The Novel Triterpenoid C-28 Methyl Ester of 2-Cyano-3, 12-Dioxoolen-1, 9-Dien-28-Oic Acid Inhibits Metastatic Murine Breast Tumor Growth through Inactivation of STAT3 Signaling

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Abstract

We and others have reported that C-28 methyl ester of 2-cyano-3, 12-dioxoolen-1, 9-dien-28-oic acid (CDDO-Me) effectively inhibits the growth of multiple cancer cell types. Our previous studies indicated that prolonged CDDO-Me treatment inactivated extracellular signal-regulated kinase signaling in acute myelogenous leukemia cells. Whether treatment with CDDO-Me has an earlier effect on other proteins that are important for either signal transduction or oncogenesis is unknown. Constitutively activated signal transducer and activator of transcription 3 (STAT3) is frequently found in human breast cancer samples. Constitutively activated STAT3 was shown to up-regulate c-Myc in several types of cancer and has a feedback effect on Src and Akt. To examine the effects of CDDO-Me on STAT3 signaling in breast cancer, we used the murine 4T1 breast tumor model, which is largely resistant to chemotherapy. In vitro, after treatment of 4T1 cells with 500 nmol/L CDDO-Me for 2 h, we found (a) inactivation of STAT3, (b) inactivation of Src and Akt, (c) 4-fold reduction of c-Myc mRNA levels, (d) accumulation of cells in G2-M cell cycle phase, (e) abrogation of invasive growth of 4T1 cells, and (f) lack of apoptosis induction. In in vivo studies, CDDO-Me completely eliminated 4T1 breast cancer growth and lung metastases induced by 4T1 cells in mice when treatment started 1 day after tumor implantation and significantly inhibited tumor growth when started after 5 days. In vivo studies also indicated that splenic mature dendritic cells were restored after CDDO-Me treatment. In summary, these data suggest that CDDO-Me may have therapeutic potential in breast cancer therapy, in part, through inactivation of STAT3.

Introduction

Signal transducer and activator of transcription 3 (STAT3) has frequently been found in human breast cancer samples (1). STAT3-mediated up-regulation of c-Myc levels was proposed to be one of the important mediators of STAT3 oncogenesis (2). c-Myc is a potent oncoprotein that has been found to play a critical role in tumorigenesis (3). The use of an inducible c-Myc model showed tumor growth or suppression, respectively, by controlling c-Myc expression in a murine hepatocarcinoma model (4). Our previous studies and that of others have shown that c-Myc expression is regulated by constitutive activation of STAT3 in mouse breast cancer and other types of cancer cells (5–7). As the immediate upstream regulator of STAT3, Src has been considered to play an important role in breast carcinogenesis. However, we found that Src can be inactivated by STAT3 knockdown in 4T1 cells (5). We have also shown that Akt can be inactivated following STAT3 knockdown in 4T1 cells (5), suggesting that STAT3 is required for maintaining the activation status of Src and Akt. As a downstream target of Src, Akt plays an important role in regulating antiapoptotic proteins and proteins that regulate cell cycle progression (8). However, it is unclear how these intracellular signal transduction proteins interact with each other, and the biological consequences of these interactions remain uncertain. Recent reports provided convincing evidence that constitutively activated STAT3 in breast cancer cells is associated with impaired cell-mediated immunity (9) and that STAT3 activation in cancer cells may allow them to evade host immune surveillance (10). In agreement with these observations, we reported that knockdown of STAT3 completely abolished breast tumor formation in an immunocompetent mouse model (5).

The 4T1 cell line was originally derived from a spontaneous mammary carcinoma in BALB/c mice (11). It has been reported that 4T1 cells mimic the effects of human mammary carcinoma in that morbidity is due to the outgrowth of spontaneous micrometastatic tumor cells that migrate to distant organs relatively early during primary tumor growth (12, 13) and are notoriously chemoresistant (14). Therefore, 4T1-driven breast cancer in BALB/c mice constitutes an appropriate in vivo system for modeling human breast cancer with regard to tumor growth and metastasis. As such, the 4T1/BALB/c mouse system also provides a useful experimental model for exploring the biological effects in mammary tumorigenesis and for studying the effects of small molecules that target signal transduction pathways in vivo.

2-Cyano-3, 12-dioxoolen-1, 9-dien-28-oic acid (CDDO) and its derivative CDDO-Me are synthetic triterpenoids proven to be effective agents in controlling cancer cell growth in preclinical models of leukemias, multiple myeloma, lymphomas, and solid tumors, such as breast, pancreatic, and colon cancer (15–18). CDDO-Me has been shown by us to (a) promote apoptosis in several leukemic cell lines, (b) induce proapoptotic Bax protein as a...
prelude to caspase activation, and (c) suppress the activation of extracellular signal-regulated kinase (ERK) 1/2 with concomitant inhibition of Bcl-2 phosphorylation (18). In the present study, we intended to examine early changes induced by CDDO-Me in mouse breast cancer cells. Our findings indicate that CDDO-Me effectively inhibits STAT3 signaling, which contributes to profound antitumor efficacy of this compound in the in vitro and in vivo models of highly metastatic 4T1 breast cancer cells. These observations hence highlight the novel mechanism of CDDO-Me activity in breast cancer, which is potentially applicable to other types of tumors driven by the activation of STAT3 signaling.

Materials and Methods

Cell line, antibodies, and reagents. The 4T1 cell line was obtained from the American Type Culture Collection. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Anti-phosphorylated Tyr-STAT3 (phosphorylated Tyr705), anti-STAT3, anti-phosphorylated Src (phosphorylated Tyr416), anti-Src, anti-phosphorylated Akt (phosphorylated Ser473), and anti-c-Myc were purchased from Cell Signaling Technology, Inc.; 3D1 and MHC class II antibodies were obtained from eBioscience; anti-β-actin was obtained from Sigma Life Sciences. The Cell Invasion kit was purchased from Chemicon International, Inc. Coelenterazine was obtained from Biotium, Inc.; Trizol was purchased from Invitrogen, Inc. Primers for c-Myc real-time PCR were purchased from Applied Biosystems, Inc. CDDO-Me was provided by Reata Pharmaceuticals, Inc.

Animals. Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratories, Inc. and maintained in M. D. Anderson conventional animal facility.

Construction of lentivirus for Renilla luciferase cDNA transduction. The plasmid was created by inserting the 2-kb Renilla luciferase cDNA fragment from the pMOD-LucSH plasmid (InvivoGen). The lentiviral gene transfer vector pWIP/GFP was a generous gift from Dr. Didier Trono (Geneva University, Geneva, Switzerland). Lentivirus preparation, gene transduction by lentiviral infection, and cell sorting were done as described previously (5).

Western blotting. Western blotting was done as described previously (19).

Cell proliferation assay. Cell viability was determined using a Cell Counting Kit from Eastman Kodak.

Intratumoral CDDO-Me measurements. The tumor samples were placed in 1 mL methanol after being weighed. The samples were homogenized and centrifuged at 4,000 rpm for 10 min. The supernatants containing CDDO-Me were saved, lyophilized, and further reconstituted with 200 μL of 1:1 methanol/water. Samples were spun for 10 min at 13,200 relative centrifugal force, and CDDO-Me concentrations were determined by liquid chromatography-tandem mass spectrometry (LC/MS/MS; ref. 15).

Histologic analysis. For the histologic analysis, breast tumor and lungs from mice treated with/without CDDO-Me were excised and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with H&E. The slides were analyzed under a Nikon Optiphot microscope with a digital capture camera (Microscopy Documentation System 290, Eastman Kodak).

Results

Renilla luciferase is stably expressed in 4T1 cells after lentiviral infection. A lentivirus gene transfer plasmid was constructed as indicated in Fig. 1A, in which Renilla luciferase was expressed from the EF-1α promoter in the pWIP/GFP vector. 4T1 cells were infected with lentivirus carrying both cDNA of Renilla luciferase and GFP. After a single exposure to the virus, 4T1 cells started expressing both GFP and Renilla luciferase.

Figure 1. Transduction of Renilla luciferase into 4T1 cells by lentiviral infection. A, a lentiviral gene transfer vector was constructed. 4T1 cells were infected with lentivirus carrying both cDNA of Renilla luciferase and GFP. B and C, after a single exposure to the virus, 4T1 cells started expressing both GFP and Renilla luciferase. D, image of the luciferin signal as detected by the Xenogen imaging system.
and green fluorescent protein (GFP) were designed in a bicistronic fashion, which allows GFP to function as the marker of gene transduction. After a single exposure to the lentivirus that was carrying cDNAs of Renilla luciferase and GFP, 4T1 cells expressed both Renilla luciferase and GFP. As shown in Fig. 1B and C, 4T1 cells started expressing GFP 72 h after lentiviral infection. Meanwhile, the cells were also expressing high levels of Renilla luciferase (Fig. 1D). After fluorescence-activated cell sorting for selection of GFP(+) cells, the population of Renilla luciferase expressing 4T1 cells was enriched to >90% and used for further studies after expansion.

CDDO-Me blocks 4T1 intracellular signal transduction pathways. We previously showed that, as a major oncogenic regulator, STAT3 was constitutively activated in 4T1 cells (5). In this study, we first examined whether CDDO-Me had an effect on constitutively activated STAT3. As shown in Fig. 2A, we found that STAT3 activity was significantly inhibited by 500 nmol/L CDDO-Me after 2 h, whereas total STAT3 levels remained unchanged. We also examined the effect of CDDO-Me on Src and Akt. We found that both Src and Akt were inactivated, but total Src and Akt levels were not affected. We then examined effects of CDDO-Me on the mRNA level of c-Myc, a known downstream target of STAT3. We found that the level of c-Myc in treated cells was only one third of that in untreated 4T1 cells (Fig. 2B). Using small interfering RNA (siRNA), we observed similar effects, specifically which knocking down STAT3 led to the inactivation of both Src and Akt and reduction of the expression of c-Myc (Fig. 2C).

CDDO-Me does not induce apoptosis in 4T1 cells but blocks cell proliferation in vitro. To determine the functional consequences of STAT3 inhibition by CDDO-Me, we examined the ability of CDDO-Me to induce apoptosis and/or growth inhibition in 4T1 cells in vitro. We observed no significant apoptosis induction in 4T1 cells after CDDO-Me treatment as assessed by Annexin V flow cytometry (Fig. 3A). Furthermore, no significant apoptosis was
detected even at higher doses of CDDO-Me after 6, 24, and 48 h (Fig. 3B; data not shown). However, we found that CDDO-Me completely blocked cell growth at the 500 nmol/L dose level as indicated in Fig. 3C. Therefore, we further analyzed the changes in cell cycle distribution of 4T1 cells after treatment with 500 nmol/L CDDO-Me. As indicated in Fig. 3D and E, 4T1 cells were blocked in G2-M after treatment with CDDO-Me for 24 and 48 h. Rather unexpectedly, when STAT3 was depleted in 4T1 cells using siRNA technology, CDDO-Me induced a profound apoptotic response, with no viable cells remaining at 48 h (Fig. 4F).

**CDDO-Me inhibits invasion of 4T1 cells.** It has been reported that 4T1 cells are highly invasive, which reflects their metastatic ability in vivo. To determine whether CDDO-Me can affect this property of 4T1 cells, we used an in vitro matrix gel invasion assay to evaluate effects on 4T1 cells in the presence of 500 nmol/L CDDO-Me. As shown in Fig. 4, 500 μmol/L CDDO-Me virtually abrogated the invasion of 4T1 in the assay.

**Effect of CDDO-Me on 4T1 breast cancer growth and metastasis in immunocompetent mice.** It has been well established that 4T1 cells generate aggressive primary breast tumors and metastatic tumors in BALB/c immunocompetent mice (21). To study the effect of CDDO-Me on breast tumor formation, we first established the 4T1/rLuc breast tumor model to prove that 4T1 cells expressing Renilla luciferase would not be rejected by the host. As shown in Fig. 5A and B, the ability of 4T1/rLuc cells to form breast tumors was indistinguishable from those formed by parental 4T1 cells (5). We then examined the effect of CDDO-Me on the ability of 4T1 cells to form tumors. CDDO-Me treatment was

![Figure 3.](image)

**Figure 3.** CDDO-Me does not induce 4T1 cell apoptosis but inhibits cell proliferation and cell cycle progression. **A,** induction of apoptosis was analyzed by Annexin V flow cytometry. 4T1 cells did not undergo apoptosis when treated with 500 nmol/L CDDO-ME for up to 6 h. The control cells were treated with the same amount of the solvent DMSO. **B,** dose-finding study indicates that CDDO-Me does not induce apoptosis in 4T1 cells even at 1,000 nmol/L for 6 h. A longer time treatment was also conducted (up to 72 h). However, no significant apoptosis was observed (data did not show). **C,** CDDO-Me at 500 nmol/L blocks the growth of 4T1 cells. **D,** CDDO-Me results in G2 arrest (**p** < 0.01). **D,** effect of CDDO-Me on 4T1 cell cycle after 24 h (middle) and 48 h (right). **E,** statistical analysis of cell cycle effects of CDDO-Me on 4T1 cells. **F,** effects of CDDO-Me in STAT3 knockdown 4T1 cells.
initiated 1 day after inoculation of mice with 4T1 breast tumor cells \((7 \times 10^4\) per mouse), at the dose of 200 mg/mouse at 2-day intervals, for a total of five injections. The results presented in Fig. 5B showed that 4T1 breast tumor formation was completely inhibited by CDDO-Me in five mice (Fig. 5B, right). In contrast, four mice without CDDO-Me treatment developed aggressive breast tumors at the injection site (Fig. 5B, left), which required sacrifice at 35 days. No tumors or tumor metastases were observed in CDDO-Me–treated mice over a 90-day observation period, whereas numerous metastatic tumors in the lung were observed in control animals (Fig. 5C). In addition, the lungs from four untreated mice and from five CDDO-Me–treated mice were analyzed histologically. The results indicated that extensive (50%) metastatic lesions were seen in lungs that were isolated from all control mice, whereas no metastasis was detected in lungs isolated from five CDDO-Me–treated mice (Table 1; Fig. 6). These results show striking antitumor and antimetastatic activity of CDDO-Me treatment in this aggressive murine model of breast tumorigenesis.

Next, we extended our observations by delaying initiation of CDDO-Me treatment from day 1 to day 5 after the breast tumor inoculation. As shown in Fig. 5D, five control mice developed aggressive breast tumors (Fig. 5D, left). In contrast, at day 30 after tumor inoculation, breast tumors were significantly smaller in 10 of 15 CDDO-Me–treated mice in comparison with controls \((P < 0.0001; \text{Fig. 5E})\). Further, 5 of 15 mice were completely tumor-free after CDDO-Me treatment for up to 95 days. CDDO-Me was given only nine times at 2-day intervals (200 mg/mouse). No significant toxicity was observed in CDDO-Me–treated animals. We measured intratumor CDDO-Me concentration from a total six tumor samples, which averaged from 3.8 to 6.4 nmol/L (Table 2).

The effect of CDDO-Me on splenic mature dendritic cells. To assess potential immunologic effects of CDDO-Me on tumor growth, we analyzed mature dendritic cells isolated from the spleens of control or CDDO-Me–treated tumor-bearing mice. As shown in Fig. 7, the number of mature dendritic cells from untreated tumor-bearing mice was two thirds lower than that from normal mice (Fig. 7C). In contrast, the number of mature dendritic cells in spleens from CDDO-Me–treated mice (Fig. 7B) was almost identical to that of normal untreated control mice (Fig. 7A) without tumors. These results suggest that, in the immunocompetent murine 4T1 breast cancer model, CDDO-Me maintained the mature dendritic cell population, which possibly contributed to the potent antitumor and antimetastatic effect of this agent.

Discussion

Our results indicate that CDDO-Me effectively inhibits growth and metastases of the aggressive mouse breast tumor cell line 4T1 both in vitro and in vivo (Figs. 3–7). Mechanistic studies showed that CDDO-Me inactivated STAT3 in 4T1 cells after 2 h (Fig. 2A). In our previous studies, we were able to show that CDDO-Me affected ERK and other intracellular components 6 h after treatment (22). Our present data suggest that STAT3 may be the earliest intracellular target to be affected by CDDO-Me. To our knowledge, this is the first report to show that CDDO-Me is capable of inactivating STAT3.

Our investigations also showed that inactivation of phosphorylated STAT3 by CDDO-Me was associated with inactivation of two other potent oncoproteins, Src and Akt (Fig. 2A). The finding that STAT3 inactivation in breast cancer cells resulted in the inactivation of Src and Akt is consistent with findings of our previous study (5), in which knockdown of STAT3 led to the inactivation of Src and Akt in 4T1 cells. We hypothesize that this phenomenon is associated with the requirement of STAT3 in the complex formed by Src and Akt. It is believed that STAT3 relocalizes from the cytoplasm into the nucleus after the protein is phosphorylated (16). However, phosphorylated STAT3 may also remain and function in the cytoplasm, interacting with other signal transducers, such as Src and Akt, and maintain their function. The mechanisms of the activation of Src and Akt by phosphorylated STAT3 or by STAT3 remain largely unknown. Clearly, further studies are needed to clarify this coactivation and its biological sequelae on breast carcinogenesis.

In addition, we showed that CDDO-Me reduces c-Myc mRNA levels. Overexpressed c-Myc has been reported in many types of cancers and cancer cell lines, and the reduction of c-Myc has been shown to suppress tumor growth in vitro (4). In our model, 4T1 cells expressed a detectable amount of c-Myc, and the mRNA of c-Myc was reduced 4-fold by 500 nmol/L CDDO-Me, suggesting that CDDO-Me down-regulated c-Myc through the STAT3 pathway. In parallel experiments, we observed similar results using siRNA technology, whereby a depletion of STAT3 in 4T1 cells resulted in the inactivation of Src, Akt, and c-Myc down-regulation (Fig. 2C).

Curiously, in STAT3-depleted 4T1 cells, CDDO-Me induced profound apoptosis as shown in Fig. 3F, suggesting that either (a) complete inactivation of STAT3 by combined CDDO-Me and siRNA exposure is required for the execution of the apoptosis program or (b) CDDO-Me has additional intracellular targets, inactivation of which combined with STAT3 blockade causes synergistic apoptotic response. Some of these targets have been elucidated (e.g., nuclear factor-κB and peroxisome proliferator-activated receptor-γ; refs. 23–25). Constitutively activated STAT3 has been widely reported to function as a potent oncoprotein for human breast cancer (1, 21, 26). Clinical data indicate that a large percentage of human breast cancer samples contain activated STAT3 (1). It has been reported that STAT3 promotes the expression of proteins that are directly involved in cancer cell invasion and metastasis, such as glycogen synthase kinase (GSK)-3β and matrix metalloproteinases (MMP; refs. 28, 29). We here provide evidence that CDDO-Me inactivates STAT3 and blocks breast tumor metastases. The mechanisms of this antimetastatic...
effect of CDDO-Me require further investigation; GSK and MMPs are potential candidate targets. In addition, cumulative evidence indicates that constitutively activated STAT3 in cancer cells prompts cancer cells to produce factors that impair the host immune response to cancer cells, suggesting that constitutively activated STAT3 plays an important role not only in cell transformation but also in the interaction between cancer cells and host immune cells (30, 31). A recent study indicated that dendritic cell maturation was inhibited by breast cancer treatment (9, 32). To assess the potential effects of CDDO-Me on established breast tumors, we delayed the timing of treatment initiation from day 1 to day 5 after the tumor cell inoculation. D, left, all untreated mice developed aggressive breast tumors, shown here on day 26 after tumor cell inoculation; right, in contrast, mice that received CDDO-Me (200 μg/mouse, i.v. at 2-d intervals, for a total of eight injections) developed smaller breast tumors or were completely tumor-free after the treatment. E, the difference between the treated and untreated groups was significant (P < 0.001).

Figure 5. CDDO-Me blocks breast tumor growth. A, left, mice were inoculated with 4T1/rLu/GFP cells at the indicated dose, and tumors were visualized using the Xenogen imaging system; right, breast tumors reached about 1.5 to 2.5 cm within 25 to 30 d of injection of 7 × 10^6 cells into the mammary gland fat pad. The experimental group was treated with CDDO-Me beginning 1 d after the tumor cell inoculation at the dose of 200 μg/mouse, i.v. at 2-d intervals, for a total of five injections of CDDO-Me. Mice that received empty liposomes developed breast tumors (left), whereas the CDDO-Me–treated mice did not develop tumors (right). This image was taken on day 20 after the tumor cell inoculation. The treated mice remained tumor-free at the end of the study period, which was 90 d after the tumor inoculation. C, metastatic lung tumors were observed in mice that did not receive CDDO-Me (left), whereas the lung from CDDO-Me–treated mouse was tumor-free (right). To assess the potential effects of CDDO-Me on established breast tumors, we delayed the timing of treatment initiation from day 1 to day 5 after the tumor cell inoculation. D, left, all untreated mice developed aggressive breast tumors, shown here on day 26 after tumor cell inoculation; right, in contrast, mice that received CDDO-Me (200 μg/mouse, i.v. at 2-d intervals, for a total of eight injections) developed smaller breast tumors or were completely tumor-free after the treatment. E, the difference between the treated and untreated groups was significant (P < 0.001).
tumor-bearing mice was only one third of that in normal control splenic tissue; in contrast, in CDDO-Me–treated and tumor-free mice, the mature dendritic cell population was the same as in normal control splenic tissue. These results in conjunction with our previous studies suggest that breast tumors can be suppressed by inactivating STAT3, in part, via restoration of dendritic cell maturation.

The antitumor efficacy of CDDO-Me has been extensively investigated in several other studies (15, 18, 25, 33). In our murine

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<th>Table 1. Mouse histopathology</th>
<th>CDDO-Me treated</th>
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<tr>
<td>Total no. mice</td>
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<td>4</td>
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<tr>
<td>Lung metastatic tumor observed</td>
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<td>Histology diagnosis</td>
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<td>No metastases in lung</td>
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<td>No metastases in liver</td>
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<td>Histology diagnosis</td>
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NOTE: Histopathology of 4T1 breast cancer-bearing mice with and without treatment with CDDO-Me.

<table>
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<th>Table 2. Intratumor levels of CDDO-Me</th>
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NOTE: Duplicate samples from murine breast tumor were homogenized and processed for LC-MS/MS analysis. Standard curve was made by adding 1 mL methanol to similar mass blank mouse kidney tissue and measured by LC-MS/MS analysis.

Figure 6. CDDO-Me blocks lung metastasis. Histologic analysis indicated that massive lung metastatic tumors were seen in lungs from untreated mice. Magnifications, ×100 (A) and ×250 (B). In contrast, no metastatic tumors were seen in lung section from CDDO-Me–treated animals. Magnifications, ×100 (C) and ×250 (D). E and F, primary breast tumor that was induced by 4T1 cells. Magnifications, ×100 (E) and ×250 (F).
model, we observed no induction of tumor cell apoptosis by CDDO-Me but complete inhibition of tumor cell growth, most likely as a consequence of cell signaling and cycle blockade (Fig. 3). Preclinical toxicity study results indicated that CDDO-Me is well tolerated in monkeys and rodents. This finding is supported by our study where no apparent toxicity was observed despite profound antitumor effects. Notably, intratumor CDDO-Me concentrations were above 3 μmol/L levels (Table 2), which by far exceeded concentrations of CDDO-Me required for growth-inhibitory effects in vitro, hence showing excellent tumor tissue accumulation of the compound without obvious systemic toxicity.

To our knowledge, the results presented here are the first evidence that CDDO-Me can inactivate STAT3 in breast cancer cells and can abolish tumor development and metastasis in immunocompetent mice. These results have potential clinical implications because STAT3 is an important target for breast cancer therapy and because CDDO-Me would be an effective agent for inactivating STAT3 and further inhibiting breast tumor growth in vivo. CDDO-Me is currently in phase I clinical trial in patients with solid tumors at M. D. Anderson Cancer Center, and the results of our study strongly suggest breast cancer patients as prime candidates for this novel therapy. Because STAT3 plays an important role in tumorigenesis in different tumor types, we propose that inactivation of STAT3 by CDDO-Me may have therapeutic benefit in other tumors driven by the activation of STAT3 signaling.

Acknowledgments

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References

The Novel Triterpenoid C-28 Methyl Ester of 2-Cyano-3, 12-Dioxoolen-1, 9-Dien-28-Oic Acid Inhibits Metastatic Murine Breast Tumor Growth through Inactivation of STAT3 Signaling

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