CDDO-Methyl Ester Delays Breast Cancer Development in Brca1-Mutated Mice

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Abstract

The breast cancer–associated gene 1 (BRCA1) is the most frequently mutated tumor suppressor gene in familial breast cancers. Mutations in BRCA1 also predispose to other types of cancers, pointing to a fundamental role of this pathway in tumor suppression and emphasizing the need for effective chemoprevention in these high-risk patients. Because the methyl ester of the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Me) is a potent chemopreventive agent, we tested its efficacy in a highly relevant mouse model of BRCA1-mutated breast cancer. Beginning at 12 weeks of age, Brca1Co/Co; MMTV-Cre;p53+/− mice were fed powdered control diet or diet containing CDDO-Me (50 mg/kg diet). CDDO-Me significantly (P < 0.05) delayed tumor development in the Brca1-mutated mice by an average of 5.2 weeks. We also observed that levels of ErbB2, p-ErbB2, and cyclin D1 increased in a time-dependent manner in the mammary glands in Brca1-deficient mice, and CDDO-Me inhibited the constitutive phosphorylation of ErbB2 in tumor tissues from these mice. In BRCA1-deficient cell lines, the triterpenoids directly interacted with ErbB2, decreased constitutive phosphorylation of ErbB2, inhibited proliferation, and induced G0–G1 arrest. These results suggest that CDDO-Me has the potential to prevent BRCA1-mutated breast cancer. Cancer Prev Res; 1–9. ©2011 AACR.

Introduction

The need to find better ways to prevent breast cancer, especially in premenopausal women is self-evident. Therapy of “triple negative” (estrogen receptor, progesterone receptor, and Her2 expression) cancers, which are often associated with a mutation in the BRCA genes, has been particularly disappointing (1, 2). Women who are newly diagnosed with BRCA mutations have no realistic options, other than the personally unsatisfactory possibility of bilateral prophylactic mastectomy or “watchful waiting” with all of its attendant anxieties (3–5). There is thus a major need for new approaches to prevention, especially the development of new drugs that would be safe, free of undesirable side effects, and effective for chemoprevention when given to women over prolonged periods of time (2, 6, 7).

Over the past 30 years, many drugs have been developed for chemoprevention of ER-positive breast cancer, and 2 of these, tamoxifen and raloxifene, are approved by the Food and Drug Administration for clinical use as preventive agents (8, 9). The problem of chemoprevention of ER-negative breast cancer has been more difficult because drugs that block estrogen synthesis or estrogen action have limited or no use for ER-negative malignancies. However, some success has been achieved in experimental models, especially with rexinoids (selective ligands for the nuclear receptors known as RXR) using the MMTV-Her2 neu transgenic mouse model (10–13). The newer development of mice with mutations in both BRCA1 and p53 (14–16) has now made it possible to study chemoprevention (17–19) that is relevant to the disease caused by a BRCA mutation in women. For the past 10 years, our laboratory has been developing new synthetic triterpenoids as agents for both chemoprevention and chemotherapy of cancer (20–22), and in the present article, we now report, for the first time, the successful use of a triterpenoid, namely, CDDO methyl ester (CDDO-Me), for inhibition of breast carcinogenesis induced by mutations of both BRCA1 and p53. Moreover, we also show mechanistic interactions between CDDO-Me and phospho-ErbB2, a known marker for ER-negative mammary tumorigenesis.

Materials and Methods

Reagents and in vitro assay

CDDO-Me and biotinylated triterpenoid (Bt-CDDO) were synthesized as described (23–25). For cell culture studies, compounds were dissolved in dimethyl sulfoxide (DMSO), and controls containing equal concentrations of DMSO (V<0.1%) were included in all experiments. Sources
of reagents and antibodies were as follows: antibodies against p21Waf1/Cip1 and Cdk4 from Santa Cruz Biotechnologies; ErbB2 from Lab Vision; p-ErbB2 and γH2AX from R&D Systems; and cyclin D1 from Cell Signaling Technology. The Brca1 mutant cell line W780 was derived from a mammary tumor in a Brca1Co/Co;MMTV-Cre;p53+/−/C0 mouse containing a targeted deletion of full-length Brca1 (15). W780 cells were cultured in Dulbecco’s Modified Eagle’s Media (DMEM) with 5% FBS (Invitrogen) and were treated with CDDO-Me at the concentrations indicated in the text and in the figure legends. No additional cell testing was done by the authors. For the immunoprecipitation experiments, W780 cells were treated with 3 mmol/L biotinylated triterpenoid for 1 hour and lysed in 100 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100. Total protein (1 mg) was incubated with 50 μL DynaBeads MyOne Streptavidin T1 (Invitrogen) for 1 hour, pelleted, and washed 4 times with Tris-HCl, 1% Triton X-100 buffer. Samples were resuspended in 40 μL Laemmli loading buffer, boiled for 5 minutes to remove the bound proteins from the beads, and analyzed by Western blotting (26).

**Cell-cycle analysis**

Cells were treated with CDDO-Me or DMSO. After trypsinization, cells were fixed in 70% ethanol for 30 minutes at 4°C. The cells were washed twice with PBS, and then incubated for 30 minutes in the dark at 37°C in 1 mL of PBS containing 100 μg propidium iodide and 100 μg RNase A.
After flow cytometry, histograms were generated using Cell Quest and Mod-Fit software.

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. For the serial sacrifice study, mammary glands from Brca1Co/Co;MMTV-Cre;p53 mice (15) at 12, 16, 20, 24, and 28 weeks of age were harvested. For the prevention studies, 12-week-old female Brca1Co/Co;MMTV-Cre;p53 mice (15) were fed powdered 5002 rodent chow (PMI Feeds) or powdered diet containing CDDO-Me (50 mg/kg diet). After 4 days on diet, mammary glands were harvested and whole blood was collected into heparinized tubes. Mammary glands were homogenized in PBS and then all samples were extracted in acetonitrile, separated by reverse-phase liquid chromatography and detected by mass spectrometry as previously described (27). Standard curves were generated by serially diluting known concentrations of CDDO-Me in control blood or tissue homogenate. All samples were within the linear range of the standard curve.

Tissue levels

Six female Brca1Co/Co;MMTV-Cre;p53+/− mice were fed CDDO-Me in diet (50 mg/kg diet). After 4 days on diet, mammary glands were harvested and whole blood was collected into heparinized tubes. Mammary glands were homogenized in PBS and then all samples were extracted in acetonitrile, separated by reverse-phase liquid chromatography, and detected by mass spectrometry as previously described (27). Standard curves were generated by serially diluting known concentrations of CDDO-Me in control blood or tissue homogenate. All samples were within the linear range of the standard curve.

Statistical analysis

When appropriate, data were expressed as means ± SD of at least 3 independent experiments. Statistical analysis for single parametric comparisons was done using the Student t test; the nonparametric data in Fig. 3 were analyzed by the Kruskal–Wallis one-way ANOVA on ranks followed by the Dunn method. Fig. 4A was analyzed using a χ2 test and the Wilcoxon signed rank test, and percentages were analyzed using a Z-test. The criterion for statistical significance was P < 0.05.

Results

CDDO-Me inhibits the phosphorylation of ErbB2 and reduces the expression of cyclin D1 in BRCA1-deficient cells

Deng and colleagues have previously shown that the ErbB2 (Her-2/neu) and cyclin D1 cell-cycle regulatory proteins are overexpressed in the mammary tumors of Brca1 conditional knockout mice (15), and we have shown that synthetic triterpenoids inhibit proliferation and induce apoptosis in cell lines from these knockout mice (22). To determine whether the triterpenoids alter cellular and molecular proteins that regulate proliferation, we treated W780 breast cancer cells with CDDO-Me for 24 hours. CDDO-Me inhibited the expression of p-ErbB2, cyclin D1, and CDK4, an important complex for cell-cycle G1 phase progression, and markedly induced the expression of p21, a well known cell-cycle inhibitor at the G1 phase, in a concentration- and time-dependent manner (Fig. 1A and B). This reduction is ErbB2 phosphorylation is not specific to CDDO-Me, as

Figure 2. CDDO-Me directly interacts with ErbB2. A, W780 cells were treated with 3 μmol/L of biotinylated triterpenoid (BT-CDDO) for 1 hour, triterpenoid-protein complexes were precipitated from cell lysates with strepavidin Dynabeads, and the levels of ErbB2 were assessed by Western blot analysis. Cyclin D1 was used as a negative control. B, the thiol modifying agents NAC and DTT abrogated the interaction between ErbB2 and BT-CDDO-Me. W780 cells were treated with NAC or DTT for 1 hour and/or BT-CDDO-Me for an additional 1 hour. NeutrAvidin precipitations were done as described, and BT-CDDO-ErbB2 complexes were detected by Western blot analysis.
the synthetic triterpenoid CDDO-Imidazolide (CDDO-Im), which inhibits proliferation in BRCA1-deficient cell lines (22), also inhibited the expression of p-ErbB2 and induced the expression of p21 in the same cell line (Fig. 1C). Although CDDO-Im is equipotent to CDDO-Me, the stability and pharmacokinetic profile of CDDO-Me make it more suitable for in vivo studies. CDDO-Me showed similar effects on the same cell-cycle regulators in E18-14C-27 breast cancer cells (data not shown), which express wild type BRCA1 and constitutively overexpress ErbB2 (10). CDDO-Me also induced G0–G1 arrest in the BRCA1-deficient cell lines, thereby reducing the percentage of cells in G2–M (Fig. 1D).

**CDDO-Me directly interacts with ErbB2**

On the basis of a binding model, cysteine-805, located within the catalytic cleft of ErbB2, is ideally positioned for covalent interaction with irreversible inhibitors of ErbB2 that dock in the ATP-binding pocket (28, 29). Because CDDO-Me could potentially form covalent bonds with sulfhydryl groups through Michael addition at carbon 1 or 9 (20, 30, 31), we determined whether a biotinylated analogue of CDDO (Bt-CDDO) could directly interact with ErbB2 (26). W780 cells were treated with Bt-CDDO, and lysates were precipitated with immobilized NeutrAvidin to isolate Bt-CDDO-protein complexes prior to Western blotting. As shown in Fig. 2A, Bt-CDDO directly interacts with ErbB2, p-ErbB2, cyclin D1, and γH2AX.
ErbB2 but not with cyclin D1, which was used as a negative control. To further investigate the role of sulfhydryl groups in the interaction of CDDO-Me with ErbB2, we pretreated with the thiol modifying agents, N-acetylcysteine (NAC) and dithiothreitol (DTT) before treatment with CDDO-Me. As expected, pretreatment of W780 cells with the reducing agents NAC or DTT blocked Bt-CDDO binding to the ErbB2 protein (Fig. 2B), suggesting that CDDO-Me can form covalent adducts with cysteine thiols in ErbB2. The effects of CDDO-Me on the expression of p-ErbB2 and p21 were also significantly reversed when cells were pretreated with DTT (data not shown), indicating that CDDO-Me targets reactive cysteine residues in the ErbB2 protein.

**p-ErbB2 is overexpressed in Brca1-deficient mice**

Our *in vitro* data indicate that CDDO-Me inhibits procession through the cell cycle and reduces phosphorylation of ErbB2, a receptor tyrosine kinase encoding the *Her2/neu* proto-oncogene that stimulates cell growth and differentiation. We next examined the expression of various proteins in the mammary glands of 12- to 28-week-old *Brca1*−/− mice and found increased expression of these molecules with age (Fig. 3A). Blinded analysis revealed that the levels of p-ErbB2, as detected by immunohistochemistry, were significantly increased in the mammary glands from the *Brca1*-deficient mice after 20 weeks of age (Fig. 3B), suggesting that this molecule could be a potential biomarker in breast cancer induced by mutations in *BRCA1*. Phosphorylation of histone H2AX (γH2AX), an early marker of DNA damage, was also increased at 20 and 28 weeks. Although highly variable, Western blot analysis confirmed that these markers were upregulated in the mammary glands from *Brca1*-deficient mice in a time-dependent manner (Fig. 3C) and that p-ErbB2 is expressed at very high levels in tumors from these mice.

**CDDO-Me delays tumor development in the mammary glands and extends survival of Brca1-deficient mice**

Because CDDO-Me is a potent chemopreventive agent in a variety of experimental models (20, 31), we investigated the ability of CDDO-Me to prevent mammary tumorigenesis in *Brca1*-deficient mice. Mice were fed control diet or a diet containing CDDO-Me (50 mg/kg diet), beginning at 12 weeks of age. Mammary tumors were first detected at an average of 30.8 weeks of age in the control group, whereas the average first detection of tumors was significantly (*P < 0.005*) delayed to an average of 36 weeks in the mice fed CDDO-Me (Fig 4A). The average number of tumors per mouse was also reduced (*P < 0.05*) in the mice fed CDDO-Me, with an average of 2.5 tumors per mouse compared with an average of 3.2 tumors per mouse in the control group (Fig. 4B). The average tumor burden per mouse (Fig. 4C) was only 2.9 g in the mice fed CDDO-Me versus 5.1 g in the control mice (*P < 0.001*), and the average lifespan was significantly (*P < 0.005*) higher in the mice fed CDDO-Me compared with the controls (34.2 and 39.3 weeks, respectively). In a pilot feeding study, an average of only 60 ± 30 nmol/L CDDO-Me was detected in whole blood but 1.3 ± 0.6 μmol/L CDDO-Me was detected in the mammary gland.

**CDDO-Me attenuates the expression of p-ErbB2 in tumors from Brca1-deficient mice**

To test the ability of CDDO-Me to inhibit the expression of p-ErbB2 and cyclin D1 *in vivo*, *Brca1*-mutated mice were fed control diet or CDDO-Me (50 mg/kg diet) for 18 to 24 weeks. Fig. 5A shows that the levels of ErbB2, p-ErbB2, cyclin D1, and γH2AX were reduced by almost 50% in the mice fed CDDO-Me; the expression of p-ErbB2 was significantly (*P < 0.05*) decreased as characterized by H&E staining and Western blotting (Fig. 5A and B).

**Discussion**

We have shown the utility of a synthetic triterpenoid, CDDO-Me for inhibition of the process of carcinogenesis in a mouse model that is highly relevant to the development...
of invasive malignancy in women with a mutated BRCA1 gene. The suppression of malignancy in the mouse model (Fig. 4), although only partial, is nevertheless significant, and if it could be translated into clinical practice, would provide meaningful benefit to women who presently have no desirable therapeutic options. The dosage of triterpenoid that has been used in these experiments is apparently free of undesirable side effects in the mice, which continued to gain weight during the course of an almost year-long experiment.

Notably, a recent study has found that the EGFR inhibitor erlotinib delays tumor development in Brca1-deficient mice (17), but erlotinib may be too toxic for clinical chemoprevention studies. CDDO-Me is not a conventional cytotoxic drug and has an excellent safety profile in humans (32). The well-tolerated dose used in these studies was apparently free of undesirable side effects in the mice, which continued to gain weight during the course of an almost year-long experiment.

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Mechanistically, we have shown important interactions between CDDO-Me and the protein target, ErbB2. ErbB2 is a validated target for drugs that are used in cancer chemotherapy, such as Herceptin. Many of the advances in chemotherapy of breast cancer have relied on the ability of drugs to modulate the activity of ErbB2, which undergoes phosphorylation to become biologically active. The biological relevance to carcinogenesis induced by BRCA1 mutation is shown here in Fig. 3A which indicates that increased phosphorylation of ErbB2 occurs in mice as early as 16 weeks of age. From a pharmacologic perspective, we have shown that CDDO-Me inhibits phosphorylation of ErbB2 in cell cultures of breast cancer cells having a BRCA1 mutation; doses between 300 and 1,000 nmol/L are effective in vitro, and concentrations of 1.5 μmol/L can be obtained in the mammary glands in vivo. Furthermore, CDDO-Me was able to suppress the expression of phospho-ErbB2 in actual tumors in mice that received this drug by chronic administration in the

![Graph A](Image 1)

**Figure 4.** CDDO-Me delays tumor development in Brca1-deficient mice. A, beginning at 12 weeks of age, Brca1Co/Co;MMTV-Cre;p53+/− mice were fed powdered control diet or CDDO-Me (50 mg/kg diet). Mice were palpated weekly, and no tumors were found before the mice were 20 weeks old. The CDDO-Me–treated groups was significantly (P < 0.05) different than the control group for weeks 26 to 47. n = 33 mice in the control group and n = 15 in the CDDO-Me group. *, P < 0.05 versus control; Me, CDDO-methyl ester. Mean ± SE of the average number of tumors per mouse (B) and the average tumor burden per mouse (C).
diet; levels of p-ErbB2 were reduced almost 50% in tumors obtained from treated mice (Fig. 5A and B). Finally, in experiments with a biotinylated analog of CDDO-Me, we have also shown that the triterpenoid directly interacts with ErbB2, presumably by Michael addition with a reactive cysteine at the catalytically active ATP binding pocket of this protein. It is well established that CDDO-Me and related triterpenoids are potent agents for Michael addition, although this is not a random process. Rather, the pentacyclic scaffold of CDDO-Me, together with the exocyclic methyl groups of this triterpenoid, provide a highly stereospecific platform for interaction with unique cysteine residues on target proteins (20, 30, 31). This is undoubtedly an important consideration in the relative safety of the use of these triterpenoids for chemoprevention.

In addition to the above mechanism, there are undoubtedly other mechanisms that contribute to the useful chemopreventive activity of CDDO-Me. It has previously been shown in both cell culture and in vivo experiments that CDDO-Me is a potent antiangiogenic agent (38). Furthermore, recent proteomic studies have shown that the same biotinylated analog of CDDO-Me used here has multiple protein targets, most notably the mTOR complex and several of the nuclear receptors in the steroid receptor superfamily (39). The separate and individual contributions of each of these targets are difficult to determine in an in vivo context. Moreover, we cannot be certain how much of the chemopreventive activity of CDDO-Me is due to effects on stromal cells that comprise a particularly large fraction of the total cells in a carcinoma of the mammary gland (40–42).

The ultimate application of the results we have obtained here for prevention of breast cancer in women at exceptionally high risk still requires further development. It is most likely that CDDO-Me will be most effective if used in combination with other chemopreventive agents (6, 21). Whether such agents will turn out to be PARP inhibitors, rexinoids, or other anti-inflammatory agents remains to be determined. The practical use of CDDO-Me (generically known as bardoxolone methyl) for successful treatment of advanced diabetic nephropathy in phase 2 clinical trials (32, 43) indicates that synthetic oleanane triterpenoids can be given safely to patients in a useful manner. Considering the clinical problems facing young women who are newly diagnosed with a BRCA mutation, there is now a compelling need to push this area of research to the point that it becomes clinically practical.

Disclosure of Potential Conflicts of Interest

M.B. Sporn has commercial research grant in Reata Pharmaceuticals, Inc.; M.B. Sporn and K. Liby have patent interest in synthetic triterpenoids. No potential conflicts of interest were disclosed by other authors.

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