CDDO-Me inhibits tumor growth and prevents recurrence of pancreatic ductal adenocarcinoma

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Abstract. Methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) has shown potent antitumorigenic activity against a wide range of cancer cell lines in vitro and inhibited the growth of liver, lung and prostate cancer in vivo. In the present study, we examined the antitumor activity of CDDO-Me for pancreatic ductal adenocarcinoma (PDAC) cells with and without activating K-ras mutations. Treatment of K-ras mutant MiaPaCa-2 and K-ras normal BxPC-3 cells with CDDO-Me elicited strong antiproliferative and proapoptotic responses in both cell lines in culture. The inhibition of cell proliferation and induction of apoptosis was accompanied by the inhibition of antiapoptotic/prosurvival p-Akt, NF-κB and p-mTOR signaling proteins. For testing efficacy of CDDO-Me in vivo heterotopic and orthotopic xenografts were generated by implanting BxPC-3 and MiaPaCa-2 cells subcutaneously and in the pancreatic tail, respectively. Treatment with CDDO-Me significantly inhibited the growth of BxPC-3 xenografts and reduced the levels of p-Akt and p-mTOR in tumor tissue. In mice with orthotopic MiaPaCa-2 xenografts, treatment with CDDO-Me prolonged the survival of mice when administered following the surgical resection of tumors. The latter was attributed to the eradication of residual PDAC remaining after resection of tumors. These preclinical data demonstrate the potential of CDDO-Me for treating primary PDAC tumors and for preventing relapse/recurrence through the destruction of residual disease.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for >90% of all exocrine pancreatic cancers and is almost uniformly lethal with an estimated annual number of 45,220 new cases ~38,460 annual deaths and a 5-year survival rate of <5% (1-3).

Late initial diagnosis, aggressive metastatic behavior and resistance to chemo-radiotherapy render pancreatic cancer one of the most difficult to treat of all malignancies. Systemic chemotherapy with either gemcitabine or FOLFIRINOX (5-FU, irinotecan and oxaliplatin) is the current standard of care for advanced PDAC, providing short-term symptomatic improvement with minor impact on survival (4-6). Surgical resection is potentially curative; however, nearly 80% of the patients are diagnosed with locally advanced disease, precluding surgical intervention. Even in early stage PDAC patients in whom cancerous tissue is surgically resected (Whipple procedure), a majority of them relapse within two years after surgery. Thus, there is a tremendous need for novel therapeutics and treatment strategies for treating primary and residual PDAC for preventing relapse after pancreatic cancer surgery.

Aberration of apoptosis has been implicated in carcinogenesis and resistance to conventional anticancer therapies (7). Thus, promotion of apoptosis in cancer cells with novel agents could lead to tumor regression and improved prognosis. Triterpenes or triterpenoids are members of a large family of structurally related compounds known as cyclosqualenoids that are widely distributed in nature (8). Oleanolic acid is naturally occurring triterpenoid that has been used in traditional medicine as an anticancer and anti-inflammatory agent (9-11). In order to increase the anti-inflammatory and anticancer activity of oleanolic acid several synthetic analogues have been synthesized. Synthetic lealane triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its C-28 methyl ester derivative methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Me) and C-28 imidazole derivative 1-(2-cyano-3,12-dioxooleana-1,9-dien-28-yl) imidazole (CDDO-Im) exhibit stronger anti-inflammatory activity than oleanolic acid (12,13). CDDOs have also shown potent antiproliferative and proapoptotic activity in diverse types of tumor cell lines through the inhibition of MAPK (Erk1/2), NF-κB and PPARγ signaling pathways and they induce cytoprotective phase 2 response through Nrf2 signaling (14-20). In previous studies, we have demonstrated that the antiproliferative and apoptotic inducing activity of CDDO-Me in prostate cancer cell lines is mediated through the inhibition of antiapoptotic (prosurvival) Akt/NF-κB/mTOR signaling pathways (21). Although the antiproliferative activity of CDDOs in PDAC cell lines in vitro has been reported their therapeutic efficacy for PDAC in vivo has not been adequately studied.
We have investigated the therapeutic efficacy of CDDO-Me against heterotopic and orthotopic tumor xenografts generated with human PDAC cell lines with a focus on preventing or delaying relapse/recurrence. Our data show therapeutic efficacy of CDDO-Me in treating primary tumor growth as well as in preventing/delaying recurrence of PDAC when administered after surgical removal of tumors.

**Materials and methods**

**Reagents.** CDDO-Me was obtained from the National Cancer Institute, Bethesda, MD, USA through the Rapid Access to Intervention Development Program. Antibodies against p-Akt (ser473), p-mTOR (Ser2448), PARP-1 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). CellTiter 96 AQueous One Solution Proliferation assay system was from Promega (Madison, WI, USA) and Annexin V-FITC apoptosis detection kit II was obtained from BD Pharmingen (San Diego, CA, USA).

**Cell culture.** Human PDAC cell lines MiaPaCa-2 and BxPC-3 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cell lines were cultured at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air and maintained by subculturing cells twice a week.

**Mice.** Six-week-old female Scid/Ncr mice were purchased from the Charles River Laboratories, Inc., Frederick, MD, USA. Mice were maintained in a temperature-controlled room (68-72°F) with a 12-h light/dark cycle and provided semi-purified AIN-76A mouse chow and water *ad libitum*. Mice were acclimated for one week before starting the experiment. All animal treatments were according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC).

**Measurement of cell viability.** Tumor cells (1x10⁴) were seeded into each well of a 96-well plate in 100 µl of tissue culture medium. After incubation at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air for 24 h, cells were treated with CDDO-Me at concentrations of 0.625-5 µM for 72 h. Cell viability was then determined by the MTS assay using CellTiter 96 AQueous One Solution Proliferation assay system.

**Measurement of apoptosis.** Apoptosis was assessed by the binding of Annexin V-FITC to phosphotidylserine, which is externalized to the outer leaflet of the plasma membrane early during induction of apoptosis. Briefly, untreated cells and cells treated with CDDO-Me for 24 h were resuspended in the binding buffer provided in the Annexin V-FITC apoptosis detection kit and reacted with 5 µl of Annexin V-FITC reagent and 5 µl of propidium iodide (PI) for 30 min at room temperature in the dark. Stained cells were analyzed by flow cytometry using Accuri C6 flow cytometer (Accuri Cytometers Inc, Ann Arbor, MI, USA). The induction of apoptosis by CDDO-Me was confirmed by the cleavage of PARP-1 by western blotting.

**Western blotting.** Cell lysates were prepared using NP 40 cell lysis buffer. Lysates were clarified by centrifugation at 14,000 x g for 10 min at 4°C and protein concentrations were determined. Samples (50 µg) were boiled in an equal volume of sample buffer [20% glycerol, 4% SDS, 0.2% bromophenol blue, 125 mM Tris-HCl (pH 7.5), and 640 mM 2-ME] and separated on pre-casted Tris-glycine polyacrylamide gels using the XCell Surelock™ Mini-Cell, in Tris-glycine SDS running buffer, all from Novex (Invitrogen, Carlsbad, CA, USA). Proteins resolved on the gels were transferred to PVDF membranes. Membranes were blocked with 5% milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl with 0.05% Tween-20 (TPBS) and probed using specific antibodies against proteins of interest or β-actin (loading control) and HRP-conjugated secondary antibody. Immune complexes were visualized with enhanced chemiluminescence. Protein bands were imaged and band densities analyzed using the NIH/Scion image analysis software. The protein band densities were normalized to the corresponding β-actin band densities and percent change in signal strength was calculated.

**Generation of tumor xenografts.** Heterotopic PDAC xenografts were generated by implanting 1x10⁶ BxPC-3 cells in 50 µl of PBS subcutaneously in the right flank of 7-week-old Scid-Ncr female mice. Cells were allowed to form tumors for 7 days before initiating treatment with CDDO-Me. Orthotopic PDAC xenografts were generated by implanting MiaPaCa-2 cells stably expressing luciferase gene (MiaPaCa-2-Luc cells) in the pancreatic tail of Scid/Ncr mice. Briefly, mice were anesthetized with ketamine/xylazine (100/8 mg/kg i.p.) and a 2-cm long subcostal laparotomy was performed after clipping hair. Pancreases were exposed and 1x10⁶ MiaPaCa-2-Luc cells in 20 µl of PBS were injected in the pancreatic tail with a 30 G needle. Proper deposition of cells was confirmed by bleb formation at the site of injection. The fascia and skin incisions were sutured following implantation of tumor cells.

**Residual disease.** To generate residual disease, 1x10⁶ MiaPaCa-2-Luc cells in 20 µl of PBS were injected in the pancreatic tail as described above. Cells were first allowed to form tumors for 28 days and then mice were anesthetized with ketamine/xylazine (100/8 mg/kg i.p.) and partial pancreatectomy was performed to remove all visible tumor growth. After resection of tumors, incisions were sutured and mice were either treated or not with CDDO-Me.

**Treatment with CDDO-Me.** Two treatment paradigms were followed. To treat primary tumor growth, treatment was started on day 7 after implantation of BxPC-3 cells subcutaneously. To treat residual disease, treatment was started one day after resection of tumors on day 28 after implantation of MiaPaCa-2-Luc cells in the pancreas. Mice in both treatment paradigms were administered CDDO-Me at a dose of 15 µmol/kg (7.5 mg/kg), 5 days a week in 0.1 ml of vehicle consisting of cremophor-EL:DMSO:PBS (1:1:8) by oral gavage until day 40 (heterotopic xenografts) to treat primary tumor growth or day 100 (orthotopic xenografts) to treat residual disease. Control mice were treated with vehicle alone without CDDO-Me.

**Bioluminescent imaging (BLI).** The growth progression of orthotopic PDAC xenografts generated by implanting MiaPaCa-2-Luc cells in the pancreas was measured...
non-invasively by BLI imaging using Kodak Multispectral Imaging system (Carestream Health, Woodbridge, CT, USA). Briefly, mice were anesthetized with isofluorane and anesthesia was maintained by inhalation of a mixture of isofluorane (2%) and oxygen during the imaging procedure. First an X-ray image was taken and then BLI was obtained for 2 min, 10 min after i.p. injection of sodium luciferin (150 mg/kg). The X-ray and BLI images were overlaid and peak photon flux (photon/sec/mm²) from the tumors was plotted against time to obtain tumor growth curve.

Statistical analysis. Most data are presented as means ± SD and outcomes for treated and untreated cells were compared by Student's t-test. Differences were considered significant at p<0.05.

Results

CDDO-Me inhibits proliferation and induces apoptosis in PDAC cells. To test the effect of CDDO-Me on the proliferation of PDAC cells, 1x10⁴ MiaPaCa-2 or BxPC-3 cells were plated in 96-well microtiter plates for 24 h and then treated with CDDO-Me for 72 h at concentrations ranging from 0 to 5 µM. Viability of cultures was assessed by MTS assay and percent reduction in viability of cultures was determined. As shown in Fig. 1, significant decrease in the viability of cell cultures was observed in both cell lines treated with CDDO-Me at concentrations of 1.25-5 µM (MiaPaCa-2: 44-73%; BxPC-3: 57-79%).

To determine whether inhibition of proliferation of PDAC cells by CDDO-Me was associated with induction of apoptosis, binding of Annexin V-FITC to cells treated with CDDO-Me was measured. For this, MiaPaCa-2 or BxPC-3 cells were treated or not with CDDO-Me (0-5 µM) for 24 h and analyzed for Annexin V-FITC binding by flow cytometry. As shown in Fig. 2A, the percentage of Annexin V-FITC binding MiaPaCa-2 cells increased from <2% (untreated cells) to 17, 24, 37 and 62% following treatment with CDDO-Me at 0.625, 1.25, 2.5 and 5 µM, respectively. The percentage of Annexin V-FITC binding BxPC-3 cells was 1, 9, 16, 56 and 67% at 0, 0.625, 1.25, 2.5 and 5 µM CDDO-Me.
The induction of apoptosis by CDDO-Me was confirmed by the cleavage of native PARP-1. As shown in Fig. 2B, treatment with CDDO-Me caused the emergence of an 89 kDa cleaved PARP-1 fragment in both cell lines. A representative western blotting analysis of cleaved PARP-1 levels is shown in Fig. 2B. Together, increased binding of Annexin V-FITC and cleavage of PARP-1 demonstrated induction of apoptosis by CDDO-Me in PDAC cells.

CDDO-Me inhibits p-Akt, NF-κB and p-mTOR signaling proteins in PDAC cells. Akt, NF-κB and mTOR are pro-survival signaling proteins that are constitutively active in a variety of human cancers, providing survival advantage to cancer cells. We investigated whether PDAC cells express constitutively active Akt (p-Akt) and mTOR (p-mTOR) and the effect CDDO-Me has on the expression of these signaling proteins. Cell lysates were prepared from MiaPaCa-2 and BxPC-3 cells treated or not with CDDO-Me (0-5 µM) for 24 h and analyzed by western blotting for the levels of these proteins. Fig. 3 shows that both PDAC cell lines expressed p-Akt, NF-κB and p-mTOR and treatment with CDDO-Me significantly or completely inhibited their expression at concentrations of 1.25-5 µM. These data demonstrated that induction of apoptosis by CDDO-Me is attributable at least in part to the inhibition of pro-survival Akt, NF-κB and mTOR.

CDDO-Me inhibits the growth of BxPC-3 xenografts. Next we determined the tumor inhibitory activity of CDDO-Me in vivo against heterotypic BxPC-3 xenografts. One week after injection of 1x10⁶ BxPC-3 PDAC cells in the flanks of 7-week-old female Scid/Ncr mice, mice were treated with CDDO-Me (15 µmol/kg) or vehicle only by oral gavage, 5 days/week for 5 weeks (n=5) and experiment was terminated on day 40. To measure the effect of CDDO-Me on progression of tumor growth, tumor dimensions (length and width) were measured with a caliper at weekly intervals. Data are plotted as tumor volumes vs days past tumor cell implantation ± SD. As shown in Fig. 4A, treatment with CDDO-Me significantly inhibited the growth of BxPC-3 xenografts compared to vehicle control animals (tumor volume (mm³ ± SD): control, 53±10, 98±23, 226±21 and 284±39; CDDO-Me, 22±6, 37±10, 79±16 and 111±19 on days 14, 21, 28 and 35 after implantation of tumor cells). Furthermore, treatment with CDDO-Me also reduced p-Akt and p-mTOR levels in the tumor tissue (76 and 54% reduction, respectively) (Fig. 4B). These data demonstrated therapeutic efficacy of CDDO-Me against PDAC in vivo and provided evidence that activated Akt and mTOR are therapeutic targets of CDDO-Me in PDAC tumors.

Generation of orthotopic PDAC xenografts and efficacy of CDDO-Me for residual disease. To closely mimic physiological microenvironment of PDAC in patients for treatment with CDDO-Me we generated orthotopic xenografts by injecting MiaPaCa-2-Luc cells in the pancreatic tail in Scid/Ncr mice. The development and progression of orthotopic xenografts...
was monitored non-invasively by bioluminescence imaging using Kodak Multispectral Imaging System (Carestream Health, Woodbridge, CT, USA). Fig. 5 shows BLI images of a xenograft on days 15 and 35 (Fig. 5A and B) and a metastatic lesion in the liver (Fig. 5B, arrow). The growth curve of xenografts was created by plotting photon flux emission (photons/sec/mm²) vs time (days) after implantation of MiaPaCa-2-Luc cells (C). Size and histology of a tumor harvested on day 35 is also shown (D and E). (F) To determine efficacy of CDDO-Me for preventing relapse, all visible tumor growth was resected on day 28 after implantation of MiaPaCa-2-Luc cells and mice were treated with CDDO-Me at a dose of 15 µmol/kg orally, 5 days a week until day 100. Control mice were treated with vehicle alone without CDDO-Me. In a separate group, mice were also treated with gemcitabine i.p., twice a week at a dose of 2 mg/kg to compare the activity of CDDO-Me to that of a standard agent for PDAC. Kaplan-Meier plots (F) represent survival data of vehicle only, gemcitabine or CDDO-Me treated animals.

As shown in Fig. 5F, six of the nine mice in the vehicle control group died during this period (33% survival; mean survival time =81.6±22 days). Five of the nine mice treated with gemcitabine survived during the same period (56% survival; mean survival time =81±25 days, p>0.05). However, the group of mice treated with CDDO-Me showed the best survival rate, i.e., eight of the 12 mice treated with CDDO-Me revealed the presence of tumors in 2 of the 3 vehicle control, 2 of the 5 gemcitabine-treated and 1 of the 8 CDDO-Me-treated surviving mice on day 100.

Discussion

Pancreatic ductal adenocarcinoma is an intractable malignant disease with limited therapeutic options. Discovering effective novel agents with defined therapeutic targets might provide urgently needed therapeutics to treat this cancer. Natural products have long been recognized as a rich source of chemical diversity and compounds with a wide spectrum of anticancer activities. From vincristine to paclitaxel, the effective antitumor activity and novel mechanisms of action of many natural compounds and their synthetic derivatives have been described (22,23). CDDO and CDDO-Me are synthetic triterpenoids derived from oleanolic acid with
potent antiproliferative and pro-apoptotic activity. Earlier we demonstrated efficacy of CDDO and CDDO-Me in preventing/delaying the development and progression of prostate cancer in the transgenic TRAMP mouse model of prostate tumorigenesis (24,25). Others have shown efficacy of CDDOs for breast, lung and colon cancers (26,27). On the other hand, therapeutic efficacy of CDDOs for pancreatic cancer has not been adequately investigated. Studies described in this report were intended to determine therapeutic efficacy of CDDO-Me for pancreatic ductal adenocarcinoma. Since Kras is mutated in >90% of human PDAC and mutation of Kras at codon G12 leads to constitutive activation of Kras oncoprotein and more aggressive PDAC phenotype (28), we investigated the anticancer activity of CDDO-Me in vitro and in vivo using two PDAC cell lines with and without activating K-ras mutations (MiaPaCa-2 and BxPC-3, respectively). CDDO-Me strongly inhibited the proliferation of both cell lines in vitro independent of K-ras mutations. Since inhibition of cell proliferation induces apoptosis in cancer cells, we assessed induction of apoptosis by CDDO-Me in PDAC cells. Induction of apoptosis was measured by the binding of Annexin V-FITC to phosphotidylserine which is externalized to the outer leaflet of the plasma membrane early during induction of apoptosis. Treatment with CDDO-Me significantly increased the percentage of Annexin V-FITC binding cells in both cell lines. Induction of apoptosis was confirmed by the cleavage of PARP-1, a hallmark of cells undergoing apoptosis.

PI3K/Akt/mTOR and Akt/NF-κB are major antiapoptotic/prosurvival signaling pathways that are constitutively active in most cancers including pancreatic cancer. Akt promotes cell growth and survival by inactivating downstream substrates such as Bad, proaprase-9 and forkhead transcription factors (28,29). Antiapoptotic NF-κB and progrowth mTOR signaling pathways are downstream targets of activated PI3K/AKT. The NF-κB family of transcription factors controls the expression of genes involved in immune, inflammatory and oncogenic responses and play a critical role in resistance of cancer cells to anticancer therapies by protecting them from apoptosis (30). mTOR is a serine-threonine kinase, which controls cell growth, survival and ribogenesis (31). CDDO-Me inhibited the expression of p-AKT, NF-κB and p-mTOR in MiaPaCa-2 and BxPC-3 cells. The inhibition of these anti-apoptotic and survival-promoting signaling proteins suggested that they are involved in mediating the antiproliferative and proapoptotic activity of CDDO-Me in PDAC cells.

The results of in vitro studies showing strong antiproliferative and proapoptotic activity of CDDO-Me for PDAC cells prompted us to evaluate its therapeutic efficacy for PDAC in vivo, since many times antitumor effects observed in cell lines in vitro do not pan out in vivo. We first examined CDDO-Me for therapeutic efficacy against heterotopic xenografts generated with BxPC-3 cells with normal K-ras expression. Treatment with CDDO-Me significantly inhibited the growth of heterotopic xenografts (p<0.01). Western blot analysis of the tumor tissue extract showed that the inhibition of tumor growth was associated with the inhibition of anti-apoptotic/prosurvival p-Akt, NF-κB and p-mTOR signaling proteins. These data also demonstrated that in vitro effects of CDDO-Me in PDAC cells does translate into therapeutic efficacy in vivo.

To further analyze therapeutic efficacy of CDDO-Me for PDAC, we tested it for preventing relapse/recurrence when administered after surgical resection of tumors. This was accomplished by creating a model of PDAC residual disease wherein orthotopic PDAC xenografts generated by implanting MiaPaCa-2-Luc cells in the pancreas were surgically removed after four weeks of tumor growth. Treatment with CDDO-Me in this adjuvant setting significantly prolonged survival of mice compared to untreated animals. In fact, the survival advantage with CDDO-Me was even better than that with gemcitabine, a standard drug for the treatment of patients with advanced PDAC. Thus, therapeutic outcomes in xenografts models generated with PDAC cells expressing either normal K-ras (BxPC-3) or activated K-ras (MiaPaCa-2) correlated with responses to CDDO-Me in vitro. Although the exact mechanism of the antitumor activity of CDDO-Me in vivo remain to be investigated, our data suggest that downregulation of p-Akt, NF-κB and p-mTOR signaling proteins plays a role in inhibition of cell proliferation and induction of cellular apoptosis in tumor cells by CDDO-Me. Since treatment with gemcitabine or CDDO-Me alone improved survival when administered after removal of tumors suggests that combinatorial regimen of gemcitabine and CDDO-Me might be even more beneficial in preventing/delaying recurrence. This inference of course needs more rigorous analysis in these preclinical models of PDAC.

In conclusion, our investigations show that CDDO-Me exhibits therapeutic efficacy in xenograft models of PDAC (heterotopic and orthotopic) for inhibiting primary tumor growth and delaying/preventing relapse by destroying residual disease. Because of its ability to inhibit p-AKT, NF-κB and p-mTOR in tumor tissue, we speculate that CDDO-Me inhibits tumor growth by inhibiting cell proliferation and promoting apoptosis through the inhibition of anti-apoptotic/prosurvival AKT/NF-κB/mTOR signaling pathways. Collectively, these findings unveiling antitumor potential of CDDO-Me for PDAC warrant further studies for development of CDDO-Me as an adjuvant to existing PDAC therapies to obtain more durable responses and improved outcomes.

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