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CDDO-Imidazolide induces DNA damage, G2/M arrest and apoptosis in BRCA1-mutated breast cancer cells

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Abstract

Breast cancer-associated gene 1 (BRCA1) protein plays important roles in DNA damage and repair, homologous recombination, cell-cycle regulation, and apoptosis. The synthetic triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Imidazolide, CDDO-Im) is a promising anticancer and chemopreventive agent with potent anti-proliferative and apoptotic activities against a wide variety of cancer types. However the mechanisms responsible for the selective apoptotic effects of CDDO-Im in cancer cells remain elusive. In the present work, CDDO-Im induced G2/M arrest and apoptosis in BRCA1-mutated mammary tumor cell lines. Prior to the induction of apoptosis, CDDO-Im induced DNA damage and the phosphorylation of H2AX followed by activation of the DNA damage response. Moreover, CDDO-Im also induced the generation of reactive oxygen species (ROS), which is associated with the induction of DNA damage, in both mouse and human tumor cells containing a *BRCA1* mutation. The inhibition of ROS generation by uric acid prevented the induction of DNA damage by CDDO-Im. Furthermore, treatment with CDDO-Im did not induce ROS in non-malignant MCF-10A breast epithelial cells or in E18-14C-27 breast cancer cells with wild-type *BRCA1* genes and was not cytotoxic to normal mouse 3T3 fibroblasts, highlighting a selective therapeutic potential of CDDO-Im for BRCA1-associated breast cancer cells. Altogether, our results demonstrate that CDDO-Im induces ROS and subsequent DNA damage, thereby facilitating the activation of the DNA damage checkpoint, G2/M arrest and finally apoptosis in BRCA1-mutated cancer cells. The particular relevance of these findings to the chemoprevention of cancer is discussed.

Introduction

The *breast cancer-associated gene 1* (*BRCA1*) is the most frequently mutated tumor suppressor gene found in familial breast cancers, and 27 missense variants, affecting 15 codons and over 670 truncating mutations have been identified for *BRCA1* (1). *BRCA1* mutations also predispose to other types of cancers, pointing to a fundamental role of this pathway in tumor suppression. BRCA1 is a large protein with multiple functional domains and associates with a number of proteins that are involved in many important biological processes/pathways (2). BRCA1 plays important roles in DNA damage and repair response,

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Disclosure of Potential Conflicts of Interest

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homologous recombination, cell-cycle regulation, protein ubiquitination and apoptosis (3). Loss of BRCA1 causes a defective DNA repair response and G2/M cell cycle checkpoint. Because of their high risk for developing invasive cancer, BRCA1-mutation carriers have special needs for effective preventive and therapeutic agents.

We used primary cell lines from mammary tumors of *Brca1* $^{Co/Co}$*; MMTV-Cre; p53*$^{+/-}$ mice containing a targeted deletion of full-length Brca1 in this study as the tumors and cell lines from these mice mimic human *BRCA1*-mutated breast cancer as shown by extensive microarray experiments comparing mouse and human breast tumors. Gene set enrichment analysis (GSEA), used to search for shared relationships between mouse and human *BRCA1* tumors, showed that functional gene sets exhibit similar levels of correlation between gene expression and the class labels (i.e., BRCA1-proficient vs. BRCA1-deficient) in both species. Hence, gene sets that were predictive for the human tumor samples were also likely to be predictive for the mouse samples (4). Therefore, it is likely that studies using primary cell lines from mammary tumors of *Brca1* $^{Co/Co}$*; MMTV-Cre; p53*$^{+/-}$ mice should have significant value for preclinical evaluation of new drugs.

Synthetic triterpenoid derivatives of oleanolic acid, including 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), CDDO-imidazolide (CDDO-Im) and CDDO-methyl ester (CDDO-Me), have strong anti-inflammatory and apoptotic activity in various cancer cells and are effective for the prevention and treatment of cancer in a variety of preclinical animal models (5). Among them, CDDO-Im is known as one of the most potent cytoprotective agents to induce phase 2 enzymes via Nrf2-ARE signaling in several tissues (5). In this study, CDDO-Im inhibits proliferation and induces apoptosis in BRCA1-deficient mammary cancer cells. However, precise molecular mechanisms that initiate the selective induction of apoptosis by triterpenoids in cancer cells remain incompletely clarified (6). Some reports propose that triterpenoids induce apoptosis via the intrinsic cell death pathway, whereas others have found that other triterpenoids act through the extrinsic apoptotic pathway. Here, we have investigated the relationship among CDDO-Im, reactive oxygen species (ROS), DNA damage and apoptosis in BRCA1-mutated breast cancer cells. Our results demonstrate that CDDO-Im rapidly and selectively induces intracellular ROS, which causes DNA damage and the activation of checkpoint signaling. In particular, we show that a target of CDDO-Im is checkpoint kinase1 (Chk1), which functions as a feedback link between ROS and DNA damage. This is the first report to show that the ability of CDDO-Im to inhibit BRCA1 mutant tumor cell growth is associated with oxidative DNA damage followed by the activation of the G2/M checkpoint pathway, triggering selective apoptosis of the tumor cells. Thus, CDDO-Im would be expected to have beneficial effects in the prevention or treatment of BRCA1-mutated breast cancer.

Materials and Methods

Reagents

CDDO-Im and other derivatives (CDDO-Me, CDDO-EA, and CDDO-TFEA) were synthesized as described (7–9). For cell culture studies, compounds were dissolved in DMSO, and controls containing equal concentrations of DMSO (<0.1%) were included in all experiments. Sources of reagents and antibodies were as follows: H_2DCFDA from Molecular Probes (Eugene, OR); Chk1 siRNA, scrambled siRNA and antibodies against $p21^{Waf1/Cip1}$ from Santa Cruz Biotechnologies, (Santa Cruz, CA); γH2AX from R&D systems (Minneapolis, MN); pChk1, pChk2 and pCdc2 from Cell Signaling Technology (Beverly, MA); PARP from Upstate (Millipore, Billerica, MA). UCN-01 and uric acid were obtained from Sigma Co (St. Louis, MO). Uric acid was solubilized in 1 M NaOH with heat.

Cell lines and cell culture

The Brca1 mutant cell lines W0069 and W780 were derived from mammary tumors of *Brca1* $^{Co/Co}$*; MMTV-Cre; p53*$^{+/-}$ mice containing a targeted deletion of full-length Brca1 (10). To generate a new cell line from mammary tumors of *Brca1* $^{Co/Co}$*; MMTV-Cre; p53*$^{+/-}$ mice, a tumor was minced with a scalpel and digested in phosphate buffered saline (PBS) supplement with collagenase (300 units/ml, Sigma) for 30 min at 37 °C with gentle agitation from a stir bar. The cell suspension was filtered through a 40 μm Cell Strainer (BD Bioscience), centrifuged at 220 g for 10 min, and plated in DMEM + 20 % fetal bovine serum. After 10 passages in this medium, a stable cell line (B8701) was obtained and then used for mechanistic studies. All mouse Brca1 mutant cell lines (10) were cultured in DMEM with 5 % FBS and supplemented with antibiotics (Invitrogen, Carlsbad, CA); HCT1937 cells (ATCC) were grown in RPMI with 10% FBS; MCF-10A cells (ATCC) were cultured in DMEM/F12 + 5% HS; and E18-14C-27 cells (provided by Powel Brown, M.D. Anderson Cancer Center) and 3T3 cells (ATCC) were grown in DMEM with 10% FBS. Cells were treated with CDDO-Im dissolved in DMSO in a 10 mmol/L stock solution and used at the final concentrations indicated in the text and in figure legends.

Cell proliferation, cytotoxicity and apoptosis assay

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were seeded into 48-well plates at 1–2 × 10^4 cells/well overnight before synthetic triterpenoids were added. Then the cells were incubated with different concentrations of triterpenoids for 24 h. Cells were incubated with MTT for 2 h (Sigma, St. Louis, MO) and read at OD570. For proliferation assays, cells were treated with compounds for 24 h, pulsed with ^{3}H-thymidine for 2 h, and its incorporation into cells measured by scintillation counting. Apoptosis was analyzed by fluorescence-activated cell sorting using the TACS Annexin V-FITC Apoptosis Detection kit (R&D Systems, Minneapolis, MN) and Cell Quest software (Becton Dickinson, San Jose, CA) or by Western blotting with PARP antibodies.

Cell cycle analysis

Cells were treated with CDDO-Im or DMSO as a control. After trypsinization, cells were fixed in 70 % ethanol for 30 min at 4 °C. The cells were washed twice with PBS, and then incubated for 30 min under dark condition at 37 °C in 1 ml of PBS containing 100 μg propidium iodide and 100 μg RNase A. The flow cytometric analysis was performed and the proportion of cells was assessed by the histograms generated using the computer program, Cell Quest and Mod-Fit.

Small interfering RNA

Small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) and transfected into W780 cells using Lipofectamine™ 2000 according to the manufacturer's procedure (Invitrogen, Carlsbad, CA).

Determination of cellular ROS

Cellular ROS contents were measured by incubating the control or drug treated W0069, W780, B8701, or NIH3T3 cells with 10 μM H_2DCFDA for 30 min, followed by flow cytometry using a FACS Calibur equipped with Cell Quest software.

Comet assay

DNA damage was measured using single cell gel electrophoresis (comet assay) (Trevigen, Gaithersburg, MD). After electrophoresis, the slides were stained with silver and then covered with 100 μl of 5 % acetic acid to stop the reaction prior to drying in the dark overnight. Measurement of mean tail moment was from 100 cells/slide from 15–20

randomly selected fields representing the whole area of each slide. Comet tail moments were determined using Comet Assay IV software (Perceptive Instruments; Suffolk, UK).

Statistical analysis

When necessary, data were expressed as means ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's *t* test. The criterion for statistical significance was $p < 0.05$.

Results

Triterpenoids inhibit proliferation and induce apoptosis in BRCA1-mutated cancer cells

To determine the cytotoxic activity of synthetic triterpenoids against cultured BRCA1-mutated breast cancer cells, W780 and W0069 cells were treated with CDDO-Im, CDDO-Me and CDDO- trifluoroethyl amide (TFEA) for 24 hours. CDDO-Im was the most potent inhibitor of cellular proliferation, as measured by ^{3}H-thymidine incorporation (Fig. 1A). CDDO-Im inhibited proliferation in a concentration-dependent way in all three BRCA1-mutated human breast cancer lines tested (Fig. 1B). To clarify whether the inhibition of cell growth by CDDO-Im is attributed to its ability to induce apoptosis, we examined the effect of CDDO-Im on the staining of FITC-Annexin V, a biochemical hallmark of apoptosis. CDDO-Im induced apoptosis in three different BRCA1-deficient cancer cells but had no effect in normal mouse 3T3 fibroblasts (Fig. 1C). W780 and B8701 were more sensitive to the apoptotic stimuli of CDDO-Im than W0069 cells. Treatment of W780 cells with CDDO-Im caused cleavage of poly(ADP-ribose) polymerase (PARP) (Fig. 1D) and significantly inhibited PARP activity (data not shown). We also performed the proteome profiler antibody array analysis (R&D Systems) which can measure the relative levels of expression of 35 apoptosis-related proteins. We found that CDDO-Im significantly induced cleavage of caspase-3 and the expression of HSP70, and down-regulated the expression of claspin, survivin, Bclx and Bad (supp. 1).

CDDO-Im induces DNA damage signaling and G2/M arrest in BRCA1-mutated cancer cells

To investigate further the inhibitory effect of CDDO-Im on cell growth, we examined the cell cycle distribution in W780 cells by flow cytometry. As shown in Fig. 2A and B, quantitation of cells in G1, S, and G2/M phase of the cell cycle showed that W780 cells accumulated in G2/M phase after exposure to CDDO-Im, whereas the cell population in G1 was decreased in a concentration-dependent manner. Collectively, these observations suggest that CDDO-Im causes G2/M arrest in W780 cells. Chk1 has emerged as the part of the cellular machinery that recognizes and responds to DNA damage, the signaling cascade that specifically regulates cell cycle arrest after DNA damage (11). Because Chk1 is known as one of the most important member in the G2/M DNA damage checkpoint (12), the effects of UCN-01 on cell cycle delay were investigated in W780 cells exposed to CDDO-Im (Fig. 3A). The staurosporin analog UCN-01 is a potent inhibitor of Chk1 but not Chk2 (13). Quantification of the percentage of cells in each phase of the cell cycle showed that UCN-01 decreased the proportion of CDDO-Im-treated W780 cells in G2/M. To confirm that Chk1 is required for G2/M arrest, W780 cells were transfected with either control or Chk1 small interfering RNA (siRNA) before CDDO-Im-stimulated cell cycle analysis (Fig. 3B). Protein immunoblotting confirmed siRNA specificity for Chk1 in W780 cells (data not shown). Chk1 siRNA abolished the effect of CDDO-Im to induce G2/M arrest in these cells (Fig. 3C), indicating that activation of Chk1 might be crucial for G2/M arrest induced by CDDO-Im.

To assess further the involvement of the G2/M checkpoint pathway, we conducted immunoblotting using antibodies recognizing phospho-specific Chk1, Chk2, Cdc2 or p21

(Fig. 3D). We chose these particular proteins because they are known to regulate cell-cycle arrest. Treatment of BRCA1-mutated breast cancer cells with either CDDO-Im or CDDO-Me markedly increased phosphorylation of Chk1 at Ser345 and Chk2 at Thr68. Several reports suggest that $p21^{waf1/Cip1}$ regulates the entry of cells at the DNA damage-induced G2/M checkpoint as an inhibitor of cyclin-dependent protein kinases (CDK) and induces apoptosis (14,15). Western analysis revealed that treatment with synthetic triterpenoids for 24 h resulted in a marked induction of the expression of $p21^{waf1/Cip1}$, indicating its involvement in CDDO-Im-mediated G2/M arrest. The activation of Cdc2 (also named as CDK1) is the rate-limiting factor for cells to enter into mitosis, whereas its inactivation leads to G2/M arrest (15). Cdc2 is activated by phophorylation of Thr160 and the dephosphorylation of Thr14 and Tyr15. As shown in Fig. 3D, CDDO-Im and CDDO-Me induced dephosphorylation of Cdc2 at Tyr15 in W0069 and W780 cells. Synthetic triterpenoids also induced the activation of G2/M checkpoint signaling in our new primary cell line B8701 (data not shown). Next, we determined the kinetics for this activation of the G2/M checkpoint by treatment with CDDO-Im for varying times in W780 cells. As early as 2 h treatment with CDDO-Im resulted in an increase in the phosphorylation of Chk1 protein (data not shown). Up-regulation of p21 and phosphorylation of Chk2 were observed 4 h after treatement with CDDO-Im during the time course (data not shown). Collectively, these results indicate that synthetic triterpenoids activate Chk1 and Chk2 kinases and block the G2/M phase transition.

CDDO-Im induces DNA damage in W780 cells

To investigate the mechanisms of G2/M checkpoint signaling activation by CDDO-Im, the genotoxicity of this compound was investigated using the alkaline comet assay to measure DNA damage and Western blotting to monitor phosphorylation of the nuclear histone H2AX, a sensitive marker for breaks of double stranded DNA. As shown in Fig. 4A, the BRCA1-deficient W780 cells showed greatly increased levels of DNA damage after 1 h of exposure to CDDO-Im. However, CDDO-Im failed to induce DNA damage in normal mouse 3T3 fibroblasts. Interestingly, CDDO-Me did not induce DNA damage in W780 cells under identical conditions (data not shown). Tail Moment, a damage measurement method combining the amount of DNA in the tail with the distance of migration was used as the measure of DNA damage. We also focused on the phosphorylation on serine 139 of histone H2AX (γH2AX), which is an early molecular event in response to DNA damage and a marker of double strand breaks (16). As shown in Fig. 4B, induction of γH2AX was barely detectable in CDDO-Im-treated NIH3T3 cells while it was clearly induced by CDDO-Im in W780 cells (Fig. 4B). These observations suggest that CDDO-Im causes DNA damage which is recognized by the cell prior to triggering G2/M DNA damage response pathways.

The induction of ROS generation is required for the induction of DNA damage by CDDO-Im in BRCA1-mutated cancer cells

To define the mechanisms contributing to the DNA damage caused by CDDO-Im, we first examined the effect of synthetic triterpenoids on intracellular ROS levels in W780 cells. As shown in Fig. 5A, exposure of cells to CDDO-Im (0.1 – 3 μM) for 1 h caused a significant concentration-dependent increase in ROS accumulation. In contrast, CDDO-Me did not cause any increase in cellular ROS levels. The ability of CDDO-Im to elevate intracellular ROS was also observed in two other BRCA1-mutated breast cancer cell lines, W0069 and B8701 (Fig. 5B) and in HCT1937 human BRCA1-mutated breast cancer cells (Supp. 2). Accumulating evidence suggests that, compared with their normal counterparts, many types of cancer cells have increased levels of ROS and a decreased capacity to eliminate ROS (17). Compared to NIH3T3 cells, W780 cells exhibited higher basal ROS content, as quantified by flow cytometry (Fig. 5C). Importantly, CDDO-Im caused ROS production in W780 cells, whereas there was no significant change in H_2DCFDA fluorescence after

treatment with CDDO-Im in NIH3T3 cells. Although CDDO-Im also induced apoptosis of E18-14C-27 breast cancer cells, which were derived from transgenic mice overexpressing the erbB2 protein in the mammary gland but have wildtype BRCA1 expression, it did not induce ROS formation or G2/M arrest in these cells (Supp. 3). We also found similar effects on ROS generation with this triterpenoid in MCF-10A and MCF-10AT1 (18), human breast epithelial and Ha-*ras* transfected cells, respectively. MCF-10AT1 cells transformed with Ha-*ras* exhibited a significant increase of ROS after treatment with CDDO-Im compared to their parental MCF-10A cells (data not shown). However, CDDO-Im does not induce ROS in the MCF-10A cells (Supp. 4), which also express wildtype BRCA1. These results support previous reports showing that oncogenically transformed cells are more sensitive to ROS-mediated damage due to their high basal ROS generation (19). Transformation of ovarian epithelial cells with H-*Ras* and *Bcr-Abl* led to increased basal ROS content and elevated ROS generation in response to a ROS-inducing agent compared to their parental cells (19).

The above observations suggest that ROS might mediate the anticancer activity of CDDO-Im in BRCA1-mutated breast cancer cells. To further test whether CDDO-Im could induce ROS-mediated DNA damage in BRCA1-mutated cells, we treated W780 cells with uric acid, an antioxidant acting as an electron donor followed by CDDO-Im. Indeed, uric acid decreased ROS levels induced by CDDO-Im (Fig. 6A) and abrogated CDDO-Im-induced DNA damage (Fig. 6B) in W780 cells, suggesting DNA damage induced by this triterpenoid was a response to ROS stress. To determine whether Chk1 is also responsible for the accumulation of ROS, as well as G2/M arrest in CDDO-Im-treated W780 cells, we employed Chk1 siRNA to knock down the c*hk1* gene expression (Fig. 6C). Loss of Chk1 prevented the accumulation of ROS in response to CDDO-Im in W780 cells, showing that Chk1 is required for the CDDO-Im-induced increase in ROS levels.

Discussion

Although CDDO-Imidazolide was first shown in 2003 (8) to inhibit DNA synthesis and to be useful for treatment of melanoma and leukemia in mice, this is the first report that any synthetic oleanane triterpenoid induces DNA damage and activates cell cycle checkpoint signaling in BRCA1-mutated breast cancer cells. In the present study, we have demonstrated that CDDO-Imidazolide induced ROS accumulation, causing DNA damage, G2/M arrest and finally apoptosis in these cancer cells. Cancer cells usually exhibit increased levels of intracellular ROS, which in turn can initiate a vicious cycle in which ROS induce gene mutations leading to further metabolic malfunction and ROS generation (17,20). ROS cause oxidative damage to DNA, proteins, lipids and other cellular components and therefore are a significant cellular stress (20). In line with this notion, a proposed therapeutic strategy against cancer is to treat cancer cells with pharmacological agents that have pro-oxidant properties, which increase the intracellular ROS generation to a toxic threshold that triggers cell death in the cancer cells without harming normal cells (17). This hypothesis is strengthened by the recent findings that ROS-generating agents showed selective toxicity in tumor cells with increased endogenous ROS, raising oxidative stress over the threshold of toxicity as antioxidant systems become overwhelmed both *in vivo* and *in vitro* (19,21,22).

In this study, we used a relatively high dose of CDDO-Im (1 μM for 1 h) compared to much lower doses used in previous reports that have focused on the anti-inflammatory properties of the triterpenoids (23,24). However, this higher dose is commonly used to induce apoptosis of cancer cells (25,26). The purpose of this study is to show the ability of CDDO-Im to induce apoptosis through generation of ROS and DNA damage. As noted previously, low concentrations of triterpenoids are cytoprotective and anti-inflammatory, whereas high concentrations are apoptotic (5,24). These differences in activity appear to be correlated with increasing induction of oxidative stress as concentrations of triterpenoids are increased

(5). These higher concentrations of triterpenoids are still relevant as we have reported concentrations of CDDO-Im in tissue of nearly 1 μM (27), and tissue levels well above 1 μM can be safely achieved *in vivo* with other triterpenoids with better pharmacokinetic properties.

Furthermore, CDDO-Im selectively increased intracellular ROS levels and subsequently induced apoptosis in mouse and human BRCA1-mutated breast cancer cells, but was unable to induce ROS production in non-malignant MCF-10A breast epithelial cells, in NIH3T3 fibroblasts, or in E18-14C-27 cells; these three cell lines all express a wildtype BRCA1 gene. Moreover, CDDO-Im induced a significant increase in ROS in Ha-*Ras* transfected human breast epithelial cells but did not increase ROS in the parental MCF-10A cells. Therefore, CDDO-Im could be considered as a possible therapeutic compound to selectively cause ROS stress overload in cancer cells.

The G2/M checkpoint plays an important role in cellular response to genotoxic stimuli. The G2/M checkpoint prevents cells from entering mitosis when DNA is damaged, providing an opportunity for repair and stopping the proliferation of damaged cells, which helps to maintain genomic stability (28). As the signal transducer to mediate DNA damage signal to the downstream effectors, Chk1 is required for cell cycle arrest in response to damaged DNA. Transfection with Chk1 siRNA revealed that Chk1 is essential to cell cycle arrest in G2/M phase induced by CDDO-Im in W780 cells. This is consistent with the observations with UCN-01, a Chk1 inhibitor. Interestingly, knock down of *chk1* gene expression using Chk1 siRNA prevented ROS generation in CDDO-Im-treated BRCA1-mutated breast cancer cells. This result supports the hypothesized vicious cycle of ROS production that, once initiated, leads to accumulation of gene mutations, which in turn exacerbates ROS generation. In this study, the high level of ROS induced by CDDO-Im in BRCA1-deficient cells caused DNA damage, which subsequently activated Chk1 and G2 checkpoint. However, the signal to repair damaged DNA may not be transmitted to downstream effectors such as p53, cdc2 or cdc25 in Chk1 siRNA-transfected cells. Accordingly, the cells without Chk1 would not recognize or restore DNA damage, and would not trigger a vicious cycle. In addition, it is reported that Chk1 is associated with chromatin, and that a phosphorylation event is likely to take place on chromatin when chk1-containing complexes and the sensor complexes come together on DNA (29). Based on this observation, it is likely that Chk1 has a role in detection of DNA damage and monitoring genomic integrity.

Over the past few years, evidence has accumulated to support the role of synthetic triterpenoids in the induction of apoptosis in many types of cancer cells. In addition to their pivotal role in inhibiting cell proliferation and inducing apoptosis, CDDO and its derivatives, at high concentrations, show pro-oxidative properties which are strongly reflected by rapid oxidation of the intracellular GSH pool (25). The molecular links between the generation of ROS and apoptosis by the triterpenoids, however, remain incompletely defined. The generation of ROS induced by CDDO and CDDO-Im has been reported to result in apoptosis of leukemic (26) and multiple myeloma cells (30). Furthermore, several reports demonstrated that CDDO (31) and CDDO-Me (32), again at high concentrations, promoted the release of cytochrome *c* from mitochondria and inhibited mitochondrial electron transport via perturbations in inner mitochondrial membrane integrity, indicating that this organelle is a direct target of these agents. In addition, CDDO and CDDO-Im have been reported to form unregulated, constitutively open, permeability transition pores by the formation of high molecular weight protein aggregates through direct interaction with numerous mitochondrial protein thiols (33). Interestingly, different mechanisms have been proposed for the cytotoxic activity of CDDO-Im and CDDO-Me in various types of cells. CDDO-Me has been reported to induce apoptosis by down-regulation of the Akt pathway (34,35), inhibition of IKK (36) and the mitochondrial pathway (26,32,37), whereas CDDO-

Im has been reported to induce apoptosis in a caspase- (38) or ROS-dependent manner (26,30).

Cancer chemoprevention has emerged as an effective and rational approach for modulating multi-stage carcinogenesis (39). An effective chemopreventive agent should intervene early in the process of carcinogenesis to suppress or eliminate premalignant cells before they become malignant. New evidence indicates that some chemopreventive agents can trigger apoptosis in transformed cells *in vivo* and *in vitro*, which appears to be associated with their effectiveness in controlling the process of carcinogenesis (40,41). Our observations suggest that CDDO-Im, at high concentrations, induces ROS and subsequent DNA damage which is strengthened through a feedback loop, thereby facilitating the activation of the DNA damage checkpoint, G2/M arrest, and finally apoptosis in BRCA1-mutated cancer cells. BRCA1-mutated cells are defective in repairing their DNA damage, which makes the mutant cells more sensitive to apoptosis induced by CDDO-Im, whereas normal cells with normal BRCA1 alleles have a normal cell cycle checkpoint, and they repair their DNA effectively. In this scenario, cells with a BRCA1 mutation selectively undergo apoptosis induced by CDDO-Im, whereas normal cells are spared from this action of the drug. In addition, because malignant cells have high levels of intrinsic ROS (17), CDDO-Im may be considered even further as a selective chemopreventive agent for BRCA1 mutation carriers. Our findings thus now indicate that there is a new rationale to use CDDO-Im as a chemopreventive agent. We are now exploring this possibility with new *in vivo* studies in mice bearing BRCA1 mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Fackenthal JD, Olopade OI. Breast cancer risk associated with BRCA1 and BRCA2 in diverse populations. Nat Rev Cancer. 2007; 7(12):937–48. [PubMed: 18034184]
2. Kennedy RD, Quinn JE, Mullan PB, Johnston PG, Harkin DP. The role of BRCA1 in the cellular response to chemotherapy. J Natl Cancer Inst. 2004; 96(22):1659–68. [PubMed: 15547178]
3. Deng CX. BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. Nucleic Acids Res. 2006; 34(5):1416–26. [PubMed: 16522651]
4. Liu X, Holstege H, van der Gulden H, et al. Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. Proc Natl Acad Sci U S A. 2007; 104(29):12111–6. [PubMed: 17626182]
5. Liby KT, Yore MM, Sporn MB. Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. Nat Rev Cancer. 2007; 7(5):357–69. [PubMed: 17446857]
6. Hyer ML, Shi R, Krajewska M, et al. Apoptotic activity and mechanism of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic-acid and related synthetic triterpenoids in prostate cancer. Cancer Res. 2008; 68(8):2927–33. [PubMed: 18413762]
7. Honda T, Rounds BV, Bore L, et al. Synthetic oleanane and ursane triterpenoids with modified rings A and C: a series of highly active inhibitors of nitric oxide production in mouse macrophages. J Med Chem. 2000; 43(22):4233–46. [PubMed: 11063620]
8. Honda T, Honda Y, Favaloro FG Jr, et al. A novel dicyanotriterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production. Bioorg Med Chem Lett. 2002; 12(7):1027–30. [PubMed: 11909709]

9. Place AE, Suh N, Williams CR, et al. The novel synthetic triterpenoid, CDDO-imidazolide, inhibits inflammatory response and tumor growth in vivo. Clin Cancer Res. 2003; 9(7):2798–806. [PubMed: 12855660]
10. Brodie SG, Xu X, Qiao W, Li WM, Cao L, Deng CX. Multiple genetic changes are associated with mammary tumorigenesis in Brca1 conditional knockout mice. Oncogene. 2001; 20(51):7514–23. [PubMed: 11709723]
11. Ashwell S, Zabludoff S. DNA damage detection and repair pathways--recent advances with inhibitors of checkpoint kinases in cancer therapy. Clin Cancer Res. 2008; 14(13):4032–7. [PubMed: 18593978]
12. Chen Y, Sanchez Y. Chk1 in the DNA damage response: conserved roles from yeasts to mammals. DNA Repair (Amst). 2004; 3(8–9):1025–32. [PubMed: 15279789]
13. Zhou BB, Anderson HJ, Roberge M. Targeting DNA checkpoint kinases in cancer therapy. Cancer Biol Ther. 2003; 2(4 Suppl 1):S16–22. [PubMed: 14508077]
14. Dash BC, El-Deiry WS. Phosphorylation of p21 in G2/M promotes cyclin B-Cdc2 kinase activity. Mol Cell Biol. 2005; 25(8):3364–87. [PubMed: 15798220]
15. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature. 2004; 432(7015):316–23. [PubMed: 15549093]
16. van Attikum H, Gasser SM. The histone code at DNA breaks: a guide to repair? Nat Rev Mol Cell Biol. 2005; 6(10):757–65. [PubMed: 16167054]
17. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009; 8(7):579–91. [PubMed: 19478820]
18. Worsham MJ, Pals G, Schouten JP, et al. High-resolution mapping of molecular events associated with immortalization, transformation, and progression to breast cancer in the MCF10 model. Breast Cancer Res Treat. 2006; 96(2):177–86. [PubMed: 16319984]
19. Trachootham D, Zhou Y, Zhang H, et al. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. Cancer Cell. 2006; 10(3):241–52. [PubMed: 16959615]
20. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell. 2009; 136(5):823–37. [PubMed: 19269363]
21. Alexandre J, Batteux F, Nicco C, et al. Accumulation of hydrogen peroxide is an early and crucial step for paclitaxel-induced cancer cell death both in vitro and in vivo. Int J Cancer. 2006; 119(1): 41–8. [PubMed: 16450384]
22. Bey EA, Bentle MS, Reinicke KE, et al. An NQO1- and PARP-1-mediated cell death pathway induced in non-small-cell lung cancer cells by beta-lapachone. Proc Natl Acad Sci U S A. 2007; 104(28):11832–7. [PubMed: 17609380]
23. Reddy NM, Suryanaraya V, Yates MS, et al. The triterpenoid CDDO-imidazolide confers potent protection against hyperoxic acute lung injury in mice. Am J Respir Crit Care Med. 2009; 180(9): 867–74. [PubMed: 19679692]
24. Liby K, Hock T, Yore MM, et al. The synthetic triterpenoids, CDDO and CDDO-imidazolide, are potent inducers of heme oxygenase-1 and Nrf2/ARE signaling. Cancer Res. 2005; 65(11):4789–98. [PubMed: 15930299]
25. Samudio I, Konopleva M, Hail N Jr, et al. 2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) directly targets mitochondrial glutathione to induce apoptosis in pancreatic cancer. J Biol Chem. 2005; 280(43):36273–82. [PubMed: 16118208]
26. Ikeda T, Sporn M, Honda T, Gribble GW, Kufe D. The novel triterpenoid CDDO and its derivatives induce apoptosis by disruption of intracellular redox balance. Cancer Res. 2003; 63(17):5551–8. [PubMed: 14500394]
27. Liby K, Yore MM, Roebuck BD, et al. A novel acetylenic tricyclic bis-(cyano enone) potently induces phase 2 cytoprotective pathways and blocks liver carcinogenesis induced by aflatoxin. Cancer Res. 2008; 68(16):6727–33. [PubMed: 18701497]
28. Lobrich M, Jeggo PA. The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nat Rev Cancer. 2007; 7(11):861–9. [PubMed: 17943134]

29. Jiang K, Pereira E, Maxfield M, Russell B, Goudelock DM, Sanchez Y. Regulation of Chk1 includes chromatin association and 14-3-3 binding following phosphorylation on Ser-345. J Biol Chem. 2003; 278(27):25207–17. [PubMed: 12676962]
30. Ikeda T, Nakata Y, Kimura F, et al. Induction of redox imbalance and apoptosis in multiple myeloma cells by the novel triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid. Mol Cancer Ther. 2004; 3(1):39–45. [PubMed: 14749474]
31. Konopleva M, Tsao T, Estrov Z, et al. The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces caspase-dependent and -independent apoptosis in acute myelogenous leukemia. Cancer Res. 2004; 64(21):7927–35. [PubMed: 15520199]
32. Samudio I, Konopleva M, Pelicano H, et al. A novel mechanism of action of methyl-2-cyano-3,12 dioxoolean-1,9 diene-28-oate: direct permeabilization of the inner mitochondrial membrane to inhibit electron transport and induce apoptosis. Mol Pharmacol. 2006; 69(4):1182–93. [PubMed: 16410408]
33. Brookes PS, Morse K, Ray D, et al. The triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid and its derivatives elicit human lymphoid cell apoptosis through a novel pathway involving the unregulated mitochondrial permeability transition pore. Cancer Res. 2007; 67(4):1793–802. [PubMed: 17308122]
34. Deeb D, Gao X, Jiang H, Dulchavsky SA, Gautam SC. Oleanane triterpenoid CDDO-Me inhibits growth and induces apoptosis in prostate cancer cells by independently targeting pro-survival Akt and mTOR. Prostate. 2009; 69(8):851–60. [PubMed: 19189297]
35. Vene R, Larghero P, Arena G, Sporn MB, Albini A, Tosetti F. Glycogen synthase kinase 3beta regulates cell death induced by synthetic triterpenoids. Cancer Res. 2008; 68(17):6987–96. [PubMed: 18757413]
36. Shishodia S, Sethi G, Konopleva M, Andreeff M, Aggarwal BB. A synthetic triterpenoid, CDDO-Me, inhibits IkappaBalpha kinase and enhances apoptosis induced by TNF and chemotherapeutic agents through down-regulation of expression of nuclear factor kappaB-regulated gene products in human leukemic cells. Clin Cancer Res. 2006; 12(6):1828–38. [PubMed: 16551868]
37. Samudio I, Kurinna S, Ruvolo P, et al. Inhibition of mitochondrial metabolism by methyl-2-cyano-3,12-dioxooleana-1,9-diene-28-oate induces apoptotic or autophagic cell death in chronic myeloid leukemia cells. Mol Cancer Ther. 2008; 7(5):1130–9. [PubMed: 18483301]
38. Elsawa SF, Novak AJ, Grote D, et al. CDDO-imidazolide mediated inhibition of malignant cell growth in Waldenstrom macroglobulinemia. Leuk Res. 2008; 32(12):1895–902. [PubMed: 18468679]
39. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. Science. 1997; 278(5340): 1073–7. [PubMed: 9353183]
40. Sun SY, Hail N Jr, Lotan R. Apoptosis as a novel target for cancer chemoprevention. J Natl Cancer Inst. 2004; 96(9):662–72. [PubMed: 15126603]
41. Rendi MH, Suh N, Lamph WW, et al. The selective estrogen receptor modulator arzoxifene and the rexinoid LG100268 cooperate to promote transforming growth factor beta-dependent apoptosis in breast cancer. Cancer Res. 2004; 64(10):3566–71. [PubMed: 15150113]

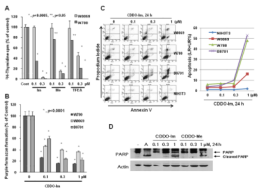

Figure 1. Triterpenoids inhibit proliferation and induce apoptosis in BRCA1-mutated cancer cells

A, To measure proliferation, BRCA1-mutated breast cancer cells were treated with triterpenoids for 24 h and then evaluated using a ^{3}H-thymidine incorporation assay. ***B***, The ability to reduce MTT was assayed 24 h later and expressed as percent relative to controls. Mean from three independent experiments run in sextuplicate; bars, SD. ***C***, BRCA1-mutated breast cancer cells were treated with CDDO-Im (0.1, 0.3 and 1 μM) for 24 h and were analyzed by flow cytometry for FITC-Annexin V and propidium iodide staining. *X* axis, Annexin V; *Y* axis, propidium iodide. ***D***, W780 cells also were treated for 24 h, and Western blots were probed with PARP and β-actin antibodies to verify the induction of apoptosis. Me, methyl ester; Im, imidazolide; TFEA, trifluoroethyl amide of CDDO; A, anisomycin (10 μg/mL; positive control for PARP cleavage).

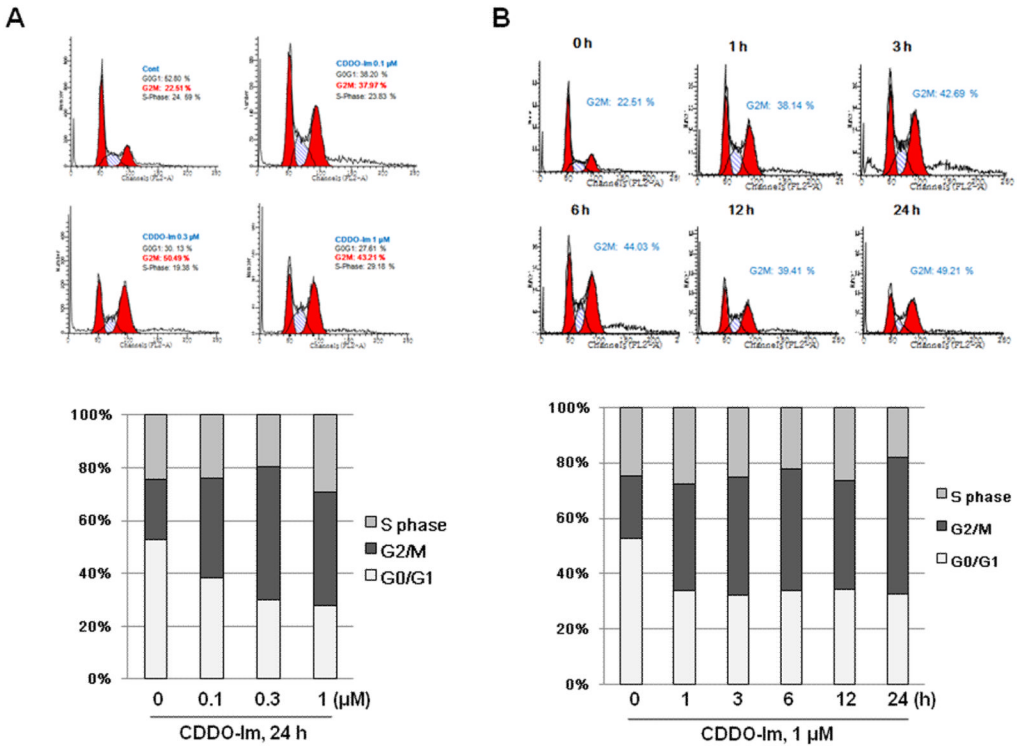

Figure 2. CDDO-Im induces G2/M arrest in BRCA1-mutated cancer cells

Flow cytometry analysis of W780 cells synchronized at the G1/S transition by serum starvation and released into the cell cycle in the absence or presence of CDDO-Im at different concentrations (***A***) and times (***B***). The percentage of cells in different phases of the cell cycle was determined by using the Mod-Fit program (Materials and Methods).

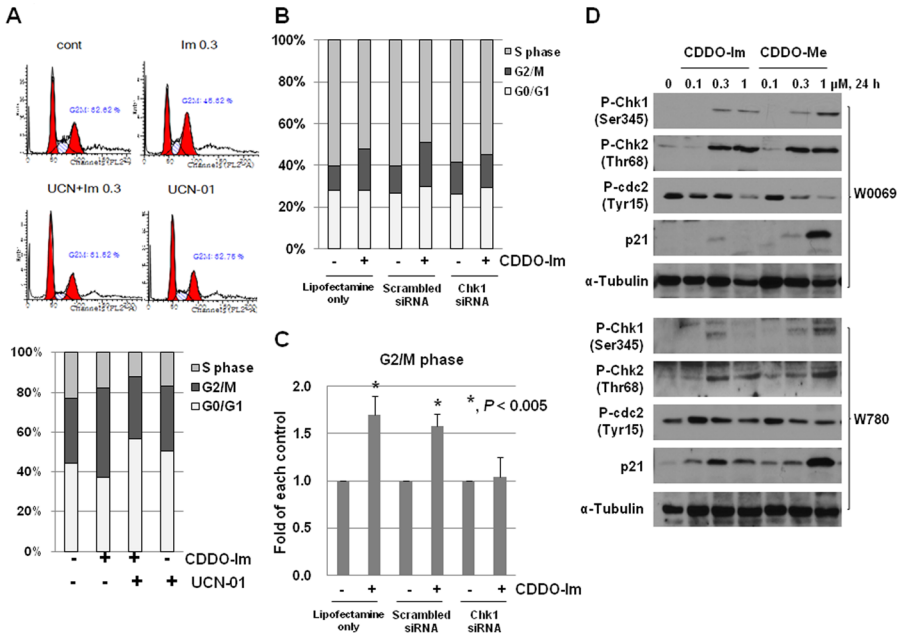

Figure 3. CDDO-Im activates DNA damage signaling in BRCA1-deficient cells

A, UCN-01, a Chk1 inhibitor abrogated G2/M arrest induced by CDDO-Im. Flow cytometry analysis of synchronized W780 cells were released into the cell cycle in the absence or presence of 100 nM UCN-01 for 2 h pretreatment and/or 0.3 μM CDDO-Im for 24 h. The percentage of cells in different phases of the cell cycle was determined by using the Mod-Fit program (Materials and Methods). ***B***, W780 cells were transfected with control or Chk1 siRNA. After serum starvation, flow cytometry analysis of synchronized W780 cells were released into the cell cycle in the absence or presence of 1 μM CDDO-Im for 3 h. The percentage of cells in different phases of the cell cycle was determined by using the Mod-Fit program (Materials and Methods). ***C***, Chk1 siRNA abrogated G2/M arrest induced by CDDO-Im. Mean from three independent experiments; bars, SD. ***D***, W0069 and W780 cells were exposed to different concentrations (0.1, 0.3 and 1 μM) of synthetic triterpenoids for 24 h and soluble protein extracts made were subjected to SDS-PAGE and Western blotting with the indicated antibodies. Representative immunoblots show the effect of synthetic triterpenoids treatment on the phosphorylation of Chk1 (Ser345), Chk2 (Thr168), and Cdc2 (Tyr15) and $p21^{Waf1/Cip1}$. α-Tubulin were used as a loading control.

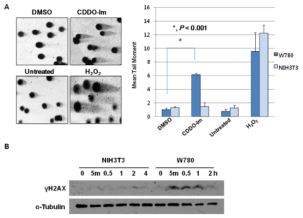

Figure 4. CDDO-Im induces DNA damage in W780 cells

A, Comet assay expressed as average tail moment on W780 or NIH3T3 cells untreated, after incubation for 30 min in medium containing 100 μM H_2O_2 in 4 °C, and after incubation for 1 h in medium containing DMSO or 1 μM CDDO-Im. Measurement of mean tail moment was from 100 cells/slide from 15–20 randomly selected fields representing the whole area of each slide. Comet tail moments were determined using Comet Assay IV software. ***B***, NIH3T3 and W780 cells were treated with 1 μM CDDO-Im for indicated time periods before total protein isolation and Western blot analysis for phospho-histone H2AX.

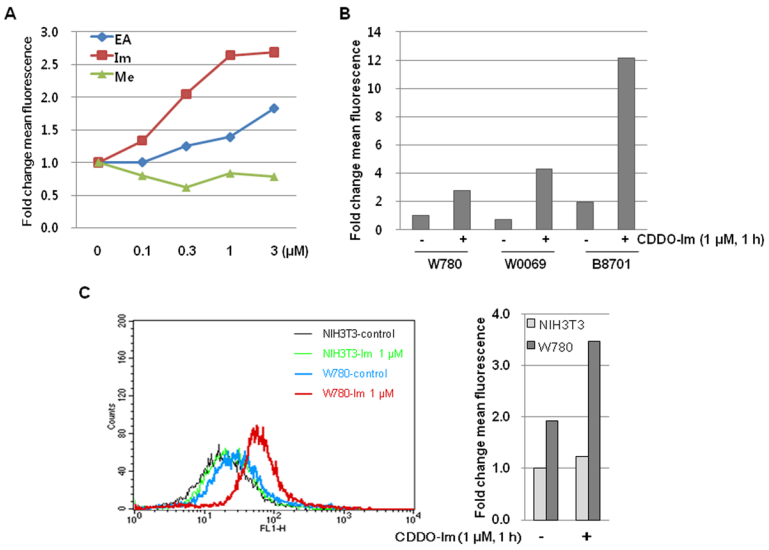

Figure 5. Triterpenoids induce ROS levels in BRCA1-mutated cancer cells

A, W780 cells were treated 0 – 3 μM triterpenoids for 1 h, harvested, and loaded with H_2DCFDA. The mean fluorescence intensity of 20,000 cells per group was detected by flow cytometry, and results are expressed as fold of control. EA, ethyl amide; Im, imidazolide; Me, methyl ester. ***B***, Increased ROS by CDDO-Im were detected by flow cytometry in BRCA1-mutated cancer cell lines W780, W0069 and B8701. ***C***, W780 and NIH3T3 cells were treated with 0 or 1 μM CDDO-Im for 1 h, harvested, and loaded with H_2DCFDA. The mean fluorescence intensity of 20,000 cells per group was detected by flow cytometry, and results are expressed as fold of control.

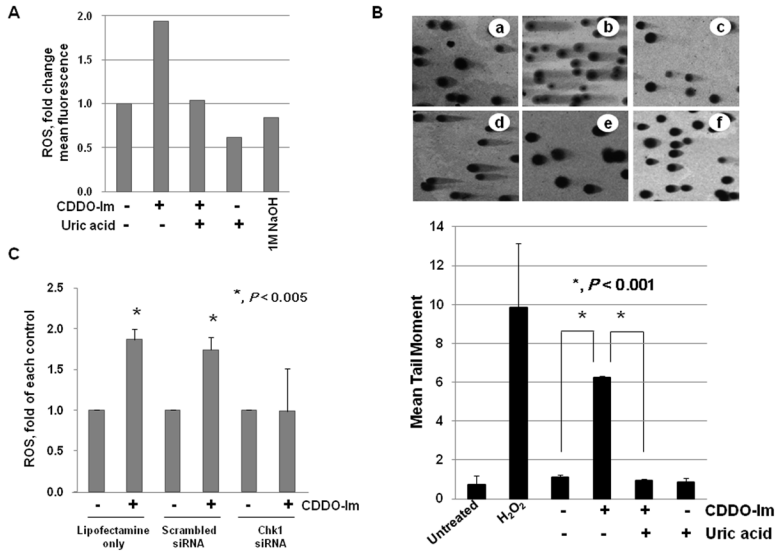

Figure 6. The induction of ROS generation is critically required for DNA damage by CDDO-Im and abrogated by loss of Chk1

A,W780 cells were incubated with an antioxidant, 1 mM uric acid for 1 h and treated with 0 to 1 μM CDDO-Im for an additional 1 h, harvested, and loaded with H_2DCFDA. The mean fluorescence intensity of 20,000 cells per group was detected by flow cytometry, and results are expressed as fold of control. ***B***, Comet assay expressed as average tail moment on W780 cells untreated, after incubation for 30 min in medium containing 100 μM H_2O_2 in 4 °C, and after incubation for 1 h in medium containing 1 μM CDDO-Im with or without 1 mM uric acid for 1 h pretreatment. Measurement of mean tail moment was from 100 cells/slide from 15–20 randomly selected fields representing the whole area of each slide. Comet tail moments were determined using Comet Assay IV software. **a**, untreated; **b,** H_2O_2; **c,** DMSO control; **d,** CDDO-Im; **e,** CDDO-Im + uric acid; **f,** uric acid alone. ***C***, After transfection with control or Chk1 siRNA for 24 h, W780 cells were incubated with 1 μM CDDO-Im for 1 h, harvested, and loaded with H_2DCFDA. The mean fluorescence intensity of 20,000 cells per group was detected by flow cytometry, and results are expressed as fold of each control. Mean from three independent experiments; bars, SD.