

RESISTANCE TO TAMOXIFEN-INDUCED APOPTOSIS IS ASSOCIATED WITH DIRECT INTERACTION BETWEEN Her2/neu AND CELL MEMBRANE ESTROGEN RECEPTOR IN BREAST CANCER

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Overexpression of Her2/neu is implicated in the development of resistance to the antiestrogen tamoxifen (TAM) that exerts its inhibitory effect through interaction with estrogen receptor (ER). Whereas Her2/neu and ER are believed to be important cell survival/death factors in human breast cancer cells, if and how they interact to confer resistance to hormone therapy is not known. This prompted us to investigate whether modulation of the effect of TAM occurs via the Her2/neu pathway and whether targeting the interaction between the Her2/neu pathway and the ER pathway is beneficial. There are 2 forms of ER that are localized to the cell membrane and to the nucleus. For the first time, we found that Her2/neu directly interacts with ER at the cell membrane. We then investigated the role of Her2/neu overexpression in the regulation of the cell membrane ER pathway in TAM-resistant breast cancer cells and the nature of this interaction in apoptotic signaling. Relief of TAM resistance was associated with Her2/neu downregulation and ER upregulation. TAM-induced apoptosis occurred immediately after dissociation of Her2/neu from cell membrane ER. These results demonstrate a novel mechanism by which Her2/neu regulates the cell membrane ER-coupled apoptosis and the possible involvement of the Her2/neu in TAM resistance of breast cancer cells. Moreover, the antiproliferative activity of TAM should rely on the integration between the signal transduction from the cell membrane ER and the gene regulation by the nuclear ER. Coordinated modulation on the cell membrane ER/Her2/neu pathway and the nuclear ER/RAR pathway may provide a new approach for treatment of ER-positive, Her2/neu overexpressing breast cancer.

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More than 60% of human breast cancers are ER-positive.¹ These tumors are supposed to respond to antiestrogenic therapy with TAM, which has fewer side effects than chemotherapy. Not all patients with positive ER, however, benefit from endocrine therapy.² It is being recognized increasingly that a variety of other growth factors, their receptors and their signaling pathways interact with ER signaling to suppress or stimulate the growth of ER-positive breast cancers. Clinical studies have shown that the response rate to TAM is reduced from 50% in ER-positive breast cancers with normal Her2/neu expression to 17% in ER-positive breast cancers with Her2/neu overexpression.³ Suppression of Her2/neu enhances the antiproliferative activity of TAM.⁴ In addition, antiestrogens and retinoids have been found to be additive or synergistic in their antitumor effects on ER-positive and retinoic acid receptor (RAR)-positive breast cancers.^{5,6} Therefore, use of coordinated modulations on signaling pathways such as Her2/neu, ER and RAR may provide an efficient alternative to classical endocrine therapy.

The antiestrogen TAM, a nonsteroid ligand of ER, is effective in breast cancer prevention and treatment by inhibiting the proliferative effects of estrogen that are mediated through the ER. The roles of ER are known to act not only as a steroid hormone nuclear receptor but also as a cell surface receptor linked to several intracellular mitogen-activated protein kinase (MAPK) pathways.^{7–12} Other non-ER-mediated mechanisms of

TAM effects on antiproliferation have been suggested to induce transforming growth factor-beta (TGF- β) synthesis,¹³ lower the circulating levels of insulin-like growth factor-1 (IGF-1),¹⁴ and inhibit the protein kinase C (PKC) pathways.¹⁵

All-trans retinoic acid (RA) binds and activates the nuclear RAR, which reveals a striking homology to the steroid receptor and acts as a transcriptional factor. RAR regulates the transcription of specific genes and controls cellular growth, death and differentiation.¹⁶ RA has been shown to enhance the antiestrogen TAM effect in ER-positive breast cancer cells *in vivo*.¹⁷ The RA/RAR complex exerts the antiestrogenic effect by impairing the binding of ER to the estrogen response element.¹⁸ Tumor response to RA also appears to correlate with the level of ER expression.⁶ Additionally, the RA/RAR complex is a repressor of Her2/neu expression at the transcriptional level.¹⁹

Phenylbutyrate (PB), β -oxidized to phenylacetate *in vivo*, represents a new nontoxic class of differentiation agents with therapeutic potential in different cancer patients.^{20–25} The mechanisms reported so far include inhibition of histone deacetylation,²⁴ of DNA methylation²⁰ and of protein isoprenylation.²³ Many studies have shown that PB upregulates several tumor suppressors²⁶ and nuclear receptors such as RAR²⁷ and downregulates or inhibits oncogenes.^{20,22,23} PB has been used to synergize with RA by modulating the RAR expression and the retinoic acid response element.^{25,27} Resistance to the differentiating actions of RA could be overcome by co-treatment with PB.²⁴ In addition, the combination of PB and antiestrogen TAM can enhance apoptosis in ER-positive breast cancer cells.²⁸

Although PB, RA, or TAM has been shown to have inhibitory effects on breast cancers, preclinical or clinical trials involving either single agents or different 2-drug combinations have been suboptimal in terms of antitumor activity or clinical benefit.^{17,28–30} In our study, we clearly demonstrate the enhanced effects of the 3-drug combination on growth inhibition and apoptosis in ER-positive and Her2/neu overexpressing breast cancer cells by modulating the RAR, Her2/neu and ER pathways interactively.

Abbreviations: ER, estrogen receptor; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PB, sodium phenylbutyrate; PKC, protein kinase C; RA, all-trans retinoic acid; RAR, retinoic acid receptor; TAM, tamoxifen.

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MATERIAL AND METHODS

Cell lines, reagent and growth inhibition experiments

The ER-positive breast cancer cell lines, BT-474 overexpressing Her2/neu and MCF-7 normally expressing Her2/neu, were obtained from ATCC and plated in 24-well plates (10^4 cells/well) in RPMI 1640 medium (GIBCO, Gaithersburg, MD) containing 10% heat-inactivated FCS (GIBCO) at 37°C in an atmosphere of 5% CO₂. After an overnight incubation, the cells were treated with various concentrations of estrogen (E₂), PB, RA, or TAM or different combinations in the presence or absence of specific inhibitors such as staurosporine (a PKC inhibitor) (Sigma, St. Louis, MO), SB203580 (a p38 MAPK inhibitor) (Calbiochem, La Jolla, CA), or a caspase-3 inhibitor (Calbiochem) as indicated in context. The cells were allowed to grow until the indicated time. The medium was removed. Tetrazolium dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) (Sigma) (1 ml) was added. After a 1-hr incubation at 37°C, MTT was removed and isopropanol/1N HCl (96:4) was added. Absorbance at 570 nm was determined on an automated microtiter plate reader. Values represent the mean of at least 2 experiments performed in triplicate; SD was less than 10%. Results are expressed as the percentage of the control cells. For MCF-7_{Her2/neu} cells, MCF-7 cells were transfected with pcDNA3-Her2/neu by the Lipofectamine reagent and protocol (GIBCO) and grown in culture medium containing 800 µg/ml G418 for 2 weeks, with 1 change in medium and G418 addition weekly. Then G418 resistant cells were pooled together and incubated in the presence of G418. The expression level of Her2/neu in MCF-7_{Her2/neu} cells was compared to that in parental MCF-7 cells by FACS as described below.

Cell cycle analysis

After exposing cells to different drug combinations as indicated in context, the cells were fixed in cold 100% ethanol for 1 hr and stained with a PBS solution containing RNase A (100 µg/ml) and propidium iodide (25 µg/ml, Sigma) at room temperature for 1 hr in the dark. The proportion of cells in G0-G1 was determined by flow cytometric analysis of DNA content by using an Epics Profile Analyzer (Coulter Corp., Hialeah, FL).

Flow cytometric staining (FACS)

BT-474 cells (5×10^4 cells/ml) were plated and treated as indicated in context. After treatment, the cells were removed from the plates by using a disposable cell scraper and centrifuged at 1,500 rpm for 5 min at 4°C. Fixation and permeabilization were carried out by incubating cell pellets for 15 min at 4°C with a solution containing 2% v/v paraformaldehyde and PBS-S (Sigma) and incubated for 30 min at 4°C with the specific antibody and the respective isotype-matched control antibody. The cell pellets were washed in PBS-S and incubated for 30 min at 4°C in the dark with a 1:150 dilution of goat antimouse immunoglobulin FITC (Sigma) or a 1:200 dilution of goat antirabbit FITC (Oncogene Research Products, Boston, MA) secondary antibody. After incubation, the cells were washed twice in PBS-S and then suspended in 2% paraformaldehyde at 4°C in the dark. The background fluorescence from matched isotype control sample was determined and each specific fluorescence intensity was expressed in % of control. Membrane antigens were assessed using the same protocol with the following minor modifications: the cells were gently detached with EDTA (0.5 µM) and fixed after incubation with the Her2/neu antibody and the FITC-conjugated secondary antibody.

Subcellular fractionation

The cells were washed with PBS and pelleted by centrifugation, then incubated on ice for 15 min in hypotonic buffer A (10 mM HEPES pH7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride) and homogenized in a loose fitting glass homogenizer with 15 strokes. Nuclei were separated from the cytosolic fraction by centrifugation at 5,000 rpm for 5 min at 4°C. Then the cytosolic fraction was collected from the supernatant by centrifugation at 40,000 rpm for 30 min to pellet the crude membrane fraction that was then

rehomogenized by sonication in lysis buffer/protease inhibitor solution (4 mM EDTA, 2 mM EGTA, 50 mM TrisCl pH 7.4 and 0.2 mM PMSF) containing 1% Triton X-100 and centrifuged for 1 hr at 100,000g (4°C). The resultant Triton X-100-soluble fraction was collected as the cell membrane fraction. For the mitochondria fraction, the cells were washed once in cold PBS and homogenized in the buffer (250 mM sucrose, 0.2 mM phenylmethylsulfonyl fluoride [PMSF] and 10 mM TrisCl pH 7.4), then the mitochondria fraction was separated by differential centrifugation sedimentation as described.³¹ Each subcellular fractions were resuspended in the sample buffer and subjected to the Western blot assay. The purity of the nucleus, cytosol and mitochondria extracts was assessed by immunoblotting the extracts with antibodies against the nuclear protein such as PARP, the cytosol protein such as α -tubulin and the mitochondria protein such as Bcl-2.

Western blot assay

Equal amount of protein (100 µg) was electrophoresed in 10% SDS-polyacrylamide gel (PAGE) and blotted onto polyvinylidene-difluoride (PVDF) membranes (Millipore, Bedford, MA). The blotted membranes were treated with 5% skim milk in PBS/0.1% Tween-20 and incubated with the different primary antibodies of rabbit polyclonal anti-Bax (P19, Santa Cruz, Santa Cruz, CA), anti-cytochrome c (H-104, Santa Cruz), anti-PARP (H-250, Santa Cruz), anti-ER- α (H-184, Santa Cruz) anti-PKC- α (C-20, Santa Cruz) and mouse monoclonal anti-phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) (New England Biolabs, Beverly, MA), anti-phospho-SAPK/JNK (Thr¹⁸³/Tyr¹⁸⁵) (Cell Signaling, Biolabs, Beverly, MA), anti-RAR- γ (M-454, Santa Cruz), anti-Her2/neu (9G6, Santa Cruz) and anti-Bcl-2 (100, Santa Cruz), respectively, for 16 hr at 4°C. The membranes were then incubated with the peroxidase-labeled second anti-rabbit or mouse antibody (Amersham, Arlington Heights, IL) for 1 hr at room temperature. The membranes were rinsed, treated with enhanced chemiluminescent (ECL) reagent (Amersham) and exposed to RX100 films (Fuji, Tokyo, Japan).

DNA fragmentation assay

The cells were harvested at the indicated time after different drug treatment and digested in lysis buffer (10 mM EDTA/50 mM Tris/0.5% N-Laurosacosine, pH 8.0/200 µg/ml of RNaseA treatment (37°C, 1 hr). Genomic DNAs were isolated by phenol/chloroform extraction and ethanol precipitation and dissolved in Tris-EDTA buffer (pH 7.5). DNA ladders were separated on 0.8% agarose gel and visualized by staining with ethidium bromide.

Immunofluorescence and confocal studies

Cells growing on glass coverslips were fixed in methanol at -20°C for 10 min. Several dilutions of antibodies of mouse anti-Her2/neu (9G6, Santa Cruz) and rabbit anti-ER- α (H-184, Santa Cruz) were used to obtain the optimal results. For staining of Her2/neu and ER, BT-474 cells were treated with mouse anti-Her2/neu and rabbit anti-ER- α antibodies followed by secondary rhodamine-labeled (red) anti-mouse and FITC-labeled (green) anti-rabbit antibodies (Dako, Carpinteria, CA). Confocal microscopy was performed using a Zeiss laser scanning confocal microscope. Each image represents Z-sections at the same cellular level and magnification.

Immunoprecipitation (IP)

The cells were incubated with the ice-cold lysis buffer (50 mM TrisCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 µg/ml PMSF, 1 µg/ml aprotinin, 1% NP-40 and 0.5% sodium deoxycholate) for 20 min and centrifuged at 12,000g for 2 min at 4°C. The supernatant proteins (400 µg) were incubated with anti-Her2/neu (5 µg) (9G6, Santa Cruz), anti-ER- α (5 µg) (H-184, Santa Cruz) and anti-RAR- γ (5 µg) (M-454, Santa Cruz), respectively, in 0.5 ml of NET-gel buffer (50 mM TrisCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA pH 8.0, 0.25% gelatin and 0.02% sodium azide) on a rocking platform for 1 hr at 4°C. Then the protein A-Sepharose beads (Pharmingen, San Diego, CA) were added for an 1-hr incubation at 4°C. The immunoprecipitate

Sepharose beads containing protein A-antigen-antibody complexes were collected by centrifugation at 12,000g for 20 sec at 4°C and then washed 5 times with NET-gel buffer. The immunoprecipitates were resuspended in the sample buffer and subjected to the Western blot assay. For the IP tests between Her2/neu and cell membrane ER, the same protocol was used with the following modification: nuclei were first extracted as mentioned above to avoid the nuclear ER interference.

Immunocytochemistry

DAKO LSAB kit was used. In brief, the cells were fixed by immersing in -20°C methanol and acetone (1:1) for 1 min, treated with 3% H₂O₂ for 10 min and incubated with the mouse anti-human Her2/neu antibody (9G6, Santa Cruz) for 1 hour at room

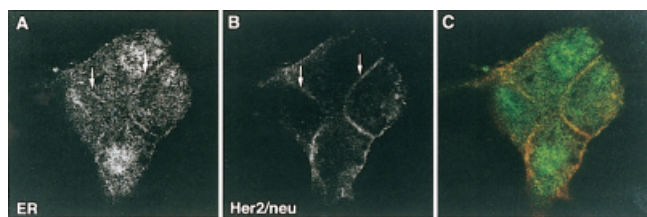


FIGURE 1 – Immunofluorescence and confocal imaging to demonstrate the colocalization of cell membrane ER- α and Her2/neu. BT-474 cells growing on glass coverslips were fixed in methanol at -20°C for 10 min. For staining of Her2/neu and ER, cells were treated with mouse anti-Her2/neu (9G6, Santa Cruz) and rabbit anti-ER- α antibodies (H-184, Santa Cruz) followed by secondary rhodamine-labeled (red) anti-mouse and FITC-labeled (green) anti-rabbit antibodies (DAKO). Confocal microscopy was performed using a Zeiss laser scanning. Each image represents Z-sections at the same cellular level and magnification. (a,b) Immunostaining. Arrows point out the cell membrane localization of ER- α and Her2/neu, respectively. (c) Confocal imaging. Cell membrane ER- α (green), Her2/neu (red) and co-localization (yellow).

temperature, then with the biotinylated anti-mouse immunoglobulins in PBS for 10 min and then with streptavidin conjugated to horseradish peroxidase in PBS for 10 min. AEC Chromogen was added for 15 min. The cells were rinsed with distilled water.

CPP32/Caspase-3 activity assay

The cells were washed 3 times with cold PBS and scraped with a rubber policeman. The cell pellet was suspended in the ice-cold hypotonic cell lysis buffer (25 mM HEPES pH 7.5, 5 mM MgCl₂, 5 mM EDTA, 5 mM DTT, 2 mM PMSF, 10 μ g/ml Pepstatin A and 10 μ g/ml Leupeptin) at a concentration of 10⁸ cells/ml. The cells were frozen and thawed 4 times and centrifuged at 16,000g for 20 min at 4°C. The supernatant fraction was collected. The activities of CPP32/Caspase-3 were measured according to the protocol of fluorometric CaspACE™ assay system (Promega, Madison, WI). In brief, 100 μ g of the supernatant fraction, 2 μ l of 2.5 mM CPP32/caspase-3 substrate, 2 μ l of DMSO, 10 μ l of 100 mM DTT, 32 μ l of ICE-like enzyme assay buffer (312.5 mM HEPES pH 7.5, 31.25% sucrose and 0.3125% CHAPS) and deionized water were incubated in final 100 μ l vol for 1 hr at 37°C. The fluorescence of each reaction at an excitation wavelength of 360 nm and an emission wavelength of 460 nm were measured by a fluorescence plate reader. The results were calculated according to the protocol formula. Each treatment was tested in 3 separate wells per assays and the same assay performed 3 times.

RESULTS

Direct interactions between Her2/neu and cell membrane ER and between RAR and nuclear ER

To determine whether the association between Her2/neu overexpression and the low efficacy of TAM therapy is related to the Her2/neu-mediated ER interference, we first examined the *in situ* localization of Her2/neu and ER in BT-474 cells by indirect double-immunofluorescence and confocal microscopy (Fig. 1). ER- α was detected in both membrane and nuclear compartments but the number of nuclear receptors greatly exceeded those localized to the cell membrane (Fig. 1a). Her2/neu was primarily found

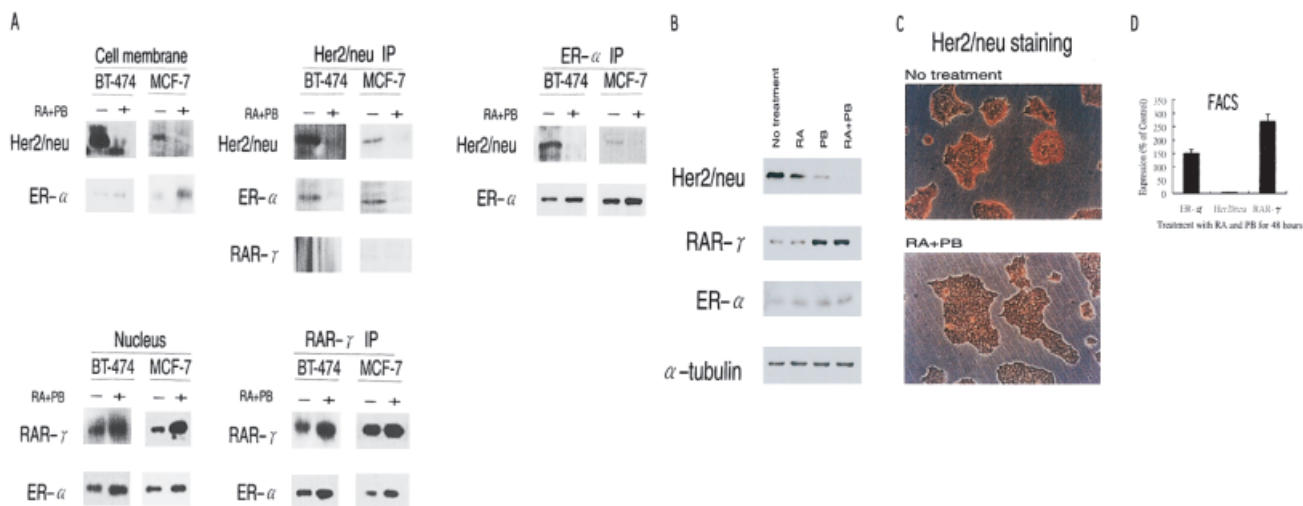


FIGURE 2 – Direct physical associations between Her2/neu and cell membrane ER and between RAR and nuclear ER. (a) For immunoprecipitation (IP) tests between Her2/neu and the cell membrane ER, nuclei were first extracted as mentioned in Material and Methods to avoid the nuclear ER interference. The cells treated with or without RA and PB for 48 hr were incubated with the ice-cold lysis buffer and centrifuged. The supernatant proteins (400 μ g) were incubated with anti-Her2/neu (5 μ g) (9G6, Santa Cruz), anti-ER- α (5 μ g) (H-184, Santa Cruz) and anti-RAR- γ (5 μ g) (M-454, Santa Cruz), respectively. Then the protein A-Sepharose beads were added. The immunoprecipitates and subcellular fractions as described in Material and Methods were subjected to the Western blot assays. For subcellular fractions, 10 times more cell membrane protein was loaded, compared to nuclear protein. (b) BT-474 cells treated with or without RA or PB for 48 hr were subjected to the Western blotting with anti-Her2/neu, RAR- γ , ER- α and α -tubulin. Lane 1: no treatment; lane 2: RA (2 μ M); lane 3: PB (2 mM); lane 4: the combination of RA and PB. (c) Immunocytochemistry. BT-474 cells treated with or without RA and PB for 48 hr were stained with anti-Her2/neu antibody (9G6, Santa Cruz). (d) FACS. BT-474 cells treated with or without RA and PB for 48 hr were labeled with anti-Her2/neu, RAR- γ and ER- α as described in Material and Methods, then subjected to flow cytometric analysis. The results of treated cells compared to control cells (no treatment) were expressed in % of control. Each experiment was repeated at least twice.

in the cell membrane (Fig. 1*b*). The co-localization of cell membrane ER- α (green) and Her2/neu (red) is shown in yellow (Fig. 1*c*).

We then carried out the immunoprecipitation (IP) studies to determine whether physical association exists between cell membrane ER and Her2/neu (Fig. 2*a*). ER- α was co-immunoprecipitated by the anti-Her2/neu antibody. Her2/neu was also detected by the reciprocal co-IP using the anti-ER- α antibody. That Her2/neu IP did not co-immunoprecipitate RAR- γ shows the specificity of the observed interaction between Her2/neu and ER.

By using that PB synergizes with RA by upregulating RAR to augment the RA effect on Her2/neu downregulation, the combination of PB and RA suppressed Her2/neu expression completely in BT-474 cells (Fig. 2*b*).^{19,25} Immunocytochemistry further confirmed the complete Her2/neu downregulation by PB and RA (Fig. 2*c*). In contrast to Her2/neu protein level almost down to zero, the combination of PB and RA increased ER protein up to 150% of control (Fig. 2*d*). Once Her2/neu was downregulated completely and ER- α was upregulated, the anti-Her2/neu antibody did not capture ER even in the presence of increased cell membrane ER and the anti-ER- α antibody captured more ER- α but no Her2/neu (Fig. 2*a*).

The nuclear ER was co-immunoprecipitated by the anti-RAR antibody. Once RAR was upregulated by PB (Fig. 2*b,d*), there was an increase in interaction between RAR and nuclear ER (Fig. 2*a*). This finding is consistent with the previous reports that show differential modulation of transcriptional activity of ER by direct protein-protein interactions with RAR³² and that the antiestrogenic effect of RA occurs at the estrogen response element level.¹⁸

Enhancement of TAM sensitivity occurs with the combination of PB and RA

We then hypothesized that downregulation of Her2/neu to relieve cell membrane ER can enhance the TAM-initiated signaling transduction from the cell membrane ER and promote reversion of the sensitivity to TAM. To support this idea, we first compared the TAM sensitivity between BT-474 cells and MCF-7 cells with or without PB or RA treatment. MCF-7 cells are characterized by higher levels of ER (20,000 sites/cell) and lower levels of Her2/neu (5,500 sites/cell) (Fig. 2*a*).³⁸ BT-474 cells express lower levels of ER (5,000 sites/cell) and higher amount of Her2/neu (110,000 sites/cell) (Fig. 2*a*).³⁸ MTT assays and flow cytometry were used to measure the cell growth inhibition and cell death, respectively. As shown in Figure 3*a*, BT-474 cells have similar sensitivity of estrogen to MCF-7 cells but show marked resistance to TAM. This at least suggests that the ERs in MCF-7 and BT-474 cells are functional and the effect of TAM may not solely on the basis of the level of ER expression. Both cell lines demonstrated no significant difference in sensitivity to either PB or RA in a dose range of 0.5–5 mM and 0.1–5 μ M, respectively.

We then treated cells with relatively low inhibitory concentrations of single agent alone of PB (2 mM), RA (2 μ M) or TAM (2 μ g/ml), or different 2-combinations including TAM and RA, TAM and PB or RA and PB for 72–96 hr. Each single agent and 2-drug combination showed only mildly to moderately inhibitory effects and minimal apoptosis induction (Fig. 3*b,c*). In contrast, only the three-drug combination of PB, RA and TAM produced 50% of growth inhibition in MCF-7 and in BT-474 cells at about 2.2 days and 3.2 days, respectively (Fig. 3*b*) and started increase of sub-G1 cell cycle distribution in MCF-7 and in BT-474 cells at about 12 hr and 36 hr, respectively, after treatment (Fig. 3*c*).

TAM induces apoptosis signaling immediately in the absence of Her2/neu overexpression

The function of cell membrane ER is now known to be involved in regulating a wide variety of biological processes through different MAPK signaling cascades.¹¹ Activation of the p38 MAPK pathway by TAM is coupled to non-genomic ER-induced apoptosis.¹² To test if the TAM-initiated apoptosis signaling starting from the cell membrane ER is functional in BT-474 cells, we checked the apoptotic pathways inducible by TAM.¹² We found that PB, RA or TAM alone, or each 2-drug combination at these low

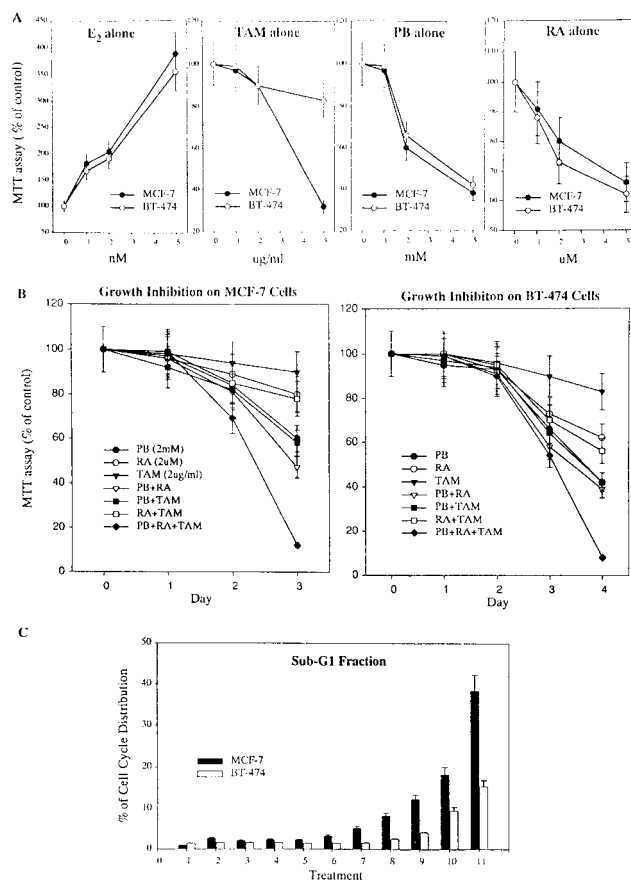


FIGURE 3 – The combination of PB and RA promotes reversion of TAM resistance. The ER-positive breast cancer cell lines, BT-474 overexpressing Her2/neu and MCF-7 normally expressing Her2/neu, were plated in 24-well plates (10^4 cells/well). The growth curves were measured by the MTT assays as described in Material and Methods. (a) Cells were exposed to the agents (E₂, TAM, PB, RA) with various concentrations for 3 days. (b) Cell growth after 3–4 days of exposure to PB (2 mM), RA (2 μ M), TAM (2 μ g/ml), PB + RA, PB + TAM, RA + TAM, or PB + RA + TAM. (c) Sub-G1 fraction in cell population exposed to various agents for various times was measured by flow cytometry. 1: No treatment (48 hr); 2: PB (2 mM) (48 hr); 3: RA (2 μ M) (48 hr); 4: TAM (2 μ g/ml) (48 hr); 5: PB + RA (48 hr); 6: PB + TAM (48 hr); 7: RA + TAM (48 hr); 8: PB + RA + TAM (12 hr); 9: PB + RA + TAM (36 hr); 10: PB + RA + TAM (48 hr); 11: PB + RA + TAM (60 hr). Mean \pm SE of triplicate measurements. Each experiment was repeated at least twice.

concentrations had only minimal effects on activation of apoptotic pathways in BT-474 cells (Figs. 3*c*, 4*a,d*). With PB and RA pretreatment for 48 hr, however, TAM could activate PKC- α , p38 MAPK and Jun N-terminal kinase (JNK) within 30 min (Fig. 4*a*). Moreover, Bax translocation to mitochondria, cytochrome c release into cytosol and increase of CPP32/caspase-3 activity accompanied by PARP cleavage into 85 Kd fragments occurred at the first 4 hr exposure to TAM (Fig. 4*a–c*). No change of Bcl-2 expression level was noted. α -tubulin was used as an internal control. The purity of the cytoplasmic, mitochondria and nuclear fractions was confirmed with the nuclear protein PARP, with the mitochondria protein Bcl-2 and with the cytoplasmic protein α -tubulin (Fig. 4*b*). TAM-induced apoptosis in BT-474 cells pretreated with PB and RA was also confirmed by the DNA fragmentation assay (Fig. 4*d*).

Furthermore, the inhibitor of the PKC pathway (staurosporine), the p38 MAPK pathway (SB203580) or the caspase pathway (CPP32/caspase-3 inhibitor) blocked the TAM-mediated growth

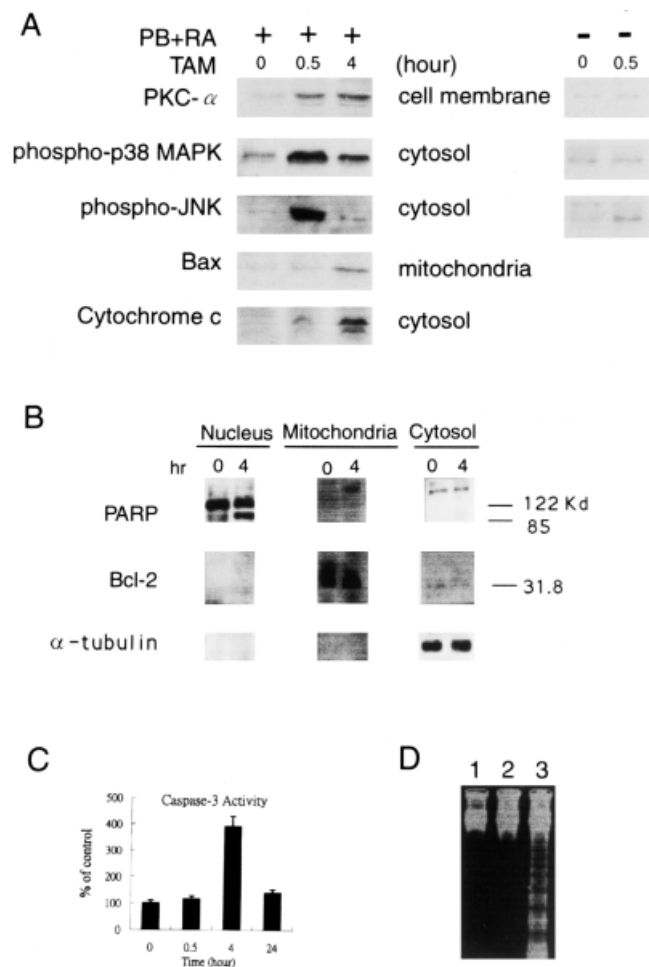


FIGURE 4 – TAM-induced apoptosis occurs immediately after dissociation of Her2/neu from cell membrane ER by PB and RA. Subcellular fractionation from the BT-474 cells incubated with or without PB (2 mM) and RA (2 μM) for 48 hr followed by TAM (2 μg/ml) for various times was prepared as described in Material and Methods. (a,b) Equal amount of different subcellular fraction (100 μg) was electrophoresed, blotted onto PVDF membranes and incubated with the different primary antibodies of rabbit polyclonal anti-Bax (P19, Santa Cruz), anti-cytochrome c (H-104, Santa Cruz), anti-PARP (H-250, Santa Cruz) and anti-PKC-α (C-20, Santa Cruz) and mouse monoclonal anti-phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) (New England Biolabs), anti-phospho-SAPK/JNK (Thr¹⁸³/Tyr¹⁸⁵) (Cell Signaling), anti-RAR-γ (M-454, Santa Cruz) and anti-Bcl-2 (100, Santa Cruz), respectively, for 16 hr at 4°C. (c) The activities of CPP32/Caspase-3 were measured according to the protocol of fluorometric CaspACE™ assay system (Promega). (d) DNA fragmentation. The genomic DNAs of treated cells were extracted as described in Material and Methods. Lane 1: TAM (2 μg/ml) alone for 3 days; lane 2: PB (2 mM) and RA (2 μM) for 3 days; lane 3: the cells pre-treated with PB (2 mM) and RA (2 μM) for 2 days, followed by exposure to TAM (2 μg/ml) alone for 1 day.

inhibition (Fig. 5a). This directly links apoptosis to the low dose TAM treatment in BT-474 cells pre-treated with PB and RA.

Her2/neu downregulation after PB and RA-conditioning is a key factor for TAM enhancement

We further tested if the PB and RA-conditioning is essential for the enhancement of TAM effects in BT-474 cells. The cells pre-treated with PB and RA for 2 days followed by PB, RA and TAM for another 2 days showed marked growth inhibition by 90% (Fig. 5a), which was significantly enhanced when compared to those treated with various combinations (Fig. 3b). Moreover, only a

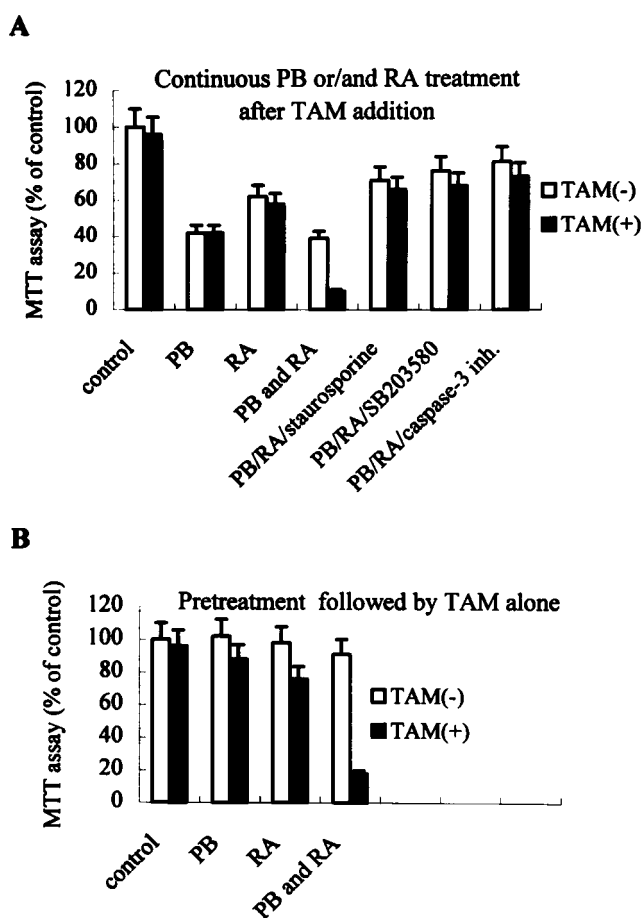


FIGURE 5 – The PB and RA-conditioning is essential for relief of TAM resistance. BT-474 cells were plated in 24-well plates (10⁴ cells/well). After various treatment, the cell growth was measured by the MTT assay as described in Material and Methods. The results of treated cells compared to control cells (no treatment) were expressed in % of control. Each experiment was repeated at least twice. (a) Continuous treatment with PB or RA: 2 days PB (2 mM) or RA (2 μM) followed by 2 days PB (2 mM) or RA (2 μM) ± TAM (2 μg/ml) ± the PKC inhibitor (100 nM of staurosporine), the p38 MAPK inhibitor (20 μM of SB203580), or the CPP32/caspase-3 inhibitor (7.5 μM). (b) Pretreatment by PB or RA: 2 days PB (2 mM) or RA (2 μM) followed by 2 days ± TAM (2 μg/ml) alone.

2-day PB and RA preincubation was sufficient to sensitize the cells to TAM alone without the concurrent presence of PB and RA (Figs. 4d, 5b). Compared to Figure 3a, Figure 5b demonstrates that dose-response to TAM in BT-474 cells is shifted by the pretreatment with PB and RA. Although the effect of growth inhibition by PB or RA was mild and cytostatic (Figs. 2c, 5b), the low dose TAM addition led to irreversible cell death noted by the growth kinetics, sub-G1 distribution of cell cycle, apoptotic signaling and DNA fragmentation (Figs. 2c, 3–5).

We asked if the effect of PB and RA-conditioning on TAM enhancement disappears in cells with constitutive Her2/neu overexpression, or if TAM enhancement is associated with Her2/neu downregulation but not just other effects induced by PB and RA. MCF-7_{Her2/neu} (MCF-7 cells transfected with Her2/neu) is suitable for this purpose because PB and RA cannot suppress exogenous Her2/neu overexpression (Table I).⁴⁴ After treatment with PB and RA, the levels of ER-α and Her2/neu were upregulated and downregulated, respectively, in parental MCF-7 cells. In contrast, only ER upregulation but no Her2/neu downregulation was noted in MCF-7_{Her2/neu} cells (Table I). There was no significant difference in growth inhibition in the presence of PB and RA between MCF

and MCF-7_{Her2/neu}. After medium change to contain TAM alone, however, the growth inhibition was correlated with the Her2/neu expression level. MCF-7_{Her2/neu} cells having constitutive Her2/neu overexpression even after PB and RA treatment was still resistant to TAM.

DISCUSSION

Why Her2/neu overexpression causes estrogen-dependent, antiestrogen TAM-resistant tumorigenic growth of breast cancer cells is unclear.³⁹ Because 40–50% of Her2/neu overexpressing breast cancers are also ER-positive,⁴⁰ treatment designed to block estrogen's effects remains one of the most challenging areas of study.⁴¹ This is the first report demonstrating that the combination of PB and RA enhances the antiproliferative effects of TAM in ER-positive, Her2/neu overexpressing breast cancer by modulating simultaneously the interactions between Her2/neu and cell membrane ER and between RAR and nuclear ER. Our study also provides evidence that Her2/neu may directly interfere with the TAM effect on the cell membrane ER. As a result, the ratio of Her2/neu to the cell membrane ER can be regarded as an indicator for TAM sensitivity and even prognosis.

Previous studies have shown that there are 2 forms of ER that are localized to the cell membrane and to the nucleus.¹⁰ The existence of a cell membrane ER was reported more than 20 years ago but most studies of ER had focused on its action as a ligand-regulated nuclear transcription factor.^{33–37} For the first time, we found a cell membrane ER-signaling pathway involving a direct interaction with Her2/neu (Figs. 1,2). Because ERs in both membrane and nuclear compartments have near-identical affinities for the 17- β -E₂ (an estrogen),^{10,11,34–37} the cell membrane and nuclear ERs are also supposed to be available to TAM (an estrogen analogue). It is possible that Her2/neu interferes with the TAM effect by either modulating the cell membrane ER signaling or influencing the TAM-binding to cell membrane ER. Whether there is difference in binding affinity between Her2/neu bound membrane ER and non-Her2/neu bound membrane ER needs a further experiment using the labeled-TAM. Anyway, the TAM-initiated effects appeared soon after Her2/neu downregulation, suggesting these are non-genomic actions via the cell membrane ER according to the time course of these acute events (Fig 4a).^{11,12} Although the fraction of cell membrane ER is much less than that of nuclear ER (Figs. 1, 2),¹⁰ the rapidly TAM-mediated non-genomic action may have a larger effect during early period of treatment. The longer treatment time the cells receive, the more important role the nuclear ER is supposed to play.

The demonstration of direct physical associations between Her2/neu and cell membrane ER and between RAR and nuclear ER results in speculation that the antiproliferative effects of TAM should rely on the integration of both the cell membrane ER signaling and the nuclear ER function. This implies that TAM may not work well even in the presence of functional nuclear ER if the cell membrane ER signaling does not favor

growth inhibition or cell death, or epidermal growth factor receptor pathways are dominant. That is, the persistently activated Her2/neu may directly block the cell membrane ER-initiated apoptosis and indirectly offset the nuclear ER effect on growth inhibition. In our study, cells with exogenously constitutive Her2/neu overexpression even after PB and RA treatment still showed TAM resistance (Table I). Cells with Her2/neu downregulation by PB and RA but without subsequent TAM addition, however, only produced reversible cytostasis (Fig. 5b). Therefore, these observations may indicate that suppressing the Her2/neu signaling, enhancing the cell membrane ER-coupled apoptotic pathway (early response) and modulating the nuclear ER activity (late response) are required for effective breast cancer treatment. Consistent with the results, the enhancement of TAM effect associated with Her2/neu suppression is also seen by treatment with RA or with the 4D5 anti-Her2/neu antibody.^{4,17,19}

The 3-drug combination of PB, RA and TAM was the most effective treatment when compared to single agents or 2-drug combinations in both MCF-7 and BT-474 cells but the onset of antiproliferative effect in BT-474 cells was about 12–24 hr later than that in MCF-7 cells (Fig. 3b,c). The time course difference in the effects of TAM in MCF-7 and BT-474 cells with or without PB or RA treatment is a reflection of their Her2/neu and cell membrane ER status and is not simply a function of time or other PB and RA effects when comparing the effects of PB or RA on the expression level of Her2/neu and the association between Her2/neu and cell membrane ER (Figs. 2–5, Table I). We can see that because the combination of PB and RA cannot suppress the exogenous Her2/neu overexpression in MCF-7_{Her2/neu} cells, the effect on TAM enhancement is low. Moreover, the relatively slower onset in BT-474 cell growth inhibition is compatible with the time that cells need for Her2/neu downregulation and increase of the free form of cell membrane ER.

The balance of activities among the Her2/neu, RAR and ER pathways should be an important factor in the decision of breast cells to proliferate, remain quiescent or undergo apoptosis. Using differential modulations of the pathways of Her2/neu, RAR, cell membrane ER and nuclear ER to lead to growth inhibition and apoptosis in ER-positive, Her2/neu overexpressing breast cancers, however, has not been adequately studied before. In our study, downregulation of Her2/neu by PB and RA to dissociate cell membrane ER from Her2/neu inhibition may shut down the Her2/neu-initiated MAPK pathways such as Ras-MAPK for cell cycle stimulation (Fig. 2)¹¹ and make other cell membrane ER-linked apoptotic MAPK pathways such as p38 MAPK and JNK become dominant (Fig. 4a).¹² In addition, PB can further inhibit Ras function by influencing Ras isoprenylation.²³ Upregulation of RAR by PB to increase direct protein-protein interactions between nuclear ER and RAR will exert the differential transcriptional activities of ER and RAR on modulations of certain genes related to the cell growth control (Fig. 2a).³²

Non-ER-mediated biochemical interactions that might also contribute to the antitumor activity of antiestrogens have been described.⁹ TAM is known as an inhibitor of PKC.¹⁵ In our study, however, PKC- α activity increased immediately once TAM was added to the cells pre-treated with PB and RA (Fig. 4a). The PKC

TABLE I—PROTEIN EXPRESSION LEVELS ER- α , HER2/NEU) AND TAM EFFECTS AFTER PRE-TREATMENT WITH PB AND RA ON MCF-7 CELLS TRANSFECTED WITH HER2/NEU AS COMPARED TO PARENTAL MCF-7 CELLS

Breast cancer	Expression levels (x-fold) ¹		Mean fraction of cell growth (% of control) ²	→ PB and RA for 48 hr	Expression levels (x-fold) ¹		Mean fraction of cell growth (% of control) ²	→ then TAM alone for 48 hr	Mean fraction of cell growth (% of control) ²
	ER- α	Her2/neu			ER- α	Her2/neu			
MCF-7	1	1	100		1.83	00.10	80		28
MCF-7 _{Her2/neu}	0.84	18.25	100		1.25	21.37	84		82

¹Protein levels were determined by FACS as described in Material and Methods, with relative values (x-fold as compared to MCF-7) as shown.²Cell growth with or without PB (2 mM) and RA (2 μ M) for 48 hr, and then TAM (2 μ g/ml) alone for another 48 hr was measured by MTT assays as described in Materials and Methods, with mean fractional values expressed relative to vehicle treated control cells. At least two experiments performed in triplicate. SD was less than 10%.

inhibitor staurosporine did protect cells from death (Fig. 5a), indicating that PKC pathway is involved in this synergy. A previous study has shown a large increase in PKC activity was observed after sequential treatment with non-toxic levels of bryostatin (a PKC activator) followed by TAM.⁴² Consistent with this result, our previous study revealed that PB is a PKC activator.⁴³ It is likely that treatment with PB followed by TAM results in difference in activation, inhibition and intracellular translocation of individual PKC isoforms.

In our findings, it warrants further investigations *in vivo* to coordinately modulate the Her2/neu, RAR, nuclear ER and cell membrane ER pathways by the combination of PB, RA and TAM on growth inhibition in human ER-positive breast cancers, especially with Her2/neu overexpression. PB, RA and TAM, all FDA-approved drugs, have been currently used in certain inherited diseases and cancers. Their low toxicity, clinical availability, oral acceptability and low cost allow them to become good candidates in clinical trials.

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