ORIGINAL RESEARCH

Synergistic Anti-Breast-Cancer Effects of Combined Treatment With Oleuropein and Doxorubicin In Vivo

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ABSTRACT

Context • Breast cancer is a leading cause of cancer fatalities among women worldwide. Of the more than 80% of patients who receive adjuvant chemotherapy, approximately 40% relapse. The majority of these patients die of disseminated metastatic disease, which emphasizes the need for new therapeutic strategies

Objective • The study intended to investigate the anticancer effects of oleuropein (OL) and doxorubicin (DOX) individually and in combination on breast tumor xenografts and also to evaluate the molecular pathways involved.

Design • The research team designed in vivo (animal) and in vitro (cell culture) studies.

Setting: The study was performed in the College of Science of King Saud University in the University Center for Women Students (Riyadh, Saudi Arabia).

Animals • The study involved 40 female, nude mice (BALB/c OlaHsd-foxn1).

Intervention • The mice were injected subcutaneously with MDA-MB-231 human breast cancer cells. After the growth of tumors, the animals were randomly divided into 4 groups to receive intraperitoneal injections: (1) group 1 (control group)—dimethyl sulfoxide, (2) group 2 (intervention group)—50 mg/kg of OL, (3) group 3 (intervention group)—2.5 mg/kg of DOX, and (4) group 4 (intervention group)—1.5 mg/kg of DOX, immediately followed by 50 mg/kg of OL. The OL was extracted from Manzanillo olive trees (*Olea europaea*) grown in Tabouk, Saudi Arabia.

Outcome Measures • The measures included the isolation and primary culture of the tumor xenografts, apoptosis analysis by annexin V, cellular lysate preparation, and immunoblotting.

Results: The volume of the tumor increased aggressively, reaching 173 mm^3 in the control animals in a time-dependent manner. On the other hand, a sharp drop, to 48.7 mm^3, in the volume of the tumor was observed with the 2 drugs combined, a more than 3-fold decrease. The effect was mediated through the induction of apoptosis via the mitochondrial pathway. The combined treatment downregulated the antiapoptosis and proproliferation protein, nuclear factor-kappa B, and its main oncogenic target cyclin D1. Furthermore, it inhibited the expression of BCL-2 and survivin. This inhibition could explain the cooperative suppression of the proliferation of breast tumor xenografts and the induction of apoptosis by the combined effect of the compounds used.

Conclusions • The key findings clearly indicate the synergistic efficacy of DOX with natural and nontoxic OL against breast tumor xenografts. (*Altern Ther Health Med*. [E-pub ahead of print.])

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One in 8 women develop breast cancer at some stage in their lives, and it may be fatal if not caught in time.[1] The risk of the metastatic development of breast cancer in patients cannot be accurately predicted. Of the more than 80% of patients that receive adjuvant chemotherapy, approximately 40% of them relapse and die of metastatic breast cancer.[2] The survival rates in the last 10 years have been better, but many patients do relapse, and the majority of those patients die of disseminated metastatic disease, which emphasizes the need for new therapeutic strategies.[2]

Several epidemiological studies encompassing various countries, including Italy, Spain, and Greece, have shown that the Mediterranean region has a significantly reduced, all-cause death rate.[3-5] This reduction was mainly due to a decreased incidence of cardiovascular disease and cancer.[6] The protective effects were attributed to the Mediterranean diet, which is rich in fruits and vegetables, whole grains, and olive products.[7] Olive oil is rich in oleic acid and other monounsaturated fats with various biological actions.

Olive oil intake has been shown to induce significant levels of apoptosis in various cancer cells.[8] However, the anticancer properties are thought to be mediated by polyphenols present in the olive, namely oleuropein (OL) and its metabolite, hydroxytyrosol.[9] The concentration of this phenolic fraction is several times higher in the olive leaf than in the oil.

OL is the most abundant of the phenolic compounds in olives.[10] It is the main constituent of the leaves and unprocessed olive drupes of *Olea europaea*. The majority of the polyphenols found in olive oil or table olives are products of its hydrolysis.[11] Several studies have shown that OL possesses a wide range of pharmacological and health-promoting properties including antiatherogenic, antiviral, antimicrobial, hypotensive, and antidiabetic effects.[12,13] Many of these properties have been described as being the result of the antioxidant characteristic of OL.[14]

The current research team has previously investigated the anticancer properties of OL in vitro on basal-like and luminal breast cancer cells.[15] The results have shown that the molecule triggers apoptosis mainly through the mitochondrial pathway via the upregulation of the proapoptotic BAX protein and downregulation of the level of the antiapoptosis BCL-2 protein. It inhibits cell proliferation by delaying the cell cycle at the S phase. Furthermore, OL inhibits nuclear factor-kappa B (NF-κB) and its major target, cyclin D1.[15]

Doxorubicin (DOX) is an effective and frequently used chemotherapeutic agent for various malignancies, such as breast cancer, prostate cancer, and multiple myeloma.

Its action on cancer cells could be through its insertion into DNA and its disruption of topoisomerase-2-mediated DNA repair and of the generation of free radicals, which damage the cellular membranes, DNA, and proteins.[16] Its major adverse effect is cardiotoxicity, which may limit its use.[17]

In the present study, the research team investigated the anti-breast-cancer properties of OL in vivo, both separately and in combination with DOX. The effect of each agent individually as well as the agents in combination on target oncogenes and tumor suppressor genes has been investigated.

METHODS

Animals

Forty female, nude mice (BALB/c OlaHsd-foxn1) were purchased from Harlan Laboratories (Livermore, CA, USA). The nude mice were kept in cages with proper ventilation and given food and water ad libitum.

The study was performed at the College of Science of King Saud University in the University Center for Women Students (Riyadh, Saudi Arabia). All procedures used for animal experimentation were approved by the institutional animal ethics committee of the university.

Procedures

Cell Lines, Chemicals, and Cell Culture. MDA-MB-231 human breast cancer cells were obtained from ATCC (Manassas, VA, USA) and were cultured according to the protocol provided by the company. Cells were maintained in RPMI-1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA); L-glutamine 1%, fetal bovine serum (FBS) 10%; and 1% antibiotic/antimycotic—penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA). Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

The MCF10-A (nontumorigenic epithelial cell line used as control) was also obtained from ATCC and was cultured following the company's instructions. They were maintained in a universal medium: 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (Gibco), supplemented with 5% FBS, 1% antibiotic antimycotic, 20 ng/mL of epidermal growth factor, 100 ng/mL of choleratoxin, 10 mcg/mL of insulin, and 500 ng/mL of hydrocortisone. The cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Olive Leaf Extract Preparation. Green leaves were collected from Manzanillo olive trees (*O europaea*) grown in Tabouk, Saudi Arabia. The plant was identified by members of the Department of Botany of the Faculty of Science at King Saud University. The olive leaves were dried and then powdered. A mixture of 400 mL of methanol and water at 4:1 v/v was added to a sample of 100 g of olive-leaf powder. The mixture was left to stand under agitation for 24 hours and then was filtered. The extract was concentrated by evaporation to dryness at 30°C, and the residue obtained was stored in glass vials at 0°C in the dark until the high-performance liquid chromatography (HPLC) analysis. The chromatograph was obtained from Shimaduz (Kyoto, Japan).

HPLC Analysis. A reversed-phase (HPLC) technique was developed to identify and quantify the OL obtained from the extract under the following conditions: a flow rate of 1.0 mL/minute and a monitoring wavelength of 280 nm. The mobile phase used was 0.1% phosphoric acid in water (A) and 70% acetonitrile in water (B) for a total running time of 30 minutes. The column was 150×4.0 mm ID of Cosmosil 5C18 (Nacalai Tesque, Kyoto, Japan), and the temperature

was maintained at 35°C. Standard OL was purchased from Sigma-Aldrich. The OL extracted from the olive leaves was used for treatment of the mice.

Injection of MDA-MB-231. After acclimatization for 7 days, each mouse was injected subcutaneously with 1×10^6 of MDA-MB-231 human breast cancer cells in the right flank region. When the tumor size was approximately 50 mm^3, the mice were divided into 4 treatment groups, each containing 10 mice, and the mice were treated intraperitoneally (IP) with DMSO and DOX from Sigma-Aldrich and the OL extract. The tumor sizes were measured with a caliper using the following formula: length × width × height.

Intervention

The animals were randomly divided into 4 groups to receive the IP injections once per week for 4 weeks: (1) group 1 (control group)—dimethyl sulfoxide (DMSO), (2) group 2 (intervention group)—50 mg/kg of OL, (3) group 3 (intervention group)—2.5 mg/kg of DOX, and (4) group 4 (intervention group)—1.5 mg/kg of DOX, immediately followed by 50 mg/kg of OL.

Outcome Measures

Tumor Xenografts, Isolation, and Primary Culture. After 4 weeks of treatment, xenografts were excised and collected in tubes containing RPMI-1640 medium. Under sterile conditions, the tumors were placed in tissue culture plates with a few drops of RPMI-1640 media, before being minced with sharp scalpel blades into fine pieces of less than 1 mm in size.

The resulting mixture was further pipetted to obtain maximal cell suspension. Subsequently, the mixture was transferred to sterile, 60-mm culture plates, in the form of a drop in the center of each plate. Each drop was then trapped under a sterile, glass cover slip. Media was added to cover the minced tissue. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The media were changed every 3 to 4 days.

Apoptosis Analysis by Annexin V. Confluent cells were either treated with DMSO and used as control or challenged with the different agents, whereupon cells were incubated in the medium with supplements. The detached and adherent cells were harvested 72 hours later and centrifuged and resuspended in 1 mL of phosphate buffered saline (PBS).

The cells were then stained with propidium iodide (PI) and Alexa Fluor 488 annexin V, using a Vibrant Apoptosis Assay Lit No. 2 (Molecular Probes, Eugene, OR, USA). The stained cells were analyzed by flow cytometry. The percentage of cells was determined by the FACS cadibur apparatus (Becton Dickinson, San Jose, CA, USA) and the Cell Quest Pro software from (Becton Dickinson). For each cell culture, 3 independent experiments were performed.

Cellular Lysate Preparation. The cells were washed with PBS and then scraped in radioimmunoprecipitation assay buffer—150 mM of sodium chloride NaCl, 1 mM of EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM of Tris-hydrochloride at pH 7.5, supplemented with protease inhibitors. The lysates were homogenized and then centrifuged at 14 000 RPM at 4°C for 15 minutes in an Eppendorf microcentrifuge (Thermo Fisher Scientific). The supernatant was removed, aliquoted, and stored at -80° C.

Immunoblotting. The SDS polyacrylamide gel electrophoresis (PAGE) was performed using 12% separating minigels, and an equal amount of protein extract (50 mcg) was loaded. After protein migration and its transfer to polyvinylidene difluoride membrane, the membrane was incubated overnight with the appropriate antibodies. The antibodies included survivin (D-8), NF-κB (F-6), p21 (F-5), BAX (B-9), BCL-2 (C-2), cyclin D1 (HD11), and COX-2 (29), which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cleaved caspase-3 (Asp175) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling (Danvers, MA, USA). Visualization of the second antibody was performed using the super Signal West Pico Chemiluminescent substrate according to the manufacturer's recommendations (Thermo Fisher Scientific).

Statistical Analysis

Significance was determined using the paired 2-tailed Student's *t* test. Data are presented as standard deviations. Tumor sizes are the group's mean tumor sizes. Overall, the results show the effects of the drugs on tumor sizes (in vivo study) and cell apoptosis (in vitro study, in which 3 independent experiments were performed for each cell culture). The effects of the drugs on oncogenes and tumor suppressor genes were also studied and the results are shown by Immunoblotting (results are also shown in the figures). A probability of $P \leq .05$ was considered statistically significant. The graphs were generated using SigmaPlot computer software, version 11 (Systat Software Inc, San Jose, CA, USA).

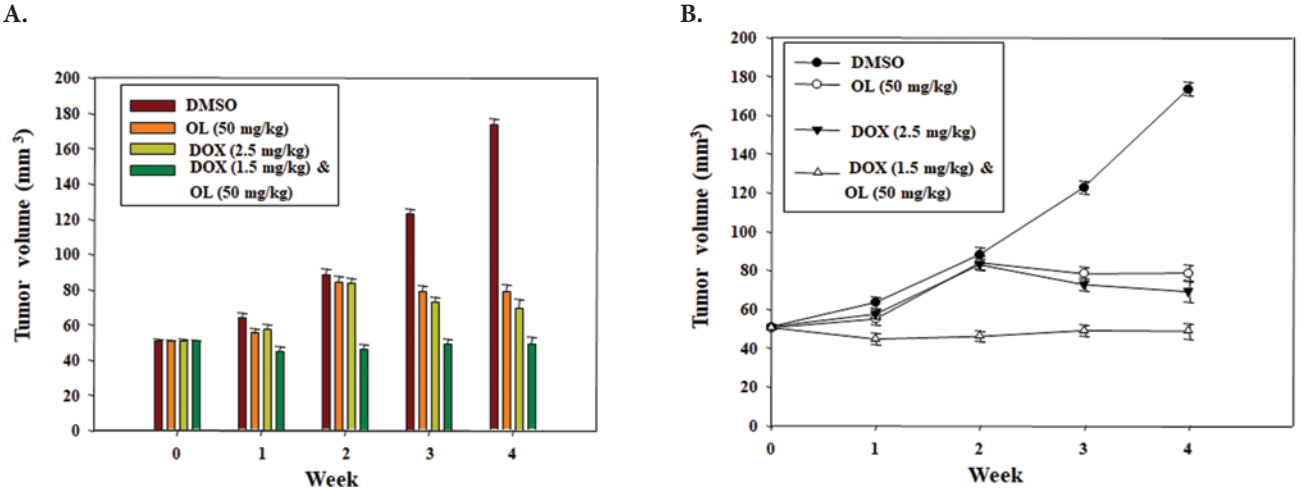
RESULTS

Effect of Combined Treatment on Breast Tumor Growth

The volume of the tumors increased aggressively in the control group in a time-dependent manner, becoming more than 3-fold bigger than the initial size at baseline after 4 weeks of treatment with DMSO. That group's mean tumor size increased significantly after 4 weeks of treatment, growing from approximately 50 mm^3 to 173 mm^3, with $P < .001$ (Figure 1A and Figure 1B).

On the other hand, tumor growth was inhibited in the intervention groups, but the effect was more pronounced in the group that was treated with the combination of drugs. The treatment with either DOX or OL individually reduced the mean size of the tumors gradually, with the OL reducing its size significantly, to 79 mm^3, compared with the control group, with $P < .001$. The mean size decreased to 69 mm^3 when DOX alone was used, with a significant difference when compared with the control group, with $P < .001$.

Figure 1. Effects of OL and DOX, Administered Individually or in Combination, on Tumor Growth in Nude Mice



Note: The tumor sizes were measured with a caliper. In Figure 1A, each error bar represents the standard deviations of at least 3 experiments. In Figure 1B, each point represents the same measurement. The combined dose of 1.5 mg/kg of DOX and 50 mg/kg of OL reduced the tumor size significantly compared with the control group ($P < .001$).

Abbreviations: OL, oleuropein; DOX, doxorubicin; DMSO, dimethyl sulfoxide.

A sharp drop in the volume of the tumor to 48.7mm^3 was observed with use of the DOX followed by the OL, resulting in a more than 3.5-fold decrease after 4 weeks of treatment, and the difference was significant when compared with the control group, with $P < .001$. The dose reduced the mean tumor size to that of its initial size before treatment (Figure 1A and Figure 1B). The results show that OL synergistically increased the DOX-induced inhibition of the proliferation of breast cancer cells in vivo.

Combined Treatment Triggers Apoptosis Through Mitochondrial Pathway

To confirm the synergistic growth-inhibition action of DOX and OL as well as to identify the apoptotic pathway involved, the annexin V/PI staining technique, followed by flow cytometry, was used. Figure 2A shows 4 groups of cells: (1) bottom left quadrant—viable cells that excluded both annexin V and PI (annexin V-/PI-); (2) bottom right quadrant—early apoptotic cells that were stained only with annexin V (annexin V+/PI-); (3) top right quadrant—late apoptotic cells that were stained with both annexin V and PI (annexin V+/PI+); and (4) top left quadrant—necrotic cells that were stained only with PI (annexin V-/PI+).

The proportion of apoptosis was considered to be the sum of both the early and the late apoptosis after deduction of the proportion of spontaneous apoptosis. Although MCF-10A showed only marginal sensitivity to the drugs, MDA-MB-231 exhibited high sensitivity. The intervention with only OL triggered apoptosis in more than 40% of the MDA-MB-231 cells compared with 6% in the control group, with a significant difference between them, with $P < .001$. However, the intervention with DOX only induced apoptosis in approximately 60% of the cells compared with 6.5% in the control group, and the difference was significant as well, with $P < .001$. The synergistic effect of the combined DOX and OL was more potent on the MDA-MB-231 cells, for which the apoptotic rate reached more than 84%, which was significantly higher than the 6.2% for the control group, with $P < .001$ (Figure 2B).

Combined Treatment Downregulates NF-κB, Cyclin D1, p21WAF1

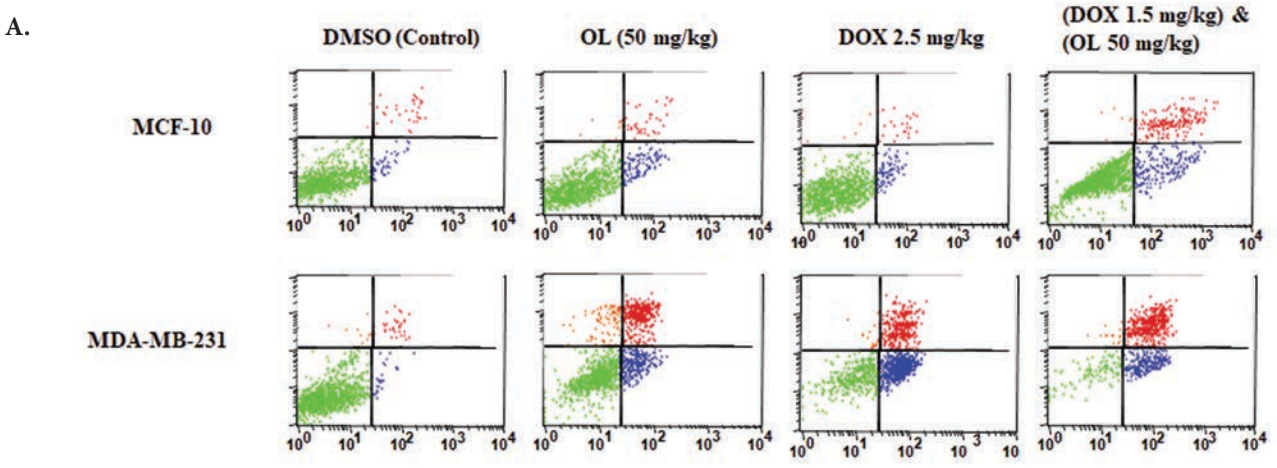
The molecular pathway responsible for the synergistic inhibition of the proliferation of breast cancer cells in vivo by the combined DOX and OL was studied. NF-κB is one of the most important procarcinogenic molecules, which enhances cell proliferation and inhibits cell death.[18]

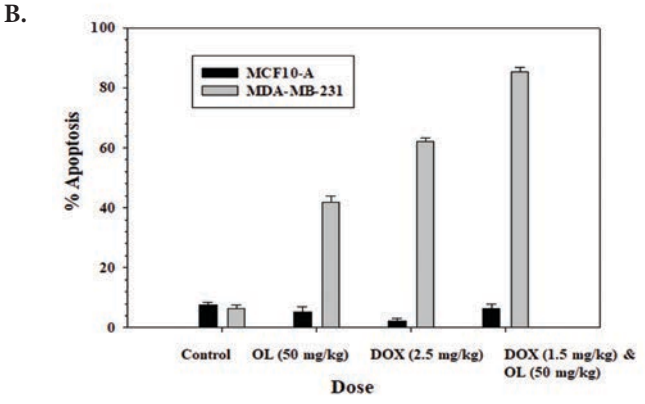
Treatment with either the DOX or OL individually decreased the expression of NF-κB. With OL alone, a 1.4-fold decrease occurred in NF-κB expression, whereas an approximate 12-fold decrease occurred in NF-κB expression when DOX only was used. More important, the combined treatment with DOX and OL induced an approximate 33-fold decrease in NF-κB expression (Figure 3).

To confirm the inhibitory effect of the combined treatment with DOX and OL on NF-κB, the research studied its effects on the main NF-κB downstream effector, cyclin D1. DOX alone decreased the cyclin D1 level by 5-fold; however, only a 2-fold decrease was induced when OL was used alone. The combined treatment with DOX and OL showed a sharp, 100-fold downregulation of cyclin-D1 expression.

Another breast cancer oncogene, COX-2, was also investigated. OL alone increased the expression level of COX-2, whereas DOX alone had no pronounced effect on the oncogene. When combined, COX-2 was downregulated 100-fold as compared with the basal level.

Figure 2. Effects of OL and DOX, Administered Individually or in Combination, on the Apoptosis of Breast Cancer Cells

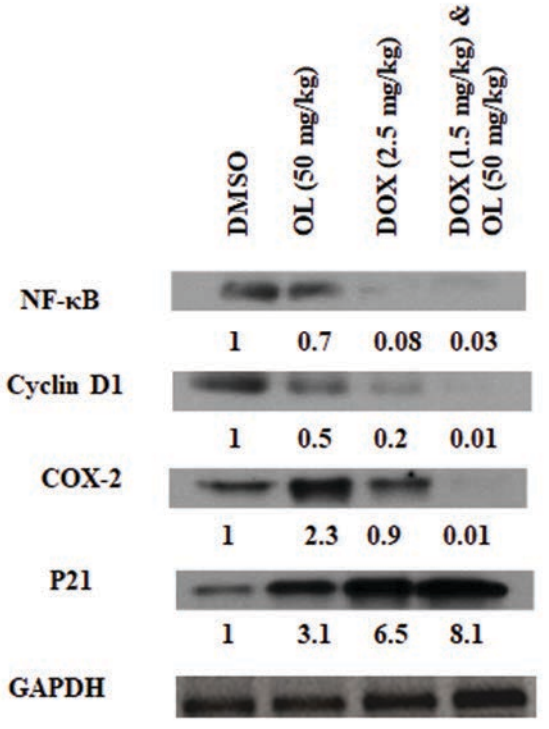




Note: Figure 2A shows 4 groups of cells: (1) bottom left—viable cells that excluded both annexin V and PI (annexin V-/PI-); (2) bottom right—early apoptotic cells that were stained only with annexin V (annexin V+/PI-); (3) top right—late apoptotic cells that were stained with both annexin V and PI (annexin V+/PI+); and (4) top left—necrotic cells that were stained only with PI (annexin V-/PI+). The histogram in Figure 2B shows the proportions of apoptotic cells. The error bars represent the standard deviations of at least 3 experiments. The DOX and OL combination induced apoptosis in the MDA-MB-231 cells in a significantly different manner when compared with the control and the other 2 intervention groups, with $P < .001$.

Abbreviations: OL, oleuropein; DOX, doxorubicin.

Figure 3. Effects of OL and DOX, Administered Individually or in Combination, on the Expression of Some Oncoproteins and the Tumor Suppressor Gene



Note: Tumors were excised, and protein extracts were prepared and used for immunoblotting analysis using the indicated antibodies. The numbers below the bands represent the corresponding expression levels as compared with the control group and after normalization against GAPDH.

Abbreviations: OL, oleuropein; DOX, doxorubicin; DMSO, dimethyl sulfoxide; NF-κB, nuclear factor kappa B; COX-2, cyclooxygenase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The level of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ was also investigated, and its level increased 3-fold and 6.5-fold when OL alone and DOX alone were used alone, respectively. An 8-fold increase occurred when the drugs were combined. This finding indicates that the synergistic effects of the drugs were more pronounced on the inhibition of oncogenes and the upregulation of $p21^{WAF1}$, one of the important tumor suppressor genes.

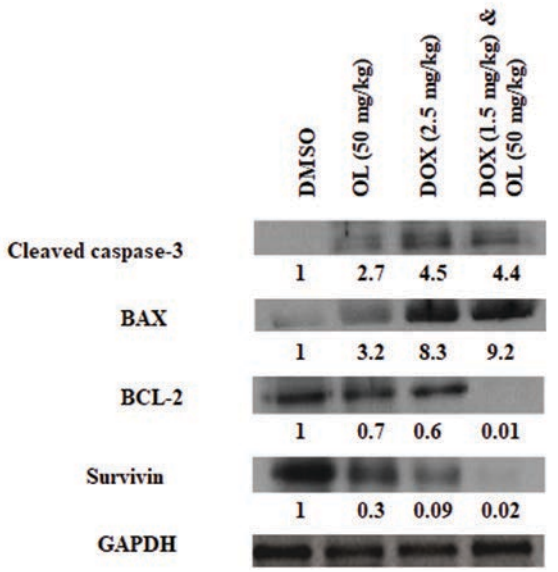
To confirm the synergistic effects of DOX and OL on apoptosis in breast cancer cells and to determine their apoptotic route in vivo, 50 mcg of extracted proteins were used to evaluate the levels of proapoptotic and antiapoptotic proteins, using the immunoblotting technique and specific antibodies. GAPDH was used as internal control.

OL alone upregulated the active form of caspase-3 to a level 2.7-fold higher than the basal; however, DOX alone increased the caspase-3 level up to 4.5-fold, which was similar to that induced by the 2 drugs combined (Figure 4).

To investigate the possible involvement of the mitochondrial pathway, the research team assessed the effects of the drugs on the level of BAX and BCL-2 proteins. Treatment with OL alone increased the level of the pro-apoptotic BAX protein 3.2-fold; however, an 8.3-fold increase occurred in BAX level when DOX was used separately. When combined, the drugs showed 9.2-fold upregulation in the level of BAX. Almost similar effects on BCL-2 levels were observed when using OL or DOX separately, around a 1.5- fold decrease. BCL-2 was highly downregulated, by 100-fold, when the drugs were combined.

Furthermore, the research team studied the effects of the agents on the level of survivin, an anti-apoptosis protein.[19,20] An approximate 3-fold decrease occurred in the survivin level when OL was used individually; however, DOX individually induced a greater effect, a 11-fold decrease on the survivin level. On the other hand, the synergistic action of the drugs was highly pronounced, which was observed as a 50-fold downregulation in the survivin level.

Figure 4. Effects of OL and DOX, Administered Individually or in Combination, on the Expression of Some Apoptotic and Antiapoptotic Proteins



Note: Tumors were excised, and protein extracts were prepared and used for immunoblotting analysis using the indicated antibodies. The numbers below the bands represent the corresponding expression levels as compared to the control group and after normalization against GAPDH.

Abbreviations: OL, oleuropein; DOX, doxorubicin; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

DISCUSSION

The Mediterranean diet, which is rich in fruits and vegetables, has been associated with a lower incidence of disease and an overall improvement in health. These results have been attributed to a high consumption of olive oil in addition to olive leaves (*O europaea*).[21]

The present study showed that a combined treatment with DOX followed by OL, a natural phenolic compound, exhibited a strong anti-breast-cancer effect in vivo. The findings clearly indicated that using either 50 mg/kg of OL or 2.5 mg/kg of DOX effectively reduced breast tumor growth in nude mice. When used individually, the effects of the 2 agents individually on tumor growth were comparable, with a mild, pronounced, greater level of effectiveness for DOX after 4 weeks of treatment (Figure 1A and Figure 1B).

The combined treatment using 1.5 mg/kg of DOX and 50 mg/kg of OL reduced the mean size of the tumors up to more than 3-fold after 4 weeks of treatment. Using high doses of OL in vivo was found to be safe, which has also been reported in earlier studies.[21,22] In fact, it has been previously shown that OL has an outstanding safety profile in rodents. Indeed, a single dose as high as 100 mg/kg of OL was given to rats in one study with no side effects observed.[22] In another study, 1000 mg/kg of olive-leaf extract was administered orally twice daily for 30 weeks to inhibit skin carcinogenesis and tumor growth in mice, and that high dose was found to be safe and significantly effective.[21]

In a previous study, the current research team had shown that OL exhibits specific cytotoxicity against breast cancer cells, with a higher effect on the basal-like MDA-MB-231 cells than on the luminal MCF-7 cells.[15] The team has also shown that OL inhibits cell proliferation of MDA-MB-231 cells by delaying the cell cycle at the S phase. In addition, it has been previously reported that OL inhibits cell proliferation of MCF-7 breast cancer cells.[8] In an in vivo study, it was observed that the OL-treated mice had tumors of a noncohesive, crumbly consistency, unlike tumors from untreated animals, which were more fibrous and solid.[23]

OL is known as an antiangiogenic agent; it can prevent tumor progression.[23-25] It directly inhibits cancer cells leading to tumor regression.[22] On the other hand, DOX is also known

as anti-angiogenic agent.[26,27] Those abilities could partially explain the synergetic effects of OL and DOX in inhibition of breast xenograft growth.

Antiangiogenic compounds have been reported to potentiate antineoplastic therapies against primary and metastatic disease, presumably through increased delivery of antineoplastic agents into the tumor mass.[28] That effect could also explain the anticancer effects of the combined OL and DOX treatment. Furthermore, it has been reported that antiangiogenic therapies can also be effective in late stages of cancer treatment by preventing further growth of the tumor.[29]

The findings of the present study demonstrated that the combined therapy with DOX and OL was significantly more effective at inducing apoptosis in breast cancer cells than when the compounds were used individually. Indeed, using OL singly induced apoptosis of more than 40% of breast cancer cells, while DOX alone increased the apoptotic rate up to 60%. When they were combined, a sharp increase in cell death occurred; it reached around 85% after 72 hours of treatment. In a previous study, the current research team found that 200 μM of OL could trigger apoptosis in more than 50% of MDA-MB-231 cells and could induce necrotic death of around 20% of the same cells in vitro.[15]

It has been reported that OL could irreversibly round ovarian cancer cells, preventing their replication, motility, and invasiveness; these effects were reversible in normal cells. The OL induced cell rounding, causing disruption of the actin cytoskeleton.[23] On the other hand, DOX has been clinically applied to various types of cancer[30]; it has shown induction of cell death in a variety of cancer cells.[31,32]

DOX has been shown to induce apoptosis in ovarian cancer cells due to reorganization of cytoskeletal proteins, including actin, tubulin, and vimentin.[33] OL and DOX share the same feature of affecting cytoskeletal proteins, which may partially explain the synergistic action of the agents in inducing apoptosis in breast cancer cells. The reorganization of cytoskeletal proteins appears to be linked to features of apoptosis.[33,34] Moreover, targeting the cytoskeleton network may attenuate the metastatic activities of tumor cells.[35,36]

In the current study, the combined DOX and OL treatment downregulated 2 major oncoproteins related to breast cancer, namely NF-κB and cyclin D1 (Figure 3). The inhibition of NF-κB activation and its signaling pathway offers a potential cancer therapy strategy. The combined treatment can induce the expression of diverse target genes that promote cell proliferation, regulate apoptosis, facilitate angiogenesis, and stimulate invasion and metastasis.[37,38] It has been found that NF-κB upregulation is implicated not only in tumor growth and progression but also in the resistance to chemotherapies and radiotherapies.[39] One of the most important targets of NF-κB is cyclin D1, which plays important roles in cell proliferation and survival.[40]

In the current study, the combined treatment with DOX followed by OL exhibited a 33-fold downregulation of NF-κB; however, it induced a 100-fold downregulation in cyclin D1. The current research team has previously reported that OL can inhibit the expression of both NF-κB and cyclin D1 in breast cancer MDA-MB-231 cells in vitro.[15] Cyclin D1 overexpression has been found in more than 50% of human breast cancers and causes mammary cancer in transgenic mice. It also plays an important role in cell cycle progression.[41] Therefore, the combined, treatment-related downregulation of NF-κB and its common downstream target cyclin D1 could have a great inhibitory effect on breast-cancer growth.

In the current study, COX-2 was downregulated 100-fold when DOX and OL were combined (Figure 3). Overexpression of COX-2 is considered to be an ubiquitous driver of mammary carcinogenesis, and, reciprocally, COX-2 blockade has a strong potential for breast cancer prevention and therapy. COX-2-positive tumors are associated with markers of a worse breast cancer prognosis.[42] It has been found that COX-2-positive tumors were associated with a 35% increased risk of breast cancer death in simple models.[43]

In the present study, the combined treatment of DOX and OL triggered the cleavage of caspase-3, which led to a significant increase in its active form, confirming its synergistic induction of apoptosis in breast cancer cells. The current research team has previously shown that OL could induce caspase-3 cleavage in MDA-MB-231 cells in vitro.[15]

The combined treatment in the current study also induced overexpression of BAX by 9.2-fold. Furthermore, it exerted a strong inhibitory effect on the 2 major apoptosis-inhibitor proteins, BCL-2 and survivin, which are both related to breast-cancer pathology and therapeutic outcome.[20,44,45]

Survivin is a multifunctional protein that controls cell division, inhibition of apoptosis, and promotion of angiogenesis. It is overexpressed in the majority of breast cancers and its expression has been found to confer resistance to chemotherapy and radiation.[20,46] The current research team has previously shown that OL can trigger apoptosis through the mitochondrial pathway in MDA-MB-231 breast cancer cells in vitro.[15]

The present study showed that the combined treatment of DOX and OL upregulated $p21^{WAF1}$ in a p53-independent manner, because the action occurred in MDA-MB-231 cells, which are p53-defective.[47] This result is supported by the current research team's previous finding that OL increased the expression of $p21^{WAF1}$ in MDA-MB-231 cells in vitro. Overexpression of $p21^{WAF1}$ can block both the G1/S and G2/M transitions of the cell cycle.[48] Moreover, $p21^{WAF1}$ is a modulator of apoptosis in a number of systems.[49]

Thereby, the combined treatment with DOX and OL inhibited both NF-κB and its common downstream target cyclin D1 in tumor xenografts as well as had a strong inhibitory effect on the 2 major apoptosis-inhibitor proteins, BCL-2 and survivin. These findings indicate potent anti-breast-cancer properties for the compounds. To confirm the anti-breast-cancer activity of the DOX-OL combined therapy, additional human clinical studies are needed, especially on the clinically aggressive ER-negative types, for which the prognosis is still poor and which are clinically characterized as less responsive to standard treatments.[50,51]

In the present study, using a low dose of DOX combined with OL exhibited higher cytotoxic and apoptotic effects than using a high dose of DOX individually. Therefore, OL can protect against the DOX-induced cardiotoxicity in mice. Its cardiomyopathic effect has been dose-related, the total cumulative dose being the only criteria currently used to predict the toxicity.[52]

CONCLUSIONS

A broad assessment of all the variables evaluated in the present study could explain the combined treatment-dependent suppression of cell proliferation and the induction of apoptosis in breast cancer cells. OL serves as a promising anticancer agent and further studies may provide important leads toward its clinical application.

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