

The broad-spectrum metalloproteinase inhibitor BB-94 inhibits growth, HER3 and Erk activation in fulvestrant-resistant breast cancer cell lines

TOVE KIRKEGAARD¹, CHRISTINA W. YDE¹, MARIE KVEIBORG² and ANNE E. LYKKESFELDT¹

¹Breast Cancer Group, Cell Death and Metabolism, Danish Cancer Society Research Center; ²Department of Biomedical Sciences and Biotech Research and Innovation Centre (BRIC), University of Copenhagen, Copenhagen, Denmark

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Abstract. Breast cancer cells can switch from estrogen receptor α (ER)- to human epidermal growth factor receptor (HER)-driven cell growth upon acquiring antiestrogen resistance. HER ligands are cleaved by metalloproteinases leading to release of active HER ligands, activation of HER receptors and consequently increased cell growth. In this study, we investigated the importance of HER receptors, in particular HER3, and HER ligand shedding for growth and signaling in human MCF-7 breast cancer cells and MCF-7-derived sublines resistant to the antiestrogen fulvestrant. The HER3/HER4 ligand heregulin 1 β induced phosphorylation of HER3, Akt and Erk, and partly rescued fulvestrant-inhibited growth of MCF-7 cells. HER3 ligands were found to be produced and shed from the fulvestrant-resistant cells as conditioned medium from fulvestrant-resistant MCF-7 cells induced phosphorylation of HER3 and Akt in MCF-7 cells. This was prevented by treatment of resistant cells with the metalloproteinase inhibitor TAPI-2. Only the broad-spectrum metalloproteinase inhibitor BB-94, and not the more selective inhibitors GM6001 or TAPI-2, which inhibited shedding of the HER ligands produced by the fulvestrant-resistant cells, was able to inhibit growth and activation of HER3 and Erk in resistant cells. Compared to MCF-7, fulvestrant-resistant cells have increased HER3 phosphorylation, but knockdown of HER3 had no inhibitory effect on resistant cell growth. The EGFR

inhibitor gefitinib exhibited only a minor growth inhibition, whereas the pan-HER inhibitor CI-1033 exerted growth arrest. Thus, neither HER3 nor EGFR alone are the main driver of fulvestrant-resistant cell growth and treatment should target both receptors. Ligand shedding is not a treatment target, as receptor activation occurred, independent of release of ligands. Only the broad-spectrum metalloproteinase inhibitor BB-94 could abrogate HER3 and Erk activation in the resistant cells, which stresses the complexity of the resistance mechanisms and the requirement of targeting signaling from HER receptors by multiple strategies.

Introduction

The antiestrogen tamoxifen has for several decades and until recently been recommended as first-line endocrine treatment for women with primary estrogen receptor α (ER)-positive breast cancer. Although many patients respond well to the treatment, resistance to tamoxifen is inevitable in advanced disease and therefore a major clinical challenge. Upon progression on tamoxifen, the pure antiestrogen fulvestrant (ICI 182,780 or faslodex) is a treatment option (1). However, as for tamoxifen, resistance to fulvestrant will eventually occur in patients with advanced disease. Although the molecular mechanisms leading to antiestrogen resistance are still not fully clarified, it is well recognized that breast cancer cell growth, upon acquired antiestrogen resistance, can switch from being ER-driven to be mediated by members of the human epidermal growth factor receptor (HER) family (2-6).

The HER receptor family comprises four type-1 transmembrane receptor tyrosine kinases: EGFR/HER1, HER2, HER3 and HER4 (7,8). Increased HER expression and HER signaling is a frequent phenomenon in various human cancers (9). In breast tumors, the expression and phosphorylation of EGFR, HER2 and HER3 have been associated with poor prognosis, in contrast to the expression of HER4, which has been linked to a better disease outcome (10-12). Several ligands have been identified for EGFR, HER3 and HER4, whereas no ligands have been identified for HER2 (7). The HER ligands are all synthesized as transmembrane precursors that are proteolytically cleaved and released (shed) from the membrane by metalloproteinases (7,13). In the absence of ligand, the HER

Correspondence to: Dr Tove Kirkegaard, Breast Cancer Group, Cell Death and Metabolism, Danish Cancer Society Research Center, Strandboulevarden 49, DK-2100 Copenhagen, Denmark
E-mail: tki@cancer.dk

Abbreviations: ADAM, a disintegrin and metalloproteinase; ER, estrogen receptor α ; HER, human epidermal growth factor receptor; MMP, matrix metalloproteinase; TACE, tumor necrosis factor α converting enzyme

Key words: antiestrogen resistant breast cancer, HER receptor, HER ligands, metalloproteinase inhibitors, ligand shedding

receptors are monomeric. However, upon ligand activation of the HER receptors, changes in the receptor conformation leads to formation of homo- or heterodimers and consequently activation of the receptors via tyrosine phosphorylation (except for HER3, which lacks a functional kinase domain) (7). Activated HER receptors can induce multiple downstream signaling pathways, which leads to increased cell proliferation and reduced cell death (7). Accordingly, as the HER receptors represent important therapeutic targets for cancer treatment, several therapies have been developed. These include antibodies such as trastuzumab (directed against HER2) and cetuxumab (directed against EGFR) as well as small tyrosine kinase inhibitors like gefitinib (targeting EGFR) or lapatinib (targeting EGFR/HER2). Although the HER targeted therapies have shown promising results in the adjuvant and advanced setting, still many patients exhibit *de novo* or acquired resistance (14,15). It has been suggested that targeting the HER receptors individually leads to treatment resistance as increased production of the HER ligands may circumvent the loss of the function of a single receptor (16,17). Thus, preventing HER ligand shedding with metalloproteinase inhibitors may be an alternative and more efficient way to prevent HER-mediated signaling and cancer cell growth than targeting the HER receptors individually.

To investigate the mechanisms behind antiestrogen resistant breast cancer cell growth, we have established a model system with MCF-7 breast cancer cell lines with acquired resistance to fulvestrant (18). The fulvestrant-resistant MCF-7 cell lines have increased protein expression or phosphorylation of EGFR and HER3 and can be growth inhibited by targeting the HER receptors or signaling molecules downstream of the HER receptors (2,6,19). Thus, the breast cancer cell lines can switch from ER- to HER-driven cell growth upon acquiring resistance to fulvestrant. In agreement with the high levels of phosphorylated HER3, the level of the HER3 activating ligands heregulin 2 α and -2 β was also increased in resistant cell lines. Collectively, our study corroborate the importance of the HER system in signaling and growth of fulvestrant-resistant breast cancer cells (2). The work presented here was carried out to unravel the importance of the HER receptors, in particular HER3, and HER ligand shedding for growth and signaling in fulvestrant-resistant MCF-7 cell lines and to explore whether inhibition of ligand shedding could be a new treatment strategy for resistant cells.

Materials and methods

Cell lines, culture conditions and reagents. The MCF-7 cell line was originally obtained from the Human Cell Culture Bank (Mason Research Institute, Rockville, MD, USA). MCF-7 cells were maintained in growth medium without phenol red (DMEM/F12; Gibco) supplemented with 1% FCS, 2 mM glutamax (Gibco, Invitrogen, CA, USA) and 6 ng/ml insulin (Sigma-Aldrich, St. Louis, MO, USA). The fulvestrant-resistant cell lines, MCF-7/164^R-5 (164^R-5) and MCF-7/164^R-7 (164^R-7) were established as previously described (18) and maintained in MCF-7 growth medium supplemented with 0.1 μ M fulvestrant (Tocris, Avonmouth, Bristol, UK). For experiments, 2.5 \times 10⁵ units of penicillin and 31.25 μ g/l streptomycin (Gibco) were added to the growth medium. TAPI-2, BB-94 and GM6001 were obtained from Calbiochem (Merck Biosciences,

Nottingham, UK), CI-1033 (Canertinib) and gefitinib from Selleck (Chemicals, Munich, Germany), Ab5 from Thermo Fisher Scientific (Fremont, CA, USA) and heregulin 1 β , TGF α and EGF from R&D Systems (Minneapolis, MN, USA). Stock solutions of 12 mM TAPI-2 was dissolved in water, whereas stock solutions of 1 mM gefitinib, 10 mM CI-1033, 10 mM BB-94 and 10 mM GM6001 were dissolved in DMSO.

Cell growth assays. The cell lines were seeded in 24-well multidishes in growth medium and allowed to adhere for two days. When experiments were initiated (day 0), growth medium containing fulvestrant (0.1 μ M), HER ligands (10 ng/ml), gefitinib, CI-1033, TAPI-2, BB-94 or GM6001 were added at concentrations indicated in the figure. The control cells were added similar amount of vehicle as the treated cells. Growth medium was replaced on day three, and cell number was determined on day five, using a crystal violet colorimetric assay as previously described (20). Each experiment was performed in quadruplicate and repeated at least twice.

Western blot analysis. Western analyses were performed with cells lysed in RIPA buffer (100 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) supplemented with 1 mM DDT, 1 mM NaF, 10 mM β -glycerol phosphate, 100 μ M Na₃VO₄, 150 μ M PMSF and 1 tablet/10 ml RIPA complete mini protease inhibitor cocktail (Roche). To investigate the effect of HER ligands, HER inhibitors or metalloproteinase inhibitors on expression of total and phosphorylated forms of HER receptors or downstream signaling molecules, cells were grown in 6-well multidishes until 60-80% confluence and then treated for 15 min (HER ligands) or 24 h (HER or metalloproteinase inhibitors) before cell extracts were obtained by cell lysis in RIPA as described above. Conditioned medium was prepared from MCF-7, 164^R-5 and 164^R-7 cells grown with or without 20 μ M TAPI-2. The cells were seeded in T75 flasks and pre-treated for 24 h with 20 μ M TAPI-2 before new medium with 20 μ M TAPI-2 was added. After additional 24, 48 or 72 h, the medium from each flask was collected and concentrated ten times by centrifugation using iCON concentrators with a molecular weight cut-off of 20 kDa (Thermo Fisher Scientific, Fremont, CA, USA). MCF-7 cell cultures were grown to 60-70% confluence under standard conditions and treated for 15 min with medium to which concentrated conditioned medium from MCF-7, 164^R-5 or 164^R-7 was added. Cell extracts were obtained by cell lysis in RIPA as described above.

Determination of protein concentration in all cell lysates was done using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Copenhagen, Denmark). Total protein (15 μ g) was separated on 4-15% Tris-HCl or 3-8% Tris-Acetate resolving criterion gels (Bio-Rad Laboratories) and transferred to an ethanol-activated Immobilon-P membrane (Millipore, Bedford, MA, USA). To prevent non-specific binding, membranes were blocked in TBS containing 5% dry-milk, 0.2% FCS and 0.1% Tween-20 for 2-3 h at room temperature. Incubation with the primary antibody was performed overnight at 4°C followed by 1-h incubation with species-specific peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark). Protein bands were visualized by enhanced chemiluminescence [ECL Plus detection system (GE Healthcare, Hilleroed,

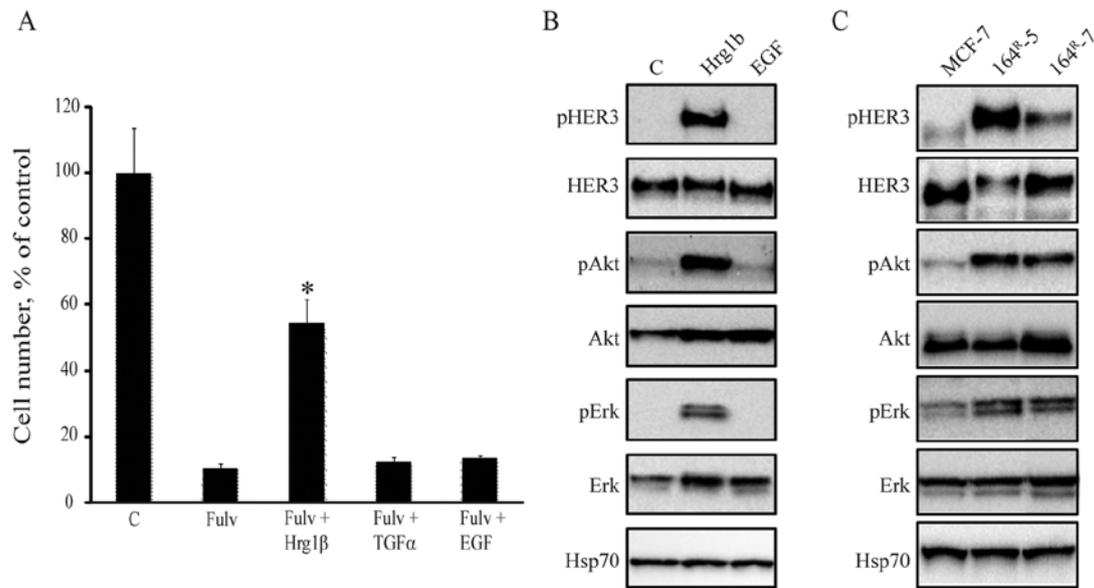


Figure 1. (A) Effect of heregulin 1 β , TGF α and EGF on cell growth of MCF-7 treated with fulvestrant (fulv). The cells were treated for five days with 0.1 μ M fulvestrant with and without 10 ng/ml of the indicated growth factors. Cell number was measured by a colorimetric assay and expressed as percent of the untreated control. A representative experiment with mean \pm SD is shown. *Significant difference between cells treated with fulvestrant alone and cells treated with a combination of fulvestrant and ligand. (B) Western blot showing expression of total and phosphorylated HER3 (Tyr¹²⁸⁹), Akt (Ser⁴⁷³) and Erk (Thr²⁰²/Tyr²⁰⁴) in lysates from MCF-7 cells grown for 15 min with or without 10 ng/ml heregulin 1 β or EGF. (C) Western blot showing expression of total and phosphorylation of HER3 (Tyr¹²⁸⁹), Akt (Ser⁴⁷³) and Erk (Thr²⁰²/Tyr²⁰⁴) in cell lysates from MCF-7, 164^R-5 and 164^R-7 cells.

Denmark)] and detected by a CCD camera (LAS-1000, Fujifilm). In order to detect multiple proteins, the antibodies were stripped from the membrane by incubation for 15 min in 62.5 mM Tris-HCl, 100 mM β -mercaptoethanol and 2% (w/v) SDS, pH 6.7, and washed before incubated with another antibody. The following antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA): phosphorylated HER3 (Tyr¹²⁸⁹, 1:500, 4791), phosphorylated Akt (Ser⁴⁷³, 1:500, 9271), phosphorylated Erk (Thr²⁰²/Tyr²⁰⁴, 1:1000; 4377), Akt (1:2000, 9272) and Erk (1:2000, 9102). HER3 (1:2000, M7297) was purchased from Dako and Hsp70 (1:500,000, MS-482) from Thermo Fisher Scientific. All western analyses were performed at least twice and representative blots are shown.

Gene silencing with small interfering RNA. HER3-targeting SMART pool siRNA (L-003127) and scrambled sequence (non-targeting) control pool siRNA (D-001810) were obtained from Dharmacon (Lafayette, CO, USA). Transfection of cells with 300 nM HER3 or scramble siRNA in 100 μ l nucleofector solution (Cell Line Nucleofector kit V) was performed using the Nucleofector device from Amaxa (Lonza, Cologne, Germany) according to the manufacturer's instructions. Transfected cells were seeded in 6- and 24-well multidishes in standard growth medium to measure protein expression and cell growth, respectively. Medium was replaced at day one and the cell number in 24-well plates were determined on day one, three and six using a crystal violet colorimetric assay as previously described (20). Three days after transfection, cells grown in 6-well multidishes were harvested in RIPA buffer and subjected to western analysis as described above.

Statistics. Three independent growth experiments were performed with quadruplicate measures. Two-sample unequal

variance t-test followed by Bonferroni's correction was used to calculate whether the observed differences in growth were statistically significant. For all experiments, a P-value <0.05 was considered significant. Representative experiments with mean and standard deviation (SD) are shown.

Results

HER ligands partially rescue fulvestrant-mediated growth inhibition. To clarify the importance of HER signaling in response to treatment with fulvestrant, we investigated whether different HER ligands could rescue fulvestrant-inhibited growth of MCF-7 cells. Five days treatment with fulvestrant alone inhibited cell growth of MCF-7 cells to 10% of the untreated control (Fig. 1A). However, when heregulin 1 β , which has the same function as heregulin 2 β e.g. activation of HER3 (2), was added, fulvestrant-mediated inhibition of MCF-7 cell growth was partially rescued. No effect on cell growth was observed when the EGFR ligands EGF or TGF α were added to MCF-7 cells (Fig. 1A). The role of the HER ligands on intracellular signaling in MCF-7 cells was investigated by western blot analysis showing that heregulin 1 β could induce HER3, Akt and Erk phosphorylation in MCF-7 cells (Fig. 1B). Thus, the HER3 ligand heregulin 1 β can partly rescue fulvestrant-inhibited cell growth via activation of HER3 and downstream signaling molecules.

We have previously shown that our fulvestrant-resistant MCF-7 cell lines produce increased levels of the heregulins (2). In line with this, the fulvestrant-resistant cell lines, 164^R-5 and 164^R-7, express increased levels of phosphorylated HER3, Akt and Erk compared with their parental MCF-7 cells (Fig. 1C).

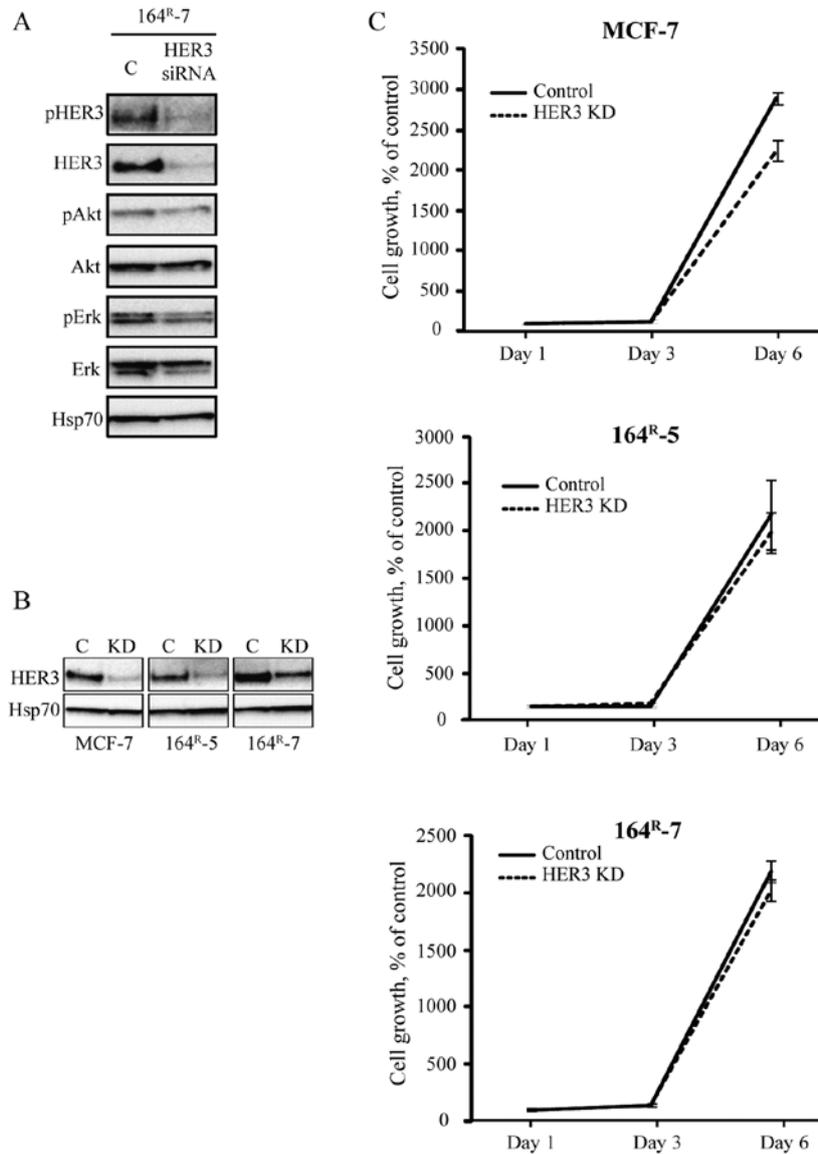


Figure 2. Effect of the HER3 knock-down on growth and signaling in fulvestrant-resistant 164^R-7 cells. (A) Western blot showing expression of total and phosphorylated HER3 (Tyr¹²⁸⁹), Akt (Ser⁴⁷³) and Erk (Thr²⁰²/Tyr²⁰⁴) in lysates from 164^R-7 cells transfected with control siRNA (designated C) or siRNA targeting HER3. Hsp70 was used as loading control. (B) Western blot showing expression of HER3 in lysates from MCF-7, 164^R-5 and 164^R-7 cells transfected with HER3 (designated KD) or control (designated C) siRNA. Hsp70 was used as loading control. (C) MCF-7, 164^R-5 and 164^R-7 cells were transfected with HER3 or control siRNA and cell number estimated at day 1, 3 and 6 after transfection by a crystal violet colorimetric assay. Cell growth is expressed as percentage of cell growth at day 1. The experiment was performed three times.

The knock-down of HER3 in parental and fulvestrant-resistant cell lines has no effect on cell growth. To further explore the role of HER3 for fulvestrant-resistant cell growth, siRNA-mediated knock-down of HER3 was performed. HER3 knock-down in 164^R-7 consequently depleted the cells of HER3 as well as reduced the level of phosphorylated Akt and Erk (Fig. 2A). Next, growth of MCF-7 and the fulvestrant-resistant 164^R-5 and 164^R-7 cells was investigated upon transfection with control (scramble) or HER3 siRNA (Fig. 2B). As shown in Fig. 2C, siRNA-mediated knock-down of HER3 had no effect on growth up to six days after transfection of the fulvestrant-resistant cell lines. In contrast, growth of the parental MCF-7 cells was inhibited after the siRNA-mediated knock-down of HER3 (Fig. 2C). Thus, although signaling via HER3 is upregulated in the resistant cells, knock-down of HER3 had no effect on resistant cell growth.

Metalloproteinase inhibitor TAPI-2 prevents release of HER3 activating ligands. Signaling in our fulvestrant-resistant cells is, at least partly, due to endogenous synthesis of ligands which activate HER3 (2). As the knock-down of HER3 does not seem to be important for resistant cancer cell growth, we investigated if inhibition of ligand shedding, and thus prevention of HER3 activation, could abolish growth and signaling in resistant cell lines. HER3 ligands are synthesized as membrane-bound proteins, which are shed by metalloproteinases and thus able to activate HER3. Initially, in order to reveal whether metalloproteinase-mediated ligand shedding takes place in fulvestrant-resistant cells, conditioned medium was collected from the fulvestrant-resistant 164^R-5 or 164^R-7 cells grown with or without TAPI-2, a potent TACE inhibitor with some activity towards other ADAMs and matrix metalloproteinases. The medium was then concentrated and

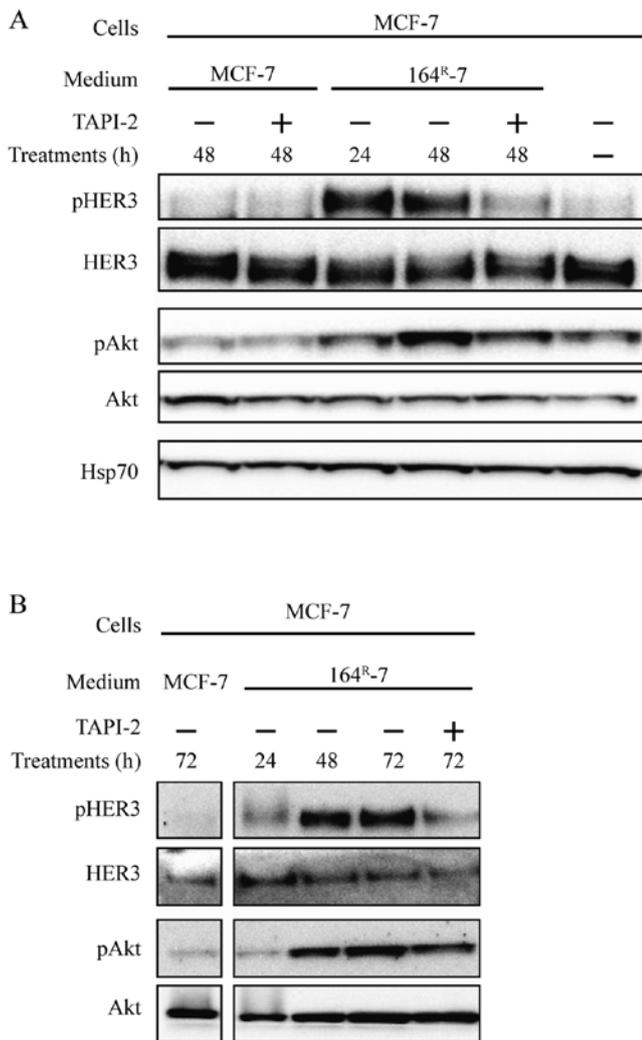


Figure 3. Effect of conditioned medium on phosphorylation of HER3 and Akt in MCF-7 cells. Western blot showing expression of total and phosphorylated HER3 (Tyr¹²⁸⁹) and Akt (Ser⁴⁷³) in lysates from MCF-7 cells treated for 15 min with concentrated conditioned medium from MCF-7, 164^R-7 (A) or 164^R-5 (B) incubated for 24, 48 or 72 h with or without 20 μ M TAPI-2 as indicated in the figure. The last lane in A is untreated MCF-7 cells. Hsp70 was used as loading control.

added to MCF-7 cells to explore the effect on HER3 and Akt phosphorylation.

Medium conditioned by 164^R-7 cells for 24 or 48 h induced phosphorylation of HER3 and Akt in MCF-7 cells (Fig. 3A). When MCF-7 cells were incubated with conditioned medium from 164^R-7 cells treated with TAPI-2 for 48 h, no phosphorylation of HER3 was seen and downstream signaling to Akt was reduced (Fig. 3A). Similar results were obtained with conditioned medium from 164^R-5 (Fig. 3B). Treatment of MCF-7 cells with conditioned medium from 164^R-5 or 164^R-7 cells treated with TAPI-2 had no effect on the expression of total HER3 and total Akt (Fig. 3A and B). Moreover, there were no differences in the expression and phosphorylation levels of HER3 and Akt when MCF-7 cells were incubated with medium conditioned by MCF-7 cells grown in the presence or absence of TAPI-2 for 48 h (Fig. 3A). Collectively, these data support that the fulvestrant-resistant cell lines 164^R-5 and 164^R-7, in contrast to MCF-7 cells, produce endogenous HER3

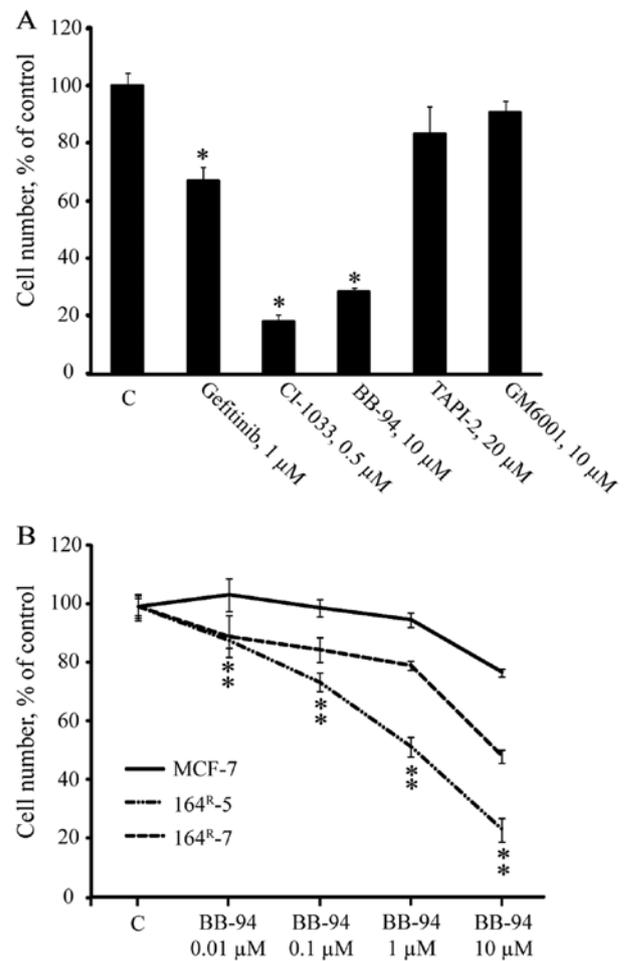


Figure 4. Effect of gefitinib, CI-1033, TAPI-2, BB-94 and GM6001 on cell growth. (A) 164^R-7 cells were treated for 5 days with the indicated concentrations of gefitinib, CI-1033, TAPI-2, BB-94 and GM6001. Cell number was measured by a colorimetric assay and expressed as percent of the untreated control. A representative experiment with mean \pm SD is shown. Significant difference (* P <0.05) between untreated controls and treated cultures. (B) MCF-7, 164^R-5 and 164^R-7 cells were treated for five days with the indicated concentrations of BB-94. Cell number was measured by a colorimetric assay and expressed as percent of untreated control (designated C). A representative experiment with mean \pm SD is shown.

ligands, which require cleavage by TACE, or another TAPI-2 sensitive metalloproteinase to be shed into the medium.

Metalloproteinase inhibitor BB-94 is able to prevent growth and signaling of fulvestrant-resistant breast cancer cell lines.

To further investigate the importance of HER receptors and HER ligand shedding for cell growth and signaling in the resistant cell lines, 164^R-7 was treated for five days with gefitinib (preferentially targeting EGFR), CI-1033 (pan-HER inhibitor), or the metalloproteinase inhibitors TAPI-2, BB-94 or GM6001 (Fig. 4A). Compared to growth of the untreated control, 1 μ M gefitinib or 0.5 μ M CI-1033 significantly inhibited growth of 164^R-7 by 30 and 80%, respectively. Moreover, the broad-spectrum metalloproteinase inhibitor BB-94 significantly inhibited resistant growth by 70%, whereas the more selective metalloproteinase inhibitors TAPI-2 and GM6001 had no inhibitory effect on growth of 164^R-7 (Fig. 4A). When the five-day dose-response growth experiments with BB-94 were

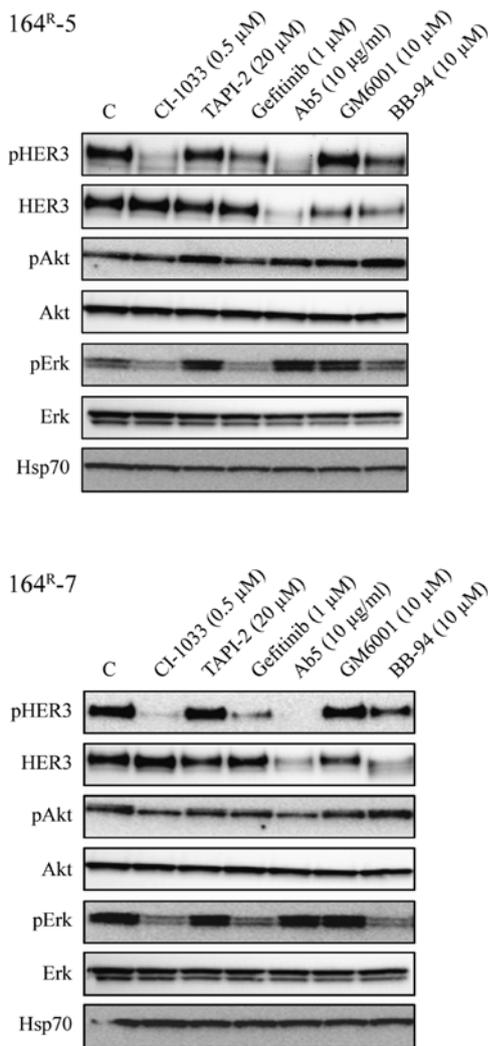


Figure 5. Effect of gefitinib, CI-1033, Ab5, TAPI-2, BB-94 and GM6001 on cell signaling. Western blot showing expression and phosphorylation of HER3 (Tyr¹²⁸⁹), Akt (Ser⁴⁷³) and Erk (Thr²⁰²/Tyr²⁰⁴) in 164^{R-5} and 164^{R-7} cells treated for 24 h with the indicated concentrations of CI-1033, TAPI-2, gefitinib, Ab5, GM6001 and BB-94. Hsp70 was used as a loading control.

performed, increasing concentrations (0.01–10 μM) of BB-94 resulted in a dose-dependent growth inhibition up to 50% and 80% compared to the untreated control for 164^{R-7} and 164^{R-5}, respectively, whereas growth of the parental MCF-7 cells in the presence of increasing concentrations of BB-94 was <20% inhibited compared to the untreated control (Fig. 4B). Thus, BB-94 preferentially inhibited growth of fulvestrant-resistant MCF-7 cell lines.

We then tested the effect of CI-1033, gefitinib, Ab5 (neutralizing antibody against HER3), TAPI-2, BB-94 and GM6001 on signaling in 164^{R-5} and 164^{R-7} cells. Treatment of the fulvestrant-resistant cells for 24 h with CI-1033 (0.5 μM) strongly inhibited phosphorylation of HER3 and Erk whereas phosphorylation of Akt was only slightly reduced (Fig. 5). Treatment with gefitinib (1 μM) reduced the level of phosphorylated HER3 and strongly reduced Erk phosphorylation, indicating that EGFR signals via Erk. Treatment with Ab5 (10 $\mu\text{g/ml}$) prevented phosphorylation of HER3, had no effect on phosphorylation of Erk and only little effect on phosphorylation of Akt. Ab5 also resulted in degradation of HER3. BB-94

(10 μM) inhibited phosphorylation of HER3 and Erk, induced degradation of HER3 but had no effect on phosphorylation of Akt. In contrast, TAPI-2 (20 μM) and GM6001 (10 μM) had no effect on phosphorylation of HER3, Akt or Erk.

Collectively, the experiments in Figs. 4 and 5 show that treatment with CI-1033, which exerted the most severe growth inhibition, completely blocked HER3 phosphorylation and resulted in severe reduction of phosphorylated Erk. Treatment with gefitinib resulted in reduced phosphorylation of Erk, but did not totally reduce phosphorylation of HER3 and had only a modest effect on cell growth. As the broad-spectrum metalloproteinase inhibitor BB-94 severely reduced phosphorylation of Erk, reduced expression and phosphorylation of HER3 and exerted strong growth inhibition while TAPI-2 and GM6001 had no effect on phosphorylation of HER3 and Erk, our data indicate that BB-94, in contrast to TAPI-2 and GM6001, targets signaling of both via EGFR and HER3.

Discussion

Several experimental studies have shown that breast cancer cell lines can switch from ER- to HER-driven cell growth upon acquiring antiestrogen resistance (2–4,6,21,22). This is in agreement with the clinical observation that response to endocrine treatment is reduced in ER-positive breast cancer patients with HER2-positive tumors compared to the response in HER2-negative tumors (23). Moreover, it is in line with the clinical benefit for patients with ER/HER2-positive breast cancer treated with a combination of endocrine therapy and the EGFR/HER2 tyrosine kinase inhibitor lapatinib, as compared to treatment with endocrine therapy alone (24). In this study, we used MCF-7 and MCF-7-derived breast cancer cell lines, which have progressed from antiestrogen sensitive to antiestrogen resistant cells during long-term treatment with the pure antiestrogen fulvestrant. The expression of ER is maintained in the resistant cells but at a reduced level. However, when grown in presence of fulvestrant, ER is degraded and the resistant cells primarily use the HER system for growth (2,6). Previous studies have shown that the fulvestrant-resistant cell lines secrete increased levels of the HER3/4 ligands heregulin 2 α and 2 β , and in agreement with this also express significantly increased level of activated HER3. The level of total EGFR, phosphorylated EGFR and phosphorylated Erk is also increased in the resistant cell lines, whereas HER4 level is severely reduced (2). The aim of the current study was to unravel the importance of HER receptors, in particular HER3, and HER ligand shedding for growth of the fulvestrant-resistant MCF-7 cell lines and to explore whether inhibition of ligand shedding could be a new treatment strategy for resistant cells.

Initially, we tested whether prevention of ER signaling and consequent cell growth arrest induced by fulvestrant in the ER-positive MCF-7 cell line could be abrogated by activation of the HER system via addition of exogenous HER ligands. We found that MCF-7 cells were able to grow in the presence of fulvestrant when the HER system was activated by the HER3/HER4 ligand heregulin 1 β but not by TGF α or EGF. Western blot analysis showed that exogenous heregulin 1 β induced phosphorylation of HER3, Akt and Erk in MCF-7 cells, supporting that activation of signaling via HER3 can abrogate fulvestrant induced growth inhibition.

To further explore the importance of HER3 for resistant cell growth, siRNA mediated HER3 knock-down was performed in two fulvestrant-resistant cell lines, 164^R-5 and 164^R-7. The HER3 knock-down resulted in close to total abrogation of expression of HER3 and consequently also phosphorylation of HER3, as well as reduced levels of phosphorylated Akt and Erk, but cell growth was not significantly inhibited. This demonstrates that Akt and Erk are downstream mediators of HER3 signaling induced by endogenous ligands in the resistant cell lines, in agreement with our finding that exogenous heregulin 1 β was able to activate HER3, Akt and Erk in parental MCF-7 cells. The lack of significant growth inhibition by HER3 knock-down in fulvestrant-resistant cell lines is in agreement with the finding that treatment with Ab5, a neutralizing antibody to HER3, only resulted in a small growth inhibitory effect in two of three tested fulvestrant-resistant cell lines (2). Ab5 exerted significant downregulation of total and phosphorylated HER3, but had no effect on the level of phosphorylated Akt or Erk. This may explain the lack of growth inhibition. We presume that upon knock-down of HER3, other HER receptors in the resistant cells may compensate for the lack of HER3. EGFR has previously been shown to play a role for fulvestrant-resistant cell growth (2,6,22), but targeting EGFR alone with the kinase inhibitor gefitinib at 1 μ M concentration had only a modest growth inhibition in the order of 20-30%. This is again in agreement with our previous finding that the EGFR neutralizing antibody cetuximab exerted only a small inhibition of growth of the resistant cell lines (6). Similarly, targeting HER2 alone with increasing concentrations of trastuzumab in fulvestrant-resistant breast cancer cells had no effect on cell growth (25). Thus, targeting the HER receptors individually had no inhibitory effect on resistant cell growth, in contrast to targeting all HER receptors with the pan-HER inhibitor CI-1033, which completely blocked growth and signaling via HER3 and Erk in the resistant cells.

It has been shown that the effect of targeting the HER receptors individually is compromised by increased expression of HER ligands, which may circumvent the loss of the function of a single receptor. For instance, the expression of the EGFR ligand TGF α was increased upon trastuzumab treatment (16) and heregulin could partly bypass the growth inhibitory effect of EGFR inhibitors (17). We therefore investigated the importance of HER ligand shedding for signaling and growth of breast cancer cells. In agreement with the lack of HER3 phosphorylation in MCF-7 cells, conditioned medium from MCF-7 cells did not contain factors able to activate HER3 in MCF-7. In contrast, HER3 and Akt were phosphorylated in MCF-7 cells treated with conditioned medium from fulvestrant-resistant 164^R-5 and 164^R-7 cells, whereas conditioned medium from antiestrogen resistant cells treated with TAPI-2, an inhibitor of TACE and MMPs, showed no HER3 activation. This clearly demonstrates that resistant cells produce HER3 activating factors, which require cleavage by a metalloproteinase to be released from the cells and supports that shedding of HER ligands could be mediated by TACE as previously suggested (13).

When further investigating the effect of TAPI-2 as well as another metalloproteinase inhibitor GM6001 on resistant cell growth, we unexpectedly found that these metalloproteinase

inhibitors had no effect on growth of the fulvestrant-resistant cell line neither on expression of activated HER3, Akt or Erk. The maintained HER3 activation upon TAPI-2 treatment of resistant cells, which was shown here to prevent shedding of HER3-activating ligands, indicates that a juxtacrine mechanism may be responsible for HER3 activation.

We have previously seen that the expression and phosphorylation levels of EGFR is increased in our resistant cells compared to MCF-7, but although increased, it is not possible to measure phosphorylated EGFR by western blotting in either parental or the resistant cell lines (2). The fact that gefitinib and cetuxumab (unpublished data) strongly inhibited Erk signaling in the resistant cells, supports that EGFR signals via Erk and the lack of effect of TAPI-2 and GM6001 on phosphorylation of Erk suggests that EGFR activation is not inhibited by these metalloproteinase inhibitors. Instead, signaling via EGFR, either as homodimer or heterodimer with HER2, appears to be sufficient to maintain resistant cell growth even though HER3 activation is abrogated. In contrast to TAPI-2 and GM6001, the broad-spectrum metalloproteinase inhibitor BB-94 exerted nearly complete growth inhibition of the 164^R-5 cells, about 50% reduction of growth of 164^R-7 cells and only 20% reduction of growth of parental cells. The growth inhibition was concomitant with reduced expression of activated HER3 and Erk. These findings support that BB-94, in contrast to TAPI-2 and GM6001, blocks activation of EGFR. HER3 may still bind ligands by a juxtacrine mechanism, but since EGFR is presumably not activated, as indicated by reduced Erk phosphorylation, HER3 will not be able to form heterodimers with EGFR, and this may explain the reduced level of phosphorylated HER3 upon treatment with BB-94. Previously, we found that the level of heregulin 2 α and -2 β is increased in 164^R-5 and 164^R-7 compared to the level in MCF-7 and that TGF α , EGF, HB-EGF and betacellulin are expressed in the resistant cells but at similar levels as in MCF-7 (2). These HER ligands have been shown to be shed by TACE (TGF α , HB-EGF and heregulins) or ADAM10 (EGF, betacellulin and HB-EGF) (13,26). However, as neither TAPI-2 nor the broader metalloproteinase inhibitor GM6001 reduced growth and signaling in the fulvestrant-resistant cell lines, our data suggest that HER receptor activation in fulvestrant-resistant cell lines can occur independent of ligand shedding. Yet, the fact that the more broad-spectrum metalloproteinase inhibitor BB-94 caused a marked decrease in both resistant cell growth and levels of HER3 and Erk phosphorylation strongly indicates some other metalloproteinase implication not targeted by TAPI-2 and GM6001.

In this study, we investigated the importance of the HER receptors and in particular HER3 as well as HER ligand shedding for growth of our fulvestrant-resistant cell lines. Compared with their parental MCF-7 cell lines, the resistant cells have severely reduced HER4 expression, unchanged level of HER2, increased level of EGFR, phosphorylated HER3, Erk and Akt and increased level of heregulin 2 α and -2 β (2). We found that neither HER3 nor EGFR alone was a key driver of growth of the resistant cells. Inhibition of growth required concomitant inhibition of HER3 activation and signaling via Erk and this was achieved with the pan-HER inhibitor CI-1033. We also found that targeting ligand shedding with a broad-spectrum metalloproteinase inhibitor prevented growth

and inhibited activation of HER3 and Erk in the resistant cells. The less potent metalloproteinase inhibitor TAPI-2, which blocked HER3 ligand shedding, did not prevent HER3 nor Erk activation, indicating that other mechanisms including juxtacrine HER activation may occur. Thus, ligand shedding is presumably not a target for treatment, and collectively, our combined data underscore the complexity of the resistance mechanisms and the requirement of targeting signaling from HER receptors by multiple strategies.

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