

## Effects of (5Z)-7-Oxozeaenol on MDA-MB-231 Breast Cancer Cells

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### **Abstract:**

**Aim:** (5Z)-7-Oxozeaenol was studied to reveal the path through which it exerts its effects on triple-negative MDA-MB-231 breast cancer cells. **Materials and Methods:** The apoptotic effect of (5Z)-7-oxozeaenol on MDA-MB-231 cancer cells was analyzed by cell flow cytometry. The effects of (5Z)-7-oxozeaenol on the expression of the nuclear factor kappa B (NF-κB) p65, p50, IκB kinase (IKKα), IKKβ and caspase-7 were analyzed by western blot. The expression of intracellular reactive oxygen species (ROS) and effects on cell adhesion were also assessed. Cell viability was determined using the 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. **Results:** (5Z)-7-Oxozeaenol down-regulated NF-κB in a dose-dependent manner. Intracellular levels of ROS increased in a dose-dependent manner when treated with (5Z)-7-oxozeaenol and potentiated in the presence of H<sub>2</sub>O<sub>2</sub>, when compared to paclitaxel which was used as positive control. Treatment with (5Z)-7-oxozeaenol resulted in G1-phase arrest of treated cells and inhibition of cell proliferation. Cell adhesion was notably affected in treated cells. (5Z)-7-Oxozeaenol also significantly enhanced apoptosis of treated cells, through the activation of caspase-7. **Conclusion:** Our findings suggest that (5Z)-7-oxozeaenol is a potent up-stream inhibitor of the NF-κB pathway, enhances the sensitivity of treated cells to apoptosis induced by ROS, and affects cell adhesion of MDA-MB-231 breast cancer cells. Thus, (5Z)-7-oxozeaenol is a potential new lead for breast cancer drug development since it might, in combination therapy, enhance the efficacy of current treatments and reduce resistance to chemotherapy of triple negative breast cancer.

**Keywords:** (5Z)-7-Oxozeaenol | MDA-MB-231 | NF-κB | caspase-7 | apoptosis | IKK | ROS

### **Article:**

**\*\*\*Note: Full text of article below**

## Effects of (5Z)-7-Oxozeaenol on MDA-MB-231 Breast Cancer Cells

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**Abstract.** Aim: (5Z)-7-Oxozeaenol was studied to reveal the path through which it exerts its effects on triple-negative MDA-MB-231 breast cancer cells. Materials and Methods: The apoptotic effect of (5Z)-7-oxozeaenol on MDA-MB-231 cancer cells was analyzed by cell flow cytometry. The effects of (5Z)-7-oxozeaenol on the expression of the nuclear factor kappa B (NF- $\kappa$ B) p65, p50, IKK kinase (IKK $\alpha$ ), IKK $\beta$  and caspase-7 were analyzed by western blot. The expression of intracellular reactive oxygen species (ROS) and effects on cell adhesion were also assessed. Cell viability was determined using the 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Results: (5Z)-7-Oxozeaenol down-regulated NF- $\kappa$ B in a dose-dependent manner. Intracellular levels of ROS increased in a dose-dependent manner when treated with (5Z)-7-oxozeaenol and potentiated in the presence of H<sub>2</sub>O<sub>2</sub>, when compared to paclitaxel which was used as positive control. Treatment with (5Z)-7-oxozeaenol resulted in G1-phase arrest of treated cells and inhibition of cell proliferation. Cell adhesion was notably affected in treated cells. (5Z)-7-Oxozeaenol also significantly enhanced apoptosis of treated cells, through the activation of caspase-7. Conclusion: Our findings suggest that (5Z)-7-oxozeaenol is a potent up-stream inhibitor of the NF- $\kappa$ B pathway, enhances the sensitivity of treated cells to apoptosis induced by ROS, and affects cell adhesion of MDA-MB-231 breast cancer cells. Thus, (5Z)-7-oxozeaenol is a potential new lead for breast cancer drug

development since it might, in combination therapy, enhance the efficacy of current treatments and reduce resistance to chemotherapy of triple negative breast cancer.

The triple-negative breast cancer cells, MDA-MB-231, are hormone independent and lack receptors for estrogen (ER), progesterone (PgR), and the human epidermal growth factor 2 (HER-2) (1). MDA-MB-231 cells are also deficient in p53 suppressor gene (2). The cells are aggressive, metastatic and do not respond to existing pharmacological treatments, such as herceptin and estrogen antagonists, and there is still need for more effective targeted treatment of malignancies caused by this type of cell (3). Thirty-five out of the 60 NF- $\kappa$ B-related genes are up-regulated in inflammatory breast cancer, which are related to apoptosis, immune response, proliferation, tumor promotion, and angiogenesis (4). Since the nuclear factor kappa B (NF- $\kappa$ B) cell signaling pathway is responsible for survival signaling, proliferative control, and promotes resistance to certain tumorigenic agents and chemotherapy (4), targeting NF- $\kappa$ B activity and inducing apoptosis might result in a promising approach to the development of more effective antineoplastic agents.

Our group has reported that the resorcylic acid lactone (5Z)-7-oxozeaenol affected cervical HeLa cells through caspase-3-induced cell death (5). It has also previously been shown by Ninomiya-Tsuji and co-workers that (5Z)-7-oxozeaenol inhibits the transforming growth factor  $\beta$ -activated kinase 1 (TAK1), a member of the mitogen-activated kinase (MAPKKK) family, likely by competitive binding at the ATP binding site (6). A closely related analog of (5Z)-7-oxozeaenol (termed: E6201) is currently in phase II clinical trials for the treatment of melanoma, by Eisai Inc. (7, 8) as well as a possible topical agent for psoriasis (9). In this study, it has been examined the apoptotic effects of (5Z)-7-oxozeaenol (Figure 1) (10) on triple-negative breast cancer cells and the inhibitory effects of this compound on the NF- $\kappa$ B pathway and on cell adhesion.

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*Key Words:* (5Z)-7-Oxozeaenol, MDA-MB-231, NF- $\kappa$ B, caspase-7, apoptosis, IKK, ROS.

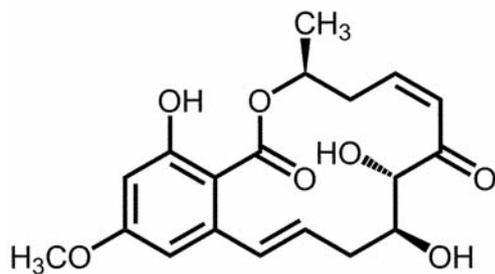


Figure 1. Chemical structure of (5Z)-7-oxozeaenol (10).

## Materials and Methods

**Reagent sources.** (5Z)-7-Oxozeaenol was previously isolated from a filamentous fungus as part of our drug discovery program (10). Vitamin C, iron sulfate ( $\text{FeSO}_4$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), propidium iodine (PI), 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and 2',7'-dichlorofluorescein-diacetate (DCFH-DA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Rocaglamide was acquired from Enzo Life Sciences, Inc. (Farmingdale, NY, USA).

**Cell culture.** Human breast cancer cell line (MDA-MB-231) was obtained from the American Type Culture Collection, Manassas, VA, USA. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine calf serum and 10% antibiotic-antimycotic from Gibco (Rockville, MD, USA). The cells were kept at  $37^\circ\text{C}$  and in an atmosphere with 5%  $\text{CO}_2$ .

**Cytotoxicity assay.** The cytotoxicity of (5Z)-7-oxozeaenol and the cell viability was assessed using 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates and treated with (5Z)-7-oxozeaenol at various concentrations. Optical density at 570 nm and detection of formazan was a measure of cell viability. Cell survival was calculated based on non-treated cells that were used as negative control. The concentration at which 50% of the cell growth was inhibited ( $\text{IC}_{50}$ ) was calculated. Paclitaxel was used as the positive control.

**Immunoblotting.** The experiments were performed following a previously described procedure (5). To confirm the effects of (5Z)-7-oxozeaenol on the NF- $\kappa\text{B}$  pathway, MDA-MB-231 cells were treated at different concentrations (0.008, 0.016, 0.4, 2.0 and 10 mM) for 24 h. Cells were also treated with rocaglamide as a positive control. Briefly, cells were lysed using PhosphoSafe Lysis Buffer (Novagen, Madison, WI, USA). Protein concentration of the cell lysate was determined by using Bradford protein assay kit and albumin standard (Thermo Scientific, Rockford, IL, USA). Absorbance of cell extract samples and of albumin standard dilutions was measured using Fluostar Optima plate reader (BMG Labtech Inc., Durham, NC, USA). Equal amounts of protein (20  $\mu\text{g}$ ) were loaded together with lithium dodecyl sulfate (LDS) sample loading buffer (Invitrogen, Carlsbad, CA, USA) and determined using Nu-PAGE 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) Bis-Tris gels together with SeeBlue® Plus2 Pre-Stained Standard Ladder (Invitrogen). Electrophoresis was accomplished using SDS-PAGE

running buffer in a Nu-PAGE XCell SureLock Module from Invitrogen. Proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane using tris buffered saline with tween-20 buffer (TBS-T). The blots were then blocked at room temperature using non-fat milk and subsequently probed with primary antibodies (1:1000) against each target protein using 1% bovine serum albumin (BSA) in TBS-T overnight. Primary antibodies to caspase-7, NF- $\kappa\text{B}$  p65 (65 kDa) and p50 (50 kDa), I $\kappa\text{B}$  kinase (IKK $\alpha$ ), and IKK $\beta$  were obtained from Cell Signaling Technologies (Beverly, MA, USA). The secondary horseradish peroxidase (HRP) conjugated antibodies (1:2000) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Conjugated antibodies were detected using Chemiluminescent Substrate Supersignal Femto LumiGLO kit (Thermo Scientific, Rockford, IL, USA).

**ROS assay.** The assay was performed following a previously described protocol (5, 11). The presence of ROS in treated cells, in the absence and presence of  $\text{H}_2\text{O}_2$ , was detected using the fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (Sigma Aldrich, St. Louis, MO, USA). Cells were seeded in a 96-well plate and treated with (5Z)-7-oxozeaenol (0.28  $\mu\text{M}$ -280  $\mu\text{M}$ ) or controls for 5 h. Cells were then either treated or not-treated with  $\text{H}_2\text{O}_2$  (1.25 mM) and  $\text{FeSO}_4$  (0.2 mM), followed by incubation for 30 minutes. In these experiments, vitamin C and  $\text{H}_2\text{O}_2$  were used as controls. Afterwards, the fluorescent probe DCFH-DA was added to detect intracellular ROS. Fluorescence was detected and measured using FLUOstar Optima fluorescence plate reader (BMG Labtechnologies GmbH, Inc., Durham, NC, USA) with an excitation wavelength of 485 nm and emission wavelength of 530 nm. All treatments were performed in triplicate and are representative of at least two different experiments.

**Cell cycle analysis.** Cell death and apoptosis were determined by using propidium iodine (PI) to stain DNA in MDA-MB-231 cells. The MDA-MB-231 cells were seeded on 10 cm plates and treated with (5Z)-7-oxozeaenol (10  $\mu\text{M}$ ) for 24 h. Treated and not-treated cells were then washed with phosphate buffered saline (PBS), treated with trypsin EDTA, centrifuged, and re-suspended in PBS. Afterwards, cells were fixed using ethanol (70%), kept on ice for 1 h, washed, re-suspended in ice-cold PBS, and treated with RNAase (20 mg/ml), followed by 1 h incubation at  $37^\circ\text{C}$ . Cells were stained with PI (1.0 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) and kept in the dark until analysis using BD FACS Canto II (San Jose, CA, USA) at 488 nm (5). Comparison of the results from both treated and not-treated cells is presented below.

**Cell adhesion assay.** Cell suspension was plated in a 96-well plate, and treated in triplicates with different concentrations of (5Z)-7-oxozeaenol for 5 h. Cells were then washed with PBS, trypsinated and re-suspended in cell media. Cells were counted using a hemocytometer and an inverted microscope (Axiovert 40 CFL, Zeiss). Cell adhesion was calculated based on non-treated cells that were used as negative control.

## Results

**Cytotoxicity.** (5Z)-7-Oxozeaenol was found to significantly inhibit the growth and cell viability of triple negative MDA-MB-231 breast cancer cells as determined by the MTT reduction assay (Figure 2). To understand the mechanism of growth inhibition, further analysis was conducted as presented below.

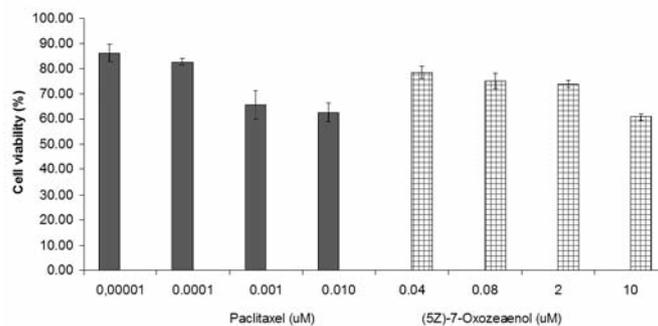


Figure 2. Cell viability was assessed using the 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Reduction of cell proliferation was observed in cells treated with increasing concentrations of (5Z)-7-oxozeaenol.

**Western blot analysis.** (5Z)-7-Oxozeaenol was identified previously as being able to inhibit NF-κB in cervical HeLa cells (5, 11). In this study, (5Z)-7-Oxozeaenol exhibited inhibitory effects on mediators of the NF-κB pathway, and more specifically on p65 (65 kDa) and p50 (50 kDa), in a concentration dependent manner in MDA-MB-231 treated cells (Figure 3). Likewise, the expression levels of up-stream mediators of the NF-κB pathway, IKKα and -β, were down-regulated in a concentration-dependent manner (Figure 4). Western blot analysis also showed that cell death might be induced by a caspase-7-dependent pathway in MDA-MB-231 cells (Figure 5).

**ROS assay.** This assay was performed on MDA-MB-231 cells in absence and presence of H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub>, respectively (Figure 6). The intracellular levels of ROS increased in a concentration dependent manner when cells were treated with (5Z)-7-oxozeaenol alone. The levels of ROS were further potentiated when cells were treated with (5Z)-7-oxozeaenol in the presence of H<sub>2</sub>O<sub>2</sub>. Vitamin C and H<sub>2</sub>O<sub>2</sub> were used as controls. The up-regulation of ROS was indicative of the oxidative stress induced by (5Z)-7-oxozeaenol in MDA-MB-231-treated cells.

**Cell cycle analysis.** A total of 37% of untreated MDA-MB-231 were found to be in the G<sub>1</sub>-phase compared to 64% of treated cells (Figure 7). Accumulation of MDA-MB-231 cells in the G<sub>1</sub>-phase after treatment was observed by cell flow cytometry. Thus, (5Z)-7-oxozeaenol induced cell death through arrest of MDA-MB-231 treated cells in the G<sub>1</sub>-phase.

**Cell adhesion.** Cell adhesion was affected in treated cells when compared to untreated MDA-MB-231 breast cancer cells (Figures 8 and 9). After 5 h of treatment with (5Z)-7-oxozeaenol (0.28 μM), 78% of treated cells were detached. Untreated cells were attached (100%) to the wells after 5 h of incubation under the same conditions as the treated cells.

## Discussion

Induction of the transcription factor NF-κB prevents cancer cells from entering apoptosis (12). Both IKKβ and NF-κB in the canonical pathway are induced by the pro-inflammatory cytokines (IL-1), tumor necrosis factor-α (TNF-α), and epidermal growth factor (EGF), as well as free radicals (13). NF-κB is sequestered in the cytoplasm, bound to IκB. After phosphorylation of IκB by the IκB kinase (IKK), NF-κB is subsequently released and translocated to the nucleus, where it exerts its effects, activating a wide range of target genes that are involved in apoptosis inhibition, cell adhesion, and cell signaling mediators (14). Inhibition by (5Z)-7-oxozeaenol would likely affect the ubiquitination of the NF-κB complex and the subsequent proteasome degradation of the transcription element. Additionally, inadequate NF-κB regulation has been linked to cancer, inflammation, and autoimmune diseases. Inflammatory breast cancer is frequently hormone receptor-negative and more aggressive than other types of cancer (4), making NF-κB an attractive target for the discovery of new NF-κB inhibitors as potential cancer chemotherapeutic agents for the treatment of triple negative breast cancer.

In previous studies, (5Z)-7-oxozeaenol was biologically evaluated in different types of cells, but to our knowledge, there is no previous report on the study of this compound using hormone independent MDA-MB-231 breast cancer cells. Since (5Z)-7-oxozeaenol exhibited a strong cytotoxic effect on this type of cell (Figure 2), attempts are being made to uncover the potential path through which this compound induces cell death in hormone-independent cancer cells. For this study, it is hypothesized that (5Z)-7-oxozeaenol is an NF-κB inhibitor that induces apoptosis through the caspase-7-mediated pathway and reduces cell adhesion of MDA-MB-231 hormone-independent breast cancer cells. As an NF-κB inhibitor, (5Z)-7-oxozeaenol treatment did not inhibit K-RAS activity induced by EGF (5), suggesting that the NF-κB pathway was affected by (5Z)-7-oxozeaenol through a different avenue. The effect of (5Z)-7-

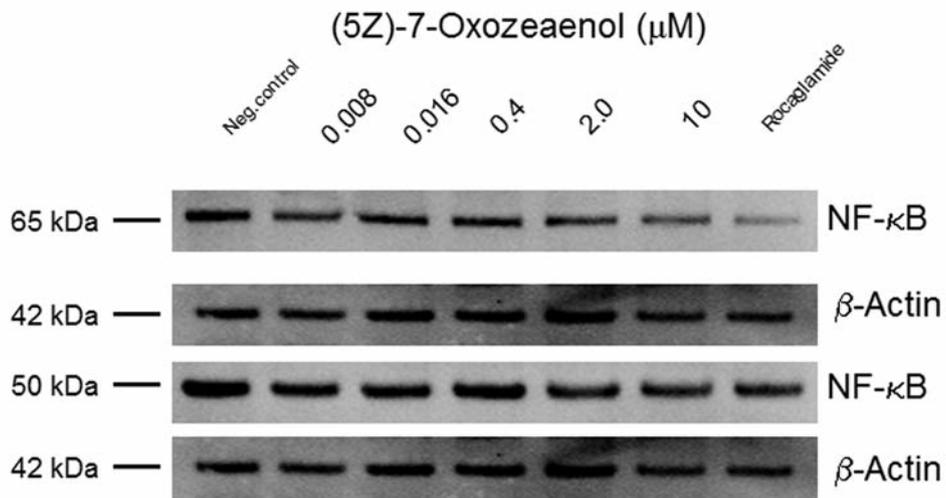


Figure 3. Western blot analysis was performed on MDA-MB-231 cellular extracts and showed that (5Z)-7-oxozeaenol suppresses NFκB-p65 (65 kDa) and -p50 (50 kDa) activation. The NFκB response was dose dependent.

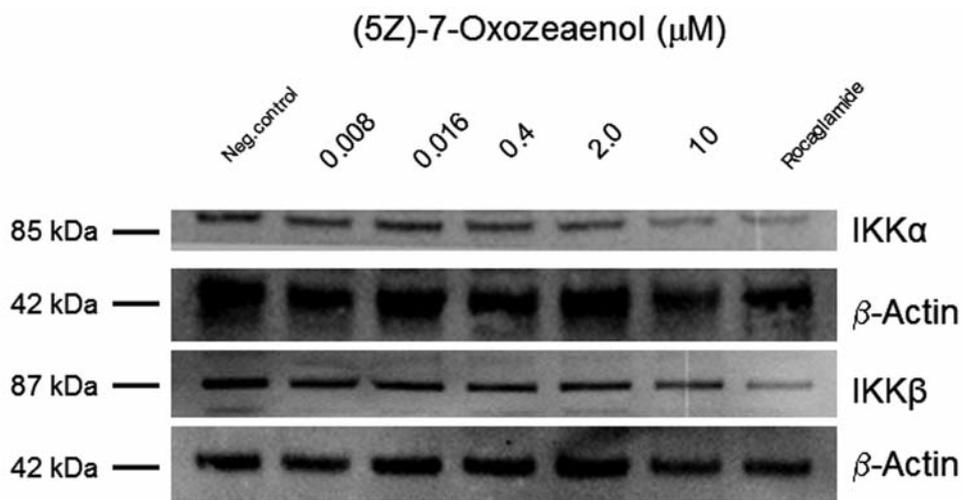


Figure 4. IκB kinase α (IKKα) and IκB kinase β (IKKβ) were down-regulated in (5Z)-7-oxozeaenol treated MDA-MB-231 cells. β-Actin was used as an internal control to establish equivalent loading.

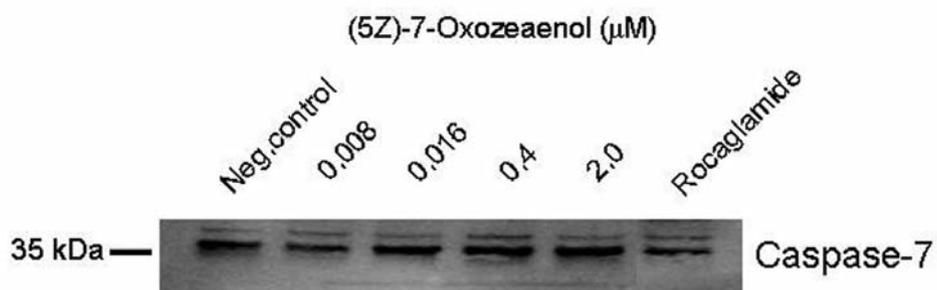


Figure 5. MDA-MB-231 cells treated with (5Z)-7-oxozeaenol expressed caspase-7 in a dose-dependent manner.

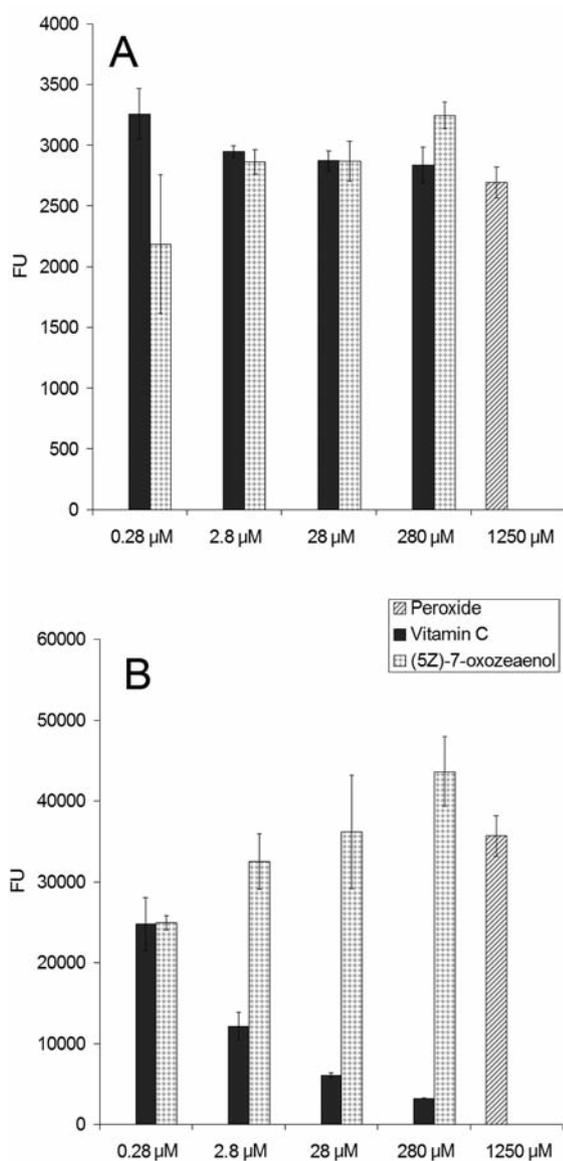


Figure 6. General evaluation of intracellular reactive oxygen species (ROS) induction by (5Z)-7-oxozeaenol on MDA-MB-231 cells in the absence (A) and presence (B) of peroxide ( $H_2O_2$ ). Cells were treated at four different concentrations for 5 h. ROS was detected with fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA) at 485 nm emission wavelength and 530 nm excitation wavelength.  $H_2O_2$  and vitamin C were used as positive and negative controls, respectively. Each experiment was carried out in triplicate and the graph represents the results of two separate experiments. Values represent the means of fluorescent units (FU)  $\pm$  SEM of triplicate samples from two independent experiments.

oxozeaenol (0.25  $\mu$ M) (10) on TNF $\alpha$  induced NF- $\kappa$ B in MDA-MB-231 cells was analyzed and confirmed by western blot analysis (Figure 3). The data presented suggests that (5Z)-7-oxozeaenol inhibits IKK $\beta$ /NF- $\kappa$ B by targeting upstream signaling mediators (Figure 4). Hence, (5Z)-7-oxozeaenol

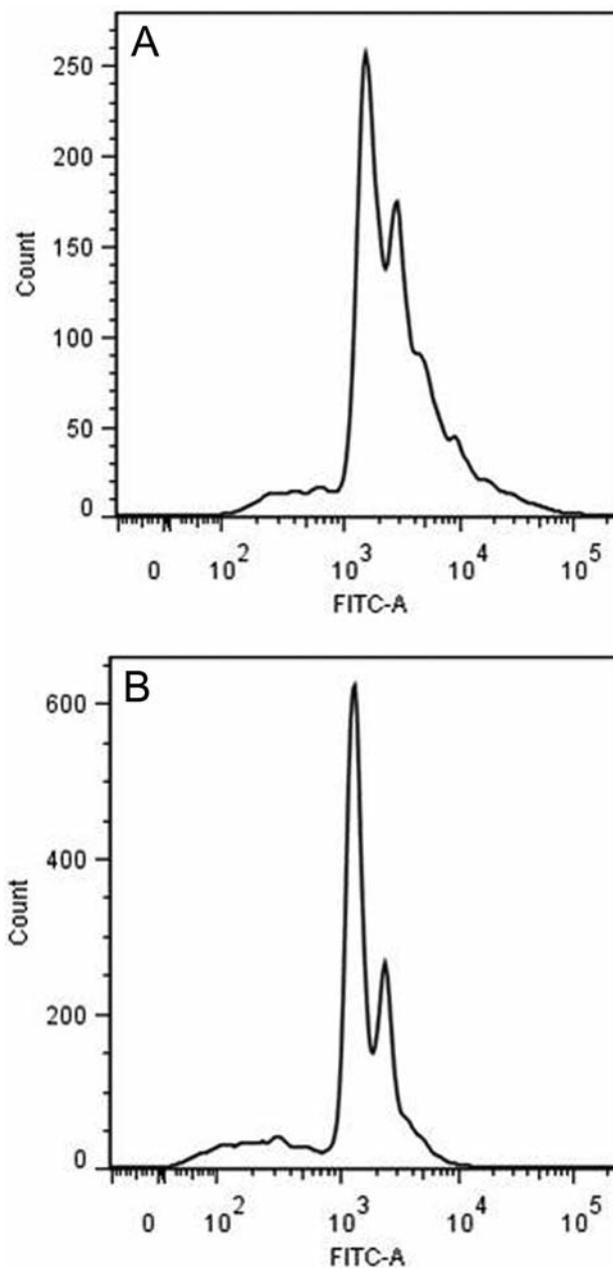


Figure 7. DNA histogram for MDA-MB-231 cells. A: Control exhibited 37% of MDA-MB-231 cells in  $G_1$ -phase. B: Treatment with (5Z)-7-oxozeaenol induced cells cycle arrest in the  $G_1$ -phase of 64% of MDA-MB-231 cells.

targets the NF- $\kappa$ B canonical pathway (7, 15) by inhibiting TNF $\alpha$  induced NF- $\kappa$ B activation (8, 16). TNF $\alpha$  plays a crucial role in NF- $\kappa$ B activation as well as in inflammatory response (13). It is also a key element in inflammatory breast cancer since it is hormone receptor-negative. ROS levels (Figure 6) were also increased in a dose-dependent fashion, suggesting that apoptosis might be mediated by high intracellular levels of ROS (17). Accordingly, inducing oxidative stress in MDA-MB-

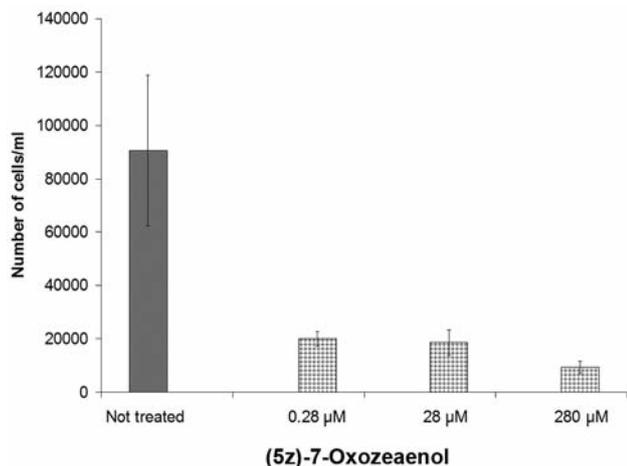


Figure 8. Effect of (5Z)-7-oxozeaenol on the adhesion of MDA-MB-231 breast cancer cells. After 5 h of treatment, 78% of treated cells were detached from the plates.

231 cells appears to affect, as well as it does the activation of NF- $\kappa$ B p50 and p65. Further, the expression of intracellular levels of ROS was potentiated when the (5Z)-7-oxozeaenol treated cells were concomitantly treated with H<sub>2</sub>O<sub>2</sub>, suggesting the potential use of (5Z)-7-oxozeaenol in combination therapy with other chemotherapeutic drugs that induce ROS expression in cytotoxic pathways for the treatment of triple negative breast cancer.

The high level of cytotoxicity exhibited by (5Z)-7-oxozeaenol also prompted analysis on how the cell cycle was being affected by this compound. The flow cytometric study performed using (5Z)-7-oxozeaenol (Figure 7) showed that this compound block the G<sub>1</sub>-phase of MDA-MB-231 cells, thus affecting the metabolic changes that prepare cells for division. Since NF- $\kappa$ B is also involved in cell adhesion, a basic analysis of the cell treatment with (5Z)-7-oxozeaenol after 5 h of incubation confirmed that this compound caused detachment of 78% of cells from the plates at 0.28 µM (Figure 8), suggesting that inhibition of NF- $\kappa$ B did reduce the adhesion of MDA-MB-231 treated cells (Figure 9). Cell adhesion is important for cell division and survival. Downstream factors correlated to NF- $\kappa$ B regulation include effects on apoptosis regulators, growth factors, receptors, cell adhesion, and on other members of the RelA/NF- $\kappa$ B family. Treatment with (5Z)-7-oxozeaenol inhibits the NF- $\kappa$ B pathway and cell proliferation, and negatively affects cell adhesion of MDA-MB-231 cells, thus strongly suggesting that the cell cycle might be interrupted by the effect of this compound on the NF- $\kappa$ B pathway. The MDA-MB-231 cell line is a highly invasive and metastatic cell line, thus the inhibitory effect of (5Z)-7-oxozeaenol on NF- $\kappa$ B and the effect on cell adhesion might reduce the metastatic progression of the disease. Therefore, (5Z)-7-oxozeaenol and

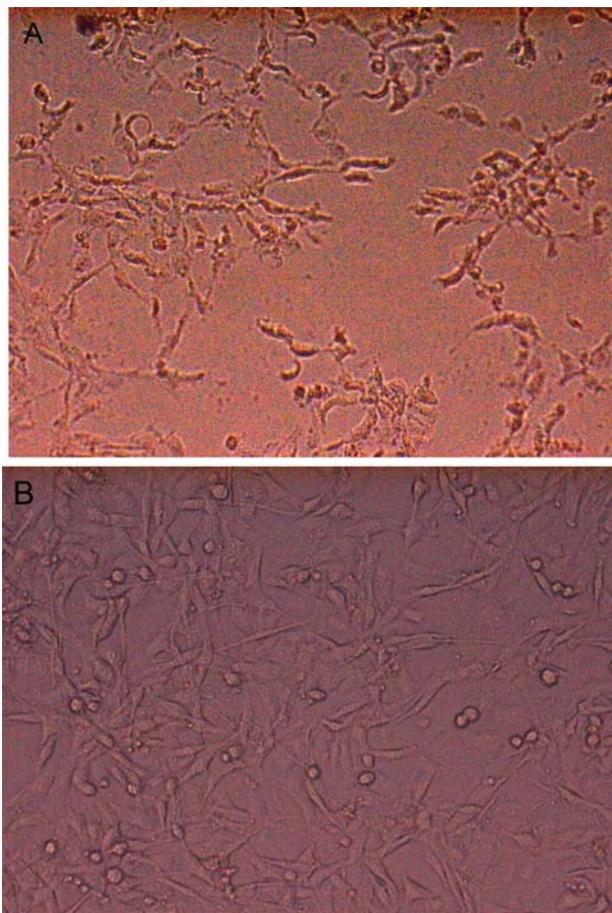


Figure 9. Representative images of cellular adhesion of MDA-MB-231 cells using an inverted microscope (Axiovert 40 CFL, Zeiss) and  $\times 20$  magnification. A: Representative image of reduced cellular adhesion of MDA-MB-231 cells after 5 h of treatment with (5Z)-7-oxozeaenol (280 µM). B: Representative image of cellular adhesion of untreated MDA-MB-231 cells after 5 h of incubation.

related analogs might represent new lead structures in the treatment of hormone-resistant breast cancer.

In a previous report, (5Z)-7-oxozeaenol exhibited a dose-dependent effect on caspase-3 in both an enzyme assay and on western blot analysis when using cervical HeLa cells (5). In this study, (5Z)-7-oxozeaenol exhibited a dose-dependent effect on caspase-7 (Figure 5). Caspase-7 expression was induced with increasing level of (5Z)-7-oxozeaenol, suggesting that apoptosis was mediated by the caspase-dependent pathway in MDA-MB-231 treated cells. These results are in agreement with a previous report (5). Findings further suggest that (5Z)-7-oxozeaenol increased the level of intracellular ROS and activated a caspase-mediated pathway which resulted in G<sub>1</sub> cell cycle arrest. In summary, an antiproliferative effect of (5Z)-7-oxozeaenol was detected in triple negative MDA-MB-231 breast cancer cells. Thus, NF- $\kappa$ B inhibitory agents, such as

(5Z)-7-oxozeaenol, are potential new anti-tumorigenic lead compounds for the treatment of triple negative breast cancer since it down-regulates and suppresses the canonical NF- $\kappa$ B survival pathway in triple negative breast cancer cells and induces cell-death by ROS accumulation.

## Conclusion

(5Z)-7-Oxozeaenol inhibits NF- $\kappa$ B, increases ROS levels as well as potentiates ROS expression in the presence of other ROS inducing agent, and reduces cell adhesion, leading to cell death by a caspase-mediated pathway. Thus, (5Z)-7-oxozeaenol, which has been found to inhibit NF- $\kappa$ B activity, potentiate ROS effect, and exert antiproliferative activity on MBA-MD-231 breast cancer cells, represents a potential chemotherapeutic agent that in combination with ROS inducing anticancer drugs might increase the efficacy of existing treatments and reduce the incidence of resistance to chemotherapy in the treatment of inflammatory breast cancer, particularly triple negative breast cancer.

## Conflict of Interest

The Authors confirm that there are no conflicts of interest.

## Acknowledgements

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