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Breast cancer cells can switch between estrogen receptor α and ErbB signaling and combined treatment against both signaling pathways postpones development of resistance

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Abstract The majority of breast cancers are estrogen responsive, but upon progression of disease other growth promoting pathways are activated, e.g., the ErbB receptor system. The present study focuses on resistance to the pure estrogen antagonist fulvestrant and strategies to treat resistant cells or even circumvent development of resistance. Limited effects were observed when targeting EGFR and ErbB2 with the monoclonal antibodies cetuximab, trastuzumab, and pertuzumab, whereas the pan-ErbB inhibitor CI-1033 selectively inhibited growth of fulvestrant resistant cell lines. CI-1033 inhibited Erk but not Akt signaling, which as well as Erk is important for antiestrogen resistant cell growth. Accordingly, combination therapy with CI-1033 and the Akt inhibitor SH-6 or the Protein Kinase C inhibitor RO-32-0432 was applied and found superior to single agent treatment. Further, the resistant cell lines were more sensitive to CI-1033 treatment when grown in the presence of fulvestrant, as withdrawal of fulvestrant restored signaling through the estrogen receptor α (ER α), partly overcoming the growth inhibitory effects of CI-1033. Thus, the resistant cells could switch between ER α and ErbB signaling for growth promotion. Although parental MCF-7 cell growth primarily depends on ER α signaling, a heregulin-1 β induced switch to ErbB signaling

rescued MCF-7 cells from the growth inhibition exerted by fulvestrant-mediated blockade of ER α signaling. This interplay between ER α and ErbB signaling could be abrogated by combined therapy targeting both receptor systems. Thus, the present study indicates that upon development of antiestrogen resistance, antiestrogen treatment should be continued in combination with signal transduction inhibitors. Further, upfront combination of endocrine therapy with pan-ErbB inhibition may postpone or even prevent development of treatment resistance.

Keywords Breast cancer · Treatment resistance · ER α · ErbB · Fulvestrant · CI-1033 · Combined treatment

Introduction

The antiestrogen tamoxifen has been first line endocrine treatment for ER α positive breast cancer patients for more than two decades. After an initial response in the majority of patients, almost all patients with advanced disease develop resistance to the therapy. Treatment with the pure antiestrogen fulvestrant has proven effective upon progression on tamoxifen both in vitro and in vivo and is approved for second line endocrine treatment [1–4]. However, as for treatment with tamoxifen, development of resistance will inevitably occur. The molecular mechanisms contributing to the development of antiestrogen resistance are still elusive, but increasing evidence suggests that aberrant activation of growth factor signaling cascades can provide proliferation and survival signals, overcoming the effect of antiestrogen treatment. Several preclinical studies of fulvestrant resistance have reported changes in the ErbB system, consisting of the EGF receptor (EGFR/HER1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4

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(HER4) receptors and at least 12 activating ligands. The changes correlated with fulvestrant resistance have been observed at the ErbB receptor level, the ligand level, and in ErbB downstream signaling molecules [5–11]. Consequently, therapies targeting the ErbB system represent an attractive strategy in breast cancer treatment. Focus has primarily been on EGFR and ErbB2 targeted treatment, but such therapies have shown limited clinical efficacy due to inherent or acquired resistance. For trastuzumab targeting the ErbB2 receptor, response rates of approximately 30% have been reported in patients with ErbB2 overexpressing tumors, whereas the benefit of EGFR targeted therapies may be as low as 5–15% (reviewed in [12]). The limited effects of EGFR and ErbB2 monotherapy could be due to the tight integration and redundancy of the ErbB system in sustaining tumor growth and progression, for example through activation of the ErbB3 receptor (reviewed in [13]). Further, the importance of both ErbB and ER α signaling in breast cancer have raised focus on interactions between these receptors and increasing amounts of data suggest that cross-receptor interactions are more common than previously anticipated and may affect growth of breast cancer cells and their response to treatment. For example, ER α may activate membrane tyrosine kinases through non-genomic interactions that may lead to activation of downstream effector molecules, which in turn may exert positive feedback to activate ER α signaling. Such a bidirectional cross-talk can augment signaling of both ER α and ErbB pathways, enhance growth and survival of breast cancer cells and may be involved in tamoxifen resistance [14, 15]. In contrast, tumor cells which escape fulvestrant treatment leading to ER α downregulation are more likely to develop ER α independent upregulation of growth factor receptor signaling. Furthermore, preclinical data have suggested that increased growth factor expression and signaling through the ErbB receptors or downstream effectors may repress ER α expression and function [16–19], potentially making breast cancer cells less sensitive to endocrine therapy. Also, clinical data have indicated an inverse relationship between expression of at least the EGFR and ErbB2 receptors with ER α and their overexpression has been correlated to decreased antiestrogen sensitivity [20–23].

We have established a model system with acquired fulvestrant resistant breast cancer cell lines developed from parental MCF-7 cells [24]. These resistant cell lines show increased expression of EGFR and the ErbB3/4 ligand heregulin-2 β , increased activation of ErbB3, Erk, and Akt, and decreased expression of ErbB4 [11]. The resistant cell lines were preferentially sensitive to treatment with the tyrosine kinase inhibitor gefitinib targeting EGFR, compared to parental antiestrogen sensitive MCF-7 cells [11]. However, in our cell lines, 1 μ M gefitinib did not only

inhibit EGFR phosphorylation but also ErbB3 and ErbB4 phosphorylation ([11] and unpublished data for ErbB4). Thus, the potent effect of gefitinib in our resistant cell lines may be due to the pan-ErbB inhibiting effects. CI-1033 is a novel irreversible pan-ErbB inhibitor that is highly effective in inhibiting the four ErbB receptors and causes prolonged suppression of ErbB kinase activity in breast cancer cells [25, 26]. CI-1033 has been suggested as a promising anti-cancer drug due to the involvement of ErbB signaling in various cancers. Preclinical data proved the efficacy of CI-1033 in two different breast xenograft models [26]. Furthermore, CI-1033 inhibited ErbB downstream signaling through Akt and Erk and induced apoptosis in BT474 breast cancer cells, whereas CI-1033 alone could not induce apoptosis in MDA-MB-453 cells [27]. Clinically, CI-1033 has entered phase I and II trials and has demonstrated evidence of target biomarker modulation and antitumor activity [26]. A few heavily pretreated breast cancer patients have been enrolled in phase I studies with CI-1033 and stable disease for more than three months was observed in a small subset of these patients [26, 28]. However, a recent phase II trial with CI-1033 in pretreated metastatic breast cancer patients expressing one or more ErbB receptors showed disappointing results [29]. Antitumor activity was only observed at doses reaching unacceptable levels of toxicity. Yet, the study was performed in heavily pretreated patients, which may have limited the effectiveness of CI-1033.

The present study was designed to clarify the importance of ErbB and ER α signaling for growth of breast cancer cells with focus on fulvestrant resistant breast cancer. In a panel of fulvestrant resistant cell lines, we investigated the growth inhibitory effect of treatment with EGFR and ErbB2 neutralizing antibodies and with the pan-ErbB inhibitor CI-1033 alone or in combination with the Akt inhibitor SH-6 and the Protein Kinase C (PKC) inhibitor RO-32-0432. CI-1033 selectively inhibited resistant cell growth compared to growth of parental MCF-7 cells. Further, the effect of CI-1033 was investigated in presence and absence of fulvestrant, which completely blocks ER α signaling. Withdrawal of fulvestrant from the growth medium of fulvestrant resistant cells resulted in re-expression of ER α and significantly reduced the sensitivity to CI-1033 treatment, indicating that the resistant cells are able to switch to ER α signaling upon blockade of ErbB signaling. Concurring, it was tested and found that activation of ErbB signaling with heregulin-1 β in parental MCF-7 cells could substitute for repression of ER α -mediated cell growth with fulvestrant. The above-mentioned experiments point toward a high flexibility for breast cancer cells to promote growth through ER α or ErbB signaling, and it was analyzed whether a concerted action against ER α and ErbB signaling results in delay or prevention of treatment resistance.

Materials and methods

Inhibitors

Fulvestrant (ICI 182,780, FaslodexTM) was obtained from Tocris (Avonmouth, Bristol, UK). The HER tyrosine kinase inhibitor CI-1033 (Canertinib) was donated by Pfizer Inc. (Groton, CT, USA), while SH-6 (Akt inhibitor) and RO-32-0432 (PKC inhibitor) were purchased from Calbiochem (Gibbstown, NJ, USA). The monoclonal antibody cetuximab (Erbix) targeting EGFR was purchased from Merck (Glostrup, Denmark). Two ErbB2 targeting antibodies were used. Pertuzumab was a gift from Genentec (San Francisco, CA, USA), and trastuzumab was purchased from Roche (Hvidovre, Denmark).

Cell lines and culture conditions

The MCF-7 cell line was originally obtained from the Human Cell Culture Bank, Mason Research Institute (Rockville, MD, USA) and adapted to grow in low serum concentration (1%) to reduce the estrogens supplied through the serum to a level resembling postmenopausal concentrations of circulating estradiol [30]. The cells were maintained in growth medium without phenol red (DMEM/F12 medium (Gibco, Invitrogen, CA, USA)), supplemented with 1% heat-inactivated fetal calf serum (FCS) (Life Technologies, Bethesda, MD), 6 ng/ml bovine insulin (Novo Nordic, Bagsvaerd, Denmark), and 2 mM glutamax (Gibco). The fulvestrant resistant cell lines MCF-7/164^R-5 (164^R-5), MCF-7/164^R-7 (164^R-7), and MCF-7/182^R-6 (182^R-6) were established as described earlier [24] and maintained in growth medium supplemented with 0.1 μM fulvestrant. For experiments, 2.5×10^5 U penicillin and 31.25 μg/l streptomycin (Gibco) were added to the growth medium.

Cell proliferation assays

All cell lines were seeded in 24-well plates (2 cm² wells) in growth medium and allowed to adhere for 1–2 days. At the onset of experiment (day 0), growth medium containing inhibitors was added as indicated in the figure legends. Untreated controls were added similar amount of vehicle (ethanol or DMSO) as the treated cultures. Growth medium was replaced on day 3, and cell number determined on day 5 as previously described using a crystal violet colorimetric assay [31]. The obtained optical density (OD) for each sample was expressed as a relative value in percent of untreated control. Each experiment was performed in triplicate. For resistance development, MCF-7 cells were seeded in 24-well plates. The cells were allowed to attach for 24 h before treatment was initiated (day 0). Growth medium was changed twice every week and cell number

was estimated with the colorimetric assay during the weeks 1, 2, 3, 4, 5, and 6 from onset of experiment. For these experiments, the actual OD was used as a measure for the cell number to the given time interval.

Western analysis

All cell lines were seeded in 6-well plates and cultured for 4–5 days in growth medium to obtain 70–80% confluent cultures. Cells were treated for 4 or 48 h with inhibitors as indicated in the figure legends and whole cell extracts were generated by lysing the cells in RIPA buffer (100 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 0.5% sodiumdeoxycholate, 0.1% SDS and 1 mM EDTA, with the addition of 1 mM DTT, 1 mM NaF, 10 mM β-glycerolphosphate, 100 μM Na₃VO₄, 150 μM PMSF, and one tablet/10 ml complete mini protease inhibitor cocktail (Roche)). Protein concentration was determined using the BioRad Protein Assay kit (Bio-Rad Laboratories, München, Germany). The proteins (10–15 μg per lane) were separated by electrophoresis on 4–12% Bis-Tris gels or 3–8% Tris Acetate gels (NuPage, Invitrogen) and transferred onto methanol-activated PVDF membranes (Immobilon-P, Millipore, Bedford, MA, USA). Non-specific binding of antibodies was prevented by incubating the membranes in TBS containing 5% dry-milk, 0.2% FCS, and 0.1% Tween-20 for 2 h at room temperature (RT). Incubation with primary antibodies was performed 1 h at RT or over night at 4°C depending on the antibody and followed by 1 h incubation with species-specific peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark). Specific binding was detected by ECL^{PLUS} (GE Healthcare, Hillerød, Denmark) and visualized using a CCD camera (LAS-1000, Fujifilm, Stockholm, Sweden). Quantification of protein expression levels was performed using the software Multi Gauge V2.2 (Fujifilm). In order to detect multiple proteins, the antibodies were removed from the membrane by incubation in 62.5 mM Tris-HCl, 100 mM β-mercaptoethanol and 2% (w/v) SDS, pH 6.7, and washed before incubation with antibody. The expression level of HSP70 (1:500,000, MS-482, Thermo Fisher Scientific, Fremont, CA, USA) was used as a loading control. The following antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): pAkt (1:500, 9271), Akt (1:2000, 9272), pErk1/2 (1:1000, 4377), and Erk1/2 (1:2000, 9102). The ERα (1:4000, RM-9101) antibody was purchased from Thermo Fisher Scientific. Bcl-2 antibody (1:2000, M0887) was purchased from Dako, Glostrup, Denmark.

Statistics

For all cell growth assays, three independent experiments were performed with triplicate or quadruplicate measures

and the data were pooled for statistical analysis. Assumption of normality and homogeneity of variance was performed by graphical inspection of residuals. A mixed model of analysis of variance, followed by pair wise comparison with Bonferroni's correction was used to analyze for differences in response to treatment within and between cell lines. The given treatment and cell line were regarded as fixed variables, whereas the individual experiments were regarded as a random variable in the model. For experiments with combination therapy, data were log transformed in order to analyze data on a multiplicative scale, allowing for evaluation of interactions between the applied inhibitors. For experiments with resistance development in MCF-7 cells, each single experiment with triplicate measures was analyzed instead of pooling data from the three individual experiments. A linear model was used, followed by pair wise comparison with Bonferroni's correction. For all experiments, results were considered significant when $P < 0.05$. Calculations were performed using SAS, version 9.1 (SAS institute, Cary, NC, USA).

Results

Cetuximab, but not trastuzumab or pertuzumab, inhibits growth of fulvestrant resistant cells

A prominent change in the fulvestrant resistant cell lines compared to parental MCF-7 cells is a significant upregulation of EGFR expression [11]. Accordingly, we examined the effect of the antibody cetuximab on growth of parental MCF-7 cells and three fulvestrant resistant cell lines. As expected, treatment with cetuximab had no effect on the MCF-7 cell line that expresses a very low level of EGFR protein. For the resistant cell lines, only a modest growth inhibition (10%) was observed when 164^R-5 cells were treated with 50 $\mu\text{g/ml}$ cetuximab ($P = 0.02$) and a 16 and 18% statistically significant growth inhibition was seen when 182^R-6 cells were treated with 5 $\mu\text{g/ml}$ ($P = 0.02$) or 50 $\mu\text{g/ml}$ ($P = 0.01$) cetuximab, respectively, Fig. 1a. No statistically significant growth inhibition was observed for 164^R-7 cells. ErbB2 is the preferred dimerization partner for the other ErbB receptors and especially the ErbB2/ErbB3 dimer has been proposed to function as an oncogenic unit [32]. Thus, we investigated the effect of ErbB2 targeting antibodies on cell growth to clarify if ErbB2 could play a role in antiestrogen resistant cell growth as a dimerization partner for ErbB3 as well as EGFR. However, there was not a significant effect on growth of MCF-7 cells or the resistant cell lines 164^R-5 or 164^R-7 when they were treated with the ErbB2 targeting antibodies trastuzumab (20 $\mu\text{g/ml}$) or pertuzumab (20 $\mu\text{g/ml}$), Fig. 1b.

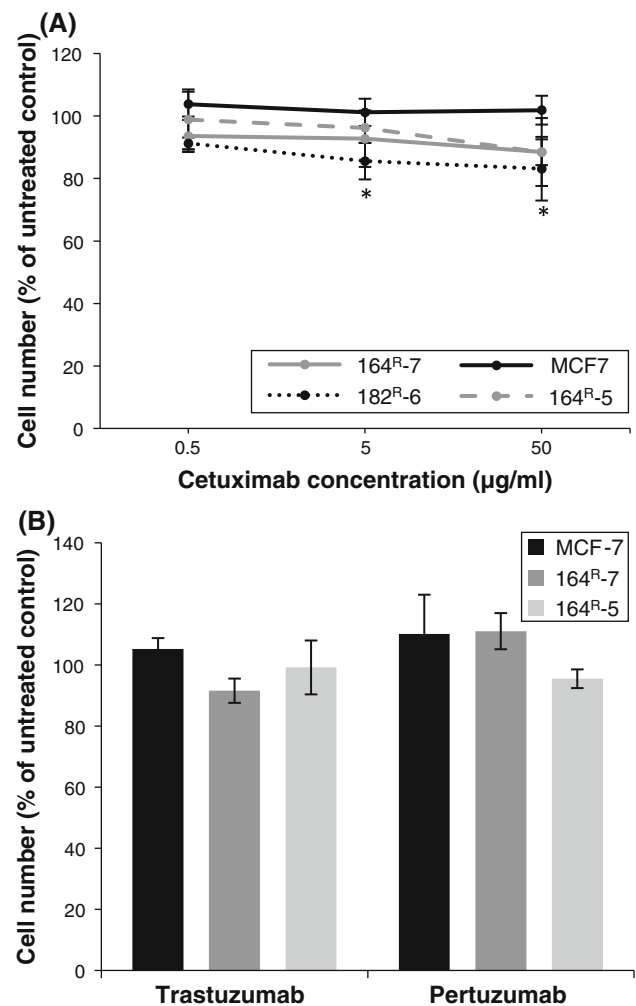


Fig. 1 **a** Effect of the EGFR directed antibody cetuximab on cell growth of MCF-7 and the fulvestrant resistant cell lines 164^R-5, 164^R-7, and 182^R-6. Cells were treated for 5 days with the indicated concentrations of cetuximab before cell number was estimated by a colorimetric assay and expressed as percent of untreated control. Values (mean \pm SD) are pooled data from three independent experiments, each performed with triplicate measures. * indicates values significantly different from the untreated control of the respective cell line. **b** Effect of the ErbB2 directed antibodies trastuzumab and pertuzumab on cell growth of MCF-7 and the fulvestrant resistant cell lines, 164^R-5 and 164^R-7. Cells were treated for 5 days with trastuzumab (20 $\mu\text{g/ml}$) or pertuzumab (20 $\mu\text{g/ml}$) before cell number was estimated by a colorimetric assay and expressed as percent of the untreated control. Values (mean \pm SD) are pooled data from three independent experiments, each performed with triplicate measures

CI-1033 preferentially inhibits growth of fulvestrant resistant cells and downregulates Erk signaling

In contrast to the limited effect of the monoclonal antibodies targeting EGFR or ErbB2, treatment with the new pan-ErbB inhibitor CI-1033 resulted in potent growth inhibition of all resistant cell lines, Fig. 2a. A preferential dose related growth inhibitory effect of the resistant cell

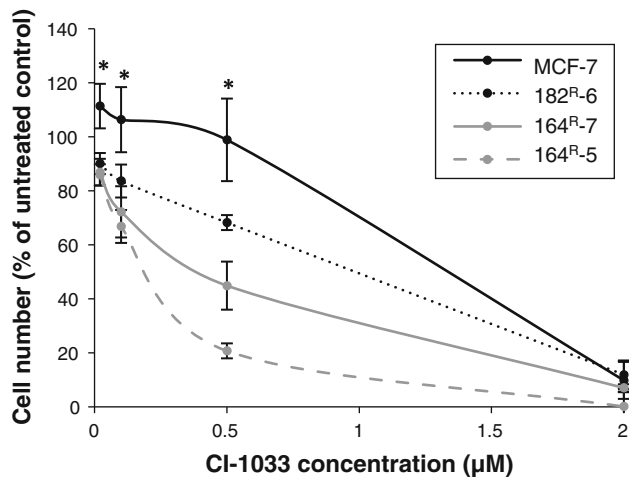


Fig. 2 Effect of the pan-ErbB inhibitor CI-1033 on cell growth of MCF-7 and the fulvestrant resistant cell lines 164^R-5, 164^R-7, and 182^R-6. Cells were treated for 5 days before cell number was estimated by a colorimetric assay and expressed as percent of the untreated control. Values (mean \pm SD) are pooled data from three independent experiments, each performed with triplicate measures. * indicates significant differences between the MCF-7 culture and three fulvestrant resistant cell lines after treatment with the indicated concentrations of CI-1033

lines compared to the unaffected parental MCF-7 cells was observed when treated with 0.02 μ M (164^R-5 $P = 0.005$, 164^R-7 $P = 0.007$, and 182^R-6 $P = 0.03$), 0.1 μ M (164^R-5 $P < 0.0001$, 164^R-7 $P < 0.0001$, and 182^R-6 $P = 0.01$), or 0.5 μ M (164^R-5 $P < 0.0001$, 164^R-7 $P < 0.0001$, and 182^R-6 $P < 0.0001$) CI. Thus, fulvestrant resistant cell lines were more dependent on ErbB signaling than the antiestrogen sensitive MCF-7 cells. 2 μ M CI-1033 inhibited growth of MCF-7 cells to the same extent as the resistant cell lines, indicating general toxicity of the compound at this concentration. Both the PI3-K/Akt and the MEK/Erk pathway are commonly accepted to be downstream of ErbB signaling [32] and we have previously shown upregulation of phosphorylated Akt (pAkt) and Erk (pErk) kinases in a panel of our resistant cell lines [8, 11]. In the present study, a marked upregulation of pErk was found in the 164^R-5, 164^R-7, and 182^R-6 cell lines and a modest upregulation of pAkt was observed in the 164^R-5 and 164^R-7 cell lines, Fig 3a. The expression of pErk in the resistant cell lines was quantified by expressing the ratio pErk/Erk in percent of the ratio for MCF-7 cells. The expression of pAkt was not quantified due to the inaccuracy obtained when relating to a very low expressing control. Treatment of cells with CI-1033 effectively downregulated pErk expression in MCF-7 cells and all three resistant cell lines at concentrations from 0.1 μ M, Fig. 3b. In order to quantify the observed changes in pErk expression, the pErk/Erk was determined for each treatment and expressed as a percent of the untreated control

(Fig. 3b). CI-1033 treatment had no or only minor effect on pAkt expression (Fig. 3b). Again, the expression of pAkt was not quantified due to the inaccuracy obtained when relating to a low expressing control.

Combination therapy with CI-1033, SH-6, and RO-32-0432 is superior to single agent therapy

We have previously shown that growth of fulvestrant resistant cell lines also depend on Akt signaling and that treatment with the Akt inhibitor SH-6 has a preferential inhibitory effect on resistant cell growth [8]. As Akt signaling appeared quite persistent despite CI-1033 treatment, we tested the effect of combined CI-1033 and SH-6 treatment on cell growth. PKC α also plays a role for growth of the resistant cell lines and the PKC inhibitor RO-32-0432 shows a preferential inhibitory effect on growth of resistant cells [33] independent of Erk or Akt signaling (Kristina Emdal, unpublished data). Therefore, we also tested the effect of a combination of CI-1033 and RO-32-0432 on growth of the cell lines. A low concentration of CI-1033 (0.1 μ M), was used in combination with increasing concentration of SH-6 or RO-32-0432. In MCF-7 cells, 0.1 μ M CI-1033 had no significant effect on cell growth alone and combination with SH-6 or RO-32-0432 was not superior to treatment with SH-6 or RO-32-0432 alone, Fig. 4a and b. In contrast, combination of CI-1033 and SH-6 was superior to treatment with each single agent in all the resistant cell lines, Fig 4a. For 164^R-5 the combination was superior at SH-6 concentrations of 1.5 μ M ($P = 0.02$) and 2 μ M ($P = 0.002$). For 164^R-7, the combination was superior at SH-6 concentrations of 1 μ M ($P = 0.03$) and 1.5 μ M ($P = 0.03$) and for 182^R-6 superior effects were seen at SH-6 concentrations of 1 and 1.5 μ M ($P < 0.0001$) and 2 μ M ($P = 0.02$). For the combination of CI-1033 and RO-32-0432, a superior effect compared to single agent treatment was observed in all resistant cell lines when CI-1033 was combined with 2 μ M RO-32-0432 (164^R-5 $P = 0.008$, 164^R-7 $P = 0.02$, and 182^R-6 $P = 0.04$), and 3 μ M RO-32-0432 (164^R-5 $P = 0.002$, 164^R-7 $P = 0.01$, and 182^R-6 $P < 0.0001$).

The growth inhibitory effect of CI-1033 is more pronounced in resistant cells grown in presence of fulvestrant

In order to investigate whether the effect of CI-1033 on the resistant cell lines was affected by the presence of fulvestrant, withdrawal experiments were performed as resistant cells are routinely cultured with fulvestrant in the medium. When fulvestrant was withdrawn from the resistant cell lines for one week prior to initiation of experiments, ER α expression was increased in all three resistant cell lines

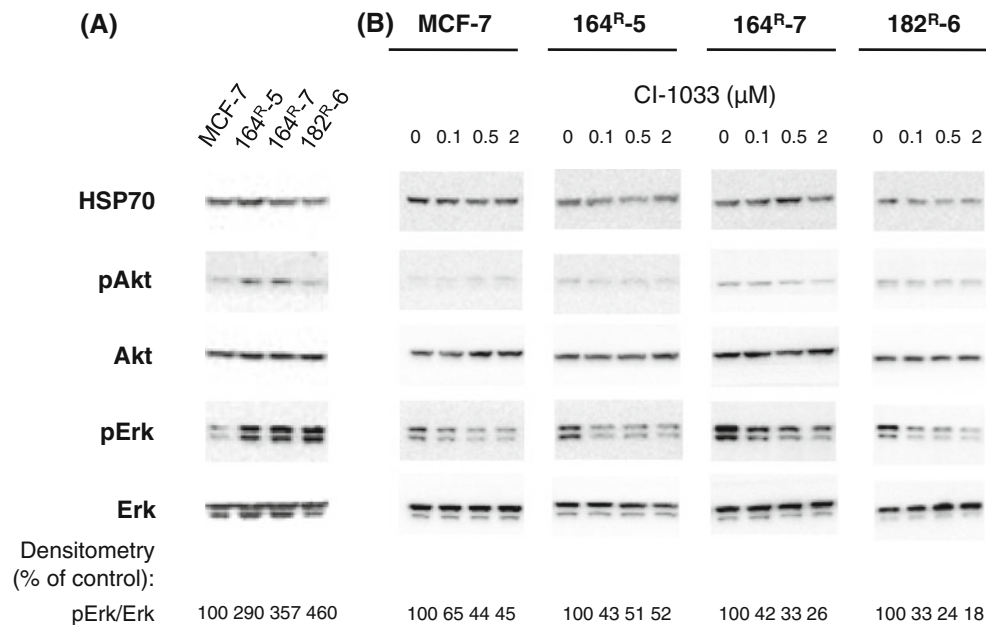


Fig. 3 a Western blots showing expression of the total and phosphorylated levels of the Akt and Erk kinases in MCF-7 and the fulvestrant resistant cell lines 164^R-5, 164^R-7, and 182^R-6. HSP70 was used as loading control. Cells were grown until approximately 70% confluence and lysed with RIPA buffer. Three independent experiments were performed and one representative blot is shown. The expression of pErk in the resistant cell lines was compared to the expression in MCF-7 by expressing the ratio pErk/Erk as a percentage of the ratio in MCF-7 cells. **b** Western blots showing the effect of CI-

1033 on total and phosphorylated forms of Akt and Erk in MCF-7 and the fulvestrant resistant cell lines 164^R-5, 164^R-7, and 182^R-6. HSP70 was used as loading control. Cells were treated for four hours with CI-1033 and lysed with RIPA buffer. Three independent experiments were performed and one representative blot is shown. To quantify the effect of CI-1033 on pErk expression, we expressed the ratio pErk/Erk as a percentage of the ratio in the untreated control for each cell line

(compare fulvestrant cultures without CI-1033 and fulvestrant withdrawn cultures without CI-1033, Fig. 5). If ER α is functional, increased expression of ER α -regulated genes would be expected to follow fulvestrant withdrawal. Expression of the progesterone receptor (PR) is often used as an indicator of active ER α signaling. However, as fulvestrant resistant cell lines express extremely low PR levels and PR is not induced by estradiol in the resistant cells withdrawn from fulvestrant [34], the estrogen inducible Bcl-2 protein was selected as an indicator of functional ER α . A substantial increase in expression of Bcl-2 upon fulvestrant withdrawal was found in 164^R-5 and 164^R-7, but not in 182^R-6 (compare fulvestrant cultures without CI-1033 and fulvestrant withdrawn cultures without CI-1033, Fig. 5). Other studies have shown that a blockade of ErbB signaling could re-establish ER α expression and function [19, 35]. In the present study, such an effect of ErbB blockade with CI-1033 was mainly expected in the fulvestrant withdrawn cultures, as the ER α protein is destabilized in the presence of fulvestrant. However, we did not observe any increase in ER α or Bcl-2 when fulvestrant withdrawn cultures were treated with CI-1033. In contrast, a slight increase in ER α expression was observed in the 182^R-6 fulvestrant cultures when treated with CI-1033, but

the increase was minimal compared to the large increase observed upon fulvestrant withdrawal, indicating that ER α signaling in our resistant cell lines is mainly regulated by presence or absence of fulvestrant. Still, CI-1033 had a more pronounced growth inhibitory effect on all three resistant cell lines when added in combination with 0.1 μ M fulvestrant at concentrations of 0.1 μ M (164^R-5 $P = 0.02$, 164^R-7 $P = 0.02$, and 182^R-6 $P = 0.04$), 0.5 μ M (164^R-5 $P = 0.002$, 164^R-7 $P = 0.01$, and 182^R-6 $P = 0.005$), or 1 μ M (164^R-5 $P = 0.02$, 164^R-7 $P = 0.01$, 182^R-6 $P < 0.0001$), Fig. 6. Thus, all resistant cell lines were able to partly escape the effects of CI-1033 treatment when released from fulvestrant-mediated blockade of the ER α receptor.

Heregulin-1 β rescues MCF-7 cells from the growth inhibitory effects of fulvestrant

The lack of growth inhibition of MCF-7 cells with CI-1033 (see Fig. 2a) indicates that MCF-7 cells are independent of ErbB signaling when grown at standard conditions. Treatment of MCF-7 cells with fulvestrant significantly inhibits cell growth in a dose dependent manner, an effect that could partly be abrogated by co-treatment with 10 ng/ml of

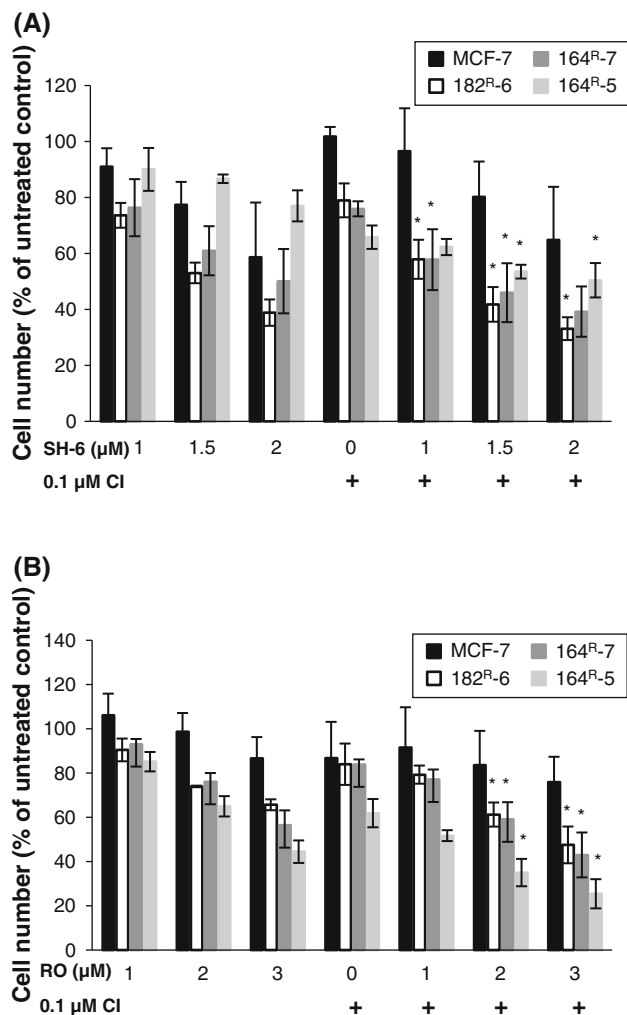


Fig. 4 **a** Effect of the Akt inhibitor SH-6 alone or in combination with the pan-ErbB inhibitor CI-1033 (CI) on growth of MCF-7 and the fulvestrant resistant cell lines 164^R-5, 164^R-7, and 182^R-6. Cells were treated for 5 days with the indicated concentrations of the inhibitors, before cell number was estimated by a colorimetric assay and expressed as percent of the untreated control. Values (mean \pm SD) are pooled data from three independent experiments, each performed with triplicate measures. * indicates superior effect of the combined treatment compared to treatment with each single agent. **b** Effect of the PKC inhibitor RO-32-0432 (RO) alone or in combination with the pan-ErbB inhibitor CI-1033 (CI) on cell growth of MCF-7 and the fulvestrant resistant cell lines 164^R-5, 164^R-7, and 182^R-6. Cells were treated for 5 days before cell number was estimated by a colorimetric assay and expressed as percent of the untreated control. Values (mean \pm SD) are pooled data from three independent experiments, each performed with triplicate measures. * indicates superior effect of the combined treatment compared to treatment with each single agent

the ErbB3/ErbB4 ligand heregulin-1 β (Hrg) at all fulvestrant concentrations tested ($P < 0.0001$), Fig. 7a. Thus, as also shown previously [36], activation of ErbB signaling can potentially substitute for the fulvestrant-mediated blockade of ER α signaling in MCF-7 cells. When fulvestrant resistant cells are developed from MCF-7 cells by

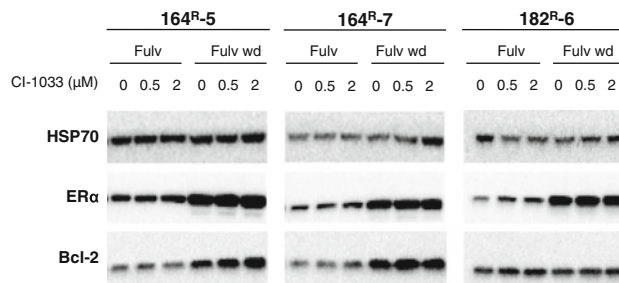


Fig. 5 Effect of fulvestrant (fulv) withdrawal (wd) and CI-1033 treatment on estrogen receptor α (ER α) and Bcl-2 protein expression in the fulvestrant resistant cell lines 164^R-5, 164^R-7, and 182^R-6. HSP70 was used as loading control. For withdrawal cultures, fulvestrant was removed from the cell culture medium one week prior to onset of experiments. Cells were treated for 48 h with the indicated concentrations of CI-1033 and lysed with RIPA buffer. Protein expression was measured by Western analysis. One representative blot of three is shown

prolonged exposure to 0.1 μ M fulvestrant, the cells show an initial arrest/death phase, followed by outgrowth of a small number of proliferating cell colonies after approximately 1 month [24]. Based on the finding that ErbB activation through Hrg treatment could rescue the effects of fulvestrant, we speculated whether a Hrg-mediated activation of the ErbB system could accelerate development of fulvestrant resistance. Therefore, MCF-7 cells were cultured for prolonged time (6 weeks) with 0.1 μ M fulvestrant with or without 10 ng/ml Hrg. The cells treated with fulvestrant and Hrg displayed exponential growth and the cultures could be split every 10–12 days with a split ratio of about 10. Thus, switching on ErbB3/4 signaling by Hrg abolishes the growth inhibiting effects of even prolonged treatment with fulvestrant. However, long-term growth in presence of Hrg and fulvestrant (6 weeks) did not result in permanent fulvestrant resistance as withdrawal of Hrg resulted in cessation of growth, Fig. 7b. In order to examine how Hrg rescued the cells from the inhibitory effect of fulvestrant, different inhibitors were added in combination with fulvestrant and Hrg. Exposure to 50 μ g/ml cetuximab had no effect on the Hrg-mediated rescue, whereas a partial inhibition of the Hrg-mediated rescue was observed when the long-term fulvestrant and Hrg cultures were treated with 20 μ g/ml pertuzumab ($P < 0.0001$) or 20 μ g/ml trastuzumab ($P < 0.0001$). A combination of 20 μ g/ml trastuzumab and 20 μ g/ml pertuzumab increased the inhibitory effect compared to single treatment with pertuzumab ($P = 0.006$) and this combination as well as treatment with CI-1033 alone, completely blocked the rescuing effect of Hrg (evaluated as not significantly different from the fulvestrant-only treated culture). Thus, the rescuing effect of Hrg was mediated through ErbB activation, likely through ErbB3 and/or ErbB4 dimerization with ErbB2.

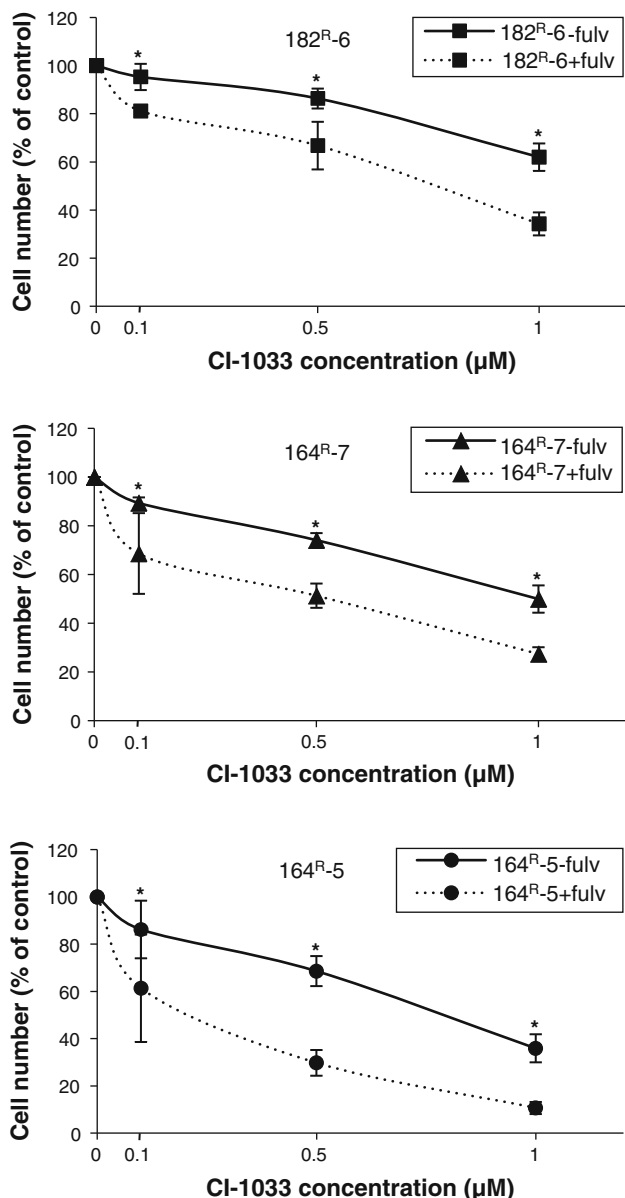


Fig. 6 Effect of the pan-ErbB inhibitor CI-1033 ± 0.1 µM fulvestrant (fulv) on cell growth of the fulvestrant resistant cell lines 164^R-5, 164^R-7, and 182^R-6. For withdrawal cultures, fulvestrant was removed from the cell culture medium one week prior to onset of experiments. Cells were treated with CI-1033 for 5 days before cell number was estimated by a colorimetric assay and expressed as percent of the respective control cultured with or without fulvestrant. Values (mean ± SD) are pooled data from three independent experiments, each performed with triplicate measures. * indicates significantly different effects of CI-1033 in cell cultures grown with and without fulvestrant

Combination of CI-1033 and fulvestrant postpones development of resistance in MCF-7 cells

Treatment resistance is a serious problem in the clinic and several approaches are investigated to postpone development of resistance, including combination therapy

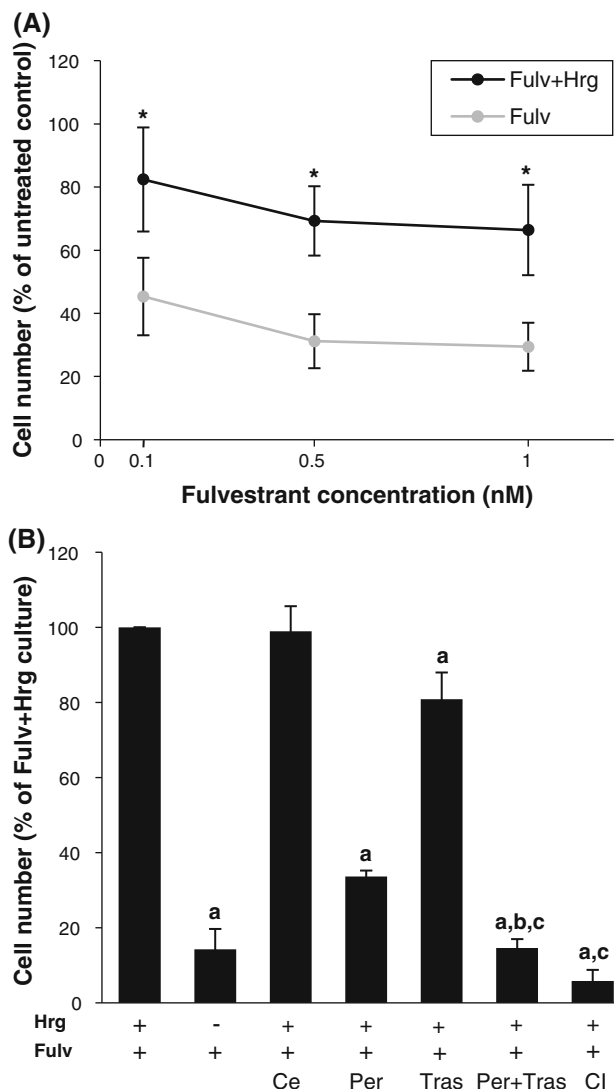


Fig. 7 a Effect of heregulin-1β (Hrg) on cell growth of fulvestrant (Fulv) treated MCF-7 cells. Cells were treated for 5 days with the indicated concentrations of fulvestrant with or without 10 ng/ml Hrg before cell number was estimated by a colorimetric assay and expressed as percent of the untreated control. Values (mean ± SD) are pooled data from three independent experiments, each performed with triplicate measures. * indicates significant difference between cultures grown with or without Hrg. **b** Effect of ErbB-receptor inhibition on growth of MCF-7 cells continuously cultured with fulvestrant (Fulv) and heregulin-1β (Hrg). MCF-7 cells continuously grown with 0.1 µM fulvestrant + 10 ng/ml Hrg were seeded in 24-well plates and treated with cetuximab (Ce; 50 µg/ml), pertuzumab (Per; 20 µg/ml), trastuzumab (Tras; 20 µg/ml), CI-1033 (CI; 0.1 µM) or pertuzumab in combination with trastuzumab (both 20 µg/ml) for 5 days. Hrg and fulvestrant were maintained in the medium except in one culture grown with fulvestrant only. Cell number was estimated by a colorimetric assay and expressed as percent of the control culture grown continuously with fulvestrant and Hrg. Values (mean ± SD) are pooled data from three independent experiments, each performed with triplicate measures. *a* indicates values significantly different from the fulvestrant and Hrg control culture. *b* indicates values significantly different from the pertuzumab treated culture. *c* indicates values equal to the fulvestrant only treated culture

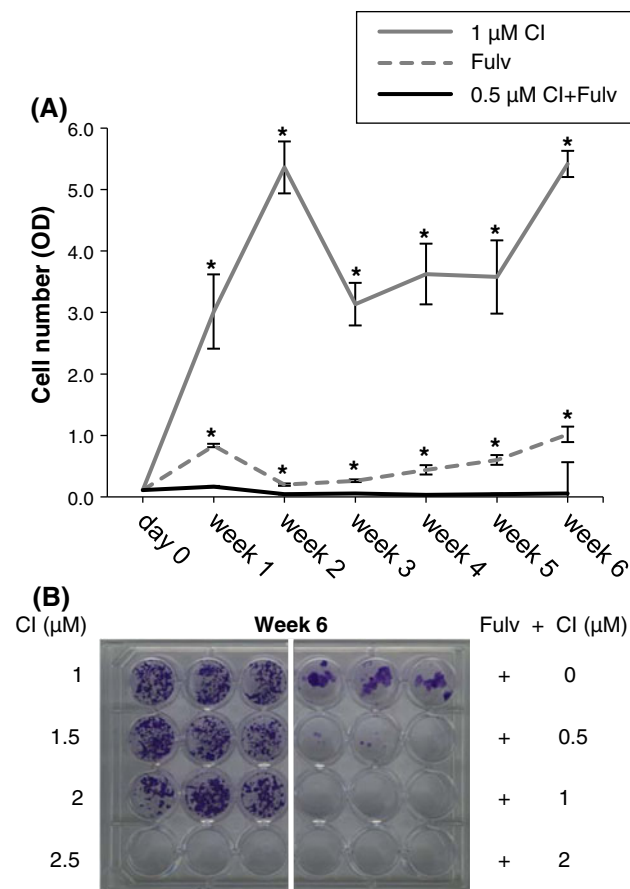


Fig. 8 a Effect of long-term treatment of MCF-7 cells with fulvestrant (Fulv) alone or in combination with CI-1033 (CI). The cells were plated in 24-well plates and treatment initiated after 24 h (day 0) with CI-1033 (1 μM) or fulvestrant (0.1 μM) alone or CI-1033 (0.5 μM) in combination with fulvestrant (0.1 μM). Treatments were performed in triplicate and the cell number was determined by a colorimetric assay at day 0 and after 1, 2, 3, 4, 5, and 6 weeks. Values are mean \pm SD from one representative out of three independent experiments, each performed with triplicate measures. * indicates cell numbers higher than the cell number at day 0. **b** Effect of long-term treatment of MCF-7 cells with fulvestrant (Fulv) and CI-1033 (CI) as single agents and in combination. The cells were plated in 24-well plates and treatment initiated after 24 h with CI-1033 and fulvestrant alone or fulvestrant in combination with CI-1033. Fulvestrant was administered in a dose of 0.1 μM and CI-1033 in doses as indicated in the figure. Treatment was performed in triplicate and the cells were stained with crystal violet at day 0 and after 1, 2, 3, 4, 5, and 6 weeks. Picture from a representative culture plate stained at week 6 is shown

[14, 37, 38]. Our findings that Hrg is able to restore growth of fulvestrant-treated MCF-7 cells and that MCF-7 sublines with acquired fulvestrant resistance are more sensitive to pan-ErbB inhibition when maintained on fulvestrant suggest that combined treatment targeting ErbB and ER α signaling could be used to prevent or delay development of treatment resistance. Prolonged exposure to 0.1 μM fulvestrant, 1–2.5 μM CI-1033, or a combination of 0.1 μM fulvestrant with 0.5–2 μM CI-1033 was performed in

MCF-7 cells. Figure 8a shows the results from treatments with fulvestrant alone, 1 μM CI-1033 alone, and 0.5 μM CI-1033 in combination with fulvestrant. The MCF-7 culture treated with fulvestrant alone displayed a small initial increase in cell number followed by cell arrest and cell death. However, after 6 weeks a minor increase in cell number was observed, Fig. 8a. This increase was due to a few colonies of proliferating cells as shown in Fig. 8b. In the presence of 1 μM CI-1033, MCF-7 cells grew until confluence, yet slower than untreated cells, where after a balance between cell growth and cell death resulted in a steady state with a near-confluent cell density during the weeks 3–6, Fig. 8a. A comparable pattern was observed for cultures treated with 1.5 and 2 μM CI-1033 resulting in a stable population of viable cells after 6 weeks of treatment, Fig. 8b. MCF-7 cells could not sustain growth in presence of 2.5 μM CI-1033 for more than 2–3 weeks. It should be mentioned that MCF-7 cells could be subcultivated continuously with 1 μM CI-1033 with a weekly split ratio of between 10 and 15, compared to a split ratio for untreated MCF-7 cells of around 15–20. Cultures treated with a combination of fulvestrant and 0.5 μM CI-1033 only showed a small increase in cell growth over the first week, followed by cell arrest and cell death, Fig. 8a. Around week 5–6, small distinct colonies of cells appeared, Fig. 8b, but the cell number did not increase significantly above the cell number for day 0 during the course of this experiment, Fig. 8a. No colonies were visible in the cultures treated with 1 or 2 μM CI-1033 in combination with fulvestrant, Fig. 8b. Thus, long-term fulvestrant treatment results in a clonal selection of resistant cells, which could be prevented or delayed by a combination treatment strategy with CI-1033.

Discussion

Data from preclinical breast cancer models indicate that signaling through the ErbB tyrosine kinase receptors can promote antiestrogen failure in ER α positive breast cancer cells, e.g., through ectopic overexpression of TGF α , heregulin-1 β , EGFR, and ErbB2 [39–42]. In concert, development of acquired resistance to antiestrogen therapy is often accompanied with changes resulting in increased signaling through the ErbB system, e.g., increased expression of ligands, receptors, or downstream effector molecules [5–11, 15]. In the clinic, response to endocrine therapy is found to be reduced in patients with high expression of TGF α or EGFR [43, 44], and ER α /ErbB2 positive tumors are less sensitive to both antiestrogen and aromatase inhibitor therapy than ER α positive/ErbB2 negative tumors [23]. In our cell culture model system comprising MCF-7 breast cancer cell lines with acquired

resistance toward treatment with the antiestrogen fulvestrant, changes in the ErbB signaling pathway were evident at ligand, receptor, and downstream effector levels [8, 11]. The present study examined the effects of ErbB inhibition in these fulvestrant resistant cell lines, alone and in combination with other signal transduction inhibitors as well as the effect of combined ErbB and ER α inhibition. The data showed that targeting either EGFR or ErbB2 with the monoclonal antibodies, cetuximab, pertuzumab, or trastuzumab, had limited or no effect on cell growth of parental MCF-7 cells and fulvestrant resistant sublines. In contrast, low concentrations of the pan-ErbB inhibitor CI-1033 showed preferential inhibition of the fulvestrant resistant cell lines ranging from 30 to 80% inhibition without affecting growth of MCF-7 cells. The superior effect of the tyrosine kinase inhibitor CI-1033 compared to antibody-based ErbB inhibition may likely be due to its pan-ErbB inhibitory effects. We have previously shown preferential growth inhibition of the resistant cell lines with 1 μ M of the tyrosine kinase inhibitor gefitinib, a concentration believed only to inhibit the kinase activity of EGFR [11]. This could appear to be in conflict with the modest/lacking effect of cetuximab observed in the present study, however in our cell lines 1 μ M gefitinib did not only inhibit EGFR phosphorylation but also ErbB3 and ErbB4 phosphorylation ([11] and unpublished data for ErbB4), reinforcing the need of pan-ErbB inhibition for repression of resistant cell growth. It is becoming increasingly clear that the ErbB system of receptors is tightly coupled, pointing to the importance of dealing with these receptors as a complex network, which should be targeted in combination, rather than as individual receptors [45]. A superior effect of targeting more than one ErbB receptor has also been shown in a breast cancer xenograft model, where a combination of pertuzumab, trastuzumab, and gefitinib more efficiently blocked tumor growth than either single agent [46]. Our resistant cell lines show increased expression of the ErbB3/4 ligand heregulin-2 β and increased activity of ErbB3 [11] and this increased ErbB3 signaling may in part explain the insensitivity to EGFR/ErbB2 antibody-based treatment as has previously been indicated in both clinical and pre-clinical studies [47, 48]. However, it should be mentioned that the potent effects of CI-1033 compared to antibody-based therapy may not be solely ascribed to its pan-ErbB inhibitory effects as off-target inhibition of other tyrosine kinases cannot be ruled out. Early studies reported a high specificity of CI-1033 for the ErbB receptors [26], but this specificity has later been questioned as CI-1033 has been shown to be somewhat promiscuous in its targets [49]. Yet, at low concentrations (0.1–0.5 μ M) CI-1033 may be rather specific in targeting the ErbB receptor system in the present model system, based on the preferential effect in cell lines depending on ErbB signaling from growth. Overall, the

potent and preferential growth inhibitory effect of low CI-1033 concentrations on antiestrogen resistant breast cancer cells clearly support the testing of CI-1033 in metastatic antiestrogen resistant breast cancer and may even suggest clinical activity in a subset of antiestrogen resistant breast cancers that do not respond to EGFR/ErbB2 monotherapy.

Erk and Akt are well known downstream effectors of ErbB signaling [45]. In our cell lines, inhibition of ErbB signaling with CI-1033 primarily downregulated pErk and had little effect on pAkt. This is in agreement with our previous study, in which gefitinib-mediated inhibition of ErbB signaling in antiestrogen resistant cell lines resulted in reduced Erk signaling, but had no effect on the increased Akt signaling in resistant cell lines [11], suggesting that this Akt activation is independent of ErbB signaling. We have previously shown that the Akt inhibitor SH-6 preferentially inhibited growth of tamoxifen and fulvestrant resistant cell lines compared to parental MCF-7 cells [8] and therefore reasoned that a combination of CI-1033 and SH-6 could augment the growth inhibitory effects on resistant cells. Further, we have found that PKC α and δ are overexpressed in tamoxifen and fulvestrant resistant cell lines and that the PKC inhibitor RO-32-0432 displayed preferential growth inhibition of resistant cell lines compared to parental MCF-7 cells [33]. As the effect of RO-32-0432 is not mediated through modulation of either Erk or Akt (Kristina Emdal, unpublished data), we also tested whether a combination of CI-1033 and RO-32-0432 treatment could augment the growth inhibitory effects on resistant cell lines. For both the combination of CI-1033 with SH-6 and the combination with RO-32-0432, a small superior effect on inhibition of resistant cell growth was observed compared to each single agent treatment. Thus, the data indicate that concomitant inhibition of signaling through Erk and Akt as well as Erk and PKC was superior to inhibition of a single pathway. The significant growth inhibition of resistant cells was seen at concentrations which had only little effect on parental cells, indicating that toxic side effects may be reduced by combination therapy.

Noteworthy, the present results indicate that both antiestrogen resistant breast cancer and presumably also a subgroup of primary antiestrogen sensitive breast cancers could benefit from adjuvant antiestrogen treatment combined with pan-ErbB targeted treatment. This is based on the observation that both our parental antiestrogen sensitive cells and the fulvestrant resistant cell lines had the ability to switch between ER α - and ErbB-mediated signaling, depending on externally applied factors. Fulvestrant is a very potent antiestrogen that downregulates ER α protein expression and has no agonistic activity [4, 50]. Fulvestrant resistance may be mediated through complete ER α repression, sometimes recognized as a loss of ER α expression and ER α independent activation of growth factor activity

[4, 51]. Our resistant cell lines display increased dependence on the ErbB pathway as evident from the increased sensitivity to the pan-ErbB inhibitor CI-1033 in the present study and to the EGFR inhibitor gefitinib in a previous study [11]. Yet, when fulvestrant was withdrawn from the resistant cells, ER α expression was restored and in two out of three cell lines, expression of the estrogen responsive protein Bcl-2 followed, demonstrating the presence of a functional ER α in the resistant cells, in agreement with previously published data [34]. Other studies have shown that ErbB signaling in itself can suppress ER α expression and signaling in breast cancer cells and that inhibition of such ErbB signaling can reestablish ER α mediated signaling [18, 19]. This was not the case in our model system where ER α signaling, as visualized by ER α and Bcl-2 expression, was repressed by the presence of fulvestrant in the medium, but unaffected by ErbB blockade with CI-1033 upon faslodex withdrawal. Thus, ErbB signaling in the resistant cell lines did not appear to block ER α expression in itself. Further, in our fulvestrant withdrawn cells, the growth inhibitory effect of CI-1033 was significantly reduced compared to fulvestrant-treated cells, suggesting that regained ER α signaling could at least partly compensate for the inhibition of ErbB signaling with CI-1033. An ability to switch between ER α and ErbB2 signaling has recently been observed in our tamoxifen resistant cell line MCF-7/TAM^R-1 when grown in presence and absence of tamoxifen [15]. This is in line with xenograft models of tamoxifen resistance [46] and letrozole resistance [52], where ErbB blockade restored the antagonistic properties of the endocrine therapy, resulting in better effect of continued endocrine therapy with ErbB blockade than of ErbB blockade alone [52]. Also, ErbB2 overexpressing breast cancer cells with acquired resistance to ErbB directed therapy with lapatinib has been shown to switch from ErbB to ER α signaling for cell survival and growth [53]. In conclusion, our fulvestrant resistant cell lines clearly possessed the potential to use both ErbB and ER α signaling for growth stimulation, switching to one or the other pathway according to the inhibitors applied. Taken to the clinic, this support combined use of ErbB inhibitors and antiestrogen upon relapse on antiestrogen therapy.

Parental MCF-7 cells also possessed the ability to switch between ER α and ErbB signaling, as it could be demonstrated that addition of the ErbB3/ErbB4 ligand heregulin-1 β (Hrg) prevented fulvestrant-mediated growth inhibition. Hrg-mediated rescue of fulvestrant inhibition of MCF-7 cell growth likely occurred through ErbB3 and/or ErbB4 dimerization with ErbB2 as both trastuzumab and pertuzumab suppressed this stimulation and combination of the antibodies completely abolished Hrg-mediated growth stimulation. Hrg-mediated growth stimulation was also completely blocked by treatment with the pan-ErbB

inhibitor CI-1033. Furthermore, we found that when treating MCF-7 cells with a combination of fulvestrant and CI-1033, outgrowth of resistant cells was delayed or prevented in the experimental period of 6 weeks. These data indicate that upfront treatment of breast cancer patients with agents targeting both ER α and ErbB signaling may delay development of resistance. A recent phase II neoadjuvant study supports the idea of combining endocrine therapy and inhibition of growth factor pathways in early breast cancer [54]. The study showed that the efficacy of the aromatase inhibitor letrozole was significantly enhanced with concomitant targeting of mTOR, a kinase in the PI3 K/Akt pathway downstream of the ErbB receptors.

In conclusion, this study demonstrated that the new pan-ErbB inhibitor CI-1033 is very effective in blocking growth of antiestrogen resistant breast cancer cell growth at concentrations that do not affect antiestrogen sensitive cells. Further, both antiestrogen sensitive and antiestrogen resistant breast cancer cells showed the ability to switch between ER α and ErbB signaling depending on the applied treatment. Accordingly, the efficacy of CI-1033 treatment was increased when maintaining antiestrogen therapy, even after development of antiestrogen resistance. For parental antiestrogen sensitive cells, combined therapy against ER α and ErbB signaling significantly postponed or prevented the appearance of resistant cells. Thus, the present study provides support for combination of signal transduction inhibitors with endocrine agents as upfront therapeutic approach in human breast cancer as well as in patients relapsing on endocrine therapy.

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