

The Synthetic Triterpenoid CDDO-Methyl Ester
Delays Estrogen Receptor-Negative Mammary Carcinogenesis
in Polyoma Middle T Mice

Kim Tran¹, Renee Risingsong¹, Darlene Royce¹, Charlotte R. Williams¹, Michael B.
Sporn¹, and Karen Liby^{2*}

Authors Affiliations: ¹Department of Pharmacology and ²Medicine, Dartmouth Medical School, Hanover, New Hampshire.

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Correspondence should be addressed to *Karen Liby, Department of Medicine, Dartmouth Medical School, Remsen 524, HB7650, Hanover, NH 03755. Phone: 603-650-1682; FAX: 603-650-1129; Email: Karen.T.Liby@Dartmouth.edu.

Abstract

Novel drugs are needed for the prevention and treatment of breast cancer. Synthetic triterpenoids are a promising new class of compounds with activity in a variety of preclinical cancer models. We tested activity of the methyl ester derivative of the synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Me), in a relevant model of ER-negative breast cancer, the polyoma-middle T (PyMT), in which the oncoprotein drives carcinogenesis. The developing tumors recapitulate key features of the human disease. Mice were fed CDDO-Me (50 mg/kg diet), starting at 4 weeks of age. CDDO-Me significantly increased the age of mice at onset of first tumor ($P < 0.001$) by an average of 4.3 weeks and overall survival ($P < 0.001$) by 5.2 weeks. The drug also inhibited the infiltration of tumor associated macrophages (TAMs) into mammary glands of PyMT mice at 12 weeks of age and reduced levels of the chemokines CXCL12 and CCL2 in primary PyMT mammary tumor cells. Treatment with this multifunctional drug also inhibited secretion of MMP-9 in primary tumor cells from PyMT mice and decreased proliferation of these cells by inhibiting cyclin D1 and decreasing phosphorylation of EGFR and STAT3.

Introduction

Breast cancer is the most widely diagnosed cancer and the second leading cause of cancer related deaths in females in the United States (1). The incidence of estrogen receptor positive (ER+) breast cancer has gradually declined largely due to the cessation of hormone replacement therapy; however, the incidence of ER negative (ER-) breast cancer has not changed in over 30 years (2, 3). To reduce the mortality rates associated with ER- breast cancer, novel drugs and drugs combinations are needed for the prevention and treatment of the disease.

Numerous studies have demonstrated the importance of the tumor microenvironment in carcinogenesis and metastasis (4, 5), underscoring it as a target for not only cancer therapy, but also for chemoprevention (6, 7). Tumor-associated macrophages (TAMs) can represent over 50% of the tumor mass in breast cancer patients, and their infiltration correlates with poor prognosis (8, 9, 10). TAMs activate angiogenesis, providing nutrients, oxygen, and growth factors for the growing tumor (11-13) and also enhance tumor cell migration, invasion, and intravasation, increasing the metastatic capacity of the malignant tumor (14, 15). Genetic depletion of macrophages results in a significant delay of tumor progression and inhibition of lung metastasis in the Polyoma virus middle T oncoprotein (PyMT) mouse model of ER- breast cancer, where TAM infiltration is a hallmark feature (16). Xenograft studies also demonstrate that decreased TAM infiltration correlates with marked reduction in tumor growth and angiogenesis (17, 18), suggesting that TAMs might be useful therapeutic targets (19-21).

The synthetic oleanane triterpenoids, including 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and CDDO-methyl ester (CDDO-Me), are a promising class of agents for the prevention and treatment of breast cancer. These compounds inhibit proliferation of ER- breast cancer cells *in vitro* and *in vivo* (22-24). CDDO-Me significantly delayed the development of mammary tumors and arrested growth of established tumors in the MMTV-neu transgenic model of ER- breast cancer (24). CDDO-Me inhibits components of commonly deregulated signaling pathways in ER- breast cancer cells such as the NF- κ B and STAT3 pathways (22-24). Additionally, CDDO and CDDO-Me suppress factors associated with the tumor microenvironment, including proinflammatory cytokines in primary peritoneal macrophages and in the RAW 264.7 mouse macrophage-like cell line and inhibited angiogenesis *in vitro* and *in vivo* (25-28). However, the effects of triterpenoids on TAMs have not been elucidated, and our hypothesis was that these drugs could prevent cancer by targeting TAMs. In these studies, we report that the synthetic triterpenoid CDDO-Me delays mammary tumorigenesis in the aggressive PyMT model of ER- breast cancer, and it does have a modest effect on TAM infiltration in the mammary glands and tumors of these mice. It also inhibits the levels of the chemokines CXCL12 and CCL2 in primary PyMT tumor cells and targets the key breast cancer biomarkers cyclin D1, EGFR and STAT3 in these cells.

Materials and Methods

Drugs. CDDO-Me was synthesized as described (29). For cell culture studies, CDDO-Me was dissolved in DMSO, and controls containing equal concentrations of DMSO (<0.1%) were included in all experiments.

***In vivo* experiments.** All animal studies were done in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Dartmouth Medical School. Mice carrying the PyMT gene under the control of the MMTV promoter were obtained from Dr. Jeffrey Pollard (Albert Einstein College of Medicine, Bronx, NY) and were genotyped as has been previously described (30, 31). Four-week-old female PyMT mice were fed powdered 5002 rodent chow (PMI Feeds) or this powdered diet containing CDDO-Me (50 mg/kg diet). Mice were palpated twice a week for detection of new tumors. Tumors were measured weekly with calipers. In accordance to IACUC regulations, death was not used as an end point, but instead mice were sacrificed when symptoms were observed such as tumor mass exceeding 10% of total body mass, obstruction of movement by the tumor or labored breathing. To determine the effects of CDDO-Me on mammapoiesis, both PyMT^{+/-} and PyMT^{-/-} mice (n = 5 per group) were fed either control diet or CDDO-Me in diet from 4 weeks of age until 8-12 weeks of age. After 4-8 weeks on diet, mammary glands were harvested and whole mounts were stained with hematoxylin. To determine drug levels in tissue, five female PyMT mice were fed CDDO-Me in diet (50 mg/kg diet) for 1 week. Mammary glands were harvested and homogenized in PBS and whole blood was collected in heparinized tubes. Samples were extracted in acetonitrile, separated by reverse phase liquid chromatography, and detected by mass spectrometry. Standard curves were generated by serially diluting known concentrations of CDDO-Me in control blood or tissue homogenates. All samples were within the linear range of the standard curve.

Macrophage isolation and analysis. Macrophages were isolated from tumors and mammary glands using a dual purification strategy including magnetic purification followed by flow sorting. Single cell suspensions were generated from tumors and mammary glands. Briefly, all tumors and mammary glands were removed and digested in DMEM with 10% fetal bovine serum (FBS) and an enzyme mixture consisting of collagenase (300 U/mL; Sigma), dispase (1.0 U/mL; Worthington), and DNase (2 U/mL; Calbiochem) for 30 min at 37°C. Cells were passed through 40 µm cell strainers (BD Bioscience) and incubated for 15 min with CD11b magnetic beads (Miltenyi Biotec), followed by successive 5 min incubations with an antibody against F4/80 (eBioscience) and a phycoerythrin conjugated goat anti rat IgG (BioLegend). 10 uL of magnetic beads and antibodies per 10⁷ cells were used with PBS washes between incubations. Total monocytes were isolated using magnetic bead selection for CD11b+ according to the manufacturer's specifications (Miltenyi Biotec). Both magnetically selected cells and the negative flow through cell fraction were then either analyzed for the percentage of F4/80 positive cells out of total mammary gland and tumor cells using a FACScan (Becton Dickinson) or flow sorted for F4/80 selection using a FACSAria (Becton Dickinson). Total cells were dual purified in order to increase accuracy of F4/80 detection and final F4/80 percentage reflects percentage of total cells. Cell lysates from magnetic and flow sorted CD11b+F4/80+ macrophages were analyzed using either the Proteome Profiler Mouse Angiogenesis Kit or the Proteome Profiler Mouse Cytokine Panel A Array Kit (R&D Systems). Quantitation of protein expression was performed using ImageJ.

Immunohistochemistry. Mammary glands and tumors removed from mice fed either control or CDDO-Me diet were sectioned, immunostained with an antibody against F4/80 (eBioscience), and processed with Vectastain ABC Kit (Vector) and Peroxidase substrate kit (Vector). Scoring was done by the first author, who was blinded as to the primary antibodies and the treatment groups.

Cell culture and *in vitro* assays. Primary PyMT cells were derived from mammary tumors of female PyMT (+/-) mice. Resected PyMT mammary tumors were minced and digested in DMEM with 10% FBS and an enzyme mixture consisting of collagenase (300 U/mL; Sigma), dispase (1.0 U/mL; Worthington), and DNase (2 U/mL; Calbiochem) for 30 min at 37°C with gentle agitation with a stir bar. The cell suspension was filtered through a 40- μ m cell strainer (BD Bioscience), centrifuged at 220 \times g for 10 min and plated in DMEM + 10% FBS. All experiments were performed within 1 week of cell isolation. To determine targets of CDDO-Me, primary PyMT mammary tumor cells were treated with either DMSO or 300 nM of CDDO-Me for 16 hours. Cell lysates were analyzed with either the Proteome Profiler Mouse Angiogenesis Kit or the Proteome Profiler Mouse Cytokine Panel A Array Kit (R&D Systems). Quantitation of protein expression was performed using ImageJ. Cells were treated with varying concentrations of CDDO-Me for varying timepoints, and the amount of matrix metalloproteinase-9 (MMP-9) or chemokine (C-C motif) ligand 2 (CCL2) released into the medium was measured using a Quantikine ELISA kit (R&D Systems). To determine additional targets of CDDO-Me, 3 μ mol/L of biotinylated CDDO-Me (32) was added for 1 hour. Cell lysates were incubated with 50 μ L DynaBeads MyOne Streptavidin (Invitrogen) as described (24, 33-35) and then analyzed by Western blotting using antibodies against epidermal

growth factor receptor (EGFR; Cell Signaling Technology), signal transducer and activator of transcription 3 (STAT3; Cell Signaling Technology), and cyclin D1 (Santa Cruz Biotechnologies). Results were validated by Western blot analysis of samples harvested from cells treated with varying concentrations of CDDO-Me at varying time points for pEGFR (Cell Signaling Technology), pSTAT3 (Cell Signaling Technology), and cyclin D1. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were seeded into 96-well plates at 2×10^4 cells/well. The next day, cells were incubated with different concentrations of CDDO-Me for 48 hrs. Cells were then incubated with MTT for 4 hrs (Sigma) and read at OD570. For the Real-Time RT-PCR assays, total RNA was isolated from primary PyMT tumor cells at the indicated time-points using the RNEasy kit (Qiagen). Reverse transcription (RT) was performed using the SuperScript III Reverse Transcriptase Kit (Invitrogen). Quantitative real-time PCR assays were performed using SYBR Green PCR Mastermix (Applied Biosystems) for detection of mRNAs using DNA Engine Opticon (MJ Research). Results were normalized to GAPDH mRNA levels. The following primers were used: murine CCL2 forward: 5'-TAAAAACCTGGATCGGAACCAAA-3', and reverse: 5'-GCATTAGCTTCAGATTTACGGGT-3'; murine CXCL12 forward 5'-TGCATCAGTGACGGTAAACCA-3', and reverse: 5'-TTCTTCAGCCGTGCAACAATC-3'; and murine GAPDH forward: 5'-AGGTCGG TGTGAACGGATTTG-3', and reverse: 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Statistical analysis. Results are described as mean \pm SEM and were analyzed by one-way ANOVA and Tukey test or by one-way ANOVA on ranks (Wilcoxon signed rank test; SigmaStat 3.5). All P values are two-sided.

Results

TAM infiltration in mammary glands and tumors of PyMT mice. In the PyMT mouse model of ER-negative breast cancer, the expression of the oncogenic PyMT protein is targeted to the mammary epithelium by the MMTV promoter (31). This mouse model mimics key features of the clinical disease, including infiltration of TAMs as shown in Fig. 1A via immunohistochemical detection of F4/80 positive macrophages in mammary tumors (additional pictures of sections stained with either hematoxylin and eosin or F4/80 are provided in Suppl. Fig. 1 and higher magnification pictures are provided in Suppl Fig. 2). Notably, quantitative analysis of mammary glands and tumors of PyMT mice using flow cytometry demonstrated that the percentage of infiltrating TAMs in mammary tumors increased with the age of the mice until week 12, where macrophage infiltration peaked at an average of 22%. Although this trend was observed in both PyMT^{+/-} and PyMT^{-/-} mice, the macrophage infiltration was significantly greater in the mammary glands of PyMT^{+/-} mice as compared to mammary glands of PyMT^{-/-} mice (Fig. 1B). Tumors and mammary glands were analyzed together as many PyMT mice do not exhibit palpable tumors until past 12 weeks of age; however, the mammary glands of these mice possess microscopic lesions. To investigate pathways engaged in tumor formation in the PyMT mouse model, primary tumor cells and tumor-infiltrating macrophages from mammary glands were collected. At 16 weeks of age, all PyMT mice had multiple visible tumor nodules in the mammary glands, whereas, we rarely observed grossly visible tumors in 12 week old mice. Expression levels of diverse cytokines (Fig. 1C) and pro-angiogenic factors (Fig. 1D) were investigated in these cells. Many of the investigated chemokines, such as CCL2 and CXCL12, and pro-

angiogenic factors, such as MMP-9, were found to be differentially expressed among primary tumor cells and infiltrating macrophages.

CDDO-Me delays development of ER-negative mammary tumors in PyMT mice. To investigate whether CDDO-Me affected tumor development, we fed female PyMT mice control diet or diet containing CDDO-Me (50 mg/kg diet) beginning at 4 weeks of age. Mammary tumors were first detected at 12 weeks of age in the control group, with 50% tumor incidence by 15 weeks. All mice fed control diet developed mammary tumors by week 22. In contrast, CDDO-Me significantly ($P < 0.001$ versus control) delayed development of these tumors with 50% tumor incidence by week 19, and 100% incidence was not reached until week 29 (Fig. 2A). This delay in tumor development by CDDO-Me is strikingly significant as PyMT mice fed control diet live on average only 21.4 weeks. No effect on tumor number or tumor size was observed. Effects of treatment with CDDO-Me on survival were also investigated. In accordance to IACUC regulations, mice were sacrificed when symptoms such as tumor mass exceeding 10% of total body mass, obstruction of movement, or labored breathing were observed. In the control group, 50% of mice were sacrificed by week 20 and 100% by week 28. In contrast, 50% of mice fed CDDO-Me diet were sacrificed by week 25, whereas 100% of these mice were sacrificed by week 42 (Fig. 2B). Thus, CDDO-Me prolonged survival ($P < 0.001$) in this mouse model of ER-negative breast cancer. CDDO-Me was well tolerated at the dose used, and the mice continued to gain weight throughout the experiment, with no statistical difference in weight between CDDO-Me fed mice and control fed mice (week 13: average weight of control group 21.4 g vs CDDO-Me group 20.7 g, $p = 0.34$).

To determine drug levels of CDDO-Me, PyMT mice were fed CDDO-Me in diet for one week. An average of $1.1 \pm 0.2 \mu\text{M}$ CDDO-Me was detected in the mammary gland but only $20 \pm 5 \text{ nM}$ was detected in whole blood. Because diets were started at 4 weeks of age and thus before full maturation of the mammary gland, we also examined the effects of CDDO-Me on mammary gland development. Both PyMT^{+/-} and PyMT^{-/-} mice were fed control diet or CDDO-Me (50 mg/kg diet) for 4-8 weeks, and CDDO-Me had no effect on mammapoiesis in mice of either genotype (data not shown). Moreover, no differences in mammary gland maturation were ever observed in sections of mammary glands used for IHC, in mice treated with CDDO-Me for 8-12 weeks (data not shown).

CDDO-Me inhibits infiltration of TAMs in ER-negative mammary tumors.

PyMT tumors are characterized by infiltration of TAMs, as shown in Fig. 1B. Inhibition of TAM infiltration by genetic approaches was previously shown to delay mammary tumorigenesis (16). Thus, we investigated whether the delay of mammary tumor development by CDDO-Me was characterized by reduced TAM infiltration. The percentage of F4/80 positive cells in mammary tumors of PyMT^{+/-} mice was assayed at 8, 12, 16, and 20 weeks of age, as detailed in the methods section. The percentage of F4/80 positive cells was significantly ($P < 0.05$) but lower in mammary glands of 12 week old mice fed CDDO-Me diet as compared to litter matched mice fed control diet (Fig. 3A). This modest decrease in TAM infiltration was observed in 8 out of 9 mice fed diet containing CDDO-Me as compared to litter matched mice fed control diet. CDDO-Me diet had no significant effects on TAM infiltration in mammary tumors of 16 and 20 week old mice. Immunohistochemical analysis confirmed the decreased infiltration of

F4/80 positive cells in mammary tumors of mice fed CDDO-Me enriched diet as compared to control diet at 12 weeks of age. Representative images are shown in Fig. 3B. When sections were analyzed in a blinded fashion, the percentages of cells positive for F4/80 were remarkably similar to the values obtained by flow cytometry, with a significant decrease in the mice fed CDDO-Me only at 12 weeks of age (Fig. 3C).

CDDO-Me treatment inhibits secretion of chemokines by PyMT tumor cells.

To investigate a possible mechanism engaged in the modestly reduced infiltration of TAMs in mammary tumors of PyMT mice fed diet containing CDDO-Me, levels of chemokines in isolated primary mammary tumors cells were evaluated. CDDO-Me treatment for 8, 16, or 24 hours resulted in a significant ($P < 0.001$) dose-dependent decrease in the levels of CCL2 and CXCL12 mRNA detected via RT-PCR as compared to vehicle control (Fig. 4A). CDDO-Me also inhibited the secretion of CCL2 in a dose-dependent manner in primary tumor cells (Fig. 4B). Thus, CDDO-Me inhibits the production of chemokines capable of attracting TAMs and other inflammatory cells into mammary tumors.

CDDO-Me inhibits numerous oncogenic pathways in ER-negative mammary tumors. To determine whether CDDO-Me treatment inhibited additional oncogenic mechanisms, levels of the proangiogenic factor MMP-9 were investigated in primary PyMT tumor cells. As shown in Fig. 5A, CDDO-Me treatment resulted in a significant dose-dependent inhibition of MMP-9 secretion by primary mammary tumor cells as compared to vehicle. Notably, CDDO-Me also inhibited proliferation of the primary tumor cells (Fig. 5B) but did not induce apoptosis in these cells as assayed via annexin staining (data not shown). To elucidate potential mechanisms for inhibition of

proliferation by CDDO-Me, primary PyMT tumor cells were treated with biotinylated CDDO-Me, and lysates were precipitated with immobilized NeutrAvidin followed by western blotting of common components of oncogenic pathways. The biotinylated CDDO-Me directly interacted with EGFR and STAT3 but not with cyclin D1 (Fig. 5C) and inhibited these diverse pathways. Levels of cyclin D1 and phosphorylated EGFR and STAT3 were reduced following CDDO-Me treatment in primary PyMT tumor cells as compared to vehicle (Fig. 5D). Effects of CDDO-Me treatment on cyclin D1 were then investigated *in vivo*. ER-negative mammary tumors of PyMT mice fed diet containing CDDO-Me showed significant ($P < 0.05$) reduction of cyclin D1, as detected by immunohistochemical analysis, as compared to tumors of litter matched mice fed control diet at 8, 12, 16, and 20 weeks of age. Representative mammary tumors and quantitation of the cyclin D1 IHC are shown in Fig. 5E.

Discussion

We show in our present study that CDDO-Me is the first known drug to significantly delay tumorigenesis in PyMT mice and that it modestly inhibits the infiltration of TAMs to the tumor site in this extremely aggressive model of ER- breast cancer. These findings parallel results from previous studies where genetic depletion of TAMs in PyMT mice delay the progression of primary tumors but has no effect on the formation or growth of these tumors (16) and indicate that a pharmacologic agent can achieve similar effects to that of genetic approaches. Macrophage infiltration to the tumor site in response to inflammatory cytokines activates them to produce chemokines and a multitude of angiogenesis-promoting factors, such as vascular endothelial growth

factor (VEGF), matrix metalloproteinases, and cyclooxygenase 2 (COX2) (21, 36). Oral administration of the COX2 inhibitor celecoxib to PyMT mice with established tumors reduces mammary tumor burden (37) and reduces VEGF levels *in vivo*; however, no such antiinflammatory agents have been reported to significantly delay tumorigenesis in the PyMT model.

CDDO-Me inhibits the infiltration of TAMs to the mammary tumors of PyMT mice only at 12 weeks of age, the peak time for macrophage infiltration into the mammary gland. Although we do not understand why this change did not occur at earlier time points, the infiltration of macrophages may be involved in the transition from adenoma to carcinoma (30). By 16-20 weeks of age, large mammary tumors are present and macrophages may no longer be necessary for tumor growth. It is also possible that CDDO-Me has an effect on macrophage activation or phenotype, and additional studies will address this important issue.

Our studies also suggest that CDDO-Me has multiple effects on cells other than macrophages. For example, CDDO-Me suppresses levels of the chemokines CXCL12 and CCL2 and the secretion of MMP-9 from primary PyMT tumor cells. Both CXCL12 and CCL2 have been previously implicated as key players not only in inducing the infiltration of TAMs into primary tumors but also in promoting the seeding and growth of breast metastases into distant sites including the lung (36, 38-41). MMP-9 plays numerous roles in carcinogenesis including tumor initiation, vascularization, invasion and metastasis (42). Previous studies have demonstrated the ability of CDDO-Me to prevent lung carcinogenesis in experimental models (43, 44), and the synthetic triterpenoid CDDO-Imidazolide inhibits metastasis in experimental liver metastasis

models (45). Ongoing studies will determine whether CDDO-Me inhibits lung metastasis in PyMT mice and whether the inhibition is due suppression of chemokines CXCL12 and CCL2 or by the metalloproteinase MMP-9.

Breast cancer is a complicated disease that involves not only tumor cells but a variety of stromal cells in the surrounding tumor microenvironment. Interactions between stromal cells and tumor cells facilitate tumorigenesis leading to malignancy and eventually metastasis to distant organs (46). Hence, novel multifunctional drugs that target diverse signaling pathways in both tumor and stromal cells are needed for the prevention and treatment of breast cancer. Recent proteomic analysis has identified numerous putative CDDO-Me target proteins from diverse but interconnected signaling networks (47). We show that CDDO-Me is a multifunctional drug that inhibits various key oncogenic pathways in primary PyMT tumor cells. CDDO-Me inhibits the phosphorylation of EGFR and STAT3, cyclin D1 protein levels, and proliferation. It is well established that EGFR signaling induces proliferation and survival in breast cancer cells thus making EGFR a popular target for breast cancer treatment (48). Additionally, increases in STAT3 phosphorylation and levels of cyclin D1 are commonly found in tumor cells of breast cancer patients and thus are breast cancer biomarkers and popular therapeutic targets for the treatment of the disease (49, 50). By targeting these pathways that are commonly altered in breast cancer, and possibly TAMS as well, CDDO-Me is able to significant delay tumor development in the aggressive PyMT model.

It is also possible that CDDO-Me is more potent for inhibiting proliferation or inducing apoptosis of premalignant cells than malignant cells, as has been shown for

other chemopreventive agents such as LG100268 (51). As previously mentioned, CDDO-Me did not induce apoptosis via annexin staining in primary PyMT tumor cells, even though it does induce apoptosis by a variety of mechanisms in cancer cells *in vitro* (25). We did not explore a wide variety of doses, especially higher doses, for effects of apoptosis in our current study or compare the effects of CDDO-Me on normal, premalignant, and malignant cells. We are beginning these studies and investigating whether CDDO-Me has an effect on established tumors in the PyMT model as CDDO-Me has been shown to induce apoptosis in a variety of human ER-negative breast cancer cells (25) and arrest tumor growth in the MMTV-neu transgenic model of ER-breast cancer (24). The effects of CDDO-Me on multiple pathways and cells undoubtedly contribute to its efficacy in the PyMT model for the prevention of experimental breast cancer, and defining all of the mechanisms of action for this drug remains an area of active investigation.

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Figure 1. Characterization of macrophage infiltration in the PyMT model of ER-negative breast cancer. *A*, Detection of TAM infiltration in mammary glands or tumors via staining of F4/80, a surface marker of macrophages, in representative samples. Age of mice as shown. *B*, Quantitation of macrophage infiltration as detected by flow cytometry analysis of F4/80 in homogenized mammary glands and tumors in PyMT^{+/-} mice and in age-matched littermate PyMT^{-/-} mice. #, *P* < 0.01 versus control; *, *P* < 0.05 and **, *P* < 0.01 versus previous age time point. Quantitation of cytokines (*C*) and pro-angiogenic factors (*D*) in macrophages isolated from mammary glands and tumors of 16 week old PyMT mice and primary PyMT tumor cells detected by a Proteome Profiler Mouse Cytokine Panel A Array Kit (*C*) and a Proteome Profiler Mouse Angiogenesis Kit (*D*). Quantitation of protein expression determined via pixel density was performed using ImageJ.

Figure 2. CDDO-Me delays the development of ER-negative mammary tumors and enhances survival of PyMT mice. Beginning at 4 wks of age, female transgenic mice were fed powdered control diet or CDDO-Me in diet (50 mg/kg). Mice were palpated twice a week. The effects of CDDO-Me on tumor development (*A*) and survival (*B*) of PyMT mice as compared to control diet is shown. *n* = 15 mice per arm. **, *P* < 0.001 versus control.

Figure 3. CDDO-Me inhibits the infiltration of macrophages to the mammary glands and tumors of PyMT mice. Beginning at 4 wks of age, female transgenic mice were fed powdered control diet or CDDO-Me diet (50 mg/kg) and sacrificed at age 8, 12, 16, or 20 wk. Quantitation of macrophage infiltration was detected by flow cytometry analysis of F4/80 in homogenized mammary glands and tumors (*A*) and by F4/80 staining in mammary glands of PyMT mice (*B*). *, *P* < 0.05 versus age-matched littermate controls,

n ≥ 6 mice. The percentage of cells positive for F4/80 as detected by IHC was scored in a blinded manner (C); *, P < 0.05 versus control.

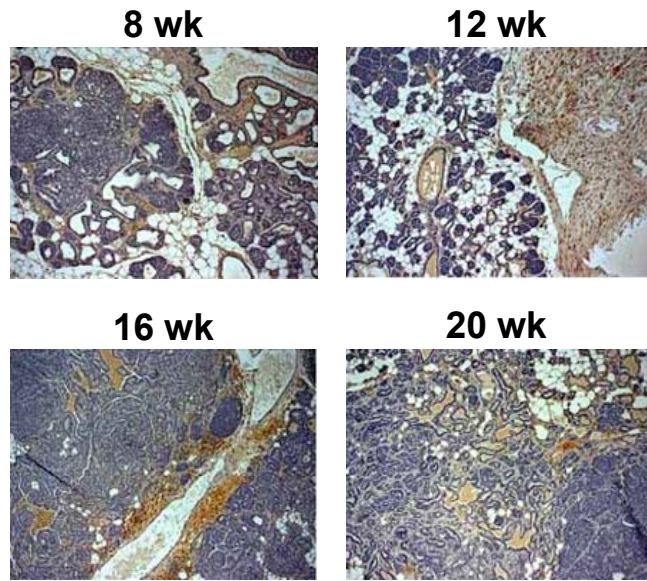
Figure 4. CDDO-Me decreases CCL2 and CXCL12 in primary PyMT tumor cells. *A*, Primary PyMT tumor cells were treated with CDDO-Me for varying time points (8, 16, 24 h) and mRNA levels of CCL2 (top panel) and CXCL12 (bottom panel) were detected by RT-PCR. *, P < 0.05 and **, P < 0.01 versus control treatment. *B*, Primary PyMT tumor cells were treated with CDDO-Me for varying time points (8, 16, 24 h) and supernatants were assayed by ELISA for CCL2 secretion. *, P < 0.05 and **, P < 0.01 versus control treatment.

Figure 5. CDDO-Me inhibits oncogenic pathways in primary PyMT tumor cells. *A*, Primary PyMT tumor cells were treated with CDDO-Me for varying time points (8, 16, 24 h) and supernatants were assayed by ELISA for MMP-9 secretion. *, P < 0.05 and **, P < 0.01 versus control. *B*, Primary PyMT tumor cells were treated with increasing concentrations of CDDO-Me for 48 h, and effects on proliferation were assayed via MTT analysis. *, P < 0.05 versus control; **, P < 0.01 versus control. *C*, Primary PyMT tumor cells were treated with 3 μmol/L of biotinylated CDDO-Me (compound 6 in ref 32) for 1 h, streptavidin DynaBeads were used to pulldown triterpenoid-protein complexes from cell lysates, and EGFR, STAT3, and cyclin D1 were detected by Western blot. *D*, Primary PyMT tumor cells were treated with CDDO-Me (0-300 nM), and lysates were immunoblotted with antibodies against pEGFR, pSTAT3, or cyclin D1. *E*, Beginning at 4 wks of age, female transgenic mice were fed powdered control diet or CDDO-Me diet (50 mg/kg) and sacrificed at age 8, 12, 16, or 20 wk. Tumor sections were analyzed for

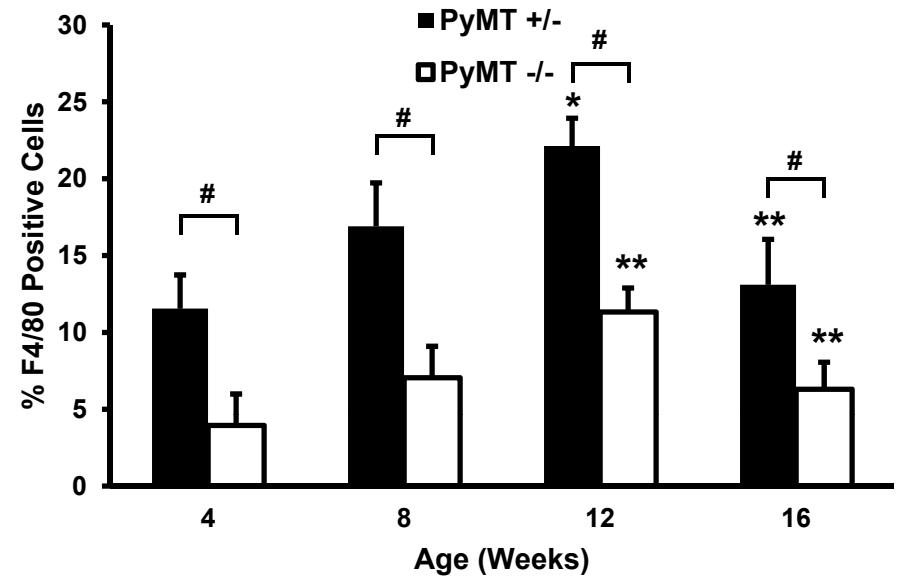
cyclin D1 levels by immunohistochemistry. Representative images are shown. The percentage of cells positive for cyclin D1 was scored in a blinded manner: *, $P < 0.05$ versus control; ** $P < 0.001$ versus control.

Fig. 1

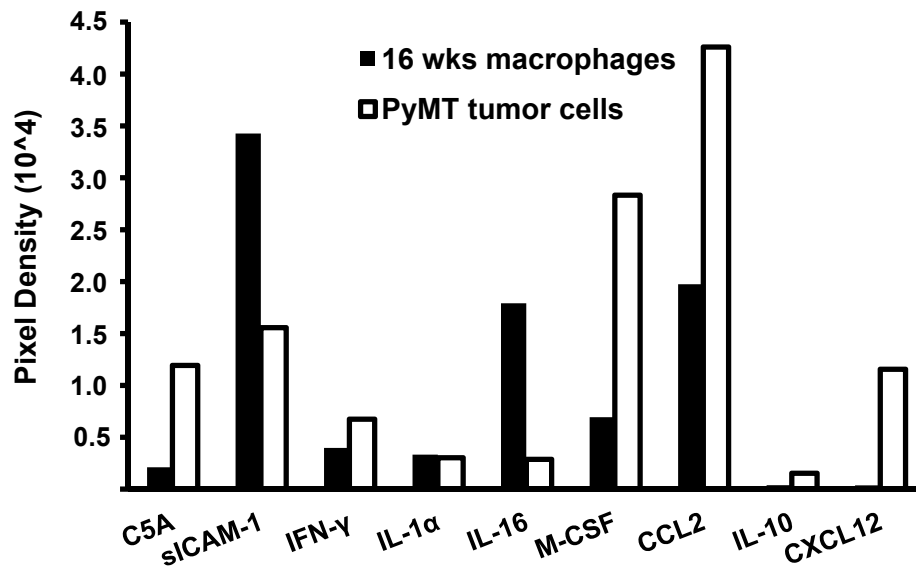
A



B



C



D

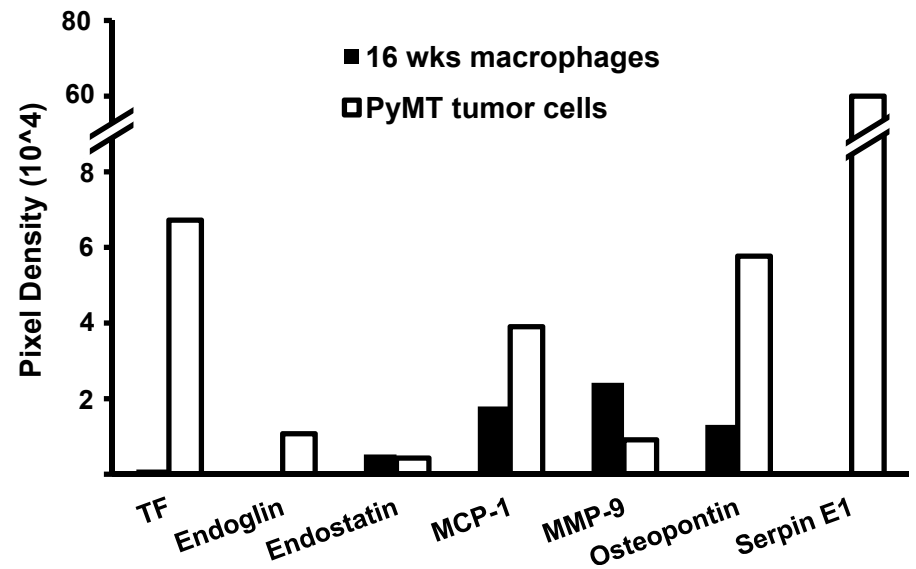
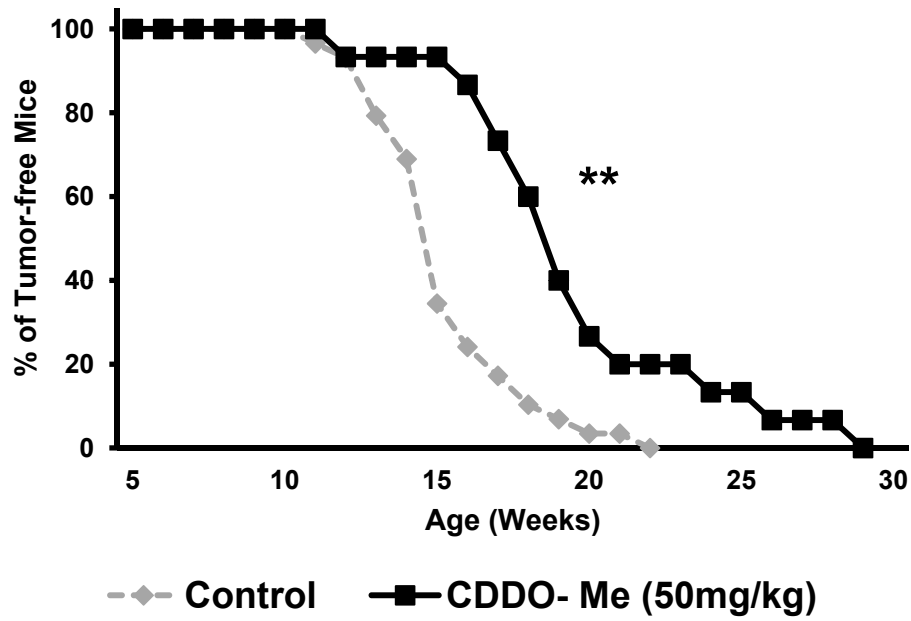


Fig. 2

A



B

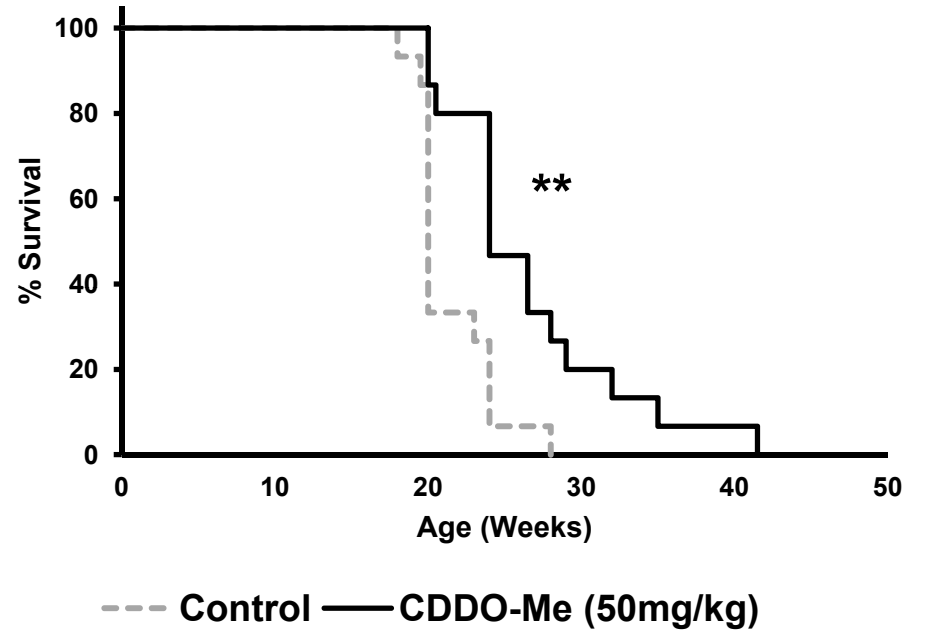
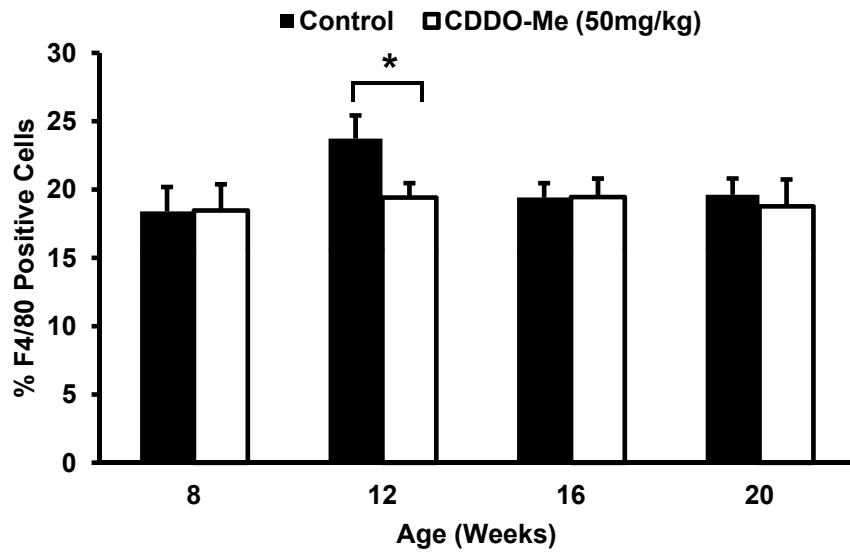


Fig. 3

A



B

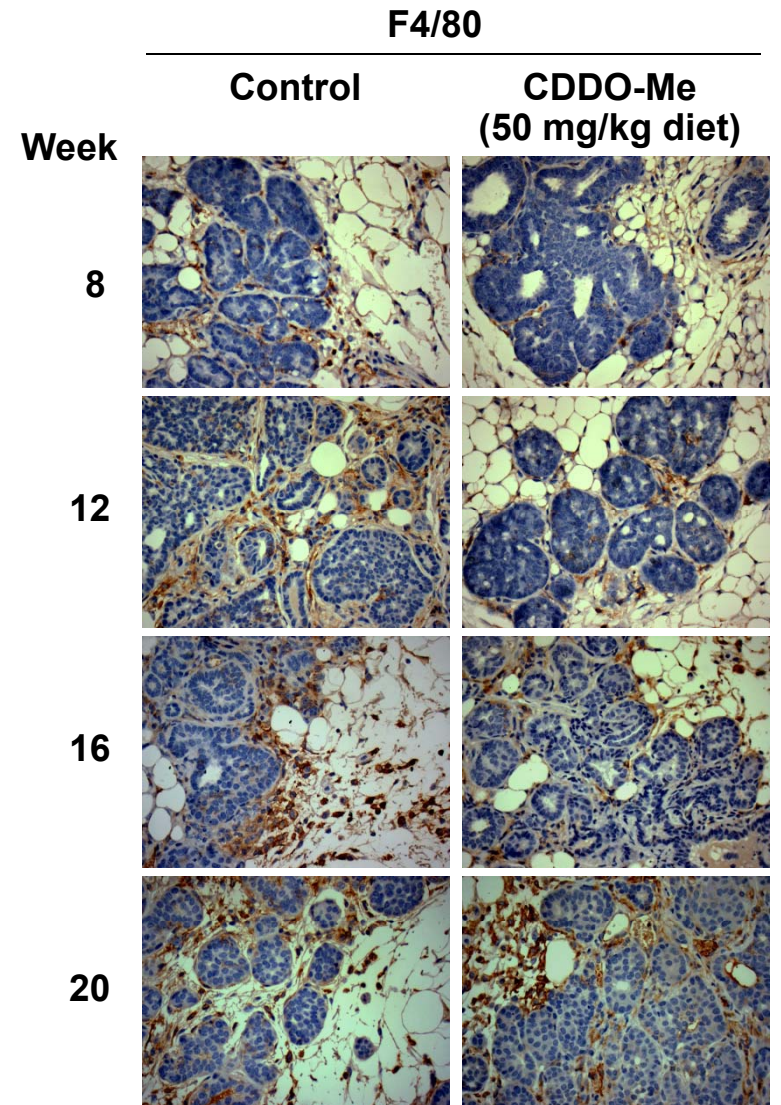


Fig. 3

C

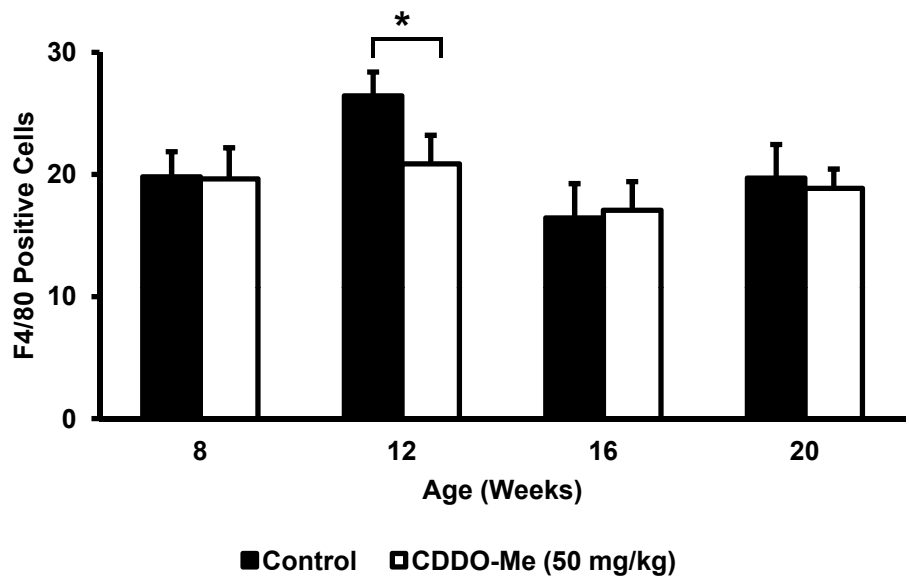
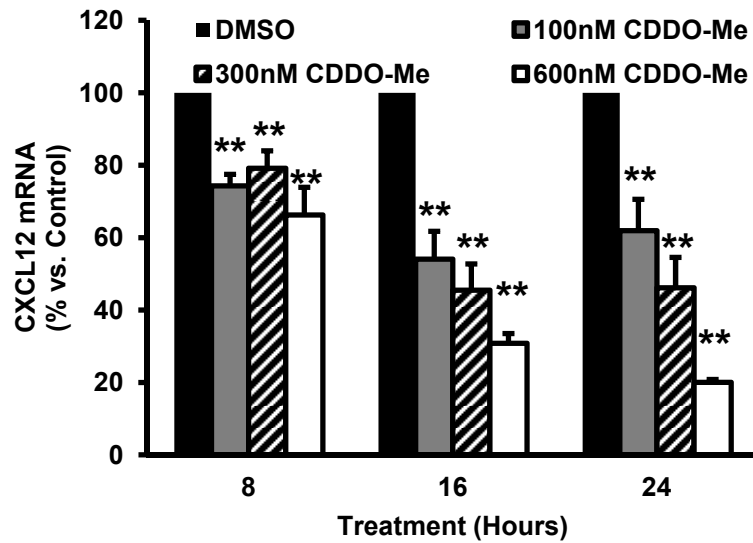
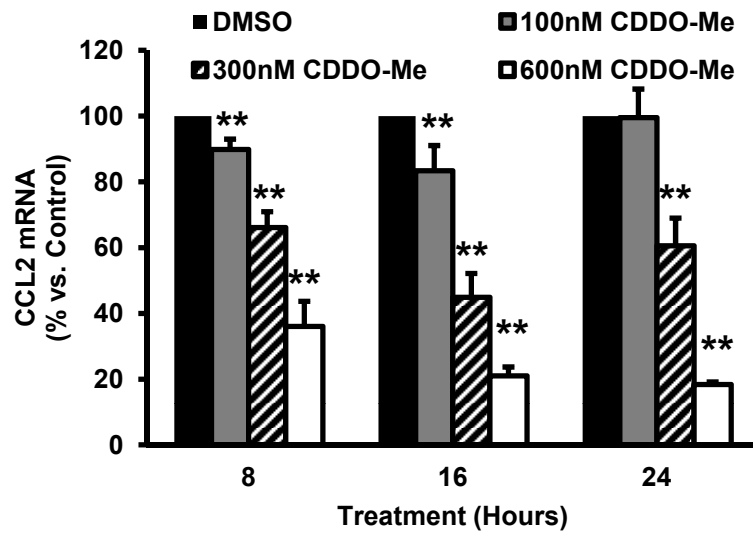


Fig. 4

A



B

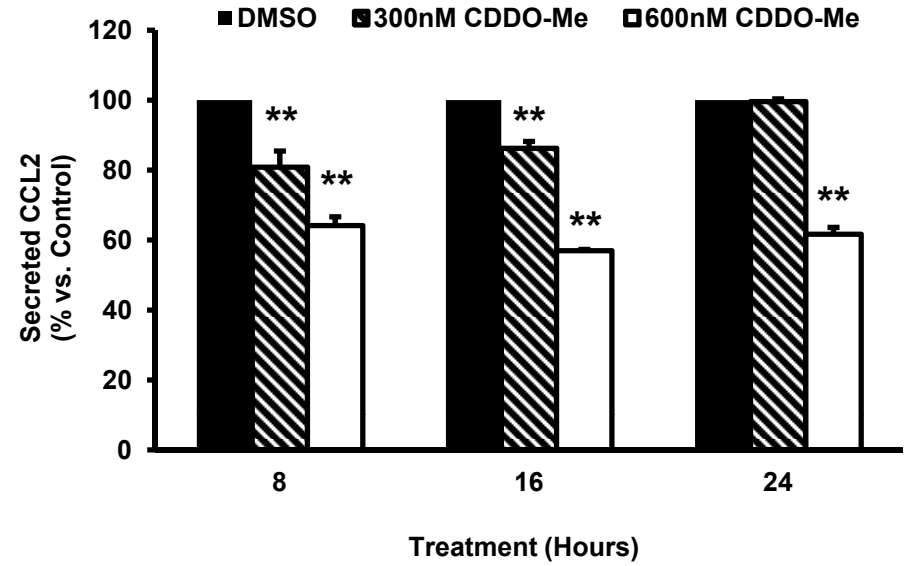


Fig. 5

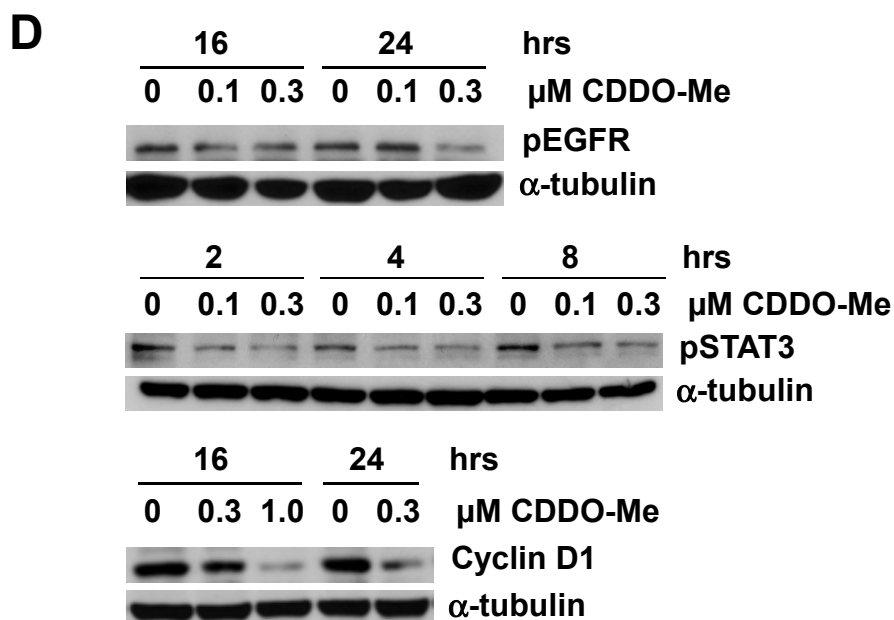
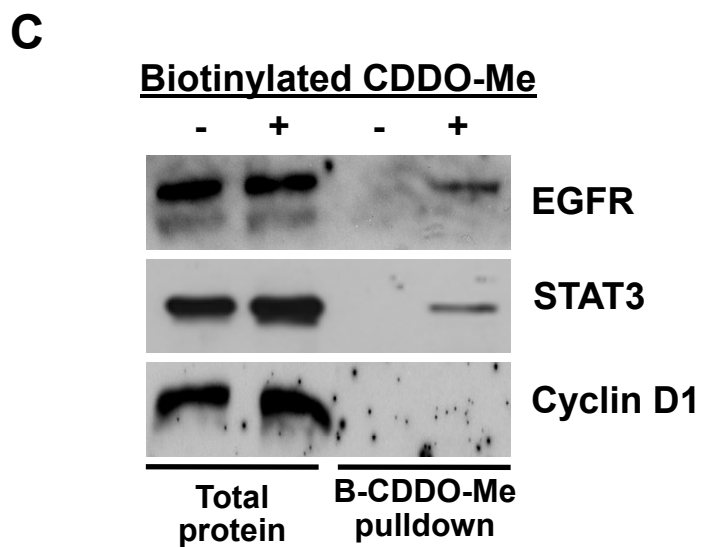
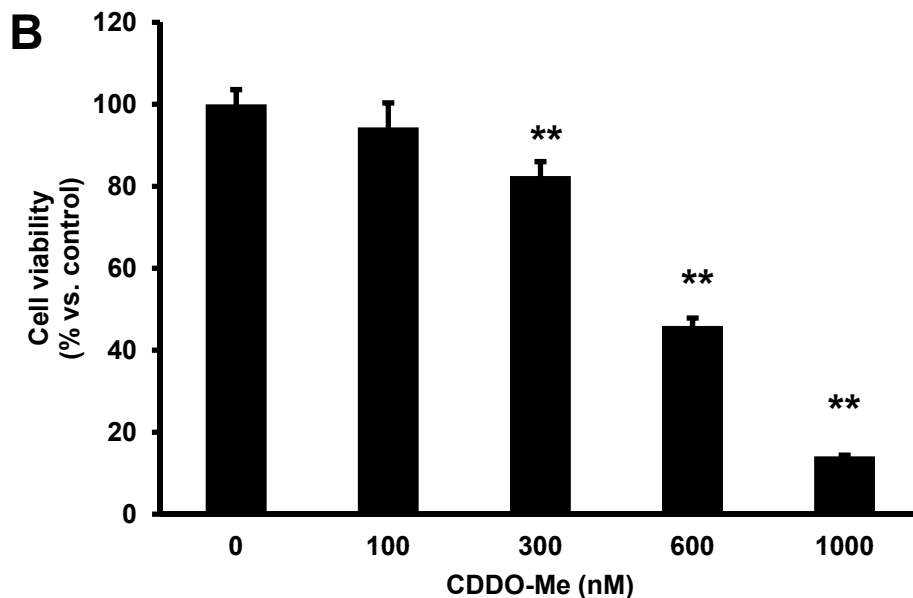
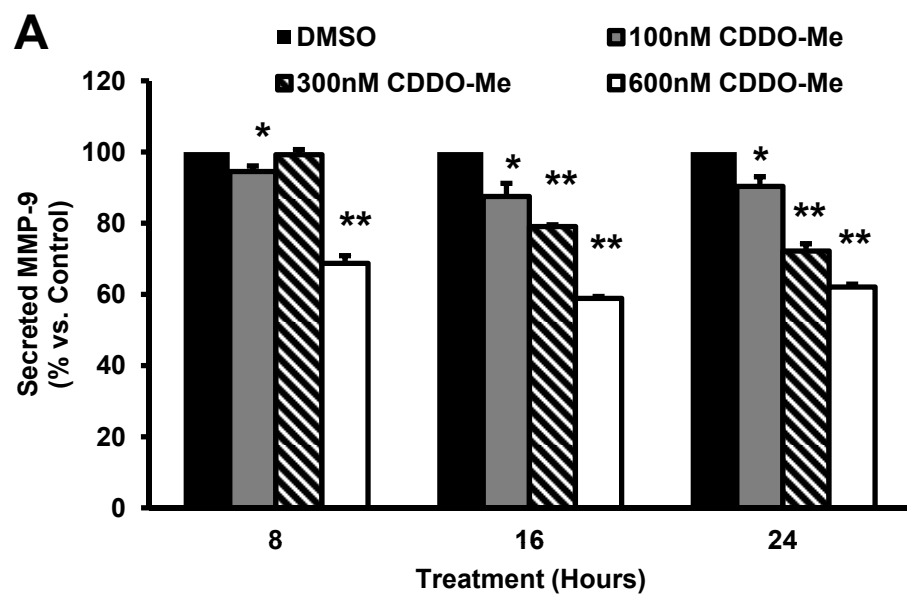
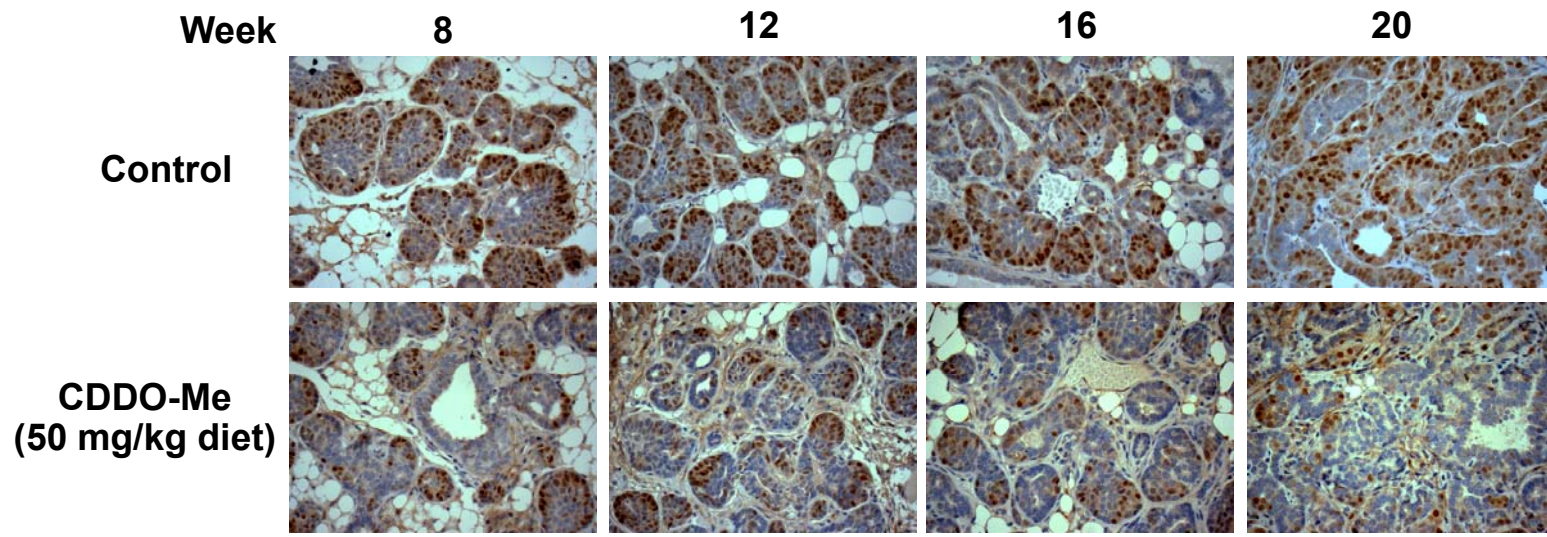
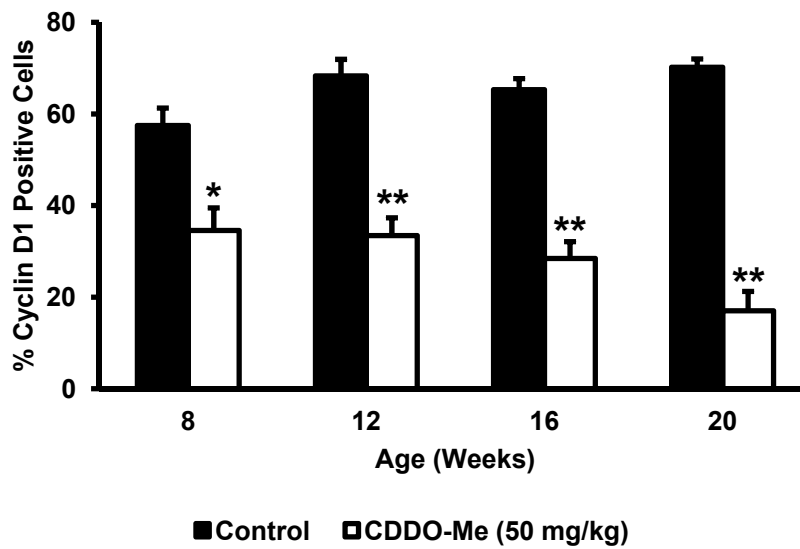


Fig. 5

E



Cyclin D1



Cancer Prevention Research

The Synthetic Triterpenoid CDDO-Methyl Ester Delays Estrogen Receptor-Negative Mammary Carcinogenesis in Polyoma Middle T Mice

Kim M. Tran, Renee Risingsong, Darlene B. Royce, et al.

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