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

Eun-Hee Kim1, Chuxia Deng3, Michael B. Sporn1, Darlene B. Royce1, Renee Risingsong1, Charlotte R. Williams1, and Karen T. Liby2

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;

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+/−/Co 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 μmol/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 Laemmlı 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.

Figure 1. CDDO-Me inhibits the phosphorylation of ErbB2 and expression of cyclin D1 in BRCA1-deficient cells. W780 cells were treated with different concentrations (A) of CDDO-Me (A, B) or CDDO-Im (C) for the indicated time periods (B, C) and soluble protein extracts were subjected to SDS-PAGE and Western blotting with the indicated antibodies. Glyceraldehyde 3-phosphate dehydrogenase and α-tubulin were used as loading controls. Cell-cycle analysis (D) of W780 cells treated with CDDO-Me for 24 hours and analyzed by flow cytometry.
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 Brcα1<sup>Co/Co</sup>;MMTV-Cre;p53<sup>−/−</sup> mice (15) at 12, 16, 20, 24, and 28 weeks of age were harvested. For the prevention studies, 12-week-old female Brcα1<sup>Co/Co</sup>;MMTV-Cre;p53<sup>−/−</sup> mice were fed powdered 5002 rodent chow (PMI Feeds) or powdered diet containing CDDO-Me (50 mg/kg diet) and were palpated weekly for tumors. Mammary glands and tumor samples were fixed in neutral-buffered formalin and embedded in paraffin blocks according to standard procedures, and sections were stained with hematoxylin and eosin (H&E). After deparaffinization and rehydration, antigen retrieval was done by boiling in citrate buffer (pH 6.0). After cooling, slides were incubated for 60 minutes at room temperature with the following mouse monoclonal antibodies: ErbB2 (1:100; Neomarkers, Lab Vision Corporation), p-ErbB2 (1:300; R&D Systems), cyclin D1 (1:100; Cell Signaling Technology), or γH2AX (1:100; Immunotech, R&D Systems). All slides were developed with 3,3′diaminobenzidine followed by hematoxylin counterstaining. Scoring was done by the first author, who was blinded as to the primary antibodies and the treatment groups. For ErbB2, p-ErbB2, cyclin D1, and γH2AX, the percentage of positively stained cells was estimated. Cases with 5% or fewer positively stained cells were scored as 1, 2 for 5%–20%, 3 for 20%–50%, 4 for 50%–80%, and 80% or more stained cells were denoted as 5.

Tissue levels
Six female Brcα1<sup>Co/Co</sup>;MMTV-Cre;p53<sup>−/−</sup> 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 χ<sup>2</sup> 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 Brcα1 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 G<sub>1</sub> phase progression, and markedly induced the expression of p21, a well known cell-cycle inhibitor at the G<sub>1</sub> phase, in a concentration- and time-dependent manner (Fig. 1A and B). This reduction is ErbB2 phosphorylation is not specific to CDDO-Me, as...
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.
ErbB2 but not with cyclin D1, which was used as a negative control. To further investigate the role of sulphydryl 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 progression 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−/−, MMTV-Cre;p53+−/− 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 tumors from 35-week-old Brca1−/− mice. Western blot analyses confirmed that these markers were upregulated in the mammary glands from mice 12, 16, and 20 weeks of age and in mammary tumors from 35-week-old Brca1−/− 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−/− 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.

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 BRCA 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
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.

Grant Support

These studies were supported by the Breast Cancer Research Foundation and the NIH (RO1 CA78814).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 14, 2011; revised August 22, 2011; accepted September 13, 2011; published OnlineFirst September 20, 2011.
References

Cancer Prevention Research

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

Eun-Hee Kim, Chuxia Deng, Michael B. Sporn, et al.

Cancer Prev Res  Published OnlineFirst September 20, 2011.

Updated version  Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-11-0359

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.