ROS Mediate Proapoptotic and Antisurvival Activity of Oleanane Triterpenoid CDDO-Me in Ovarian Cancer Cells

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Abstract. *Oleanane triterpenoids are broad-spectrum antiproliferative and proapoptotic agents. In this study, we investigated whether reactive oxygen species (ROS) play a role in the antitumor activity of methyl-2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oate (CDDO-Me) in OVCAR-5 and MDAH 2774 ovarian cancer cells. Treatment with CDDO-Me caused the generation of ROS (H_2O_2) and pre-treatment with N-acetylcysteine (NAC) prevented the generation of ROS. NAC also blocked the inhibition of cell proliferation by CDDO-Me. Likewise, NAC prevented the CDDO-Me-caused binding of fluorescein isothiocyanate (FITC)-tagged annexin V, cleavage of poly ADP-ribose polymerase-1 (PARP-1), procaspases-3, -8 and -9 and loss of mitochondrial membrane potential. CDDO-Me inhibited the expression of prosurvival phospho-AKT (p-AKT), phospho-mammalian target of rapamycin (p-mTOR) and nuclear factor-kappa B (NF-κB) (p65) signaling molecules and NF-κB-regulated antiapoptotic B-cell lymphoma-2 (BCL-2), B-cell lymphoma-extra large (BCL-xL), cellular inhibitor of apoptosis protein 1(c-IAP1) and survivin, but pre-treatment with NAC blocked the down-modulation of these signaling and antiapoptotic proteins by CDDO-Me. Together, these results indicate the pivotal role ROS play in the antiproliferative- and apoptosis-inducing activity of CDDO-Me in ovarian cancer cells; however, the role of ROS in the down-regulation of prosurvival AKT, mTOR, NF-κB and antiapoptotic BCL-2, BCL-xL, c-IAP1 and survivin warrants further investigation.*

Carcinoma of the ovary is the most common gynecological malignancy and the fifth most common cause of cancer-related death in women in the United States. The most recent estimate is for 22,280 new cases of ovarian cancer and 15,500 deaths from the disease in the United States in 2012 (1). While surgical resection of tumors confined to the ovary can result in a 5-year survival rate of approximately 90%, unfortunately, most patients have widespread disease at the time of diagnosis (2). Carboplatin/paclitaxel is the current standard-of-care for first-line treatment of ovarian cancer; however, development of drug resistance limits the effectiveness of these chemotherapeutics, underscoring the dire need for developing new strategies and agents for ovarian cancer (3, 4).

Novel agents with strong proapoptotic activity could complement chemotherapy and lead to tumor regression and improved prognosis. Triterpenoids are members of a family of structurally-related compounds known as cyclosqualenoids that are widely distributed in nature. Oleanolic acid is a naturally occurring triterpenoid that has been used in traditional medicine as an antibacterial, antifungal, anticancer, and anti-inflammatory agent (5). A synthetic derivative of oleanolic acid: methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) exhibits potent antiproliferative and proapoptotic activity against diverse types of tumor cells, including ovarian cancer cells, through the inhibition of mitogen-activated protein kinase (MAPK), extacellular signal-regulated kinases 1 and 2 (Erk1/2), nuclear factor-kappa B (NF-κB) and peroxisome proliferator-activated receptor gamma (PPARγ) signaling pathways (6-12). In a previous study, we showed that CDDO-Me induces apoptosis in multiple ovarian cancer cell lines by inhibiting prosurvival AKT/NF-κB/mTOR signaling (13). Since generation of ROS plays a role in the anticancer activity of many antineoplastic drugs, we evaluated the role of ROS in mediating the proapoptotic activity of CDDO-Me in ovarian cancer cells in the present study.

Materials and Methods

Reagents and antibodies. CDDO-Me was obtained from the National Cancer Institute, Bethesda, MD, USA, through the Rapid Access to Intervention Development Program. Anti-caspase-3, caspase-8, and caspase-9 antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Anti-p-AKT (ser^{473}) and anti-p-mTOR (ser^{2448}) antibodies were from Cell Signaling Technology

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(Danvers, MA, USA). Anti-NF-κB (p65) and anti-PARP-1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 96 AQueous One Solution Proliferation Assay System was from Promega (Madison, WI, USA).

Cell lines. Human ovarian cancer cell lines OVCAR-5 and MDAH 2774 were obtained from the American Type Tissue Collection (Rockville, MD, USA). Cells were maintained in tissue culture using fully-supplemented cell line-specific tissue culture medium.

Measurement of ROS. 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (H2DCF-DA) fluorescent probe was used to measure intracellular ROS (H_2O_2) by flow cytometry. Briefly, 1×10^6 tumor cells were plated in 6-well plates overnight and treated or not with CDDO-Me in the presence or absence of N-acetylcysteine (NAC) 3 mM. Cells were then reacted with 5 μM of H_2DCF-DA for 30 min at 37˚C, collected by trypsinization and analyzed for DCF-DA fluorescence.

MTS assay. Tumor cells (1×10^4) were seeded into each well of a 96-well plate in 100 μl of tissue culture medium. After 24 h incubation, cells were treated with CDDO-Me at concentrations of 0.625 to 10 μM for 48-72 h. Cell viability was then determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay using CellTiter 96 AQueous One Solution Proliferation Assay System (Promega).

Annexin V- Fluorescein Isothiocyanate (FITC) binding. Tumor cells treated with CDDO-Me for 20 h were suspended in the binding buffer provided in the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA) and reacted with 5 μl of annexin V-FITC reagent plus 5 μl of propidium iodide (PI) for 30 min at room temperature in the dark. Stained cells were analyzed by flow cytometry.

Mitochondrial depolarization assay. Change in mitochondrial potential was determined by flow cytometry. Briefly, after treating with CDDO-Me for 20 h, cells were loaded with mitochondrial potential sensor JC-1 (10 μg/ml) for 10 min at 22˚C and cells were analyzed by flow cytometry. In normal cells, dye is aggregated in mitochondria, fluoresces red, and is detected in the FL2 channel. In cells with altered mitochondrial potential, the dye fails to accumulate in the mitochondria, remains as monomers in the cytoplasm, fluoresces green, and is detected in the FL1 channel.

Western blotting. Total cellular proteins were obtained by detergent lysis. Samples (50 μg) were boiled in an equal volume of sample buffer [20% glycerol, 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 125 mM Tris-HCl (pH 7.5), and 640 mM 2-mercaptoethanol] and separated on pre-casted Tris-glycine polyacrylamide gels (6-10%) using the XCell Surelock™ Mini-Cell, in Tris-Glycine SDS running buffer, all from Novex (Invitrogen, Carlsbad, CA, USA). Proteins resolved on the gels were transferred to nitrocellulose membranes, which were then probed with antibody probes. Immune complexes were visualized by chemiluminescence. Protein band densities were analyzed using the Image J software (National Institute of Health Bethesda, Maryland, USA) and normalized to the corresponding β-actin band densities.

Statistical analysis. Most data are presented as the means±S.D. and outcomes for treated and untreated cells were compared by the Student's *t*-test. Differences were considered significant at $p<0.05$.

Results

CDDO-Me induces ROS generation in ovarian cancer cells. To determine whether generation of intracellular ROS play a role in the antitumor activity of CDDO-Me against ovarian cancer cells, OVCAR-5 and MDAH 2774 cells were pre-treated or not with NAC, a small-molecule antioxidant, for 2 h before treating them with CDDO-Me (2.5 μM) for 1 h. Cells were reacted with H_2DCFDA probe and DCF fluorescence was measured by flow cytometry. Treatment with CDDO-Me increased DCF fluorescence intensity in both cell lines compared to control cells (Figure 1A), which was blocked by pre-treatment with NAC. These data demonstrated that CDDO-Me induces intracellular generation of ROS in ovarian cancer cells.

NAC blocks the antiproliferative activity of CDDO-Me. Our previous studies have demonstrated that CDDO-Me inhibits the proliferation of ovarian cancer cells at 0.625 to 10 μM (13). To investigate whether ROS play a role in the antiproliferative activity of CDDO-Me in ovarian cancer cells, OVCAR-5 and MDAH 2774 cells were pre-treated or not with NAC for 2 h before treatment with CDDO-Me for 72 h and viability of cultures was determined by MTS assay. As shown in Figure 1B, CDDO-Me significantly reduced the viability of both cell lines at 1.25-10 μM ($p<0.05$). In contrast, pre-treatment with NAC completely blocked the activity of CDDO-Me at all concentrations. In addition, microscopic examination of cell cultures showed rounding and detachment of cells in cultures treated with CDDO-Me, which was blocked by pre-treatment of cells with NAC (data not shown). Thus, reversal of the antiproliferative activity of CDDO-Me by NAC indicates that ROS play a role in the growth inhibitory activity of CDDO-Me.

NAC blocks the induction of apoptosis in ovarian cancer cells. The inhibition of proliferation of ovarian cancer cells by CDDO-Me was associated with induction of apoptosis (13). Whether ROS generation by CDDO-Me plays a role in induction of apoptosis was investigated by measuring the effect of NAC on CDDO-Me-induced binding of annexin V-FITC and the cleavage of PARP-1. As shown in Figure 2A, CDDO-Me significantly increased the binding of annexin V-FITC in OVCAR-5 and MDAH 2774 cells in a dose-dependent manner (1.25 to 10 μM). In contrast, in cells pre-treated with NAC, annexin V-FITC binding was significantly reduced in both cell lines at 5 and 10 μM CDDO-Me ($p<0.05$). Pre-treatment with NAC also prevented the cleavage of PARP-1 by CDDO-Me in MDAH 2774 cells at concentrations of 1.25 to 5 μM (Figure 2B).

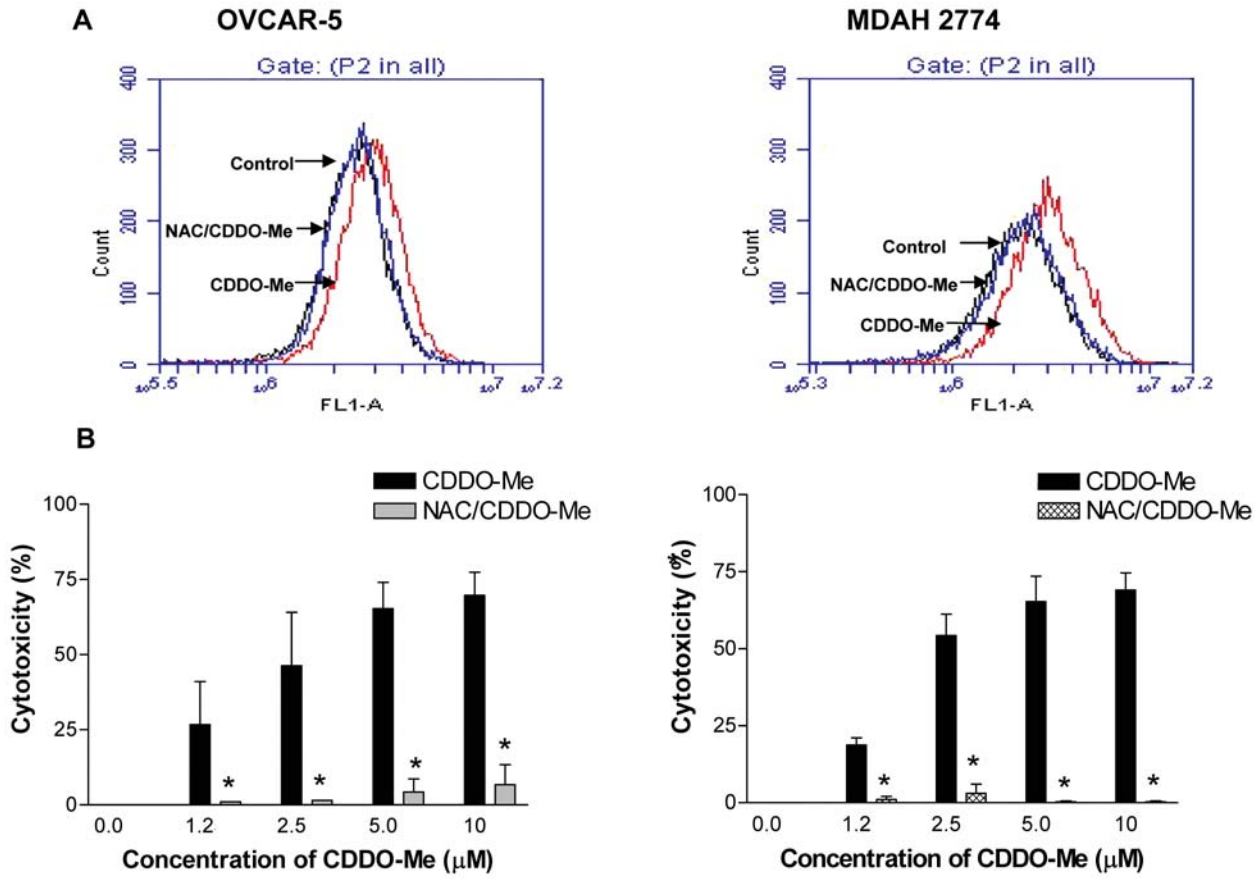

Figure 1. *A: Methyl-2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oate (CDDO-Me) induces reactive oxygen species (ROS) generation in ovarian cancer cells which is blocked by N-acetylcysteine (NAC). OVCAR-5 and MDAH 2774 cells were pre-treated with NAC (3 mM) for 2 h followed by CDDO-Me (2.5 μM) for 1 h. Cells were then treated with 5 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (H2DCFDA) for 30 min at 37°C and DCF fluorescence was measured by flow cytometry. B: CDDO-Me inhibits the proliferation of ovarian cancer cells which is blocked by NAC. A total of 1 × 10^4 OVCAR-5 and MDAH 2774 cells/well in a 96-well microtiter plate were pre-treated or not with NAC (3 mM) for 2 h before treatment with CDDO-Me (0 to 10 μM) for 72 h in triplicates. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt MTS assay using the CellTiter AQueous assay system from Promega. Data are presented as the percentage cytotoxicity obtained from three independent experiments. *p<0.05 compared to control cells.*

NAC reverses CDDO-Me-induced cleavage of procaspases and mitochondrial depolarization. To further delineate the role of ROS in induction of apoptosis by CDDO-Me, processing of procaspases-3, -8 and -9 by CDDO-Me in cells pre-treated with NAC was measured. Control cells (both cell lines) showed partial to complete processing of native procaspases-3, -8 and -9 after treatment with CDDO-Me at 5 to 10 μM CDDO-Me for 20 h (Figure 3A). Pre-treatment with NAC reduced the processing of procaspases at these concentrations in both cell lines, indicating the requirement for free radicals in the cleavage of procaspases by CDDO-Me in ovarian cancer cells.

The toxicity of CDDO-Me towards mitochondria was evaluated by measuring the change in mitochondrial membrane potential using mitochondrial-potential sensor JC-1. Treatment with CDDO-Me (2.5 μM) for 20 h significantly induced mitochondrial depolarization in OVCAR-5 and MDAH 2774 cells, which was blocked by NAC (Figure 3B). These findings demonstrated the mitochondria toxicity and role of free radicals in CDDO-Me-induced mitochondrial depolarization in ovarian cancer cells.

NAC blocks inhibition of prosurvival signaling proteins by CDDO-Me. Our previous studies have shown that induction of apoptosis by CDDO-Me is associated with the inhibition of prosurvival AKT, mTOR and NF-κB signaling proteins (13). Here we tested whether ROS play a role in the inhibition of these signaling proteins by CDDO-Me. As shown in Figure 4A, pre-treatment with NAC prevented the inhibition of p-Akt and p-mTOR and NF-κB by CDDO-Me

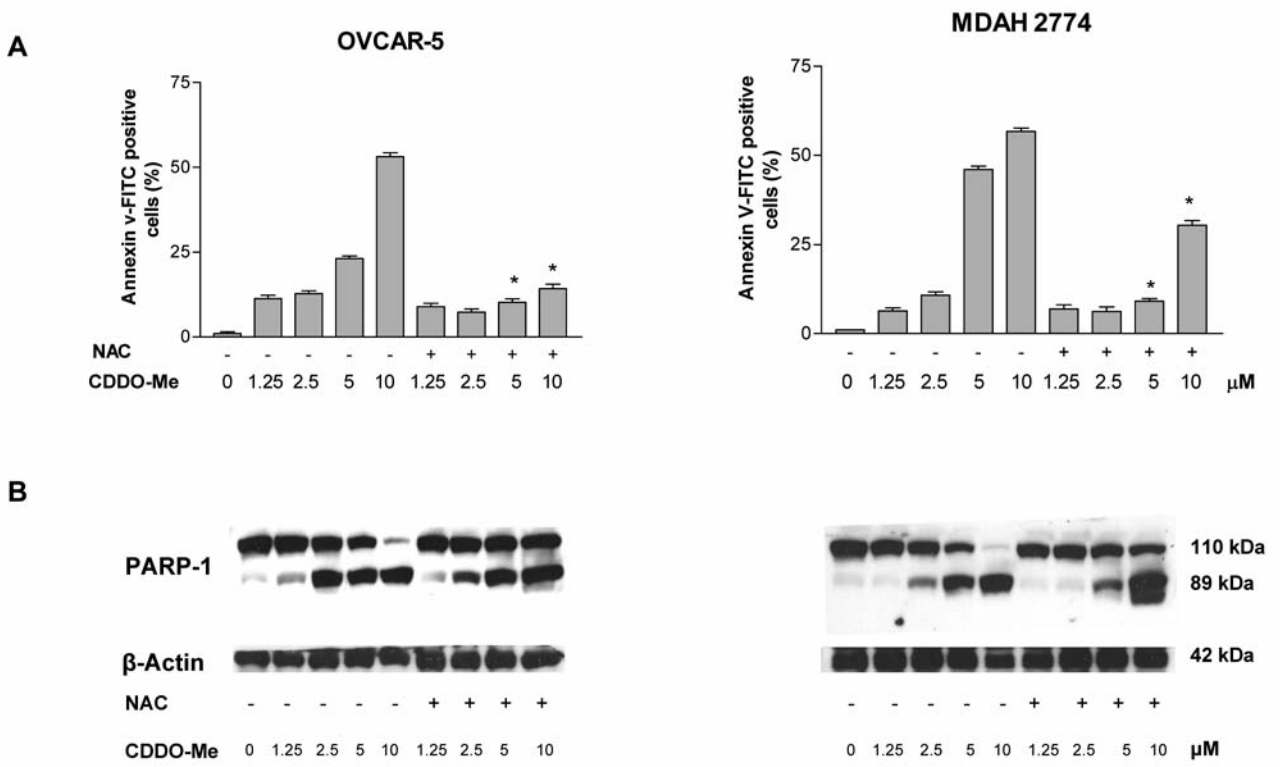

Figure 2. *Methyl-2-cyano-3, 12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) induces apoptosis in ovarian cancer cells which is blocked by N-acetylcysteine (NAC). A: Binding of annexin V- fluorescein isothiocyanate (FITC). OVCAR-5 and MDAH 2774 cells were treated or not with NAC (3 mM) for 2 h prior to treating with CDDO-Me at 1.25 to 10 μM for 20 h. Cells were then reacted with 5 μl of annexin V-FITC plus propidium iodide for 30 min at room temperature. The percentage of annexin V-FITC-positive tumor cells was determined by flow cytometry. B: NAC inhibits the cleavage of poly ADP-ribose polymerase-1 (PARP-1) by CDDO-Me.*

in OVCAR-5 and MDAH 2774 cells. Further, pre-treatment with NAC also blocked the inhibition of NF-κB-regulated antiapoptotic proteins BCL-2, BCL-xL, c-IAP1 and survivin (Figure 4B). Together, these data demonstrate the role of free radicals in the modulation of various prosurvival/antiapoptotic proteins by CDDO-Me in ovarian cancer cells.

Discussion

The present study shows that the antiproliferative and apoptosis-inducing activity of CDDO-Me in ovarian cancer cells is associated with the generation and participation of intracellular ROS, as the antitumor effects of CDDO-Me were blocked by NAC, a small-molecule antioxidant. ROS are generated as by-products of normal aerobic metabolism or as second messengers in various signal transduction pathways in response to oxidative stress (14). ROS can elicit a wide spectrum of biological responses ranging from mitogenic effects at low concentration to macromolecular damage and cell death at high concentrations (15). The generation of ROS is also part of the mechanism by which many chemotherapeutic agents and ionizing radiation kill tumor cells (16). The present study showed that ROS are generated early after treatment with CDDO-Me. CDDO-Me caused the oxidation of H2DCFDA, indicating the production of hydrogen peroxide. Pre-treatment with the general purpose antioxidant NAC blocked the oxidation of H2DCFDA. These findings are consistent with the results of previous studies showing participation of ROS in the killing of cancer cells by CDDO-Me. For example, CDDO-Me was shown to be toxic toward mitochondria in pancreatic cancer cells and generated ROS (17). In an earlier report, we showed that induction of apoptosis in prostate cancer cells by CDDO-Me was inhibited by the mitochondrial chain 1 complex inhibitors diphenyleneiodonium (DPI) and rotenone, indicating that CDDO-Me generates ROS by targeting the mitochondrial electron transport chain (7). Similarly, overexpression of antioxidant enzymes superoxide dismutase-1 (SOD-1) or glutathione peroxidase rendered prostate cancer cells resistant to CDDO-Me (7).

Cell death by CDDO-Me was due to apoptosis as characterized by the increased annexin V binding and cleavage

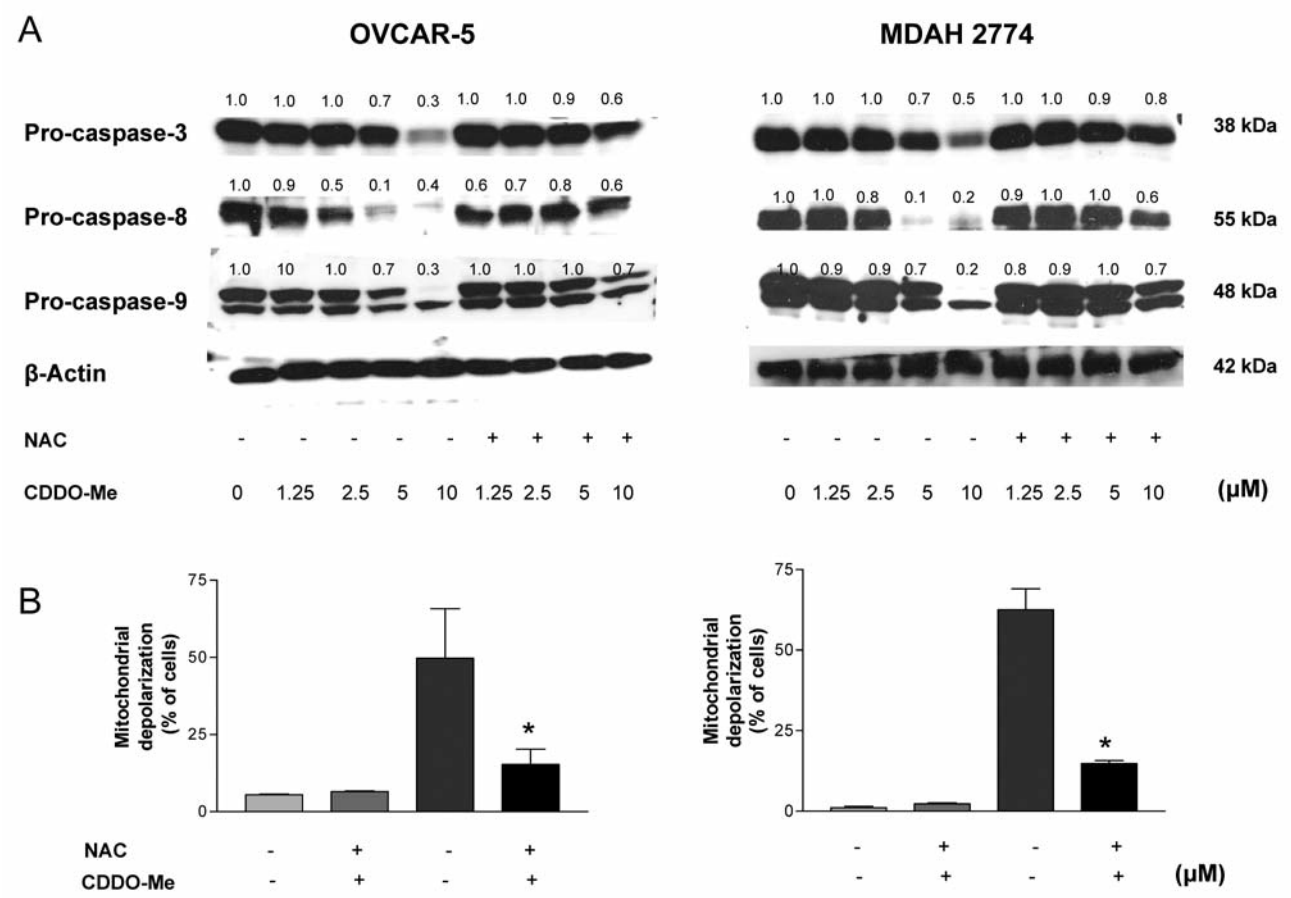

Figure 3. *A: N-acetylcysteine (NAC) prevents Methyl-2-cyano-3, 12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me)-induced processing of procaspases in ovarian cancer cells. OVCAR-5 and MDAH 2774 cells were pre-treated or not with NAC (3 mM) for 2 h prior to treating with CDDO-Me at 1.25 to 10 μM for 20 h and cleavage of native procaspases -3, -8 and -9 was analyzed by western blotting. B: NAC prevents CDDO-Me-induced mitochondrial depolarization. OVCAR-5 and MDAH 2774 cells pre-treated or not with NAC (3 mM) for 2 h prior to treatment with CDDO-Me (2.5 μM) for 20 h. Cells were then loaded with JC-1 probe (10 μg/ml) for 10 min at 22˚C and analyzed by flow cytometry for fluorescence emission. Histograms show the percentage of cells with loss of mitochondrial potential. Similar results were obtained in two separate experiments. *p<0.05 compared to cells treated with CDDO-Me-only.*

of PARP-1 and procaspases -3, -8 and -9. In addition, CDDO-Me induced mitochondrial depolarization. The reversal of these effects of CDDO-Me by NAC indicates that ROS play a role in the induction of apoptosis by CDDO-Me.

Oxidative stress is known to trigger multiple signaling pathways and cellular processes related to cell survival and death *via* interaction with proteins and lipids (18, 19). Both proapoptotic (prodeath) and antiapoptotic (prosurvival) pathways may play a role in ROS-mediated apoptosis in ovarian cancer cells by CDDO-Me. We evaluated the effect of CDDO-Me on the levels of key molecules in the AKT/NF-κB/mTOR signaling pathway. p-AKT promotes cell survival by inactivating downstream substrates such as BAD, procaspase-9, and transcription factor NF-κB (p65) that regulates the expression of genes involved cell proliferation, oncogenesis and apoptosis (20, 21). mTOR, a serine-threonine kinase, controls cell growth, survival and division (22). CDDO-Me inhibited these proteins, demonstrating that inhibition of these antiapoptotic signaling proteins was necessary for induction of apoptosis. Blocking of CDDO-Me-mediated inhibition of these proteins by NAC signifies the role of ROS in the inhibition of these prosurvival signaling proteins by CDDO-Me. Free radicals have been shown to activate AKT/NF-κB survival signaling (23) before, but what is the role of ROS is in inhibition of AKT, NF-κB and mTOR by CDDO-Me, is still unclear. However, as expected, the inhibition of NF-κB by CDDO-Me also resulted in the inhibition NF-κB-regulated antiapoptotic BCL-2, BCL-xL, c-IAP1 and survivin. Thus, it is likely that blocking of the inhibition of BCL-2, BCL-xL, c-IAP1 and

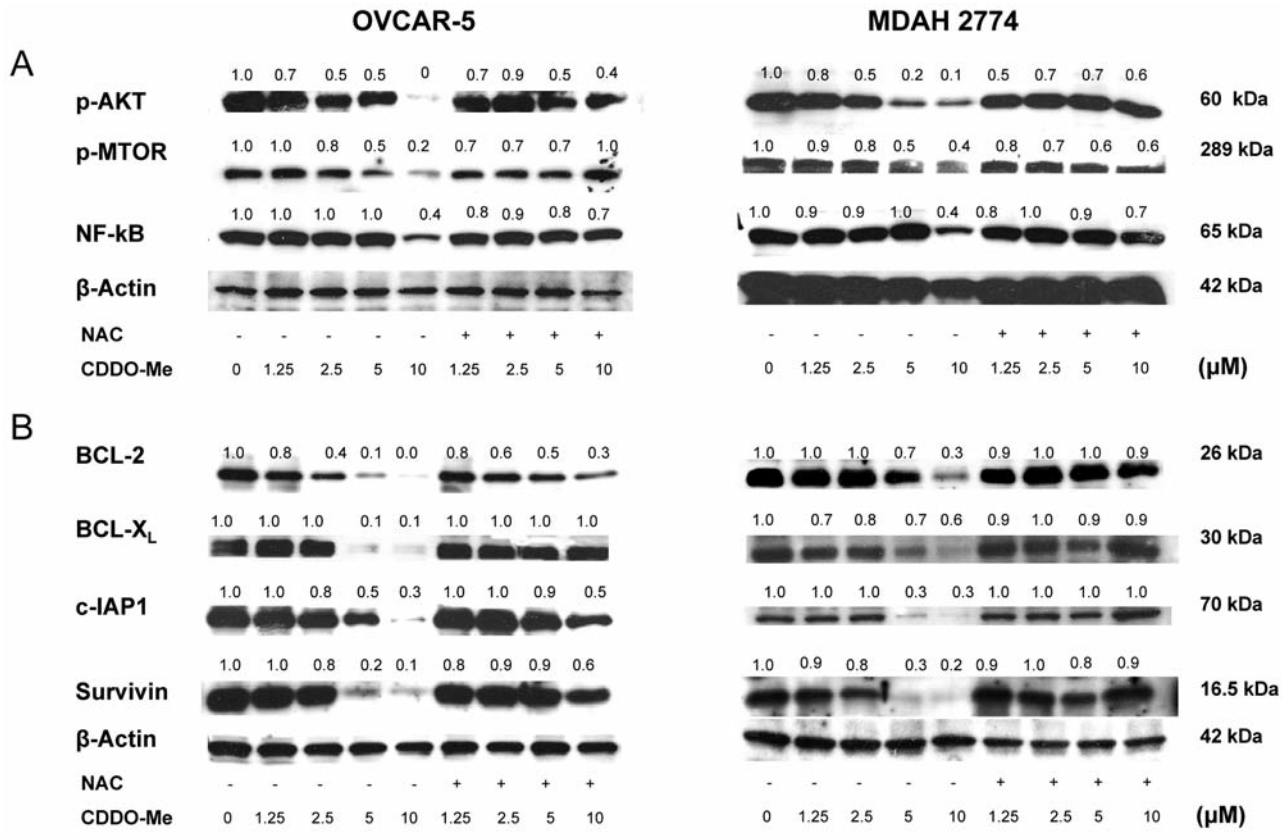

Figure 4. *N-acetylcysteine (NAC) blocks the modulation of prosurvival signaling proteins by methyl-2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oate (CDDO-Me). A: OVCAR-5 and MDAH 2774 cells were pre-treated or not with NAC (3 mM) for 2 h before treating with CDDO-Me (1.25-10 μM) for 20 h. Levels of p-AKT, p-mTOR, NF-κB (p65) and β-actin (loading control) were analyzed by western blotting. B: Immunoblots showing the effect of NAC on down-modulation of NF-κB-regulated antiapoptotic BCL-2, BCL-xL, c-IAP1 and survivin by CDDO-Me.*

survivin by NAC is attributable to the blocking of inhibition of NF-κB by CDDO-Me.

In conclusion, the present study provides an insight into the role of ROS in apoptotic death of ovarian cancer cells by CDDO-Me. Further understanding of the role of free radicals in the inhibition of AKT/NF-κB/mTOR signaling and NF-κB-regulated antiapoptotic BCL-2, BCL-xL, c-IAP1 and survivin by CDDO-Me could facilitate the development of CDDO-Me for treatment of ovarian cancer.

Acknowledgements

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