB10 overexpression in Panc1 and L3.6P cells decreased levels Sp1, Sp3, and Sp4 proteins (Fig. 5C) as observed in colon and breast cancer cells (Mertens-Talcott et al., 2007; Chintharlapalli et al., 2009). These studies also demonstrate for the first time that CDDO-Me-dependent induction of ROS is critical for downstream events because antioxidants inhibit the down-regulation of miR-27a, induction of ZBTB10 (Fig. 5D), and repression of Sp1, Sp3, and Sp4 (Fig. 4D) in Panc1 and L3.6P cells treated with CDDO-Me. Because the downstream effects of CDDO-Me are associated with decreased expression of as-miR-27a, which results in the induction of ZBTB10 and down-regulation of Sp1, Sp3, and Sp4, we also investigated the growth inhibitory/proapoptotic effects of individual steps in this pathway. Transfection of Panc1 and L3.6P cells with as-miR-27a or ZBTB10 expression plasmid or a cocktail of small inhibitory RNAs against Sp1, Sp3, and Sp4 (siSp1/3/4) inhibited cell growth and induced PARP cleavage. There were some cell context-dependent differences in the effects of individual Sp knockdown; however, these results indicate that CDDO-Me-dependent perturbation of the miR-27a:ZBTB10–Sp1/3/4 axis contributes to the growth inhibitory and proapoptotic effects of CDDO-Me.

In summary, we have shown that CDDO-Me is highly cytotoxic to pancreatic cancer cells and tumors, and this is consistent with previous reports on the potent anticancer activity of CDDO and related compounds (Honda et al., 1998, 2000; Wang et al., 2000; Ikeda et al., 2003, 2004; Melichar et al., 2004; Samudio et al., 2005, 2006; Ray et al., 2006; Yore et al., 2006; Yue et al., 2006; Liby et al., 2007). Our results demonstrate for the first time that CDDO and CDDO-Me induce the down-regulation of Sp1, Sp3, and Sp4 transcription factors and several Sp-dependent genes that are associated with cancer cell survival, growth, and angiogenesis. These observations suggest that this pathway contributes to the potent antitumorigenic activity of CDDO and related compounds and may also explain, in part, the broad spectrum

Fig. 7. CDDO-Me inhibits pancreatic tumor growth and down-regulates Sp proteins and Sp dependent genes. Tumor weights (A) and volume (B). Male athymic nude mice bearing orthotopic pancreatic (L3.6P) tumors were treated with corn oil or CDDO-Me (7.5 mg/kg) for 4 weeks, and tumor weights and tumor volumes (in cubic millimeters) were determined as described under Materials and Methods. Significant (*P < 0.05) inhibition (+) is indicated in results as means ± S.E. for five animals per treatment. Western blot analysis of tumor lysates for Sp proteins (C) and Sp-dependent proteins (D). Lysates from three mice in the treated and control groups were analyzed by Western blots as described under Materials and Methods. β-Actin served as loading control.
of activities of these drugs in cancer cell lines. CDDO-Me, like its imidazole derivative (Samudio et al., 2005), also induces transcription factors and on determining the specificity proteins.

References

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Address correspondence to: Dr. Stephen Safe, Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX 77843-4468. E-mail: ssafe@cvm.tamu.edu

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