

Improvement of sensitivity to tamoxifen in estrogen receptor-positive and Herceptin-resistant breast cancer cells

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Abstract

ERBB2 overexpression in estrogen receptor (ER)-positive breast cancer cells such as BT474 (BT) cells has been found to confer resistance to tamoxifen, and suppression of ERBB2 improves the antiproliferative effects of tamoxifen. In this study, the responsiveness to tamoxifen in the BT/HerR, Herceptin-resistant BT cell lines established through constant Herceptin exposure, was evaluated. Compared with BT cells, improvement of sensitivity to tamoxifen in BT/HerR was demonstrated by ER functional analysis and cell proliferation assay. Tamoxifen in the resistant cell line was found to inhibit 17 β -estradiol-stimulating estrogen-responsive gene *pS2* expression more effectively than in BT cells in real-time PCR assay. Western blot analysis showed that cross-phosphorylation between ER and downstream components of ERBB2 was attenuated in BT/HerR cells. ER redistribution from cytoplasm to nucleus could be found in these cells through immunofluorescence and confocal studies, and importantly, chromatin immunoprecipitation studies demonstrated that tamoxifen induced occupancy of the *pS2* promoter by ER and nuclear receptor corepressor (NCOA1) instead of coactivator NCOA3 in these cells. Finally, combination of tamoxifen and Herceptin was found to improve the sensitivity of BT/HerR cells to Herceptin. Our results suggest that the ER genomic pathway in the ER-positive and Herceptin-resistant breast cancer cells may be reactivated, allowing tamoxifen therapy to be effective again, and a combination of tamoxifen and Herceptin can be a potential therapeutic strategy for ER-positive and Herceptin-resistant human breast cancer.

Journal of Molecular Endocrinology (2008) **41**, 367–377

Introduction

The *ERBB2* oncogene encodes a 185 kDa transmembrane tyrosine kinase receptor, belonging to the epidermal growth factor receptor family that is amplified or overexpressed in 30% of breast cancers and is a poor prognostic factor (Slamon *et al.* 1989). Trastuzumab/Herceptin is a humanized monoclonal antibody directed against ERBB2, which inhibits growth and proliferation of cancer cells overexpressing ERBB2 (Carter *et al.* 1992). Herceptin has also shown efficacy in combination with conventional chemotherapeutic agents (Dieras *et al.* 2001). However, while ERBB2-overexpressing cell lines are shown to be sensitive to Herceptin, greater than 70% of patients whose tumors overexpress ERBB2 do not respond to Herceptin monotherapy (Vogel *et al.* 2002). In addition, a significant percentage of women who initially respond to Herceptin may develop acquired resistance to therapy after prolonged treatment. The precise mechanism of Herceptin resistance has not been fully characterized. Overexpression of constitutively active Akt (Clark *et al.* 2002), IGF receptor (Lu *et al.* 2001), loss of PTEN function (Nahta *et al.* 2006), or MUC4/sialomucin complex (Price-Schiavi *et al.* 2002) may potentially be involved in the resistance phenotype in breast cancer. Many strategies of combination therapy for Herceptin-resistant breast cancer cells have been considered, i.e. the combination of

Herceptin with EGFR kinase inhibitors such as AG1478 or Iressa (Lenferink *et al.* 2000, Moasser *et al.* 2001), and a COX2-inactive PDK1 inhibitor, OSU-03012 (Tseng *et al.* 2006). New potential therapeutic strategies, such as the combination of Herceptin with IGF1R kinase inhibitors or anti-IGF1R antibodies, are being evaluated.

More than 60% of human breast cancers are estrogen receptor (ER)-positive. Until now, the selective ER modulator (SERM), tamoxifen, is the most common endocrine therapy used for these cancers. While ~50–60% of ER-positive breast cancer patients benefit from tamoxifen, some of the ER-positive breast cancer patients who do not respond to tamoxifen have overexpression of ERBB2 (Slamon *et al.* 1989). There has been much experimental evidence to suggest that tamoxifen resistance in breast cancer cells involves overexpression of ERBB2 and its crosstalk with ER, which is associated with ER nongenomic activity (Pietras *et al.* 1995, Shou *et al.* 2004). Tamoxifen or estrogen (17 β -estradiol, E₂)-bound membrane/cytoplasmic ER, through multiple interactions with signaling intermediate molecules such as SH2 (Song *et al.* 2002) and modulator of nongenomic action of ER (Wong *et al.* 2002), can activate the EGFR/ERBB2 signaling pathway. Activated downstream kinases, e.g., phosphatidylinositol 3'-kinase (PI3K)/Akt and the MAPK3/1/MAPK, can phosphorylate nuclear ER and its coactivators (Kato *et al.* 1995, Sun *et al.* 2001, Schiff *et al.*

2004, Shou *et al.* 2004), thus up-regulating genomic ER activity and enhancing gene expression including genes in the EGFR/ERBB2 pathways, which in turn further augment EGFR/ERBB2 signaling, thus completing the cooperative cycle between the two activities of ER and their crosstalk with the EGFR/ERBB2 signaling pathway. In ERBB2-overexpressing cells, the resulting activation of downstream kinases can lead to tamoxifen resistance by modifying the activity of various transcription factors and blocking the inhibitory effects of tamoxifen on nuclear ER. Corresponding to this observation, a variety of *in vitro* and *in vivo* models have shown that suppression of ERBB2 improves the antiproliferative effects of tamoxifen (Pietras *et al.* 1995, Tang *et al.* 1996, Kurokawa *et al.* 2000), again demonstrating a causal association between ERBB2 overexpression and acquisition of resistance to endocrine therapy such as tamoxifen.

To explore the mechanism of Herceptin resistance and find the potential therapeutic strategy for Herceptin-resistant human breast carcinomas, several clones of BT474 (BT) human breast carcinoma cells that are resistant to Herceptin (BT/HerR) were isolated and identified over a 5-month selection process (Chan *et al.* 2005). BT is an ER-positive invasive human breast ductal carcinoma cell line with very high ERBB2 expression, which is very sensitive to Herceptin but resistant to tamoxifen, and has been used extensively for investigating the mechanisms of Herceptin and ERBB2 biology (Yakes *et al.* 2002, Zhou *et al.* 2007). BT/HerR subclones appear to have acquired a Herceptin-resistant PI3K signaling mechanism, because the downstream PI3K/Akt signaling pathway is sustained in BT/HerR cells in the presence of Herceptin, but significantly down-regulated in BT cells exposed to Herceptin (Chan *et al.* 2005). Our previous results showed that, while total and phosphorylated cellular ERBB2 protein remained high, phosphorylated Akt levels in the presence of Herceptin in BT/HerR subclones were decreased compared with wild-type BT cells in the absence of Herceptin (Chan *et al.* 2005). Based on these findings and the known crosstalk between ER and ERBB2 in tamoxifen resistance, we hypothesized that BT/HerR and BT cells might have different responses to E₂ and endocrine treatment, such as tamoxifen. This study was undertaken to explore the sensitivity to tamoxifen in the BT/HerR cells, and our results suggest that combined therapy with Herceptin and tamoxifen might be a viable therapeutic strategy for ER-positive and Herceptin-resistant human breast carcinomas.

Materials and methods

Cell culture and reagents

BT cells were routinely cultured in DMEM with 4.0 mM L-glutamine, 4500 mg/l glucose (Hyclone, Logan, UT,

USA) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 1 mM sodium pyruvate (Gibco BRL). BT/HerR0.2 clone D and BT/HerR1.0 clone E were maintained in the same medium with the continuous presence of 0.2 and 1 µmol/l Herceptin respectively (Chan *et al.* 2005). E₂-driven cell assays were performed with steroid-deprived medium, where DMEM and FBS were replaced with DMEM without phenol red and charcoal-stripped FBS (Hyclone). Herceptin (Genentech, South San Francisco, CA, USA) was purchased from the City of Hope National Medical Center Pharmacy (Duarte, CA, USA), E₂, and tamoxifen from Sigma Chemicals and the pure antiestrogen ICI 182 780 (ICI) from Tocris (Ellisville, MO, USA).

CellTiter 96 Aqueous proliferation assay

The response of BT or BT/HerR cells to tamoxifen or Herceptin alone, or a combination of both agents, was examined using CellTiter 96 Aqueous cell proliferation assay (Promega) according to the manufacturer's protocol. The cells were steroid deprived for 72 h and seeded in 96-well plates. After overnight incubation, steroid-deprived media containing 1 µM E₂, 1 µM tamoxifen or indicated concentrations of Herceptin, or combination of both agents were added to the cells and left for 6 days on the cells. Each data point was performed in triplicate. At the indicated times, cells were incubated in the presence of 20 µl CellTiter 96 Aqueous One Reagent per 100 µl medium for 1 h, and the reaction was measured colorimetrically at 490 nm in a SpectraMax M5 Reader (Molecular Devices, Sunnyvale, CA, USA). Background levels were determined in medium supplemented with DMSO at the appropriate dose, and findings were recorded as mean ± s.d.

Real time RT-PCR analysis

The cells were deprived of steroid for 72 h and incubated in the presence or absence of 1 nM E₂ with or without 1 µM tamoxifen for 24 h. Whole-cell RNA was prepared using TRIzol Reagent (Invitrogen) and first-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Real-time PCR was performed using the iQ5 Real-Time PCR Detection System (Bio-Rad). The reactions were carried out in a 96-well plate using SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Primers used were as follows: pS2, 5'-ATACCATCGACGTCCCTCCA-3' (forward), primer 5'-CACCTCAGACACGCTT-3' (reverse); β-actin, 5'-AGA-AGGAGATCACTGCCCTGGCACC-3' (forward), 5'-CCT-GCTTGCTGATCCACATCTGCTG-3' (reverse). PCRs were performed with HotStarTaq DNA polymerase (Qiagen) under the following conditions: a preheating

at 94 °C for 15 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, followed by a 72 °C extension for 10 min. Each sample was analyzed in triplicate, and β -actin was used as a control. Relative quantification of *pS2* gene expression was evaluated by utilizing the cycle threshold (C_t). The C_t values for each cell were subtracted from the respective C_t value of the β -actin control, resulting in the ΔC_t value. The ΔC_t value for DMSO-treated cells was used as a constant that was subtracted from all the other ΔC_t values to determine $\Delta\Delta C_t$ value. Fold changes were then generated for each treated cells by calculating $2^{-\Delta\Delta C_t}$.

Transient transfection and luciferase ER reporter assays

BT or BT/HerR cells were transfected with pGL3 (ERE)₃-luciferase vector using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer. A total of 1.5×10^5 cells per well were seeded onto 24-well plates and incubated overnight. A mixture of 1 μ g of the reporter plasmid, 1.5 μ l Lipofectamine 2000 and OptiMEM (Invitrogen) was added into each well and after a 4-h incubation, cells were cultured with fresh medium containing DMSO or 1 nM E₂ with or without 1 μ M tamoxifen or 10 nM ICI in phenol red-free medium with 10% charcoal-dextran-treated FBS. After a 24-h incubation following the transfection, the cells were lysed in 1 \times Reporter Lysis Buffer (Promega). Luciferase activity was measured using the Luciferase Assay System (Promega). Protein concentration was measured by the method of Bradford (Bio-Rad; Bradford 1976). The relative luciferase activity was calculated by dividing the light unit of luciferase activity by protein concentration of each sample.

Immunofluorescence and confocal studies

The cells were cultured in regular media (10% serum) or steroid-deprived media in the absence or presence of 1 nM E₂. Subcellular localization of ESRI in BT or BT/HerR was determined by indirect immunofluorescence. Cells grown on glass cover slips were fixed in ice-cold methanol:acetone (1:1) at 4 °C for 10 min. The cells were incubated with the primary antibodies, mouse ER (F-10) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or ER [6F11] antibody (Abcam, Cambridge, MA, USA) at 4 °C for overnight. After being washed three times in PBS, cells were then incubated with FITC-labeled (green) secondary antibody (Chemicon, Temecula, CA, USA). The VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used to co-stain the DNA and was color coded in blue. Confocal analysis was performed with a Zeiss laser-scanning confocal

microscope (Carl Zeiss, Oberkochen, Germany). Each image represents Z-sections at the same cellular level and magnification.

Cell extracts and western analysis

The cells were deprived of steroid for 72 h and serum starved for 24 h, and then treated with DMSO, 1 nM E₂, or 1 μ M tamoxifen for 24 h. Isolation of cell extracts and western blot analysis was done as previously described (Lou *et al.* 2007). The following antibodies were used: total ESRI and ERBB2, phospho-Ser118-ESRI, phospho-Ser167-ESRI, total and phospho-forms of Akt (Ser⁴³⁷), and MAPK (Thr²⁰²/Tyr²⁰⁴; Cell Signaling, Boston, MA, USA). β -actin (Chemicon) was used as a control for equal loading and transfer. Quantitative densitometry of the immunoblots was performed using the Model GS-700 Imaging Densitometer with Molecular Analyst Software (Bio-Rad), and expressed as the ratios to the density of β -actin bands. Phosphorylation levels of the experiments were estimated by densitometry, and were calculated as the ratio of pMAPK/tMAPK, pAkt/tAkt, or pER/tER.

Chromatin immunoprecipitation (ChIP) assays

The cells were deprived of steroid for 72 h and serum starved for 24 h, and then treated with DMSO, 1 nM E₂, or 1 μ M tamoxifen (Tam) for 45 min. ChIP assays were carried out with the ChIP assay kit (Upstate, Charlottesville, VA, USA) as previously described (Lou *et al.* 2007). One-tenth of the immunoprecipitated DNA and 1% of the input DNA were analyzed by PCR. Antibodies used for ChIP assays included: NCOA3 and NCOR1 (Affinity Bioreagents, Golden, CO, USA), ESRI and histone deacetylase 3 (HDAC3; Santa Cruz). PCRs were performed with HotStarTaq DNA polymerase (Qiagen) under the following conditions: a preheating at 94 °C for 15 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, followed by a 72 °C extension for 10 min. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel, which was then stained with ethidium bromide and photographed under u.v. illumination. Primers for the promoter region of the *pS2* gene: 5'-TTCATGAGCTCCTTCCCTTC-3' (forward) and 5'-ATGGGAGTCTCCTCCAACCT-3' (reverse). Representative experiments from at least three independent experiments are shown.

Statistical analysis

Overall differences between control and treatment groups, or differences between different treatment groups, were determined by two-way ANOVA. Direct

comparisons between control and treatment effects were assessed using a Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Different sensitivity of tamoxifen in BT and BT/HerR cells

To determine whether BT/HerR and BT cells might have different sensitivities to E_2 and endocrine treatment, we first investigated the response of BT and BT/HerR cells to endocrine therapy. Current

endocrine therapy of breast cancer includes SERMs such as tamoxifen, the pure ER antagonist ICI 182 780 (fulvestrant), and aromatase inhibitors such as letrozole. Because very low levels of aromatase activity were found in the BT and BT/HerR cells (data not shown), only the response to tamoxifen or ICI was examined. Two kinds of BT/HerR cells (BT/HerR0.2 and BT/HerR1.0 respectively) were selected to estimate the different sensitivity of BT cells to tamoxifen or ICI. In the ER functional analysis, while both the BT and BT/HerR cells were found to be responsive to E_2 , BT/HerR cells were more sensitive to tamoxifen than BT cells (Fig. 1A). In BT/HerR0.2 or BT/HerR1.0 cells, tamoxifen could significantly decrease E_2 -stimulated

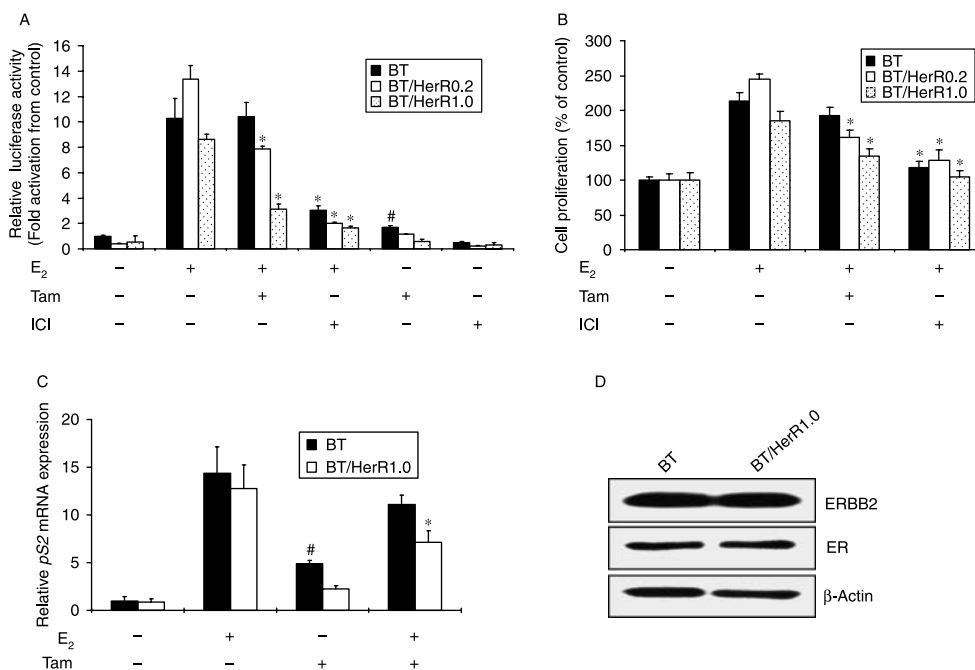


Figure 1 Different sensitivity to tamoxifen in the BT and BT/HerR cells. (A) Effect of tamoxifen and ICI on ER-mediated transcription in BT, BT/HerR0.2, and BT/HerR1.0 cells. The cells were transiently transfected with an ERE-containing luciferase reporter construct (ERE) in steroid-deprived medium, followed by a 16 h incubation in the presence or absence of 1 nM E_2 with or without 1 μ M tamoxifen (Tam) or 10 nM ICI. Normalized luciferase activity from triplicate wells was expressed relative to the luciferase activity of each cell line in the absence of E_2 , tamoxifen, and ICI. (B) Effects of tamoxifen and ICI on the proliferation in BT, BT/HerR0.2, and BT/HerR1.0 cells. The cells were deprived of steroid for 72 h and then incubated in the presence or absence of 1 nM E_2 with or without 1 μ M tamoxifen or 10 nM ICI for 6 days. Cell proliferation from triplicate wells was expressed relative to the optical density at 490 nm of each cell line in the absence of E_2 , tamoxifen, and ICI. Results in (A) and (B) are expressed as mean \pm s.d. of at least three separate experiments. * $P < 0.05$ compared with control in the presence of E_2 . # $P < 0.05$ compared with control in the absence of E_2 , tamoxifen, and ICI. (C) Real-time PCR analysis of the estrogen-responsive gene *pS2* in the BT and BT/HerR cells. BT and BT/HerR1.0 cells were deprived of steroid for 72 h and incubated in the presence or absence of 1 nM E_2 with or without 1 μ M tamoxifen for 24 h. The relative expression of *pS2* in various treated cells was analyzed using $2^{-\Delta\Delta C_t}$, which represents the expression of the *pS2* gene in various treated cells normalized to β -actin, relative to the normalized expression of *pS2* in control cells. Results are expressed as mean \pm s.d. of at least three separate experiments. * $P < 0.05$ compared with control in the presence of E_2 . # $P < 0.05$ compared with control in the absence of E_2 and tamoxifen. (D) Levels of ER and ERBB2 receptors in the BT and BT/HerR cells. BT and BT/HerR1.0 cells were cultured in regular media to 80% confluence. Lysates of cells were prepared and tested for ER and ERBB2 content by immunoblot analysis. β -actin was used as a control for equal loading and transfer. Blots are representative of three experiments.

luciferase activity, confirming its antagonistic function. However, in BT cells, tamoxifen had no effect on E₂-stimulated luciferase activity and, inversely, tamoxifen alone increased ERE-luciferase transcription to 1.7-fold above basal level in BT cells, indicating a partial agonist function. In addition, the BT and BT/HerR cells responded similarly to pure ER antagonist ICI, which could abrogate the estrogen signal in both types of cells by accelerating ESR1 degradation. To elucidate the biological significance of this difference in tamoxifen function in the BT and BT/HerR cells, we performed a cell proliferation assay and found that the BT and BT/HerR cells had the same response to ICI, but not to tamoxifen. BT cells were resistant to tamoxifen treatment, whereas E₂-driven proliferation of BT/HerR cells was inhibited by tamoxifen (57% inhibition in BT/HerR0.2 cells and 60% inhibition in BT/HerR1.0 cells; Fig. 1B). To further demonstrate the different sensitivities of tamoxifen in the BT and BT/HerR cells, levels of the estrogen-responsive gene pS2 mRNA in these two kinds of cell lines were analyzed by real-time PCR. Tamoxifen was found to inhibit E₂-stimulated pS2 expression in BT/HerR1.0 cells more effectively than in BT cells (Fig. 1C). In addition, tamoxifen alone in the absence of E₂ in BT cells could stimulate pS2 expression, suggesting again that tamoxifen in the BT cells functions as an agonist. By western blot analysis, ER and ERBB2 levels in BT and BT/HerR1.0 cells were found to be similar (Fig. 1D). These results suggest that both the BT and BT/HerR cells exhibit wild-type ER function in response to E₂, but there is a clear difference in sensitivity to tamoxifen in both growth and ER functional assays.

Attenuated cross-phosphorylation between ER and Her2 downstream components in BT and BT/HerR cells

The crosstalk between ER and ERBB2 involves ER nongenomic activity and is thought to be very important in the development of tamoxifen resistance (Pietras *et al.* 1995, Shou *et al.* 2004). To explore the mechanism of differential sensitivities to tamoxifen in the BT and BT/HerR cells, the nongenomic activity of ER in BT and BT/HerR1.0 cells was examined. Our previous studies have found that the total and phosphorylated ERBB2 levels in BT/HerR cells are not significantly changed compared with BT cells, and signaling through the PI3K/Akt pathway is sustained in the presence of Herceptin in BT/HerR (Chan *et al.* 2005). To further explore our previous findings, we examined the changes of downstream components of the ERBB2 pathway, such as MAPK and Akt, in the presence of E₂ and tamoxifen. As shown by western blot analysis (Fig. 2A and B), after a 20-min treatment with E₂ and tamoxifen, both E₂ and tamoxifen could stimulate phosphorylation of MAPK and

Akt in BT cells compared with the DMSO control. However, while the basal phosphorylated Akt was slightly elevated in BT/HerR1.0 compared with BT cells (Fig. 2B), both E₂ and tamoxifen in these cells could not stimulate phosphorylation of MAPK and Akt. These results indicate that ER nongenomic activity in BT/HerR cells is attenuated, and tamoxifen could function as an agonist in BT, but not in BT/HerR cells. To determine the effect of attenuated ER nongenomic activity on ER function in BT/HerR cells, ER phosphorylation in BT and BT/HerR1.0 cells treated with or without E₂ or tamoxifen was examined using a specific anti-phospho-Ser118 or Ser167 ESR1 antibody. In BT cells, activated MAPK by E₂ and tamoxifen could lead to potentiation of phospho-ESR1 at Ser118, which was previously reported to be phosphorylated by MAPK (Kato *et al.* 1995). However, E₂ and tamoxifen-stimulated phospho-ESR1 at Ser118 was found to be attenuated in the BT/HerR1.0 cells compared with that in BT cells. Under our experimental conditions, phosphorylation of ESR1 at Ser167, which could be phosphorylated by Akt (Campbell *et al.* 2001), could not be detected in either cell line (data not shown). Our results are similar to those of a previous study on BT cells (Wang *et al.* 2005), implying that Ser-167 phosphorylation is not abundant in the BT and BT/HerR cells. The effects of E₂ and tamoxifen treatments on phosphorylated levels of MAPK, Akt, and ER were still significant after 24 h (Fig. 2C and D), indicating that they are not transient. In addition, E₂ treatment for 24 h could down-regulate the total ER level and tamoxifen treatment for 24 h could up-regulate the total ER level in these two cell lines (Fig. 2C and D), which is consistent with other's findings (Wang *et al.* 2005).

Localization of ER in BT and BT/HerR cells

Since subcellular localization of ER has been linked to the responsiveness of breast cancer cells to tamoxifen (Yang *et al.* 2004), we analyzed the subcellular localization of ER in BT and BT/HerR1.0 cells by immunofluorescence and confocal studies. In regular media, more ER in BT cells was found to localize in the membrane/cytoplasm than in the nucleus (Fig. 3A), but in BT/HerR1.0 cells, more ER localized in the nucleus (Fig. 3B), indicating that the genomic pathway of ER in BT/HerR cells is more important than the nongenomic pathway. To further confirm these results, cells were deprived of endogenous E₂ for 3 days and then treated with 1 μM E₂ for 1 h, and the effects of E₂ on ER localization were examined. Estrogen stimulation could promote ER translocation from the nucleus to the membrane/cytoplasm in BT cells (Fig. 3A), but from the membrane/cytoplasm to the nucleus in BT/HerR1.0 cells (Fig. 3B). This different localization of ER in BT and BT/HerR1.0 cells can also be observed using another ER antibody (ER [6F11]

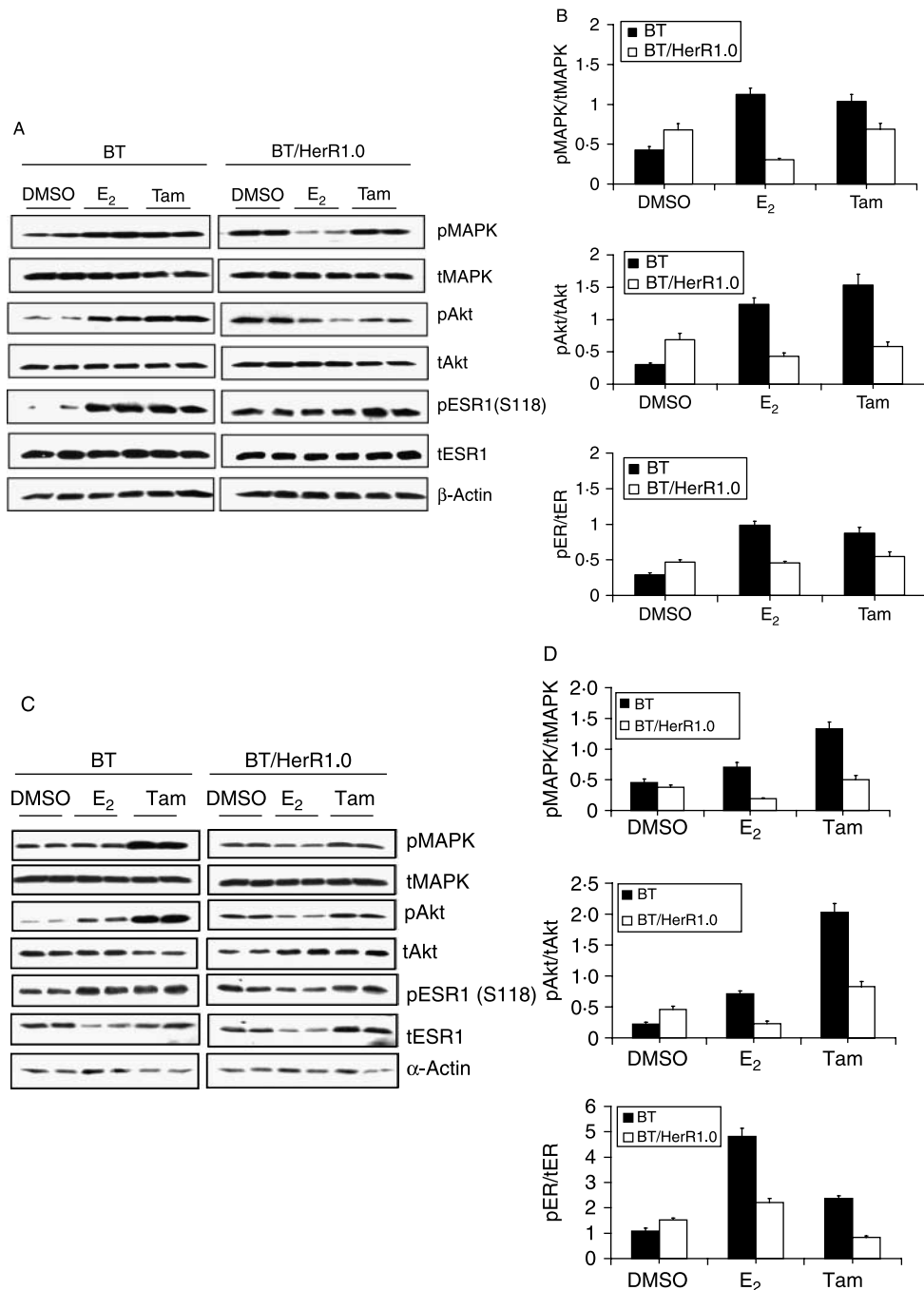


Figure 2 Cross-phosphorylation between ER and ERBB2 downstream components in the BT and BT/HerR cells. BT and BT/HerR1.0 cells were deprived of steroid for 72 h and serum starved for 24 h, and then treated with DMSO, 1 nM E₂, 1 μM tamoxifen (Tam) for 20 min (A and B) or 24 h (C and D). Levels of total (t) and phosphorylated (p) MAPK, Akt and ESR1 at Ser118 were measured in cellular extracts by immunoblot analysis. β-actin was used as a control for equal loading and transfer. Figures are representative of three experiments. Phosphorylation levels of the experiments were estimated by densitometry, and were calculated as the ratio of pMAPK/tMAPK, pAkt/tAkt, or pER/tER.

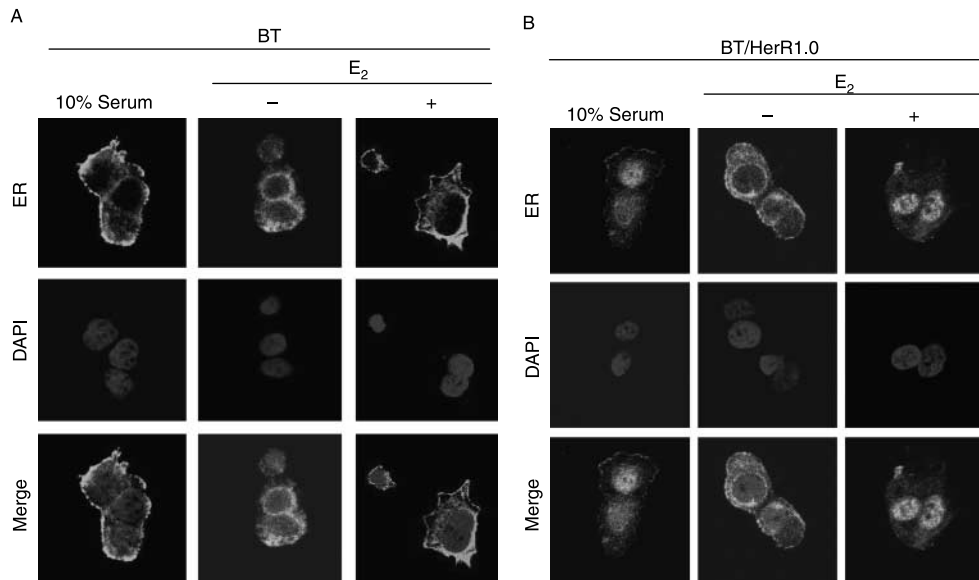


Figure 3 Subcellular localization of ER in BT (A) and BT/HerR cells (B). BT and BT/HerR1.0 cells were cultured in regular media (10% serum) or steroid-deprived media in the absence (–) or presence (+) of 1 nM E_2 . Cellular localization of ER was determined by indirect immunofluorescence as indicated in the ‘Materials and methods’. Confocal microscopy was used to show the presence of ER in the cytoplasmic compartment and nucleus. The cells were deprived of steroid for 72 h and then stimulated with E_2 for 1 h to examine the effects on ER localization. The (ER) cells labeled with ER (F-10) antibody; (DAPI) nucleus counterstained with DAPI; (merge) ER localization assessed by spectral overlap of the images in top and middle panels.

antibody, Abcam) for the same analysis (Supplementary Fig. 1, see Supplementary data in the online version of the Journal of Molecular Endocrinology at <http://jme.endocrinology-journals.org/content/vol41/issue/5>). These results show that ER subcellular localization in BT/HerR cells is different from BT cells, which suggests that enhancement of the ER genomic pathway is one of the potential mechanisms related with increased sensitivity to tamoxifen in the BT/HerR cells.

Different recruitment of nuclear receptor coactivator or corepressor on pS2 promoter by tamoxifen-bound ER in BT and BT/HerR cells

One mechanism for tamoxifen resistance may be related to a change in the balance of association of ER with nuclear receptor coactivators or corepressors (Smith *et al.* 1997). Tamoxifen had been shown to function as an agonist in BT cells but an antagonist in BT/HerR cells (Figs 1A and C and 3). Next, we employed ChIP assays to address if there was a different recruitment of nuclear receptor coactivator or corepressor on the pS2 promoter by tamoxifen-bound ER in these two kinds of cell lines. In BT cells, E_2 induced occupancy of the pS2 promoter by ER and coactivator NCOA3. Tamoxifen, like E_2 , also induced occupancy by ER and coactivator NCOA3, but not corepressor NCOR1, which is consistent with its agonist function

in these cells. In BT/HerR1.0 cells, E_2 induced occupancy of the pS2 promoter by ER and coactivator NCOA3, but tamoxifen induced occupancy by ER and corepressor NCOR1 instead of coactivator NCOA3, which is consistent with the antagonist function of tamoxifen in these cells (Fig. 4). The different recruitment of nuclear receptor coactivator or corepressor on the pS2 promoter by tamoxifen-bound ER in BT and BT/HerR cells may explain the improvement of tamoxifen sensitivity in BT/HerR cells.

Combination of tamoxifen and Herceptin improves the sensitivity of BT/HerR cells to Herceptin

The crosstalk between ER and ERBB2 signaling pathways suggests that targeting both ER and growth factor receptors simultaneously could be a fruitful approach to the treatment of breast cancer. The combination of tamoxifen and Herceptin has been reported to synergistically inhibit the proliferation of BT cells (Argiris *et al.* 2004, Wang *et al.* 2005). Although BT/HerR cells are resistant to Herceptin, the sensitivity of these cells to tamoxifen was improved. Hence, we next investigated if the combination of tamoxifen and Herceptin could improve the sensitivity of BT/HerR cells to Herceptin. Compared with Herceptin or tamoxifen treatment alone, the combination of Herceptin with tamoxifen significantly inhibited the

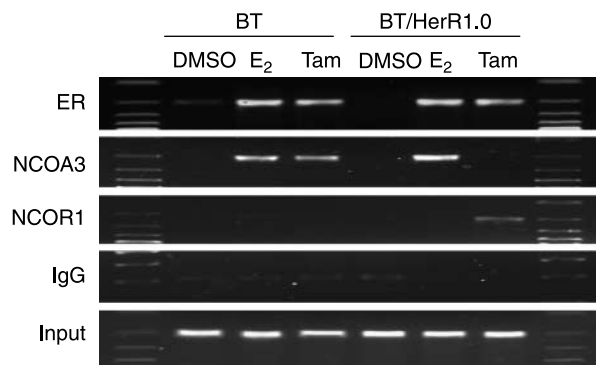


Figure 4 Different recruitment of nuclear receptor coactivator or corepressor on estrogen-responsive pS2 promoter by tamoxifen-bound ER in the BT and BT/HerR cells. BT and BT/HerR1.0 cells were deprived of steroid for 72 h and serum starved for 24 h, and then treated with DMSO, 1 nM E₂, or 1 μM tamoxifen (Tam) for 45 min. Occupancy of the estrogen-responsive pS2 promoter by ER, the coactivator NCOA3, and the corepressor NCOR1 was examined by ChIP assay as indicated in the 'Materials and methods'. Relevant pS2 promoter sequences were PCR amplified from complexes immunoprecipitated with each antibody. Input lane shows DNA that was PCR amplified from extracts before immunoprecipitation. Gels are representative of three experiments.

proliferation of the BT/HerR1.0 cells (Fig. 5A). Western blot analysis of signaling proteins showed that, consistent with other's findings (Wang *et al.* 2005), BT cells were very sensitive to Herceptin alone but not to tamoxifen alone and the combination of tamoxifen and Herceptin did not have any additional effect on phosphorylation of MAPK, Akt, or ESR1 at Ser118 (Fig. 5B). Though phosphorylation of MAPK, Akt, and

ESR1 at Ser118 was sustained in BT/HerR1.0 cells in the presence of Herceptin compared with BT cells, combination of Herceptin with tamoxifen significantly down-regulated phosphorylation of downstream signaling components of ERBB2 pathway and phospho-ESR1 at Ser118. These results indicate the combination of Herceptin with tamoxifen can sensitize BT/HerR cells to Herceptin, suggesting that the combined therapy with Herceptin and tamoxifen might be a viable therapeutic strategy for ER-positive and Herceptin-resistant human breast cancer.

Discussion

Currently, Herceptin is the only ERBB2-targeted therapy approved by the US Food and Drug Administration (FDA) for the treatment of metastatic breast cancer. While Herceptin is a powerful drug, many patients who achieve an initial response to Herceptin-based regimens develop resistance within 1 year. In addition, the expensive prices of these drugs mean it is very important to explore new potential therapeutic strategies to improve the sensitivity of Herceptin. Besides the reported strategies of combination therapy for Herceptin resistance in breast cancer, Herceptin-resistant cells have recently been shown to retain sensitivity to green tea polyphenol epigallocatechin-3-gallate, providing a novel strategy for treatment of Herceptin-resistant breast cancers (Eddy *et al.* 2007). In the present study, we demonstrated the improvement

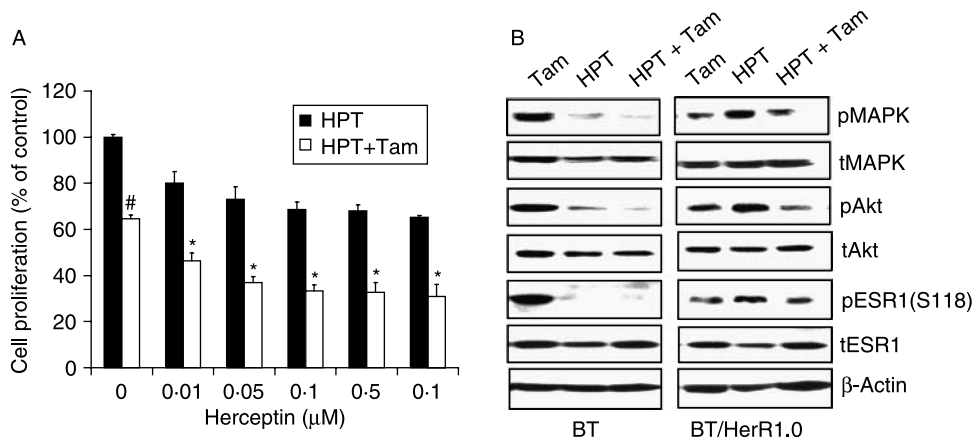


Figure 5 Combination of tamoxifen and Herceptin improves the sensitivity of BT/HerR cells to Herceptin. (A) BT and BT/HerR1.0 cells were deprived of steroid for 72 h and then incubated in the presence of 1 nM E₂ with increasing concentrations of Herceptin (HPT) or its combination with 1 μM tamoxifen (Tam) for 6 days. Cell proliferation was expressed relative to optical density at 490 nm of cell line in the presence of E₂. #*P* < 0.05 compared with control in the absence of Herceptin and tamoxifen. **P* < 0.05 combined agents versus Herceptin alone or tamoxifen alone. (B) BT and BT/HerR1.0 cells were treated with 1 μM Herceptin or 1 μM tamoxifen or both drugs (HPT + Tam) for 24 h. Levels of total (t) and phosphorylated (p) MAPK, Akt, and ESR1 at Ser118 were measured in cellular extracts by western blot analysis. β-actin was used as a control for equal loading and transfer. Figures are representative of three experiments.

of tamoxifen responses in BT/HerR cells and tried to explore the new therapeutic strategies to improve the sensitivity to Herceptin. Our results showed that there was a significant difference in response to tamoxifen between the BT and BT/HerR cells (Fig. 1A and B). BT/HerR cells were much more sensitive to tamoxifen than BT cells, and tamoxifen in these cells could function as an antagonist. By contrast, BT cells are resistant to tamoxifen, as observed by another laboratory (Zhou *et al.* 2007), and tamoxifen has a partial agonist function in these cells as well as in MCF-7/ERBB2 cells, which have been reported to be resistant to tamoxifen (Kurokawa *et al.* 2000, Shou *et al.* 2004). In addition, we observed that the two kinds of cell lines had similar sensitivity to the pure ER antagonist, ICI. The different responses to tamoxifen and ICI in these cells are associated with the different mechanisms of the two agents affecting ER function. ICI may block both the transcriptional activating function 2 (AF-2) and transcriptional AF-1 of ER by attenuating dimerization of the receptor and accelerating receptor degradation, but tamoxifen only blocks AF2 of ER by competing with the receptor and allows activation of AF1 (Dowsett *et al.* 2005). Therefore, tamoxifen in the breast cancer cells only partially inactivates ER-regulated transcription or even has a partial agonist function in ERBB2-overexpressing cells such as MCF-7/ERBB2 (Kurokawa *et al.* 2000, Shou *et al.* 2004) and BT cells (Figs 1A and C and 2). The different sensitivities to tamoxifen in the BT and BT/HerR cells are not attributable to differences in the ER and ERBB2 levels because total ER and ERBB2 levels in these two kinds of cells were similar (Fig. 1D). Considering the important role of the interaction of the ERBB2 and ER pathways in the development of tamoxifen resistance (Pietras *et al.* 1995, Shou *et al.* 2004), a change in crosstalk between ER- and ERBB2-signaling pathways might be involved in this different response to tamoxifen in these two kinds of cell lines.

In ERBB2-overexpressing tumors, the crosstalk between the ER and ERBB2 pathways is associated with ER nongenomic activity, and the nongenomic rapid ER action in these cells may become more prominent. For example, tamoxifen has been shown to rapidly activate EGFR, ERBB2, Akt, and MAPK1/MAPK in MCF-7/ERBB2 cells, establishing a cycle of cell survival and proliferation via bidirectional crosstalk between growth factor and ER signaling pathways (Shou *et al.* 2004). In BT cells, which express ER and overexpress ERBB2 and NCOA3 (Anzick *et al.* 1997, Yakes *et al.* 2002, Zhou *et al.* 2007), effects of E₂ and tamoxifen on phosphorylation of downstream effectors of EGFR/ERBB2 are similar to those in MCF-7/ERBB2 cells (Shou *et al.* 2004; Fig. 2). In MCF-7/ERBB2 cells, the EGF receptor tyrosine kinase inhibitor gefitinib could eliminate this crosstalk and restore tamoxifen's antitumor effects, and Herceptin was also demonstrated

to reverse tamoxifen resistance by reducing downstream MAPK/ESSRA/B signaling (Kurokawa *et al.* 2000). These findings suggest that Herceptin-mediated down-regulation of the ERBB2 pathway may be associated with increased sensitivity to tamoxifen. Our results showed the attenuated activation of Akt and MAPK by E₂ or tamoxifen in BT/HerR1.0 cells (Fig. 2). Consistent with the attenuated change in phosphorylated MAPK, E₂, and tamoxifen-stimulated phospho-ESR1 at Ser118 was also attenuated. These results suggest that the nongenomic activities of ER in BT/HerR cells is decreased, which leads to the attenuation of the crosstalk between growth factor and ER-signaling pathways, and may be involved in the increased sensitivity to tamoxifen in the BT/HerR cells. The reason for the attenuated activation of Akt and MAPK by E₂ or tamoxifen in BT/HerR cells is currently not clear and may be associated with chronic Herceptin exposure. For example, phosphorylated Akt levels in the presence of Herceptin in BT/HerR cells has been found to be lower than in BT cells in the absence of Herceptin (Chan *et al.* 2005). However, in the absence of Herceptin, the basal phosphorylated Akt level in BT/HerR1.0 cells was not decreased and inversely was somehow increased, compared with BT cells (Fig. 2). Hence, the precise mechanism of attenuated activation of Akt and MAPK by E₂ or tamoxifen in the BT/HerR cells will require further research.

Besides the crosstalk between ER and ERBB2, recent research has shown that subcellular localization of ER may play a mechanistic role in determining the responsiveness of breast cancer cells to tamoxifen (Yang *et al.* 2004). ERBB2 overexpression in MCF-7/ERBB2 cells promotes the relocation of ER from the nucleus to the cytoplasm, enhances interactions of ER with ERBB2, inhibits ER transactivation function, and induces resistance to tamoxifen-mediated growth inhibition of breast cancer cells. Conversely, down-regulation of ERBB2 by Herceptin leads to suppression of MAPK stimulation, restoration of ER to the nucleus, and potentiation of the growth-inhibitory action of tamoxifen. Different subcellular localization of ER in specific cells represents the different activating status of genomic pathway or nongenomic pathway of ER. Our data showed different subcellular localization of ER in the BT and BT/HerR1.0 cells, which suggest that exclusion of ER from the nucleus in BT cells might act to deprive the antiestrogenic agents of their target in the proper cellular compartment. On the other hand, entering into the nucleus of ER from the cytoplasm in BT/HerR1.0 cells might indicate a restoration of the ER genomic pathway, leading to a subsequent increased sensitivity to tamoxifen (Figs 1 and 3). The restoration of the ER genomic pathway in BT/HerR cells is not due to the changes in ERBB2 and ER levels because the steady-state ER, total and phosphorylated ERBB2 levels

in the BT and BT/HerR1.0 cells are the same (Fig. 1D). On the other hand, our results suggest that attenuated activation of Akt and MAPK by E₂ or tamoxifen could confer the suppressed cross-phosphorylation between ER and ERBB2 downstream components, which may be associated with the restoration of the ER genomic pathway and subsequent improvement of tamoxifen sensitivity in BT/HerR cells.

It is well known that ER directly binds to estrogen response element residing in the promoter region of the target gene and, by recruiting co-regulatory proteins, regulates gene transcription. In addition to the activation of ER phosphorylation, activated MAPK in tamoxifen-resistant cells can phosphorylate ER coactivator NCOA3 (Shou *et al.* 2004) and the resistant status may be related to a change in the balance of the association of ER with coactivators or corepressors (Smith *et al.* 1997). A recent report describes how the forced overexpression of ERBB2 in E₂-dependent, tamoxifen-sensitive MCF-7 cells results in tamoxifen resistance (Shou *et al.* 2004). In MCF-7 cells, tamoxifen induces occupancy of the pS2 promoter by ER, corepressor NCOR1, and HDAC3. However, in MCF-7/ERBB2 cells, tamoxifen induces occupancy by ER and coactivator complexes including NCOA3, P300, and CBP, resulting in the formation of acetylated histones, thereby explaining agonist effects of tamoxifen on steroid-regulated gene expression. Given the improvement of the ER genomic pathway and increased tamoxifen sensitivity in BT/HerR cells, recruitment of nuclear receptor coactivator or corepressor on pS2 promoter by tamoxifen-bound ER in these two kinds of cell lines should be different. Consistent with the observation in MCF-7 and MCF-7/ERBB2 cells, our results showed that tamoxifen in the BT cells induced occupancy by ER and coactivator NCOA3, which corresponds to its agonist function in these cells, whereas tamoxifen in the BT/HerR1.0 cells induced occupancy by corepressor NCOR1. The replacement of coactivator complexes with corepressor complexes in the presence of tamoxifen-bound ER is consistent with an antagonist function of tamoxifen in the BT/HerR1.0 cells. The different recruitment of nuclear receptor coactivator or corepressor on pS2 promoter in the BT and BT/HerR cells might explain the improvement of tamoxifen sensitivity in BT/HerR cells.

In summary, the results presented here show for the first time the enhancement of tamoxifen sensitivity in BT/HerR cells, relative to BT cells, and the following mechanisms are implicated: a) attenuated activation of MAPK and Akt pathway by E₂ and tamoxifen, indicating the decreased ER nongenomic activity; b) different subcellular localization from BT cells, indicating improvement of genomic pathway of ER; and c) the replacement of coactivator complexes with corepressor

complexes on ERE-containing promoters in the presence of tamoxifen-bound ER. These results serve as direct evidence for the antagonist activity of tamoxifen in the BT/HerR cells. Although there is an inverse relationship between the degree of ER positivity and ERBB2 expression, a significant proportion of human breast cancers are both ER-positive and overexpressing ERBB2 (Berry *et al.* 2000). Compared with ER-positive, ERBB2-overexpressing, and tamoxifen-resistant BT cells, the increased sensitivity to classical endocrine therapy such as tamoxifen in the Herceptin-resistant BT/HerR cells suggests that ER genomic pathway in these cells can be improved, thus allowing tamoxifen therapy to be effective again. In spite of the increased sensitivity to tamoxifen in the BT/HerR cells, our data imply that monotherapy with tamoxifen might have only modest benefits on BT/HerR cells, i.e., 60% inhibition of proliferation in BT/HerR1.0 cells compared with the control (Figs 1B and 5A). However, combination of tamoxifen and Herceptin was demonstrated to increase the sensitivity of BT/HerR1.0 cells to Herceptin (Fig. 5), suggesting that combined therapy with Herceptin and tamoxifen might be a viable therapeutic strategy for Herceptin-resistant human breast cancer cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The research project described in this paper has been supported by NIH grants ES08258 (SC), CA44735 (SC), and CA33572 (the COH Cancer Center grant).

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Received in final form 20 August 2008

Accepted 3 September 2008

Made available online as an Accepted Preprint 3 September 2008