

Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/*neu* (*erb B-2*) expression and synergistically enhances the growth inhibitory effects of trastuzumab (HerceptinTM) in breast cancer cells with Her-2/*neu* oncogene amplification

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Received 7 August 2004; revised 30 October 2004; accepted 4 November 2004

Background: The relationship between the intake of olive oil, the richest dietary source of the monounsaturated fatty acid oleic acid (OA; 18:1n-9), and breast cancer risk and progression has become a controversial issue. Moreover, it has been suggested that the protective effects of olive oil against breast cancer may be due to some other components of the oil rather than to a direct effect of OA.

Methods: Using flow cytometry, western blotting, immunofluorescence microscopy, metabolic status (MTT), soft-agar colony formation, enzymatic *in situ* labeling of apoptosis-induced DNA double-strand breaks (TUNEL assay analyses), and caspase-3-dependent poly-ADP ribose polymerase (PARP) cleavage assays, we characterized the effects of exogenous supplementation with OA on the expression of Her-2/*neu* oncogene, which plays an active role in breast cancer etiology and progression. In addition, we investigated the effects of OA on the efficacy of trastuzumab (HerceptinTM), a humanized monoclonal antibody binding with high affinity to the ectodomain of the Her-2/*neu*-coded p185^{Her-2/*neu*} oncoprotein. To study these issues we used BT-474 and SKBr-3 breast cancer cells, which naturally exhibit amplification of the Her-2/*neu* oncogene.

Results: Flow cytometric analyses demonstrated a dramatic (up to 46%) reduction of cell surface-associated p185^{Her-2/*neu*} following treatment of the Her-2/*neu*-overexpressors BT-474 and SK-Br3 with OA. Indeed, this effect was comparable to that found following exposure to optimal concentrations of trastuzumab (up to 48% reduction with 20 µg/ml trastuzumab). Remarkably, the concurrent exposure to OA and suboptimal concentrations of trastuzumab (5 µg/ml) synergistically down-regulated Her-2/*neu* expression, as determined by flow cytometry (up to 70% reduction), immunoblotting, and immunofluorescence microscopy studies. The nature of the cytotoxic interaction between OA and trastuzumab revealed a strong synergism, as assessed by MTT-based cell viability and anchorage-independent soft-agar colony formation assays. Moreover, OA co-exposure synergistically enhanced trastuzumab efficacy towards Her-2/*neu* overexpressors by promoting DNA fragmentation associated with apoptotic cell death, as confirmed by TUNEL and caspase-3-dependent PARP cleavage. In addition, treatment with OA and trastuzumab dramatically increased both the expression and the nuclear accumulation of p27^{Kip1}, a cyclin-dependent kinase inhibitor playing a key role in the onset and progression of Her-2/*neu*-related breast cancer. Finally, OA co-exposure significantly enhanced the ability of trastuzumab to inhibit signaling pathways downstream of Her-2/*neu*, including phosphoproteins such as AKT and MAPK.

Conclusions: These findings demonstrate that OA, the main monounsaturated fatty acid of olive oil, suppresses Her-2/*neu* overexpression, which, in turn, interacts synergistically with anti-Her-2/*neu* immunotherapy by promoting apoptotic cell death of breast cancer cells with Her-2/*neu* oncogene

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amplification. This previously unrecognized property of OA offers a novel molecular mechanism by which individual fatty acids may regulate the malignant behavior of breast cancer cells and therefore be helpful in the design of future epidemiological studies and, eventually, dietary counseling.

Key words: apoptosis, breast cancer, fatty acids, Her-2/*neu*, oleic acid, trastuzumab

Introduction

Epidemiological studies indicate that women in countries with high-fat diets have a risk of breast cancer that can be five-fold higher than that of women in countries with low-fat consumption [1–3], strongly suggesting that a high intake of dietary fat could increase breast cancer risk [3]. This ‘dietary fat hypothesis’ has been supported by a number of epidemiological, experimental and mechanistic data, altogether providing evidence that dietary or exogenously derived fatty acids may play an important role in the carcinogenesis, evolution and/or progression of breast cancer [3–5]. However, case–control, cohort and recent prospective epidemiological studies have generated conflicting results, and taken together do not support a strong association [6–9].

The results of studies with olive oil, the richest source of the monounsaturated fatty acid oleic acid (OA; 18:1n-9), are of particular interest. On the one hand, the strongest evidence that monounsaturated fatty acids such as OA may influence breast cancer risk comes from studies of southern European populations, in whom intake of OA sources, particularly olive oil, appear to be protective [10–13]. On the other hand, research in experimental animals has yielded inconsistent results, having reported a non-promoting or a low-promoting effect to a protective one on breast cancer [1, 14–16]. Although these conflicting results may be explained in part by the fact that olive oil is administered as a mixture of several fatty acids and other natural chemoprotectants (tocopherols, carotenoids, polyphenols, etc.) and not as individual OA [17–19], there is certainly relatively little understanding of the specific molecular mechanisms by which fatty acids such as OA may exert their effects on breast cancer. Since neoplastic development is believed to be a multi-step process involving the expression of several oncogenes, we recently hypothesized that a new molecular explanation concerning the specific promoting or inhibitory breast cancer actions of OA may be a differential regulation of breast cancer-related oncoproteins. We recently reported that, in a 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumorigenesis model in rats, a high olive oil diet acted as a negative modulator conferring to the tumor a more benign clinical behavior and a lower histopathological malignancy [20]. Interestingly, this effect was accompanied by a decrease in the mRNA coding for Her-2/*neu* [21]. At present, the Her-2/*neu* oncogene (also called *neu* and *erbB-2*) represents one of the most important oncogenes in breast cancer. Her-2/*neu* codes for the p185^{Her-2/*neu*} oncoprotein, a transmembrane tyrosine kinase orphan receptor [22, 23]. Her-2/*neu* amplification and overexpression occurs in ~20% of breast carcinomas and is correlated with unfavorable clinical outcome [24–26]. Expression of high levels of Her-2/*neu* is sufficient to induce neoplastic transformation

of some cell lines [27, 28], suggesting a role for Her-2/*neu* in the etiology of some breast carcinomas. Indeed, Her-2/*neu* is overexpressed not only in invasive breast cancer, but also in pre-neoplastic breast lesions such as atypical duct proliferations and in ductal carcinoma of the breast *in situ* [29–31]. Moreover, Her-2/*neu* is a metastatic-promoting gene, enhancing the invasive and metastatic phenotype of breast cancer cells [32, 33]. Her-2/*neu* overexpression is also associated with resistance to chemo- and endocrine therapies [34, 35], while representing a successful therapeutic target of the biotechnology era as exemplified by the drug trastuzumab (Herceptin™; Genentech, San Francisco, CA). Trastuzumab is a humanized monoclonal IgG1, binding with high affinity to the ectodomain domain of p185^{Her-2/*neu*} that has clinical activity in a subset of breast cancer patients, thus confirming the role of Her-2/*neu* in the progression of some breast carcinomas [36–39]. However, whereas these studies suggest that trastuzumab may have clear utility in select cases of advanced breast cancer, these benefits are modest and usually do not represent a cure. Moreover, not all Her-2/*neu*-overexpressing breast cancer cells respond to treatment with trastuzumab and its clinical benefit is limited by the fact that resistance develops rapidly in virtually all treated patients [40]. Although the molecular mechanisms underlying trastuzumab resistance have begun to emerge [41–43], there are no data concerning novel strategies able to sensitize breast cancer cells to the growth-inhibitory activity of trastuzumab.

Using flow cytometry, western blotting, immunofluorescence microscopy, metabolic status (MTT), soft-agar colony formation, enzymatic *in situ* labeling of apoptosis-induced DNA double-strand breaks (TUNEL assay analyses), and caspase-3-dependent poly-ADP ribose polymerase (PARP) cleavage assays, we have assessed the effects of OA on the expression of the p185^{Her-2/*neu*} oncoprotein and the efficacy of trastuzumab towards Her-2/*neu*-overexpressing human breast cancer cells. To study these issues we used BT-474 and SKBr-3 breast cancer cells, which exhibit Her-2/*neu* oncogene amplification and are Her-2/*neu*-dependent [44]. We report, to the best of our knowledge for the first time, that OA, the main monounsaturated fatty acid of olive oil, specifically suppresses Her-2/*neu* overexpression, which, in turn, interacts synergistically with anti-Her-2/*neu* breast cancer immunotherapy by promoting apoptotic cell death of breast cancer cells with amplification of the Her-2/*neu* oncogene. Although caution must be applied when extrapolating *in vitro* results into clinical practice, this previously unrecognized property of OA should help in the understanding of the molecular mechanisms by which individual fatty acids may regulate the malignant behavior of breast cancer cells, and therefore be helpful in the design of future epidemiological studies and, eventually, dietary counseling in Her-2/*neu*-positive breast cancer patients.

Materials and methods

Cell lines and culture conditions

The human breast cancer cell lines SK-Br3 and BT-474 were obtained from the American Type Culture Collection (ATCC), and they were routinely grown in phenol red-containing improved MEM (IMEM; Biosource International, Camarillo, CA) containing 5% (v/v) heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO₂. Cells were screened periodically for *Mycoplasma* contamination.

Materials

Oleic acid (18:1n-9) and vitamin E (dl- α -tocopherol) were purchased from Sigma Chemical Co. (St Louis, MO). The cultures were supplemented, where indicated, with fatty acid-free bovine serum albumin (FA-free BSA; 0.1 mg/ml) complexed with a specific concentration of OA. A BSA-OA concentrate ($\times 100$) was formed by mixing 1 ml BSA (10 mg/ml) with various volumes (1–10 μ l) of OA (200 mg/ml) in ethanol. The concentrate was mixed for 30 min at room temperature before addition to the cultures. Control cultures contained uncomplexed BSA. Trastuzumab (HerceptinTM) was kindly provided by the Evanston Northwestern Healthcare Hospital Pharmacy (Evanston, IL).

The mouse monoclonal antibodies for p185^{Her-2/neu} (Ab-3 and Ab-5 clones) were from Oncogene Research Products (San Diego, CA). Anti- β -actin goat polyclonal and anti-p27^{Kip1} rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PARP p85 fragment antibody was from Promega Corp. (Madison, WI). Anti-MAPK, anti-phosphor-MAPK, anti-AKT and anti-phosphor-AKT^{Ser473} rabbit polyclonal antibodies were from Cell Signal Technology (Beverly, MD).

Flow cytometry

Cells were seeded on 100-mm plates and cultured in complete growth medium. Upon reaching 75% confluence, the cells were washed twice with pre-warmed PBS and cultured in serum-free medium overnight. OA, trastuzumab, or a combination of OA plus trastuzumab as specified was added to the culture as specified, and incubation was carried out at 37°C up to 48 h in low-serum (0.1% FBS) media. After treatment, cells were washed once with cold PBS and harvested in cold PBS. The cells were pelleted and resuspended in cold PBS containing 1% FBS. The cells were then incubated with an anti-p185^{Her-2/neu} mouse monoclonal antibody (clone Ab-5) at 5 μ g/ml for 1 h at 4°C. The cells were then washed twice with cold PBS, resuspended in cold PBS containing 1% FBS, and incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 in cold PBS containing 1% FBS for 45 min at 4°C. Finally, the cells were washed once in cold PBS, and flow cytometric analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, San Diego, CA) equipped with Cell Quest Software (Becton Dickinson). The mean fluorescence signal associated with cells for labeled p185^{Her-2/neu} was quantified using the GEO MEAN fluorescence parameter provided with the software.

Immunoblotting

Following treatments with OA, trastuzumab, or a combination of OA plus trastuzumab, as specified, cells were washed twice with PBS and then lysed in buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 1 mM phenylmethylsulfonylfluoride] for 30 min on ice. The lysates were cleared by centrifugation in an Eppendorf tube (15 min at 14 000 r.p.m. at 4°C). Protein content

was determined against a standardized control using the Pierce protein assay kit (Rockford, IL). Equal amounts of protein were heated in SDS sample buffer (Laemli) for 10 min at 70°C, subjected to electrophoresis on either 3–8% NuPAGE (p185^{Her-2/neu}) or 10% SDS-PAGE (p27^{Kip1}, p85^{PARP}, MAPK and AKT), and then transferred to nitrocellulose membranes. Non-specific binding on the nitrocellulose filter paper was minimized by blocking for 1 h at room temperature (RT) with TBS-T [25 mM Tris-HCl, 150 mM NaCl (pH 7.5) and 0.05% Tween 20] containing 5% (w/v) non-fat dry milk. The treated filters were washed in TBS-T and then incubated overnight at 4°C with specific primary antibodies in TBS-T/5% (w/v) BSA. The membranes were washed in TBS-T, horse-radish peroxidase-conjugated secondary antibodies (Jackson Immuno-research Laboratories) in TBS-T were added for 1 h, and immunoreactive bands were detected by enhanced chemiluminescence reagent (Pierce). Blots were re-probed with an antibody for β -actin to control for protein loading and transfer. Densitometric values of protein bands were quantified using Scion Imaging Software (Scion Corp., Frederick, MD).

In situ immunofluorescent staining

Cells were seeded at a density of 1×10^4 cells/well in a four-well chamber slide (Nalge Nunc International, Rochester, NY). Following treatments with OA, trastuzumab, or a combination of OA plus trastuzumab as specified, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100/PBS for 15 min, and stored overnight at 4°C with 10% horse serum in PBS. The cells were washed and then incubated for 2 h with anti-p185^{Her-2/neu} or anti-p27^{Kip1} antibodies diluted 1:200 in 0.05% Triton X-100/PBS. After extensive washes, the cells were incubated for 45 min with FITC-conjugated anti-mouse IgG (p185^{Her-2/neu}) or tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG (p27^{Kip1}) diluted 1:200 in 0.05% Triton X-100/PBS. The cells were washed five times with PBS and mounted with VECTASHIELD+ DAPI (Vector Laboratories, Burlingame, CA). As controls, cells were stained with primary or secondary antibody alone. Control experiments did not display significant fluorescence in any case (data not shown). Indirect immunofluorescence was recorded on a Zeiss microscope. Images were noise-filtered, corrected for background and prepared using Adobe Photoshop.

Anchorage-dependent cell proliferation

SK-Br3 and BT-474 cells exponentially growing in IMEM–5% FBS were trypsinized and re-plated in 24-well plates at a density of 10 000 cells/well. Cells were incubated for 24 h to allow for attachment, after which a zero time point was determined. Cells were treated with OA, trastuzumab, or a combination of OA plus trastuzumab, as specified. Cell number was determined at days 0, 3 and 6 with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). All assays were performed at least three times in triplicate. The data are presented as mean of number cells $\times 10^4$ /well \pm SD.

In vitro chemosensitivity testing

Trastuzumab sensitivity was determined using a standard colorimetric MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) reduction assay. Cells in exponential growth were harvested by trypsinization and seeded at a concentration of $\sim 5 \times 10^3$ cells/200 μ l/well into 96-well plates, and allowed an overnight period for attachment. The medium was then removed and fresh medium, along with various concentrations of trastuzumab, OA or combinations of compounds, was added to cultures in parallel. Agents were studied in combination concurrently. Control cells without agents were cultured using the same conditions with comparable media changes. Compounds were not renewed during the entire period of cell exposure. Following treatment, the medium was

removed and replaced with fresh drug-free medium (100 μ l/well), and MTT (5 mg/ml in PBS) was added to each well at a volume of 1:10. After incubation for 2–3 h at 37°C, the supernatants were carefully aspirated, 100 μ l of DMSO were added to each well, and the plates were agitated to dissolve the crystal product. Absorbances were measured at 570 nm using a multi-well plate reader (Model Anthos Labtec 2010 1.7 reader). The cell viability effects from exposure of cells to each compound alone and their combination were analyzed, generating concentration–effect curves as a plot of the fraction of unaffected (surviving) cells versus drug concentration. Dose–response curves were plotted as percentages of the control cell absorbances, which were obtained from control wells treated with appropriate concentrations of the compound vehicles that were processed simultaneously. For each treatment, cell viability was evaluated as a percentage using the following equation: $(A_{570}$ of treated sample/ A_{570} of untreated sample) \times 100. Drug sensitivity was expressed in terms of the concentration of drug required for a 50% reduction of cell viability (IC_{50}). Since the percentage of control absorbance was considered to be the surviving fraction of cells, the IC_{50} values were defined as the concentration of drug that produced a 50% reduction in control absorbance (by interpolation). The degree of sensitization to trastuzumab by OA was evaluated by dividing IC_{50} values of control cells by those obtained when cells were exposed to OA during exposure to trastuzumab.

Determination of synergism: isobologram analysis

The interaction between OA and trastuzumab was evaluated using the isobologram technique [45], a dose-oriented geometric method of assessing drug interactions. With the isobologram method, the concentration of one agent producing a desired (e.g. 50% inhibitory) effect is plotted on the horizontal axis, and the concentration of another agent producing the same degree of effect is plotted on the vertical axis; a straight line joining these two points represents zero interaction (addition) between two agents. The experimental isoeffect points are the concentrations (expressed relative to the IC_{50} concentrations) of the two agents that, when combined, kill 50% of the cells. When the experimental isoeffect points fall below that line, the combination effect of the two drugs is considered to be supra-additive or synergistic, whereas antagonism occurs if the point lies above it. A quantitative index of these interactions was provided by the isobologram equation: $CI_x = (a/A) + (b/B)$, where, for this study, A and B represent the respective concentrations of OA and trastuzumab required to produce a fixed level of inhibition (IC_{50}) when administered alone, a and b represent the concentrations required for the same effect when the drugs were administered in combination, and CI_x represents an index of drug interaction (interaction index). I_x values <1 indicate synergy, a value of 1 represents addition, and values of >1 indicate antagonism.

Soft-agar colony-formation assays

The efficiency of colony formation in liquid culture was determined by monitoring anchorage-independent cell growth in soft-agar experiments. A bottom layer of 1 ml IMEM containing 0.6% agar and 10% FBS was prepared in 35-mm multi-well cluster dishes. After the bottom layer solidified, cells (10000/dish) were added in a 1 ml top layer containing OA, trastuzumab, a combination of OA plus trastuzumab, or vehicles (v/v) in 0.35% agar and 10% FBS, as specified. All samples were prepared in triplicate. Dishes were incubated in a humidified 5% CO_2 incubator at 37°C, and colonies measuring $\geq 50 \mu$ m were counted ~ 14 days after staining with nitroblue tetrazolium (Sigma) using a cell colony counter (Ommias 3600; Imaging Products International, Inc., Charley, VA).

Apoptosis

Detection of apoptosis in SK-Br3 and BT-474 cells treated with OA, trastuzumab, or a combination of OA plus trastuzumab, as specified, was

performed by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis using the DeadEnd™ Fluorometric TUNEL System (Promega Inc.) according to the manufacturer's instructions. Briefly, cells were split at a density of 2×10^4 cells/well in an eight-well chamber slide (Lab-Tek). After 48 h incubation the cells were treated with trastuzumab in the absence or presence of OA for 72 h. Following treatment, cells were washed twice with PBS and fixed with 4% methanol-free paraformaldehyde for 10 min. Cells were washed twice more with PBS and permeabilized with 0.2% Triton X-100 for 5 min. After two more washes, each slide was covered with equilibration buffer for 10 min more. The buffer was then aspirated, and the slides were incubated with TdT buffer at 37°C for 1 h. The reaction was stopped with $2 \times$ standard saline citrate and the slides were viewed under an immunofluorescence microscope (Zeiss). Apoptosis was quantified by determining the proportion of cells containing nuclei with complete TUNEL-associated staining. One hundred cells were assessed in triplicate for each treatment.

Statistical analysis

Statistical analysis of mean values was performed using the non-parametric Mann–Whitney test. Differences were considered significant at $P < 0.05$ and $P < 0.005$.

Results

Exogenous supplementation with OA down-regulates p185^{Her-2/neu} in Her-2/neu-overexpressing BT-474 and SK-Br3 breast cancer cells

To assess the effects of exogenous supplementation with OA on Her-2/neu expression, SK-Br3 and BT-474 cells, after a 24 h starvation period in media without serum, were incubated for 48 h with 10 μ M of OA complexed to BSA in low-serum (0.1% FBS) conditions. The cell surface-associated expression of Her-2/neu-coded p185^{Her-2/neu} oncoprotein was then determined by measuring the binding of a mouse monoclonal antibody directed against the ectodomain of p185^{Her-2/neu} (Ab-5 clone) in OA-treated BT-474 and SK-Br3 cells. Flow cytometric analysis of cell surface-associated p185^{Her-2/neu} demonstrated a significant reduction of p185^{Her-2/neu} expression levels in BT-474 breast cancer cells following OA treatment (up to 46% reduction at 10 μ M OA; Figure 1). Her-2/neu-overexpressing SK-Br3 breast cancer cells were also sensitive to the down-regulatory effects of OA on p185^{Her-2/neu} expression (up to 36% reduction at 10 μ M OA; Figure 1). These findings reveal that exogenous supplementation significantly down-regulates p185^{Her-2/neu} overexpression in breast cancer cells harboring amplification of the Her-2/neu oncogene. Indeed, this down-regulatory effect was comparable to that found following exposure to optimal concentrations of trastuzumab (up to 48% reduction at 20 μ g/ml trastuzumab).

Exogenous supplementation with OA synergistically enhances trastuzumab-induced down-regulation of p185^{Her-2/neu}

In the light of these results we next hypothesized that exogenous supplementation with OA may sensitize breast cancer cells to the well-known p185^{Her-2/neu} down-regulatory actions of

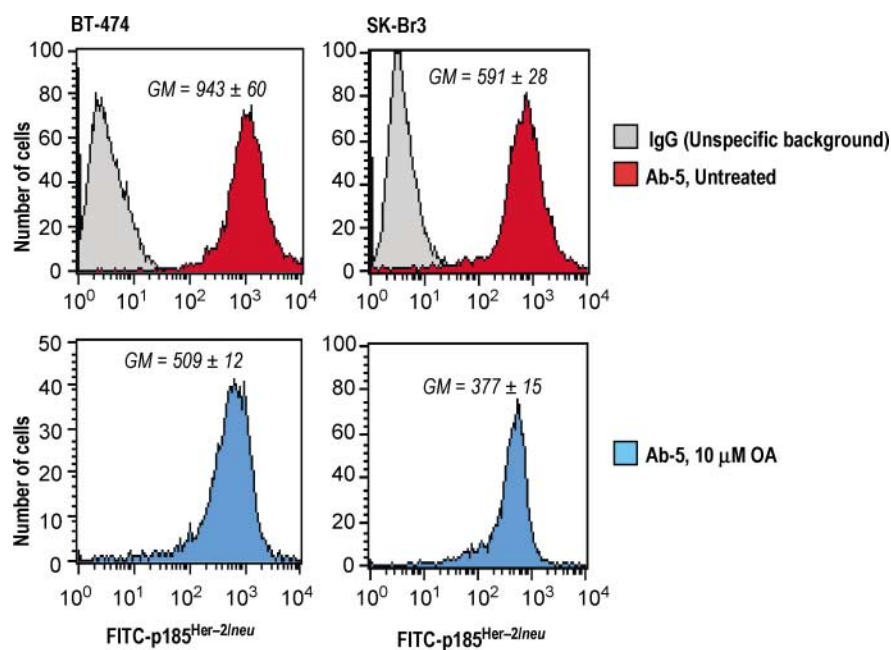


Figure 1. Flow cytometric analysis of cell surface-associated p185^{Her-2/neu} in Her-2/neu-overexpressing BT-474 and SK-Br3 breast cancer cells exogenously supplemented with OA. Overnight serum-starved BT-474 and SK-Br3 breast cancer cells were cultured in IMEM–0.1% FBS in the presence or absence of 20 μ M OA for 48 h. The specific surface expression of p185^{Her-2/neu} in OA-treated cells was determined by flow cytometry by measuring the binding of a mouse anti-p185^{Her-2/neu} monoclonal antibody directed against the extracellular domain of p185^{Her-2/neu} (Ab-5 clone) as described in Materials and methods. The mean fluorescence signal \pm SD ($n=3$) associated with cells for labeled p185^{Her-2/neu} was quantified using the geo mean fluorescence (GM) parameter provided with the Cell Quest Software (Becton Dickinson).

trastuzumab [46–48]. For this purpose, cell surface-associated p185^{Her-2/neu} was first measured by flow cytometry following treatment with low doses of OA (5 μ M) and trastuzumab (5 μ g/ml). Remarkably, the concurrent combination of OA and trastuzumab reduced p185^{Her-2/neu} expression more than when either agent was administered alone (Figure 2A). Thus, OA and trastuzumab co-treatment induced a 70% decrease of p185^{Her-2/neu} expression when concurrently combined in BT-474 breast cancer cells, whereas when used alone, OA and trastuzumab caused a 36% and 8% down-regulation of p185^{Her-2/neu}, respectively. In SK-Br3 cells, combined treatment with OA resulted in a synergistic increase in the trastuzumab-mediated down-regulation of p185^{Her-2/neu} up to 65%, whereas a 24% and 26% reduction in p185^{Her-2/neu} expression was observed following treatment with OA and trastuzumab as single agents, respectively (data not shown).

We further analyzed the impact of OA supplementation in the subcellular localization of p185^{Her-2/neu} in Her-2/neu-overexpressing breast cancer cells. To address this question, BT-474 cells, at 48 h after treatment with OA, trastuzumab, or OA plus trastuzumab, were permeabilized with Triton X-100 for the intracellular delivery of antibodies. Thereafter, p185^{Her-2/neu} cellular localization was assessed using the anti-*erbB-2/c-neu* Ab-3 mouse monoclonal antibody (Oncogene Research Products), which is directed against the C-terminal 14 amino acids of p185^{Her-2/neu}. Untreated BT-474 cells showed a prominent cell-surface staining of p185^{Her-2/neu}, whereas, upon OA treatment, p185^{Her-2/neu}-associated membrane staining was markedly reduced (Figure 2B). Indeed,

p185^{Her-2/neu} oncoprotein in OA-treated BT-474 cells displayed a cellular distribution similar to that induced by the anti-p185^{Her-2/neu} antibody trastuzumab because it was, to some extent, distributed throughout the cytoplasm. Equivalent results were found in SK-Br3 breast cancer cells (data not shown). Of note, almost a negative staining of cell surface-associated p185^{Her-2/neu} was observed following the co-exposure of BT-474 to OA and trastuzumab. Western blotting analyses further confirmed that a dramatic down-regulation of p185^{Her-2/neu} takes place in trastuzumab-treated Her-2/neu-overexpressing human breast cancer cells in the presence of increasing concentrations of OA, while the levels of β -actin remain unchanged (Figure 2C). These findings clearly demonstrate that OA, similarly to trastuzumab, selectively down-regulates expression of the p185^{Her-2/neu} oncoprotein in human breast cancer cells. Moreover, a synergistic augmentation of trastuzumab-induced down-regulation of p185^{Her-2/neu} expression occurs in OA-supplemented breast cancer cells, further supporting the hypothesis that Her-2/neu down-regulation is a novel molecular mechanism of action for OA in human breast cancer cells.

Exogenous supplementation with OA synergistically enhances trastuzumab-induced inhibition of cell growth in Her-2/neu-overexpressing breast cancer cells

We next analyzed the effects of a concurrent combination of OA and trastuzumab on the anchorage-dependent growth properties of Her-2/neu-overexpressing breast cancer cells. As expected, the anchorage-dependent cell growth of Her-2/neu overexpressors

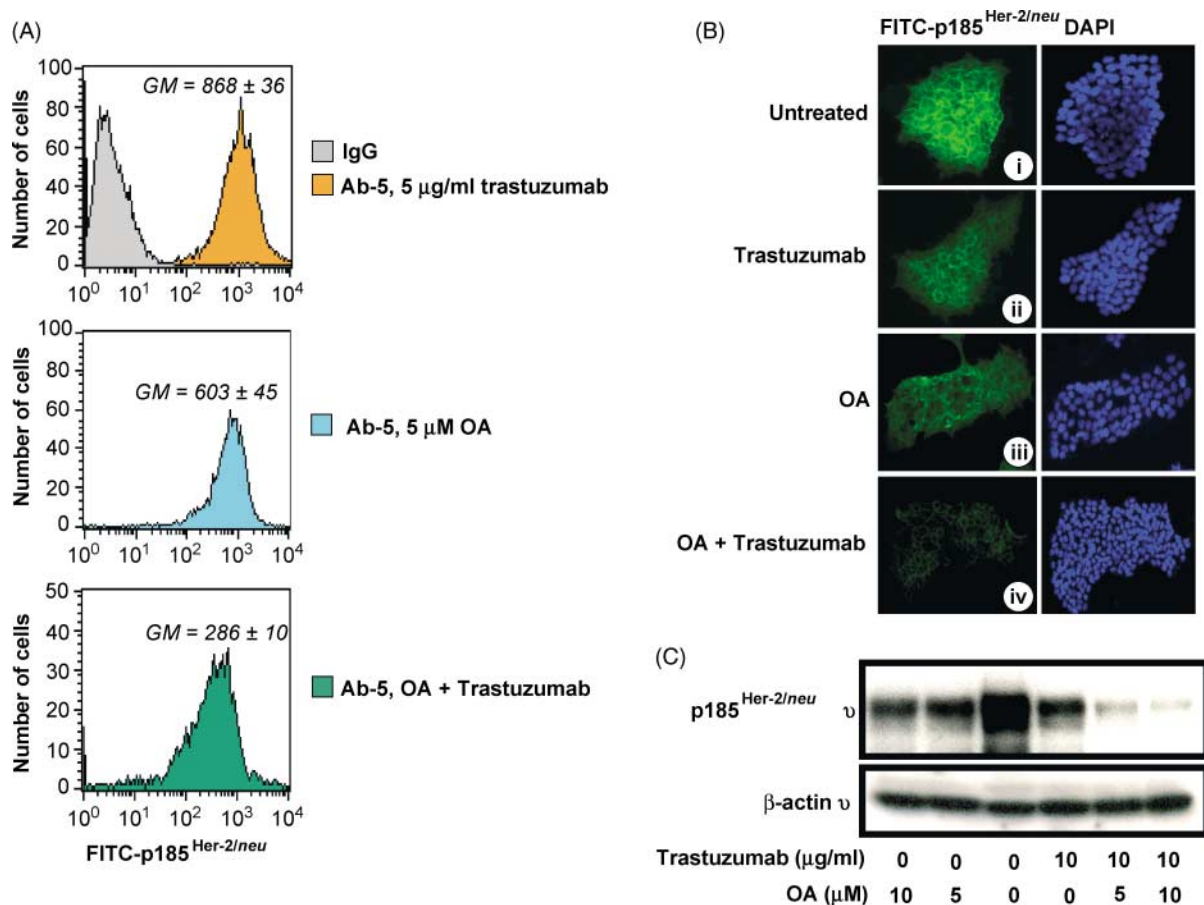


Figure 2. Exogenous supplementation with OA synergistically enhances trastuzumab-induced down-regulation of p185^{Her-2/neu}. (A) Overnight serum-starved BT-474 breast cancer cells were cultured in IMEM–0.1% FBS supplemented with trastuzumab (top panel), OA (middle panel), or a combination of OA plus trastuzumab (bottom panel) for 48 h. The amount of cell surface-associated p185^{Her-2/neu} was quantified by flow cytometric analyses using a specific antibody against the extracellular domain of p185^{Her-2/neu} (Ab-5) as described in Materials and methods. The mean fluorescence signal \pm SD ($n=3$) was quantified using the geo mean fluorescence (GM) parameter provided with the Cell Quest Software (Becton Dickinson). (B) Overnight serum-starved BT-474 breast cancer cells were cultured in IMEM–0.1% FBS (panel i) or IMEM–0.1% FBS supplemented with trastuzumab (panel ii), OA (panel iii), or a combination of OA plus trastuzumab (panel iv) for 48 h in eight-well chamber slides. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and labeled for 2 h with an anti-p185^{Her-2/neu} monoclonal antibody directed against the cytoplasmic domain of p185^{Her-2/neu} (Ab-3 clone). After labeling, cells were washed thoroughly, and localization of p185^{Her-2/neu} was detected by indirect immunofluorescence by incubating with FITC-conjugated anti-mouse IgG. After counterstaining with DAPI, cells were examined and photographed using a Zeiss fluorescent microscope equipped with a built-in camera. The figure shows a representative immunostaining analysis. Similar results were obtained in three independent experiments. (C) Overnight serum-starved BT-474 breast cancer cells were cultured in IMEM–0.1% FBS supplemented with trastuzumab, OA, or a combination of OA plus trastuzumab for 48 h, and then harvested and lysed as described in Materials and methods. Equal amounts of total protein (20 μ g per lane) were subjected to western blot analyses with a specific antibody against p185^{Her-2/neu} (Ab-3 clone), and then re-probed with a β -actin antibody. The figure shows a representative immunoblotting analysis. Similar results were obtained in three independent experiments.

was significantly decreased in the presence of increasing concentrations of trastuzumab, while exogenous supplementation with low concentrations of OA had no notable effects on breast cancer cell proliferation. Interestingly, when added in the presence of OA, trastuzumab further inhibited cell proliferation of SK-Br3 and BT-474 cells (Figure 3A, left panels). Moreover, the increase in growth inhibition with the addition of OA over that of trastuzumab itself was statistically significant (Figure 3A, right panels), suggesting a synergistic interaction between OA and trastuzumab during growth inhibition of Her-2/neu-overexpressing breast cancer cells.

Next, we examined the cytotoxic interactions between OA and trastuzumab and their effects on SK-Br3 and BT-474 cells by

evaluating the metabolic status of breast cancer cells co-treated with trastuzumab and OA, as judged by the mitochondrial conversion of the tetrazolium salt, MTT, to its formazan product (MTT assay). First, we measured the changes in cell toxicity of 10 μ g/ml trastuzumab after 72 h of co-exposure to increasing concentrations of OA. The simultaneous presence of OA during the incubation period with trastuzumab caused a significant increase in the cytotoxic effects of trastuzumab (Figure 3B, left panels). Next, we evaluated the reduction in trastuzumab concentrations needed for a 50% decrease in cell viability (IC_{50}) following OA supplementation. The IC_{50} values of trastuzumab were measured after 72 h of treatment in the presence or absence of a given concentration of OA, and the degree of potentiation of

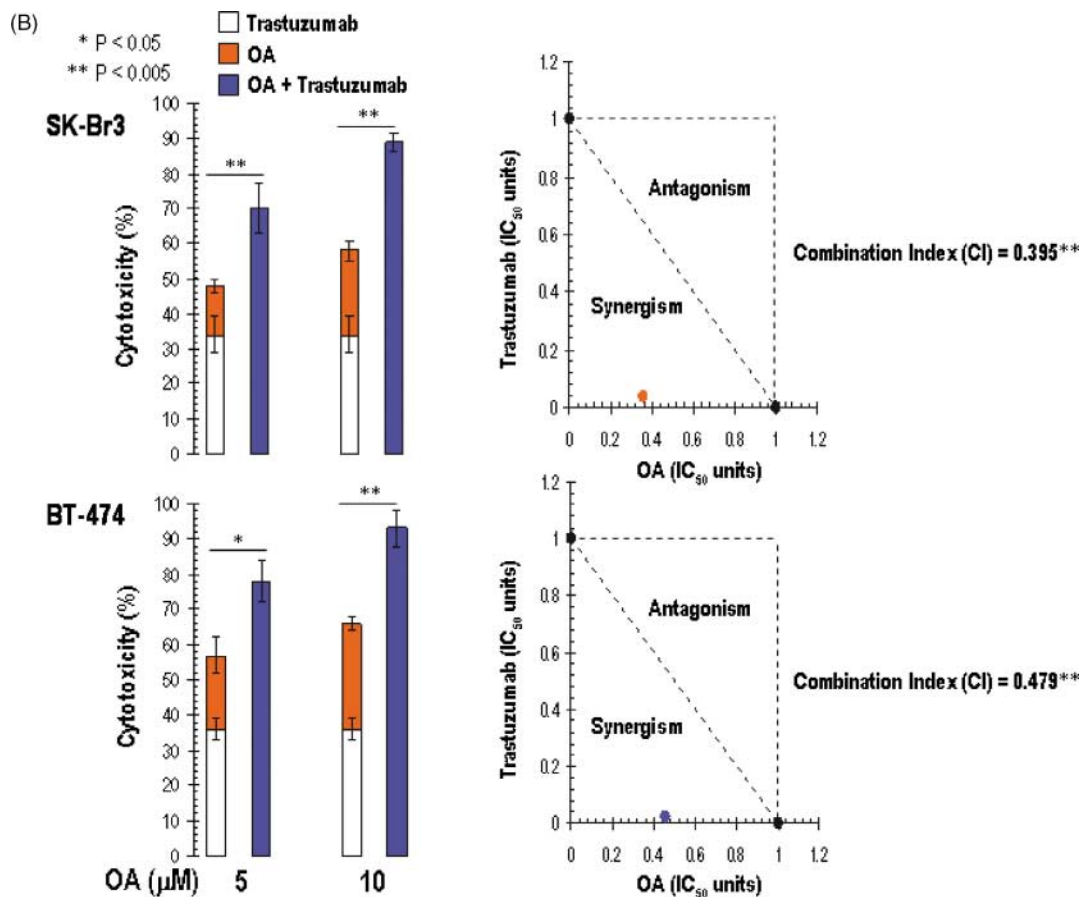
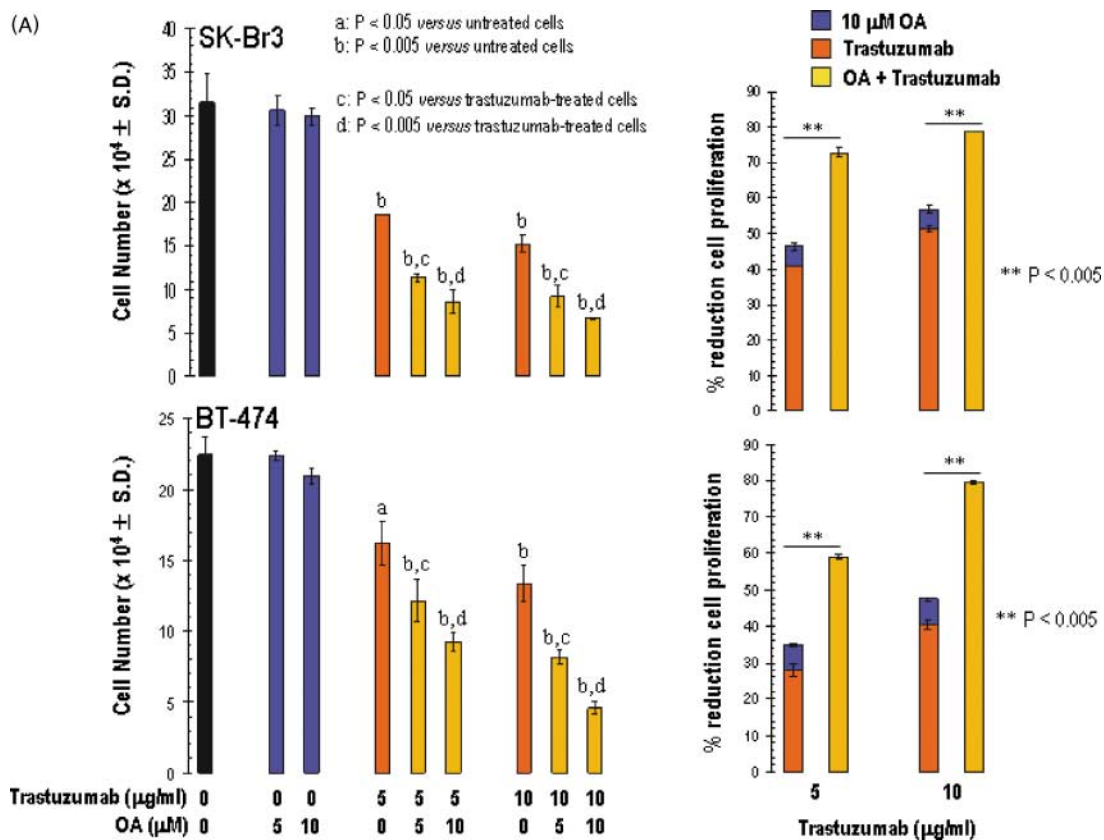


Figure 3. (Continued.)

trastuzumab efficacy was expressed as a sensitization factor by dividing the IC_{50} values in the absence of OA by those in the presence of OA. From our data we concluded that OA exposure dramatically enhanced the cytotoxic activity of trastuzumab. The most significant changes were seen in BT-474 cells, in which co-exposure to 10 μ M OA decreased the IC_{50} value of trastuzumab from $\sim 30 \mu$ g/ml to 0.75 μ g/ml (40-fold sensitization factor). For SK-Br3 cells, OA co-exposure decreased the IC_{50} value of trastuzumab from $\sim 40 \mu$ g/ml to 1.5 μ g/ml (27-fold sensitization factor). The precise nature of the interaction between trastuzumab and OA was investigated further using the classical Berenbaum isobologram analysis. When the experimental isoeffect points (the concentrations of trastuzumab and OA that, when combined, produced a 50% reduction in survival of BT-474 and SK-Br3 cells) were plotted and compared with the additive line, the data points fell to the left of the line, suggesting a supra-additive or synergistic interaction between the two agents (Figure 3B, right panels). While these figures provided a graphical representation of trastuzumab–OA interactions, the values of the mean combination index for the 50% cytotoxic level (CI_{50}) were also calculated. When statistical tests were carried out to evaluate whether significant differences in the CI_{50} means values occurred as compared with a null-hypothesized CI_{50} of 1 (additivity), and to evaluate formally whether synergism was evident, concurrent administration of trastuzumab and OA resulted in a significant synergism in BT-474 and SK-Br3 cells ($CI_{50}=0.395$ and 0.479, respectively; $P<0.005$). In other words, the combined quantity of the two agents necessary to reduce BT-474 and SK-Br3 cell viability by 50% was only ~ 0.4 times the quantity required if they demonstrated purely additive behavior ($P<0.005$ compared with a null-hypothesized interaction index of 1, i.e. additivity).

The acquisition of anchorage-independent growth is generally considered to be one of the *in vitro* properties associated with the malignancy of cells. In fact, colonization of metastatic tumor cells at a distant site may be partially modeled in soft-agar assays. Therefore, we finally evaluated the effects of concurrent exposure to trastuzumab and OA on the ability of Her-2/*neu* overexpressors to grow in anchorage-independent conditions. As a single agent, OA slightly decreased the ability of SK-Br3 and BT-474 cells to form colonies in soft agar, whereas trastuzumab, as expected, significantly blocked anchorage-independent growth of Her-2/*neu* overexpressors (Figure 3C, left panels). Interestingly, Her-2/*neu*-dependent, anchorage-independent cell growth was completely abolished in a synergistic manner following co-exposure to OA and trastuzumab (Figure 3C, left and right panels). Taken together, these results demonstrate that exogenous supplementation with OA induces synergistic augmentation of trastuzumab efficacy towards Her-2/*neu*-overexpressing breast cancer cells.

Exogenous supplementation with OA synergistically enhances trastuzumab-induced apoptotic cell death of Her-2/*neu*-overexpressing breast cancer cells

To assess if the synergistic interaction between trastuzumab and OA observed above represented cell death, we next

focused on an apoptotic effect of the combination of OA and trastuzumab as measured by the enzymatic *in situ* labeling of apoptosis-induced DNA double-strand breaks (TUNEL assay). Individually, OA (5 μ M) and trastuzumab (10 μ g/ml) caused slight increases in the number of apoptotic cells (2% and 16% of TUNEL-positive cells, respectively). Remarkably, there was an impressive increase in apoptosis when BT-474 cells were treated simultaneously with both agents (51% TUNEL-positive cells; Figure 4A, top panels). The ability of OA to synergistically enhance trastuzumab-induced apoptotic cell death in SK-Br3 cells also demonstrated a synergistic nature. To obtain further evidence that OA synergistically promotes trastuzumab-induced apoptosis, we examined whether the caspase-3-dependent proteolysis of PARP, a hallmark feature of apoptosis, also occurred in Her-2/*neu*-overexpressing breast cancer cells. Using a rabbit polyclonal antibody specific for the p85 fragment of PARP that results from caspase cleavage of the 116 kDa intact PARP molecule, a small amount of the p85 PARP degradation product was detected in trastuzumab-treated BT-474 cells, whereas an increased cleavage of the death substrate PARP was apparent in BT-474 cells co-treated with trastuzumab and OA (Figure 4A, bottom panel). Equivalent results were found in SK-Br3 breast cancer cells (data not shown). These results, taken together, establish that the combined treatment with trastuzumab and OA synergistically enhances apoptotic cell death of Her-2/*neu*-overexpressing breast cancer cells.

Exogenous supplementation with OA synergistically enhances trastuzumab-induced up-regulation and nuclear accumulation of p27^{Kip1}

Finally, we examined whether the signaling pathways downstream of Her-2/*neu* that regulate cell cycle progression and/or cell death were modified by OA. The treatment of cancer cells with trastuzumab results not only in down-regulation of p185^{Her-2/*neu*}, but also in further downstream cellular events, including accumulation of the cyclin-dependent kinase inhibitor p27^{Kip1} [42–44, 49]. Indeed, the cyclin-dependent kinase inhibitor (CDK_i) p27^{Kip1} plays a key role in the onset and progression of Her-2/*neu*-induced breast tumorigenesis and breast cancer progression, and is further involved in the development of trastuzumab resistance [42, 43, 49–51]. A slight increase in the expression of p27^{Kip1} was observed after the treatment of BT-474 cells with suboptimal concentrations of OA. In agreement with earlier studies [42–44, 51], p27^{Kip1} expression was significantly enhanced in the presence of trastuzumab. Remarkably, a dramatic up-regulation of p27^{Kip1} expression was observed in trastuzumab-treated BT-474 cells in the presence of increasing concentrations of OA (Figure 5, left panel).

We next evaluated how interruption of Her-2/*neu*-dependent signaling with OA affected the cellular localization of p27^{Kip1}. Using immunofluorescence microscopy, we found that most of p27^{Kip1} was present in the cytosol of proliferating BT-474 cells. Treatment with suboptimal concentrations of trastuzumab resulted in a significant translocation of

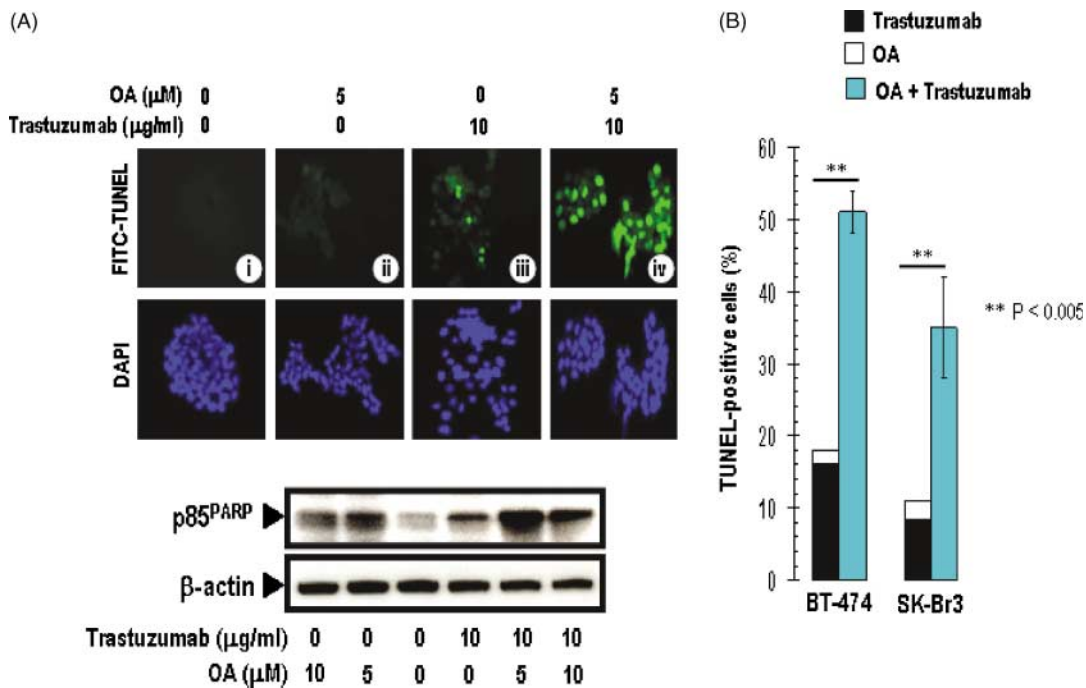


Figure 4. Exogenous supplementation with OA synergistically enhances trastuzumab-induced apoptotic cell death in Her-2/neu-overexpressing breast cancer cells. **(A)** Top panel: overnight serum-starved BT-474 cells growing in eight-well chamber slides were cultured in IMEM–0.1% FBS in the absence (panel i) or presence of 5 μM OA (panel ii), 10 $\mu\text{g/ml}$ trastuzumab (panel iii), or a combination of 5 μM OA plus 10 $\mu\text{g/ml}$ trastuzumab (panel iv). After 72 h, a TUNEL analysis was performed using the DeadEnd™ Fluorometric TUNEL System (Promega Inc.) according to the manufacturer’s protocol. The immunofluorescence photomicrographs of cells undergoing apoptosis (green staining) and the corresponding DAPI-counterstained photomicrographs are shown. Bottom panel: overnight serum-starved BT-474 breast cancer cells were cultured in IMEM–0.1% FBS in the absence or presence of trastuzumab, OA, or a combination of OA plus trastuzumab for 72 h, and then harvested and lysed as described in Materials and methods. Equal amounts of total protein (50 μg per lane) were subjected to western blot analyses with a specific antibody against the p85 fragment of PARP, and then re-probed with a β -actin antibody. The figure shows a representative immunoblotting analysis. Similar results were obtained in three independent experiments. **(B)** Analysis of the nature of the interaction between the apoptotic activities of OA and trastuzumab in SK-Br3 and BT-474 breast cancer cells. For each pair of columns, the height of the columns on the left represents the sum of the effect of each agent alone and, therefore, the expected apoptotic cell death if their effect is additive when used in combination. The total height of the columns on the right indicates the observed apoptosis when the agents are used in combination. The difference between the heights of the paired columns reflects the magnitude of synergy of apoptosis (** $P < 0.005$).

immunofluorescent p27^{Kip1} from cytosol to cell nuclei (data not shown), while co-treatment with OA resulted in an almost complete translocation of p27^{Kip1} from cytosol to cell nuclei (Figure 5, right panel). It is likely that this remarkable up-regulation and nuclear accumulation of p27^{Kip1} plays a pivotal role in determining the enhanced apoptotic cell death of trastuzumab-treated breast cancer cells following OA co-exposure.

Exogenous supplementation with OA inhibits Her-2/neu-driven MAPK and AKT phosphoproteins

We finally examined whether the signaling pathways downstream of Her-2/neu that regulate cell cycle progression and/or cell death were modified following exogenous supplementation with OA. In BT-474 cells, OA treatment inhibited active MAPK and active AKT as measured by antibodies specific to phospho-MAPK and phospho-Ser⁴⁷³ AKT, respectively, without changes in total MAPK and total AKT (Figure 6). Consistent with earlier studies [44], trastuzumab treatment in BT-474 cells significantly inhibited MAPK as well as AKT function, as measured by steady-state levels of

phosphorylated MAPK and phospho-Ser⁴⁷³ AKT, respectively (Figure 6). Although the exquisite sensitivity of MAPK and AKT signaling pathways to the down-regulatory effects of either OA or trastuzumab as single agents impeded our ability to demonstrate a synergistic blocking effect of active MAPK and AKT following co-exposure to OA and trastuzumab, low doses of OA (5 μM) were found to enhance significantly the ability of trastuzumab (10 $\mu\text{g/ml}$) to reduce the activation status of these phosphoproteins.

Discussion

Her-2/neu (*erbB-2*) is one the most commonly analyzed oncogenes in breast cancer studies. This orphan tyrosine kinase receptor regulates biological functions as diverse as cellular proliferation, transformation, differentiation, motility and apoptosis [52]. Therefore, modulation of Her-2/neu expression must be tightly regulated for normal cellular function. Accordingly, *in vitro* and animal studies clearly demonstrate that deregulated Her-2/neu overexpression plays a pivotal role in oncogenic transformation, tumorigenesis and metastasis.

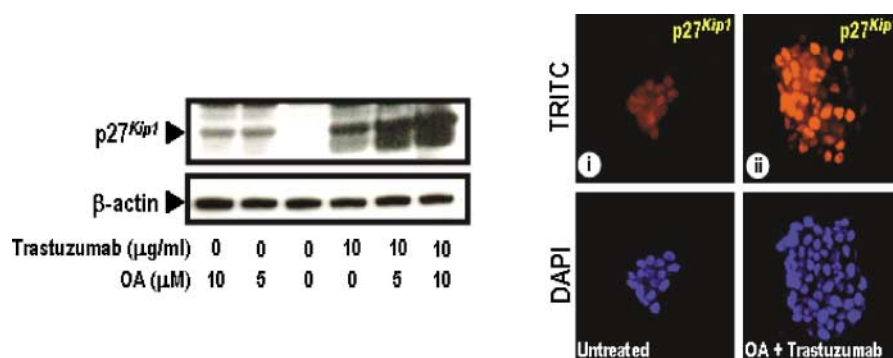


Figure 5. Exogenous supplementation with OA synergistically enhances trastuzumab-induced up-regulation and nuclear accumulation of p27^{Kip1}. Left panels: overnight serum-starved BT-474 breast cancer cells were cultured in IMEM–0.1% FBS in the absence or presence of trastuzumab, OA, or a combination of OA plus trastuzumab for 72 h, and then harvested and lysed as described in Materials and methods. Equal amounts of total protein (50 μg per lane) were subjected to western blot analyses with an anti-p27^{Kip1} rabbit polyclonal antibody and then re-probed with a β -actin antibody. The figure shows a representative immunoblotting analysis. Similar results were obtained in three independent experiments. Right panels: overnight serum-starved BT-474 cells growing in eight-well chamber slides were cultured in IMEM–0.1% FBS in the absence (panel i) or presence of 10 μM OA plus 10 $\mu\text{g/ml}$ trastuzumab (panel ii). After 72 h, p27^{Kip1} cellular localization was evaluated by immunofluorescence following 2 h incubation with an anti-p27^{Kip1} rabbit polyclonal antibody diluted 1:200 in 0.05% Triton X-100/PBS. Cellular localization of p27^{Kip1} was detected by indirect immunofluorescence by incubating with TRITC-conjugated anti-rabbit IgG secondary antibody. Cells were examined and photographed using a Zeiss fluorescent microscope equipped with a built-in camera. The figure shows a representative immunostaining analysis. Similar results were obtained in three independent experiments.

Moreover, Her-2/*neu* overexpression occurs in $\sim 20\%$ of breast carcinomas and is associated with unfavorable clinical outcome and resistance to chemotherapy [53–56]. Epigenetic factors, like dietary fatty acids, that could modify normal regulation of oncogenes such as Her-2/*neu* could have a role in the etiology of breast cancer [57]. In this regard, epidemiological and animal model studies suggest that consumption of olive oil, which is rich in the monounsaturated fatty acid OA (18:1n-9), may reduce the risk of breast cancer [10–13, 20, 21]. However, little is known about the ultimate biochemical pathways through which OA influence breast cancer risk and/or breast cancer progression. Our current findings, demonstrating that OA can repress Her-2/*neu* oncogene overexpression, represent a novel pathway through which individual dietary fatty acids may modulate both the etiology and the aggressive behavior of breast cancer.

We found that exogenous supplementation with OA dramatically down-regulates Her-2/*neu*-coded p185^{Her-2/*neu*} oncoprotein in BT-474 and SK-Br3, two natural human breast cancer models bearing amplification of the Her-2/*neu* oncogene [44]. Importantly, OA-induced suppression of Her-2/*neu* overexpression was not significantly prevented by the effective scavenger of reactive oxygen species vitamin E, thus ruling out that lipid peroxidation may be involved in this effect (data not shown). Since no toxicities have been reported or suspected with OA, it is reasonable to suggest that dietary supplementation with OA may represent a promising dietary intervention for the prevention and/or management of Her-2/*neu*-overexpressing breast carcinomas. Moreover, the present findings suggest further that dietary interventions based on OA may be even more beneficial when given in combination with novel therapies directed against Her-2/*neu*. Thus, OA co-exposure induces a dramatic increase in the sensitivity of Her-2/*neu*-overexpressing breast

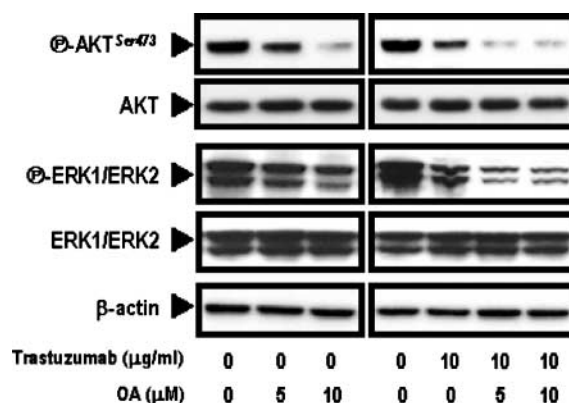


Figure 6. Exogenous supplementation with OA enhances trastuzumab-induced inhibition of AKT and MAPK phosphoproteins. Overnight serum-starved BT-474 breast cancer cells were cultured in IMEM–0.1% FBS in the absence or presence of trastuzumab, OA, or a combination of OA plus trastuzumab for 48 h, and then harvested and lysed as described in Materials and methods. Equal amounts of total protein (25 μg per lane) were subjected to western blot analyses with anti-phosphor-AKT^{Ser473} or anti-phosphor-MAPK antibodies, and then re-probed with anti-AKT, anti-MAPK and β -actin antibodies. The figure shows a representative immunoblotting analysis. Similar results were obtained in three independent experiments.

cancer cells to trastuzumab-induced cell growth inhibition upon anchorage-dependent and -independent conditions, and the nature of the interaction between OA and trastuzumab was found to be synergistic at clinically relevant trastuzumab concentrations. Importantly, exogenous supplementation with OA synergistically enhanced the ability of trastuzumab to induce down-regulation of p185^{Her-2/*neu*}. Moreover, the concurrent exposure to OA and trastuzumab was synergistically cytotoxic towards Her-2/*neu*-overexpressors by promoting

DNA fragmentation associated with apoptotic cell death, as confirmed by TUNEL staining and cleavage of the caspase-3 substrate, PARP. The sensitizing effects of OA on trastuzumab efficacy were also accompanied by the up-regulation and nuclear accumulation of p27^{Kip1}, a CDK_i playing a key role in the onset and progression of Her-2/*neu*-induced breast tumorigenesis that has recently been implicated in the development of trastuzumab resistance in breast cancer cells [42–44, 49–51]. Additionally, exogenous supplementation with OA significantly enhanced the ability of trastuzumab to inhibit the signaling pathways downstream of Her-2/*neu* that regulate cell cycle progression and/or cell death (i.e. AKT and MAPK). Although the specific mechanism through which OA molecularly modulates Her-2/*neu* expression certainly merits further investigation, preliminary results in our laboratory strongly suggest that OA is transcriptionally repressing Her-2/*neu* expression by up-regulating PEA3, an *ets* DNA-binding protein that has been shown to inhibit Her-2/*neu*-promoted tumorigenesis by down-regulating Her-2/*neu* promoter activity [58–60]. These results may demonstrate a promising therapeutic approach to Her-2/*neu*-overexpressing breast carcinomas using combinations of OA, which could indirectly repress Her-2/*neu* at the transcriptional level, and monoclonal antibodies to Her-2/*neu*, which target the ectodomain of p185^{Her-2/*neu*} and promote its degradation [46–48, 60].

In summary, our current results establish, to the best of our knowledge for the first time, that OA, the main mono-unsaturated fatty acid of olive oil, suppresses overexpression of the Her-2/*neu*-coded p185^{Her-2/*neu*} oncoprotein, which in turn interacts synergistically with trastuzumab-based breast cancer immunotherapy by promoting apoptotic cell death of breast cancer cells with amplification of the Her-2/*neu* oncogene. We recently found that exogenous supplementation with OA significantly diminishes proteolytic cleavage of the extracellular domain of Her-2/*neu* and, consequently, its activation status [61], a crucial event that determines breast cancer response to the anti-Her-2/*neu* antibody trastuzumab [62]. Although caution must be applied when extrapolating *in vitro* results into clinical practice, these findings together present the concept that a higher level of OA in breast tissue could provide an effective means of influencing the outcome of Her-2/*neu*-overexpressing breast cancer, a subset of breast carcinomas with poor prognosis. This previously unrecognized property of OA will help us to understand the molecular mechanisms by which individual fatty acids such as OA may regulate the malignant behavior of breast cancer cells and may therefore be helpful in the design of future epidemiological studies and, eventually, dietary counseling to delay or prevent trastuzumab resistance in Her-2/*neu*-positive breast cancer patients.

Acknowledgements

J. A. M. is the recipient of: a Translational Research Pilot Project (PP2) from the Specialized Program of Research Excellence (SPORE) in Breast Cancer (Robert H. Lurie

Comprehensive Cancer Center, Chicago, IL); a Basic, Clinical and Translational Award (BRCTR0403141) from the Susan G. Komen Breast Cancer Foundation (USA); and a Breast Cancer Concept Award (BC033538) from the US Department of Defense.

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