

The combination of the histone deacetylase inhibitor vorinostat and synthetic triterpenoids reduces tumorigenesis in mouse models of cancer

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Novel drugs and drug combinations are needed for the chemoprevention and treatment of cancer. We show that the histone deacetylase inhibitor vorinostat [suberoylanilide hydroxamic acid (SAHA)] and the methyl ester or ethyl amide derivatives of the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Me and CDDO-Ea, respectively) cooperated to inhibit the *de novo* synthesis of nitric oxide in RAW 264.7 macrophage-like cells and in primary mouse peritoneal macrophages. Additionally, SAHA enhanced the ability of synthetic triterpenoids to delay formation of estrogen receptor-negative mammary tumors in MMTV-polyoma middle T (PyMT) mice. CDDO-Me (50 mg/kg diet) and SAHA (250 mg/kg diet) each significantly delayed the initial development of tumors by 4 ($P < 0.001$) and 2 ($P < 0.05$) weeks, respectively, compared with the control group in the time required to reach 50% tumor incidence. CDDO-Ea (400 mg/kg diet), as a single agent, did not delay tumor development. The combination of either triterpenoid with SAHA was significantly more potent than the individual drugs for delaying tumor development, with a 7 week ($P < 0.001$) delay before 50% tumor incidence was reached. SAHA, alone and in combination with CDDO-Me, also significantly ($P < 0.05$) inhibited the infiltration of tumor-associated macrophages into the mammary glands of PyMT mice and levels of the chemokine macrophage colony-stimulating factor in primary PyMT tumor cells. In addition, SAHA and the synthetic triterpenoids cooperated to suppress secreted levels of the pro-angiogenic factor matrix metalloproteinase-9. Similar results were observed in mouse models of pancreatic and lung cancer. At concentrations that were anti-inflammatory, SAHA had no effect on histone acetylation. These studies suggest that both SAHA and triterpenoids effectively delay tumorigenesis, thereby demonstrating a promising, novel drug combination for chemoprevention.

Introduction

Lung, breast and pancreatic cancer are three of the four leading causes of cancer deaths, accounting for approximately 160 000, 40 000 and 37 000 deaths, respectively, in the USA each year (1). Despite modest

Abbreviations: CCL2, chemokine (C–C motif) ligand 2; CDDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; CI, combination index; COX-2, cyclooxygenase-2; CXCL12, chemokine (C–X–C motif) ligand 12; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; FBS, fetal bovine serum; HDAC, histone deacetylase; iNOS, inducible nitric oxide synthase; IFN- γ , interferon- γ ; M-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteinase; NO, nitric oxide; PyMT, polyoma middle T; SAHA, suberoylanilide hydroxamic acid; TAMs, tumor-associated macrophages; VEGF, vascular endothelial growth factor.

declines in overall incidence and mortality rates for most cancers in the past 5 years (1), the 5 year survival rates are still only 16% for lung cancer and 6% for pancreatic cancer. In recent years, the incidence of estrogen receptor positive (ER+) breast cancer has gradually declined in women older than 50 years of age in the United States, largely due to the cessation of hormone replacement therapy; however, overall breast cancer incidence in women is no longer declining (2,3), and the incidence of ER-negative (ER-) breast cancer has not changed in over 30 years (2). New drugs and drug combinations as well as effective approaches such as prevention will be needed to reduce both the incidence and mortality associated with these devastating diseases.

Although the importance of inflammation in the pathogenesis of many chronic diseases (4) has been known for centuries, inflammation and the tumor microenvironment are also now recognized as central hallmarks of cancer (5–8). The use of non-steroidal anti-inflammatory agents can prevent various types of cancer (9,10), but safety concerns regarding long-term administration of currently available non-steroidal anti-inflammatory agents emphasize the need for novel drugs or drug combinations that target inflammation. Tumor-associated macrophages (TAMs) are a major inflammatory component of the tumor microenvironment (11,12). Macrophages are attracted to the tumor site in response to inflammatory cytokines and in turn promote tumor cells to produce more cytokines, chemokines and a multitude of inflammatory and angiogenesis-promoting factors, such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMP), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (6,13,14), thereby making TAMs attractive therapeutic targets for cancer prevention and treatment (13,15–17).

Vorinostat [suberoylanilide hydroxamic acid (SAHA)] is the first pan-histone deacetylase (HDAC) inhibitor to be approved by the Food and Drug Administration, for cutaneous T-cell lymphoma (18). Although only approved for patients with cutaneous T-cell lymphoma, SAHA has activity in solid tumors (19–21). This drug also inhibits proliferation and migration and induces differentiation in breast cancer cells *in vitro* (19) and inhibits the growth of mammary tumors induced by carcinogens in rats. In addition to its antitumor effects, SAHA exhibits anti-inflammatory and antiangiogenic properties (22,23). SAHA disrupts VEGF signaling in human umbilical cord endothelial cells and also inhibits the production of pro-inflammatory cytokines *in vitro* and *in vivo* (22). In spite of all of these anticancer properties, clinical studies suggest that HDAC inhibitors, when used as single agents, are not sufficient to inhibit tumorigenesis in breast cancer patients (18). In addition, high doses of SAHA are associated with toxic effects in patients, suggesting that combination therapy with SAHA and other agents at lower doses may be more beneficial (18).

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 cancer (24). These compounds inhibit proliferation of ER- breast cancer cells *in vitro* and *in vivo* (25–27), and CDDO-Me delays the development of mammary tumors in the MMTV-neu and the polyoma middle T (PyMT) transgenic models of ER- breast cancer cells (27,28). Additionally, CDDO and CDDO-Me suppress the secretion of factors important to the tumor microenvironment, including cytokines and pro-inflammatory mediators such as iNOS and COX-2 in primary peritoneal macrophages and in the mouse RAW 264.7 macrophage-like cell line, and inhibit angiogenesis *in vitro* and *in vivo* (24,29–31). Furthermore, CDDO-Me suppresses TAM infiltration and inhibits levels of the chemokines, chemokine (C–X–C motif) ligand 12 (CXCL12) and chemokine (C–C motif) ligand 2 (CCL2), in the PyMT model (28). Like SAHA, the synthetic triterpenoids are effective in combination therapy, as CDDO-Me synergizes with the retinoid LG100268 to delay

mammary carcinogenesis in the MMTV-neu model (27). However, the combination of SAHA with a triterpenoid has never been tested. In this study, we report that the combination of the HDAC inhibitor SAHA with the synthetic triterpenoids CDDO-Me or CDDO-Ea is more effective for inhibiting production of the pro-inflammatory mediator nitric oxide (NO) than for suppressing cellular proliferation. SAHA also inhibits TAM infiltration and combining it with a triterpenoid delays mammary tumorigenesis in the PyMT model of ER- breast cancer. SAHA also displays slight single agent activity in mouse models of pancreatic and lung cancer, but its activity is enhanced when combined with the triterpenoid CDDO-Ea.

Materials and methods

Drugs

CDDO-Me, CDDO-Ea and SAHA were synthesized as described (32–34). For cell culture studies, drugs were dissolved in dimethyl sulfoxide, and controls containing equal concentrations of dimethyl sulfoxide (<0.1%) were included in all experiments.

Cell culture and immunoblot analysis

Primary PyMT cells were derived from mammary tumors of female PyMT (+/–) mice. Resected PyMT mammary tumors were minced and digested in Dulbecco's modified Eagle's medium (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 with gentle agitation. The cell suspension was filtered through a 40 µm cell strainer (BD Bioscience), centrifuged at 220g for 10 min and plated in DMEM + 10% FBS. All experiments were performed within 1 week of cell isolation. Raw 264.7 macrophage-like cells (ATCC) and pancreas-1343 cells, derived from a pancreatic tumor from the KPC mouse model (35), were maintained in DMEM with 10% FBS; VC-1 cells (36), derived from a lung tumor from an A/J mouse injected with vinyl carbamate, were grown in RPMI 1640 and 10% FBS. For western blotting, cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and probed with antibodies against acetyl-histone H3 (Cell Signaling), inhibitor of nuclear factor-κB alpha IκBα, cyclin D1 and tubulin (Santa Cruz).

Cell proliferation assay

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, 5×10^3 primary PyMT tumor cells/well or 5×10^4 VC-1 lung cancer cells and P1343 pancreatic cancer cells/well were seeded into 96-well plates. The following day, cells were incubated with varying concentrations of CDDO-Me, CDDO-Ea, SAHA or the combination of SAHA and triterpenoids for 48 h. Cells were then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 4 h (Sigma) and read at optical density OD570.

Nitrite analysis

Because nitrite is the stable oxidation product of NO, nitrite accumulation was used as an indicator of NO production in the medium and was assayed by the Griess reaction as described previously (29). Briefly, RAW 264.7 cells were plated into 96-well plates at 5×10^4 cells/well. The following day, cells were incubated with varying concentrations of CDDO-Me, CDDO-Ea, SAHA or the combination of SAHA and triterpenoids along with either interferon-γ (IFN-γ) (10 ng/ml) or lipopolysaccharide (LPS) (3 ng/ml) for 24 h. The cultured supernatants were then collected and mixed with equal volumes of Griess reagent for 10 min at room temperature. Plates were read at 550 nm, and nitrite concentration was calculated based on a standard curve of sodium nitrite. For the primary peritoneal macrophage studies, 10–12-week-old female PyMT mice were injected intraperitoneally with 2 ml of a 4% thioglycollate broth. Four days later, peritoneal macrophages were harvested and plated (8×10^4 cells/well). After 4 h, cells were treated with triterpenoids and SAHA for 48 h, and NO was measured.

Enzyme-linked immunosorbent assay

Primary PyMT cells were treated with varying concentrations (0–1000 nM) of SAHA for varying timepoints (8–48 h), and the amount of macrophage colony-stimulating factor (M-CSF) or MMP-9 released into the medium was measured using quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems).

PyMT studies

All animal studies were done in accordance with protocols approved by the institutional animal care and use committee 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 described previously (37,38). Four-week-old female PyMT mice were fed powdered 5002 rodent chow (PMI Feeds) or the powdered diet containing CDDO-Ea (400 mg/kg diet), SAHA (250 mg/kg diet), the combination of SAHA (250 mg/kg) and CDDO-Me (50 mg/kg), or the combination of SAHA (250 mg/kg) and CDDO-Ea (400 mg/kg). The drugs are stable in diet for over a month, and food was replaced in the cages twice a week. Mice were palpated twice a week for detection of new tumors, and tumors were measured weekly with calipers.

Macrophage analysis

Percent of infiltrating macrophages were analyzed from tumors and mammary glands using an initial purification strategy including magnetic purification followed by flow cytometry analysis. Briefly, all mammary glands and/or tumors from the mice were removed 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. 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 antirat IgG (BioLegend). Ten microliters each of magnetic beads and antibodies per 10^7 cells were used with phosphate-buffered saline 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 negative flow through cell fractions were then analyzed for the percentage of F4/80-positive cells out of total mammary gland and tumor cells using a FACScan (Becton Dickinson).

A/J mouse model of lung cancer

In a pilot experiment, female A/J mice (Jackson Laboratory) were injected intraperitoneally with 0.32 mg vinyl carbamate (Toronto Research Chemicals) per mouse in isotonic saline adjusted to pH 5. Mice were fed SAHA (250 mg/kg diet) in semisynthetic AIN-93G diet (Harlan Teklad) for 26 weeks, beginning 1 week before the injection of the carcinogen. In subsequent lung studies, mice were injected with two doses of carcinogen, and drugs were fed in AIN-93G diet for 15 weeks, beginning 1 week after the final dose of carcinogen. For all experiments, lungs were removed and inflated with formalin. The number of grossly visible lesions on the inflated lungs, and the number, size and histopathology of tumors on the lung sections were evaluated as described previously (39).

Pancreatic cancer model

KPC triple mutant mice (40) were generated by mating *LSL-Kras^{G12D/+}*, *LSL-Trp53^{R127H/+}* and *LSL-Trp53^{R127H/+}*, *Pdx-1-Cre* mice and genotyped as described (35). Four-week-old KPC mice were fed powdered 5002 rodent chow or the same powdered diet containing drugs. All mice were monitored daily and weighed weekly. Because of institutional animal care and use committee regulations, mice were killed when significant abdominal distension, weight loss or labored breathing were observed; these symptoms are consistent predictors of death within 48 h (40).

Tissue levels

A total of 5 PyMT mice were fed CDDO-Me (50 mg/kg diet), CDDO-Ea (400 mg/kg diet) or SAHA (250 mg/kg diet) for 1 week. Mammary glands and livers were harvested, and blood was collected into heparinized tubes. Tissues were homogenized in phosphate-buffered saline, extracted in acetonitrile, separated by reverse-phase liquid chromatography and detected by mass spectrometry as described previously for triterpenoids (27). For samples containing SAHA, acetonitrile extracts were loaded on a Waters Atlantis T3 5 µm column in 8% acetonitrile and 0.1% formic acid. A 6 min gradient to 35% acetonitrile and 0.1% formic acid was applied, and the SAHA eluted at 34% acetonitrile. Mass spectrometry detection was in electrospray positive mode using a cone voltage of 25. Standard curves were generated by serially diluting known concentrations of drug in control blood or tissue homogenates. All samples were within the linear range of the standard curve, and values were calculated using Waters MassLynx v. 4.1 software.

Statistical analysis

Results are described as mean ± standard error of the mean and were analyzed by one-way analysis of variance or one-way repeated measures analysis of variance and the Tukey test, by one-way analysis of variance on ranks (Wilcoxon signed rank test; SigmaStat 3.5) for multiple groups, the *t*-test/Mann–Whitney rank sum test if only two groups were compared, and Kaplan–Meier analysis for survival data. All *P*-values are two-sided. In order to test for additive or synergistic effects, the combination index (CI) was calculated using CompuSyn software (www.combosyn.com).

Results

SAHA enhances NO inhibition by CDDO-Me and CDDO-Ea in RAW 264.7 cells and primary peritoneal macrophages from PyMT mice

Synthetic triterpenoids are known potent anti-inflammatory agents that inhibit the *de novo* synthesis of the pro-inflammatory mediator iNOS and the enzyme product of iNOS, NO in primary mouse macrophages (24,30). Treatment with low nanomolar concentrations of CDDO-Me or CDDO-Ea along with IFN- γ stimulation in RAW 264.7 macrophage-like cells for 24 h resulted in a significant ($P < 0.05$) dose-dependent decrease in the levels of NO detected via the Griess reaction in cell supernatants (Figure 1A). When RAW 264.7 cells were co-treated with the combination of SAHA and varying concentrations of CDDO-Me or CDDO-Ea, SAHA alone inhibited NO production, but the combination of SAHA and triterpenoids significantly enhanced the effects of the individual drugs in a dose-dependent manner. The decrease in NO levels following SAHA and either CDDO-Me or CDDO-Ea treatment was significant compared with SAHA ($P < 0.001$ for treatment of SAHA with all doses of CDDO-Me or CDDO-Ea, except as noted on the figure legend), CDDO-Me or CDDO-Ea alone ($P < 0.05$ for treatment of SAHA with 1 nM of CDDO-Me or CDDO-Ea, and $P < 0.001$ for treatment of SAHA with 3 and 10 nM of CDDO-Me or CDDO-Ea).

Additionally, we investigated the effect of co-treatment of SAHA and triterpenoids in RAW 264.7 cells stimulated with LPS. SAHA and CDDO-Me or CDDO-Ea treatment significantly ($P < 0.001$ versus control for SAHA treatment with all doses of CDDO-Me or CDDO-Ea) reduced NO release in a dose-dependent manner (Figure 1B). The decrease in NO levels following combination treatment was significant compared with individual administration of SAHA ($P < 0.05$ for treatment of SAHA with all doses of CDDO-Me or CDDO-Ea), CDDO-Me or CDDO-Ea ($P < 0.001$ for treatment of SAHA with 3 nM of CDDO-Me or CDDO-Ea, and $P < 0.05$ for treatment of SAHA with 10 nM of CDDO-Me or CDDO-Ea).

The triterpenoids, SAHA or the combination also significantly reduced the production of NO in a dose-dependent manner in peritoneal macrophages from 10–12-week-old PyMT mice (Figure 1C and 1D). In PyMT mice, TAMs infiltrate into the mammary glands and drive tumorigenesis, and the maximum macrophage infiltration occurs at 12 weeks of age. Although these cells do not generate any NO unless stimulated with factors such as IFN- γ or LPS, the sensitivity of the primary macrophages to the drugs is slightly lower than in RAW 264.7 cells, possibly because the macrophages from the PyMT mice are already primed to secrete pro-inflammatory cytokines that drive tumor development. To determine whether these combinations of drugs act additively or synergistically, more doses of both drugs were included, and a CI was calculated using CompuSyn software. A CI = 1 is additive, and a CI < 1 indicates synergy. Combinations of SAHA and triterpenoids were additive against IFN- γ . When the macrophages from the PyMT mice were stimulated with LPS, the combination of SAHA and CDDO-Ea inhibited NO release in a synergistic manner, whereas the combination of CDDO-Me and SAHA worked in an additive fashion. Calculated CI values are listed in Supplementary Table 1, available at [Carcinogenesis Online](#).

The combination of SAHA and triterpenoids is less effective at inhibiting cell proliferation

Because the combination of SAHA and triterpenoids effectively suppressed NO induction in RAW 264.7 cells and in primary peritoneal macrophages from PyMT mice stimulated with either IFN- γ or LPS, we investigated whether this combination would also inhibit growth of primary PyMT breast tumor cells (Figure 1E). The percentage of proliferating cells was significantly lower following treatment with SAHA, CDDO-Me or CDDO-Ea. However, the effects on proliferation were modest, with only a 35% reduction in PyMT cell number at best. Low micromolar concentrations of triterpenoids are more

effective at inhibiting proliferation in these cells (28), but 0.3–1 μ M SAHA does not affect cell growth or viability (data not shown). Similar results were observed in P1343 pancreatic cancer cells and in VC-1 lung cancer cells, although SAHA did not inhibit proliferation in the lung cancer cells and the triterpenoids were more potent in these two cell lines than in the primary PyMT lines. The higher concentrations of triterpenoids required to inhibit proliferation (100–1000 nM) than to suppress NO (1–30 nM) suggests that the anti-inflammatory effects of these drugs might be a useful mechanism for inhibiting carcinogenesis.

Although the synthetic triterpenoids are potent inducers of the HO-1 cytoprotective enzyme and the anti-inflammatory Keap1/Nrf2/ARE pathway (24), SAHA alone does not induce transcription of *HO-1* or *NQO1* messenger RNA in RAW 264.7 cells, and the induction of these genes by the triterpenoids was not enhanced when co-treated with SAHA (data not shown). The triterpenoids can inhibit the inflammatory nuclear factor-kappaB pathway in tumor cells, as pre-treatment with CDDO-Ea prevents the degradation of IKB α in PyMT tumor cells challenged with tumor necrosis factor α (Supplementary Figure 1, available at [Carcinogenesis Online](#)); SAHA does not appear to alter this effect. Similar results were observed in VC-1 lung cancer cells and in pancreatic cancer cells, but concentrations of approximately 1 μ M of triterpenoids were required to inhibit this pathway (data not shown). SAHA and CDDO-Ea had little effect on IKB α in RAW 264.7 macrophage-like cells (Supplementary Figure 1, available at [Carcinogenesis Online](#)).

SAHA enhances the ability of CDDO-Me and CDDO-Ea to delay the development of ER- mammary tumors in PyMT mice

To investigate whether SAHA can enhance the effects of triterpenoids on tumorigenesis, we used the PyMT mouse model of ER- breast cancer. In this model, the expression of the oncogenic PyMT protein is targeted to the mammary epithelium by the MMTV promoter (38), and tumor malignancy is characterized by significant infiltration of TAMs (41). We previously showed that CDDO-Me (50 mg/kg diet), as a single agent, significantly delayed the development of PyMT mammary tumors in this aggressive model by 4 weeks. In the mice fed CDDO-Me, 50% tumor incidence was reached by week 19 and 100% incidence by week 29, compared with the control group, which reached 50% tumor incidence by 15 weeks and 100% incidence by 22 weeks (28). In this study, female PyMT mice were fed control diet or diet containing SAHA (250 mg/kg diet), CDDO-Ea (400 mg/kg diet) or the combination of SAHA with CDDO-Me or CDDO-Ea, beginning at 4 weeks of age. SAHA as a single agent delayed tumor development ($P < 0.05$) in PyMT mice with 50% tumor incidence reached by week 17 and 100% incidence by week 25, compared with the control group, which produced 50% tumor incidence by 15 weeks and 100% by 22 weeks (Figure 2A, right panel).

When SAHA was combined with CDDO-Me, initial tumor development was significantly ($P < 0.001$) delayed compared with the control group and also compared with SAHA or CDDO-Me as single agents (Figure 2B, left panel). Tumor incidence in 50% of the PyMT mice fed the combination diet was reached by week 22 with 100% of mice developing tumors by week 26, as compared with the control group, which reached 50% tumor incidence by 15 weeks and 100% incidence by 20 weeks. When possible, littermate-matched controls were included in all experiments, resulting in variability in the size of the respective control groups. The enhanced efficacy with the combination of CDDO-Me and SAHA was also observed when CDDO-Ea was combined with SAHA. As a single agent, CDDO-Ea is slightly less potent than CDDO-Me in most assays and had no significant effect on tumor development in PyMT mice (Figure 2A, right panel). However, the combination of CDDO-Ea and SAHA significantly ($P < 0.001$) delayed tumor development as compared with the control group and also compared with the single agents (Figure 2B, right panel) and was as effective as the combination of CDDO-Me and SAHA. The delays in tumor development by the combination of SAHA with CDDO-Me and CDDO-Ea are striking

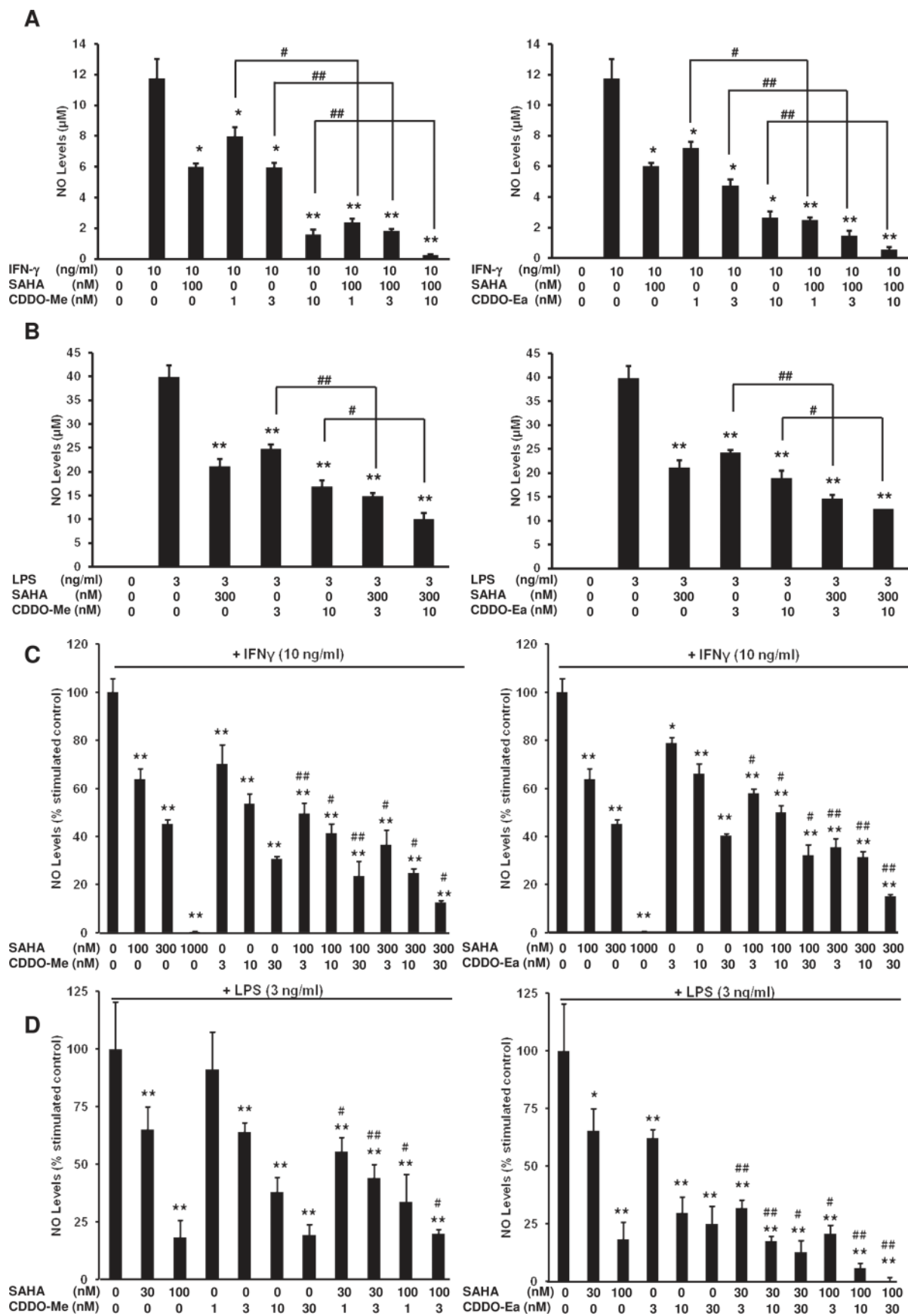
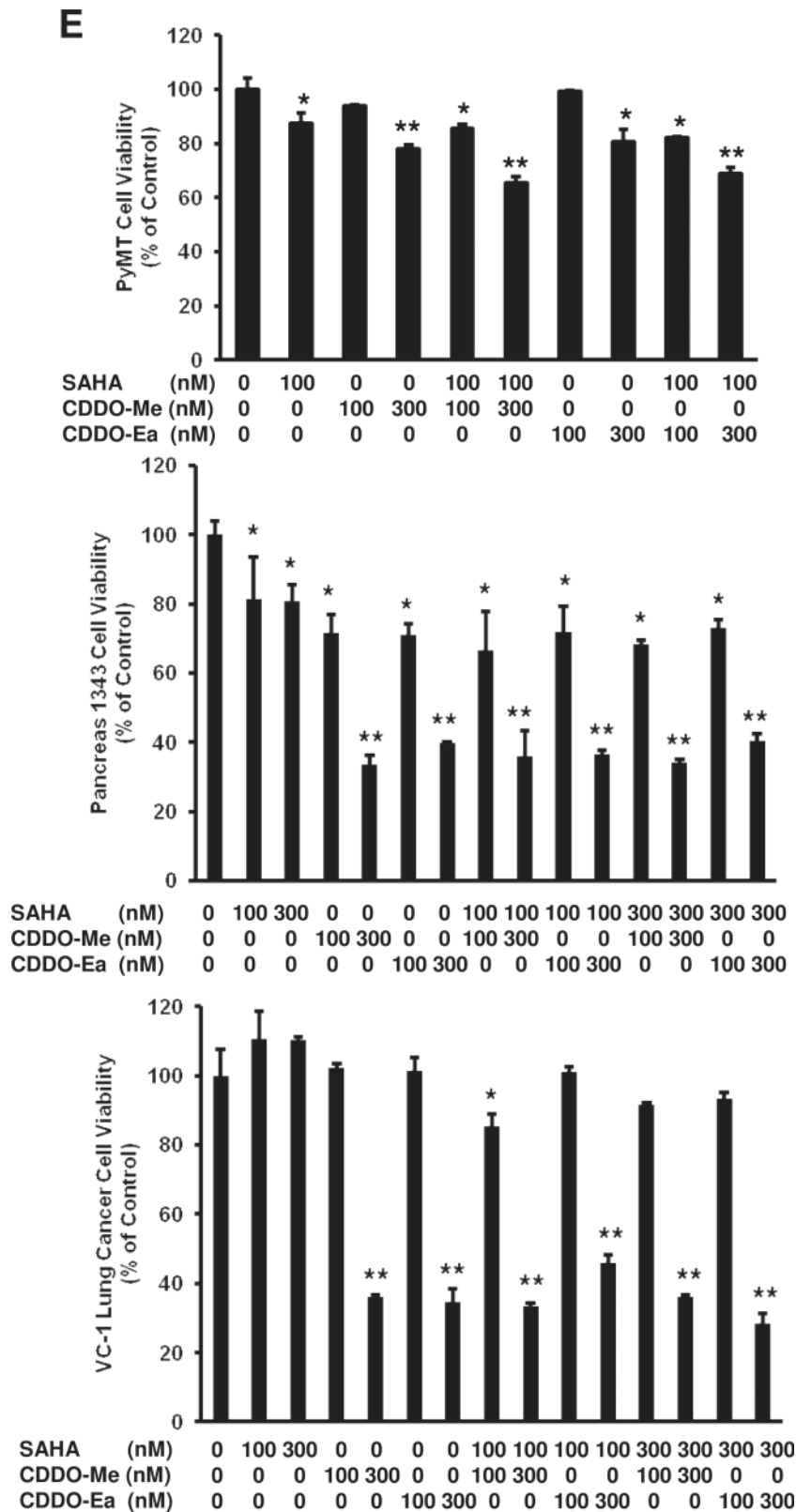


Fig. 1. SAHA enhances the ability of CDDO-Me and CDDO-Ea to suppress NO production in RAW 264.7 cells and in primary mouse macrophages. RAW 264.7 cells were treated with CDDO-Me or CDDO-Ea, SAHA or the combination of SAHA and a triterpenoid and stimulated with IFN- γ (A) or LPS (B) for



24 h. Peritoneal macrophages from PyMT mice (C, D) were treated with the same combinations of drugs for 48 h. The supernatants from the treated cells were assayed by the Griess reaction for NO production. * $P < 0.05$ and ** $P < 0.001$ versus controls stimulated with IFN- γ or LPS (A-D); # $P < 0.05$ and ## $P < 0.001$ versus single drug treatment (A, B); # $P < 0.05$ versus one single drug treatment and ### $P < 0.001$ versus both single drug treatments. (E) Primary PyMT tumor cells, pancreas-1343 and VC-1 lung cancer cells were treated with increasing concentrations of CDDO-Me or CDDO-Ea, SAHA or the combination of SAHA and triterpenoids for 48 h, and effects on proliferation were measured via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis. * $P < 0.05$ and ** $P < 0.001$ versus controls.

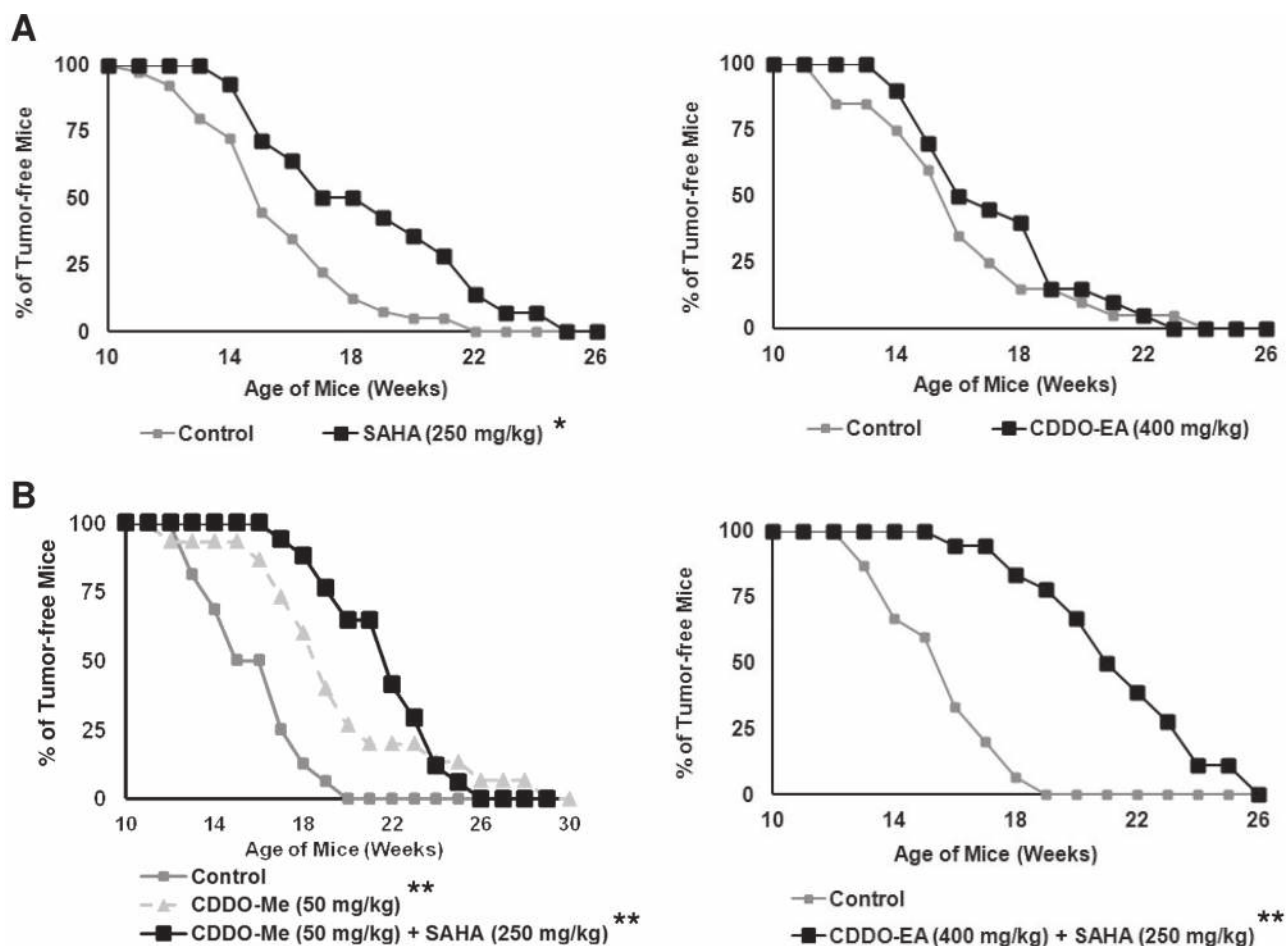


Fig. 2. SAHA enhances the ability of CDDO-Me and CDDO-Ea to delay the development of ER⁻ mammary tumors in PyMT mice. Beginning at 4 weeks of age, female transgenic mice were fed powdered control diet or diet containing SAHA (250 mg/kg diet), CDDO-Ea (400 mg/kg diet), the combination of SAHA (250 mg/kg diet) and CDDO-Me (50 mg/kg diet) or the combination of SAHA (250 mg/kg diet) and CDDO-Ea (400 mg/kg diet). Mice were palpated twice a week. The effects of single agents (A) or drug combinations (B) on tumor development in PyMT mice as compared with control diet are shown. $n = 15$ mice per arm. * $P < 0.05$ versus control in A and ** $P < 0.001$ versus control in B.

and significant as PyMT mice fed control diet live on average only 21.4 weeks versus 30.3 or 29.9 weeks when fed the combination of SAHA and CDDO-Me or SAHA and CDDO-Ea, respectively. Despite the delay in palpable tumor formation, no effects on tumor number or tumor size were observed in the treated groups by the end of the study. The modest effect of these drugs on proliferation of PyMT cells *in vitro* (Figure 1E) and the final *in vivo* tumor burden data suggest that, at least at the concentrations used in these studies, these drugs did not stop the growth of established PyMT tumors.

All drugs were well tolerated at the doses used with no signs of toxicity, and the mice continued to gain weight throughout the experiment. There were no statistical differences in weight between mice fed drugs and mice fed control diet. At week 13, when mice had been on diet for 9 weeks but before growing tumor burdens could skew weights, the average weight per group was as follows: control 21.4 g versus CDDO-Me 20.7 g, $P = 0.34$; control 22.9 g versus SAHA 22.2 g, $P = 0.31$; control 23.6 g versus CDDO-Ea 22.5 g, $P = 0.112$; control 21.9 g versus SAHA + CDDO-Me 20.6 g, $P = 0.07$; control 22.9 g versus SAHA + CDDO-Ea 21.6 g, $P = 0.09$. None of these drugs had an effect on mammary gland development or transgene expression (data not shown).

Although it was not practical to determine drug levels in the actual mice used for the tumor studies, five PyMT mice per group were fed control diet or diet containing the drugs at the doses described above for 1 week. Drug concentrations of SAHA were low in both the mammary gland and plasma and averaged 68 ± 9 nM and

48 ± 11 nM, respectively. In contrast, tissue levels of CDDO-Me were only 20 ± 2 nM in whole blood but were 1.1 ± 0.1 μ M in the mammary gland; 0.4 ± 0.1 μ M CDDO-Ea was detected in whole blood and 1.1 ± 0.2 μ M in the mammary gland. The triterpenoids are highly lipophilic and so drug levels are significantly higher in whole blood than in plasma and are usually found at high levels in adipose tissue. Because of this lipophilic nature, drug levels in mammary epithelial cells within the mammary gland are not known.

SAHA inhibits the infiltration of TAMs in ER⁻ mammary tumors and reduces M-CSF and MMP-9 levels in primary PyMT tumor cells

One of the most useful experimental properties of the PyMT mouse model is the infiltration of TAMs. We have shown previously that TAM infiltration can be quantitated via flow cytometry and that CDDO-Me modestly inhibits this infiltration (28). Because SAHA delays tumor development in this model, and the concentrations of SAHA that can be detected *in vivo* are more active in the anti-inflammatory assays than in the proliferation assays, we investigated the effects of SAHA on TAM infiltration. The percentage of F4/80-positive cells in mammary tumors of PyMT mice was assayed at 12 and 16 weeks of age, as detailed in the Materials and methods; 12 weeks is the period of maximum macrophage infiltration in this model (28). The percentage of F4/80-positive cells was significantly ($P < 0.01$) lower in mammary tumors of 12-week-old mice fed SAHA diet as compared with mammary tumors of litter-matched mice fed control diet (Figure 3A). Moreover, a decrease in the percentage of

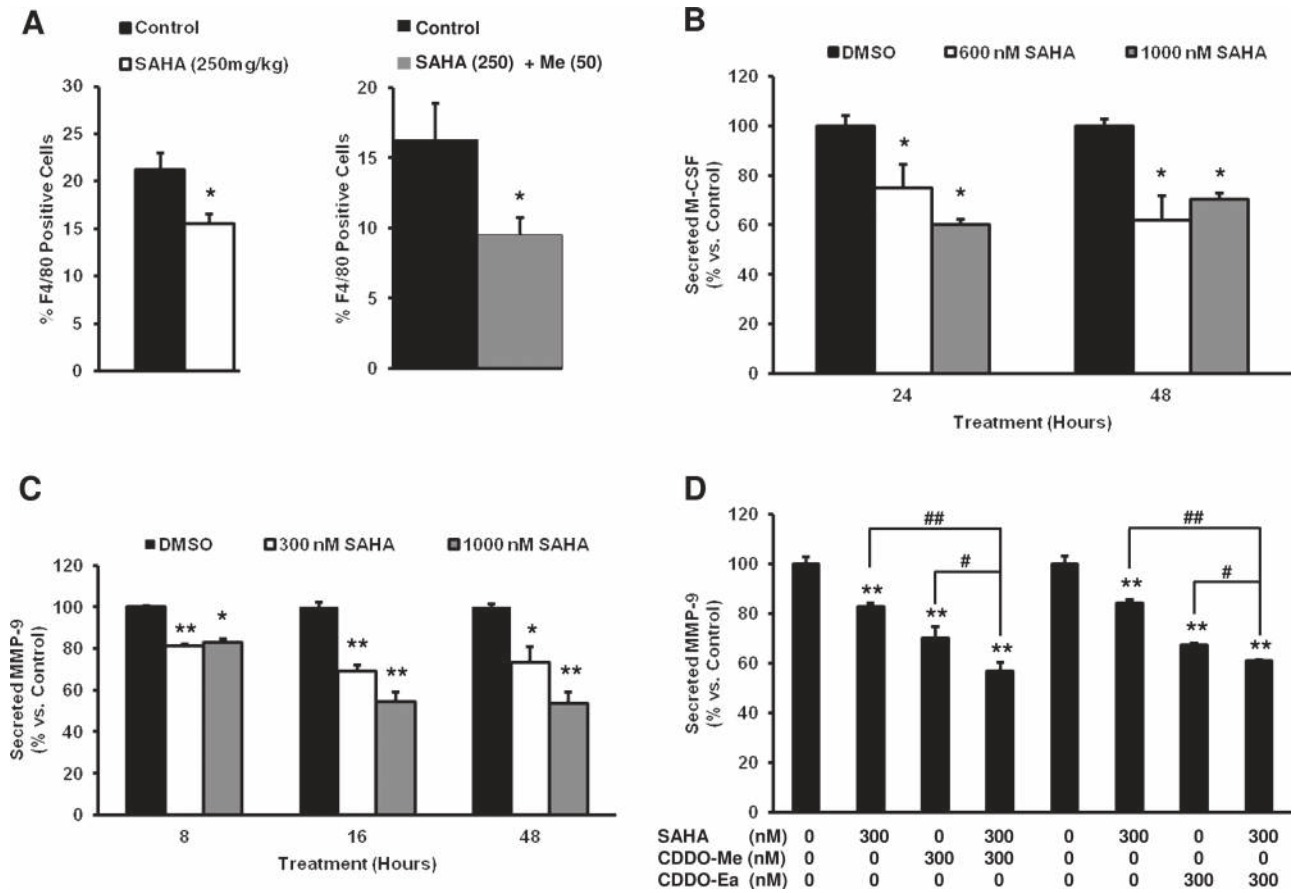


Fig. 3. SAHA, alone or in combination with CDDO-Me, inhibits the infiltration of macrophages into the mammary glands of PyMT mice and suppresses levels of M-CSF and MMP-9 in primary PyMT tumor cells. Beginning at 4 weeks of age, female transgenic mice were fed powdered control diet, SAHA diet (250 mg/kg diet) or SAHA (250 mg/kg diet) + CDDO-Me (50 mg/kg diet) and killed at 12 weeks of age (A). Quantitation of macrophage infiltration was detected by flow cytometry analysis of F4/80 in homogenized mammary glands, $n = 5$ per group for the SAHA study and $n = 19$ per group for the SAHA + CDDO-Me study. $*P < 0.05$ versus age-matched littermate controls. Primary PyMT tumor cells were treated with SAHA (0–1000 nM) for varying time points (8–48 h), and supernatants were assayed by ELISA for M-CSF (B) or MMP-9 (C) secretion. $*P < 0.05$ and $**P < 0.001$ versus control treatment. (D) Primary PyMT tumor cells were treated with the combination of SAHA (300 nM) and CDDO-Me or CDDO-Ea (300 nM) for 24 h, and supernatants were assayed by ELISA for MMP-9. $**P < 0.001$ versus control treatment; $\#P < 0.05$ and $\#\#P < 0.001$ versus single drug treatment.

TAMs in the mammary gland was observed in all of the mice fed diet containing SAHA as compared with their littermate-matched controls. SAHA diet had only minor effects on TAM infiltration in 16-week-old mice (data not shown), but by this time, palpable tumors were evident in the majority of mice, and TAMs are not required to drive continued tumor growth. The percentage of TAMs in the mammary gland was also significantly lower in PyMT mice fed the combination of SAHA and CDDO-Me than in control mice (Figure 3A right panel).

To investigate a potential mechanism responsible for the reduced infiltration of TAMs in mammary tumors, levels of the chemokine M-CSF (or CSF-1) were evaluated in isolated primary PyMT tumor cells. Overexpression of this cytokine is a poor prognostic indicator in breast cancer. M-CSF produced by tumor cells recruits macrophages that secrete cytokines and growth factors to drive tumor progression (42–44), and inhibitors of M-CSF are in development for the treatment of cancer (45). Treating primary tumor cells with SAHA for 24 or 48 h resulted in a significant ($P < 0.05$) dose-dependent decrease in the secreted levels of M-CSF, detected via ELISA, as compared with cells treated with vehicle (Figure 3B). Notably, the triterpenoid CDDO-Me had no effect on M-CSF secretion but instead reduced levels of the chemokines CXCL12 and CCL2 (28); SAHA had no effect on these two chemokines. In addition to inhibiting M-CSF, SAHA also suppressed the secretion of the pro-inflammatory and pro-angiogenic factor MMP-9 in primary PyMT tumor cells in a time-dependent manner (Figure 3C), and the combination of triterpenoids and SAHA

enhanced the suppression of secreted MMP-9 levels compared with the effects from single agents (Figure 3D). The inhibition of MMP-9 levels by the combination of either SAHA and CDDO-Me or CDDO-Ea was significant compared with individual administration of SAHA ($P < 0.001$ for treatment of 300 nM SAHA with either 300 nM CDDO-Me or CDDO-Ea versus 300 nM SAHA), CDDO-Me or CDDO-Ea ($P < 0.05$ for 300 nM SAHA with 300 nM CDDO-Me or CDDO-Ea versus either 300 nM CDDO-Me or CDDO-Ea).

SAHA and CDDO-Ea prolong survival in a mouse model of pancreatic cancer

Disease progression in a *Kras*^{G12D} transgenic mouse model of pancreatic cancer is also accompanied by the infiltration of a variety of immunosuppressive cells, including TAMs (46). Because the infiltration of these immune cells occurs in precursor pancreatic lesions and persists even in invasive tumors, drugs that inhibit these cells should be more effective when used as preventive agents rather than for treating advanced pancreatic cancer. To determine if SAHA, alone or in combination with the triterpenoid CDDO-Ea, could extend survival in an aggressive but clinically relevant model of pancreatic cancer, LSL-*Kras*^{G12D/+}, LSL-*Trp53*^{R127H/+}, Pdx-1-Cre (KPC) mice were fed test diets containing relatively low doses of drug, beginning at 4 weeks of age. In this model, when an activating mutation in *Kras* is paired with a point mutation in the *p53* tumor suppressor gene only in the pancreas, mice develop invasive and metastatic adenocarcinomas (40).

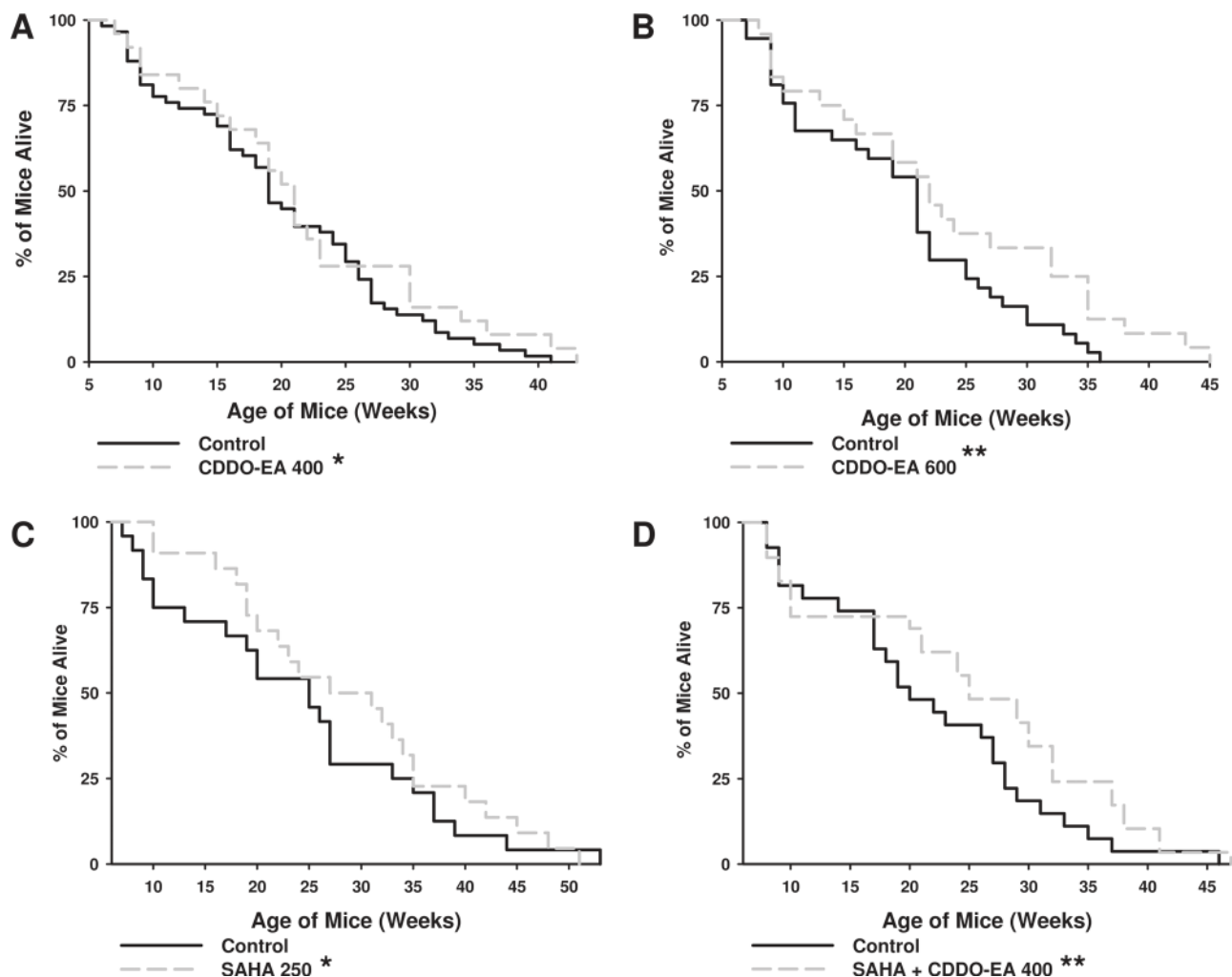


Fig. 4. Triterpenoids and SAHA extend lifespan in the KPC mouse model of pancreatic cancer. Beginning at 4 week of age, KPC transgenic mice were fed powdered control diet or diet containing CDDO-Ea (400 or 600 mg/kg diet), SAHA (250 mg/kg diet) or the combination of CDDO-Ea (400 mg/kg diet) and SAHA (250 mg/kg diet). (A) $n = 58$ for control and 25 for CDDO-Ea 400; (B) $n = 37$ for control and 24 for CDDO-Ea 600; (C) $n = 24$ for control and 22 for SAHA 250; (D) $n = 27$ for control and 29 for SAHA + CDDO-Ea. * $P < 0.05$ versus control for CDDO-Ea (400 mg/kg diet) and SAHA (250 mg/kg diet) alone in A and C; ** $P < 0.001$ versus control for CDDO-Ea (600 mg/kg diet) and SAHA + CDDO-Ea (400 mg/kg diet) in B and D.

Mutations in these two genes are found in 90% and 75% of human pancreatic cancers, respectively. Because the model recapitulates many of the symptoms, histopathology and molecular features of human pancreatic cancer, it is an appropriate model for preclinical testing of new drugs for pancreatic cancer (47).

Although the median survival time in the KPC model is only 5 months, the lifespan of these mice is highly variable, so littermate-matched controls were included when possible in this challenging breeding protocol. The triterpenoid CDDO-Ea had only a marginal, but significant ($P < 0.05$), effect on survival when fed at 400 mg/kg diet (Figure 4A), but it significantly ($P < 0.001$) increased the lifespan of the mice when fed at 600 mg/kg diet (Figure 4B). In 12 littermate-matched pairs, 10 of the 12 mice fed with CDDA-Ea 600 mg/kg lived an average of 23.2 ± 1 weeks versus only 19.1 ± 0.5 weeks in mice fed control diet ($P = 0.047$). SAHA has been shown to inhibit proliferation of pancreatic cancer *in vitro* or in xenograft models (48,49), but it has not been tested in a transgenic mouse model with an intact immune system. When fed in diet 250 mg/kg as a single agent, SAHA extended lifespan (Figure 4C, $P < 0.05$), but with the exception of the earliest weeks, the combination of SAHA and CDDO-Ea 400 mg/kg was more effective (Figure 4D, $P < 0.001$). The drug doses used in these studies were well tolerated with no differences in the average weight of the various groups (data not shown).

SAHA and CDDO-Ea reduce tumor burden in the A/J mouse model of lung cancer

Because we have shown that drugs or combinations that inhibit the production of NO and other inflammatory mediators are also effective in a model of lung cancer (36,39,50), we first tested the ability of SAHA alone to inhibit lung carcinogenesis. SAHA has been reported to be effective for prevention of lung tumors in A/J mice induced with the tobacco carcinogen 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol or with vinyl carbamate (51,52), but the doses of SAHA used in these experiments, 500–600 mg/kg diet, were at or near the maximum tolerated dose (51). In a pilot study, SAHA at only 250 mg/kg diet was fed to A/J mice, beginning 1 week before injection with vinyl carbamate, for a total of 26 weeks. The number of tumors per slide was significantly ($P < 0.001$) lower in the mice fed SAHA (1.9 ± 0.07) compared with the mice fed control diet (3.4 ± 0.03). Moreover, the average tumor burden in the lung (Figure 5A) was reduced by 70% in mice fed the SAHA diet and was only 4.6 ± 0.2 mm³/slide in the SAHA group versus 15.9 ± 0.4 mm³/slide in the control group ($P < 0.001$).

This experiment was repeated, but in the new studies, diets were not started until a week after injection with the vinyl carbamate and only continued for 15 weeks, the standard protocol we have used to evaluate triterpenoids in this model (39). As shown in Table I, SAHA

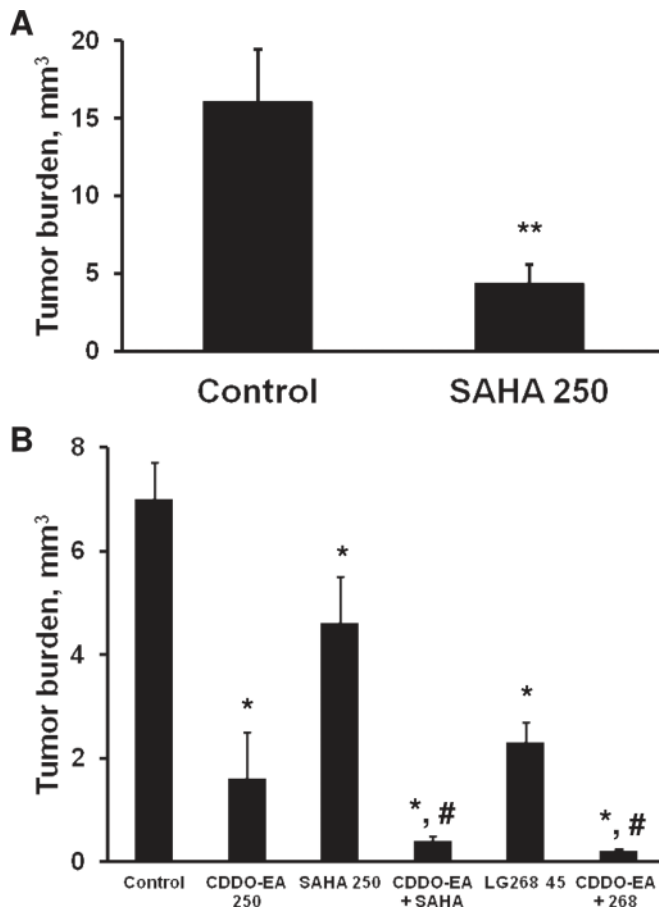


Fig. 5. SAHA or the combination of SAHA and triterpenoids inhibit lung carcinogenesis in A/J mice. (A) Beginning 1 week before injection with a single dose of vinyl carbamate, mice were fed AIN diet or AIN diet containing SAHA (250 mg/kg diet) for a total of 26 weeks; $n = 26$ mice in the control group and $n = 11$ mice in the SAHA group. (B) Female A/J mice were injected with two doses of vinyl carbamate, and drugs were fed in diet for 15 weeks, beginning 1 week after the last injection of carcinogen. * $P < 0.05$ versus control; ** $P < 0.001$ versus control; # $P < 0.05$ versus SAHA or LG268 alone.

was not as effective in this paradigm, but it did significantly ($P < 0.05$) reduce the average tumor burden by 35% ($4.6 \pm 0.9 \text{ mm}^3/\text{slide}$) compared with the control group ($7.0 \pm 0.7 \text{ mm}^3/\text{slide}$). CDDO-Ea was more effective than expected in these lung carcinogenesis studies and significantly ($P < 0.05$) reduced the number, size and histopathology of the tumors, even though the dose was reduced to 250 mg/kg diet instead of the 400 mg/kg diet used in previous studies (36,39). The combination of SAHA and CDDO-Ea was more effective than the individual drugs (Figure 5B) and reduced tumor burden by 94% compared with the controls (0.4 ± 0.09 versus $7.0 \pm 0.7 \text{ mm}^3/\text{slide}$, respectively). As a comparison, the combination of SAHA and CDDO-Ea was almost as effective as the combination of CDDO-Ea and the retinoid LG268, which is one of the most potent combinations reported for the prevention of cancer in experimental models (24).

The drug doses used in these lung cancer studies were well below the maximum tolerated dose and thus were well tolerated with no evident toxicities. Mice continued to gain weight throughout the experiment with no significant differences in the final weights per group: control $21.2 \pm 0.6 \text{ g}$, CDDO-Ea $20.1 \pm 0.2 \text{ g}$, SAHA $22.4 \pm 1.0 \text{ g}$, CDDO-Ea + SAHA $20.5 \pm 0.7 \text{ g}$, LG268 $22.9 \pm 0.5 \text{ g}$ and CDDO-Ea + 268 $20.9 \pm 0.5 \text{ g}$.

SAHA binds to the active site of HDACs, resulting in the accumulation of acetylated histones and altering the accessibility of chromatin to transcription factors. This known mechanism of action is thought to be responsible for its anticancer activity. However, concentrations of SAHA between 3 and 30 μM are required to increase acetylation of histone H3 in VC-1 lung cancer cells and in P1343 pancreatic cancer cells (Figure 6A). These high concentrations of SAHA are much higher than needed for the anti-inflammatory activity of SAHA (Figure 1). Although the triterpenoids CDDO-Me and CDDO-Ea decrease expression of cyclin D1 in both VC-1 and P1343 cells (Figure 6B), treatment with SAHA does not change expression of this regulator of cell cycle progression. These results suggest that the activity of SAHA in the animal models was at least in part the result of its anti-inflammatory properties rather than its effects on histone modification or cell proliferation.

Discussion

In this study, we show that the HDAC inhibitor, SAHA, and the synthetic triterpenoids, CDDO-Me or CDDO-Ea, each delay the development of ER- tumors in PyMT mice, prolong survival in the KPC model of pancreatic cancer and reduce tumor burden in a model of

Table I. The combination of the triterpenoid CDDO-Ea and either the HDAC inhibitor SAHA (vorinostat) or the retinoid LG100268 (LG268) prevents lung carcinogenesis in A/J mice injected with vinyl carbamate

	Control	CDDO-Ea (250 mg/kg diet)	SAHA (250 mg/kg diet)	CDDO-Ea + SAHA	LG100268 (45 mg/kg diet)	CDDO-Ea + LG268
Inflated lungs						
Number of mice/group	31	12	12	12	12	12
Average number of tumors/mouse (% control)	15.8 ± 0.7 (100)	$9.1 \pm 1.0^*$ (58)	14.1 ± 1.1 (89)	$9.0 \pm 0.8^*, \ddagger$ (57)	13.1 ± 1.0 (83)	$4.9 \pm 0.9^*, \S$ (31)
Slides						
Number of slides/group	62	24	24	22	24	24
Average number tumors/slide (% control)	3.3 ± 0.2 (100)	$1.6 \pm 0.7^*$ (48)	3.0 ± 0.7 (92)	$1.0 \pm 0.3^*, \ddagger$ (29)	2.2 ± 0.6 (66)	$1.0 \pm 0.4^*, \ddagger$ (32)
Average tumor size, mm^3 (% control)	2.1 ± 0.2 (100)	$1.0 \pm 0.6^*$ (48)	$1.5 \pm 0.2^{\text{¥}}$ (71)	$0.4 \pm 0.09^*, \ddagger$ (19)	$1.0 \pm 0.2^*$ (49)	$0.2 \pm 0.04^*, \S$ (10)
Average tumor burden, mm^3 (% control)	7.0 ± 0.7 (100)	$1.6 \pm 0.9^*$ (23)	$4.6 \pm 0.9^*$ (65)	$0.4 \pm 0.09^*, \ddagger$ (6)	$2.3 \pm 0.4^*$ (32)	$0.2 \pm 0.05^*, \ddagger$ (3)
Histopathology						
Number of low-/medium-grade tumors (% of total tumors)	81 (40)	29 (78)**	29 (40)	18 (86)**	37 (71)**	22 (88)**
Number of high-grade tumors (% of total)	122 (60)	9 (24)	43 (60)	3 (14)	15 (29) *	3 (12)

Female A/J mice were injected intraperitoneally with two doses of vinyl carbamate (0.32 mg/mouse), 1 week apart. One week after the final injection with the carcinogen, mice were fed compounds in diet for 15 weeks. Values are mean \pm standard error of the mean.

* $P < 0.05$ versus control, ** $P < 0.001$ versus control, $\ddagger P < 0.05$ versus SAHA or 268 alone, $\text{¥} P = 0.055$ versus control.

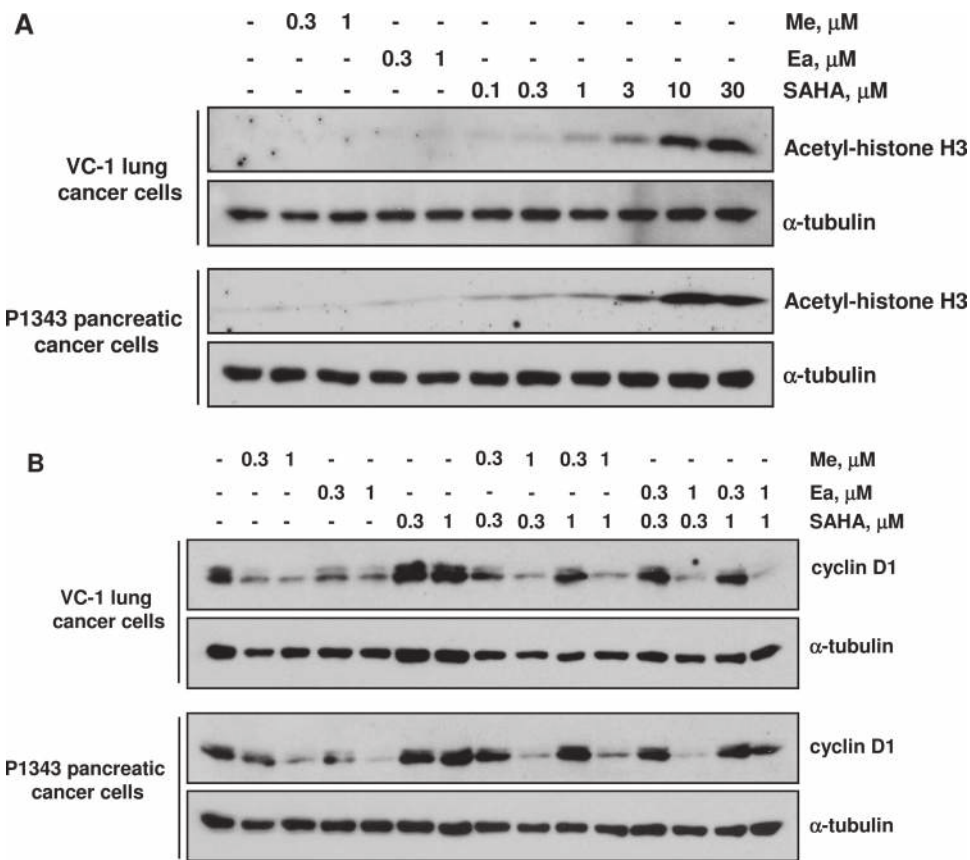


Fig. 6. The effects of SAHA and triterpenoids on histone acetylation and cyclin D1 levels. VC-1 lung cancer cells or P1343 pancreatic cancer cells were treated with the indicated concentrations of drugs for 6 h (A) or 24 h (B) and lysates were immunoblotted with antibodies against acetyl-histone H3, cyclin D1 and tubulin.

lung carcinogenesis. The combination of the two classes of drugs is even more effective than the individual drugs, especially in the PyMT model in which inflammation and TAMs are known to drive tumorigenesis. Additionally, SAHA and the triterpenoids cooperate to suppress *de novo* production of NO in RAW 264.7 cells and in primary peritoneal macrophages from PyMT mice induced with IFN- γ or LPS. SAHA, alone or in combination with CDDO-Me, also inhibits the infiltration of macrophages to the tumor site in PyMT mice while suppressing secretion of the chemokine M-CSF and the pro-angiogenic factor MMP-9 in primary PyMT tumor cells. Moreover, the combination of CDDO-Me and SAHA is more effective than either drug alone at inhibiting secretion of MMP-9.

One important mechanism that at least partially explains the delay in tumor development in PyMT mice fed SAHA is the inhibition of macrophage infiltration to the tumor site (Figure 3A). It is well known that macrophages facilitate tumorigenesis by promoting angiogenesis and metastasis, and macrophage infiltration correlates with poor prognosis in breast cancer patients. TAMs promote tumor initiation, progression and metastasis by secreting a number of pro-angiogenic factors that flip the “angiogenic switch” to drive the formation of the tumor vasculature network necessary for malignant transition (17,53). Genetic depletion of TAMs by conditional knockout of the chemotactic factor M-CSF delays tumorigenesis in PyMT mice, and we have previously demonstrated a similar delay in tumorigenesis with the pharmacologic agent CDDO-Me (28).

In this study, SAHA suppresses levels of the M-CSF in primary PyMT tumor cells (Figure 3B); M-CSF has been shown to be a crucial player in breast tumorigenesis, and inhibitors against M-CSF are currently under development (41,45). Although both SAHA and CDDO-Me inhibit TAM infiltration, SAHA targets different chemokines in primary PyMT tumor cells than CDDO-Me. We have shown that CDDO-Me

suppresses levels of the chemokines CXCL12 and CCL2 (28), but it has no effect on M-CSF. Conversely, SAHA does not affect CXCL12 or CCL2, but instead it suppresses secreted levels of M-CSF. By targeting different chemokines and thus different pathways, the combination of CDDO-Me and SAHA significantly increases the delay of tumor formation in PyMT mice (Figure 2B). Notably, the triterpenoids are potent inducers of the Nrf2 cytoprotective pathway, but SAHA does not activate this pathway. Although there are some overlapping activities, SAHA and triterpenoids appear to inhibit inflammation through different, complementary mechanisms; defining these mechanisms will be an important area of investigation in future studies.

In addition to the importance of TAMs in the PyMT model, the tumor cells themselves also secrete MMP-9, which plays numerous roles in carcinogenesis including tumor initiation, vascularization, invasion and metastasis (54–57). SAHA, CDDO-Me and CDDO-Ea as individual drugs and in combination inhibit secretion of MMP-9 in primary PyMT tumor cells. Inhibition of MMP-9 blocks tumor vascularization (13,54,58), and the MMP-9 inhibitor galardin dramatically suppresses lung metastasis in PyMT mice (54). Oral administration of the COX-2 inhibitor celecoxib to PyMT mice with established tumors also reduces tumor burden (59) and reduces VEGF levels *in vivo*. Because synthetic triterpenoids have been shown previously to suppress levels of COX-2 (24,29), VEGF (60) and angiogenesis (31), we will investigate the effects of SAHA and triterpenoids on angiogenesis and metastasis in established tumors in the PyMT model. Additional studies are also required to confirm that similar mechanisms uncovered in the PyMT model are also observed in the pancreas and lung model, although the less aggressive LSL-Kras^{G12D/+} model would be more appropriate for studying immune infiltration into pancreatic tumors than the KPC mice used in the current experiments.

Because cancer is a polygenic disease (61), multifunctional drugs and drug combinations that broadly target key regulatory processes such as inflammation will be required in order to reduce the number of deaths from breast, lung or pancreatic cancer. As such, the NO assay has proven to be an invaluable tool for screening the triterpenoids (30), rexinoids (50) and now SAHA, either alone or in combination (27), for anti-inflammatory properties that have proven useful for prevention of a variety of experimental cancers. Drug combinations can lower toxicity and enhance potency, especially if the drugs target different pathways. The triterpenoids are multifunctional drugs that are known to target multiple proteins and cell types, including macrophages and epithelial cells (62). Although developed as a HDAC inhibitor, SAHA also has effects on macrophages at lower concentrations than required to target histones or inhibit proliferation of cancer cells. In three different animal models, these drugs were effective and well tolerated, especially as SAHA was used at less than half the maximum tolerated dose reported in other animal studies (51). Although the combination effect was not as striking in the pancreas and lung model as in the PyMT model, the effectiveness of CDDO-Ea alone was more potent than expected in Figure 5B, suggesting that even lower doses of these drugs should be used in future studies. Alternatively, an intermittent approach should be considered, in which high doses of chemopreventive drugs for short, intermittent pulses are used in an attempt to apoptose premalignant cells (63). Taken together, our studies suggest that the combination of a HDAC inhibitor and a synthetic triterpenoid might be a novel, effective and well-tolerated drug regimen for chemoprevention.

Supplementary material

Supplementary Table 1 and Figure 1 can be found at <http://carcin.oxfordjournals.org/>

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