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 on HER-2/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 the 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 up-regulation. 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. 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.

Key words: Her2/neu; estrogen receptor; phenylbutyrate; retinoic acid; tamoxifen

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 cancer to 20% in HER-2/neu 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-B) 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 antitumor TAM effect in ER-positive breast cancer cells in vivo.1 A RA/RAR complex exerts the antitumor 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.20–23 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 RA/RAR expression and the retinoic acid response element.25,26 Resistance to the differentiating actions of RA could be overcome by co-treatment with PB.25 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 HER-2/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 (104 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 an 1-hr incubation at 37°C, MTT was removed and isopropanol/1N HCl (96:4) was added. Absorbance at 570 nm was determined on a determined on a 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 Analyser (Coulter Corp., Hialeah, FL).

**Flow cytometric staining (FACS)**

BT-474 cells (5 × 104 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-3× 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 pH 7.9, 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 homogenized 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.3 Each subcellular fractions were resuspended in the sample buffer and subjected to the Western blot assay. The purity of the mitochondria, 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-cytokerin c (H-104, Santa Cruz), anti-ERα (H-184, Santa Cruz), anti-ERβ (H-184, Santa Cruz) or a group of monoclonal anti-phospho-p38 MAPK (Thr 180/ Tyr182) (New England Biolabs, Beverly, MA), anti-phospho-SAPK/JNK (Thr 183/Tyr185) (Cell Signaling, Beverly, MA), anti-rabbit polyclonal anti-Bax (P19, Santa Cruz), anti-ERα (H-184, Santa Cruz) and anti-Bcl-2 (100, Santa Cruz) respectively, for 1 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 XAR100 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-Lauro sarcosine, pH 8.0/200 μg/ml of RNase A 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
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 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 µg/ml Pepstatin A and 10 µg/ml Leupeptin) at a concentration of 106 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 µg of the supernatant fraction, 2 µl of 2.5 mM CPP32/caspase-3 substrate, 2 µl of DMSO, 10 µl of 100 mM DTT, 32 µl 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 µl 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

![Image](https://example.com/image1.png)

**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 (DAKO). Confocal imaging. Cell membrane ER-α (green), Her2/neu (red) and co-localization (yellow).

![Image](https://example.com/image2.png)

**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 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) (H-184, 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 µM); 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.
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 (110,000 sites/cell) (Fig. 2a).39 BT-474 cells express lower levels of ER (5,000 sites/cell) and higher amount of Her2/neu (110,000 sites/cell) (Fig. 2a).38 MTT assays and flow cytometry were used to measure the cell growth inhibition and cell death, respectively. As shown in Figure 3a, 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 μM, respectively.

We then treated cells with relatively low inhibitory concentrations of single agent alone of PB (2 mM), RA (2 μM) or TAM (2 μg/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. 3b,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. 3b) 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. 3c).

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.14 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 concentrations had only minimal effects on activation of apoptotic pathways in BT-474 cells (Figs. 3c, 4a,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. 4a). 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. 4a–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. 4b). TAM-induced apoptosis in BT-474 cells pretreated with PB and RA was also confirmed by the DNA fragmentation assay (Fig. 4d).

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
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. 5b). Moreover, only a 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). 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-7/Her2/neu and MCF-7 cells.
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, antioestrogen TAM-resistant tumorigenic growth of breast cancer cells is unclear. Because 40–50% of Her2/neu overexpressing breast cancers are also ER-positive, treatment designed to block estrogen’s effects remains one of the most challenging areas of study. 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. 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. Previous studies have shown that there are 2 forms of ER that are localized to the cell membrane and to the nucleus. 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. Previous studies have shown that there are 2 forms of ER that are localized to the cell membrane and to the nucleus. 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.

**TABLE 1 — 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**

<table>
<thead>
<tr>
<th>Breast cancer</th>
<th>ER-α (x-fold)1</th>
<th>Mean fraction of cell growth (% of control)2</th>
<th>→ PB and RA for 48 hr</th>
<th>ER-α (x-fold)1</th>
<th>Mean fraction of cell growth (% of control)2</th>
<th>→ then TAM alone for 48 hr</th>
<th>Mean fraction of cell growth (% of control)2</th>
</tr>
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<tbody>
<tr>
<td>MCF-7</td>
<td>1</td>
<td>100</td>
<td>1.83</td>
<td>0.010</td>
<td>80</td>
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<tr>
<td>MCF-7_Her2/neu</td>
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<td>18.25</td>
<td>1.25</td>
<td>21.37</td>
<td>84</td>
<td>82</td>
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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 μM) for 48 hr, and then TAM (2 μg/ml) alone for another 48 hr was measured by MTT assays as described in Materials and Methods, with mean fractional values relative to vehicle treated control cells. At least two experiments performed in triplicate. SD was less than 10%.

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 1). Cells with Her2/neu downregulation by PB and RA but without subsequent TAM addition, however, only produced reversible cytosostasis (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.

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. 5b). 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 1). We can see that 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) and make other cell membrane ER-linked apoptotic MAPK pathways such as p38 MAPK and JNK become dominant (Fig. 4a). In addition, PB can further inhibit Ras function by influencing Ras isoprenylation. Uptregulation 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).

Non-ER-mediated biochemical interactions that might also contribute to the antitumor activity of antioestrogens have been described. TAM is known as an inhibitor of PKC. 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

In our findings, it warrants further investigations in vivo to confirm that modulating the expression of RA receptor 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.

REFERENCES