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Effect of Oncoxin Oral Solution in HER2-Overexpressing Breast Cancer

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One of the most aggressive breast cancer subtypes includes tumors with high expression of HER2. Gene expression and functional studies have shown a link between HER2 overexpression and oxidative stress. Because of this, we hypothesized that Oncoxin Oral Solution (OOS), a composite product that contains several antioxidants, could have an antitumoral effect against HER2+ tumors. Dose-response studies, biochemical and cytometric assessment of the effect of OOS on cell cycle and apoptosis, and drug combination analyses were performed on BT474 and SKBR3 cells, 2 HER2-overexpressing breast cancer cell lines. OOS reduced the proliferation of these cells, and augmented the action of lapatinib, a HER2 inhibitor used in the breast cancer clinic. Moreover, OOS decreased growth of HER2+ tumors in mice. Mechanistically, OOS provoked cell cycle blockade through upregulation of p27 expression and downregulation of cyclin D levels. OOS also caused apoptotic cell death in HER2+ breast cancer cells, as indicated by increases in PARP cleavage as well as upregulation of caspase 8 and caspase 3 activities. These results demonstrate an antitumoral action of OOS in preclinical models of HER2+ breast cancer and suggest that it can be used with anti-HER2 therapies currently adopted as standard of care in the oncology clinic.

INTRODUCTION

Breast cancer remains a leading cause of disease and death among women (1). One type of breast cancer includes tumors that bear high amounts of the transmembrane tyrosine kinase HER2. HER2 is overexpressed in an estimated 20–25% of the

1.5 million new breast cancers that are diagnosed annually worldwide and is characterized by shorter survival (2). The correlation between HER2 receptor overexpression and patient outcome, together with the demonstration of a potent oncogenic role of HER2 receptors in preclinical models (3–5), established the bases for the development of agents that target HER2 receptors for the treatment of patients bearing tumors with high HER2 receptor functionality (6). The first anti-HER2 treatment that reached the breast cancer clinic, trastuzumab, is an antibody that acts on the ectodomain of HER2 (7). Another more recently incorporated drug is lapatinib, an orally available receptor tyrosine kinase inhibitor that, like trastuzumab, is highly selective for HER2 (8). Lapatinib is especially active in refractory metastatic breast cancer patients and as a first-line metastatic treatment, with potential benefit in patients with brain metastases (9,10). Several cell-based studies have demonstrated that lapatinib combined with trastuzumab enhances breast cancer cell apoptosis in HER2 overexpressing breast cancer cells, and breast cancer cells resistant to trastuzumab monotherapy (11,12). However, most patients still relapse, indicating the need for novel combination partners to increase duration of response or to treat relapsed disease. In this respect, a novel anti-HER2 agent, the antibody Pertuzumab, has shown increased antitumor efficacy when combined with trastuzumab based therapies (13). However, despite these advances, HER2+ tumors still represent an important problem in the metastatic setting, as patients frequently relapse. Therefore, novel therapeutic strategies are required to improve efficacy of current therapies and prevent relapses. Such therapies should exploit intrinsic characteristics of the tumoral cells. For example, gene expression profiling of HER2+ tumors has revealed upregulation of genes linked to H₂O₂ generation (NADPH oxidase, Nox4), or are known to be induced upon

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oxidative stress (such as NQO1) (14). These data indicate that HER2+ overexpression may cause an oxidative stress response and suggest the possibility of using antioxidant therapies to fight against HER2+ tumors. In support of this idea is also the fact that antioxidant treatment of HER2-transformed cells inhibits their proliferation (15,16).

Multiple studies show that the coadministration of combination of various antioxidants with conventional chemotherapy or radiotherapy results in significant antitumor synergism and an inhibition of the toxicity of conventional therapy (17,18). While the pure compounds have not been licensed for use in oncology, the availability of commercial formulations, which include some of these molecules, offers the possibility to incorporate them easily and safely to the treatments commonly used to treat certain neoplasias.

Oncoxin Oral Solution (OOS) is an orally active nutritional supplement that includes recognized anti-cancer antioxidants. OOS contains green tea's polyphenols, including epigallocatechin 3-gallate, vitamin B6, and vitamin C, three products whose anti-cancer action is supported by many studies (17,19,20). It contains also cinnamic acid that plays an important role in the inhibition of tumor growth (21). Other important component is glycyrrhizinic acid, which has been described as antiinflammatory and immunomodulator (22). OOS is currently being investigated in clinical trials as part of the treatment for several types of cancer. In these studies, OOS was associated with chemotherapy and radiotherapy and showed improvement in quality of life in patients (23). However, the mechanism of action of OOS is largely unknown. Moreover, whether OOS may improve the action of novel generation targeted agents is unknown and deserves to be explored.

These antitumoral effects of OOS, together with the fact that several antioxidants have been shown to exert antitumoral actions on HER2 overexpressing breast cancer cells (24,25), led us to explore the potential antitumoral effect of OOS on breast cancer models with HER2 overexpression. We report here that OOS exerts an antiproliferative action on HER2 overexpressing breast cancer cell lines in vitro, and reduces tumor growth in 2 models of mice bearing HER2+ tumors. Moreover, combination studies aimed at evaluating whether OOS could augment the efficacy of standard of care drugs used in the breast cancer clinic showed that OOS augments the antitumoral action of lapatinib.

MATERIALS AND METHODS

Reagents and Antibodies

Cell culture medium [Dulbecco's modified Eagle medium (DMEM)], penicillin-streptomycin and serum [fetal bovine serum (FBS)] were purchased from Invitrogen (Gaithersburg, MD). OOS was provided by Catalysis, S.L. (Madrid, Spain). Lapatinib was from LC-Laboratories (Woburn, MA), respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (Madrid,

Spain), and the Annexin V-FITC Kit was from BD Biosciences (San Jose, CA). Caspase 3 and caspase 8 Fluorometric Assay Kits were obtained from Enzyme Systems Products (Livermore, CA). Other generic chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Roche Biochemicals (Hoffmann, Germany), or Merck (Darmstadt, Germany).

The origins of the different antibodies used in the Western blotting analyses were as follows: the anti-p27, anti-poly (ADP-ribose) polymerase (anti-PARP), anti-cyclin-dependent kinase 2 (anti-Cdk2), and anti-cyclin E were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-cyclin D1 was from BD Biosciences (San Jose, CA). The anti- α -tubulin antibody was from Oncogene Science (Uniondale, NY). The anti-pRb (Ser 807/811), and anti-phosphorylated (Ser139) H2A histone family member X (anti- γ H2AX) were from Cell Signaling (Beverly, MA). The horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad (Richmond, CA).

Cell Culture

Breast cancer human cell lines (BT474 and SKBR3, with overexpression of HER2 receptor) were grown in DMEM supplemented with 10% FBS, 100 mU/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine at 37°C in a humidified atmosphere in the presence of 5% CO₂/95% air. The cell lines were obtained from the American Type Culture Collection Cell Biology Collection (Manassas, VA).

In Vivo Experiments

For xenograft studies, fifteen 7-wk-old female BALB/c nude mice were obtained from Charles River Laboratories (Wilmington, MA) and housed at our Institutional Animal Care Facility. One week later, 6×10^6 of BT474 cells resuspended in 50 μ L of DMEM and 50 μ L of Matrigel were injected into 2 of the mammary fat pads of each mouse, and tumor masses measured. After 2 wk, and considering the volume of the largest of the 2 tumors in each mouse, 3 groups of mice ($n = 5$ each) were prepared. Mice with smaller tumors were included in the control group; mice with intermediate size tumors were included in a group to be treated orally with OOS (200 μ l); and a third group included mice with larger tumors which were selected to be treated intraperitoneally with OOS (200 μ l). The reason for grouping animals with larger tumors to be treated with OOS was to create an unfavorable situation for OOS to fight against the tumors. All treatments were administered with a daily schedule along 7 wk. Mice were weighed and the inoculation site was palpated at weekly intervals. Tumor diameters were serially measured by weekly measurement with a digital caliper (Proinsa, Vitoria, Spain), and tumor volumes were calculated using the following formula: $V = (L/2) \times (W/2)^2 \times 4/3 \times \pi$, where V = volume (cubic millimeters), L = length (millimeters), and W = width (millimeters). At the time of animal sacrifice

[49 days after initiation of treatment with OOS or phosphate-buffered saline (PBS)], tumor tissue was resected and immediately frozen at -80°C .

For the spontaneously tumorigenesis model, MMTV/neu transgenic mice [FVB/N-TgN (MMTVneu) 202Mul/J] were purchased from Jackson Laboratory (Bar Harbor, ME) and bred to produce multiple litters. At 6 wk of age, females were randomly assigned to 3 groups ($n = 8/\text{group}$): the mice were given regular water (control group), or solutions of OOS [with a dose of 100 μl (OOS 100 μl group) or 500 μl (OOS 500 μl group) per mouse, considering each mouse drinks 15 ml/100 g/24 h] ad libitum from 6 to 58 wk of age. Mice were weighed at weekly intervals. Mice were palpated twice weekly for mammary gland tumor nodules, and the time of appearance of the first tumor (latency period) was recorded and the growth monitored for 45 days. Animals were sacrificed when tumors reached 2 cm of diameter, or at 58 weeks of age. Mammary tissues were harvested and stored at -80°C . All animal experiments were conducted according to Institutional Guidelines for the Use of Laboratory Animals of the University of Salamanca, after acquiring permission from the local Ethical Committee for Animal Experimentation, and in accordance with current Spanish laws on animal experimentation.

Cellular Experiments

The proliferation of breast cancer cells was examined using MTT metabolization assays (26). BT474 and SKBR3 cell lines were plated at a density of 3×10^4 cells/well in 24-well plates and cultured overnight in DMEM supplemented with 10% FBS. For the dose-response studies, cells were incubated with OOS at different dilution factors (1:1000, 1:500, 1:100, 1:50, 1:10) and MTT metabolization was measured after 24 and 48 h. Briefly, at the end of the incubation period, the culture media of each well was replaced with 250 μL of fresh medium containing MTT (0.5 $\mu\text{g}/\mu\text{L}$) and incubated for 1 h. The medium was then removed and 500 μL of dimethyl sulfoxide (DMSO) were added to each well. The plate was shaken in the dark for 5 min to dissolve the MTT-formazan crystals. The absorbance was recorded at 570 nm in a multiwell plate reader (UVM-340, ASYS Hitech GmbH, Salzburg, Austria). The mean absorbance values of untreated samples from each cell line were taken as 100%. The data were plotted using mean \pm SD of quadruplicates of an experiment that was repeated twice.

For the combination studies, SKBR3 cells were cultured in the presence or absence of OOS (1/50 dilution), lapatinib (50 nM) or both and MTT metabolization values analyzed after 24 h. The results are shown as the mean \pm SD of quadruplicates of an experiment that was repeated at least twice. Statistically significant differences between drugs alone and the double combination are indicated by * ($P < 0.01$, Student's *t*-test).

For cell cycle analyses, the BT474 and SKBR3 cell lines were cultured in 100-mm culture dishes at 70% confluence, and treated with different doses of OOS (with or without

lapatinib) for 24 and 48 h. Cell monolayers were then incubated in trypsin-EDTA and resuspended in 1 ml of PBS. After 3 washes with PBS, the cell pellets were fixed in 70% ethanol at 4°C for 24 h. Cells were centrifuged at 1300 g, and pellets were treated with 1 mL of propidium iodide (PI) staining solution (PBS containing 50 $\mu\text{g}/\text{mL}$ of PI, 0.5% Tween 20, 0.1 $\mu\text{g}/\text{mL}$ RNase A). DNA content and cell cycle analyses were performed by using a FACScalibur flow cytometer and the CellQuest software (BD Biosciences, San Jose, CA).

For apoptosis analyses, the BT474 and SKBR3 cell lines were trypsinized, washed twice with cold PBS, and resuspended in binding buffer [10 mM HEPES free acid (pH 7.4), 140 mM NaCl, 2.5 mM CaCl_2] at a concentration of 10^6 cells/ml. A total of 1×10^5 cells were incubated for 15 min in the dark with 5 μL of Annexin V-FITC and 10 μL PI staining solution, and 400 μL of binding buffer were added for cytometric analyses.

Biochemical Experiments

To analyze caspase 3 and caspase 8 activation status, fluorimetric assays were performed. We treated 4×10^6 cells as indicated and then incubated the cells for 30 min at 4°C in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 20 mM EDTA, 0.2% Triton X-100, 1mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin). Lysates were cleared at 10,000 g for 15 min at 4°C . Protein (50 μg) was incubated in a 96-well plate with 0.2 mM of Ac-DEVD-AFC specific for caspase 3 or Ac-IETD-AFC specific for caspase 8. Caspase activity was measured at 410 nm in the presence or absence of *N*-acetyl-Asp-Glu-Val-Asp-CHO (1 μM). Specific caspase activity was expressed in substrate cleavage or released absorbance.

Western Blotting Analyses

BT474 and SKBR3 cell extracts were washed with PBS and scraped with ice-cold lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40, 20 mM Tris pH 7.0, 1 mM pepstatin, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 25 mM β -glycerophosphate, 1 mM PMSF, 50 mM sodium fluoride, and 1 mM sodium orthovanadate). Frozen mice tumoral samples were washed with PBS and homogenized in ice-cold lysis buffer (with $10\times$ proteases inhibitors) with a tight-fitting Dounce homogenizer (27). Samples from both origins were centrifuged at 10,000 g at 4°C for 10 min and supernatants were transferred to new tubes for protein concentration measurement by Bradford assay (28). Samples were then boiled in electrophoresis sample buffer and loaded onto SDS-PAGE gels. After transfer to polyvinylidene difluoride membranes (millipore), filters were blocked for 1 h in Tris buffered saline with 0.2% Tween 20 (TBST) and then incubated for 2 to 16 h with the corresponding antibody. After being washed with TBST, filters were incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min and bands were visualized by a luminol-based detection system with *p*-iodophenol (29) enhancement.

Statistical Analysis

The Mann-Whitney U-test was used for comparisons between means. $P \leq 0.05$ was interpreted to denote statistical significance. The statistical analyses were performed using the SPSS 14.0 statistical package (SPSS, Chicago, IL). All data were analyzed using the statistical software SPSS 14.0 (SPSS, Inc, Chicago, IL) and SigmaPlot for Windows, Version 6.0 (Systat Software, Inc. Germany).

RESULTS

Effect of OOS on the Proliferation of HER2+ Breast Cancer Cells

We measured the action of OOS in 2 breast cancer cell lines (BT474 and SKBR3) that represent in vitro models of HER2 receptor overexpression and activation (30). OOS was diluted in culture media, and its effect on the proliferation of

breast cancer cells evaluated by MTT metabolization assays. OOS decreased metabolization of MTT in the 2 cell lines studied in a dose-dependent manner (Fig. 1A). This effect was more evident at largest incubation times. In fact, the IC_{50} values of OOS were different at 24 and 48 h. At 24 h the IC_{50} values (the dilution of OOS that inhibited growth of treated cells by 50% relative to untreated cells) for SKBR3 and BT474 cells were 0.08 and 0.06, respectively, and at 48 h of 0.02 and 0.03.

In Vivo Efficacy of OOS in Breast Cancer Murine Models

Given the action of the OOS in vitro, we explored whether this in vitro effect could translate in vivo. To this end, we used 2 different murine models. One consisted of a xenograft model in BALB/c nude mice orthotopically injected with BT474 cells, and another of spontaneous tumorigenesis using MMTV/neu transgenic mice [FVB/N-TgN(MMTVneu)

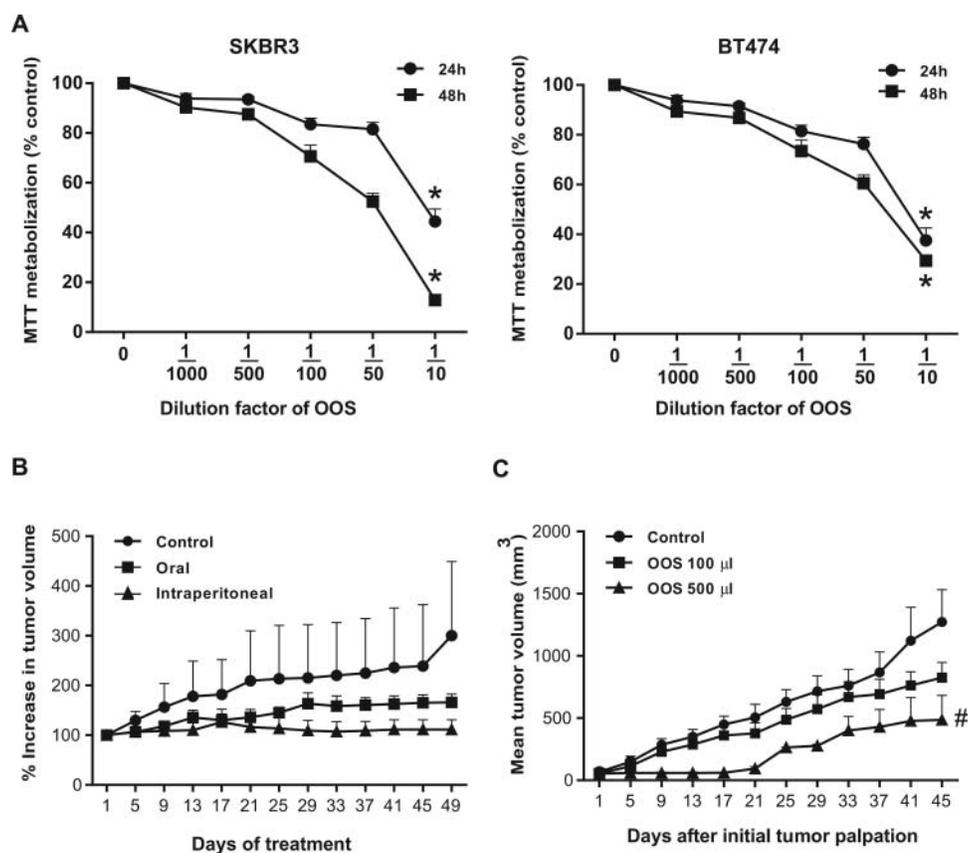


FIG. 1. Efficacy of Oncoxin Oral Solution (OOS) on HER2 breast cancer cells in vitro and in vivo. A: Dose-dependent effect of OOS on the proliferation of BT474 (right) and SKBR3 (left) cell lines in vitro. Cells were incubated with OOS at the indicated dilution factors and MTT metabolization was measured after 24 and 48 h. The mean absorbance values of untreated samples from each cell line were taken as 100%. Data are represented as mean \pm SD of quadruplicates of an experiment that was repeated twice. The asterisks indicate $P < 0.01$ (Student's *t*-test) of the values of OOS treated samples when compared to untreated control samples. B: Female BALB/c nude mice were injected with BT474 and treated either intraperitoneally or orally with OOS or vehicle phosphate-buffered saline (PBS) for 7 days/week for 7 wk. The percent increase in tumor volume for each of the 3 groups of mice with respect to Day 1 of treatment is shown. Data are represented as mean \pm SD. C: Female, MMTV-neu transgenic mice were treated with OOS (100 μ l or 500 μ l per mouse) in the diet as described in **Material and Methods**. When tumors were palpable, their growth was monitored twice weekly for 45 days. Results represent the mean tumor volume \pm SD. Statistically significant differences between control and treated group are indicated as # at Day 45 ($P < 0.05$, Mann-Whitney U-test).

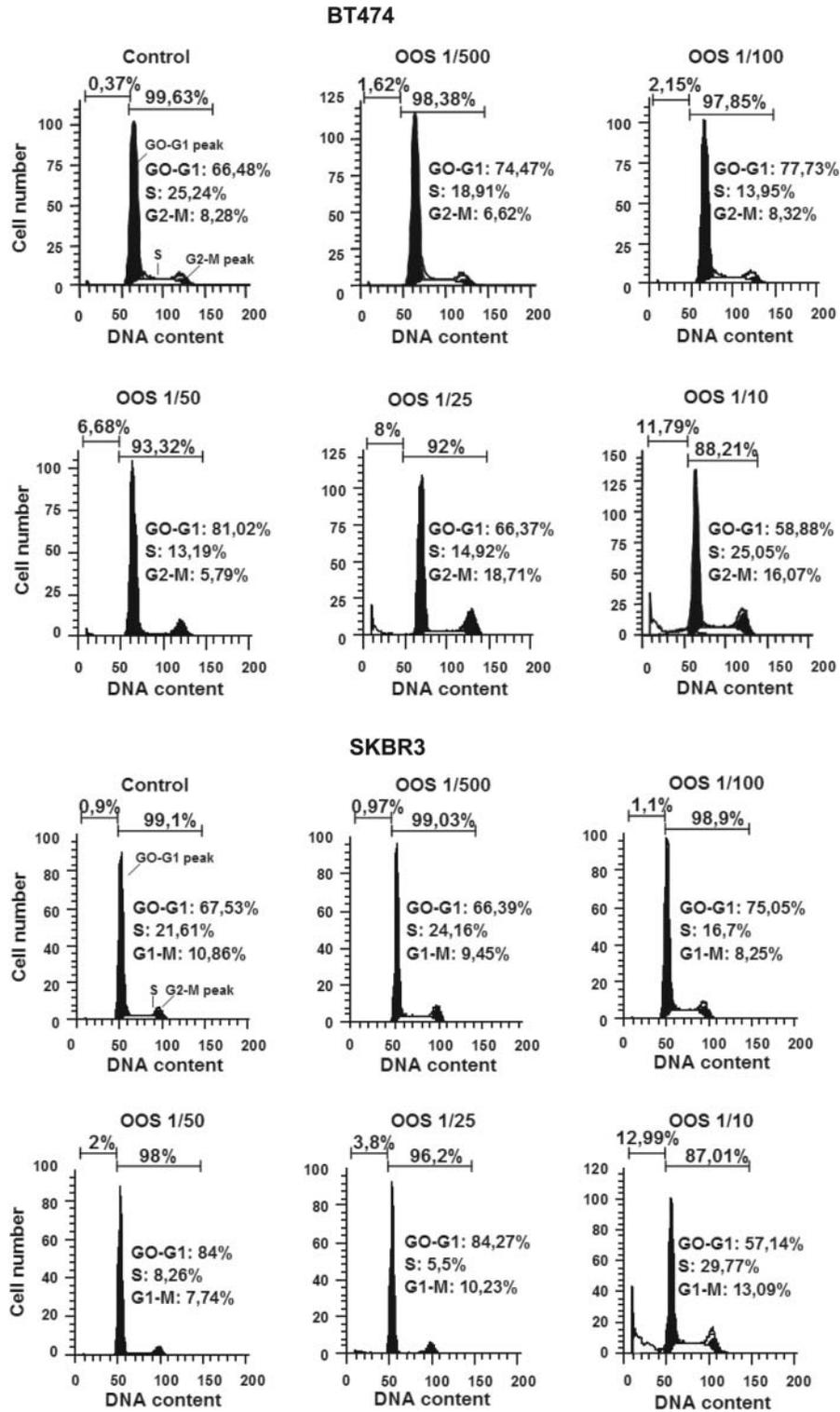


FIG. 2. (Continued)

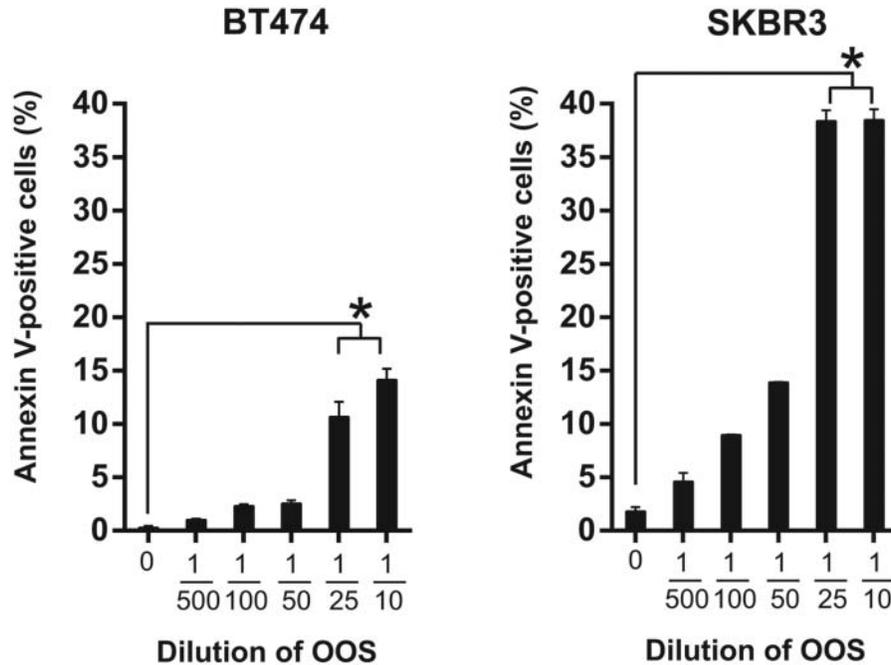


FIG. 2. Effect of Oncoxin Oral Solution (OOS) on cell cycle progression and apoptosis. A: BT474 and SKBR3 cell lines were treated with indicated dilutions of OOS for 24 h, and cell cycle progression was examined by flow cytometry after staining with propidium iodide (PI). The histograms of 1 representative experiment are shown. The percent of cells in each phase of the cell cycle (G0/G1, S, or G2/M) is indicated, as well as the sum of the percentages of cells in all of these phases. The % of cells in the subG0 region is also indicated. B: Flow cytometric analysis of apoptosis in cells treated with OOS for 24 h and stained with Annexin V. The bar graph represents the percentage of cells (mean \pm SD) positively stained with Annexin V. Statistically significant differences between control and treated samples are indicated as * ($P < 0.01$, Student's *t*-test).

202Mul/J] (31). In the first model we used BT474 cells because they represent a well-established HER2+ cell line used to evaluate the action of anti-HER2 treatments. These cells were orthotopically injected at the 2 caudal mammary glands of mice, and the growth of each individual tumor analyzed. This orthotopic model was used to assess the action of OOS once tumors had established, whereas the MMTV/neu transgenic mice model was used to evaluate the potential preventive action of OOS in breast cancer tumorigenesis driven by HER2. In the first model, BALB/c nude mice bearing human subcutaneous tumors of BT474 cells were grouped to receive PBS or 2 administration routes of OOS: oral and intraperitoneal. Mice with smaller tumors ($64.7 \text{ mm}^3 \pm 16.33$) were included in the control group, which received PBS orally as vehicle. Mice with intermediate size tumors ($89.9 \text{ mm}^3 \pm 24.61$) were included in a group to be treated orally with OOS ($200 \mu\text{l}$). A third group included mice with larger tumors ($113.1 \text{ mm}^3 \pm 17.85$), which were selected to be treated intraperitoneally with OOS ($200 \mu\text{l}$). Because of these differences in initial tumor volume, to compare growth rates of the different groups, the initial mean tumor volumes were normalized to 100%, and subsequent measures along time referred as % change with respect to the initial tumor volume of each cohort when treatment was initiated (Day 1). Treatment either orally or intraperitoneally with OOS

decreased the growth of the xenografted human tumors (Fig. 1B). At Day 49 the mean tumor volume in control mice was $300.3\% \pm 148.5$ (mean \pm SE) as compared with $166.1\% \pm 16.8$ in the oral group or $111.6\% \pm 19.4$ in the intraperitoneal group (Fig. 1B). The mean body weight of control and OOS-treated mice did not change significantly throughout the duration of the experiment. Thus, at Day 49 the mean body weight in control mice was 20.6 ± 0.5 grams as compared with 20.4 ± 0.5 g in the oral group or 20.4 ± 0.5 g in the intraperitoneal group.

We then analyzed the effect of OOS on tumor progression in an animal model of HER2-driven metastatic breast cancer, the MMTV-neu mice, which overexpress c-Neu (the rat homolog of human ErbB2) in the mammary epithelium. These mice develop hyperplastic glands and focal mammary carcinomas (32). MMTV/neu transgenic mice were given regular water (control group) or solutions of OOS in drinking water (with a dose of $100 \mu\text{l}/\text{day}$ or $500 \mu\text{l}/\text{day}$ per mouse, considering each mouse drinks $15 \text{ ml}/100 \text{ g}/24 \text{ h}$) ad libitum. Addition of OOS to the drinking water started at 6 wk of age. Mice receiving water developed tumors at 238 ± 25 days. Mice receiving OOS developed tumors later. Those treated with $100 \mu\text{l}/\text{day}$ developed tumors at 250 ± 23 days, whereas those receiving $500 \mu\text{l}/\text{day}$ developed tumors after 278 ± 16 days after initiation of the treatment. Once tumors had established, their

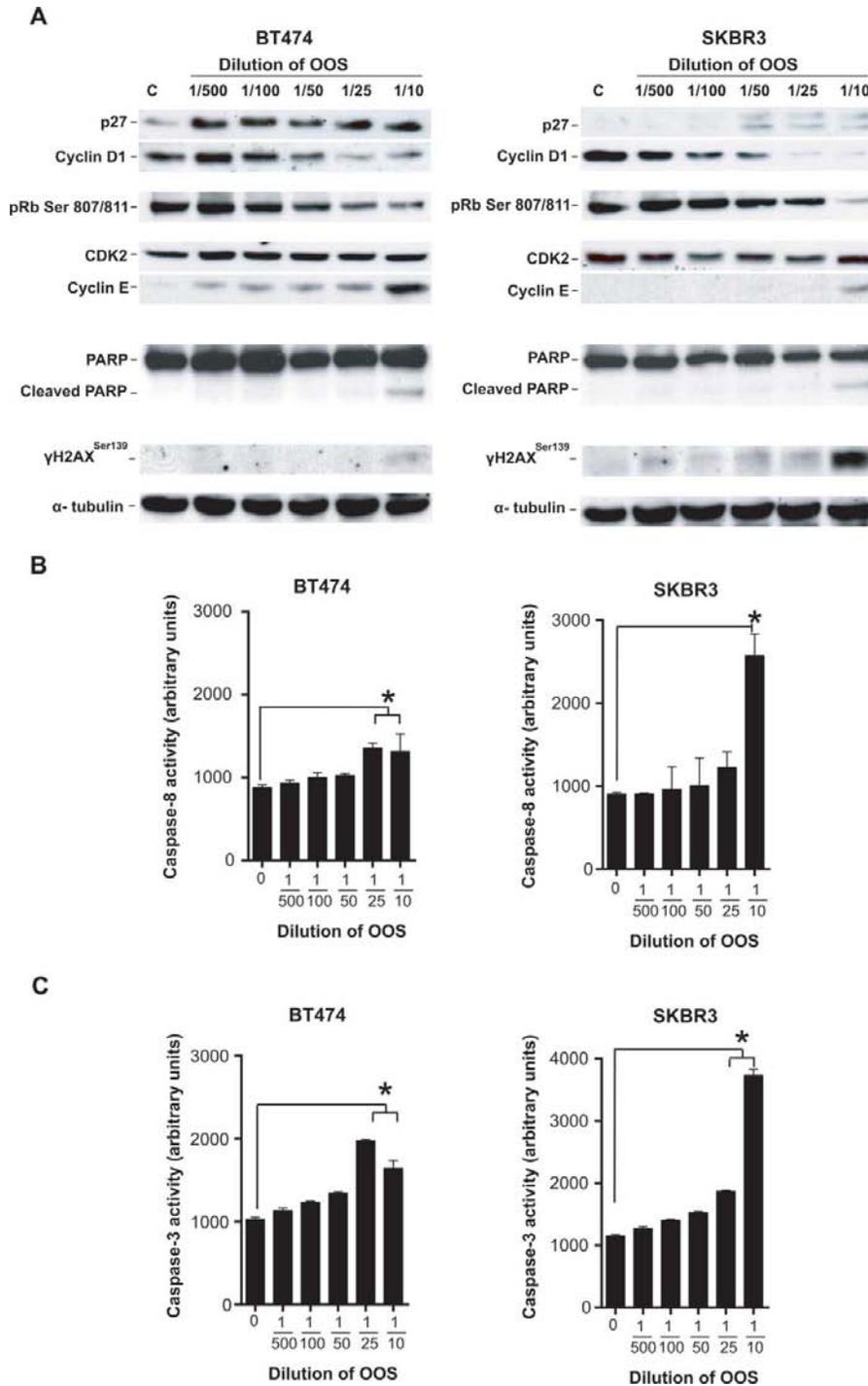


FIG. 3. Action of Oncoxin Oral Solution (OOS) on cell cycle and apoptotic proteins. A: BT474 and SKBR3 cells were treated with the indicated dilutions of OOS for 24 h, and the levels of cell cycle (p27, cyclin D1, pRb, Cdk2, cyclin E), apoptotic (PARP), and DNA damage response (γ H2AX) related-proteins were analyzed by Western blotting. α -Tubulin levels were assessed and used as loading controls. B: OOS enhances caspase 8 activity. BT474 or SKBR3 cells were treated with the indicated dilutions of OOS for 24 h, and caspase-8 activity was assessed as described under the **Materials and Methods** section. Results are presented as mean \pm SD of 3 replicates for each OOS dilution. Statistically significant differences between control and treated samples are indicated as * ($P < 0.01$, Student's *t*-test). C: OOS enhances caspase 3 activity. BT474 or SKBR3 cells were treated with OOS as above, and caspase-3 activity was assessed as described under the **Materials and Methods** section. Results are presented as mean \pm SD of 3 replicates for each OOS dilution. Statistically significant differences between control and treated samples are indicated as * ($P < 0.01$, Student's *t*-test).

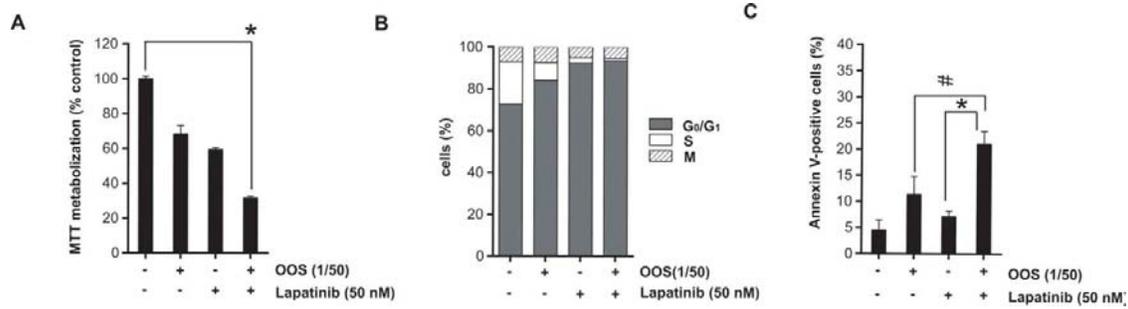


FIG. 4. Effect of Oncoxin Oral Solution (OOS) and lapatinib combination on proliferation and apoptosis. A: SKBR3 cells were cultured in the presence or absence of OOS (1/50 dilution), lapatinib (50 nM), or both and MTT metabolization values analyzed after 24 h. The results show the mean \pm SD of quadruplicates of an experiment that was repeated at least twice. Statistically significant differences between drugs alone and the double combination are indicated by * ($P < 0.01$, Student's *t*-test). B: Analysis of the percentage of cells in the different cell cycle phases in SKBR3 cells treated with OOS, lapatinib, or the combination of both for 24 h. C: Annexin V staining of SKBR3 cells treated with OOS, lapatinib, or the combination of both for 24 h. The results are presented as the mean \pm SD percentage of cells stained with Annexin V in 2 independent experiments. Statistically significant differences between drugs alone and the double combination are indicated by * ($P < 0.01$) or # ($P < 0.05$) (Student's *t*-test).

growth was monitored for 45 days. After those 45 days the mean \pm SE of tumor volumes were $1272.9 \pm 259.6 \text{ mm}^3$ (control group), $825.7 \pm 125.4 \text{ mm}^3$ (group treated with $100 \mu\text{l}$ per mouse) and $485.4 \pm 196.7 \text{ mm}^3$ (group treated with $500 \mu\text{l}$ per mouse). OOS demonstrated a clear benefit slowing

tumor growth, and statistically significant differences were seen for the group treated with a dose of $500 \mu\text{l}$ per mouse, as compared with the control group (Fig. 1C). Of note, while 25% of control animals developed 3 or more tumors (up to 4) OOS-treated animals never developed more than 3 tumors.

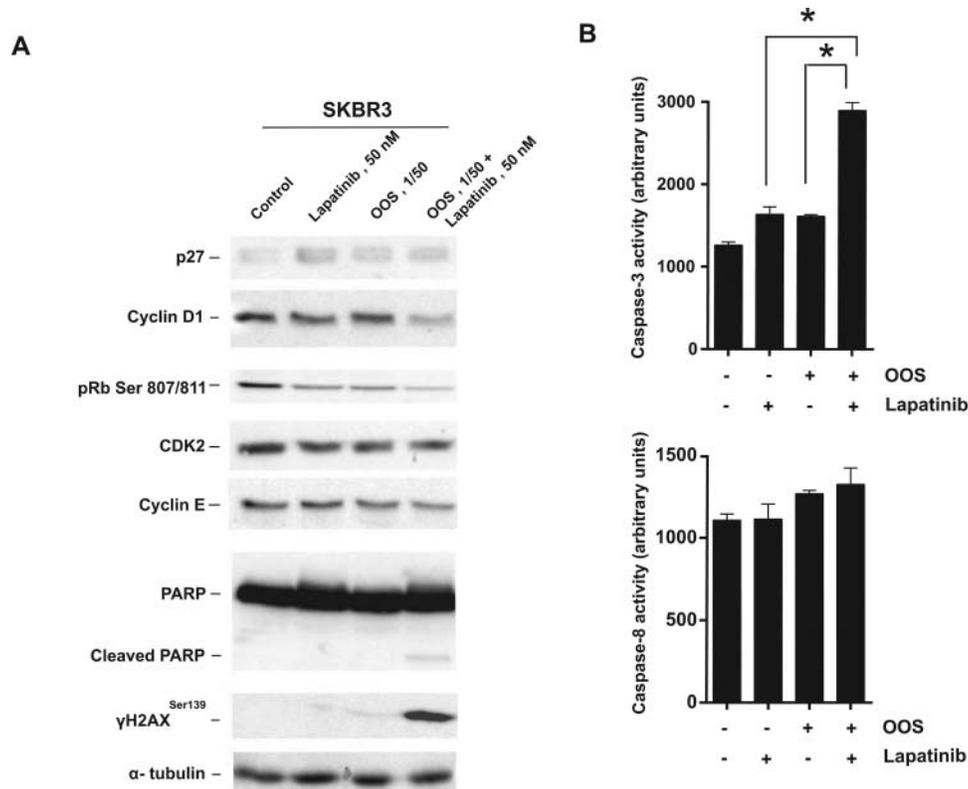


FIG. 5. Effect of Oncoxin Oral Solution (OOS) and lapatinib combination on apoptosis, cell cycle and DNA damage response pathways. A: SKBR3 cells were treated with OOS (1/50 dilution), lapatinib (50 nM), or both for 24 h, and immunoblotting analysis was done for cell cycle (p27, cyclin D1, pRb, Cdk2, cyclin E, apoptotic (PARP), and DNA damage response (γ H2AX) related proteins. α -Tubulin levels were assessed and used as loading controls. B: SKBR3 cells were cultured in the presence or absence of OOS (1/50 dilution) or lapatinib (50 nM) or a combination of both drugs for 24 h and cell lysates were subjected to caspase 8 and 3 activity assays. The graphs show the mean \pm SD of 3 replicates for each condition. Statistically significant differences between drugs alone and the double combination are indicated by * ($P < 0.01$, Student's *t*-test).

Effect of OOS on Cell Cycle Progression and Apoptosis

Because OOS decreased the number of breast cancer cells when compared to untreated controls, we questioned whether this was due to stimulation of cell death, cell cycle arrest, or both. Cell cycle analysis in BT474 cells and SKBR3 cells treated with increasing concentrations of OOS at 24 h showed a small increase of cells in G₀-G₁ phase at OOS dilutions from 1:500 to 1:50. At high concentrations of OOS (1:25) the cell cycle studies indicated the presence of a subG₀ region, characteristic of cells suffering cell death (33) (Fig. 2A). In fact, annexin V/PI assays confirmed that OOS induced apoptosis in SKBR3 and BT474 cell lines and in a dose-dependent manner (Fig. 2B). This effect was more pronounced in SKBR3 cells than in BT474 cells.

To gain insights into the mechanism of action of OOS in BT474 and SKBR3 cells we analyzed apoptotic and cell cycle signaling pathways (Fig. 3A). Analyses of several proteins implicated in cell cycle progression indicated that OOS decreased the levels of pRb and cyclin D1, but increased the levels of p27, especially in BT474 cells. In addition, at higher concentrations, OOS caused an increase in cyclin E. No major changes in CDK2 were observed. We next investigated apoptotic cell death. OOS provoked cleavage of PARP-1 at the highest dose tested. This effect was accompanied by phosphorylation of histone H2AX (γ H2AX), an established marker for DNA damage, caused by double-strand breaks (34). OOS showed analogous results in both SKBR3 cells and BT474 cells, although the SKBR3 cell line was more sensitive to the treatment with OOS than BT474 cells.

As caspases are important mediators of apoptosis (35), we assessed the activity of caspases 3 and 8, in BT474 and SKBR3-treated and untreated cells using a caspase fluorometric assay. OOS exposure for 24 h induced a dose-dependent activation of initiator caspase 8 (Fig. 3B), together with executioner caspase 3 (Fig. 3C). This effect was more evident in SKBR3 cells.

OOS Potentiates the Antitumoral Action of Lapatinib

We next investigated the potential effect of OOS in combination with other drugs that target HER2 and that are used in the therapy of patients with HER2+ tumors. For these experiments, OOS at 1/50 dilution, lapatinib at 50 nM, or trastuzumab at 10 nM were added to BT474 or SKBR3 cells for 24 h in monotherapy, and in double combinations. These experiments showed that the double combination of OOS with lapatinib reduced the MTT metabolization of SKBR3 cells, and this reduction was statistically significant when compared to the individual treatments (Fig. 4A). In contrast, combination of OOS with trastuzumab did not increase the antitumoral action of the individual treatments (data not shown).

Treatment with OOS in combination with lapatinib for 24 h induced an increase in the percentage of cells in G₀/G₁ with a decrease of those in proliferative phases (S and G₂/M phases)

(Fig. 4B). In these experiments, the effect of the drug combination was slightly higher than the effect of the individual treatments. Combination of OOS and lapatinib also induced higher apoptosis than the individual treatments (Fig. 4C). Taken together, these results indicated that the combination of OOS and lapatinib improved the antitumoral efficacy of the respective monotherapies.

To understand the molecular mechanisms by which OOS and lapatinib combination caused cell cycle arrest and apoptosis in human HER2+ breast cancer cells, SKBR3 cells were treated with suboptimal concentrations of lapatinib, or OOS, or their combination for 24 h. Western blot analyses of whole-cell lysates showed that OOS and lapatinib combination decreased the levels of cyclin D1, and pRb. No major differences in p27 and CDK2 levels were observed. This combination of OOS + lapatinib induced a slight cleavage of PARP in SKBR3 cells, and caused clear increases in γ H2AX, when compared to the individual treatments (Fig. 5A).

OOS and lapatinib combination induced caspase activation as evidenced by fluorometric caspase assays (Fig. 5B). OOS and lapatinib combination exposure for 24 h induced a slight activation of initiator caspase 8, together with executioner caspase 3.

DISCUSSION

In this study we have evaluated the antitumoral action of OOS in HER2 breast cancer using several *in vitro* and *in vivo* models. In BT474 and SKBR3 cells, treatment with OOS provoked a dose-dependent decrease in their MTT metabolization values, indicative of an antiproliferative/proapoptotic action. We extended these observations *in vivo* by using 2 murine models. In 1 of them, BT474 cells were orthotopically xenografted in mice. We used these cells instead of SKBR3 cells because our former experience indicates that BT474 cells xenograft better than SKBR3 cells. OOS slowed down tumor growth in mice treated either orally or intraperitoneally with OOS. Treatment with OOS did not apparently have a toxic effect since the weights of the control or treated mice were analogous, and no substantial changes in their behaviors were observed. Moreover, dose translation algorithms (36) indicate that a 100- μ l dose of OOS in mice corresponds to 28 ml of OOS in humans, which falls within the daily recommended dose of OOS in humans (25 ml twice daily). The second murine model we used consisted of a genetically engineered mouse model which expresses HER2 in the mammary gland. These mice develop tumors with a latency of 6-8 mo. Using this strain of mice we evaluated the preventive action of OOS on HER2-driven breast tumorigenesis. Treatment with OOS before the onset of tumors delayed their appearance and growth, particularly in the group fed with higher doses of OOS in their drinking water. Moreover, mice treated with OOS had a trend towards the development of less tumors, as compared to control mice. Therefore, OOS may prevent not only tumor

growth but also tumor initiation. Together, these data indicate that OOS exerts antitumoral actions *in vitro* and *in vivo* on breast cancers overexpressing HER2.

The mechanism of action of OOS is complex and involves effects on the cell cycle and cell survival. The effects on the cell cycle were evidenced by an increase in the proportion of cells in the G₀/G₁ phases of the cell cycle, suggesting an arrest in the progression of cells from G₁ into S. Biochemical analyses of cell proteins involved in transition through G₁ indicated that treatment with OOS caused an increase in p27, together with decreases in cyclin D1 and pRb. These biochemical effects are compatible with a blockade in cell cycle progression. In fact, reduced p27 levels and elevated cyclin D1 expression accelerate cell cycle progression through G₁ (37). Moreover, genetic studies of p21/cyclin D1 double deficient mice demonstrate that p27 and cyclin D1 cooperate *in vivo* to regulate cell cycle control (38,39).

In addition to the cell cycle effect, the antitumoral effect of OOS included stimulation of cell death. This was evidenced by increased annexin V staining, as well as an increased staining of DNA in the Sub-G₀ region of the propidium iodide histograms, especially at high OOS concentrations. Biochemically, OOS caused cleavage of PARP, indicative of caspase activation (35). Caspase activation represents a hallmark of apoptosis, and we confirmed activation of caspases 8 and 3 in cells treated with OOS. Interestingly, EGCG has shown caspase 3-activating properties and this component of OOS could underlie the apoptotic properties of OOS.

Apoptotic cell death is characterized by the fragmentation of cellular DNA. This event, which causes double-strand DNA breaks, is sensed by the DNA repair machinery which initiates a phosphorylation cascade increasing the phosphorylation of histone H2AX (40). In fact, γ H2AX represents another biochemical event that is linked to apoptotic cell death. We interpret the increases in γ H2AX upon treatment with OOS as an indication of a DNA damage response secondary to apoptotic DNA laddering.

Most antitumoral therapies rely on the combination of agents to increase the antitumor action of the individual agents. In this respect, a relevant finding of the studies here-with reported refers to the potentiation of the antitumoral action of lapatinib by OOS. In addition to this biological effect, the combination for OOS and lapatinib induced a clear increase in γ H2AX when compared to cells treated with the individual agents. Considering that HER2 inhibitory drugs such as lapatinib are standard of care for the therapy of HER2+ tumors, the potentiation of their efficacy by OOS may be clinically relevant. Furthermore, these results open the possibility of exploring the use of OOS in combination with other agents used to treat HER2+ tumors. In this respect, it is also interesting to mention that one of the most widely used agents, the antibody trastuzumab, also induces increases in p27 (41). However, our data indicated that OOS did not augment the antitumoral action of trastuzumab in BT474 and SKBR3 cells.

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